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PROCEEDINGS OF THE FNCA WORKSHOP ON PLANT MUTATION BREEDING 2001 – MOLECULAR BIOLOGICAL TECHNIQUES – AUGUST 20-24, 2001, BANGKOK, THAILAND

February 2002

(Eds.) Tamikazu KUME, Kazuo WATANABE
and Shigemitsu TANO*

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The FNCA Workshop on Plant Mutation Breeding 2001

August 20-24, 2001, Bangkok, Thailand

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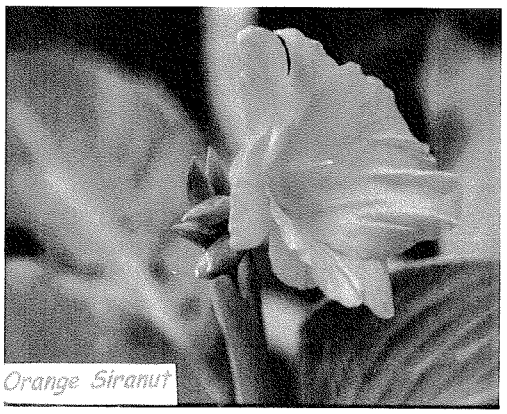
Opening


ION BREEDING AND BIOFERTILIZER
20-24 AUGUST 2001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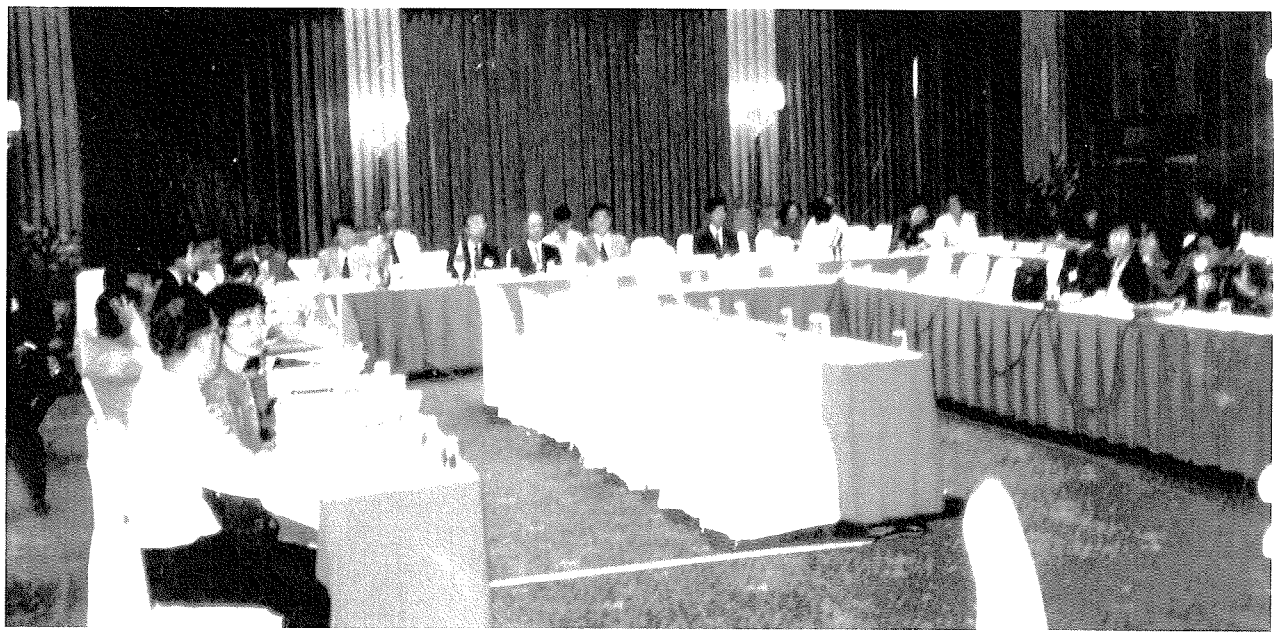

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Participants

Japan

China

Indonesia

Korea

The Philippines

Malaysia

Vietnam

Thailand

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Presentation

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Discussion

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Technical Visit

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Proceedings of the FNCA Workshop on Plant Mutation Breeding 2001 – Molecular Biological Techniques – August 20-24, 2001, Bangkok, Thailand

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(Received January 4, 2002)

The FNCA (Forum for Nuclear Cooperation in Asia) Workshop on Plant Mutation Breeding was held on 20-24 August 2001 in Bangkok, Thailand. The Workshop was sponsored by the Ministry of Education, Culture, Sports, Science and Technology (MEXT). The Kasetsart University (KU), the Office of Atomic Energy for Peace (OAEP) and Department of Agriculture (DOA) acted as local host and the organizer with the cooperation of the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, the Japan Atomic Industrial Forum (JAIF) and Japan Atomic Energy Research Institute (JAERI).

The Workshop was attended by two participants, a Project Leader and an expert on molecular biological techniques for plant mutation breeding, from each of the participating countries, i.e. China, Indonesia, Malaysia, the Philippines and Vietnam. One participant from the Republic of Korea, nine participants from Japan and thirteen participants from Thailand including three invited speakers attended the Workshop.

Eleven papers including three invited papers on the current status of molecular biological techniques for plant mutation breeding were presented. Discussions were focused to further regional cooperation, to review and discuss results of past activities. The Medium-Term Plan of the project on the application of radiation and radioisotopes for agriculture in participating countries of Regional Nuclear Cooperation Activities (RNCA) was formulated and agreed.

This proceeding compiles the invited and contributed papers that were submitted from the speakers.

Keywords: Mutation Breeding, Radiation, Plants, Molecular Biology

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*Project Leader of FNCA Mutation Breeding, Former Professor of the University of Tokyo

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「FNCA植物突然変異育種ワークショップ2001」論文集
—分子生物学的手法—
2001年8月20日～24日　　タイ・バンコク市

日本原子力研究所高崎研究所材料開発部
（編）久米　民和・渡辺　和夫・田野　茂光*

(2002年1月4日受理)

「アジア原子力協力フォーラム（FNCA）植物突然変異育種ワークショップ2001-分子生物学手法-」が、文部科学省の主催、開催国のカセトサート大学（KU）、タイ原子力庁(OAEP)、タイ農業庁(DOA)の共催、農林水産省（MAFF）、日本原子力産業会議(JAIF)及び日本原子力研究所（JAERI）の協賛により、2001年8月20日から24日にタイ・バンコク市において開催された。

本ワークショップには、中国、インドネシア、マレーシア、フィリピン、ベトナムからプロジェクト・リーダーと分子生物学的手法を用いた突然変異育種の専門家の各国２名が参加した。また、韓国から1名、日本から9名、タイからは招待講演者3名を含む計13名が参加した。

本ワークショップでは、植物突然変異育種における分子生物学的手法に関して、3件の招待講演を含め計11件の発表があった。円卓討議では、これまでの活動結果の見直しと検討、さらに今後の地域協力についての討議を行った。また、アジア地域原子力協力活動(RNCA)参加各国における「RI･放射線の農業利用プロジェクト」について討議され中期計画案が合意された。本論文集は、これら各発表者からの投稿原稿を収録したものである。

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Contents

1. Opening Address

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1.1 Welcome Address

Thira Sutabutra

President of Kasetsart University

Participants and Guests
Ladies and Gentlemen

On behalf of the local host organizing committee which is composed of three organizations, the Kasetsart University, the Office of Atomic Energy for Peace and the Department of Agriculture, I would like to extend my warm welcome to all participants attending "The 2001 FNCA Workshop on Plant Mutation Breeding and Biofertilizer".

I would like to express our sincere thanks to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Japan Atomic Energy Research Institute (JAERI) and the Japan Atomic Industrial Forum (JAIF) for their support for this Workshop.

The application of nuclear techniques in agriculture has been widely well accepted in Thailand especially in plant mutation breeding. The leading mutant rice varieties namely RD6 and RD15 generated from gamma irradiation of Khao Dok Mali 105 have been released to farmers in 1978 and since then their planting areas have been increased rapidly. These two mutant varieties and their original variety 'Khao Dok Mali' had occupied 4.9 million hectare which was 53 percent of total area devoted to rice in the rainy season in 2000.

New released variety of mungbean variety 'Chai Nat 72' which is resistant to pod borer emphasizes the significance of the induced mutation technology to the food security in Thailand.

Not only in food crops, this induced mutation technology has been intensively applied to the improvement of ornamental plants such as chrysanthemum, orchids, canna, curcuma and others. Several mutant varieties of canna have been released to the general public. Many beneficial mutants of chrysanthemum are being under field testing for uniformity, stability and market acceptance.

It is our deep appreciation to the 2001 FNCA Workshop on Agriculture for including biofertilizer as an additional aspect of the Workshop. Thailand recognizes the importance, utility and the valuation of biofertilizer to farmers. We strongly support the future cooperation in this field with our FNCA member countries.

Again to all participants we welcome you and hope that this Workshop will be an excellent opportunity for you to exchange and share experience, and to co-operate more closely for strengthening the use of plant mutation breeding and biofertilizer in agriculture. I wish this Workshop the great success and rewarding outcome to all of us.
Thank you.

1.2 Opening Remarks

Tsutomu Imamura

Director-General
Research and Development Bureau
Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan
(delivered by Manabu Hamasaki, MEXT)

It is my great pleasure to have this opportunity of saying some words on behalf of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan on the occasion of the 2001 FNCA Work Shop on Agriculture.

First of all, I would like to extend my sincere gratitude to the Royal Thai government, especially to the Office of Atomic Energy for Peace (OAEP) and Kassetsart University for preparing this Workshop.

At the initiative of the Atomic Energy Commission of Japan, the FNCA was established last year as an effective mechanism to promote the regional nuclear cooperation, by reorganizing the former International Conference for Nuclear Cooperation in Asia (ICNCA). It is still fresh in our mind that the memorable first Meeting of FNCA was held here in Bangkok.

As a result from the major reorganization of Japanese central government offices that took place this January, we, MEXT, have taken over the former STA's duties. And we are determined to actively support the implementation of cooperative activities, as the responsible ministry for promoting research and development on nuclear science and technology.

The application of radiation or radioisotopes to agriculture, which is the subject of this workshop, is one of the most important fields of nuclear science and technology application with large potential to contribute to enhance the quality of life in the region. The cooperative activities in this field began with the Feasibility Study on the Sterile Insect Technique (SIT), followed by the Work Shop on Plant Mutation Breeding continuing to date. And this year, the application of the nuclear science and technology to the bio-fertilizer utilization is taken up as a new topic and its feasibility will be discussed, I understand.

Not excepting nuclear field, the socio-economic impact from science and technology can be appreciated only when the outcome of research and development reaches its end-users, I believe. So, I expect that all the scientific discussions among the experts participating in this workshop will properly meet the needs in respective countries.

Thank you very much for your attention.

1.3 Opening Address

Kriengkorn Bejraputra

Secretary -General, Office of Atomic Energy for Peace

Distinguish Guests
Distinguish Participants,
Ladies and Gentlemen,

On behalf of the Royal Thai Government, We are pleased to welcome all of you to Bangkok and to the 2001 FNCA Workshop on Plant Mutation Breeding and Biofertilizer. We would like to take this opportunity to thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Japan Atomic Energy Research Institute (JAERI) and the Japan Atomic Industrial Forum (JAIF) for supporting and sponsoring this workshop. I would also like to thank Kasetsart University and the Department of Agriculture for co-hosting this workshop, especially the Organizing Committee for their best effort in arranging this workshop.

The meeting here fosters our common intention to co-operate with one another for application of nuclear techniques in the field of agriculture. I am pleased to say that this co-operative event extends not only to the 8 participating countries but also to the other organizations sharing our interest as well.

During this workshop, attempts should be made to exchange the experiences and knowledge in plant mutation breeding and biofertilizer developed in each country, beside fostering good will and better understanding among the representatives. With close cooperation of countries in the region, it gives us confidence to face the challenges in advancement of this field of nuclear application for the benefit of our population.

Finally, I wish you all the pleasant stay and at this moment, I would like to declare open the 2001 FNCA Workshop on Plant Mutation Breeding and Biofertilizer.

Thank you.

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2. Outlines of Mutation Breeding Workshop

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2.1 General Report

Based on the agreement of the 2nd FNCA Coordinators Meeting held on Mach 14-16, 2001 in Tokyo, the FNCA workshop on Agriculture, "Plant Mutation Breeding and Biofertilizer" took place in Bangkok, Thailand. This workshop was sponsored by the Ministry of Education, Culture, Sports, Science and Technology (MEXT). The Kasetsart University (KU), the Office of Atomic Energy for Peace (OAEP) and Department of Agriculture (DOA) of Thailand acted as the local organizers with the cooperation of the Ministry of Agriculture, Forestry, Fisheries (MAFF) of Japan, the Japan Atomic Industrial Forum (JAIF) and Japan Atomic Energy Research Institute (JAERI) as the coordinators.

The number of the participants in the workshop totaled 51 people including the guests and observers. Eleven papers including three invited papers on the current status of Molecular Biological Techniques for Mutation Breeding in the participating countries were presented. Eight country papers and four invited papers were presented for Biofertilizer. The participants had the opportunity to discuss and exchange the opinions sharing their experiences on the related subjects, which each country was facing and seeking for solutions.

The workshop was attended by two participants, a Project Leader and an expert on molecular techniques for mutation breeding from each of the participating countries, i.e. China, Indonesia, Malaysia, the Philippines and Vietnam. One participant from the Republic of Korea, twelve participants from Japan, five participants, eight observers and three invited speakers from Thailand including a Project Leader, a Keynote speaker attended in the Workshop.

At the Round Table Discussion on the second, third and last day of the workshop, discussion were made to further regional cooperation, review and discuss of the past activities. The Medium-Term Plan of the project on the Application of Radiation and Radioisotopes for Agriculture in participating countries of Regional Nuclear Cooperation Activities (RNCA) was formulated and agreed.

Opinions were exchanged among the participants mainly on how to organize further regional cooperation in the field of plant mutation breeding. The participants agreed that the cooperation through the "Regional Nuclear Cooperation Activities" scheme including annual meeting i.e. workshop, technical visits and discussions to initiate multilateral research program are very important for the participating countries.

The main points of the comments for future plans, proposals and agreed expressed opinion by the participants at the Round Table Discussions include the following:

(1) Conclusion of Phase II

1) Participants in the Workshop exchanged opinions on the drafts of "Regional Cooperation Plan on Application of Radiation and Radioisotopes for Agriculture"

which were described by Japanese side. This document is to be modified and results of the discussion of the Workshop should be incorporated and submitted to the Third Coordinators Meeting to be held in Mach, 2002 in Tokyo, Japan.

2) Results and summary of Phase Ⅱ activities were presented from each country. New varieties produced by mutation breeding during Phase Ⅱ were reported by each country including the information exchanged in the workshop were highly appreciated.
3) During Phase Ⅱ, Mutation Breeding Database (MBDB), Mutant Stock Repository (MSR) and Mutation Breeding Laboratory Manual (MBLM) have been discussed and following agreements were made to support the activities:
   a. MBDB will be published on FNCA homepage (AsiaNNet) as semi-closed system.
   b. MSR has been successfully initiated with the collaboration of Thailand and the Philippines.
   c. MBLM has been formatted, details of contents and final draft will be submitted.
4) After successful conclusion of Phase Ⅱ, the participants agreed to proceed to Phase Ⅲ with the basic theme of "Technique on Reproductive Pattern". The topic for the next workshop is "Vegetatively Propagated Crops and Radiation Breeding ", which will be held in Beijing, China in September, 2002.

(2) Proposal in Phase Ⅲ

The participants agreed on two major activities running concurrently.

1) Workshop with basic theme of "Technique on Reproductive Pattern". The annual workshop will discuss the following sub-themes.
   a. Vegetatively Propagated Crops (2002)
   b. Root Crops (2003)
   c. Clonally Propagated Crops (2004)
2) Multilateral Research Program
   a. Drought Tolerance in Sorghum, sugar cane, soybean (2002 onward)
   b. Three other topics were also thoroughly discussed and considerated.

(3) Others

1) The workshop included technical tours to the following institution:
   Mutation Breeding Group; Gamma Irradiation Service and Nuclear Research Center (GISC), KU, Office of Biotechnology Research and Development and Nuclear Techniques (DOA), Pathum Thani Rice Research Center, Rice Research Institute (DOA).
   Biofertilizer Group: N-15 Laboratory (DOA), Soil Microbiology Section of Soil Science division (DOA), Microbial and Isotope Laboratories (KU).

The participants expressed their sincere gratitude to the organizers and hosting institution.

2) Concerning on the next Workshop on Mutation Breeding and on Biofertilizer in 2002, China expressed willingness to be the host in Beijing, China. Following 2002 Workshop in China, all participants appreciated the candidature of the Philippines as the venue for 2003 Workshop.

2.2 Outlines of Country Reports

2.2.1 Thailand

Soybean is one of the important crops in Thailand. Constraints to soybean production include low yield potential, susceptibility to diseases and insects, and non-adoption of appropriate management practices. Mutation induction has been used to improve soybean yield and resistance to major diseases such as rust, purple seed, crinkle leaf, anthracnose and green seed.

The first breakthrough by mutation breeding against soybean-rust disease was the successful development of 'Doi Kham' by Dr. Sumin Smutkupt. The country report included some details of this mutant cultivar, released in 1984, five years after the gamma irradiation (150 and 300 Gy) in 1979. Under the advantage of tropical condition, two generations a year was helpful in shortening of the period necessary for the mutation breeding. The paper reviewed previous work and achievements of induced mutations in soybean. Besides 'Doi Kham' mentioned above, successful examples like development of a mutant CM 60-10kr-71, which was also resistant to rust disease, was presented. The paper included other diseases like purple seed, crinkled leaf disease, and one of the most serious diseases of soybean, i.e. anthracnose disease. Early maturity and lowering of green seed percentage were other objectives in improvement of agricultural factors. The presentation was extended to the help with molecular techniques, for example, the use of soybean Simple Sequence Repeat (SSR) markers to identify Quality Trait Loci (QTL) and Marker Assisted Selection (MAS) for aluminum tolerance, pod shattering, and other problems. Thus emphasis of the integration of mutation techniques and marker assisted selection for soybean improvement was the major concept in the presentation. The report included lists of 28 SSR markers and their linkage to the period to maturity and pod shattering character, and a figure to show the map of the markers.

Dr. Sumin Smutkupt, who developed the mutant variety 'Doi Kham', now retired from Kasetsart University, attended this workshop as an observer.

2.2.2 China

Rice quality is one of the primary rice-breeding objectives and is receiving more and more attentions lately. Rice starch mutants were developed by using different levels of markers or indices, i.e. endosperm phenotypic marker, the microsatellite marker of *Wx* gene, and RVA index. These markers or index reflect exterior, molecular and physical/chemical properties of rice starch, respectively. The phenotypic marker is a distinct endosperm described as misty, dull or snowy trait, which is specific to breeding materials with the amylose content below 15%. RVA index is the major characteristics of starch viscosity that indicates changes in rice texture during cooking determined by Rapid Visco Analyser (RVA). Using the Wxup2/485 primer, the microsatellite marker of *Wx* gene that encodes the granule-bound starch synthesis

(GBSS) was assayed to differentiate rice depending on the polymorphic (CT)n microsatellite repeats. Several endosperm mutants, RVA mutants, and GT mutants were successfully identified, and a combined assisted selection method was established.

2.2.3 Indonesia

Mutation breeding has become a proven method of improving crop varieties. Most research on plant mutation breeding in Indonesia is carried out at the Center for Research and Development of Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN). Nowadays, a biotechnological approach has been incorporated in some mutation breeding researches in order to improve crop cultivars. This approach is simply based on cellular totipotency, or the ability to regenerate whole, flowering plants from isolated organs, pieces of tissue, individual cells, and protoplasts. Tissue culture technique has been extensively used for micro propagation of disease-free plants. Other usage of this technique involves in various steps of the breeding process such as germplasm preservation, clonal propagation, and distant hybridization. Mutation breeding combined with tissue culture technique has made a significant contribution in inducing plant genetic variation, by improving selection technology, and by accelerating breeding time as for that by using anther or pollen culture.

In Indonesia, research on mutation breeding combined with tissue culture techniques has been practiced in different crop species including rice, ginger, banana, sorghum etc. In ginger, the best growth performance of explants was for those irradiated with Gamma rays with the dose of 9 Gy. Almost banana cultivars grown in Indonesia are susceptible to a plant disease caused by Fusarium oxysporum Cubense. Mutation breeding for this disease resistance has been conducted in combination with tissue culture techniques. Plantlets irradiated with gamma rays with the dose levels of 5-35 Gy were grown in MS basal media. Some healthy plants were selected at 6, 7 and 8 months after transplanting in the field.

Indonesia has so far been very much dependent on rice as a staple food. In order to guarantee food safety in the country, it is necessary to extend food diversification utilizing other food resources available. Sorghum plant is considered to be successful in attempt to make the food diversification program. Seeds of sorghum were irradiated with different dose levels of gamma rays. The embryo was then separated from the endosperm and used as an explant in a tissue culture technique. Plantlets resulted from subculturing were transferred to grown in greenhouse for producing seeds. Plant selections were simply based on visually phenotypic performance of important agronomic traits such as head size, head form, seed size, and yield.

Especially in rice, a research on identification of DNA markers linked to blast disease resistance is now still progressing. Based on the genotypic and phenotypic data, the DNA markers were detected linking to the resistance genes on Chromosome 6 and chromosome 7.

2.2.4 Korea

Cross plant breeding has played an important role in developing new varieties in agriculture. Recently the molecular techniques get accepted as an alternative tool in both developing the new cultivars and identification of a gene. Using the molecular technique, several genetically modified (GM) crops appeared in the global market since 1996. These new crops have also caused the controversial debate on the safety of GM crops as human consumption and environmental release as well.

Nevertheless, molecular techniques are widespread and popular in both investigating the basic science of plant biology and breeding new varieties.

The mutated AGPase was transformed into lettuce via gene transfer. Some of transgenic lettuces showed higher productivity. The modified Bt gene (cryIAc1) was transformed into Chinese cabbage and assayed its toxic action against diamond moth. According to the bioassay with transgenic rice, it acquired the strong resistance to leaf blight disease in rice.

Radioactive isotope (RI) is prerequisite for current molecular biology. It has been extensively used for the isolation of genes, for the visualization of gene expression of transgenic plant. Mutation breeding by irradiation has been applied to develop a new variety in agriculture, particularly in the area of disease resistance, quality improvement and floral mutation.

2.2.5 The Philippines

In the Philippines, current Project Leader Dr. Florencio Isagani S. Medina III will soon retire and Ms. A. G. Lapade of the same Philippine Nuclear Research Institute (PNRI) as Dr. Medina was nominated as the new Project Leader. In this Mutation Breeding Workshop held in Bangkok, following report was presented by Dr. Medina.

The report summarizes the status of biotechnology with emphasis on molecular techniques for plant breeding in the Philippines. Several molecular and *in-vitro* culture techniques are integrated in plant breeding work for crop improvement at Philippine Nuclear Research Institute (PNRI), University of Philippines at Los Banos (UPLB), Philippine Rice Research Institute (PhilRice) and International Rice Research Institute (IRRI). Among them, IRRI is one of the international agricultural institutions located all over the world to study agricultural system, to develop and disseminate promising crop lines on behalf of related countries. To do such important tasks, at IRRI, molecular methodologies like Polymerase Chain Reaction (PCR) techniques, Restriction Fragment Length Polymorphism (RFLP) and Randomly Amplified Polymorphic DNA (RAPD) are well developed and utilized foe example in search of Quantitative Trait Loci (QTLs).

By the development of such molecular methods, establishment of high density molecular maps, determination of breadth and diversity of germplasm and characterization of alien gene introgression were investigated. The molecular maps have identified DNA sequence of resistance genes of High Yield Varieties (HYVs) and

New Plant Types (NPTs) to abiotic and biotic stresses. The major achievement was the development of high density molecular map in rice with at least 2000 markers. These achievements were well communicated to the Philippines' domestic institutions.

PhilRice has the funds and the facilities to carry-out transgenic work, anther culture and molecular marker analyses. The biotechnology program at PhilRice for varietal improvement includes: (1) utilization of molecular marker technology for gene mapping of desired traits in rice, (2)analysis of genetic relationships of germplasm materials and breeding lines through DNA fingerprinting, (3)genetic diversity studies, (4) development and application of marker aided selection for disease resistance (Rice Tungro Disease(RTD) and Bacterial Leaf Bright (BLB)); (5) application of *in-vitro* techniques in the development of lines with tolerance to adverse conditions; (6) molecular cloning of important genes for RTD resistance; (7) genetic transformation for male sterility, resistance to sheath blight and stem borers; and (8) transfer of disease resistance from wild species to cultivated varieties.

In Institute of Plant Breeding of the University of the Philippines at Los Banos (IPB-UPLB), molecular markers, e.g. micro-satellite analysis, AFLP or other powerful molecular methods are being used for mapping and diversity studies in agriculturally important crop species other than rice, e.g. coconut, mango, banana, mungbean, corn, tomato *etc.*

Mutation breeding at PNRI using gamma irradiation has resulted in the development of crop varieties with desirable traits. The use of AFLP analyses coupled to PCR is being used to study polymorphism in plant variants of radiation-induced mutants of rice, pineapple and ornamentals. Good collaboration with PhilRice has been kept to develop practical rice cultivars by mutation technique. Other efforts include crops e.g. soybean, mungbean, sweet potato, pineapple and some foliage ornamentals.

The country report included listings of mutant cultivars in rice, soybean, mungbean, and four other crops, together with lists of fields available and actively pursued molecular work.

2.2.6 Malaysia

From Malaysia, research work on representative ornamental plants of this country, staff members reported i.e. orchid mutation breeding supported by molecular techniques from the Malaysian Institute for Nuclear Technology Research (MINT)

Orchid breeders have always been dependent on hybridization helped by *in vitro* culture technology to grow hybrid plant from very fine seeds. They very often made remote crosses, even expanding beyond species group, i.e. intra-genus and multi-genus crosses. The technology has proven very reliable and easy to use, and has produced wide range of successful cultivars with attractive combinations of spray length, bud number, flower colour and form, vase life, fragrance, seasonality, and compactness.

By the introduction of mutagenesis techniques, further wide variations of flower

colours, form and size can still be obtained in addition to overcoming the problem of sexual incompatibility and sterility. However, the life-cycle time necessary for the Mendelian segregation in M2 generation might be the barrier to develop extreme mutant phenotype. Up to now, reports on the mutagenesis of orchid plants are rather scarce. Recent development of combined mutagenesis with tissue culture techniques in other vegetatively propagated crops stimulated also in this crop group and gradually mutant clones are appearing.

In addition, complementary use of molecular techniques will allow breeders to target more specific characteristic changes and cut short the breeding time necessary to incorporate a new character. PCR-based analytical techniques for DNA revealed that some polymorphic fragments could be developed as molecular markers in the mutagenized clones. These results will be very useful and enhance orchid breeding efforts.

In their report, 11 mutant clones in *Dendrobium* 'Sonia' were reported together with RAPD analysis profiles. According to their report, flower colour genes have been cloned/extracted and recent gene transfer technologies have well developed, ready to apply. *Dendrobium* and *Oncidium* orchids will be the target plants in their research.

2.2.7 Vietnam

Biotechnology provides a powerful tool for crop improvement. It assists in raising the efficiency of rapidly creating new crop varieties with high yield, good tolerance and quality. Up to now, several molecular markers have been available for using to comprise RFLP and the ones based PCR such as: RAPDs, SSR, AFLP, RGA.

Analytical studies of plant genetic diversity have concentrated to assess the total genetic variation to aim at line identification, determination of pedigrees and variety protection. And recently, the molecular markers have been also effectively applied in mutation breeding. The studies have mainly looked for the differences at genome structure level between original variety and mutant lines.

So far, many genes have been identified and tagged with various kinds of molecular markers. Studies on gene tagging can provide the information on the number of genes controlling characters and the map location of those genes. Such information is very valuable in using genes in crop breeding. The mapped genes have been used to pyramid into crops via marker aided selection.

In Vietnam, many crops have been released and popularly grown by the farmers, they generated via mutation breeding. However, most of varieties were selected based on the morphological parameters which much depended on the environmental conditions, plant physiological status and stage of gene expression. Therefore, in order to resolve these limitations when using traditional methods, we have planned to apply the molecular techniques to characterize the mutant genotypes obtained in mutation breeding. In this

study, we carried out to analyze mutant genotypes of soybean, green pea and rice. DNA fingerprints of soybean and green pea using RAPD markers indicated the genetic distance to original variety among mutant lines. Linkage analysis using AFLP markers indicated that TGMS(thermosensitive genic male sterility)gene in rice located on chromosome 2.

2.2.8 Japan

The paper that was presented by Dr. Yano, national Institute of Agrobiological Sciences, described the new strategy and tools for the genetic analysis of agronomically important traits in plants. Many traits with economic importance are often controlled by many genes and they are also affected by several environmental factors. These traits are often referred to as quantitative trait or complex traits. Recent progress for the genome analysis of plants, including development of molecular markers, a large scale EST analysis and genome sequencing, has enabled us to analyze such complex traits of plants.

Rice genome analysis provided us a launching pad for new genetics in rice. More than 3000 DNA markers, EST database, genomic libraries, sequence data, can be used to analyze the complex traits in rice. Heading date was genetically and molecularly analyzed using these research resources. Quantitative trait locus (QTL) analysis revealed that 14 QTLs were found to be involved in determination of heading date in rice. 9 of those have been mapped as a single Mendelian factors by use of advanced backcross progenies. To characterize gene effect of detected QTLs, marker-assisted selection (MAS) was used to develop nearly isogenic lines (NILs). Five QTLs were found to confer photoperiod sensitivity. NILs can also be used for the analysis of epistatic interaction among QTLs. It was proved allele specific epistatic interaction among 5 photoperiod-sensitive QTLs. It was clearly demonstrated that the complex inheritance of quantitative traits is explained mainly by multigenic control, but epistatic interaction is also an important factor for such complexity.

This paper also demonstrated that gene at QTLs can be cloned by map-based strategy. Three genes, Hd1, Hd2 and Hd3a have been identified. Molecular cloning of QTLs and genetic analysis of epistatic interaction allowed us to make up genetic control model of heading date in rice.

Finally the presenter, Dr Yano, emphasized the importance of plant materials, chromosome segment substitution lines or nearly isogenic lines for the analysis of the complex traits. It would not possible to perform comprehensive genetic and molecular analysis of the complex traits without such plant materials. It will be important for the plant breeders and geneticist to develop not only primary mapping populations, such as recombinant inbred lines or doubled haploid lines, but also secondary mapping populations, such as chromosomal segmental substitution lines. Precise and reliable phenotype assays will also be important for that analysis of complex traits. Combining old techniques such as crossing and selection with new tools such as DNA markers and sequences will be high priority for the next decade of plant molecular genetics.

2.3 Outlines of Invited Reports

In the 2001 August Mutation Breeding Workshop held in Bangkok, three invited papers were presented by researchers in Thailand.

2.3.1 Invited Paper-1.

The first invited lecture was presented by Dr. Apichart Vanavichit of Kasetsart University (KU). The title as follows :

"Discovering Genes Underlying QTL"

The Kasetsart University has a separate campus in Kampansaen, some 100 km apart from the main campus in Bangkok. During this and in the previous occasion of the mutation breeding Workshop/Seminar, there was no excursion visit to the campus, but facilities in this separate campus is fully equipped for tissue cultures and molecular biology work. The presenter was in charge of various molecular biology studies. In the presentation, he reported present situation of studies of Quantitative Trait Loci (QTL). Previously, the concept of quantitative traits was discussed as polygenic effects, i.e. the character like crop yield is controlled by several or at least plural genes. By the development of fine structure mapping, or molecular level dissection of chromosome by the use of molecular markers, locating of gene or genes, which control the quantitative character, became reasonably possible. Basically, search of QTL needs statistical work but molecular dissection will prove the estimation by gene transfer techniques. Presently the work is still preparatory level, but detailed investigation typical laboratory model plant, *Arabidopsis thaliana* and other plant species have made the analysis in rice reasonably possible. The presentation was to review the field and explained their strategies, utilizing current reports and internet databases *etc.*

The rice agronomical characters to be studied included flood tolerance, applying experiments of flash flooding or slow flooding. For such characters, including floating rice, which is very often grow to several meters long to reach surface of flooded water, relation to "G-protein" was strongly suggested. Also physiological signal through gibberellins and/or ethylene reaction system was suggested.

Sometimes, many abbreviations in the presentation, like Yeast Artificial Chromosome (YAC), Bacteria Artificial Chromosome (BAC) or Sequence Tag Connectors (STC) are annoying to follow the talk, but the reviews and their own reports presented indicated that they are really working in one of the frontiers in the molecular biology studies.

2.3.2 Invited Paper -2.

The second invited lecture was presented by Dr. Suchirat Sakuanurungsirikul of

the Department of Agriculture (DOA). The title as follows :

"Molecular Technique for Detection of Genetic variation in Horticultural Crops"

The report was a continuation from her presentation in the Mutation Breeding Seminar held in Bangkok in 1996. The topic was cultivar identification methodology on tropical fruit trees, the model being *Salacca* sp., a kind of palms to eat its seed, and *Lansium domesticum* (Engl.:Lansat). The *Salacca* plants had been introduced from Indonesia and are monoaecious, male plants being 80 % and agriculturally productive female plants only 20 % or so, when seeds were sown. Although the plant species has such difficulty as fewer females in the progenies, in the agricultural practices, still seven cultivars, or accessions, have been observed. To develop the identification method and to study relationship among them, isoenzyme analyses and DNA Amplification Finger-printing (DFP) were applied. In isoenzyme analyses, a list of 13 loci of isozyme related genes was shown, ten loci showing polymorphism. All of the 13 loci were effective in discrimination of cultivars, but only three of them were effective for further intra-typing work.

The second case studied was another favoured tropical fruit tree, *Lansium domesticum* (Engl.:Lansat), which is said to be parthenocarpy. The pollen being sterile due to this strange reproductive nature, the plant still shows some small differentiation and three major groups have been reported. In the report, further 18 local lines were listed under the three major groups. DNA Amplification Finger-printing was used to analyze these local lines. Eight DNA primer sequences, each constituted with ten or 15 bases listed in the report were used for the analyses. This DNA analysis was effective to examine small differences in the local lines, which expressed small differences in phenotypes of the plant or of fruit. Further investigation will reveal the relationship of the each local line.

2.3.3 Invited Paper -3.

In the host institution, Kasetsart University, a gamma irradiation room became available to study the effect of, and apply chronic irradiation of gamma radiation, utilizing tissue culture methodology. The topic of the third Invited Lecture was about gamma irradiation on *Chrysanthemum* sp. Presentation was made by Dr. Siranut Lamseejan. The title was as follows :

"Mutation Induction in Chrysanthemum through *In Vitro* Acute and Chronic Irradiations with Gamma Rays"

In vegetatively propagated plants, induced variability may strongly depend on the original genotype, the more heterozygous the higher possibility to yield many mutant phenotype. In the present case, this condition seemed to meet with the requirement,

producing various colour and morphological variation. Large gamma irradiation room was helpful to have many dosage classes in search of appropriate dose-rate applied.

In Vitro culture of chrysanthemum variety 'Reagan Dark Splendid' was established using explants from axillary buds and ray florets. Shoots produced *in vitro* culture from axillary buds were irradiated with acute gamma rays of 30 Gy. While multiple shoots produced from *in vitro* culture of ray florets were irradiated with chronic gamma rays of 112 and 140 Gy. The irradiated shoots were then multiplied two times by single-node cuttings from M_1V_1 to M_1V_3. Controls and irradiated shoots were rooted, transferred to soil in the greenhouse, and finally transplanted in the field. Observation and measurement were made at flowering time for height, number of nodes per plant and flower colour mutation frequency. Flower colour mutation frequency among the irradiated plants with 30, 112 and 140 Gy was 8.2, 9.3 and 15.3% respectively. Eleven variants were selected among the M_1V_3 plants from 30 Gy dose treatment. Selected variants had flower colour varying from red, red-purple and yellow-orange. Nine variants were selected among the M_1V_3 plants from 112 Gy dose treatment. Selected variants had flower colour varying from red, red-purple, purple and yellow-orange. Sixteen variants were selected from 140 Gy dose treatment. They had flower colour varying from red-purple, purple, orange-red, yellow-orange and yellow. These variants were multiplied through *in vitro* culture as well as by conventional cutting. They will be evaluated for their performance, stability and uniformity in the field, and for market value during the next planting season.

Various color and/or flower shape changes were shown in four figures printed in colour. Colour hard copies, now available in PC-Word-Processor system, attached to the report, was very effective to demonstrate the induced variation in chrysanthemum.

2.4 Summary of Round Table Discussion

2.4.1 Round Table Discussion-1

(1) The Database of Mutation Breeding

1) Publication of Database

Although publication of Outline of Mutation Breeding Database was decided to prepare in homepage of AsiaNNet (www.fnca.jp), the reason why we should publish the database in internet were many users more than 500 researchers in all member countries could access the data source easily and instantly, and almost no expense would be required to distribute the data. In this seminar, the propriety about publicity of Mutation Breeding Database to the homepage of AsiaNNet was discussed that special consideration should be paid on publicity of private data, intellectual property right and security of the Data.

2) Security systems of Database

Appropriate security system against hackers is the most important to maintain the Database on internet. There are three systems to be adopted on the case according to the required security level.

a. Closed system: Conventional system to provide the data by means of diskette or CD-ROM to the users. No problem is arisen on security of the data. Software to operate the data is requires in each user.
b. Semi-closed system: The user of database should be registered and given ID number to access the database. Some measures are required to maintain the security. No software is needed in each user.
c. Open system: The database is opened to the public. It is usually difficult to keep the security in the case. No software is also required in user's side.

It was discussed which security system we should choose to maintain the security against hackers. In conclusion, semi-closed system was supported by the delegates of member countries.

3) New version of the Database

The Database can be operated by Access Ver. 2 based on MS-DOS not on Windows, therefore version up was requested to avoid inconvenience. A CD-ROM of version up to Access 2000 in current data is provided to each delegate of the member county.

4) Revision of the data

As the current data had been compiled in 1999, the data is proposed to revise. Recently, National Institutes in Japan have shifted to agencies, and have been reorganized. The proposal of revision was supported by all member countries, and was requested to submit revised data to the secretary in JAERI until the end of the year 2001.

5) Further use of the Database

It was proposed that the list of published reports relating to mutation breeding would be collected on the Database, Keywords and abstract of the reports should be added step by step. Such data would be utilized not only scientists but students.

The data on available mutants and their passport data in Mutant Stock Repository could be compiled in the Database for the users' convenience. More discussion should be required at next seminar concerning with further use of M.B. Database.

(2) Mutant Stock Repository (MSR)

The idea of "mutant gene bank" has been proposed in the 1995 Mutation Breeding Seminar held in Quezon City, the Philippines and positively supported by participants of Mutation Breeding Seminars /Workshops since then. To avoid confusion with general gene bank activities, the system was named as Mutant Stock Repository (MSR). It will be specialized to accommodate induced mutant lines as analytical standard, the reference paper being an important part for each stock. This basic concept will be useful to compare newly induced mutant characters with standard mutant lines. Possible repository sites for rice seeds have been suggested in Philippines (PhilRice : through PNRI) and in Thailand (Rice Research Center at Pathumthani : through Kasetsart Univ.). The passport data format has been discussed at the January, 2000, Jakarta Seminar and agreed as Form 99A.

The difficulty regarding the "Intellectual Right" or "Genetic Resources" may be managed by voluntary submission of materials where possible, and putting necessary restriction or conditions in the requesting form, Form 00A, which was discussed and agreed at the Hanoi Seminar in October, 2000. The condition indicated was that the stock received, or product derived from the mutant stock received, must not be patented by recipient(s). Understanding and collaboration of the member countries and researchers are requested for successful establishment and operation.

Basically, mutation work is a reproducible technique in terms of gene locus affected, even if detailed site of alteration in the DNA (gene) base sequence, or type of alteration, may be different. Comparison and detailed analyses with standard genetic stocks becomes necessary for detailed genetic studies and for strategic planning in mutagenesis. The way of utilizing the Mutant Stock Repository system must be a purely academic/scientific collaboration among researchers of the member countries. Voluntary submission of well defined mutant lines as genetic analytical standard should be encouraged where submission is regally possible. The Repository and expected recipient user, geneticist/researcher, must respect plant quarantine and other legal procedures necessary to obtain the living materials.

The Mutant Stock Repository must be a long term project to support

crop improvement. Therefore, the system must be operated not on the short term specific project but on a long term voluntary base. Information on stored lines and some characteristics should be disseminated to related researchers, favorably as a news-letter type periodicals. To make it possible, active participation by the researchers is strongly requested.

In the 2001 mutation Breeding Workshop held in Bangkok, the first sample group, six lines of japonica rice waxy mutant lines and its parental line, has been submitted as a test run and handed out to representatives from Thailand and to the Philippines. Small modification on submission procedure of seed materials seemed to be necessary, i.e., instead of some hundred grams of samples in each line, several healthy seeds to pass the plant quarantine examination and to be proliferated at the repository. To respect the plant quarantine regulation of the host countries, this is a necessity. In turn, submission of fewer seeds will encourage the submission from the donor researcher. Fortunately, the present host institutions showed good understanding and collaboration of the MSR activity.

Thus, rice mutant seed seems to be successfully accepted in the MSR. However, for other kinds of seeds, e.g., soybean and other legumes, other repository sites are needed, because the present MSRs are both specialized to rice. Presently, Institute of Plant Breeding, Univ. of the Philippines at Los Banos, in the Philippines, has been suggested as the one possible site. Another possibility suggested previously was the gene bank in the Rural Development Administration (RDA) in Republic of Korea. These possibilities should be examined with gratitude for their collaboration. More than two sites for each group of samples will be necessary for the security of this long term activity.

For the time being, mutant clone of vegetatively propagated plant species are put out of the MSR system, as it needs too much care to maintain. These should be listed on the Mutation Breeding Database in some way, if their mutant clone is available to spare.

Appendix 1: Form 99A
Appendix 2: Form 00A
Appendix 3: Test-run-data

Appendix 1

Form 99A

Passport Data Format for Mutant Stock Repository

Donor : Please fill in BOLD Places.

Name or code of mutant gene/stock :_____

1) Accession No. in Repository : _____
2) Registration Date in Repository : _____
3) Registration Status in Repository : _____
4) Status of sample (Status) : _____
5) Amount in stock (Original :_____<y>) _____ g mg
(Propagation :_____<y>) _____ g mg
(Propagation :_____<y>) _____ g mg
(Propagation :_____<y>) _____ g mg
6) Country Code of Donor :_____
7) Name and address of donor Institution :_____
_____
8) Name of researcher(s) to contact :_____
9) Academic Name :_____
10) Common (English) Name :_____
11) Local Name / other name :_____
12) Original cultivar/line used :_____
13) Original character already exist :_____
14) Code in the Donor Lab. :_____
15) Mutagen 1 used :_____
_____
_____
16) Mutagen 2 (or other) used :_____
_____
_____
17) Treated tissues, condition *etc.* :_____
_____
_____
18) Major mutant character induced :_____
_____
19) Mutant line or registered cultivar? :_____
20) Reference (Mutagenesis) :_____
_____
_____
21) Reference (Genetic) :_____
_____
_____
22) Reference (Molecular) :_____
_____
23) Restriction for distribution* :_____
_____
24) Remarks :_____
_____

_____

**: The result of, or product of, improvement, using the mutant stock received, must not be patented by a second country.*

Appendix 2

Form 00A

Request Form to Obtain Mutant Stock

To request : Fill in BOLD Places cleary.
(Mutant line seeds may be supplied only for the purpose of analytical comparison with standard mutant.)

Name and mailing address of requesting person and/or Institution :

_____
_____
_____
_____
_____
_____
_____

Name of mutant gene/stock requested :_____
1) Accession No. in Repository :_____
2) Amount requested :_____<unit(s)>)
(One unit : about 20 seeds)
3) Name of researcher(s) to contact, if different from the addressee
:_____
4) Purpose of the use :_____
_____
_____
_____
_____

Condition of receiving mutant line seeds :
The purpose of the Mutant Stock Repository is to serve for genetic analyses of mutant gene(s) to compare with the standard tester gene(s). The supplied mutant gene(s), result, or product which is developed from the mutant gene received, must not be patented by the recipient or by other related party.

I hereby agree with the conditions and limitations set above as a requirement to obtain mutant stock from the Repository

Date : _____ ***Signature :*** _____

Name (print) : _____

M.S.Repository use

Received	Checked	Prepared	Sent out

Remark

Test-run-data

Seed stocks planted and harvested in 2000 season in Awara, Fukui, JAPAN to submit to the Mutant Stock Repository in East Asia.

Table of Mutant Stocks:

No.	Line Name	Original line	Character	Mutagen	Harvest kg	Sent kg	Left kg
MSR-1*	Norin 8	Original line	Non-waxy	(Original)	1.8	1	0.8
MSR-2*	73wx1N1	waxy mutant	Complete waxy	EMS	1.55	1	0.55
MSR-3*	74wx8N1	waxy mutant	Interm.waxy	EMS	1.6	1	0.6
MSR-4*	75KURwx4N1	waxy mutant	Deletion waxy	Th.neutron	1.9	1	0.9
MSR-5*	75KURwx5N1	waxy mutant	Interm.waxy	Th.neutron	1.87	1	0.87
MSR-6	Cal-mochi	USA waxy	variety waxy	gammarays	0.96	0.86	0.1
MSR-7	Mangetsumochi	JPN waxy variety	Complete waxy	Tradit.breeding	1.35	1	0.35

*: Major line for the MSR. MSR-6 and MSR-7 are for reference.
In term : Intermediate phenotype, between complete waxy and complete non-waxy.
The neutron : Thermal neutrons from an atomic research reactor (KUR) was used as radiation source.

In the proliferation field, each line was planted in four rows by a transplanting machine, a single plant for a hill. At harvesting, the terminal sections and outer rows were omitted, harvesting only inside of the zone. Panicles were dried in a glass-house under natural condition.

(3) Mutation Breeding Laboratory Manual (MBLM)

In the 1998 Mutation Breeding Seminar held in Kuala Lunpur, Malaysia, need of a laboratory manual was strongly suggested at the round table discussion. It should be a practical laboratory bench-side manual, rather than a textbook for lecture. There had been a good textbook published by IAEA in mid 1970s, but it was too old to meet the recent development of genetics research. In 1999, a good text book on mutation breeding was published by Dr. Ton van Halten, but the MBLM suggested in the Seminar was much more practical one, to be placed on the laboratory bench, rather than Library book shelf. The idea has been supported by the participating members since then. The manual should be in English, the common language of this Seminar/Workshop group, Dr. Florencio Isagani S. Medina III of the Philippines was nominated as the main editor. In the following periods, he appealed to the members to submit materials to him. The major idea has been presented in the following MB Seminar and Workshop. In the 2001 MB Workshop held in Bangkok, Thailand, a general outline was presented and the way of publication or dissemination was discussed at the round table discussion.

As for the dissemination method, to utilize the ample capacity of the "AsiaNNet", the home page of JAIF, is suggested. Hard copies reproduced by the Project Leader of the member country may be distributed to related laboratory to be used at the laboratory bench.

The materials for the Manual submitted as of 21 August, 2001, were from Japan, Philippines, Thailand and Malaysia. Further submission of materials was asked to the participating members by Dr. Medina. Each presented papers in the previous MB Seminar-/Workshop may be also incorporated, if copy right is not restricting to do so.

The Editor, Dr. Florencio I. S. Medina III is going to retire, but the Institute kindly extended the work period for him, so that he can concentrate for and complete the Manual editing.

Appendix 4 : Planned content list

Appendix 4

Planned content list (Dr. F. I. Medina Ⅲ)

Mutation Breeding Manual

1. Introduction
   a. Brief Introduction
   b. Description of the Commodity
   c. Rationale (purpose)
2. Objectives
   a. General
   b. Specific
3. Materials and Methods
   a. How to Prepare the Materials
      1) Seed, Pollen Grains
      2) Vegetative Parts
      3) *In vitro* Materials
   b. Moisture Content
   c. Suggested Irradiation Modalities
      1) Physical Mutagens (X-rays, gamma-rays, ion beams)
      2) Chemical Mutagens
      3) Dose, Dose Rate (acute, chronic, recurrent)
   d. Cultural Approach
      1) *In vivo* Culture
      2) *In vitro* Culture
   e. Mutation Breeding Scheme
      1) M_0 Generation
      2) M_1 Generation (sterility, chimera *etc.*)
      3) M_2 Generation (Laboratory or field screening)
      4) M_3, M_4 generations (mutant evaluation, purification)
      5) Selection
      6) Certification as a Mutant Variety/Registration

2.4.2 Round Table Discussion-2

(1) Proposal Phase Ⅲ-Project

The themes for Phase Ⅲ followed by Phase Ⅰ and Ⅱ, were discussed and proposed to promote further mutation breeding in Asian countries. Also, multi-lateral project themes were proposed. Cooperation activities will be conducted according to high priority crops in each country.

1) Basic themes for Phase Ⅲ: "Techniques on Reproductive Pattern"
Mutation breeding techniques of the following plant species should be discussed because their specific reproductive pattern is often inconvenient to improve varieties.

a. Vegetatively propagated crops (The crop is propagated by cutting and/or grafting) Cut flowers, fruit trees and so on.
b. Clonally propagated crops (Parthenogenic seeds with the same genotype as original are produced) Citrus, mangosteen, ranthium, mango, etc.
c. Root crops (Roots are used for propagation and products)
Sweet potato, potato, cassava, ginger, yam, taro, etc.

There still remain botanically important factors for future discussion.

2) Self-incompatible crops (Radish, cabbage, vegetables of *Brassica* sp., buckwheat)
3) Self-compatible / out-crossing crops (melon, cucumber, maize, etc.)
4) Polyploidy crops and species difficult to deal with (Wheat, palms, orchids, etc.)

(2) Multi-lateral project themes
The following themes will be initiated year by year according to priority of member countries, and the research results achieved for several years will be reported at the Seminar to share the knowledge with all.

1) Drought tolerance

Crop	Proposed country
Sorghum	Indonesia
Sorghum	The Philippines
Sugarcane	Vietnam
Upland rice	Not specified

2) Disease resistance

Crop	Disease	Proposed country
Banana	Bunch top virus	The Philippines
Banana	*Fusarium* root rot	Malaysia

2.4.3 Round Table Discussion-3

How to Promote Further the Regional Cooperation in the Field of Agriculture

At the Round Table Discussion in Session 5, three main subjects were discussed to propose as PhaseⅢ. At first, Techniques on Reproductive Patten in Mutation Breeding was proposed as the main theme of Workshop in PhaseⅢ. Annual themes should be as follows:

(1) Vegetatively propagated crops (cutting, grafting)
(2) Clonally propagated crops (parthenogenic seeds)
(3) Root crops (root propagation)

Botanically important factors, such as self-incompatible crops, self-incompatible but out crossing crops and ploypoid crops, were picked up but left for future discussion.

At the second, multilateral projects were proposed as a new regional activity from Japan and discussed on the themes. Following themes will be initiated year by year having a few years to reportable results. 1) Drought resistance (Sorghum and sugar cane), Disease resistance (Bunchy Top Virus and Fusarium disease of banana).

At the third, concerning on Mutation Breeding Database, current data was distributed by CD-ROM and database will be put on the Homepage of JAIF, but security level of accession to the database is still required to be discussed.

On Mutant Stock Repository, IN and OUT Format of stock seeds have been agreed and rice mutant lines were subjected to a test run. Repository sites for rice mutant lines are Rice Research Institute in Thailand and Phil Rice in the Philippines. Deposited seeds should be proliferated at the repository.

Level of Plant Quarantine clealance may be different in each country, therefore, each member country was asked to make clear all Plant Quarantine procedures. Repository site of seeds of mutant stock line other than rice and handing method is still to be discussed further.

Concerning on Mutation Breeding Manual, general outline was explained by the Philippines. The publication may be on the Homepage of JAIF.

On the venues for Mutation Breeding Workshop, China kindly accepted to be the host in 2002 and the Philippines offered the host tentatively in 2003.

2.5 Technical visit

(1) Gamma Irradiation Service and Nuclear Technology Research Center

Construction of this center began in 1996, and ^{60}Co gamma ray source were installed in the irradiation building in 1997. The operation was tested by experts from Britain in April 1998. Gamma ray sources were constructed by three kinds of ^{60}Co intensity, one source of 400Ci and two sources of ^{200}Ci. Compact irradiation instrument was equipped with ^{137}Cs source.

Main operations are 1. Supports instruction and research in applications of radiation and nuclear technology of Kasetsart University for undergraduate and graduate students, 2. Serves as gamma irradiation service center for researchers in plant breeding at low cost, 3. Serves as a center for training researchers from Thailand and other nations on induced mutation techniques and 4. Organizes conferences and seminars to advance research in mutation techniques.

(2) Office of Biotechnology and Nuclear Technique Research and Development, Department of Agriculture

Organization is constructed from three division, gene bank, agricultural biotechnology and nuclear techniques. In gene bank main objectives are 1. Plant and microorganism collections, 2. Plant and microorganism evaluations and 3.Culture and information services. In divisions of agricultural biotechnology and nuclear techniques, The objectives are 1.improve plant productivity and quality, 2. reduce detrimental environmental impact 3. increase value of agricultural products and 4. certification services for plant diseases and non-GMO.

(3) Pathum Thani Rice Research Center, Department of Agriculture

This center is one of seven regional Rice Research Center, located on 52km north of Bangkok. Main mission is to research on all aspects of rice production in central region of Thailand. There are 72 permanent and 271 temporary workers. There are 6 ph.D., 22 M.Sc., and 13 Diploma researchers. This center is organized by five research sections, plant science, agronomy, plant protection, seed technology and post harvest.

The National Rice Seed Storage Laboratory for Genetic Resources Center is established side by side aided by Japan. At present about thirty thousand accessions are maitained in the short, medium and long term seed storage. It is expected that approximately 500 accessions will be added to seed storage each year.

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3. Country Reports

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3.1 Induced Mutations and Marker Assisted Breeding in Soybean

Somsong Chotechuen [1], Somsak Srisombun [2] and Siranut Lamseejan [3]

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Abstract

Soybean is one of the important crops in Thailand. Constraints to soybean production include low yield potential, susceptibility to diseases and insects, and non-adoption of appropriate management practices. Mutation induction has been used to improve soybean yield and resistance to major diseases such as rust, purple seed, crinkle leaf, anthracnose and green seed. This paper reviews previous work and achievements of induced mutations in soybean. Successful examples are the release of a soybean variety, Doi Kham, and the development of a mutant CM 60-10kr-71; both are resistant to rust disease. The paper also gives example of the use of soybean SSR markers to identify QTL associated with pod shattering, and emphasizes the integration of mutation techniques and marker assisted selection for soybean improvement.

1. Introduction

Soybean has become economically important crop in Thailand since 1970. About 70 % of annual soybean production is used for vegetable oil extraction; 20 % is used as source of protein for various food products; 10 % is used as seed for next planting. During crop year 1998-1999, the total planted area was 234,720 ha with the production of 321,000 t and the average yield of 1.37 t/ha. Approximately 75-80 % of soybean planting area is in the northern provinces mainly in Chiang Mai (dry season) and Sukhothai (rainy season). Major constriants to soybean production include low yield potential, susceptibility to diseases and insects, and non-adoption of appropriate management practices.

In order to increase soybean production, it is necessary to increase yield and reduce production cost. Development of a variety with high yield and resistance to major pests is regarded as the most appropriate technology for adoption of farmers. Soybean breeding program has been established in Thailand since 1951. The release of four high yielding cultivars (SJ. 4, SJ. 5, Sukhothai 1 and CM 60) during 1976-1987 contributed to the increase in national average yield at the rate of 22 kg/ha/year from 1982 to 1991.

Mutation techniques are recognized as one of the effective means to create genetic variability required for crop improvement. In Thailand, mutation induction has been used as supplementary method of conventional breeding since 1955. Successful examples are the release of three rice varieties, one soybean variety, three ornamental plant variety, one banana variety, and one mungbean variety. Previous work and achievements of induced mutations in soybean will be briefly reviewed below.

In recent years, molecular marker technology emerges as a promising approach for plant improvement and has a great impact on the development of plant breeding strategies, including mutation induction method. This paper will give an example of the use of

molecular markers in soybean improvement. Integration of mutation techniques and marker-assisted selection is also emphasized.

2. Soybean improvement via induced mutations

Induced mutations have been used for soybean improvement in Thailand since 1971 (Na Lampang, 1993). The main objectives are to improve disease resistance and some agronomic traits.

2.1 Improvement for resistance to rust disease

Soybean rust is caused by *Phakopsora pachyrhizi* Syd., and is widely distributed in rainy season in the northern provinces such as Chiang Mai, Chiang Rai, Mae Hong Son, Tak, etc. It is also destructive on soybean growing in dry season in the central provinces under a continuous rainfall (high humidity) and low temperature condition. Symptom of soybean rust is the chlorotic to gray-brown or reddish brown spots appeared on the lower surface of leaves. The disease causes premature defoliation, early maturity, and lower seed weight. Yield loss of 60 % is reported in SJ. 5 cultivar when infection is severe (Nanthapunt, 1992).

Figure 1 shows an achievement of improving rust resistance in soybean by induced mutations. A new soybean variety, Doi Kham, was developed by irradiating 11 soybean lines/varieties with 150 and 300 Gy gamma rays in 1979. Selection was done in M_3 using the International Working Group on Soybean Rust (IWGSR) rating system. Yield trials over the years 1982-1985 showed that line no. 81-1-038, obtained from irradiating SJ. 4 with 150 Gy gamma rays, gave the highest yield under naturally infected condition. Later, the line 81-1-038 was released and named 'Doi Kham' in order to acknowledge the financial support of the Royal Project. Doi Kham is agronomically similar to SJ. 4, but it is different from SJ. 4 in reaction to rust disease. Doi Kham gave higher yield (about 15 %) and larger seed size (about 11 %) than SJ. 4 under rust infection, but the yields of both varieties were not different under normal condition (Wongpiyasatid *et al.*, 1987; Smutkupt *et al.*, 1988).

Recently, a mutant line CM 60-10kr-71 has been developed from irradiating CM 60 with 100 Gy gamma rays. Screening for rust resistance was carried out in Chiang Mai during 1989-1993 rainy season. In 1995 and 1996 trials, CM 60-10kr-71 had lower rate of rust development and a low percentage of infected leaves, compared with recommended varieties, SJ. 5 and CM 60 (Nanthapunt *et al.*, 1998). Yield loss of 0-17 % was observed in CM 60-10kr-71, compared with 37-43 % of SJ. 5 and 12-41 % of CM 60; the average yield of CM 60-10kr-71 was about 50-81 % higher than CM 60 under rust infection (Table 1). This line is under testing for rust resistance in the lower northern provinces, and is currently used as a parent in soybean breeding program.

2.2 Improvement for resistance to purple seed

Pink or light to dark purple seed coat colors of soybean are caused by *Cercospora kikuchii* (T. Matsu & Tomoyasu) Gardner. The disease does not affect yield but seed germination is reduced to 64 %, compared with 81 % of the normal seeds (Poonpholkul and Srisombun, 1987).

A susceptible variety, Sukhothai 1, was gamma irradiated at 150 and 300 Gy in 1982. Selection was undertaken during 1983 to 1988 early rainy season. Later, 10 lines from the 150 Gy and 1 line from the 300 Gy doses were selected (Srisombun *et al.*, 1988). The selected lines performed lower percentage of purple seeds (0.3-3.5), compared with 7.7 % of Sukhothai 1 (Table 2). However, these lines have not been released to farmers due to their susceptibility to bacterial pustule disease.

Year	Activity	Location
1979 rainy season	Seeds of 11 soybean lines/varieties were gamma (150 and 300 Gy) irradiated; the M_1 seeds were grown	Suwan Farm, Nakhon Ratchasima province
1980 dry season	Obtained seed increase of M_2	Mae Jo Field Crops Expt. Stn., Chiang Mai
1980 rainy season	Grew M_3 seeds and selected for rust resistance using IWGSR rating system; 121 plants selected	Mae Jo Field Crops Expt. Stn.; Doi Nong Hoi Expt. Stn., Chiang Mai
1981 dry season	Plant-to-row increase of M_4 lines; 90 lines selected	Experimental field, Kasetsart University
1981 rainy season	Screening for rust resistance; 16 lines selected	Doi Nong Hoi Expt. Stn., Chiang Mai
1982 dry season	Obtained seed increase of the 16 lines; 8 high yielding lines selected	Mae Jo Institute of Agricultural Technology, Chiang Mai
1982 rainy season 1983 dry season	Preliminary yield trials of selected lines; line no. 81-1-038 gave the highest yield under naturally infected condition	Mae Jo Institute of Agricultural Technology, Chiang Mai
1983 rainy season 1984 dry, rainy season 1985 dry, rainy season	Yield trials of the line 81-1-038 and recommended varieties; ---------- Variety release ---------- ↓ 81-1-038 or 'Doi Kham'	Mae Jo Institute of Agricultural Technology; Irrigation Research Stn. Amphoe Mae Tang, Chiang Mai; Suwan Farm, Nakhon Ratchasima

Fig. 1 Summary of the development of a soybean variety 'Doi Kham'

Table 1 Lesion type, rate of disease development, infected area on leaves, and yield of soybean line/varieties at Chiang Mai Field Crops Research Center in 1995 and 1996

Lines/ Varieties [1/]	Lesion Type [2/]	Rate of disease	Infected area (%)	Yield (t/ha) [3/] Spray [4/]		No spray		Yield reduce (%)
1995 rainy season								
CM 60-10kr-71	RB	0.10	1	2.58	Ab	2.80	a	0
SJ. 5	T	0.14	89	2.72	A	1.72	b	- 37
CM 60	T	0.25	89	1.76	B	1.55	b	- 12
CV (%)					21.5			
1996 rainy season								
CM 60-10kr-71	RB+T	0.12	6	2.99	A	2.49	a	- 17
SJ. 5	T	0.14	53	2.39	A	1.38	b	- 43
CM 60	T	0.23	67	2.82	A	1.66	ab	- 41
CV (%)					15.4			

Source : Nanthapunt *et al.* (1998)

[1/] SJ. 5 and CM 60 are recommeded varieties

[2/] RB = Reddish brown type, resistant to rust; T = Tan type, susceptible to rust

[3/] Means having a common letter are not significantly different at 5% level (DMRT)

[4/] Spraying of Triadimefon 25 WP (0.5 g/l of water) at 25 and 40 days after emergence

Table 2 Percentage of purple seeds of soybean lines derived from gamma radiation, and their rating scores for bacterial pustule and downy mildew diseases in 1986 early rainy season at Srisamrong Field Crop Experiment Station, Sukhothai

Lines/varieties	Purple seeds (%) [1/]	Bacterial pustule [2/]	Downy mildew [2/]
Irradiated with 150 Gy gamma rays (10 lines)	0.3-2.0	1.0-2.3	2.5
Irradiated with 300 Gy gamma rays (1 line)	3.5	0.3	2.0
SJ. 5	0.3	1.7	2.0
Sukhothai 1	7.7	0	2.0

Source : Srisombun *et al.* (1988)

1/ Number of purple seeds was taken at random from 500 seeds/plot;
SJ. 5 = resistant check; Sukhothai 1 = susceptible check

2/ Rating score was recorded at pod filling stage as follows:
0 = no symptom of disease
1 = 1-10 % of the total leaves infected
2 = 11-20 % of the total leaves infected
3 = 21-50 % of the toal leaves infected
4 = > 50 % of the total leaves infected
Sukhothai 1 was used as resistant check for bacterial pustule disease.
SJ. 5 and Sukhothai 1 are moderately susceptible to downy mildew.

2.3 Improvement for resistance to crinkle leaf disease

White flies (*Bemisia tabaci* Gennadius) is an insect vector of soybean crinkle leaf virus, a causal agent of the disease. Infected plants are stunted with shortened petioles and internodes. Leaves are generally misshapen and puckered, occasionally with dark green enations along the veins. Diseased pods are often stunted, flattened and curved. Yield may be reduced by 50 % in any one field (Nanthapunt, 1992). Crinkle leaf disease was widely distributed in Sukhothai province in 1984. Mutation induction was used to improve the resistance of some recommended varieties. Seeds of NS 1, SJ. 5, Sukhothai 1 and CM 60 were irradiated with 150 and 250 Gy gamma rays. Selection was carried out in Sukhothai province in 1987 and 1988 rainy season, but none of mutant lines were found to be resistant to the disease (Kornthong *et al.*, 1991).

2.4 Improvement for resistance to anthracnose disease

Anthracnose is a fungal disease caused by *Colletotrichum truncatum* Schw. It is distributed to soybean planting at all stages. The fungus can infect seeds, causing dark brown, sunken lesions on cotyledons of emerging seedlings. In advanced stages of anthracnose, infected tissues are covered with black fruiting bodies. Foliar symptoms that can develop after prolonged periods of high humidity include leaf rolling, necrosis of laminar veins,

petiole cankering, and premature defoliation. Anthracnose causes serious losses on maturing plants, particularly during rainy periods, when shaded lower branches and leaves are killed. Nanthapunt (1992) reported a reduction in seed quality, germination percentage, and a yield loss of 16-26 %.

Due to the lack of resistance source, seeds of soybean varieties/line SJ. 1, SJ. 2, SJ. 4, SJ. 5, Sukhothai 1, NS 1, CM 60 and 7608-25-4 were irradiated with 100, 150 and 200 Gy gamma rays in 1988. Artificial inoculation was applied to 122 M_4 lines in 1990 rainy season; 11 lines were selected (Nanthapunt *et al.*, 1993). Field test and artificial inoculation of the 11 M_5 lines were conducted in 1991 rainy season and only 1 line was selected. Symptoms did not appear on pods of the selected line, and only 6 % of the seeds were infected (Nanthapunt *et al.*, 1994).

2.5 Improvement for short plant type and early maturity

Doi Kham is a rust resistant soybean variety developed by mutation induction in Thailand. However, it possesses the undesired traits, lodging and late maturity. To improve these traits, seeds of Doi Kham were irradiated with 200 Gy gamma rays in 1987. Screening for short plant type and early maturity was conducted in M_3 and line no. 58608 was selected. It performed short plant type and early maturity, compared with Doi Kham; however, the average yield was about 6 % lower than Doi Kham (Wongpiyasatid *et al.*, 1994).

2.6 Improvement for low percentage of green seed

A recommended soybean variety, CM 60, has a high percentage of green seeds. This results in low seed quality and reduction in seed germination and vigor. To overcome this problem, seeds of CM 60 were irradiated with 250 Gy gamma rays. M_2 seeds were grown in 1994 dry season under a stress (less water and high temperature) condition, and 307 M_2 plants without green seeds were selected (Jan-orn *et al.*, 1994). Selection based on a low percentage of green seeds was carried out in later generations. The selected lines, however, performed high lodging and susceptibility to purple seed disease; therefore, further selection was terminated.

3. Identification of SSR markers associated with aluminum tolerance and pod shattering in soybean

Molecular markers have been used for genetic mapping of various traits in crop plants. In the recent past, several moderate density (average marker spacing of < 10 cM) and high density (average marker spacing of < 5 cM) genetic maps of soybean have become available (Shoemaker and Specht, 1995; Keim *et al.*, 1997). In 1992, Soybean SSR (simple sequence repeat) markers were introduced (Akkaya and Cregan, 1992). The soybean SSR markers are highly allelic (Cregan, 1992; Morgante *et al.*, 1994; Akkaya *et al.*, 1995). Each pair of SSR primers produces an unambiguous DNA products that maps to a fixed location in the soybean genome. Thus, the SSR information can be used across soybean populations making them useful for marker assisted selection (MAS) of various soybean traits (Staub *et al.*, 1996).

A study was carried out in order to use SSR markers to fine-map QTL corresponding to aluminum (Al) tolerance and to identify SSRs associated with pod shattering in soybean (Somsong Chotechuen, unpublished data). Soybean population used as genetic materials in this study consisted of 256 single seed descent recombinant inbred lines ($F_{6:7}$) from a cross of Benning x PI 416937. Benning is a recommended variety well-adapted to the southeastern USA production environment. It is highly productive and non-shattering, but are susceptible

petiole cankering, and premature defoliation. Anthracnose causes serious losses on maturing plants, particularly during rainy periods, when shaded lower branches and leaves are killed. Nanthapunt (1992) reported a reduction in seed quality, germination percentage, and a yield loss of 16-26 %.

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to high levels of soil Al. The plant introduction PI 416937, on the other hand, is Al tolerant but exhibits relatively high levels of pod shattering. Recently, molecular mapping identified six QTL that condition Al tolerance in the population derived from Young x PI 416937; however, one QTL was linked to the RFLP marker associated with shattering (Bianchi-Hall *et al.*, 2000). Previous studies indicated that pod shattering in soybean is highly heritable and is conditioned by one large and a few minor QTL (Bailey *et al.*, 1997).

Based on results of previous studies, five SSRs (Satt409, Satt114, Satt414, Sat_093 and Satt495) were selected due to their proximity to RFLP markers associated with Al tolerance, and four SSRs (Satt414, Satt132, Satt380 and Sat_093) were selected as associated with QTL that condition pod shattering. Additional SSR markers were chosen that mapped within 10 cM from such RFLP markers (Table 3). Markers were tested for polymorphism by amplifying parent DNA (Benning and PI 416937) on the Perkin Elmer 9700 PCR machine and the PCR products were run on gels. Markers that appeared to be polymorphic were used for testing of the lines.

DNA from the cross of Benning x PI 416937 was amplified on the Perkin Elmer 9700 PCR machine and 12 inch-polyacrylamide gels were used to run the PCR products on the 377 DNA Sequencer. All gels were analyzed using GeneScan® and Genotyper®, and the resulting scores confirmed manually. Genotypic and phenotypic data (maturity and pod shattering) were combined and analyzed using SAS. GMendel 3.0 software was used to construct a linkage map.

Only two phenotypic traits, maturity and shattering score, were available in this study since testing for Al tolerance had not been carried out. Based on gel results, 14 SSR markers were selected for analysis. Results showed that 3 markers (Satt547, Satt409, Satt554) were associated with maturity at $P<0.05$. Interestingly, 8 markers (Satt596, Satt414, Satt456, Satt380, Sat_093, Satt547, Satt114 and Satt510) were potentially associated with pod shattering at $P<0.01$. R^2 values, which are the proportion of the phenotypic variation among line means accounted for by a SSR marker in a single factor analysis, were relatively high for Satt596, Satt380, Sat_093, Satt547 and Satt510 (Table 4).

Using GMendel 3.0 software, linkage maps of SSR markers from linkage groups (LG) J, A2 and F were constructed as shown in Figure 2. Five marker loci associated with QTL that condition pod shattering mapped to USDA linkage group J. Two other significant markers (Satt114, Satt510) were found on LG F. The five marker loci in LG J accounted for 3.7 % (Satt456) to 16.5 % (Satt596) of the variation in pod shattering, whereas the two marker loci on LG F accounted for 4.3 % (Satt114) and 9.2 % (Satt510). Results were consistent with previous studies (Bailey *et al.*, 1997) in which major QTL that condition pod shattering were found on LG J, and the order of these markers matched the USDA linkage map.

Table 3 SSR markers selected for 256 $F_{6:7}$ lines from the cross of Benning x PI 416937

No.	Marker	Dye	LG	PIC (%)	Size		Polymorphism
					Benning	PI 416937	
1	Satt 380	y	J	58	140	130	Yes
2	Sat_093	b	J	80	204	201	Yes
3	Satt 132	b	J	58	252	242	Yes
4	Satt 456	b	J	54	292	280	Yes
5	Satt 409	y	A2	78	180	250	Yes
6	Satt 529	b	J	54	213	227/242	Yes
7	Satt 455	g	A2	46	256	259	Yes
8	Satt 414	y	J	72	313	310	Yes
9	Satt 426	y	B1	48	200	197	Yes
10	Satt 547	b	J	54	216	233	Yes
11	Satt 183	b	J	48	242	250	Yes
12	Satt 596	y	J	74	261	250	Yes
13	Satt 228	b	A2	46	248	214	Yes
14	Satt 362	y	F	66	268	258	Yes
15	Satt 406	y	J	72		155-175	
16	Satt 334	y	F	54	213	-	
17	Satt 431	b	J	70		230-250	
18	Sct_188	y	F	48		270-295	
19	Satt 510	y	F	-		90-135	
20	Sat_071	y	L	80		155-180	
21	Satt 072	b	F	54		195-205	
22	Satt 232	y	L	46		240-252	
23	Satt 114	g	F	74		95-120	
24	Sct_065	y	J	54		160-170	
25	Satt 182	y	L	80		195-200	
26	Satt 554	y	F	64		260-265	
27	Satt 495	y	L	48		230-245	
28	Satt 509	b	B1	78		175-240	

Note : The blank data indicates that no information was obtained until polymorphism test.

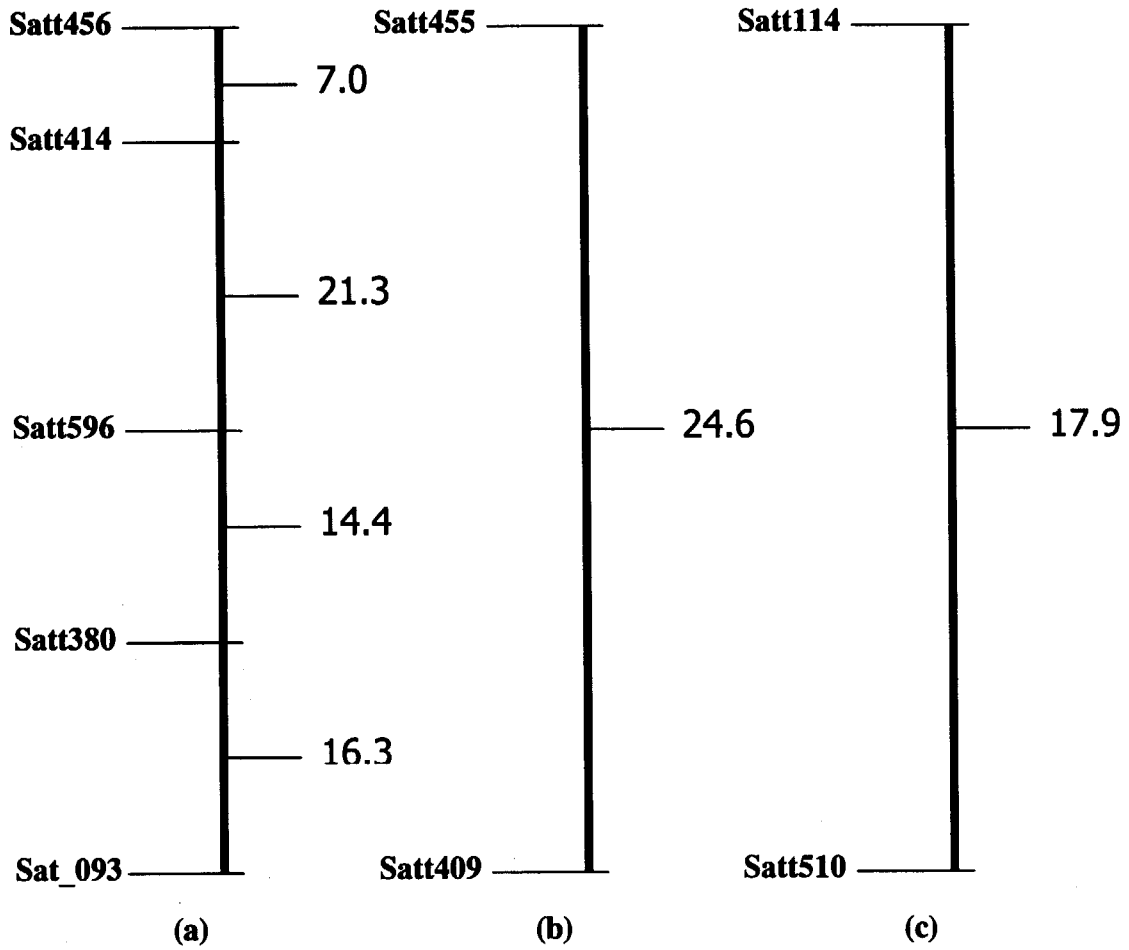

Fig. 2 Mapping of SSR markers on (a) linkage group J, (b) linkage group A2 and (c) linkage group F

Table 4 Single factor analysis of 14 SSR markers on maturity and shattering score of 256 F6:7 soybean lines from the cross Benning x PI 416937

Marker	Dye	LG	Size		No. of obs.	Maturity					Shattering *				
			Bennin g	PI 416937		P	R^2 (%)	Allele mean			*P*	R^2 (%)	Allele mean		
								B	B/PI	PI			B	B/PI	PI
Satt 456	B	J	292	280	221	.5148	0.61	57.34	57.23	57.75	.0169	3.67	0.92	1.46	1.19
Satt 414	Y	J	313	310	199	.7656	0.27	57.47	57.44	57.76	.0174	4.05	0.90	0.92	1.27
Satt 596	Y	J	261	250	188	.2018	1.72	57.19	57.33	57.92	.0001	16.50	0.70	1.50	1.38
Satt 380	Y	J	140	130	256	.8401	0.14	57.34	57.60	57.43	.0001	13.45	0.77	1.44	1.36
Sat_093	B	J	204	201	175	.1618	2.09	57.31	58.75	57.75	.0001	13.89	0.82	1.25	1.49
Satt 547	B	J	216	233	228	.0481	2.66	57.01	58.00	57.89	.0001	9.09	0.81	1.33	1.33
Satt 431	B	J	233	226	153	.2471	1.85	57.06	57.38	57.80	.1377	2.61	0.85	1.25	1.14
Satt 455	G	A2	256	259	164	.2981	1.49	57.28	58.00	57.89	.1575	2.27	0.89	0.96	1.17
Satt 409	Y	A2	180	250	178	.0321	3.85	58.00	57.07	56.82	.1454	2.18	0.94	1.14	1.24
Satt 114	G	F	109	106	220	.7878	0.22	57.60	57.68	57.35	.0083	4.32	0.91	1.23	1.28
Satt 510	Y	F	378	369	139	.6642	0.59	57.43	58.00	57.77	.0014	9.18	0.78	1.12	1.34
Satt 554	Y	F	258	264	172	.0278	4.15	57.36	56.33	58.18	.7010	0.42	0.98	1.17	1.07
Satt 495	Y	L	247	228	147	.1160	2.95	56.93	57.25	57.93	.8653	0.20	1.10	1.22	1.09
Satt 509	B	B1	191	235	174	.7985	0.26	57.66	57.50	57.84	.6088	0.58	0.96	1.05	1.11

* Shattering scale : 0 = 0 %, 1 = 10 %, 2 = 20 %, 3 = 30 %, 4 = 40 %, 5 = 50 %

4. Prospects of induced mutations vis-à-vis marker assisted breeding

Mutation induction has been recognized as an effective way to improve the selected cultivars of various crops, especially when the desired character is not available. Number of valued plant varieties developed from mutation breeding in many countries have proved that mutation induction is an important and useful means to crop improvement. Mutation techniques can be used as a supplementary method of conventional breeding, or in combination with biotechnoloy such as *in vitro* culture techniques for crop improvement and creating new germplasms. Successful application of induced mutations, however, is associated with the probability to obtain desirable mutant for particular trait, and the ability to select or distinguish between the mutant and its donor.

In recent years, technological advancements in molecular biology have created the opportunity for genetic analysis of several plant characteristics. Utilization of gene amplification by polymerase chain reaction (PCR) facilitates screening of polymorphism among a wide variety of crops. The potential for MAS to improve the efficiency of trait selection in crop plants is being widely studied (Mohan *et al.*, 1997). Molecular markers such as RFLP, RAPD, DAP, SSR were introduced to the genetic studies of soybean; they have been used to map the genomic location of both major genes and QTL for many agronomic, physiological, pest resistance, and seed composition traits in soybean. The discovery and mapping of more than 600 SSR markers on the public soybean maps make MAS in soybean more efficient and cost effective (Cregan *et al.*, 1999).

Marker assisted breeding can be used in combination with induced mutations to increase the efficiency of selection in a population. It is helpful to identify mutant plants that have inherited the desirable gene together with as few of the undesired genes as possible. Molecular markers can be very helpful for the understanding on genetic construction of induced mutations as well as for early selection of useful gene mutations. DNA-based markers can be visualized from seed or seedlings in rapid screening tests, performed by automated robotic systems in advanced application, and plants lacking the desired trait can be discarded before moving to more expensive or lengthy greenhouse and field trials.

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3.2 Screening Rice Starch Mutants via Endosperm Phenotypic Marker, Physical/Chemical Index, and Microsatellite Marker

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Summary

Rice quality is one of the primary rice-breeding objectives and is receiving more and more attentions lately. Current presentation, as partial achievement of the IAEA TC Project CPR/05/013 "Induced Mutation to Improve Rice Quality", covers the development of rice starch mutants by using different levels of markers or indices, i.e. endosperm phenotypic marker, the microsatellite marker of *Wx* gene, and RVA index. These markers or index reflect exterior, molecular and physical/chemical properties of rice starch, respectively. The phenotypic marker is a distinct endosperm described as misty, dull or snowy trait, which is specific to breeding materials with the amylose content below 15%. RVA index is the major characteristics of starch viscosity that indicates changes in rice texture during cooking determined by Rapid Visco Analyser (RVA). Using the Wxup2/485 primer, the microsatellite marker of *Wx* gene that encodes the granule-bound starch synthesis (GBSS) was assayed to differentiate rice depending on the polymorphic (CT)n microsatellite repeats. Several endosperm mutants, RVA mutants, and GT mutants were successfully identified, and a combined assisted selection method was established.

Keywords: *Oryza sativa*; Marker-assisted selection; Endosperm phenotypic marker; Microsatellite marker; Starch viscosity; Gelatinization temperature

Introduction

Rice endosperm is composed primarily of starch. Rice starch, as with other starches, consists of two polymeric forms of glucose, amylose and amylopectin. The amylose content is the most important characteristics influencing eating and cooking qualities and the major determinant used across the world to define rice market classes (Juliano and Villareal, 1993). Unlike other cereals, people of various regions prefer to eat certain types of rice (Kumar & Khush, 1987). This obviously means that precise levels of amylose have to be bred into new varieties to satisfy local preferences.

Control of amylose in rice has been extensively investigated in the numerous genetic studies. It was reported that the amylose content in nonwaxy varieties is regulated by a single dominant gene with major effect and several modifying genes with minor effect (Bollich and Webb, 1973; Deivedi and Nanda, 1979; Pooni et al., 1993). Kumar et al (1987) reported that the amylose content in nonwaxy varieties was determined by multiple allelic at the *wx* locus. The inheritance of amylose content is more complex in part due to epistasis, cytoplasmic effects, and to the triploid nature of endosperm (Okuno, 1978; Pooni et al., 1993). Environmental effects were known to cause amylose content to vary up to six percentage points for a given cultivar (Adoracion et al., 1977; Asaoka et al., 1985; Resurreccion et al., 1977). Due to these issues, it is difficult for rice breeders to make selection, depending on the

results of the single year and location (Khush and Juliano, 1985). Also, the heterozygotes could not be identified by using endosperm phenotype and chemistry determination of amylose content (Mckenzie and Rutger 1983; Pooni, 1993). Current report presents some progress in the development of rice starch-quality mutants by using different levels of markers or indices.

1. Screening the misty, dull mutants with endosperm phenotypic marker

Varietal differences in amylose content have been investigated in rice breeding programs for improving grain quality (Williams et al., 1958; Inatsu et al., 1974; Juliano, 1979). These reports indicted that a wide diversity in amylose content existed among nonwaxy rice varieties. Several mutants affecting the amylose content of starch in rice endosperm were produced by mutagenesis (Asaoko et al., 1993; Okuno et al., 1983; Omura, 1981; Yano et al., 1980 and 1985). Among them, several mutants with low amylose content were characterized by *wx, dull, sugary* and higher amylose content. Recently, Wu et al (2000) established a protocol to identify low-amylose-associated *misty* and *dull* endosperms. These amylose-associated endosperms help rice breeders to identify plant lines or mutants at early generation.

Depending on endosperm phenotypic marker, more than 20 mutants with apparently decreased amylose were identified from high amylose content early indica rice 'Jinzao47' with 300 Gy gamma irradiation. Several misty mutants (Fig.1) were also isolated from early maturing mutants, which were induced from indica rice 'Jingxian89' by space technology (Wu et al., 2000). In addition, several *wx* cultivars were officially released, such as 'Hangyu No. 1' (Xue et al., 1999), 'Yang-Fu-Nuo No. 1'(Shi, 1992).

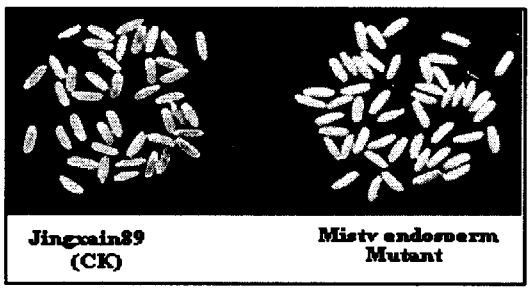


Fig.1. The endosperm phenotype of misty mutant

2. Isolating the starch viscosity and GT mutants

Starch viscosity indicates changes in rice texture during cooking and have been used as an indicator to evaluate rice grain quality (AACC, 1998). During the heating, higher temperature, and cooling process, rice flour has a pasting viscosity profile, which can be determined by a Rapid Visco Analyser (RVA) manufactured by Newport Scientific Inc (Warriewood, Australia). Although the peak viscosity value is variable and affected by many factors, but the breakdown and setback value were significantly related to gel consistency and texture in subspecies of rice, respectively (Shu et al., 1998; Wu et al., 2001). RVA analysis can distinguish the amylose levels of different cultivars and evaluate eating quality, based on the

setback value and breakdown value. Genetic studies revealed that peak viscosity, hot pasting viscosity, cool pasting viscosity, breakdown, setback, and consistence were mainly controlled by *Wx* gene (Bao et al., 1999 and 2000). Furthermore, it has been demonstrated that RVA analysis has the potential as a tool for rice breeding program (Wu et al., 2001).

In M_3 population of Xie-qing-zao B, three RVA mutants were identified with a remarkably increased breakdown and setback value (Fig.2). However, these mutants had the identical amylose content to the original parent, Xie-qig-zao B, a leading hybrid rice maintainer.

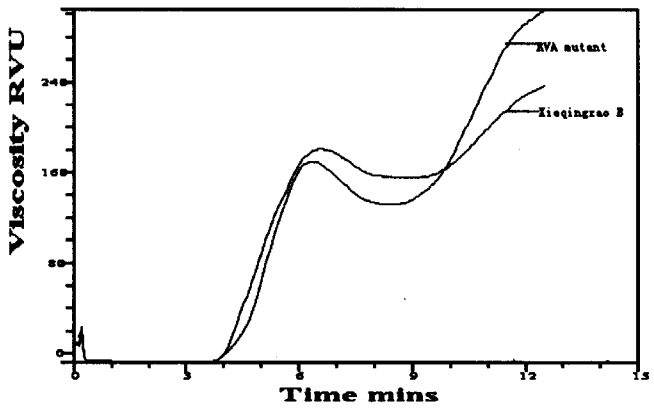
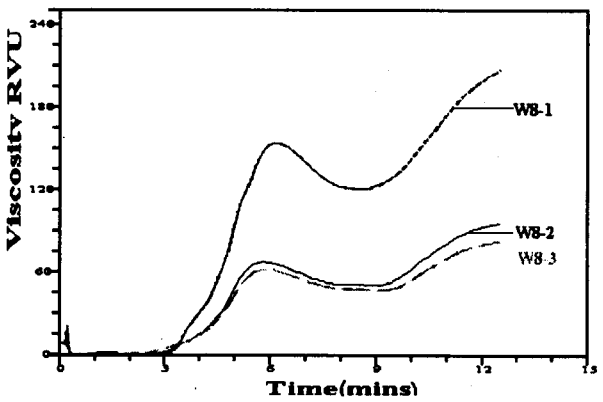


Fig.2. RVA mutants with a remarkably reduced setback value and increased breakdown value

Meanwhile, two mutants with a decreased viscosity and obviously increased digestibility were also isolated from an advanced population (Fig.3).

Fig.3. Two distinct mutants with decreased viscosity and obviously increased digestibility



Gelatinization temperature (GT) of starch is one of the most important factors influencing the cooking quality of rice (Juliano, 1979). It is the critical temperature when starch granules lose their birefringence and crystallinity in hot water and swell irreversibly. GT is often measured indirectly by an approximate test, the alkali spreading value (ASV), where milled rice grains are immersed in potassium hydroxide solution (Little et al., 1958). Samples with low GT requires less time to cook than those with high GT, so low GT is a preferred characteristic of edible rice, and is sometimes an essential trait for brewing industry.

In a previous study, a series of chlorophyll-deficient mutants induced from gamma

irradiation, of which one mutant was found to be a low GT mutant (Fig.4), named *Mgt-1* (ASV 6-7, compared to 2-3 for its parent variety II-32B (Chen et al., 2001). Further studies indicate that *Mgt-1* is the first GT mutant unrelated to amylose content, which implied that GT and AAC were not controlled by the same gene locus and could be useful to analyze the genetic basis of GT.

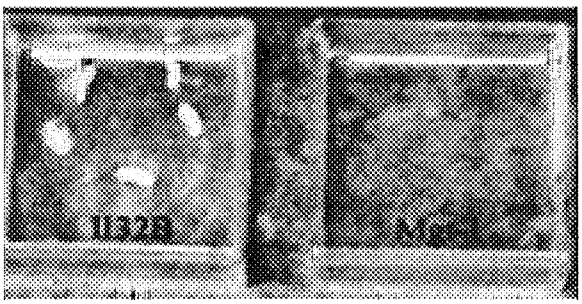


Fig.4. The alkali spreading value of the GT mutant *Mgt-1* induced from the CMS maintainer II32 B.

3. Microsatellite marker-assisted selection and prediction of *Wx* mutants

Using RFLP analysis, two alleles of the *Wx* locus have been identified (Sano et al., 1986). These alleles largely correspond to the indica and japonica rice subspecies, however, these are not adequate to explain all the observed variations in amylose content among commercial rice cultivars. Wang et al (1995) recently observed that the amylose and levels of *waxy* proteins were correlated with the cultivar's ability to excise the leader intron of the *Wx* transcript in 31 Chinese rice cultivars. High-amylose cultivars contained only the mature 2.3 kb *Wx* mRNA, while lower amylose cultivars contained varying ratios of both the mature 2.3 kb *Wx* mRNA and a 3.3 kb pre-mRNA. Bligh et al (1995) has reported a polymorphic microsatallite in the *waxy* gene, which encodes for granule bound starch synthase. This (CT) n (cytosine and thymine) repeat explained a large portion of the variations of amylose content in 89 U.S. nonwaxy cultivars (Ayres et al., 1997). Shu et al (1999) detected nine polymorphic microsatallites among subspecies of rice. Using the Wxup2/485 primer, assays for the microsatellite marker of *Wx* gene showed the potential of differentiating amylose classes and evaluating the genetic stability of new lines. Under indica/indica or indica/javanica genetic backgrounds, the *Wx* microsatellite marker was a co-dominant marker and exhibited Mendelian inheritance (Bao et al., 1998; Wu et al., 2001). These results indicated that this marker is suited for assisted-selection and shortening the development time of new cultivars.

Several *misty* endosperm mutants were characterized via microsatellite marker assay. It was found that there were two types of polymorphism between the *misty* mutants and the wild parent. The prediction of potential *Wx* mutations via microsatellite marker assay was explored when using DNA samples from M_0 seedlings, and some *misty* endosperm or *wx* mutations were subsequently confirmed in M_2 and M_3 grains (Fig. 5).

Recently, a combined assisted selection strategy was developed for superior grain quality breeding program, based on the integration of microsatellite marker with endosperm phenotypic marker and RVA index. Applied in the irradiated populations of Z95-210/Lemont, a low AAC early indica and American traditional long grain rice with superior grain quality, several elite lines that keep the Lemont starch character were bred and upgraded to attend the

regional trials in Zhejiang and Anhui Provinces.

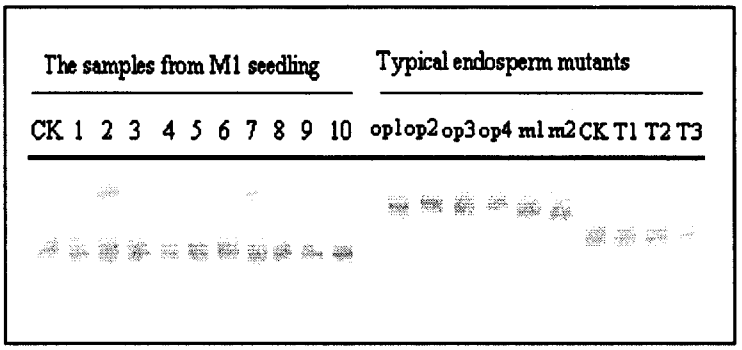


Fig.5. The *Wx* microsatellite markers of M_1 seedlings and M_2 kernels with opaque, mist and transparent endosperm

Acknowledgments

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3.3 Biotechnological Approach in Crop Improvement by Mutation Breeding in Indonesia

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Abstract

Mutation breeding has become a proven method of improving crop varieties. Most research on plant mutation breeding in Indonesia is carried out at the Center for Research and Development of Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN). Nowadays, a biotechnological approach has been incorporated in some mutation breeding researches in order to improve crop cultivars. This approach is simply based on cellular totipotency, or the ability to regenerate whole, flowering plants from isolated organs, pieces of tissue, individual cells, and protoplasts. Tissue culture technique has been extensively used for micro propagation of disease-free plants. Other usage of this technique involves in various steps of the breeding process such as germplasm preservation, clonal propagation, and distant hybridization. Mutation breeding combined with tissue culture technique has made a significant contribution in inducing plant genetic variation, by improving selection technology, and by accelerating breeding time as for that by using anther or pollen culture. In Indonesia, research on mutation breeding combined with tissue culture techniques has been practiced in different crop species including rice, ginger, banana, sorghum etc. Specially in rice, a research on identification of DNA markers linked to blast disease resistance is now still progressing. A compiled report from some research activities is presented in this paper.

Introduction

By definition, biotechnology is the application of a number of techniques and tools of cell biological and molecular biological origin in crop plants for economic purposes (Van Harten, 1998). Such techniques may help us to better understand genetic processes and be of value for plant breeding research as well as directly utilized for the production of new, genetically improved cultivars. It was also noted by Van Harten (1998) that basically there are two groups of biotechnological activities can be distinguished in plants. The first group is all activities related to in-vitro culture and the second group is the various recombinant DNA methods. Techniques belonging to both groups are often combined and several ways of using recombinant DNA would not even possible without having suitable in-vitro systems.

Basically, in-vitro culture refers to growing different plant organs, explants, tissues, cell suspensions, single cells, and protoplasts under control and aseptic conditions. Application of in-vitro methods has become practice in many recent mutation breeding projects. In Indonesia, for instance, tissue culture techniques have been practiced to generate mutant lines of several crop species including rice, ginger, banana, sorghum etc.

The most important recombinant DNA technology is genetic transformation which implies the transfer and incorporation of foreign DNA into the DNA of a recipient plant cell, followed by recombination of that DNA into the cell's genome. For useful application of this technique in plant breeding, it is essential that the newly integrated foreign DNA is expressed in a controlled way in the recipient cell and that transformed cells can be properly regenerated.

The application of recombinant DNA technology in plant mutation breeding has not been practiced yet in Indonesia. Anyhow, research on the application of other DNA technology i.e. the use of DNA markers for helping selection in a mutation breeding program has been initiated in rice. This research is now still under state of identifying DNA markers linked to blast disease (Pyricularia grisea Cav.) resistance in rice. Some experimental results including both the application of in-vitro techniques in plant mutation breeding programs and the preliminary research on identification of DNA markers are summarized in this paper.

Mutation Induction in General

Plant breeding requires genetic variation of useful traits for crop improvement. Often, however, desired variation is lacking. Mutagenic agents, such as radiation and certain chemicals, then can be used to induce mutations and generate genetic variations from which desired mutants may be selected. Mutation induction has become a proven way of creating variation within a crop variety. It offers possibility of inducing desired attributes that either cannot be found in nature or have been lost during evolution. When no gene or genes for resistance to a particular disease or for tolerance to stress can be found in the available gene pool, plant breeder have no obvious alternative but to attempt mutation induction.

Treatment with mutagens alters genes or breaks chromosomes. Gene mutations occur naturally as errors in deoxyribonucleic acid (DNA) replication. Most of these errors are repaired, but some may pass the next cell division to become established in the plant offspring as spontaneous mutations. Spontaneous mutations happened in nature with a very slow rate. Induced mutation by using a certain mutagen has been proven to be able to speed up mutation in plants and it has become an alternative tool in plant breeding for increasing plant genetic variability.

Artificial induction of mutations by ionizing radiation dates back to the beginning of the 20th century. But it took about 30 years to prove that such changes could be used in plant breeding. Initial attempts to induce mutations in plants mostly used X-rays, but later on gamma and neutron radiation were employed as these types of ionizing radiations became readily available from newly established nuclear research centers.

Biotechnology in Plant Breeding

Breeding for improved plant cultivars is based on two principles i.e. genetic variation and selection. The process is extremely laborious and time consuming with high inputs of intellectual and manual work. However, the development of plant cell and tissue culture has made it possible to transfer part of the breeding work from field to laboratory conditions.

Extensive research has resulted in a new areas of plant breeding namely "plant biotechnology" and "gene engineering". They are based on cellular totipotency or the ability to regenerate whole, flowering plants from isolated organs (meristems), pieces

of tissue, individual cells, and protoplasts. The isolated plant parts are aseptically grown in test tubes on artificial media of known chemical composition (in vitro culture). Under strictly controlled conditions, they form plantlets that subsequently can be transferred to soil where they grow to maturity.

In plant breeding, tissue culture techniques have been exploited commercially for micro propagation of disease-free stocks of horticultural crops such as strawberry, potato, banana, ornamentals etc. In vitro techniques are also used in various steps of the breeding process, such as germplasm preservation, clonal propagation, and distant hybridization (Van Harten, 1998).

Radiation mutation breeding and isotope techniques, combined with tissue culture, have made a significant contribution to plant breeding. They have introduced new techniques for inducing genetic variation, by improving selection technology, and by accelerating breeding time (Novak and Brunner, 1992).

Other methods, known as anther or pollen culture, make it possible to regenerate plants from male gametes with half the number of chromosomes (haploids). Compared to plants with full chromosomal content (diploids), the use of haploids in mutation breeding is advantageous since it allows detection of mutations immediately after their induction. Haploid methods have proven to significantly speed up the breeding of new varieties of rice, barley, and vegetables (Novak and Brunner, 1992).

Genetic engineering procedures allow the transfer of genetic material (DNA) from the cell of one species to that of another genetically unrelated organism. For example, a piece of DNA from a bacterial cell may be integrated into the genome of a plant cell to form a transgenic plant. The new DNA (gene) expresses itself in the plant phenotype regenerated from the transgenic cell. Nuclear techniques, based on nucleic acid bases labeled by isotopes, are employed in genetic engineering, to identify and isolate suitable genes for transfer, as delivery system to introduce genes into recipient cells, and to detect new genetic material in recipient organisms. Genetic engineering already has resulted in the production of plants with new desirable traits, such as insect resistance, virus disease resistance, and better ripening properties. However, early enthusiasm is being tempered by the growing discussion of the potential hazards of releasing transgenic plants to the environment (Novak and Brunner, 1992).

Mutation Breeding in Indonesia

Mutation breeding in Indonesia was started in rice in 1972, initially in an attempt to improve protein content of rice grain (Ismachin and Hendratno, 1972). This research activity was carried out under the IAEA Research Coordination Program. In 1974, rice mutation breeding activities were focused on searching mutant lines resistant to brown plant hopper (BPH) because, at that time, serious epidemics were recorded in some important rice-producing areas in Indonesia. Following subsequent selection process for resistant plants, a number of mutant lines resistant to BPH were obtained (Mugiono, 1990). Some of the promising mutant lines were then tested for their yield potential at several locations.

In 1982, rice mutant line No. 627/4-214/Psj and 627/10-3/Psj were submitted for official release by the Department of Agriculture. However, only the former was received approval for release as a new variety by the name of Atomita-1. This new variety had a better grain quality, early maturing, high yield, resistant to BPH biotype-1 and green leaf hopper. Unfortunately, Atomita-1 was not resistant to BPH biotype-2

so that this variety was not grown widely in the country because of the endemic situation of BPH biotype-2 in the rice field.

Following Atomita-1, other new mutant varieties of rice by the name of Atomita-2, Atomita-3, and Atomita-4 were subsequently released by the Department of Agriculture (Mugiono and Hendratno, 1995). However, these mutant varieties were not grown widely by farmers. It was suspected that the reasons might be due to naming "Atomita" gave a frightening impacts to farmers who might think the mutant varieties contained a "dangerous atom" inside them. Therefore, since 1992, naming a new mutant variety for rice had been proposed to use a river name instead of Atomita, and it did give a significant impacts to farmer's acceptance.

It was proven by the release of new mutant variety of upland rice with the name of Situgintung. Beside resistant to BPH biotype-1 and biotype-2 and blast disease, this variety had high yield under upland condition. In 1996, another new mutant variety "Cilosari" was released by the Department of Agriculture. This variety was suitable for irrigated areas or lowland condition in Indonesia (Mugiono, 1997). At present, Situgintung and Cilosari are popular as they are now grown widely by farmers in almost all provinces in Indonesia.

Beside in rice, research on mutation breeding in Indonesia had also resulted some new mutant varieties of soybean and mungbean (Soeranto et. al., 2000). Three mutant varieties of soybean by local name of Muria, Tengger, and Meratus and one mutant variety of mungbean by the name of Camar had officially been released by the Department of Agriculture. These mutant varieties were also grown widely by farmers in several provinces in Indonesia. Meanwhile, some promising mutant lines of other crop species including peanuts, banana, onions, ginger, patchouly, and sorghum were still under field investigation for official release as new cultivars.

Combined Biotechnology and Mutation Breeding

1) Tissue Culture Techniques in Ginger

Ginger plant (Zinger officinale Rosc.) is an important spice crop which demand is always increasing year by year. In Indonesia, ginger is generally used in various medicinal and culinary preparation in most local communities. Ginger is vegetatively propagated through the underground rhizomes but, unfortunately, its multiplication rate is very low. Hosoki et.al. (1977) reported heavy losses in ginger production in some plantations due to some disease attacks caused by bacterial wilt (Pseudomonas solanacearum) and soft rot (Pythium aphanidermatum). Because the diseases are mainly transmitted by rhizomes propagated every year, a production of disease-free clones are necessitated in order to get a successful ginger cultivation. Micro propagation by using tissue culture techniques can be a proper alternative to produce disease-free clones of ginger plant.

Problems faced in ginger breeding has so far been the very low genetic variation in ginger plant. This is because ginger is vegetatively propagated crop and hybridization is not effective since its flower biology has not been properly observed yet. Wide genetic variation is needed in plant breeding in order to search ideal plant types during the process of selection (Simmonds, 1986). In a breeding for disease resistance, the narrow genetic variation will slow down the selection process due to the risk of susceptibility to the disease.

A choice to increase plant genetic variability can be through somaclonal variation derived from tissue culture techniques, or through induced mutation techniques. Larkin (1981) stated that the somaclonal variation was a genetic variation

produced from a tissue or cell cultures. Meanwhile, Reisch (1983) mentioned that somaclonal variation happened in differentiation stage of tissue or cell cultures can be increased by applying either chemical or physical mutagen.

A research on induced mutation in ginger plant combined with tissue culture techniques has been conducted at BATAN. The explants used were the shoot tips sized 0.4 - 0.5 cm long taken from the rhizomes. The explants were exposed to gamma rays emitted from Cobalt-60 source with different level of doses. Gamma chamber is available at BATAN with initial activity of about 10980 curries. The irradiated explants were then sterilized with 70 % alcohol and 0.2 % HgCl2, rinsed 5 times with distilled water, plated in the modified MS basal media, and subcultured every 4 weeks. After 8 weeks, observations were done for the variable survival growth rate, number of shoot emerged, shoot height, and abnormality. Results of the observations were presented in Table 1.

As shown at Table 1, the best growth performance of ginger explants was for those irradiated with Gamma rays with the dose of 9 Gy. It seemed that dose inhibited bacterial and fungal contamination and stimulated explant growth. Lower doses as to 3 and 6 Gy did not show any better growth performance and it might be due to bacterial and fungal contamination. The Gamma ray doses higher than 9 Gy seemed to cause significant abnormalities as shown by dwarf plant with pale leaves. Based on Table 1, data analysis for best fitting models found the relationship between Gamma ray irradiation doses with the survival rate follows the Sinusoidal equation i.e. $Y = 69.14 + 40.33 \cos(1.33X + 0.48)$ with coefficient of regression $r = 0.921$.

Table 1. The effects of Gamma rays on ginger tissue culture after 2nd subcultured.

Gamma rays doses (Gy)	Survival rate (%)	Number of shoots	Shoot height (cm)	Abnormality
0	85.0	2-7	1.5-8.0	None
3	62.5	4-8	1.5-8.0	None
6	43.8	2-8	2.0-8.0	None
9	87.5	2-16	1.5-9.0	None
10	85.0	2-6	1.5-6.0	Dwarf, pale leaf
12	25.0	2-3	1.5-5.0	Dwarf, pale leaf
15	50.0	2-5	1.0-5.0	Dwarf, pale leaf
20	37.5	1-2	1.0-2.0	Dwarf, yellow leaf

Modification in MS basal media with thidiazuron (TDZ) hormone significantly improved explant growth as shown in Table 2 (Ismiyati et. al., 2000). Following the next subcultures, plantlets were transplanted to the field for screening against bacterial wilt (Pseudomonas solanacearum) and soft rot (Pythium aphanidermatum) diseases

Table 2. Growth performance of ginger explant culture in modified MS basal media with TDZ hormone.

Gamma ray doses (Gy)	Average			
	Explant growth (%)	No. of plantlets/explant	No. of leaves/plantlet	Plantlet height (cm)
0	100	3.3	3.0	5.9
6	80	2.6	3.0	6.9
8	100	2.5	2.7	6.9
10	97	2.8	3.5	6.7
15	92	3.7	3.1	7.0
20	86	2.1	3.4	6.4

2) Tissue Culture Techniques in Banana

Banana is an important fruit crops, ranked as second most important fruit in the world after citrus (Swennen & Rosales, 1994). The world production of banana in 1993 reaches 74 million tons (FAO, 1993). Banana production is concentrated in Central and South America, Africa, and Asia-Pacific with their contribution to the world production of 36, 35, and 29 %, respectively.

In Indonesia, the majority of edible banana is cultivated around the backyard with high soil fertility. In a country with densely increased population, like Indonesia, it is difficult to maintain banana cultivation around the backyard. Shifting its cultivation to another large-scale arable land may also compete with annual food crops or with other horticultural crops. Thus, banana production in the arable land is low and insignificant.

Banana is traditionally propagated by separating the suckers from their mother plant, which is then planted individually to establish a new mother plant. Because of that nature of propagation system, and the fact that banana flowers are mostly sterile, breeding banana by mean of hybridization is difficult to be conducted. Induced mutation combined with tissue culture techniques can be used as an alternative to increase plant genetic variation in a breeding program. Subsequent breeding procedures through selection processes can theoretically shorten breeding time of banana to reach the desired objectives.

Almost banana cultivars grown in Indonesia are susceptible to a plant disease caused by Fusarium oxysporum Cubense (FOC). Mutation breeding for this disease resistance has been conducted in combination with tissue culture techniques (Ismiyati et. al., 1998). A local banana variety Ambon Kuning (cavendish type) was used as the plant material. Its plantlets with height of about 5 cm were irradiated with Gamma rays emitted from Cobalt-60 source with the dose levels of 5-35 Gy. The irradiated plantlets were grown in MS basal media modified with adding growth regulators 4 ppm BAP and 2 ppm IAA. The grown plantlets were then subcultured up to M1V5 generation, acclimatized, and transferred to a hot spot field for Fusarium disease. Observations were done for plantlet performances during in-vitro stage and severity of Fusarium attacks in the field. Results of the observations were presented in Table 3, 4, and 5.

Table 3. Number of survival plantlets at different plant age after Gamma rays irradiation treatments.

Gamma ray doses (Gy)	Number of survival plantlets			
	3 months	6 months	9 months	12 months
0	312	423	511	554
5	324	414	482	538
10	272	347	392	468
15	275	351	412	471
20	94	0	0	0
25	96	0	0	0
30	78	0	0	0
35	45	0	0	0

Table 4. Plant performances in the field at 3 months after transplanting.

Gamma ray doses (Gy)	Average		
	Plant height (cm)	No. of leaves	No. of suckers
0	69.17	12.3	1.56
5	88	13.82	2.65
10	87.51	12.61	2.39
15	88.75	11.4	1.82

Table 5. Number of healthy and infected plants at 6, 7 and 8 months after transplanting in the field.

Gamma ray doses (Gy)	6 months		7 months		8 months	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
0	19	31	16	34	8	42
5	12	38	9	41	7	43
10	25	25	22	28	15	35
15	35	15	34	16	28	22

3) Tissue Culture Techniques in Sorghum

Indonesia has so far been very much dependent on rice as a staple food. In order to guarantee food safety in the country, it is necessary to campaign food diversification utilizing other food resources available. Sorghum plant (Sorghum bicolor L.) is thought of among the ones need to be considered in attempt to make the food diversification program be successful. Sorghum's potential is primarily due to its high production, low-input crop (required less fertilizer), more tolerant to drought and other adverse conditions, and its high nutrition values. In many countries, sorghum has been used as either food, feed, or industrial crops. According to FAO (1994) sorghum is the world's fifth food after wheat, rice, corn and barley. As a food crop, sorghum was reported to have good nutrition values (Direktorat Gizi DEPKES RI, 1992). Unfortunately, sorghum has so far been grown very limited by Indonesian

farmers who are used to grow rice in their lands. This limitation may be due to some problems such as availability of qualified seeds of sorghum varieties.

Farmers usually grow local variety in multiple cropping system with other crops where sorghum is used as an alley between crops. Normally, farmers use uncertified seeds of their own because certified ones are not available in the local market. From agronomic point of view, the local variety has some disadvantages as its characters of having high stature, late maturity, uninteresting seed color (brown), dropped head, and low production. The character of having high stature tends to make this variety be susceptible to lodging, and that of brown color is less desirable than that of the white one. For being used directly as food or in a food industry, white sorghum is usually more preferable.

In Indonesia, sorghum breeding was aimed at improving the local varieties for suitable use as food, feed, or industrial purposes. Unfortunately, variability of sorghum genotypes found in Indonesia is quite narrow and, therefore, different methods of breeding need to be operated. Meanwhile, Some improved sorghum lines as results of breeding by using mutation techniques have been produced, released and listed as new varieties (Wang, 1998 and Maluszynski et. al., 2000). Combining tissue culture techniques with induced mutations was one method that has been conducted at BATAN.

Seeds of sorghum varieties Keris, Durra, and Ethio-95 was irradiated with different dose levels of Gamma rays emitted from Cobalt-60 source. The embryo was then separated from the endosperm and used as an explant in a tissue culture technique. The irradiated embryos were sterilized with 70 % alcohol and 0.2 % HgCl2, rinsed 5 times with distilled water, plated in the modified MS basal media, and subcultured after 4 weeks. Plantlets resulted from subculturing were transferred to polybags and grown in greenhouse for producing seeds. All the seeds produced were grown in the field for selections. Plant selections were simply based on visually phenotypic performance of important agronomic traits such as plant height, plant age, head size, head form, seed size, seed color, productive tillers, and yield. Selected plants were grown in the next subsequent generations for further selections if they were still segregating. The promising mutant lines were then tested against drought in a drought-prone area i.e. in Gunung Kidul District of Yogyakarta Province. The hypothesis was that some promising mutant lines could adapt well in such conditions so that they could be developed further in the region. It is our hope that drought tolerant sorghum can help local farmers overcome food and feed crisis during dry season and also as an alternative plant in producing biomass for supporting development of a sustainable agriculture. The scheme of combined tissue culture techniques and mutation breeding in sorghum was presented at the attached Figure.

4) Identification of DNA Markers in Rice

Blast disease caused by fungi Pyricularia grisea Cav. is one of the most important disease of rice. In the tropics, blast attacks are commonly found in both lowland and upland rice fields (Bonma and Mackill, 1998). The use of resistant variety has so far been the most effective way of controlling the disease. Unfortunately, the resistant variety generally cannot be long lasting because of out breaking the resistance by the new disease races (Kiyosawa, 1982).

Plant breeding for blast disease resistance has been aimed at using molecular markers for helping breeders incorporate complete and partial resistance genes into a new rice variety. A number of more than 30 resistance genes have so far been

identified in rice (Kinoshita, 1998), and some of those genes have been mapped by using RFLP markers (Miyamoto, et. al., 1996; Monna, et. al., 1997; Wang, et. al., 1994; and Yu, et. al., 1996).

The local rice variety Laka was used as a resistance source in a plant breeding program for blast disease resistance. This variety is resistant to almost all races of Pyricularia grisea found in Indonesia. Meanwhile, Kencana Bali variety was used as susceptible control. This variety is susceptible to almost all disease races found in Indonesia. The contrast attributes between these two varieties are also found for their agronomic characteristics such as plant height, plant age, growth type, seed form etc. Inbred lines resulted from crossing between these two varieties will be of useful as permanent mapping population in identifying and analyzing genes controlling different characters found in the two varieties. RFLP analysis has been done for the inbred lines in order to search RFLP markers linked to blast-disease-resistance genes.

For identifying the combination of enzyme-markers showing polymorphism between Laka and Kencana Bali varieties, DNA was extracted from the two varieties by Dellaporta method (Dellaporta, et. Al., 1983). Another variety, Asahan, was also included in the identification. The extracted DNA was then digested with four restriction enzymes i.e. Bam HI, BglII, DraI, and HindIII. The digested DNA samples were electophorized at 0.8 % agarose gel and transferred to nitrocellulose membranes by using alkalic solution 0.4 N NaOH. Afterwards, the transferred DNA were hybridized with 146 different RFLP markers from Rice Genome Research Program in Japan (Harushima et. al., 1998).

Hybridizing, labeling, and detecting the DNA band on X-ray film were done by using ECL kits from Amersham. The combination of enzyme-markers showing polymorphism was used for genotyping the inbred lines. RLFP procedure in genotyping the inbred lines was the same as that in the identification of enzyme-marker combination showing polymorphism.

From that research work, some results have been obtained. A number of 144 inbred lines of rice have been resulted from crossing the Laka and Kencana Bali varieties. These inbred lines can be used for identifying DNA markers linked to the blast disease resistance, and also for being used in studying the inheritance of some important agronomic traits derived from the two varieties.

Based on the genotypic and phenotypic data, the DNA marker R2171 was linked to Chromosome 6, and the DNA marker R3089 to chromosome 7, with the resistance genes derived from Laka variety. Meanwhile, for detecting location of identified and other resistance genes, it is necessary to use additional markers and to test the inbred lines against blast disease with other races of Pyricularia grisea. This research is now still progressing under cooperation with the Plant Breeding Laboratory, Kyushu University, Japan.

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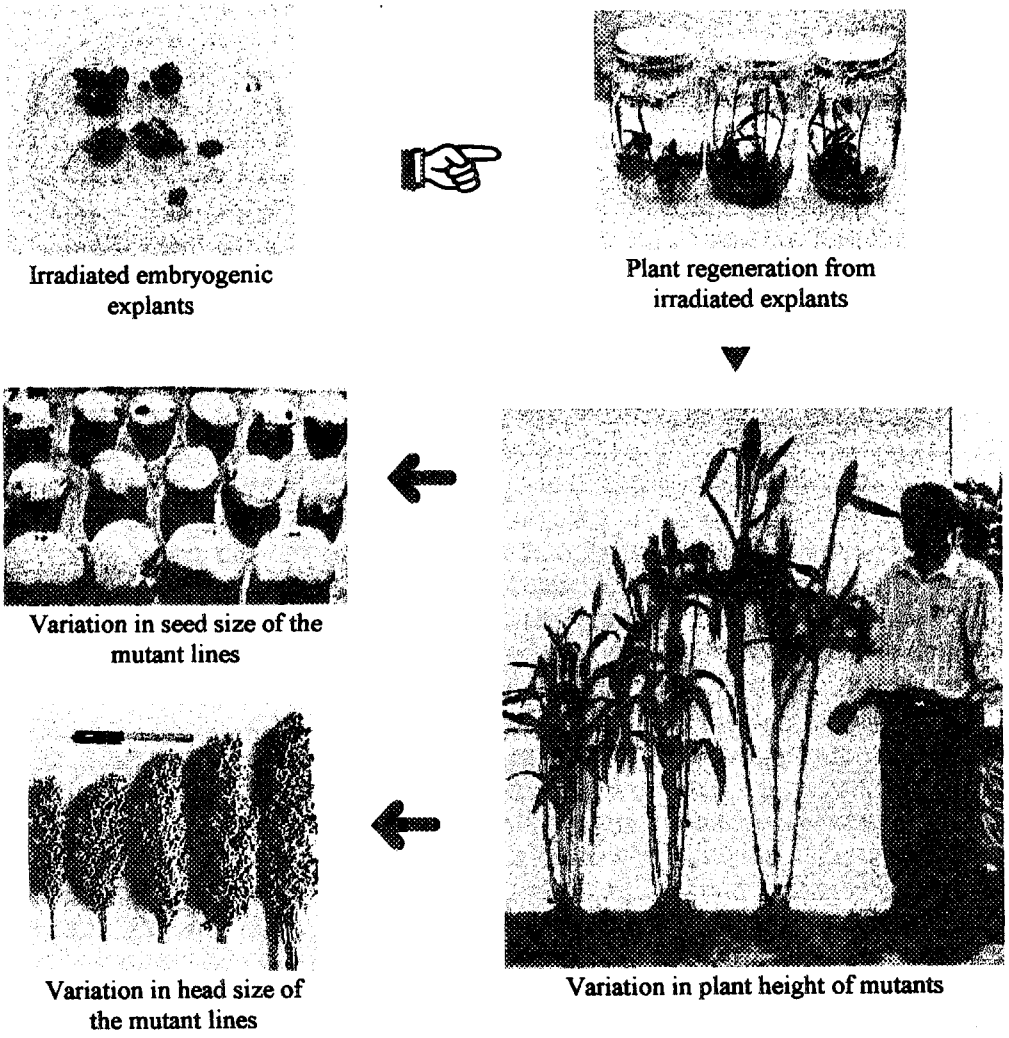

Figure 1. The scheme of combined tissue culture techniques and mutation breeding in sorghum.

3.4 Current Status of Molecular Biological Techniques for Plant Breeding in the Republic of Korea

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Classical plant breeding has played an important role in developing new varieties in current agriculture. For decades, the technique of cross-pollination has been popular for breeding in cereal and horticultural crops to introduce special traits. However, recently the molecular techniques get widely accepted as an alternative tool in both introducing a useful trait for developing the new cultivars and investigating the characteristics of a trait in plant, like the identification of a gene. Using the advanced molecular technique, several genetically modified (GM) crops (e.g., Roundup Ready Soybean, YieldGard, LibertyLink etc.) became commercially cultivated and appeared in the global market since 1996. The GM crops, commercially available at the moment, could be regarded as successful achievements in history of crop breeding confering the specific gene into economically valuable crops to make them better. Along with such achievements, on the other hand these new crops have also caused the controversial debate on the safety of GM crops as human consumption and environmental release as well.

Nevertheless, molecular techniques are widespread and popular in both investigating the basic science of plant biology and breeding new varieties compared to their conventional counterparts. Thus, the Department of Bioresources at the National Institute of Agricultural Science and Technology (NIAST) has been using the molecular biological techniques as a complimentary tool for the improvement of crop varieties for almost two decades.

1. The researches of Bioresources Department

Bioresources department currently consists of five divisions. Molecular techniques are being mainly used at three divisions of the five. The major works can be divided into six categories according to the procedure of research; cloning of useful genes, characterization of cloned genes, plant transformation of the genes, analysis of transgenic plants and rice genomics. Our ultimate goal is to develop the transgenic crops with new traits for higher yield productivity and better agronomical character.

2. Researches by Molecular Techniques

A. Analysis of starch unregulated lettuce

ADP-glucose pryophosphorylase (AGPase) is a key enzyme in metabolic pathway to starch synthesis in plant chloroplast during photosynthesis. AGPase is a holozyme, which consists of two large and two small subunits for the enzymatic activity. The regulatory site is considered to locate at large subunit and the catalytic site at small subunit. In order to develop more productive crops, AGPase gene, purified from potato, was mutated at allosteric site of large subunit not to inhibit the enzyme activity by its product (ADP-glucose, a building block of starch), called the feedback inhibition. After then, the mutated AGPase was transformed into lettuce via *Agrobacterium* gene transfer. Some of transgenic lettuces showed higher productivity in leaf size and number than wild type. The total fresh weight increase up to 140% compared to the wild type at six weeks after sowing in the greenhouse.

B. Expression of modified cryIAc1 gene

Bacillus thuringiensis (Bt) produces a crystal protein toxic to insect. Several Bt genes were already cloned and then developed for stable expression in eukaryotes. Transgenic maizes, transformed with such Bt genes, were commercialized by the companies of Monsanto, Aventis and Novartis. Recently a few kinds of insect-resistant maizes are commercially cultivated and shown in the agricultural food trade. As the way to control diamond moth (*Plutella xylostella*) in Chinese cabbage, the nucleotide-sequence of wild Bt gene was accurately modified for the efficient expression in plant without change of amino-acid sequence. The modified Bt gene (cryIAc1) was transformed, under the control of CaMV 35S promoter, into Chinese cabbage via *Agrobacterium* and assayed its toxic action against diamond moth. The transgenic plant showed strong resistance against insect.

C. Transgenic rice resistant to leaf blight disease

To confer the antibacterial resistance to rice, a gene of glucose oxidase (GO) was transformed into rice (cv. Nagdongbyeo) via *Agrobacterium*. GO is known to oxidize β-D-glucose, and then produce glucose-δ-lactone and hydrogen peroxide (H_2O_2). Of the two products, hydrogen peroxide is the substance that is toxic itself to pathogen and can also activate the plant defense response before the pathogen invades plant. According to the bioassay with transgenic rice, it acquired the strong resistance to pathogen (*Xanthomonas oryzae*, race K1) which causes leaf blight disease in rice.

D. Induction of male-sterility

Male-sterility is commercially important trait since it could be used to produce easily the hybrid (F1) in crop breeding. As the first step for developing the way to manipulate the

male-sterility, anther-specific promoter gene (BcA9) was cloned from cabbage (*Brassica campestris*). The BcA9 turned out to be specifically active in tapetum-tissue in which the pollen is differentiated at the reproductive stage of anther. To inhibit the pollen production, diphtheria toxin gene (DTx-A) was fused at the end of BcA9 to control its selective expression in tapetum. The DTx-A protein generally has the cytotoxic effect, for that reason the cell comes to be killed in the end by the toxin. The transgenic cabbage, introduced with BcA9::DTx-A *via Agrobacterium*, showed the exactly normal phenotype compared with the wild-type in vegetative growth, however in reproductive growth, the tissue of tapetum in anther was undifferentiated, killed and finally disappeared by the action of the toxin. On the other hand, female organ, pistil and ovary, did not show any visible damage. As for the anther of transgenic cabbage, the size decreased severely and the development was obviously inhibited when the pollen is about to differentiate from tapetum tissue. Observing the anther under microscope, no pollen has been observed in transgenic cabbage.

E. Radioactive Isotope in Molecular Biological Technique

Radioactive isotope (RI) is prerequisite for current molecular biology. It has been extensively used for the isolation of genes, for the visualization of gene expression espcially at the level of transcription and for the selection of transgenic plant. Several different types of the RI are currently used in this area. Among them, P-32 dNTP would be main RI to label nucleic acids. At NIAST, P-32 dNTP takes the first position in importance and occupies the 90% of total consumption for the past couple of years. There are also a few non-radioactive labeling systems (e.g., DIG Roche) to substitute for the RI. However, despite of the possible risk to human and enviroment, many researchers prefer to use the RI over the other labeling systems in molecular biological research.

3. Mutation Breeding by Irradiation

Mutation breeding by irradiation has been applied to develop a new variety in agricul ture, particularly in the area of disease resistance, quality improvement and floral mutation. I n order to induce the mutation of the phenotypes in *Chrysanthemum* species, γ-ray of 0-50 gray (Gy) was irradiated to the plantlets (M1V1) that were cultured *in vitro*. After then, th e plantlets were maintained *in vitro* for next third generation (M1V3) to observe the growth p attern and the phenotypic differences. In M1V3 generation, some differences (e.g., non-branching, abnormalities of leaf, flower and stem) appeared more frequently as the dose of γ-ray increased at the generation of M1V1. The frequency of the phenotypic mutation showed to increase comparatively along with the increase of doses. And the number of mutants in flo ral color and shape were generally most abundant at the dose of 25 Gy. By irradiation, variou s kinds of mutants were obtained easily.

4. Concluding Remarks

Molecular biological techniques have been developed so fast for the past decades and have already taken an important position in the most areas of biological science including the current agriculture. In the near future, numerous kinds of GM crops should be seen easily in the field and the global market more frequently than now. There is no objection that modern biotechnology must be developing further continuously and widening the area to apply to. Actually, most people have been thinking of the technique that is one of the promising ways for well being of human society. However, recently controversial debate is growing on the globe about the GM crops bred by molecular techniques. Even though most scientists consider the basic principle of molecular technique as well as that of traditional breeding in terms of introducing specific traits (gene) in order to improve the quality and quantity in crop production. Regarding the situation on such techniques, scientists should be responsible for demonstrating the safety of GM crops properly before the release of them.

3.5 Status of Biotechnology with Emphasis on Molecular Techniques for Mutation Breeding in the Philippines

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Abstract

This paper summarizes the status of biotechnology with emphasis on molecular techniques for plant breeding in the Philippines. Several molecular and in-vitro culture techniques are integrated in plant breeding for crop improvement at PNRI, UPLB, IRRI and PhilRice. At IRRI, PCR techniques, RFLP and RAPD, PCR techniques, RFLP and RAPD were developed to establish high density molecular maps, determine breadth and diversity of germplasm and characterize alien introgression. The molecular maps have identified DNA sequence of resistance genes of HYVs and NPTs to abiotic and biotic stresses. the major achievement is the development of high density molecular maps in rice with at least 2000 markers.

The biotechnology program at PhilRice for varietal improvement includes: (1) utilization of molecular marker technology such gene mapping of desired traits in rice, analysis of genetic relationships of germplasm materials and breeding lines through DNA fingerprinting and genetic diversity studies and development and application of marker aided selection for disease resistance (RTD and BLB); (2) application of in-vitro techniques in the development of lines with tolerance to adverse conditions; 93) molecular cloning of important genes for RTD resistance; (4) genetic transformation for male sterility and resistance to sheath blight and stem borers; and (50 transfer of disease resistance from wild species to cultivated varieties.

In IPB, molecular markers:microsatellites or SSR, AFLP and RGA are being used for mapping and diversity studies in coconut, mango, banana, mungbean, corn and tomato.

Mutation breeding at PNRI using gamma radiation has resulted in the development of crop varieties with desirable traits. The use of AFLP coupled to PCR is being used to study polymorphism in plant variants of radiation-induced mutants of rice, pineapple and ornamentals.

Introduction

Feeding the hungry and attaining food sustainability and biodiversity are the battle cries in the turn of the millenium to cope-up with the ever increasing world population. Such demand for food and accelerated population growth occurs largely in the undeveloped and rapidly developing countries of Asia, Africa and Latin America (Eyzaguirre, 1996). Though world food production more than doubled in a 40-year period from 692 million tons in 1950 to 1.9 billion tons in 1990, still hundred of millions of people are in starvation. In rice growing countries alone, population pressure is so intense that 80 to 100 million additional people must be fed each

year. Likewise, world's annual unmilled rice production must increase by about 65 % over the next 31 years to keep up with population growth and income induced demand for food (IRRI, 2001).

To match these pressures, unprecedented increases in production in terms of expanded areas for production and extraordinary increases in yield per crop per unit area must be attained. Failure to provide such rapid rates of growth will result in rising food prices, economic instability, unemployment and consequently depressed food consumption and nutritional status for low income populace.

In order to avert these conditions researchers must employ science tools that enhance the process already occurring in nature to increase production beyond the current potential crop yields (Sebastian, 2001). Biotechnology such as in-vitro cell culture, improved diagnostic for crop diseases, gene mapping and genetic transformation are now available and invaluable tools in conventional and mutation breeding works for crop improvement.

Biotechnology is now included in agriculture modernization in the Philippines through research and development in government and private research institutions. It is recognized that biotechnology has enormous potential in the increase yield of domestic staple crops and enhancement of yield and by-products of the country's export winner commodities such as fruits and ornamentals (Uriarte, 2001). Several molecular/ biotechnological techniques are being integrated in plant breeding works at the Philippine Nuclear Research Institute (PNRI), University of the Philippines at Los banos (U.P.L.B.), International Rice Research Institute (IRRI) and Philippine Rice Research Institute (PhilRice). Application or use of such techniques in crops are as follows: Coconut-gene mapping and hybridity testing, maize-fingerprinting, mungbean-disease resistance, tomato-polymorphism, papaya-resistance to ringspot virus and onion-hybrid verification. In rice-varietal improvement molecular markers coupled with polymerase chain reaction (PCR) have been developed to identify varieties carrying multiple genes for disease reistance , transfer of bacterial blight resistance genes to new plant type (NPTs), molecular mapping of genes and quantitative trait loci (QTLs), including resistance to stem borer, brown plant hopper, gall midge, tungro, salinity tolerance and photoperiod sensitivity. Likewise, amplified fragment length polymorphism (AFLP) coupled to PCR is being used to indentify plant variants of radiation - induced mutants of rice, pineapple, and ornamentals. The purpose of this report is to summarize the status of biotechnology with emphasis on molecular techniques for crop improvement in the Philippines.

A. Molecular and *In-Vitro* Culture Techniques for Mutation Breeding at the Philippine Nuclear Reserch Institute (PNRI)

The Mutation Breeding Program is presently being undertaken by the Agricultural Research Section of the Atomic research Division, PNRI.

In recent years, induced mutations have played an increasing role in the breeding programs of rice (*Oryza sativa* L.) soybean (*Glycine max* L.) mungbean (*Vigna radiata* Wilczek), sweet potato (*Ipomea batatas* L. Poir), pineapple (*Ananas comosus* (L.) Merr.), and foliage ornamentals (*Murraya exotica* and *Dracaena sanderiana*). Several improved varieties with desirable traits were successfully developed through induced mutation breeding at our Institute (Table 1).

Research on the mutation breeding of rice using gamma rays has resulted in the development of 5 new varieties (PARC 2 Milagrosa Mutant, Bengawan Mutant, Azmil Mutant and the Sigadis -Milagrosa Mutant, otherwise known as PSBRc 78 or Pampaga) that has been approved and released by the Seed Board.

These mutant varieties exhibited two or more of the following desirable traits: good eating quality, improved grain characteristics, high yielding, early maturing, reduced height and resistance to diseases.

The mutants developed are being used in crosses with other rice varieties/traditional cultivars like Denorado,Azmil and Azucena.

In the crosses using the mutants, exciting selections have been identified like black hull lines which came from the cross between Denorado and Bengawan Mutant. Black hull and aleurone were also selected from the cross between Denorado and Azmil Mutant. Most of the lines are early, short and non-lodging. Their yields have yet to be tested however in a replicated yield test.

Brown hulls with black aleurone were likewise isolated from the cross between Denorado and Azmil Mutant.

In the cross between Malagkit Sungsong Mutant and the original cultivar, good lines were selected but with certain degree of sterility. In the cross between Milagrosa mutant and IR52, early, short and fine-grained selections are about to be yield tested.

Advances in biotechnology such as the use of *in-vitro* culture in conjunction with gamma irradiation is being used in enhancing genetic variability in rice. The embryo and anther culture are being incorporated in the mutation induction using gamma radiation.

Regeneration of plantlet has been obtained in C4-63G, Azmil, Fortuna and Milfor 6(2) calli derived from the embryos that have been irradiated with gamma rays. Desirable variants from-gamma irradiated calli 0f C4-63G and Sinandoming were isolated.

In C4-64G, a reduction in maturity by 4 days and yellowish-green plants were obtained. In Sinandoming, narrow leaf and pale green mutants were generated. In other cultivars, variants with pale green leaves and various sterilities were noted except for Fortuna wherein an early mutant was isolated.

Anther cultured from crosses between mutants and other rice varieties resulted in the selection of plants with desirable traits in the F_2 generation (Table 2).

Molecular markers, AFLP-PCR was used for DNA fingerprinting in radiation-induced rice mutants in Bengawan and in PARC-1, PARC 2 and PARC 3.

Graphic DNA database of mutant lines of rice submitted to PhilRice for gene bank will be done using the same molecular technique.

The *in-vitro* technology is being integrated in the varietal improvement of asexually propagated crops as a tool to induce mutations and as a rapid means of propagation.

In sweet potato (*Ipomea batatas* L.) the single-cell approach through leaf propagation (cut-leaf technique) has been successfully developed in the Kinastanyas variety to induce mutations. Cut-leaves were irradiated with different doses of gamma radiation. This involves inducing the leaves to produce roots and eventually shoots or complete plantlets. This technique eliminates the problem of somatic competition between the mutated cells and the non-mutated ones.

A mutant with increased yield was induced from a selection derived from 5 Gy gamma irradiation of Kinastanyas sweet potato. It yielded 49% more than the original parent variety. pPlanting materials of the mutant are being increased to be used for preliminary yield trial. Another mutant that has been induced is the chlorophyll mutant. This mutant is presently being propagated in preparation to the evaluation of the alleged mutation for shade tolerance.

In the tissue culture of Kinastanyas callus was successfully induced from tuber, shoot tip, midrib and petiole explants and well-developed roots from calli derived from petiole and tuber sections that are grown in Murashige & Skoog's medium with naphthalene acetic acid. No shoot regeneration was obtained.

In BNAS variety of sweet potato, the protocol for *in-vitro* culture system for plantlet regeneration was established using CS-5 medium with kinetin.

Preliminary study on the radiosensitivity of the calli to gamma radiation showed a decrease in the mean weight of calli irradiated with doses higher than 30 Gy.

A very slight increase in weight was noted at 10 Gy, 20 days after irradiation Studies on the effects of gamma radiation on in-vitro cultured explants of yam (*Dioscorea alata* L.) cv. Kinampay was conducted with the objectives of : (1) establishing protocol for callus induction and plantlet regeneration and (2) the determination of the optimum dose of gamma radiation to be used for mutation induction using the in-vitro culture system for trhe development of improved variety of yam that is bushy, non-seasonal and early to develop storage roots that are of good quality.

Various explants of yam were irradiated with gamma rays at doses ranging from 5-50 Gy. Induction of callus was obtained in tuberous root and other vegetative explants: petiotle, node, internode and shoot apex. Callus induction was observed in the irradiated ands unirradiated fleshy or tuberous root explants grown in Murashige and Skoog's (MS) medium with benzyl adenine (BA) in combination with naphthalene acetic acid (NAA) ranging from 2-6 ppm. Stimulation of callus growth was obtained from tuberous root explants irradiated with 5 and 10 Gy and grown in MS medium supplemented with 2 ppmBA in combination with 6 ppm NAA. Similar callus growth (as indicated by their weight) was observed at higher doses of 30 and 40 Gy, provided that higher levels of 4 ppm BA and 6 ppm NAA were incorporated into the MS medium. Similarly, induction of callus was enhanced in tuerous root sections of putative mutant lines (for dwarfness and earliness) that were cultured in hgiher levels of BA and NAA in MS medium.

Regeneration of plantlets was obtained from callus-derived shoot apex irradiated with 40 Gy and from calli-derived unirradiated nodal and tuberous root explants.

Three varieties of ubi (Kinampay, Bohol and Zambal) were irradiated with different doses of gamma radiation ranging from 5-40 Gy.

In the MV_2 generation, putative mutant with short internode were identified in the Kinampay variety that was irradiated with 10 Gy. Changes in the color of the flesh of the tuber was noted in both Bohol and Zambal varieties. The color of the flesh of Bohol tuber, which is originally purple, changes into creamish, while the Zambal variety that receives the same dose of gamma radiation has a change in color from purple to deep purple. Multiple tubers were also induced in Bohol variety that was recurrently irradiated.

In the induction of mutations using gamma rays in pineapple (*Ananas comosus* L. Merr.), one of the problems encountered is diplontic selection and chimera formation. This problem can be avoided by developing a single-cell approach which can be made possible by developing the cloning technique through tissue culture.

At present, PNRI has successfully developed the tissue culture technique for pineapple Queen Variety and the use of axillary buds as a means of propagation.

The tissue culture technique and the use of axillary buds are being used as tools for mutation induction with the objectives of: (1) reducing or removing the spines of the leaves in the Queen variety (2) inducing chlorophyll mutations which can be the bases of ornamental types.

Irradiation of the axillary buds resulted in the development of improved Queen variety of pineapple with reduced spines or thorns along the margin of the leaves (66 to 85% reduction and a chlorop[hyll mutant that look-like ornamental bromeliad. These mutants are being purified and propagated using the axillary bud and tissue culture techniques. The protocol for the tissue culture of pineapple Queen variety has been established and is now being used as a tool for mutation induction. Induction of callus from crown section is a continuing activity so that callus tissues are made available for irradiatioin. These calli are being grown in Murashige and Skoog's (MS) medium with benzyl adenine and naphthalene acetic acid. Studies on the radiosensivity callus tissues to gamma radiation were undertaken. Irradiation of callus tissues with 20 Gy induced the development of profuse and vigorous regenerated plantlets. Somaclonal variation which resulted in the reduction of thorns were also observed from *in-vitro* culture.

The molecular marker, AFLP-PCR technique was used to characterize the pineapple chlorophyll mutant and the unirradiated control.

Mutation breeding through the use of gamma radiation coupled with tissue culture techniques and related biotechnology is being undertaken for the improvement of foliage ornamentals (*Dracaena sanderiana* and *Murraya exotica* L.) and cutflowers (*Chrysanthemum morifolium* and orchid *Vanda sanderiana*, *Dendrobium* Pattaya Beauty and *Phalaenopsis schilleriana).* Research on the mutation breeding of ornamental crops has resulted in the induction of beneficial mutations in foliage ornamentals (1) Dwarf mutants in *Murraya exotica* (2) Chlorophyll mutants in *Dracaena sanderiana*. The Chlorophyll *Dracaena* mutant and the *Murraya* mutants were highlighted and test marketed during the Atomic Energy Week.

At present there are 298 Dwarf *Murraya* mutants propagated (159 about to flower and 139 flowering plants ready for sale to interested clients. This mutant was submitted for registration and was approved by the Technical Working Group on ornamentals.

Multiplication of the chlorophyll *Dracaena* mutant was done. To date, there are 165 plants ready for "test marketing". This mutant will be submitted for registration.

Tissue culture studies in orchids and chrysanthemum are being done as a tool for mutation induction. Immature embryo of orchids, *Dendrobium* sp. and *Vanda sanderiana* were used as explants for *in-vitro* culture experiments. Protocorms derived from immature embryo were irradiated with gamma rays ranging from 5-50 Gy. Profuse growth of protocorm tissues was observed in all doses except 50 Gy with repeated sub-culturing of Dendrobium Pattaya Beauty orchids. Shoots were formed from protocorm irradiated with 10-30 Gy as well as in the control. In Vanda sp., protocorm irradiated with 10 Gy and grown in KC medium resulted in development of plantlets thatr are bigger and more vigorous than those at 20 Gy and the control.

reflasking of plantletr was done until roots were well-developed and ready for community potting. Seedlings were compotted in orchid pots containing osmunda fiber and charcoal and grown under greenhouse conditions. A decrease in seedling height was observed with increasing dose of gamma radiation.

Chrysanthemum stem section was irradiated with 10-20 Gy gamma rays and micropropagated in Murashige and Skoog's medium. Whorling and changes in leaf color were observed at 10 Gy and doubling of the leaf growth at the node in 20 Gy of the 3rd vegetative generation. Morphological changes observed in the 4th generation were multiple branching per node in the 10 Gy dose as well as in the 20 Gy. From the 5th vegetative generation plantlets with well-developed roots will be transfered in the soil for acclimatization and later grown in greenhouse condition. Selection and evaluation of the chrysanthemum plants for improved characteristics were undertaken.

Data base of gamma induced variants of foliage ornamentals (*Murraya exotica* and *D.* sanderiana) and putative mutants for earliness to bear flowers were studied using AFLP coupled to PCR technology. Development of the AFLP-PCR protocol was established for variants od ornamentals with specific restriction enzyme pairs to produce a clear DNA fragment pattern in acrylamide gel. AFLP-PCR fragment produced specific DNA pattern in gels for each variant that was used to identify gamma radiation-induced mutation.

B. Molecular Biotechnology at the University of the Philippines (U.P.L.B.)

The Institute of Plant Breeding is the center of major biotechnology activity at the University of the Philippines, College, Laguna. Several markers such as microsatellites or simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and resistance gene analog (RGA) being developed for mapping and diversity studies in coconut (*Cocos nucifera* L.), mango (*Mangifera indica* L.), banana (*Musa acuminata* L.) mungbean (*Vigna radiata* L. Wilezek), corn (*Zea mays* L.) and tomato (*Lycopersicon esculentum,* Mill.) (Table 3).

In coconut, corn and irradiated banana clones, microsatellite at SSR is used to establish diversity and genomic profiles. An initial survey of polymorphic SSRs in the TAC X BAO coconut progenies generated 5 mappable SSR markers which segregated into ten expected Mendelian backcrosses ratio. In corn, microsatellite profiles from 80 local bred corn inbred lines involving 32 loci was developed. SSRs in banana was able to differentiate Lacatan from Latundan clones but none was able to select irradiated and normal clones within Lacatan or Latundan.

Amplified fragment length polymorphism (AFLP) is tried for mapping pests and diseases in mungbean and tomato. Earlier results showed polymorphism in each crop: 42.5% for mungbean and 18.4% for tomato. The same marker is being established for finger printing Philippine Mango cv. Carabao and irradiated banana. Polymorphism ranged from 12 to 83% for mango and 51.1% for banana. AFLP for banans is shown to differentiate Lacatan from Latundan and clones among the Latundan.

Resistance gene analog (RGA) primers were used to amplify fragments from bacterial wilt resistant and susceptible tomato genotypes and bruchid resistant and susceptible mungbean genotypes. In all these tests, polymorphism was obtained for both crops.

In rice, PCR based molecular markers (RAPD) and Microsatellites) are being used to determine breadth and diversity in 78 HYVs released from 1965 to 1995 and their 81 ancestral lines. As compared with the morphology based analysis, these techniques are much preferred in determining diversity of improved varieties most especially on genotypes with unknown ancestry (Caldo, R. 1996).

Studies were conducted to develop a protocol for *in-vitro* culture of mucellar embryos in citrus to be used for mutation induction. Results showed that the optimum medium for support of *in-vitro* growth and plantlet regeneration consisted of the basis Murashige and Skoog's medium supplemented with myo-inositol (100 mg/l), pyridoxine-HCI (1 mg/l), nicotinic acid (1 mg/l) thiamine (0.2 mg/l) Fe-EDTA (25 mg/l), sugar (20g/l), agar-agar (7g/l) and benzyladenine (mg/l) with a pH of 5.7. LD_{50} for gamma irradiation was 100-150 Gy. For ethyl methanesulfonate, concentrations up to 24.6 ml/l were tolerable. Screening for resistance to leaf mottling organism among the resulting seedlings gave promising results. A few plants had been recovered exhibiting a certain degree of resistance to this pathogen.

C. Molecular Biotechnology at the International Rice Research Institute (IRRI)

In the Philippines, IRRI is the leading agency on the application of molecular/biological techniques for varietal improvement due to its financial capability and collaboration with Asian Rice Biotechnology Network (ARBN). The molecular marker technologies coupled with PCR techniques being done in the institute has made possible to identify varieties carrying multiple genes by the use of closely linked molecular markers as in the case of resistances to B_1 and BB (Table 4) (Magpantay et al. 1996). Using marker assisted selection (MAS), BB resistance genes xa5, xa13 and Xa2 are successfully transferred to NPT(new plant types) rice lines IR 65598-112, IR6500-42 and IR 6500-96. DNA sequences of the resistance genes are made using RFLP and RAPD while detection of polymorphism is done by polymerase chain reaction (PCR) (Sanchez et al. 2000). Fifty nine BC3F2 near isogenic lives (NILs) in the three NPT backgrounds containing one to three BB resistance genes in various combinations are developed through MAS for the resistance genes and phenotypic selection for the NPTs. The BC3F3 NILs having more than one BB resistance genes showed a wider resistance spectrum and manifested increased level of resistance to X00 races of *Xanthomonas oryzae* pv. *Oryzae* as compared with those having a single BB resistance gene. Likewise, emplyoing 200 PCR based markers for molecular genotyping about 200 lines are placed in the advance trials for the 2000 cropping seasons. These lines are part of the BC3F1 and BC2F2 populations of HYVs IR64, IR 58025B, IR 68897B and several NPT lines (Sebastian, 2001).

At IRRI, RFLP and RAPD techniques are developed to determine the extent and process of introgression of desired genes (resistance to BPH, grassy stunt, BB, blast, salinity, drought and cold tolerance, Fe toxicity and submergence, from wild species to HYVs NPTs and hybrids (Brar and Khush, 1997). RFLP analysis revealed introgression from 11 of the 12 chromosomes of C genome species into rice. Introgression has also been obtained from other distant genomes (EE,FF,GG) and in majority of cases RFLP markers are introgressed. RAPD markers are also found to be linked to Pi-9t, a gene for blast resistance introgressed from *Oryzae minuta* into HYVs (Amante-Bordeos et al. 1992).

Among the major achievement at IRRI's biotechnology program is the development of a high density molecular map with at least 2000 markers. Molecular mapping of genes and quantitative trait loci (QTLs) is actively pursued in numerous traits including resistance to stem borer, brown plant hopper, gall midge, bacterial blight and tungro, resistance to lodging, days to heading fertility, salinity tolerance, elongation ability and photoperiod sensitivity (Sebastian, 2001). Genes for earliness in rice has also been mapped using RFLP and found to be recessive (Brar and Khush, 1997).

The advent of genetic engineering gives way to the development of a number of transgenic plants with desired traits (Brar and Uchymiya, 1990). IRRI has again taken the lead in thre development of genetically engineered rice. Transgenic rice free of antibiotic resistance marker may now be produced by either co-transformation with the gene of interest followed by segregation or use of the marker gene encoding 6-phosphomannase isomerase. Maintainer and restorer lines for hybrid rice production were rendered resistant to stem borers and leaf-folders by transformation with Bt genes (Sebastian, 2001). Further development of this exciting field of discipline is however hampared by environmental issues and social relevance.

At present, IRRI's genomic project takes pride of more than 18,000 deletion mutant lines. The collection is still growing and is expected to hit 40,000 by year 2002. A large collection of introgression lines is also being developed carrying wide range of beneficial chromosomes introgressed from wild rice to conventional HYVs and NPTs.

The whole gamut of these enormous collection of genes and chromosomes are already mapped and identified through the use of PCR techniques, MAS, RFLP and RAPD.

D. Molecular Biotechnology at the Philippine Rice Research Institute

The Philippine Rice Research Institute is tasked to develop rice varieties under Philippine conditions. One of the strategies is to use biotechnology in area where conventional breeding has been shown not very efficient, The biotechnology program is attuned to the country's concern of rice self-sufficiency. Its application in varietal improvement as shown in Table 5 is as follows: 1. Utilization of molecular marker technology such as genetic mapping of important traits in rice, analysis of genetic relationships of germplasm materials and breeding lines through DNA fingerprinting and genetic diversity studies and development and application of marker-aided selection for rice tungro disease (RTD) and bacterial leaf blight (BLB) resistance, 2. Utilization of in-vitro techniques in the development of lines with tolerance to adverse condition, 3. Molecular cloning of important genes such as those for RTD resistance; 4. genetic transformation of rice plants for male sterility and resistance to sheath blight and stem borers; and 5. transfer of resistance genes for tungro, sheath blight and stem borers from wild Oryza species to cultivated varieties (Sebastian and Obien, 1999).

PhilRice has the funds and the facilities to carry-out transgenic work, anther culture and molecular marker analyses such as micro satellites, amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP). In direct seeded irrigated paddy rice, molecular mapping of quantitative loci (QTLs) for seedling vigor of HYVs has been developed using RFLP, RAPD and AFLP markers. In transplanted irrigated rice cultures, introgression of resitance genes for tungro, green leaf hopper (GLH), Brown plant hopper (BPH), bacterial leaf blight (BLB), sheath blight and

yellow stem borers has been done from wild rice to HYVs. Effect is being done to map the resistance genes using RFLP for isolation and purification. DNA markers was also developed in the marker aided pyramiding of BLB resistance genes (Xa-5, Xa-21 and O.minuta gene) in modern cultivars and in the genetic mapping of blast resistance in a Tequing-Lemont recombinant inbred population.

In the hybrid rice program, three varieties PSB Rc 26H, PSB Rc 72H and PSB Rc 76H are already released and under cultivation in different parts of the country. Thousands of rice lines with different traits have been developed in the process. DNA fingerpringting is being done in cytoplasmic sterility (CMS) lines using RAPD, microsatellites and AFLP to determine the diversity of the existing germplasm.

In resistance to salinity, two salt tolerant varieties PSB Rc 50 and PSB Rc 48 have been developed. Molecular maps microsatellite and RFLP markers of recombinant inbred salt tolerant lines and BC3F4 population are constructed to determine the major gene and quantitative traits low Na obsorption, high k and low Na/K absorption ratio.

Prospects/Futureplans

Mutation breeding program at PNRI has contributed to crop improvement in the Philippines with the development of mutant crop varieties and several variants of ornamental plants with desirable traits. Radiation induced mutants are being used in crosses with other traditional varieties/cultivars for varietal improvement program. Integration of biotechnology on the use of *in-vitro* culture and molecular techniques can greatly enhance the efficiency of mutations. Molecular markers are being incorporated in the conventional breeding at IRRI, PhilRice and UPLB to determine the breadth and diversity of germplasm in rice and other food crops. At PNRI, AFLP-PCR technology is presently being used to study polymorphism in gamma radiation induced mutants of food crops as well as ornamental variants. At present, PNRI has collaboration with UPLB and PhilRice on mutation breeding research activities. This collaboration, should be maintained and further linkages with IRRI, PCARRD and the Department of Agriculture should be established, so that there will be sharing of resources, training in molecular techniques for plant breeding that is necessary to avoid duplication of research activities and will further enhance the attainment of breeding objectives for national development.

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Table 1. Crop Varieties That Have Been Improved Through induced Mutation Breeding at the Philippine Nuclear Research Institute.

Crop	Parent Material/ Variety	Mutant Variety/ Year Developed	Mutant Dose	Mutated Character/ Main Attributes of the Variety	Status of Mutant
1. Rice	IRS-283-8 (LOCAL)	PARC-2 or Atomic Rice (1971)	Gamma Ray 400 Gy	*Longer grains with less chalky area, better eating quality and has tungro resistance comparable to C4-63 and IR 20,Comparable to parent variety	Approved by the Seedboard in 1973 for release to farmers
	Milagrosa (Local)	Milagrosa Mutant (1973)	Gamma rays 200 Gy	*non-photoperiod sensitive, longer grains,yields 32% more than the parent variety	*Approved by the Philippine Rice Working Group in 1974
	Bengawan (Indonesian variety)	Bengawan mutant (1981)	Gamma rays 300 Gy	*Yields 35% more than the parent variety, lodging resistant due to its reduced height, retains tungro disease resistance,drought resistance, non-photoperiod sensitive	*Multiplied and distributed by Maligaya Rice Research and Training Center in 1983-1984
	Azmil (local)	Azmil mutant (1976)	Gamma rays 200Gy	*yielded 40% more than the parent variety, retains drought resistance	*Used in crosses with other varieties
	Sigadis Milagrosa	Sigadis Milgrosa Mutant or PSB RC78 or Pampanga	Gamma rays 250 Gy	* yielded 3.7% more than the check variety IR72,intermediate resistance to blast, bacterial leafblight,sheath blight & rice tungro virus resistant, to all biotypes of the brown plant hopper, matures 14days earlier than the original Sigadis Milagrosa, grain are long & slender,intermediate amylose content & high milling recovery	* Approved as a variety in 1999 Produced through the cooperative effort between PHILRICE & PNRI

Crop	Parent Material/ Variety	Mutant Variety/ Year Developed	Mutant Dose	Mutated Character/ Main Attributes of the Variety	Status of Mutant
Soybean	L114 (US variety)	L114 mutant (1976)	fast neutrons	*yielded 15% more than the parent material, reduction in height by 40% very elusive character	*Viability was lost due to lack of cold storage
Mungbean	Native Variety	Multi-foliata	EMS	*very leafy has 5,7,9,11 or 13 leaflets per leaf instead of 3	*Can possibly compete with weeds
		PAEC 1* (1975)		*non-shattering pods, moderately resistant to powdery mildew,resistant to Cercospora leafspot, large seeded	
		PAEC 2*		non-shattering pods, large seeded,resistant to Cercospora leafspot	
		PAEC 3*		non-shattering pods with very shiny yellow seeds, large seeded	
		PAEC 5*		resistant to Cercospora leafspot, non-shattering pods	
		PAEC 9*		long pods, non-shattering pods	

Crop	Parent Material/ Variety	Mutant Variety/ Year Developed	Mutant Dose	Mutated Character/ Main Attributes of the Variety	Status of Mutant
Sweet Potato	Kinastanyas	Kinastanyas mutant	5 Gy gamma rays	yielded 49% more than the original parent variety chlorophyll mutant	
Pineapple	Queen	Queen mutant	30 Gy reccurently irradiated with 30 Gy gamma rays	Chlorophyll mutant which look like "ornamental bromeliad" induction of multiple shoots	Chlorophyll mutant will be submitted for registration
Murraya exotica (Kamuning)	*M. exotica*	*Murraya* 'Ibarra Santos' Dwarf mutant	30 Gy gamma rays	Dwarf mutant	Submitted for registration and was approved by the Technical Working Group for ornamentals Will be submitted for registration
Dracaena sanderiana	*D. sanderiana*	*D. sanderiana* chlorophyll mutant	20 Gy gamma rays	Chlorophyll mutant	

Table 2. Anther culture from crosses using mutants and other varieties in the selected F2 plants with desirable traits.

Code	Parentage
PNRI 15	Azucena/Bengawan Mutant
PNRI 94	PARC 2/ Malagkit Sungsong /Sinandoming Mutant
PNRI 243	I R8/Malagkit Sungsong mutant
PNRI 248	Sigadis-Milagrosa Mutant/Denorado
PNRI 249	Sigadis-Milagrosa Mutant/Basmati
PNRI 254	Bengawan Mutant/Basmati
PNRI 255	Bengawan Mutant/Wild Rice
PNRI 257	Malagkit Sungsong Mutant/Denorado
PNRI 258	Azmil Mutant/Azucena//Purple Rice

Table 3. Integration of molecular and biotechnological techniques for conventional Breeding

Crop	Molecular Techniques	Use/Application	Institution
Coconut	Microsatellite or SSR	Diversity analysis mapping & hybridity testing	IPB-CA UPLB
Maize	- do -	For fingerprinting	-do-
Mungbean	AFLP	For mapping pest & disease resistance(*Cercospora leaf spot)*	-do-
Tomato	AFLP	To detect polymorphism between Hawaii 7996(Lycopersicum *esculentum*) resistant parent & Wva 700 (L. pimpenellifolium susceptible parent)	
Allium cepa	Random amplifield polymophic DNA (RAPDS)	To verify the interspecific hybrids of *Allium cepa* L. and *Allium fistulosum* L.	-do-
Mango	Microsatellites or SSR, AFLP	For mapping and diversity studies	IPB, CA UPLB
Papaya(*Carica papaya)*	Genetic transformation (microprojectile bombardment)	To develop ringspot virus resistant papaya	-do-
Corn	Adaption of B+ transgenic technology for Asiatic corn borer resistance	For resistance to corn borer	-do- -do-

Table 4. Status of biotecnology activities in IRRI (Hossain et al, 1999)

Trait	Bioscience	Biotechnology	Breeding Application
Biotic Stress			
Tungro			
Bacterial blight			
Blast			
Sheath blight			
Stem borer			
BPH			
WBPH			
Gall midge			
Nematodes			
Abitic Stress			
Salinity			
Drought			
Submergence			
Fe toxicity			
Al tixicity			
High light			
New Frontier			
Apomixis			
N2 fixation			
Perennial rice			
Others			
Aroma			
CMS			
TGMS			
Yield			
Fe content			

DNA marker _____

Transformation ----------

Table 5. Rice biotechnology projects/activities in the different programs (after Sebastian, LS and SR Obien, 1999)

Projects/Activities	Programs			
	Direct seeded	Transplanted	Hybrid	Adverse
1. Use of the DNA marker technology for rice improvement		x	x	
a. Pyramiding bacterial blight resistance gene				
b. Mapping/tagging	x	x	x	x
-resistance genes (tungro, bacterial blight,blast)	x			
-seedling vigor			x	
-CMS		x	x	
c. Varietal fingerprinting & biodiversity analysis	x	x	x	
2. Cloning of tungro resistance gene				
3. Genetic transformation-resistance to BPH, blast, and bacterial				
blight, salt tolerance, male sterility, seedling vigor, and beta	x	x	x	x
carotene.		x		
a. Xa21, chitinase, pin2 and hva1 genes				
b. beta glucanase gene	x	x	x	x
4. Screening of GMO (bacterial blight, tungro, beta carotene)				
5. Anther culture (salt tolerance & cold tolerance)				
6. Wide hybridization (tungro resistance)				

3.6 Molecular Techniques as Complementary Tools in Orchid Mutagenesis

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Abstrack

Orchid breeders have always been dependent on hybridization technology to produce new orchid hybrids and varieties. The technology has proven very reliable and easy to use and has produced wide range of successful cultivars with attractive combinations of spray length, bud number, flower colour and form, vase life, fragrance, seasonality, and compactness.

By introducing mutagenesis however, wide variations of flower colours, form and size can still be obtained in addition to overcoming the problem of sexual incompatibility and sterility.

In addition, complementary use of molecular techniques will allow breeders to target more specific characteristic changes and cut short breeding time. PCR-based techniques used to analyse the DNA of mutagenic clones found polymorphic fragments that can be developed as molecular markers.

This paper describes how mutagenesis and molecular techniques can be used to enhance orchid breeding efforts.

Background

Orchid breeding has been dependent mainly on sexual hybridisation. Ever since the first man-made orchid hybrid the *Calanthe domini* flowered in 1856, thousands of hybrids have been produced. From this first inter-species hybrid of *Calanthe furcata* and *Calanthe masuca*, hybridizations have been made not only inter- or intra species but between 2 and up to 4 genera. Wide range of successful cultivars with attractive combinations of spray length, bud number, flower colour and form, vase life, fragrance, seasonality, and compactness have been produced. By combining the dominant colour of one parent with that of another, hybridists were able to increase the intensity of each and in some cases come up with a blend of the two or even a completely new colours that can not be found naturally (Griesbach, 1984). When crosses proved to have superior qualities and to breed true, any future crosses made by hybridists from them had not only superior flowers but also plants that were more vigorous. The possibilities for genetic variations from the hybridisation techniques are seemingly so vast, that more colors and forms are expected from various combinations of sexual hybridisation.

Inherently however, hybridizations are limited by the availability of the desired genotype and sexual compatibility of the genetic sources. Breeding time is also very long due to long sexual cycle (generally 3 years from first seed to first flower). The only advantage in this long breeding time is that it provides sufficient time for hybridists to keep accurate records, so that it

is possible to trace the ancestry of any hybrid right to the original species. It is also true that undesirable characteristics could be inherited as well.

Although the ability to hybridise orchids across several genera has to a certain extent overcome the limitation of gene pool, the heterozygous nature of the material makes the alteration of individual traits impracticable. Furthermore, certain traits of potential value such as resistance to viral, bacterial, and fungal diseases, unique colour such as rose red and blue are rare or absent among the breeding sections.

Thus, even though hybridization technology has been very successfully applied in orchid breeding, very reliable and easy, an introduction of new technology is still essential to ensure the sustenance and expansion of the orchid industry. Over the years there are a few technological landmarks that has significantly influenced the breeding procedures of orchids and thus accelerated the growth of the orchid industry.

One of the most significant technological landmarks is *in vitro* technology. Orchid is one of the most successful plants to be propagated through tissue culture. It was Dr Lewis Knudson who first developed an asymbiotic method of culture and successfully germinated orchid seeds on a growing medium containing sugar as source of carbohydrate. This has then been the standard method of germinating seeds and propagating seedlings for the industry. Dr Georges Morel then pioneered the meristeming technique that revolutionized orchid propagation. Through this method thousands of plants can be grown in a relatively short period of time and every regenerated plant is exactly like the parent plant in every aspect. The meristem cloning procedures has become a very important procedure in clonally producing hybrids of choice in the orchid industry. Various other tissue cultures manipulations or procedures have also been developed that contribute as an important basis for the applications of the next technological landmark.

Like in any other fields in the life sciences, research in orchids is not exclusive to the impact of the discovery of the DNA structure by James Watson and Francis Crick in the 1960s. The science of molecular biology is beginning to be appreciated in the studies of orchids, especially in the understanding of orchid phylogeny and classification. The utilization of molecular techniques for orchid conservation has been described and reviewed (Qamaruz-Zaman *et al.*, 1988a, b). Further applications of molecular techniques present powerful approaches for the understanding of orchids, their biology and molecular genetics. In complementation with hybridization these techniques offer more controlled trait introgression and shortened breeding time to improve existing successful cultivars with desired traits.

In Malaysian context, another technology that has been found useful and effective for generating new varieties is mutagenesis. There are not many published reports on mutagenesis work on orchids. The lack of application of mutagenesis technology in orchid improvement may be due to many reasons such as the ease of getting wide range of variation from sexual hybridization, the wide range of crosses that can be made between species and genera, polyploidy level of orchids, the specialized facilities and high capital associated with the setting up of mutagenesis work.

This paper relates the Malaysian experiences in orchid mutagenesis and how molecular techniques can complement the breeding approach for orchid improvement.

Orchid Mutagenesis

Induced mutation technique is thought to be an effective tool to complement the hybridization technique especially in cases where sterility or incompatibility is encountered. The idea of using this technique was first proposed in 1980 but it was not until 1990 that this idea was earnestly implemented at MINT. Radiosensitivity of *Dendrobium, Cymbidium* and *Vanda* was investigated. Consequently, exploratory experiments conducted on *Dendrobium* Ekapol resulted in changes in colour, shape and size of the flowers (unpublished data). At the *in vitro* stage leave variegations was observed on shoots regenerated from the irradiated protocorms. Higher doses were observed to inhibit growth of protocorm into shoots and plantlets. Based on these preliminary results and observations, an *in vitro* mutagenesis studies on a commercially popular *Dendrobium* 'Sonia' were conducted to develop new range of flower colour and shape.

Selection of induced mutations was done after the first flowering of the plants regenerated from the irradiated protocorms.

Following the first flowering of each plant, the spray was harvested when it was at 70% blooming stage. As a preliminary classification and to simplify maintenance as well as data collection, plants that produced flowers with similar morphology are grouped together. Table 1 illustrates the morphological characteristic of some of the mutants that have been selected. To date, mutants that showed stable characteristics changes have been grouped into 27 categories based on flower colour and form. Data such as the horizontal and vertical measurement of fully bloomed flower, thickness of petals, sepals and lip of the flowers were taken. Colour grading was based on the colour chart by the Royal Horticultural Society of London (RHS colour chart). Wide range of variations in flower form and colour were observed. The variations consist of numerous combinations of colour and shape variations in the petals, sepals and lip of the flower (Sakinah *et al.*, 2000).

The mutant flowers also show a single characteristic change such as flower colour while the other characteristics remain the same as the control. One mutant, DS 35-White which has totally white flower, has similar flower shape as the control but smaller in size. Most mutants show a wide range of purple shades in the flower colour as characterised according to RHS colour chart. The different color variations are expressed as whole or gradual distribution of intensity from pale to darker shade in the petals or sepals.

The mutant flowers also show differences in size. They can be larger or much smaller than the control. Flower size of mutant can be 20% larger although the shape of the flower is similar to the control *Dendrobium* 'Sonia'. The smallest mutant flower measuring 5.5 cm horizontally and 5.7 cm vertically is 31.25% smaller than the control.

The arrangement of the flowers along the spray is also affected by the irradiation treatment. Some sprays are observed to have a very tight and neat flower arrangement on the stalk. While others may have flowers that are widely spread laterally or arranged on a zigzag stalk. The length of the stalk and number of flowers on the stalk varies between plants but this could be due to the plant age rather than mutations.

There are a few unstable variations in flower colour and form which were observed after the first flowering. One mutant plant for example, produced spray with darker coloured flowers at the base of the spray but gradually became lighter coloured towards the tip. However, on the second flowering the spray did not exhibit the same characteristics.

Several other mutant plants produced spray that has mixed of deformed and normal flowers. The deformities observed included loss of petal or sepal, fused petals, and two flowers attached together by the sepals. These deformed flowers often appeared only at one blooming point of a single spray, and did not recur in the subsequent flowering.

DNA analysis of orchid mutants

The variations exhibited by these mutants created an interest in the molecular biology of orchids. A PCR-based analytical tool is thought to be appropriate for DNA analysis of the mutant lines. Several mutants were then selected for DNA analysis to detect polymorphisms as result of irradiation and develop marker that can be used for fingerprinting or to isolate useful genes. Mutants were selected based on flower colour or flower form for PCR-based RAPD analysis using arbitrary 10-mer primers (Promega) which mostly constitute of at least 50% GC content.

Random amplification of the DNA isolated from mutant lines and the control plant was carried out using the Perkin Elmer Cetus GeneAmp PCR Kit on the Perkin Elmer GeneAmp PCR System 9600. The amplification products were run on two sets of 2.0% agarose gel, stained with ethidium bromide and photographed.

Table 1. Flower characteristic of selected mutants and control

Mutant	Description	Horizontal (a) (cm)	Vertical (b) (cm)	Petal (cm)	Sepal (cm)	Lip (cm)
Control	Red purple (71A) petals, sepals and lip. Narrow petals. Long and narrow lip with curled at the edges.	8.0	7.3	L : 4.7 W: 3.0 T : 0.075	L : 4.0 W: 1.6 T : 0.062	L: 3.7 W: 2.0 T : 0.067
DS 35-C	Purple (78B) narrow petals with curled at the edges. Smudge of purple (78B) sepals and purple (78A) long and narrow lip with curled at the edges.	9.0	8.2	L: 5.0 W: 3.5 T : 0.077	L: 4.6 W: 1.7 T : 0.067	L: 4.0 W : 1.9 T : 0.053
DS 35-I	Purple (78C) narrow and elongated petals. Petal colour is paler around the edges and more intense towards the center region of the petal. Smudge of purple (78C) sepals and purple (78B) long and broad lip.	9.5	9.6	L: 6.2 W: 3.6 T: 0.079	L: 5.4 W: 1.7 T: 0.074	L 4.2 W: 2.3 T: 0.075
DS 35-R	Purple (78B) petals, sepals and lip. Narrow petals. Long and narrow lip.	6.7	6.5	L: 4.5 W: 2.7 T: 0.063	L: 4.2 W: 1.4 T: 0.061	L: 3.0 W: 1.4 T: 0.044
DS 35-M	Purple (78C) petals, sepals and lip. Narrow and broad petals. Veinous petals and sepals. Long and broad lip with curled at the edges.	7.4	7.1	L: 4.4 W: 3.3 T: 0.068	L: 4.0 W: 1.7 T: 0.057	L: 4.0 W: 3.9 T: 0.064
DS 35-U	Purple (78B) petals and lip. Smudge of purple (78B) with green tip sepals. Short and broad lip with curled at the edges.	6.5	5.6	L: 3.7 W: 3.3 T: 0.070	L: 3.3 W: 1.7 T: 0.056	L: 3.0 W: 2.1 T: 0.074
DS 35-N	Smudge of purple (76B) petals and sepals. Rounded petals. Purple (76A) short and broad lip with curled at the edges.	5.5	5.7	L: 3.1 W: 3.0 T: 0.064	L: 2.9 W: 1.8 T: 0.055	L: 2.7 W: 2.0 T: 0.049

Table 1 contd.

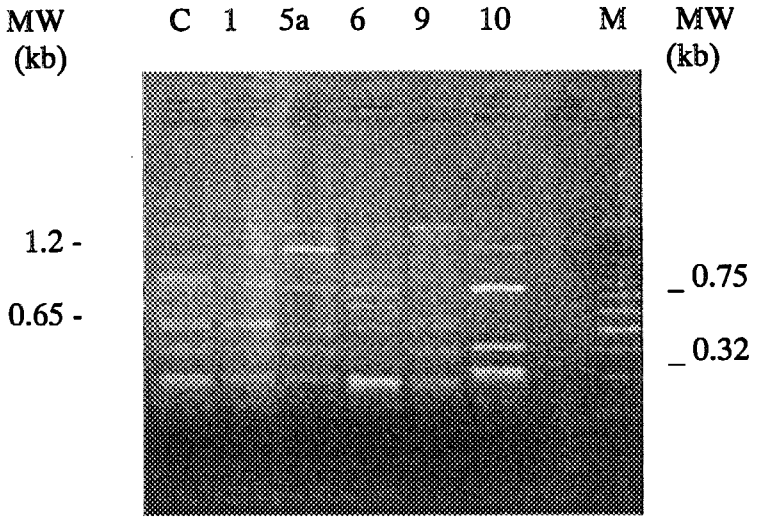
Mutant	Description	Horizontal (a) (cm)	Vertical (b) (cm)	Petal (cm)	Sepal (cm)	Lip (cm)
DS 35-White	Pure white petals, sepals and lip. Narrow petals.	7.3	6.7	L: 5.0 W: 3.4 T: 0.063	L: 4.5 W: 1.7 T: 0.051	L: 3.8 W: 1.7 T: 0.058
DS 35-135	Purple (78A) veinous, broad and elongated petals. Smudge of purple (78B) sepals. Purple (78A) long and broad lip with curled at the edges.	8.2	7.2	L: 4.5 W: 3.7 T: 0.052	L: 3.9 W: 1.7 T: 0.044	L: 3.6 W: 2.4 T: 0.047
DS 35-1/B	Purple (78B) broad petals. Petals is white at the center but gradually becomes purple towards the edges. Smudge of purple (78D) sepals. Purple (78B) narrow and curled backward lip.	8.5	7.5	L : 4.7 W: 3.5 T: 0.068	L: 4.3 W: 1.8 T: 0.056	L: 3.5 W: 1.9 T: 0.059
DS 35-1/O	Red-purple (71A) petals, sepals and lip. Rounded petals. Short and broad lip	7.0	6.5	L: 3.8 W: 3.5 T: 0.060	L: 3.5 W: 1.9 T: 0.050	L: 3.5 W: 2.4 T: 0.060
DS 35-2/L	Purple (78B) petals, sepals and lip. Narrow and curled backward petals. Veinous sepals. Short and broad lip.	6.2	6.5	L: 4.0 W: 3.1 T: 0.065	L: 3.5 W: 1.6 T: 0.052	L: 3.5 W: 2.1 T: 0.057

*Colour description-according to RHS colour chart
(L) : Length
(W) : Width
(T) : Thickness

Figure A shows that amplification by duplex arbitrary primer combination 10-92 has produced polymorphic DNA fragment of approximately 750 bp in lane10, 6 and 5a, fragment of approximately 320 bp in lane 10 and another DNA fragment of 1.2 kb present in lane 5a and lane 10. All these fragments are not present in the non-irradiated parent orchid (lane C). DNA polymorphism is detected between mutants as well as between mutants and parent (M. Nazir *et al.*, 1998).

In another analysis, two fragments had been sequenced and were found to have 80% homology to a retrotransposon (Ling, 1998). This is an interesting finding that warrants further research. A confirmation on the presence of transposable element in orchid genome may provide explanation on observed changes in characteristics through the process of tissue culture as well as the unstable characteristics observed amongst the mutants in this studies. It may also open up a new research front in orchids that can lead to gene isolation by gene tagging or insertion mutagenesis.

Fig. A RAPD profiles obtained with duplex arbitrary primer combination primer-10-92



Molecular breeding of flower

Complementing the mutagenesis technology to improve colour in orchid, is an approach utilising advance molecular techniques. In general, these techniques are applied for the manipulations of flower colour, modifications of plant and flower architecture, creation of longer-life flowers and fragrance modifications. Molecular approaches such as these, have allowed researchers to alter individual traits in agronomic and ornamental crops if the genes encoding the desired trait are available in a clone form, if the target crop can be transformed and regenerated, and if the inserted gene can be expressed in a predetermined manner.

The availability of cloned genes has increased as a direct result of the *Arabidopsis* and rice genome sequencing projects; this is particularly true for those genes that are difficult to clone by conventional means. At the same time, the biochemistry and molecular genetics of anthocyanin production which responsible for flower color, the homeotic genes for flower

architecture, and biosynthesis of ethylene that primarily responsible for post-harvest life have been extensively researched into and reviewed. These advances have formed a good foundation for molecular approach in orchid breeding. The available tools and knowledge can cater for specific breeding objective and in some cases shorten the breeding time.

Based on the above considerations, the current orchid mutagenesis work has been complemented with genetic manipulation of flower color. The objective is to be able to manipulate and control the expression of the genes responsible for flower colour. However, to be able to do this it was also imperative that the gene transfer system for orchid is developed. Thus both approaches were developed concurrently.

Flower color manipulations

Flower colour is due to three different pigments which are located in different organelles in the cells. The green color is produced by the pigment chlorophyll which is located in the chloroplasts found throughout the sepal and petal cells. The pigment carotenoids in the chromoplasts on the other hand, are responsible for yellow and orange colours. While these two are lipid soluble, the other pigment the flavonoids are water soluble and found within the cellular vacuole. Flavonoids are responsible for the blue and red colour.

The mixing of these three pigments (flavonoids, chlorophyll and carotenoids) in different proportions gives the resultant colour of the flower. For example, a cross between *Phalaenopsis amboinensis* and *Doritaenopsis* Grebe produced a new bronze colour which could not be found in nature as a result from combining a gold carotenoid with magenta anthocyanin (Griesbach, 1984).

While the biochemistry of carotenoids and chlorophyll in relation to flower colour is little known, the flavonoids has been subjected to manipulations to change flower colour. The flavonoids can be subdivided into several groups: anthocyanins, flavonols, aurones, chalkones and gossypetins. In addition, there are six major anthocyanins – pelargonidin, cyanidin, delphinidin, malvidin, petunidin and peonidin.

Enzymes like Chalcone synthase (CHS), Flavanone 3-Hydroxylase (F3H) and Dihydroflavonol Reductase (DFR) have been identified to play an important role in the flavonoid biosynthesis pathway. The Phytoene Synthase on the other hand is the first enzyme in the carotenoid biosynthesis pathway that determine the pigment for yellow and orange colours. Since each pigment is the result of a different sequence or pathway of biochemical reactions and the production of each pigment is independent of the each other, blocking the synthesis of the one pigment will have no effect on the synthesis of the other pigments. However, it will result in flower colour change since flower colour is a result of mixing of the pigments. Accordingly, it was found that the antisense reduction in expression of CHS and other key flavonoid biosynthetic enzymes has resulted in the production of white flowers in transgenic plants (van der Krol *et al.*, 1988).

Following a similar approach, antisense constructs for CHS, F3H and DFR are made and used to transform callus and PLBs of *Dendrobium* and *Oncidium* hybrids. The antisense constructs are made from partial gene sequences of flavanon-3-hydroxylase (F3H), chalcone synthase (CHS), dihydroflavanol-4-reductase (DFR) and Phytoene synthase (PSY). These partial gene sequences were isolated by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method from *Oncidium* flowers. They showed 67% to 89% homology to homologues from other plant species. Antisense transformation vectors driven by a 35S promoter was constructed firstly by cloning into pHK17 to generate the antisense expression

and secondly to subclone the expression cassette into pCAMBIA. The sequence was inserted in a reverse orientation with the 3' end of the gene joint to the 35S promoter (Sugumaran *et al.*, 1999).

Another approach that is being considered is the reverse genetics approach, utilizing the mutant lines obtained from the mutagenesis work. Gene sequences such as the F3H, CHS, and DFR are already available and can be used to identify orthologs responsible for the corresponding traits in various orchid mutants that display various shades colors including total absence of pigment. Reverse genetics approach such as this will lead to the cloning, isolation and characterisation of the corresponding genes in orchid, the understanding of patterns of expression, and identifying the differences in the control of expression or the functions of the genes through the analysis of gene malfunction in the mutant lines.

Gene transfer in orchids

The development of efficient gene transfer technique for orchids is considered as an important tool for the breeding of resistance to viral, bacterial and fungal diseases as well as unique flower colors such as rose red and blue. Direct introduction of desired gene will also shorten breeding time.

Studies on wide range of gene transfer techniques by Nan and Kuehnle 1995 showed that electroinjection and microprojectile bombardment are suitable for *Dendrobium* while seed imbibition and the pollen-tube pathway were ineffective. Successful transfer using the biolistic method (microprojectile bombardment) has also been reported by several other groups using luciferase (Chia *et al.*, 1994), β-glucuronidase (GUS) and neomycin phosphotransferase (Kuenhle and Sugii, 1992; Yang et al., 1999) as marker genes for screening and selection. Neomycin phosphotransferase which confers resistance to antibiotic kanamycin however, has been found to be leaky and very ineffective as a selectable marker for orchid transformation (M. Nazir unpublished; Pillai personal communication; Wee *et al.*, 1999).

Based on these work an efficient and reproducible transformation procedure was developed using the biolistic method on *Oncidium* and *Dendrobium* using hygromycin phosphotransferase gene as selectable marker (Pillai *et al.*, 2000). Hygromycin was used as selective agents as it gives a very clear selective level for the transformants. The procedure has been used to deliver the antisense construct of the ACC oxidase gene into Dendrobium Savin White, and other antisense constructs for controlling flower colors (CHS, F3H, DFR and PSY) into different hybrids of *Oncidium* and *Dendrobium*. PCR analysis and Southern Blotting have showed that these genes have been successfully transferred into orchids. Transgenic plantlets are currently being grown to maturity in the greenhouse.

Discussion

The approaches adopted for flower colour manipulations are based on genetics. However, it is well known that anthocyanin coloration is influenced not only by genetic basis but also an environmental basis. A considerable amount of research has been devoted to studying the effects of flavonoids on flower colour as well as how the environment (pH, metals, light intensity, temperature, co-pigments, etc.) can modify the expression of flavonoid colour.

In most orchids fortunately, the flowers are buffered. Soil pH has no effect on flower colour as in the case of hydrangea, which produces pink flowers when the soil pH is 6.0 and blue flower when the pH is 5.5. The pH of the orchid flowers is predominantly under genetic control with very little environmental interaction. The pH of petals appears to be controlled by a small number of genes, thus one can breed for pH to obtain colour variations. Breeding for such trait as increased or decreased pH of flavonols, may create an almost endless range of different flower colours.

Very little is known about how carotenoids influence flower colour or how combinations of the plastid pigments and flavonoids can change the colour of flowers.

A thorough knowledge of both parentage and cultural conditions are necessary to adequately judge flower colour in orchids as there are many other environment factors affecting the colour.

Challenges

The efforts for modifications of flower color of orchids through mutagenesis in complementation with molecular techniques have produced some interesting results. Genes needed for the modifications of flower color, have been cloned, sequenced and successfully introduced into *Dendrobium* and *Oncidium* orchids. The expression of these introduced genes will be analysed. These manipulations are done by introducing conventional anti-sense genes constructs using the particle gun approach that relies on antibiotic marker genes for selection. Greater awareness in biotechnological approaches however, has generated a lot of concerns among the public and the scientific community regarding the use of antibiotic marker genes, the interspecies genes transfer, the introduction and manipulation of large gene sequences without in-depth knowledge of the long-term effects, as well as the non-specificity of the insertion sites. Ways are sought/researched into to find alternatives that do not rely on antibiotic marker genes in the gene transfer procedures as well as for a more precise insertion and minimum genetic alterations.

Two recent advances in introducing a more specific change to a specific gene are described in the homologous recombination-dependent gene targeting and chimeric oligonucleotide-dependent mismatch repair techniques. In the later, recombinant heritable DNA/RNA hybrid oligonucleotides are introduced using standard techniques (biolistic, electroporation etc.) into cultured cells where they bind to the DNA region of interest. The introduction of 'mutated' chimeric oligonucleotides will result in change in the function of the targeted gene with no other effect on the metabolism or other gene functions in the plant. Although the efficiencies of these procedures is still not favorable for recovery of targeted events in genes that cannot be selected for, this will be the general approach for gene alteration in the future.

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3.7 Application of Molecular Techniques to Crop Mutation Breeding in Vietnam

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1. Introduction

Biotechnology provides a powerful tools for crop improvement. It assists in raising the efficiency of rapidly creating new crop varieties with high yield, good tolerance and quality. Up to now, several molecular markers have been available for using to comprise RFLP and the ones based PCR such as: RAPDs, SSR, AFLP, RGA...(Mullis et al. 1987; Foolad et al., 1995; Tansley et al., 1989, Vos et al., 1995; Chen et al.1998). Those markers are being extensively exploited in genotype identification, pathogen identification, taxonomy, genome structure analysis, ecology and population biology.

Analysis of plant genetic diversity has been carried out in several recent decades. Almost studies have concentrated to assess the total genetic variation to aim at line identification, determination of pedigrees and variety protection (Mackill et al. 1995; Maheswaran et al. 1996). And recently, the molecular markers have been also effectively applied in mutation breeding (Chu, 2001). The studies have mainly looked for the differences at genome structure level between original variety and mutant lines (Ladin et al. 1984; Masato et al. 1989; Chu 2001). So far, many genes have been identified and tagged with various kinds of molecular markers (McCouch et al. 1988; Tanksley et al. 1989; Becker et al. 1997, Vu et al. 2000). Studies on gene tagging can provide the information on the number of genes controlling characters and the map location of those genes. Such information is very valuable in using genes in crop breeding. The mapped genes have been used to pyramid into crops via marker aided selection (MAS). Many genes were cloned from different creatures, some genes controlling good agronomic traits such as: Bt, Xa-21...which have been introduced into crops to aim at creating new crops carrying designed traits (Zheng et al. 1995). MAS can enable the breeder to rapidly identify individuals that possess the genes affecting the traits (Tanksley et al., 1989; Wang 1992; Rafalski and Tingrey 1993; Mauleon 1995; Mendoza 1996).

In Vietnam, many crops have been released and popularly grown by the farmers, they generated via mutation breeding. Among those crops, rice comprising varieties: DT10, DT11, A20, CM1, DT33.., maize consisting of DT6, DT8..., peanut: B5000, V74, 4329, D332, D329, DPC9102.., soybean: DT84, DT90, DT95, M103, V48, A5...(Tran et al. 2000). However, almost of aforementioned varieties only selected based on morphological parameters which much depended on the environmental conditions, plant physiological status and stage of gene expression...Therefore, in order to resolve the limitations when using traditional methods, we have planned to apply the molecular techniques to characterize the mutant genotypes obtained in mutation breeding. In this study, we carried out to analyze mutant genotypes of soybean, green pea and map TGMS gene in mutant rice line using molecular techniques.

2. Materials and Methods

Materials were used in this analysis to comprise the mutant rice, soybean, green pea lines which obtained by gamma treatment. For rice, the TGMS-1 mutant rice line was obtained by treating gamma ray on a Chiem Bau rice variety of Vietnam, this mutant line was confirmed to carry a TGMS gene. The mutant soybean lines consisting of ML10, ML48, ML59, ML61, ML70, ML81, and mutant green pea lines including MXU21, MX1103, TXU21 and TX16012.

Three markers were mainly used to comprise RFLP, AFLP and RAPD. RFLP and AFLP markers were used to map TGMS gene that located in genome of the TGMS-1 mutant rice line, and RAPD markers helped to analyze the mutant genome of soybean and green pea lines.

3. Results and Discussions

1) Analysis of Soybean and Green Pea Genomes

The mutant soybean lines were subjected to DNA fingerprint using RAPD primers. The PCR products were electrophoresed in 1.5% agarose gel to analyze genetic variation among lines (figure 1). Three RAPD primers such as: RA31, RA36 and 142 showed the polymorphism between the mutant soybean lines and original variety. The polymorphism among them were demonstrated by present of new DNA bands and absent DNA bands. This suggests that the genotype of original soybean variety had quite change after treating gama ray leading to the high polymorphism between it and mutant lines. The genetic relationships among lines were analyzed using NTSYS software (table 1). The result indicated that the mutant lines and their original variety clustered in 5 groups and had highly genotypic differences ranging 14% to 47%. Among them, two lines: ML81 and ML48 showed highest similarity but the different index reached 0.14. Line ML59 differentiated from ML48 and ML81 to be 0.18 and 0.23 respectively. The genetic distance of ML48, ML81 and ML59 to their original variety was 0.22, while line ML61 was far from its original variety and ML59, ML48, ML81 a distance of approximately 0.36. Different index between ML10 line and line ML70 was 0.23 and both had genetic distance to be far from their original variety was 0.47 so they clustered in a group.

For green pea, three out of 10 primers (consisting of RA36, RA45 and 142) gave polymorphism among mutant green pea lines tested (figure 1). Total number of DNA bands obtained for three primers ranged from 25 to 34. The DNA band number of original variety were less than those of two mutant lines: TXU21 and TX16012. Comparison of differences between original variety and mutant lines indicated that the lines: MXU21 and MX1103 differentiated from original variety a distance of 0.28 and 0.35 respectively, and among these two lines had genetic distance of 0.40 (table 2). Finally, the results of this study indicated that among mutant lines of green pea obtained to have different genotypes and they also differentiated from original varieties.

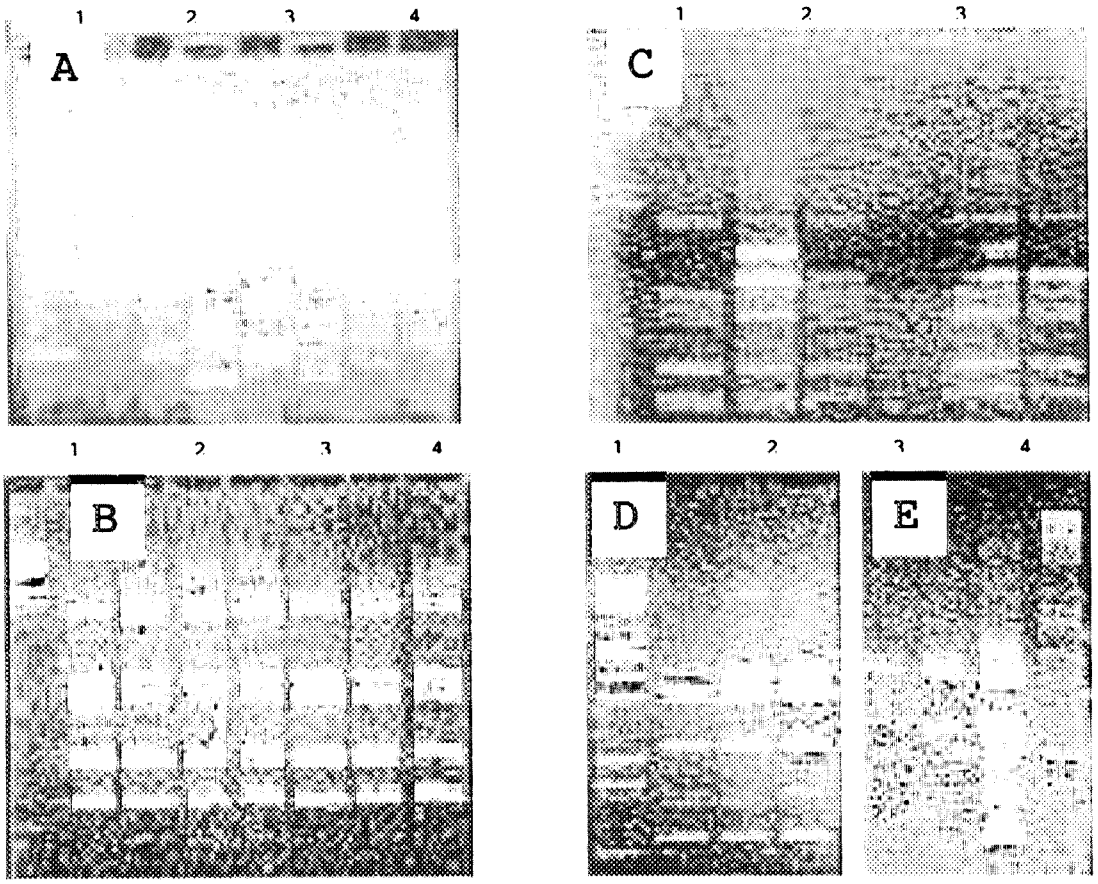

Figure 1. DNA fingerprints of soybean and green pea using RAPD markers

A: DNA fingerprints of soybean using primer: RA31. Lane 1: λDNA EcoRI+HindIII, lane 2: original variety, Lanes 3-8: mutant lines.

B: DNA fingerprints of soybean using primer: RA36. Lane 1: λDNA HindIII, lane 2: original variety, Lanes 3-8: mutant lines.

C: DNA fingerprints of "Mo" green pea using primer: RA45. Lane 1: λDNA EcoRI+HindIII, lane 2: "Mo" original variety, Lanes 3: MXU21, 4: MX1103, lane 5: "Moc tieu" original variety, lane 6: TXU21, lane 7: TX16012.

D: DNA fingerprints of "Mo" green pea using primer: RA36. Lane 1: λDNA EcoRI+HindIII, lane 2: "Mo" original variety, Lanes 3: MXU21, 4: MX1103,

E: Lane 1: "Moc tieu" original variety, lane 2: TXU21, lane 3: TX16012, lane 4: λDNA EcoRI+HindIII

Table 1. Similarity coefficient between original soybean variety and mutant soybean lines

	Original variety	ML81	ML10	ML61	ML70	ML59	ML48
Original variety	1.00						
ML81	0.80	1.00					
ML10	0.50	0.57	1.00				
ML61	0.57	0.73	0.70	1.00			
ML70	0.60	0.48	0.77	0.52	1.00		
ML59	0.80	0.77	0.43	0.60	0.52	1.00	
ML48	0.15	0.86	0.48	0.70	0.52	0.82	1.00

Table 2. Similarity coefficient between original green pea varieties and mutant green pea lines

"Mo" green pea			
	Original variety	*MXU21*	*MX1103*
Original variety	1.00		
MXU21	0.72	1.00	
MX1103	0.65	0.60	1.00
"Moc tieu" green pea			
	Original variety	*TXU21*	*TX16012*
Original variety	1.00		
TXU21	0.70	1.00	
TX16012	0.63	0.60	1.00

2) Mapping of TGMS Gene

In order to map TGMS gene located in genome of the TGMS-1 mutant rice line, we used AFLP markers to look for the close linkages between markers and TGMS gene. Bulked segregant analysis was employed to identify AFLP markers linked to the TGMS gene. A total 200 AFLP primer combinations were used to screen the polymorphism two bulks and two parental DNA samples. Four markers (E2/M5-200, E3/M16-400, E5/M12-600 and E10/M12-

200) were polymorphic between both the bulks as well as the parents. Those AFLP markers were named by using the name of enzyme combination, the primer combination and molecular weight of the AFLP fragment. Co-segregation analysis with AFLP markers using 124 F2 individual progenies was performed. To look for the linkages between four putative AFLP markers and TGMS gene, those markers were radio-labeled and hybridized to blots containing two parents DNA that digested with 17 enzymes. Three out of them consisting of E3/M16-200, E5/M12-600 and E10/M12-200 were single copy, and E2/M5-200 contained repeated sequences. Among four markers, E5/M12-600 showed the polymorphism between parents with ApaI enzyme. The distance between the TGMS gene and E5/M12-600 was estimated to be 3.3 cM based on the recombination data observed from F2 population. This result was consistent with that obtained from AFLP analysis and co-segregation data using AFLP and RFLP blats (Fig 2)

The doubled haploid IR64xAzucena mapping rice population was used to determine chromosome site of the TGMS gene. E5/M12-600 marker detected the polymorphism between parents of mapping population with PstI and EcoRI enzymes while no polymorphism was observed for E3/M16-400 and E10/M12-200 markers. 70 selected doubled haploid lines of this mapping population were surveyed using PstI and EcoRI enzymes. Both combinations E5/M12-600-proble/PstI-enzyme/EcoRI-enzyme showed the segregation in Mendelian fashion. E5/M12-600 marker was mapped on chromosome 2 near the marker RG437 at distance of 9.1. This marker was re-mapped with a DHL mapping population from the cross CT-9993xIR-6226, it confirmed that E5/M12-600 located on chromosome 2. 6 selected RFLP markers (RG437, RZ643, RG157, RZ599, RG171 and RG83) and 5 microsatelite markers (RM8, RM27, RM28, RM53 and RM262) closely linked to E5/M12-600 on chromosome 2 were used for a parental polymorphism survey. One microsatelite (RM27) showed co-segregation with TGMS gene. Based on the result of study, we concluded that the TGMS gene located on chromosome 2 (Fig 3).

4. Conclusion

Application of molecular markers to crop breeding is a necessary and effective approach in Vietnam. This orientation helps to analyze at genotypic level of mutant crops. Therefore, we could effectively use mutant genotypes to aim at creating crops with designed agronomic traits.

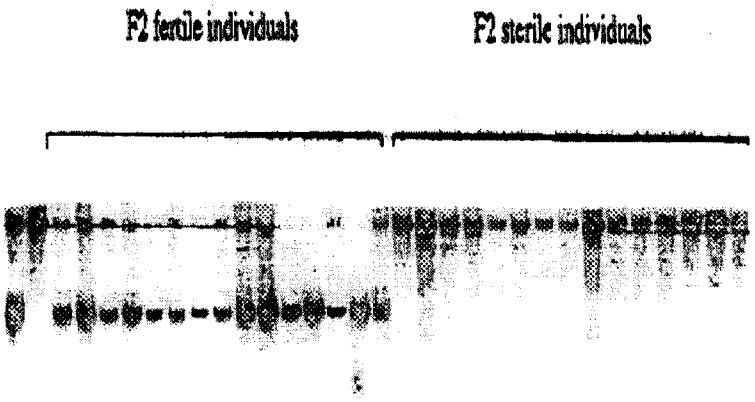

Figure 2. DNA fingerprints of the parents and F2 individuals using marker E5/M12-600. Lane 1: CH1 variety, lane 2: TGMS-1 line, remaining lanes: F2 individuals

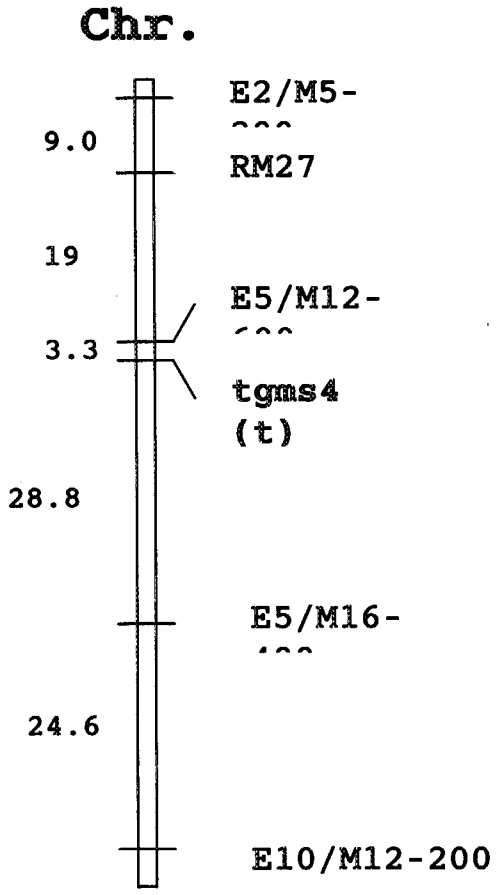

Figure 3. Chromosome site of tgms4 (t) gene.

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3.8 Genetic and Molecular Dissection of Naturally Occurring Variations in Rice

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Introduction

Structural analysis of the rice genome has progressed much in the last decade. More than 40 000 ESTs have been sequenced and deposited in the public DNA databases (Sasaki et al. 1994, Yamamoto and Sasaki 1997). Moreover, a high-density restriction-fragment-length polymorphism (RFLP) linkage map has been constructed and about 60% genome coverage of yeast artificial chromosome (YAC) clone contigs has been achieved (Harushima et al. 1998, Kurata et al. 1997). This progress has allowed us to embark on the sequencing of the whole rice genome (Sasaki and Burr 2000). Resources—genetic markers, sequence data, and genomic clones—derived from such efforts will be used for the functional analysis of rice genes in the next decade. Although artificially induced variations, such as mutants, have been used mainly for genetic and physiological studies in rice and other plant species, the development of DNA markers has made possible access to naturally occurring allelic variations underlying complex traits (Tanksley 1993, Paterson 1995, Yano and Sasaki 1997). Such analysis is often referred to as quantitative trait locus (QTL) analysis. Many QTLs have been mapped for many complex traits in rice (McCouch and Doerge 1995, Yano and Sasaki 1997).

During the analyses of several quantitative traits by the DNA marker-assisted strategy, 2 questions about QTL analysis have been raised: 1) Does a QTL represent a single Mendelian locus or a cluster of multiple loci? 2) Is it possible to precisely map a QTL and identify QTLs at the molecular level using map-based or other strategies? To answer these questions, we have performed a series of analyses on heading date as a model for complex traits. This paper describes a comprehensive analysis of QTLs for heading date, including the identification of putative QTLs, characterization and fine mapping of QTLs using nearly isogenic lines (NILs), and identification of genes at QTLs for heading date by the map-based strategy. In addition, we have developed several primary permanent mapping populations and secondary genetic resources, such as chromosomal segmental substitution lines, to facilitate the genetic analysis of naturally occurring allelic variations. What we have learned so far during this work clearly indicates that naturally occurring allelic variations will be a new resource for functional genomics in rice.

Launching pad for new genetics in rice

The most important aspect in the genetic dissection of complex traits is the effective production of genetic markers in the target chromosome regions. Fig. 1 summarize potential resources for the production of chromosomal region-specific markers in rice. To this end, 2 high-density RFLP linkage maps with RFLP markers were constructed to provide a

framework of markers for the detection of individual factors controlling complex traits (Causse et al. 1994, Harushima et al. 1998, reviewed by Nagamura et al. 1997). Kurata et al. (1997) have also achieved about 60% genome coverage of yeast artificial chromosome (YAC) clone contigs. P1-derived artificial chromosome (PAC) and bacterial artificial clone (BAC) libraries have also been constructed in rice genome sequencing activities (Sasaki and Burr 2000). These YAC, PAC, and BAC clones are also very useful materials for developing region-specific genetic markers. Moreover, about 5000 cDNA clones have been already mapped on YAC contigs that were landed on the genetic linkage map (Wu et al. 2000). These cDNA clones can be used as a potential candidate for genetic markers in target chromosomal regions. Furthermore, the release of rice genome sequence data has been started (Sasaki and Burr 2000). Although completion of the sequencing of the rice genome will need additional efforts, the existing sequence data have already provided and will continue to provide an effective basis for the development of new region-specific genetic markers. These resources will become a launching pad for the analysis of naturally occurring allelic variations in rice.

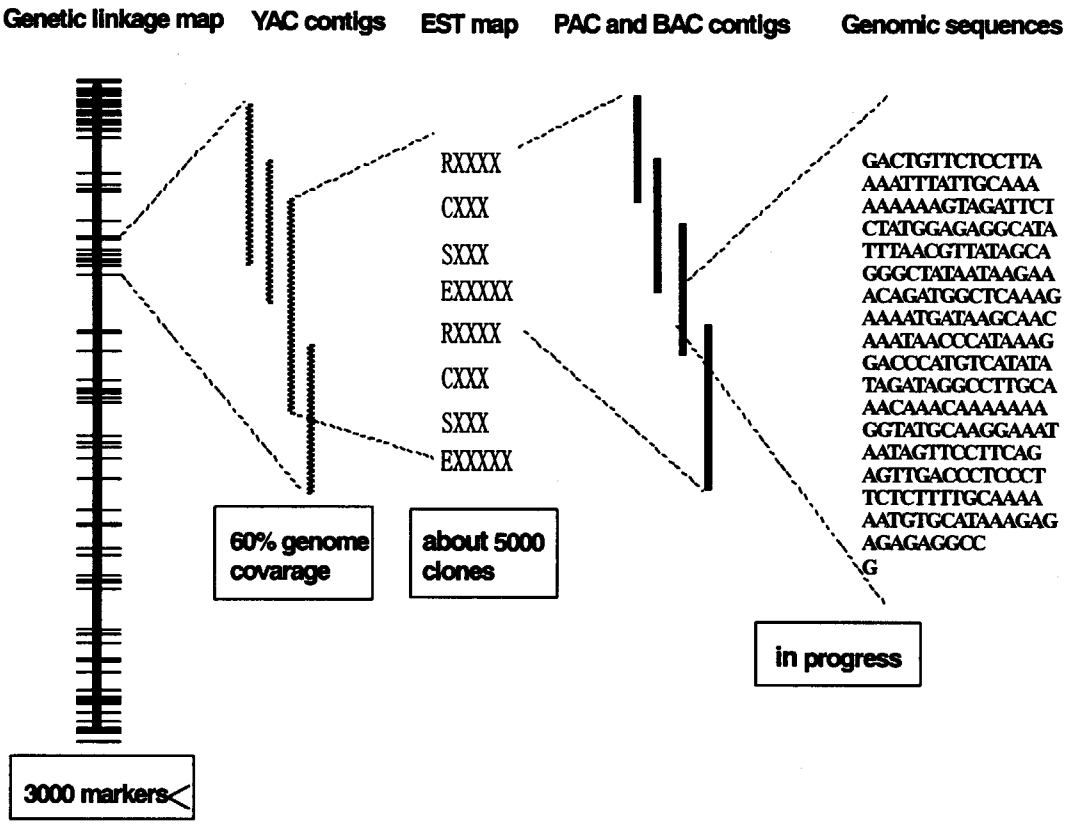

Figure 1. Potential resources for the production of chromosomal region-specific markers in rice.

Detection of QTLs controlling heading date

We have performed comprehensive analyses of heading date as a model trait for complex traits using the resources described above. Thirteen QTLs controlling heading date

have been identified by using several progeny derived from Nipponbare (*japonica*) and Kasalath (*indica*) (Fig. 2). Five QTLs—*Hd1–Hd5*—have been mapped based on analysis of the F_2 population (Yano et al. 1997), and an additional 2—*Hd7* and *Hd11*—have been detected by using BC_1F_5 lines (Lin et al. 1998). In addition, new loci involving heading date—*Hd6*, *Hd8–Hd10*, *Hd12*, and *Hd13*—have been detected by using advanced backcross progeny, such as BC_3F_2 or BC_4F_2, but not F_2 or BC_1F_5 (Yamamoto et al. 2000, M. Yano unpublished data). These results clearly indicate that not all factors involved in the target traits could be detected by using primary mapping populations, such as F_2 and recombinant inbred lines. One reason is the statistical limitation of QTL analysis. It is often difficult to detect QTLs with small phenotypic effects in the analysis of a primary population, because of the noise of QTLs with a large effect or environmental variation (Tanksley 1993, Yano and Sasaki 1997). Another reason may be due to the existence of epistatic interaction among QTLs (Yamamoto et al. 2000, Lin et al. 2000). This point is described later.

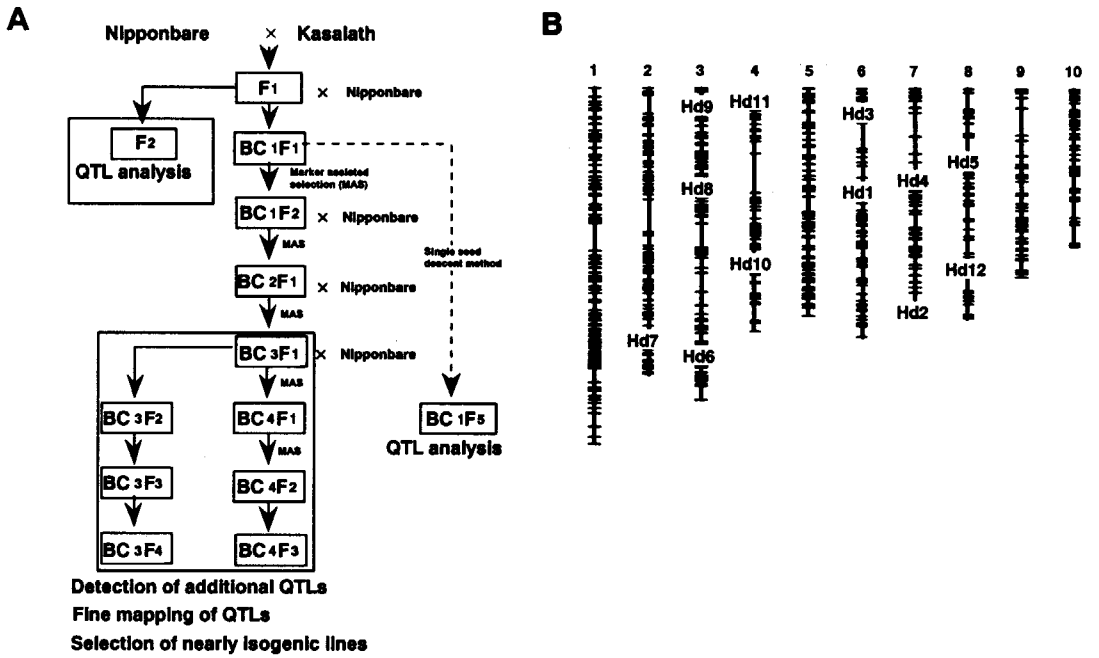

Figure 2. Plant materials used for the detection of QTLs and chromosomal locations of QTLs controlling heading date. A: Mapping populations derived from a cross between Nipponbare and Kasalath. QTL mapping was performed by using F_2, BC_1F_5, and BC_3F_2 lines. Fine mapping and selection of nearly isogenic lines were conducted using advanced backcross progeny. B: A high-density RFLP linkage map showing chromosomal locations of putative QTLs (*Hd1–Hd13*) for heading date.

Fine mapping of QTLs using advanced backcross progeny

Because of the statistical nature of QTL analysis, the chromosomal location of a QTL is not accurate and allows no information about the function of the genes. Populations used in previous QTL analyses of rice were mainly F_2, BCF_1, recombinant inbred lines, or doubled haploid lines (McCouch and Doerge 1995, Yano and Sasaki 1997). These populations, which segregate multiple genetic factors on the whole genome simultaneously,

cannot be used for the precise mapping of 1 of multiple QTLs. Moreover, determination of the true genetic action of the QTL is more difficult, because the genetic parameters of a given QTL are often affected by the segregation of other QTLs.

In plant genetics, nearly isogenic lines (NILs) developed by backcrossing have been widely used to perform accurate genetic analysis, because the segregating populations obtained from crossing NILs and their recurrent parents simplify genetic variation by excluding extra-genetic factors. To get an answer for the main question about QTLs—does a QTL represent a single locus or multiple loci?—we performed fine mapping of a QTL as a single Mendelian factor using advanced backcross progeny. So far, we have shown that 9 of 13 QTLs for heading date could be mapped precisely on the RFLP linkage map as single factors (Yamamoto et al. 1998, 2000, H. X. Lin and M. Yano unpublished data). Only 1 chromosomal region could be dissected into 2 factors with different functions in the control of heading (Monna et al. 2001).

Characterization of genes at QTLs using nearly-isogenic lines

To characterize QTLs, we used marker-assisted selection (MAS) to develop NILs differing only in the presence of a single specific QTL (QTL-NILs) for heading date in rice (Fig. 3). Each line contained the chromosomal region of the target QTL from the donor variety Kasalath in the genetic background of variety Nipponbare. Days to heading was investigated under various controlled-daylength conditions. The QTLs were classified into 2 groups based on response of QTL-NILs to daylength. Five QTLs—*Hd1*, *Hd2*, *Hd3*, *Hd5*, and *Hd6*—were found to confer photoperiod sensitivity (Yamamoto et al. 2000, Lin et al. 2000). However, 3 NILs—for *Hd7*, *Hd8*, and *Hd9*—did not vary in response to daylength. This result indicates that these 3 loci do not confer photoperiod sensitivity. Functions of other QTLs are under investigation.

Analysis of epistatic interaction among QTLs

Many studies have been performed to detect epistatic interactions among QTLs by using primary populations, such as F_2 and recombinant inbred lines, but successful examples of detection seem to be relatively few (Tanksley 1993, Yano and Sasaki 1997). The more the number of contributing QTLs increases, the more difficult it is to detect significant differences to distinguish individual QTLs without using a huge population size. Yano et al. (1997) predicted an interaction between

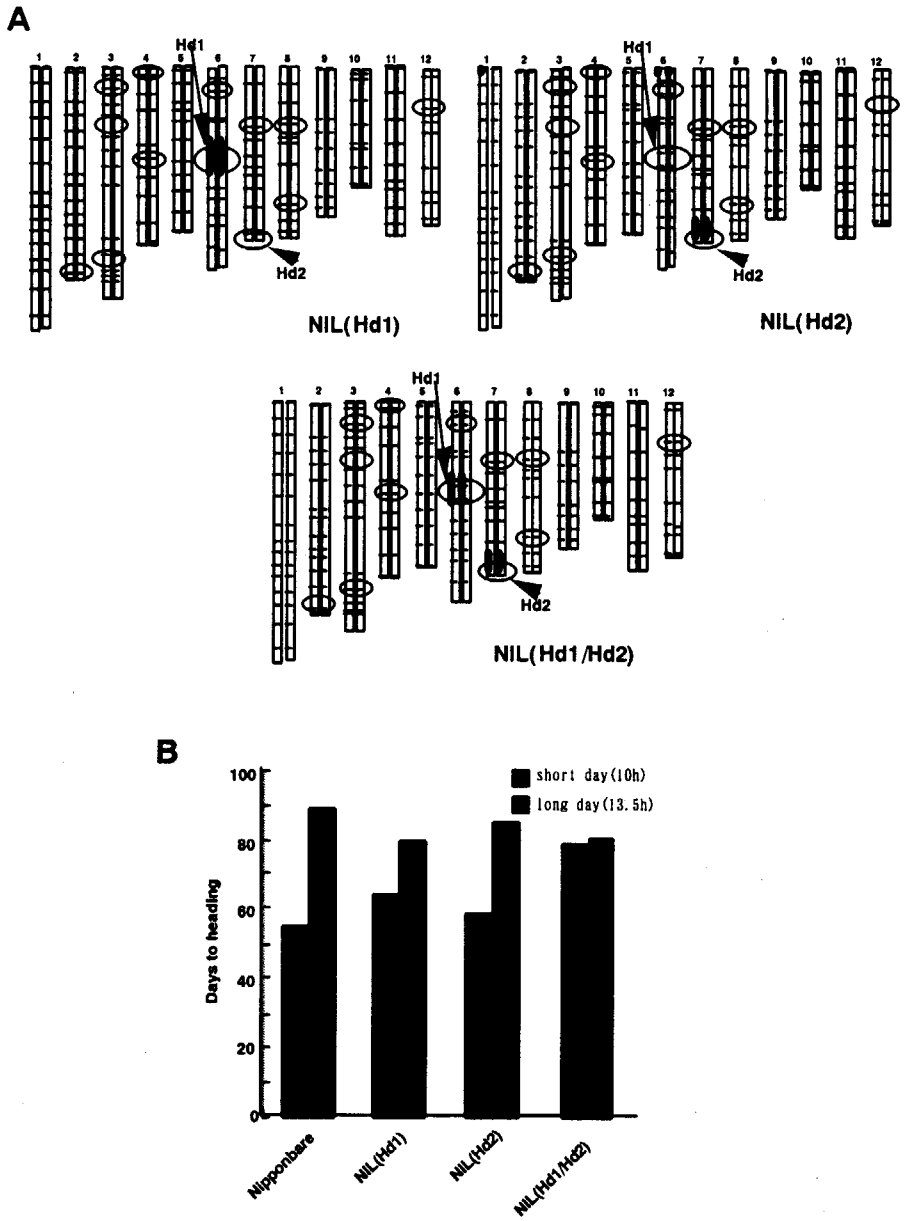

Figure 3. (A) Graphical representation of genotypes of nearly isogenic lines for *Hd1*, *Hd2*, and *Hd1*/*Hd2*, and (B) their days to heading under short- and long-day conditions. The 12 pairs of bars represent the chromosomes, numbered at the top. The horizontal lines on the bars show positions of marker loci used in marker-assisted selection (MAS). The 13 circles on the chromosomes represent regions for heading date QTLs detected. Open bars and solid bars show segments of the chromosomes derived from Nipponbare and Kasalath, respectively.

Hd1 and *Hd2*, the 2 largest QTLs. But the existence of *Hd6* and its interaction could not be detected in their analysis of an F_2 population (Yamamoto et al. 2000). This result suggests that many epistatic interactions do exist among minor QTLs that are not detected in the primary population. Thus, it is necessary to develop new experimental materials, such as chromosomal substitution lines or NILs, for a better understanding of epistasis among QTLs.

The effectiveness of this strategy was proved by the analysis of photoperiod sensitivity QTLs in 2 studies (Lin et al. 2000, Yamamoto et al. 2000), and in other work (Doebley et al. 1995, Eshed and Zamir 1996). It was possible to detect epistatic interactions

among QTLs in 3 ways: constructing QTL-NILs for each detected QTL by MAS based on the results of primary QTL analysis; combining QTLs by crossing all QTL-NILs; and comparing each phenotype with each combining QTL genotype. In addition, secondary F_2 populations derived from a cross between QTL-NILs were used for the detection and confirmation of epistasis between QTLs. The results showed that 3 photoperiod-sensitive QTLs, *Hd1*, *Hd2*, and *Hd3*, interacted with each other (Lin et al. 2000). Based on these results, we suggest that the Kasalath allele of *Hd3* does not affect photoperiod sensitivity by itself but that it is involved in enhancing the expression of the Nipponbare alleles of *Hd1* and *Hd2*. We also showed that digenic epistatic interaction prevented us from detecting major QTLs in a primary mapping population, such as F_2 (Yamamoto et al. 2000).

In the QTL analysis of 186 F_2 plants by Yano et al. (1997), the phenotypic difference caused by segregation of the chromosomal region where *Hd6* is located was surveyed under the simultaneous segregation of 2 major photoperiod-sensitive QTLs, *Hd1* and *Hd2*. This situation could not secure a large enough population to detect the gene effect of *Hd6*, which shows epistatic interaction. As a result, the variance due to the difference in genotypes might not be distinguishable from the variance due to the segregation of other QTLs and to environmental error. On the other hand, the existence of an additional QTL, *Hd6*, and epistatic interaction between *Hd2* and *Hd6* were clearly detected in the analysis of the advanced progeny.

In summary, in QTL analysis of a population in which a QTL with a large effect will segregate, a putative gene effect of an epistatic QTL can be recognized only as a small effect, even if the actual gene effect is large. The complex inheritance of quantitative traits is probably explained mainly by multigenic control, but epistatic interaction is also an important factor for such complexity.

Map-based cloning or candidate identification of genes at QTLs

Figure 4 shows a procedure for the molecular identification of genes at QTLs, from detection to cloning. After the detection of putative QTLs with major effects in a primary mapping population, the construction of special genetic stocks, such as NILs or substitution lines, can be effectively and efficiently achieved by using MAS. For some traits that can be evaluated with high reliability, such as heading date and culm length, high-resolution and fine-scale mapping of putative QTLs as Mendelian factors will be feasible by combining the use of NILs and ordinal linkage mapping based on progeny testing. In the case of traits whose expression and performance are affected largely by environmental factors, it would be more difficult to employ simple progeny testing, such as F_2 plant /F_3 lines. In such a case, advanced progeny testing might be effective at determining the genotypes of target QTLs. We should raise generations beyond the second to obtain fixed lines of a recombinant chromosome for more reliable phenotyping.

Once a given QTL can be mapped as a single Mendelian factor using advanced backcross progeny, chromosomal walking and landing methods can be used for the identification of genes at QTLs (Tanksley 1993, Tanksley et al. 1995, Yano and Sasaki 1997). It will be necessary to increase the population size enough to minimize the candidate genomic region. In our analysis of heading date QTLs, high-resolution linkage mapping allowed us to define a genomic region of < 50-kb as a candidate using 1000–2000 plants. We have developed a new PCR-based marker, the cleaved amplified polymorphic sequence (CAPS) marker, and have used it to facilitate these labor-intensive experiments.

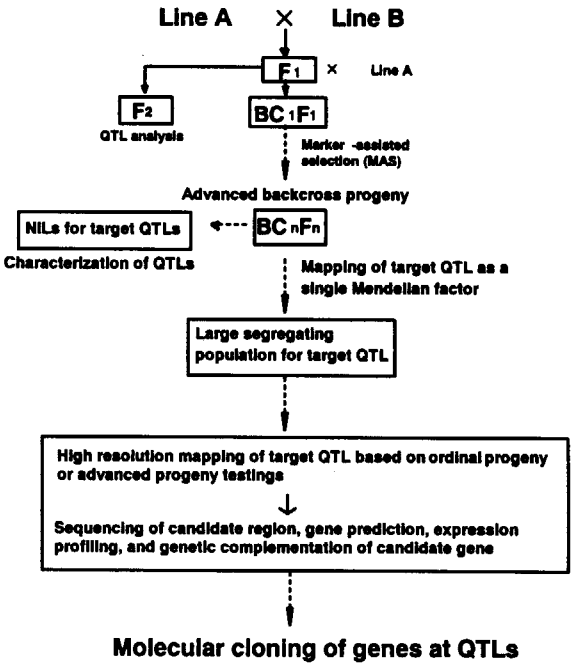

Figure 4. Schematic representation of a strategy for map-based cloning of genes at QTLs.

The production of region-specific markers is another crucial factor in the fine-scale mapping of QTLs. As mentioned above, a large number of resources—YAC, PAC, and BAC clones and about 5000 ESTs—are available for the production of new markers. Combining the analysis of a large segregating population and region-specific markers allowed us to define a candidate genomic region of < 50-kb. After defining a candidate genomic region, we can use several molecular approaches, such as sequencing of the candidate region, gene prediction, expression profiling, and genetic complementation of the candidate gene.

A major QTL for photoperiod response, *Hd1*, was recently isolated and found to encode a protein with a zinc finger structure (Yano et al. 2000). This protein shows high similarity with *CO*, a gene for photoperiod response in *Arabidopsis*. The most probable candidates of *Hd3a* and *Hd6* were also identified (Takahashi et al. 2001, Kojima et al. 2001). We have also conducted high-resolution mapping for *Hd2*, *Hd3b*, *Hd5*, and *Hd9*. The development of genomic clone contigs and identification of candidate genes are in progress for these QTLs.

Plant materials available for the analysis of quantitative traits

To facilitate the genetic analysis of complex traits in rice, permanent mapping populations based on *japonica* × *indica* or *japonica* × *japonica* crosses are being constructed. Recombinant inbred lines (RILs), backcross inbred lines (BILs), and doubled haploid lines have been developed, and framework linkage maps for permanent use are being constructed (Table 1) (Tsunematsu et al. 1996, Lin et al. 1998, M. Yano and S. Y. Lin unpublished data). These permanent-mapping populations will facilitate the detection of naturally occurring allelic variations for target traits. However, these materials are not enough to allow us to proceed with further analysis, such as fine mapping and characterization of target QTLs. Secondary mapping populations, such as chromosomal substitution lines or NILs, will be required to facilitate more comprehensive analysis of target QTLs. To this end, we have been developing chromosomal substitution lines derived from crosses between Nipponbare and

Kasalath, and between Koshihikari (*japonica*) and Kasalath (Table 1). The construction of a series of intraspecific substitution lines of rice with overlapping chromosomal segments is progressing by marker-assisted selection (MAS) (Table 1). In addition, some accessions of wild relatives have been used as donor parental lines to develop chromosome segmental introgression lines at Kyushu University (Doi et al. 1997, Sobrizal et al. 1999). A wide range of cross combinations will be very important for the detection of naturally occurring allelic variations, because wild relatives and ecotypes are adapted to specific environmental conditions, and variations in genes give advantages for adaptation. We might find a wider range of allelic variation in these wild relatives than in cultivated species.

Table 1. Permanent mapping populations for the analysis of naturally occurring allelic variations

Cross combination	Population structure[1]	Generation	No. of lines	Genotype data	Institution involved[2]
Nipponbare/Kasalath	BIL	BC_1F_{10}	98	245 RFLPs	NIAR, STAFF, Hokuriku NAES
Nipponbare/Kasalath	SL	BC_3F_7/BC_4F_6	115	125 RFLPs	NIAR, STAFF, Hokuriku NAES
Asominori/IR24	RIL	F_{12}	71	375 RFLPs	Kyushu Univ., NIAR, STAFF
Koshihikari/Akihikari	DH	A_4	214	108 RFLPs 48 RAPDs	Ishikawa Agr. College, NIAR, STAFF
Sasanishiki/Habataki	BIL	BC_2F_6	85	238 RFLPs	Hokuriku NAES, NIAR, STAFF
Koshihikari/Kasalath	BIL	BC_1F_7	183	135 RFLPs	NIAR, STAFF
Koshihikari/Kasalath	SL	BC_3F_2/BC_4F_1	under development		NIAR, STAFF

1) RIL: Recombinant inbred line, BIL: Backcross inbred line, SL: Chromosome segment substit
DH: Doubled haploid line
2) NIAR: National Institute of Agrobiological Resources, STAFF: Institute of Society for Te innovation for Agriculture, Forestry and Fisheries, Hokuriku NAES: Hokuriku National Ag Experiment Station

Future prospects

Although many mutants have contributed to our understanding of gene functions so far, the application of mutational approaches in rice, compared with the model plant *Arabidopsis thaliana*, is limited for several reasons, including large plant size, long life cycle, and large seed size. As described in this paper, the use of naturally occurring allelic variations will be an alternative resource for functional genomics in rice (Yano 2001), as well as in *Arabidopsis* (Alonso-Blanco and Koornneef 2000).

There are several advantages to the use of naturally occurring allelic variations compared with mutational approaches. First, naturally occurring allelic variations may determine not only the presence or absence of allele function, but also leaky or weakly functional alleles. This would be more informative for the analysis of an important gene; for example, when the gene function is completely lost, the plant does not survive. Multi-copy genes (redundant genes), whose function is always complemented by other members, cannot

be easily analyzed by simple gene disruption analysis, because genes with no or very small phenotypic effect cannot be analyzed genetically. On the other hand, various kinds of allele combinations of redundant genes will occur in the progeny of different ecotypes or wild relatives. Dramatic phenotypic changes will be detected in this type of progeny, even if the frequency is very low. An example is gametic abortion causing seed sterility.

Second, phenotype assays often require large-scale field or greenhouse experiments and need a long period. Applications of these types of assays are limited to mutational approaches based on chemical, physical, and transposon mutagenesis. It is currently not possible to perform such large-scale assays on transgenic mutagenesis, such as T-DNA tagging or Ac/Ds mutagenesis. In contrast, the use of naturally occurring allelic variations needs the survey of only 100–200 lines or individuals from the primary mapping population. This approach will be a very powerful strategy for analyzing yield performance, abiotic and biotic stress tolerance, eating and cooking qualities, and so on. Although there are several disadvantages to the analysis of naturally occurring allelic variations compared with mutational analysis, such as small allelic differences in phenotypes, the approach will complement mutational approaches in the functional analysis of rice genes.

We are now employing this strategy to analyze several complex traits, such as seed shattering habit, plant height, seed size, and pollen sterility; tolerance to abiotic stresses such as cold temperature and ultraviolet irradiation; and tolerance to nutrient imbalances. Only 1 cross combination has given us a wide range of resources for functional genomics. However, it is obvious that the most important issue for the effective use of naturally occurring allelic variations is to provide a wide mapping population derived from wild relatives and different ecotypes. We will need to develop not only primary mapping populations, such as recombinant inbred lines or doubled haploid lines, but also secondary mapping populations, such as chromosomal segmental substitution lines. In addition, precise and reliable phenotype assays will be necessary in the use of naturally occurring allelic variations in functional genomics. Combining old techniques such as crossing and selection with new tools such as DNA markers and sequences will contribute greatly to the functional analysis of rice genes.

Acknowledgments

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4. Invited Reports

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4.1 Discovering Genes Underlying QTL

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1. Introduction

Rice is the most important cereal feeding developing countries for centuries and continue to be more important as fertile land has now become limited. New rice varieties that can combat well with stresses, responsive to production inputs and highly nutritious are the most desirable for the next century. In the case of rice, conventional breeding still play important roles in generating new types of rice cultivars. Therefore, technology that can improve breeding efficiency will have great impact on making new strains of rice. In the past few years, molecular markers and copies of expressed genes have been identified and mapped to segregating populations. With those technologies, genes responsible for biotic and abiotic stress resistance, yield components and cooking quality have been mapped in several chromosomes. Innovative breeding programs that utilize tightly linked markers to genes of interest are collectively called marker-assisted selection. However, the new breeding technologies have not been implemented successfully in every case. One of the most limiting factor was the use of molecular markers that were not physically linked to the responsible genes. Secondly, cost of detection is too expensive for conventional breeders. If genes controlling the traits were used directly, marker-assisted selection would have been the most effective.

A map-based approach has allowed scientists to discover few genes at a time. In addition, the reproductive barrier between cultivated rice and wild relatives has prevented us from utilizing the valuable germplasm by a map-based approach.

Most genetic traits important to agriculture or human diseases are manifested as observable, quantitative phenotypes called Quantitative Trait Loci (QTL). In many instances, the complexity of the phenotype/genotype interaction and the general lack of clearly identifiable gene products render the direct molecular cloning approach ineffective, thus additional strategies like genome mapping are required to identify the QTL in question. Genome mapping requires no prior knowledge of the gene function, but utilizes statistical methods to identify the most likely gene location. To completely characterize genes of interest, the initially mapped region of a gene location will have to be narrowed down to a size that is suitable for cloning and sequencing. Strategies for gene identification within the critical region have to be applied after the sequencing of a potentially large clone or set of clones that contains this gene(s). Tremendous success of positional cloning has been shown for cloning many genes responsible for human diseases, including cystic fibrosis and muscular dystrophy as well as plan t disease resistance genes (Martin et al., 1993; Bent et al., 1994; Grant et al., 1995; Song et al., 1995).

2. Genome and QTL Mapping

Genome mapping is used to identify the genetic location of mutants, or qualitative and

quantitative trait loci (QTL). Linking the traits to markers using genetic and family information of a recombinant population can identify the gene location. Through mapping, we can answer how many loci are involved, where the loci are positioned in the genome, and what contribution each allele may have to the trait. To determine relationships between marker loci and the target trait, mapping requires the following: • segregating populations (genetic stocks), • marker data set(s), and • a phenotypic data set.

Genetic mapping can be narrowed down to 1-3 cM in optimal cases of human diseases or as tight as 0 cM in plants (Pillen et al., 1996; Ashikari et al., 1999). The corresponding physical size, however, may vary widely due to genome size as well as regional and sex-specific differences in recombination rates. In humans, 1 cM is typically corresponding to roughly 1- 3 Mb. In plants, the physical to genetic distance per 1 cM varies with genome size (e.g., 100 kb in *Arabidopsis*, 250 kb in rice, 1,000 kb in maize). In recombination hot spots, the physical to genetic distance may be particularly small and such regions have been frequently associated with gene richness. Choices of strategies for positional cloning are depending on the tools available for the particular organisms.

3. Positional Cloning: The Pre-genomics Era

Information about map positions of genes is used to conduct chromosome walking in positional cloning. In the initial step, flanking markers which, are tightly linked to the target gene must be identified. These tightly linked markers are then used as initial points for the development of the high-resolution map around the target region, using highly polymorphic content markers. When the flanking markers are narrowed down, the next step is to construct a physical map around the target region. The candidate region can subsequently be narrowed down further, sometimes to a region being covered by a single large insert clone. After the genes were characterized by sequencing, the functional analysis by complementation in transformed plants is the most important piece of evidence for the successful identification and cloning (Gibson and Somerville, 1994)

3.1 Successful Positional Cloning

To date more than 100 inherited disease genes in humans have been isolated (*http://genome.nhgri.nih.gov/clone/*). Significant progress in positional cloning in plants was achieved, however, due in most part to the development of high density maps and large insert libraries in major crops such as rice (Kurata et al., 1994), *Arabidopsis* (Kornneef, 1994), tomato (Tanksley et al., 1992), and barley (Sherman et al., 1995). Two classic examples for gene identification were the cloning of the *Pto* gene in tomato (Martin et al., 1993) and the *Xa21* gene in rice (Song et al., 1995). These genes are responsible for resistance against bacterial pathogens. Arabidopsis has become a model plant for map-based cloning, due to the simplicity of the identification of mutations, comprehensive genetic and physical maps, and the ease of gene transformation. Examples for disease resistance genes from *Arabidopsis* that have been cloned include the *RPM1* against *P. syringae* (Grant et al., 1995), the *RPS2* against a different strain of *P. syringae* (Bent et al., 1994), *RPP13* against downy mildew fungus (Bittner-Eddy, 1999), *Mlo,* against the broad spectrum fungal attack in barley (Buschges et al., 1997), and *I2* against fusarium wilt in tomato (Simons et al., 1998). Another set of disease resistance genes

will soon be identified, such as *Tm2a* against *TMV* in tomato (Pillen et al., 1996), *Asc* against alternaria stem cancer in tomato (Mesbah et al., 1999), *Pi-b* against rice blast (Monna et al., 1997), *Pi-ta2* also against rice blast (Nakamura et al., 1997), and *Rar-1* against powdery mildew in barley (Lahaye et al., 1998). Dietrich et al. (1997) have also reported positional cloning of a negative regulator for hypersensitive response in *Arabidopsis*. Successful positional cloning has been achieved for the gene responsible for the resistance to beet cyst nematode, $Hs1^{pro-1}$, (Cai et al., 1997). The *Br* gene responsible for resistance to bruchid, grain weevil that destroys mungbean seeds, was cloned by Kaga and Ishimoto (1998).

Cloning attempts of plant genes responsible for stress resistance to abiotic factors such as cold, drought, flooding, etc. have faced more challenges due to their complex interaction with several genetic and non-genetic factors. This is particularly true for genes related to phytohormone activity. Many *Arabidopsis* mutants with defects in signal transduction pathways were used for map-based cloning. Successful gene isolation by map-based cloning was reported for genes responsible for insensitivity to abscisic acid, *ABI1* (Leung et al., 1994), *ABI2* (Leung et al., 1997), *ABI3* (Giraudat et al., 1992), and *ABI4* (Finkelstein et al., 1998), elongation-regulating hormone, auxin, *AXR1 & 2* (Leyser et al., 1993), senescence-promoting ethylene, *ETR* (Chang et al., 1993), *EIN2* and *CTR1* (Kieber et al., 1993), gibberellic acid, *GAI* (Peng et al., 1997) and *GA1* (Sun et al., 1992).

Positional cloning has been successfully used to isolate genes involved in development that are important to agriculture such as, *d1*, dwarfism in rice (Ashikari et al., 1999) and a *MADBOX* gene controlling fruit dehiscence (*jointless*) in tomato (Mao et al., 2000). In rice, three genes controlling flowering time were cloned. By candidate cloning, *SE5*, a phytochrome gene, was cloned and demonstrated that it encodes a putative heme oxygenase (Izawa et al., 2000). A major QTL controlling flowering time in rice, *Hd1*, mapped on rice chromosome 6, is a homologue of Arabiodopsis *CONSTANS* (Yano et al., 2000). Recently, *Hd6*, a rice QTL involved in photoperoid sensivity, encodes an alpha subunit protein kinase *CK2* (Takahashi et al., 2001).

3.2 Refining the Critical Region: Physical Approaches

The decision to start the physical mapping process depends on the mapping tools available for a particular organism. In humans, typically a 1-3 Mb interval can be reached using YAC physical mapping. In other cases, where the physical map is not well refined, the critical region must be narrowed down to as small distance as possible, using the wealth of polymorphic markers in the particular region. Typically, mapping the closet genetic markers is used to initiate clone isolation (e.g., YAC, BAC, or PAC). If necessary, new markers can be generated from the ends of the clone which can then be used to screen the next adjacent overlapping clones, a strategy called "chromosome walking". Additional markers such as STS and EST, which were identified to be located in the critical region, can be used to assist clone isolation. In an ideal case the entire critical genomic interval between the flanking markers can be isolated in YAC or BAC clones. Fine mapping with such specific markers can significantly narrow down the critical region, and gene isolation can be done with less effort than physical mapping.

3.3 Identification of Genes within the Refined Critical Region

The most challenging step in positional cloning is the identification of genes in the critical region. There are strategies for the identification of genes in large genomic clones such

as YAC, BAC, or PAC clones. In the ideal case the trait is cosegregated with the marker, or an EST derived from high-resolution mapping. In cases where the critical region cannot be narrowed down further and no other known marker is available, small DNA fragments from a YAC or BAC clones containing the gene can be used to hybridize in the orthologous region in such systems as the human/mouse/rat system, *Arabidopsis*-tomato-brassica, or the rice/maize/wheat system. Detection of cross-hybridization reveals conserved sequences between these species and, therefore, genes or ESTs identified in such syntenic region are likely to have biological function. The presence of CpG islands nearby often marks the 5' ends of genes and can be subsequently used for gene isolation. CpG islands, exon trapping and direct cDNA selection are complementary approaches that can be used to identify exons in genomic sequences.

Gene Detection by CpG Island. Its unusual G+C% rich DNA first distinguished CpG islands from other genomic sequences (Bird, 1987), more than 60% of human genes contain CpG islands, both in promoters and at least a part of one exon (Larsen et al., 1992).

Exon Trapping. The presence of consensus sequences at "splice junctions" allows the isolation of adjacent exons by "exon amplification" or "exon trapping" (Dyke et al., 1990). In some cases, the entire internal exon can be captured (Nehls et al., 1994). In a similar fashion, several genes causing human diseases have been isolated by trapping their terminal exons using the poly A tail signal (Krizman and Berget, 1993).

Direct cDNA selection. The candidate genomic clones can be used either as template to screen cDNA libraries constructed from the target tissues or subtractive hybridization. Using a method called "direct cDNA selection", the target genomic clones are fixed on an affinity matrix to capture cDNAs by homology-based hybridization. The success of the method relies on the source and quality of the cDNAs that are about to be captured. The genes can only be captured, if they are expressed in the tissue from which mRNA or cDNA libraries were isolated. Some genes showing low levels of expression or are absent in the target tissue may be difficult or impossible to isolate by direct cDNA selection.

4. Comparative Approaches to Gene Identification

Comparative mapping combines genetic information accumulated from related species. Usually, linkage maps of each species have been constructed with various kinds of molecular markers and used independently for particular genetic purposes. Comparisons among maps of related species, using low copy number sequences as a probe to hybridize with genomic DNA indicated the substantially conserved orders of the DNA sequences among their genomes. This is well documented in the grass family, which diverged ~ 60 million years ago (Ahn and Tanksley, 1993; Van Deynze et al., 1995; Guimaraes et al., 1997). The mammals including human and mouse evolved from a common progenitor ~ 70 million years ago and are also documented to show conserved orders of DNA sequences (Carver and Stubbs, 1997). The existence of conserved gene orders (colinearity) and contents in related species indicates that new genes are rarely created within evolutionary time frames of at least ten million years. Most new genes probably arise from gene duplication and/or gene modification of currently existing genes (Bennetzen, 2000). Therefore, the colinearity can allow gene predictions across families and the extrapolation of mapping data from one organism to the other. Candidate genes can thus

also be easily isolated and predicted from species for which well established linkage maps do not exist.

Genome Cross-Referencing in the Grass Family

During evolution, rice (430 Mb), maize (3,500 Mb) and wheat (16,000 Mb) have been separated 50-60 million years ago (Moore et al., 1993). The only genes which are extensively conserved in the orthologous region of genomes, are those that diverged several million years ago (Avramora et al., 1996). Comparative maps have been constructed between rice and maize (Ahn and Tanksley, 1993), oat and maize (Ananiev et al., 1997), among rice strains, as well as wheat and maize based on a common set of cDNAs (Ahn et al., 1993), and rice and barley (Dunford et al., 1995, Kilian et al., 1995). Being phylogenetically 60 million years apart, members of the grass family still share extensive syntenies in a number of regions. Therefore, the idea that the map position of one species can be used to identify and compare orthologous alleles across species is feasible for grasses (Bennetzen and Freeling, 1993; Paterson et al., 1995).

In positional cloning experiments, microcolinearity can be extremely useful for cloning genes from species with large genomes, such as wheat and maize, using information from small genome species like rice based on their synteny. Kilian et al. (1995) compared a 6.5 cM region in barley's chromosome 1 containing the barley rust resistance gene *Rpg1* with the 2.5 cM syntenic region in rice chromosome 6 and found that the order of RFLP markers was conserved. In the case of the *Adh1* locus, composition and arrangement of genomic DNA fragments were compared between maize YAC and sorghum BAC clones, containing the orthologous loci (Springer et al., 1994, Avramova et al., 1996). Because of a 75 kb stretch of highly repetitive elements in maize, chromosome walking to the *Adh1* gene was made possible by cross-referencing to the sorghum BAC. In a similar case, synteny was reported in the *sh2-a1* homologous regions between maize, sorghum, and rice, where the distance between the two genes was 140 kb in maize but only 19 kb in rice and sorghum (Chen et al., 1997). These studies reveal that small rearrangements, including frequent insertions of transposons or retrotransposons can occur without significant rearrangement of the orthologous region (Bennetzen, 2000).

Another striking demonstration of colinearity in the grass family is the gene which, causes dwarfism. The so-called "green revolution" genes were found in wheat (*Rht*, reduced height), maize (*d8*, dwarf) and rice (*d*, dwarf). The sequence comparison revealed the *Rht* and maize *d8* were orthologs of the *Arabidopsis GAI*, which is the gibberellin-insensitive mutant (Peng et al., 1999). Comparative mapping between wheat and rice using RFLP markers linked to *Rht-D1b* and between rice and maize using RFLP markers linked to D8 clearly showed colinearity among wheat chromosome D4, rice chromosome 3, and the maize chromosome 1. Additionally, one of the spontaneous rice dwarf mutants, *d1*, was isolated by positional cloning and was found to be an ortholog or the alpha subunit of G-protein, which is related to the *GAI* mutant found in *Arabidopsis* (Ashikari et al., 1999). However, the *d1* mutant was mapped to chromosome 5. Due to the fact that in rice at least 54 dwarf mutants have been identified, at least one of them may actually be located in the synthenic region of chromosome 3. Although comparative mapping is a powerful tool for gene finding in large syntenic regions, the extensive

gene or segmental duplication in the reference genome may obstruct such comparison and may lead to the false assignment of a syntenic region.

5. Positional Cloning: the Genomics Era

Genome projects can dramatically simplify the long, tedious process of positional cloning. Physical mapping can also be tedious and costly. For most genome projects, BAC-end sequences or sequence tag connectors (STC) from a 10-20 X BAC library can tremendously simplify the physical map construction. "End walking" can be accomplished *in silico*, initiated by several rounds of *Blast* searching the sequences of flanking markers or the next BAC end sequences against the respective BAC-end sequence database until the critical region is totally overlapped with a BAC contig. In addition, a BAC fingerprint database, where contigs are being assembled, can be used to confirm and anchor the genetic map by using well-mapped molecular information.

5.1 ESTs: Key to Gene Identification in the Critical Region

As a sequence-based marker, ESTs play a crucial role in both gene-based physical map construction and candidate gene identification. In recent years, a massive scale of EST production has been conducted at the Institute for Genomic Research, TIGR (*http://www.tigr.org/*), and over 100,000 ESTs were released by TIGR and others to be maintained in the 'dbEST' database (*http://www.ncbi.nlm.nih.gov/dbEST/*) at NCBI. Among the largest publicly available EST collections, more than 500,000 ESTs have been produced at Washington University in St. Louis, supported by Merck and Company (*http://genome.wustl.edu/est/esthmpg.html*) . Because the current ESTs are only 97% accurate and short, unedited, single-pass reads, they are clustered into "Tentative Consensus Sequences" at TIGR (*http://www.tigr.org/*) or "Uniquegene Cluster" at NCBI (*http://www.ncbi.nlm.nih.gov/UniGene/*).

ESTs have been also produced from organisms other than human. One of the largest collections is the 300,000 mouse ESTs funded by Howard Hughes Medical Institute (*http://genome.wustl.edu/est/mouse_esthmpg.html*). Other model organisms are *C. elegans* (*http://ddbj.nig.ac.jp/htmls/c-elegans/html/*), *Arabidopsis thaliana* (*http://genome-www.stanford.edu/Arabidopsis/*), rice (*http://ww.staff.or.jp/*), and *Drosophila melanogaster* (*http://fly2.berkeley.edu/*). These homologous or orthologous resources are curated at 'HomoloGene' at NCBI (*http://www.ncbi.nim.nih.gov/HomoloGene/*).

Identification of ESTs on physical and RH maps of a critical region can to immediately help identifying candidate genes and simplifying the positional cloning of particular genes by leaping across taxonomic boundaries due to the conserved protein sequences.

5.2 Single Nucleotide Polymorphism (SNP)

Genetic variation within coding sequences is very conserved and can rarely be detected by length polymorphism. Polymorphisms corresponding to differences at a single nucleotide level, which are caused either by deletion, insertion, or substitution, are biallelic in diploids, occurring frequently and uniformly in most genomes at roughly one in every 500-1,000 bp. As genomic and EST sequences are increasing at an exponential rate in the past few years, SNP discovery in coding regions is projected for at least 100,000 markers with the aim of identifying

and cataloging all human genes and creating more or less complete human SNP maps. Because SNPs residing within coding region are rare, these mutations may correspond to defects that are associated with polymorphism at the protein level, diseases, or other phenotypes. To prove that SNPs are associated with a particular disease, haplotype analysis of SNPs in the candidate genes must be conducted among the affected and unaffected individuals from the same family. In this case, the sampling size must be large enough to reveal the statistical differences between the affected and unaffected pools and show a linkage disequilibrium. Positional cloning can be more effective and less time-consuming using SNPs not only to refine the critical region, but also to confirm the position of the real candidate genes.

5.3 Predicting Candidate Genes from Genomic Sequence

The most direct approach to gene identification in a genomic region involves the analysis of DNA sequences. Such high-quality sequence are available for human, *C elegans*, *Arabidopsis*, including rice, among others. Taking the human genome project as an example, the availability of the comprehensive high resolution map and physical map that assembles EST, STC, STS, STR into large genomic contigs allows human disease genes to be assigned precisely to the critical region in a short timeframe. Genes can be predicted more accurately where genomic sequences are available using modern computational tools including GenScan, GeneMarkHMM, Xgrail, and Glimmer. All the genes identified by homology or by prediction in the critical region can become candidates. It is essential to understand how those candidate genes function. To prove that one of those candidate genes is the responsible gene, it has to be demonstrated that the mutation in the gene is genetically associated with the phenotype. At this stage the availability of single nucleotide polymorphism (SNP) will improve the rate of mutation discovery in the candidate genes tremendously. However, the ultimate proof that the candidate gene is functionally correct, one requires evidence that the normal phenotypic form can be complemented by the susceptible gene or vice versa.

5.4 Rice: A Model for Gene Discovery and Molecular Breeding

The International Rice Genome Sequencing Program has aimed to completely sequence the genome within 2004. Recently, 97.8 Mb of the 430 Mb of the genomic sequence is available at the Rice Genome Research Program (RGP), Japan for public uses. In 2002, a high quality draft with 99.9% sequence accuracy will be released from RGP and Monsanto altogether. The basic goal for the rice genome project is to completely sequence the genome and to provide a more direct route to uncover genes *en masse* from rice and related species. There are several reasons why rice is suitable for the whole genome project. Rice has the smallest genome size and the highest gene density among grain cereals. In addition to genomic sequence, rice EST in the NCBI's database has reached 80,099 accessions of which 6605 EST were physically mapped on the rice genome using YAC-based physical map (RGP). For functional genomics, several laboratories have been working on Agrobacterium-based gene knock-out systems and Tos-17, a unique retrotransposon-based knock-out system in rice. With well colinearity among cereal genomes, optimized transformation systems, vast number of mutations available and the ease of functional analysis, rice has become a gold mine for gene hunting in maize, sorghum, wheat, etc. Following the construction of comparative mapping among cereals, genes that were more difficult to be cloned in one species can now be done in rice.

5.5 Discovering genes underlining QTL: A cast study

The major QTL for submergence tolerance mapped onto a 6 cM subcentromeric region on chromosome 9 has been one of the largest QTL ever reported. One way to learn how the genes located on the QTL regulate submergence tolerance is to sequence the whole genomic region and looking for genes of interest. We reported here the 1.5 Mb finished sequences of chromosome 9 from 4 PAC and 5 BAC clones. The average physical to genetic distance of 170 kb per cM was surprisingly high considering the sequencing area is proximal to the centromere. Within the first megabase region, more than 80 genes were predicted and confirmed by homology search. High gene density with small physical to genetic distance makes this subcentromeric region particularly gene-rich and hotspot for recombination. One feature that might contributed to high recombination hot spot is high number of retrotransposon flanking genes. In total, 10 genes are hypothetical proteins, 9 genes have no match, 5 genes are transcription factors and 7 genes plays roles in signal transduction. No genes encoding enzymes involved in fermentation pathway such as ADH, PDC, etc located in the QTL region. Regulatory genes were clustered into two distinct subgenomic regions surrounded by large number of retrotransposons forming two gene-dense islands. These two gene islands were essential for maintaining submergence tolerance. Genes located on the first island were involved in G-protein signal transduction such as a hypothetical protein containing G-protein coupled factor, a Gamma response I protein, and a small Ras GTP-binding protein. On the second island, two EREBP, ethylene response element binding proteins, a GTPase regulator and transcription factors containing zinc-finger motif and WRKY were located. The roles of these regulatory proteins in submergence tolerance will be elucidated. At least in the case of submergence tolerance, genes underlining the QTL plays important roles in signal transduction pathways that involve in plant hormones, gibberellin, ethylene, ABA and auxin.

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4.2 Molecular Techniques for Detection of Genetic Variation In Horticultural Crops

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Summary

The application of molecular techniques in cultivar identification and classification of some horticultural fruit crops are briefly reviewed in this paper. Two distinct approaches have been utilized including electrophoresis of polymorphic isozymes and DNA Amplification Fingerprintings; DAFs. Such markers were successfully employed in distinguishing genetic variability and generated genetic relatedness dendrogram among closely related cultivars of *Salacca species,* and *Lansium domesticum* Correa.

Introduction

Cultivar identification of most tropical fruit trees in Thailand, generally, based on an evaluation of morphological characteristics. Although several characteristics of these trees are in common, there are yet considerable variations among individuals. It becomes even more difficult when quantitative traits involve. This is where multiple genes interaction play their roles, and visual traits could come from cascade expression of many genes (Torres, 1983).

As the status of our agricultural markets and consumer behaviors have changed, horticultural fruit products become more prominent in both local and international trades. There are urges for crops with horticulturally superior characters. As a consequence, many new cultivars are registered and released in response to these trends. Many of these modern cultivars are morphological similar to each other. Some are very difficult to distinguish using their visual traits, especially the hybrids between closely related parents. The discrimination is even impossible if evaluation is carried out on seedling of some plant species.

Several biochemical methods especially isozyme analysis had exhibited their usefulness in the identification of individual genotype of many fruit crops for many years (Huang, et al., 1997; Cousineau and Donelly, 1989). In addition, during the past decade many new and efficient discrimination techniques have been offered by the fast-moving technology of DNA markers. The utilization of specific probes, such as RFLP, or PCR (polymerase chain reaction) based have already proved to be valuable for cultivar identification in many plant species (Fang, et al., 1997 ; Lanham and Brennan, 1999) .

The objective of this paper is to briefly review the achievements of the works conducted in our laboratory using two different classes of markers, i.e. isozyme and DNA markers, in discrimination of genetic variability among closely related cultivars of two locally important fruit crops.

Salacca sp.

Salacca sp. is a dioecious plant in the Family of Palmae, with male and female inflorescenses developing on separate tree. The main cultivation areas are centered on

eastern and southern Thailand. It was previously common fruit in the local market but has gained much popularity over the last decade when superior varieties were introduced. Recently these plants have been propagated to other cultivation areas throughout Thailand while the eastern provinces, especially Chantaburi, are the source of seedling distributors.

Among 7 accessions of *Salacca* plants collected for this study, only two accessions were classified as *S. walliciana* Mart. and *S. zalacca.* Others were recorded as *Salacca sp.* and not yet given a taxonomic classification (Table 1). The favorite type at present is referred as "Sala" which fruits and seedlings are of higher value. Two cultivars of Sala type ; Nern-wong and Maw, are closely related since it was reported that Nern-wong were originated from seedling of Maw (Polpasid, 1992). The origin of Sakum, the spineless type, is obscured, while Rakum-krang was presumably derived by chance-seedling of Rakum.

Accession	Type	Cultivar	Species
1	Sala	Nern-wong	*Salacca sp.*
2	Sala	"sumalee"	*Salacca sp*
3	Sala	Maw	*Salacca sp*
4	Rakum	Rakum	*S.wallichiana* Mart.
5	Rakum	Rakum-krang	*Salacca sp*
6	Unknown	Sakum	*Salacca sp*
7	Salak	salak	*S.zalacca*

Table 1. *Salacca* plant materials using for analysis of genetic variation by isozyme polymorphism

Salacca palm is naturally propagated by seed, and generally there is a chance of 80% that a seedling develops into a male plant. Moreover germination by seed also disperses into variation. Therefore vegetative propagation such as stem section and separating rooted sucker are common commercially. *Salacca* palms are morphologically similar during their young seedlings. Visual identification of these plants is difficult and confusion exists especially among the two types of Sala and Rakum.

In our study, electrophoresis of polymorphic isozymes were found to detect useful amounts of variation in this *Salacca* palms and unique banding patterns were obtained for the majority of cultivars analysed (Sakuanrungsirikul, et al, 1999). In this study, extracts of young leaves were analysed for 25 isozyme markers, using polyacrylamide gel eletrophoresis (PAGE) (Shield, et al. 1983). 13 polymorphic loci were produced by 6 isozymes, i.e., Peroxidase (PRX), Glutamate oxalotransaminase (GOT), Superoxide dismutase (SOD), Phosphoglucomutase (PGM), Malate dehydrogenase (MDH), and Laccase (LC) (Table 2).

The unique alleles were detectable in Sala group while variation of other varieties were not readily detectable by this protocol. Some cultivars such as Rakum and Sakum, which were apparently phenologically diferent, could not be separated, and produced completely identical banding patterns. While Salak showed unique profiles. However, these observable genetic variation maybe under-estimate because the number of available allozymes were limited. These isozyme similarities were probably only a coincidental. Among these 6 isozymes investigated, the largest variabilities were found in the loci produced by PRX. Culture environment including ages of tree and leaf, cultivation location,

and season, were found not to show any effect on PRX banding patterns. This was also true for their preserving sample of leaf extracts that were frozen for a period of a month. In the inception of discriminating effectiveness, polymorphisms of these four PRX loci could be used solely in differentiation of these *Salacca* palms.

Table 2 Polymorphic isozyme loci detected in *Salacca sp.*
Showing effectiveness of discrimination among cultivars.

locus	# polymorphic	discriminating effectiveness	
		Inter-typing	Intra-typing
PRX-1	2	yes	yes
PRX-2	3	yes	yes
PRX-3	2	yes	no
PRX-4	3	yes	no
GOT-1	3	yes	no
GOT-2	1	yes	no
SOD-1	1	yes	no
SOD-2	2	yes	no
SOD-3	3	yes	yes
PGM-1	2	yes	no
PGM-2	1	yes	no
MDH	3	yes	no
LC	3	yes	no

Further investigation was conducted in addition, to determine the assessment of PRX banding pattern that could be used as a core-banding profile for future identifying of such commercially recognized cultivars. The number of PRX staining patterns investigated in the population of over 300 *Salacca* trees, sampled from different cultivation areas in eastern Thailand, were found to be of eight phenotypes. Among these polymorphisms, unique banding profiles were found consistently correlated with morphological identities (Table 3). This finding indicated that major patterns such as A, C, D and G could be used as the profile to identify Nern-wong, Maw, Rakum and Salak, respectively. Deviations from its corresponding group were generally correlated with those originated from seeds. The results of this study is therefore, clearly demonstrated that the application of isozymes can be used to identify *Salacca* palms and feasible to discriminate commercially valuable cultivars.

Table 3 Percentage of the frequency of *Salacca sp.* population recorded in each PRX-banding pattern of A to H

Variety	Numbr of trees	%Frequency of population in each PRX banding pattern							
		A	B	C	D	E	F	G	H
Nernwong	171	85.9	0.6	0	12.9	0.6	0	0	0
Maw	86	1.2	1.2	86	11.6	0	0	0	0
"sumalee"	8	100	0	0	0	0	0	0	0
Rakum	43	0	0	0	100	0	0	0	0
Rakumkrag	2	0	0	0	100	0	0	0	0
Sakum	54	0	0	0	85.2	3.7	11.1	0	0
salak	27	0	0	0	0	0	0	88.9	11.1

***Lansium domesticum* Correa**

This species is another important fruit tree in Southeast Asia. It is among one of the favorite fruits in the region, and the product for the market is still under supplied. *L.domesticum* Corr. is reported to be parthenocarpy. According to some of the studies; flower do not form pollen due to degeneration of androecium (Yaacob and Bamroongrugsa, 1992). However, they exhibit morphological variation in the nature. Classification of this plant species so far, is still tentative. Some taxonomists have classified those deployed clones as separate species, while others defined them as varieties within this species. In Thailand there are three major groups of variations reported referred as; longkong, langsat, and luku (Isaragraisil, 1993). Each group also varies into many subgroups, which were referred to as cultivar in our study (Table 4). Morphological differences between groups can be observed in the phenology of tree canopy and fruit characteristics, which include fruit shape, size, thickness of the skin, latex exudate, and flavor. Among these three groups, longkong gains much attention from the consumers, as fruit tastes are much more pleasant and yet the fruits are almost seedless. This is again also applied to the cost of seedlings and fruits which relatively high compare to the other two groups.

L. domesticum was originally distributed from southern to eastern Thailand. It is generally propagated from seed, with the exceptional for longkong, that seed per fruit is limited. Propagation by grafting is thus preferable, and seedling of luku is generally used for rootstock (Mameevattana, 1993). There are a lot of confusions within the nomenclature of this plant species. Some names were arbitrarily chosen by the growers, and some cultivars were appeared by the different names in different locations. On top of that there are yet difficulties in discrimination of the seedlings which are consequently cover-up their identities. We have studied 18 cultivars, not all of them were commercially accessible (Table 4). These cultivars were collected from various cultivation sources in the regions of Chantaburi and Trad provinces. All of them, except two accessions, represented the three major groups. Those accessions, though sharing common characteristics, but exhibited slight to fairly extreme levels of phenotypic variation, especially in the group of longkong. Some of them derived from seeds and many of them were from misidentified or mixed seedlings.

Our objective was to determine whether such variations could be observed through the application of molecular markers. Assessment was made using the DNA Amplification Fingerprinting Technique (DAF) which involved PCR amplification of DNA using a single primer (Bentley and Bassam, 1996). This approach has proved to be useful in identifying many other plant species (Scott, et. al., 1996 ; Caetano-Anolles, 1995).

Table 4 . Description of *Lansium domesticum* cultivars in DAF studied.

Major group	Sub-group	Cultivar
longkong	Dry-fruit type Watery-fruit type Kalamae	Lonkong
		See-po
		Nam-pheung
		Kaew # 1
		Kaew # 2
		Som # 1
		Som # 2
		Sai-nampheung # 1
		Sai-nampheung # 2
		Luku-nam
		Luku-big seed
		Luku-small seed
langsat	-	Langsat
		Chum-kaw
luku	Pae-mae Watery-fruit type	Luku-ban-ang
		kalamae
-	-	Unknown # 1
		Unknown # 2 (male)

Genomic DNA was extracted from leaf tissues and amplified using shor oligonucleotide primers. Amplification products were separated on polyacrylamide gels an DNA profiles were visualized using silver staining. The result of this method provide considerable amount of polymorphic loci in a single reaction. In this study, 8 selected primers were successfully employed to demonstrate variations of all accessions (Table 5). Among 200 of clear and reproducible bands generated by these primers,151 loci (75.5%) were polymorphic, with the average of 18.9 polymorphic bands per primer. Information of the fragments produced in this experiment was subjected for cluster analysis by the package program ; Bio1D++ (Bioprofil, Vilber, Loumat, France) using UPGMA with Nei & Lee similarity coefficient. At the level of 85% genetic relatedness, the grouping of all investigated materials based on DAF banding information, were in general agreement with morphological classification. At the relation of closer distance of 85-95%, variation within each group was readily revealed.

Table 5. Oligonucleotide primers used and their genetic polymorphism detected in studying of *Lansium sp.*

Primer sequence (5'-3')	Total interpretable bands	Number of polymorphic bands
1. TCC-GAT-GCT-G	12	7
2. AGT-CGG-GTG-G	24	16
3. TTC-CGA-ACC-C	23	13
4. GAA-TAC-AAA-GAA-TTG	28	19
5. CTA-CTG-CGC-T	28	27
6. AGG-GGG-TTC-C	25	17
7. GTG-ACG-TAG-G	17	13
8. TCT-GGA-CGG-A	43	39

The result in assessment of the commercially named cultivars ; sai-nam-phueng, obviously showed two deployed clones, and could be detected both by their phenology and their molecular information. Clustering of these two accessions, however, found to fall into a group that was rather diversified from longkong. Molecular examination of a well-known cultivar of langsat ; referred as Chum-kaw, revealed close genotypic variation of 95%, among its core group. Investigation of the two accessions designated as "Unknowns" ; one of which was distinctive phenology and of unknown source of origin, the second one with a tentative "male" plant characteristic, also fell into a unique grouping. It was noticeable that the grouping of "luku" , were obviously distinct from the other two major groups. This was appeared in both at the molecular and morphological levels. According to this information, perhaps they need to be recognized as a separate taxon. Moreover, no case of homonym detected in this study. This is because plant materials were collected from only small part of the whole cultivation area. An evaluation of more accessions of this species exist in other location of Thailand would allow a better comparison of this molecular phenology with traditional morphological classification.

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4.3 Mutation Induction in Chrysanthemum through *In Vitro* Acute and Chronic Irradiations with Gamma Rays

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Abstract

In Vitro culture of chrysanthemum variety 'Reagan Dark Splendid' was established using explants from axillary buds and ray florets. Shoots produced *in vitro* culture from axillary buds were irradiated with acute gamma rays of 30 Gy. While multiple shoots produced from *in vitro* culture of ray florets were irradiated with chronic gamma rays of 112 and 140 Gy. The irradiated shoots were then multiplied two times by single-node cuttings from M_1V_1 to M_1V_3. Controls and irradiated shoots were rooted, transferred to soil in the greenhouse, and finally transplanted in the field. Observation and measurement were made at flowering time for height, number of nodes per plant and flower colour mutation frequency. Flower colour mutation frequency among the irradiated plants with 30, 112 and 140 Gy was 8.2, 9.3 and 15.3% respectively. Eleven variants were selected among the M_1V_3 plants from 30 Gy dose treatment. Selected variants had flower colour varying from red, red-purple and yellow-orange. Nine variants were selected among the M_1V_3 plants from 112 Gy dose treatment. Selected variants had flower colour varying from red, red-purple, purple and yellow-orange. Sixteen variants were selected from 140 Gy dose treatment. They had flower colour varying from red-purple, purple, orange-red, yellow-orange and yellow. These variants were multiplied through *in vitro* culture as well as by conventional cutting. They will be evaluated for their performance, stability and uniformity in the field, and for market value during the next planting season.

Key words: chrysanthemum, acute irradiation, chronic irradiation, gamma rays, *in vitro* culture.

Introduction

In Thailand, chrysanthemum is a major floral crop for small-land holders. The main planting area for chrysanthemum is widely found in different provinces such as Chiang Mai, Chiang Rai, Nong Khai, Udon Thani, Khon Kaen and Ubon Ratchathani, covering approximately 224 ha of land (DOAE, 1999). The present demand for cut chrysanthemums outstrips the current national production. It has been reported that the import of chrysanthemum from Malaysia alone stands at 550 million baht (US$10.5). Therefore, there is an urgent need to produce new exotic varieties for growing in the country and to meet the demand of the local market. There are numerous reports on successful use of induced mutation technique in combination with *in vitro* culture to obtain new varieties of

chrysanthemum (Nagatomi, 1991; 1993; Ahloowalia, 1992; Datta and Banerji, 1993; Matsumoto and Onozawa, 1990; Neto and Latado, 1996). The purpose of this study is to improve the chrysanthemum variety 'Reagan Dark Splendid' in order to produce desirable mutants with diversed colour, shape and size for local consumption to reduce importation of this ornamental.

Materials and Methods

1. Acute irradiation

Growing shoots of chrysanthemum variety 'Reagan Dark Splendid' flower form Daisy petal colour purple, colour centre green were cut into small pieces, each with a terminal or side bud (axillary bud) and cultured in aseptic condition on half-strength MS medium (Murashige and Skoog, 1962). Growing shoots with 4-5 nodes produced *in vitro* culture were irradiated with acute gamma rays of 30 Gy (18.7 Gy/min).

2. Chronic irradiation

Ray florets of 'Reagan Dark Splendid' chrysanthemum were cultured in the MS medium supplemented with 10 mg/l N6-Benzyladenine (BA). The procedure for culturing ray florets was previously described (Jompuk *et al*, 2001). The resulting multiple shoot buds were cultured in half-strength MS medium for three weeks. Then the cultures were irradiated with chronic gamma rays of 112 and 140 Gy (~0.149 Gy/hr).

The irradiated shoots from both acute and chronic gamma rays treatments were multiplied two times by single-node cuttings at a monthly interval. Controls and irradiated shoots were rooted, transferred to the soil in the greenhouse and finally transplanted in the field at Chiang Mai. Observation and selection were done at flowering time in order to screen for various useful characteristics. Ornamental specialists and chrysanthemum growers were invited to help us in selecting desirable characters at field level. The Royal Horticulture Society colour chart was used to classify the flower colours of mutants and controls.

Results and Discussion

Phenotypic characters observed and measured on M_1V_3 plants derived from acute and chronic irradiation treatment are shown in Table 1. Average height of M_1V_3 plants irradiated with chronic gamma rays did not differ from each other and from the controls. However the average height of M_1V_3 plants irradiated with acute gamma rays were shorter than the controls. When the number of nodes per plant was counted, the controls and M_1V_3 plants treated with acute gamma rays had very similar node numbers. M_1V_3 plants treated with chronic gamma rays of 112 and 140 Gy had lower number of nodes per plant than the controls. From this finding it is interesting to note that chronic gamma rays irradiation reduced the number of nodes but increasing the internode length and thus resulting in similar height of both controls and treated plants.

Wide variation in flower shape and size, plant height, leaf morphology and branching capacity was observed in M_1V_3 plants both in acute and chronic irradiation experiment. We were interested in selecting for flower colour changes, therefore, in this study only colour mutation frequency was investigated. Slight change in flower colour was also observed in control plants. Colour mutation frequency in controls and in plants irradiated with 30 Gy, 112 Gy and 140 Gy was 2%, 8.2%, 9.3% and 15.3% respectively.

1. Acute irradiation

One control plant and eleven variants were selected from M_1V_3 plant derived from *in vitro* culture irradiated with 30 Gy acute gamma rays. The flower characters of selected variants are shown in Table 2 and Figures 1 & 2. The flowers selected from 30 Gy-treated plants had diameters ranging from 5.6 to 7.3 cm while the flowers selected from the control had diameters of 7.2 cm. The floret-disc diameters of 30 Gy-treated plants varied from 1.5 to 2.0 cm while that of the selected control plant was 1.8 cm. The length of floret rays of irradiated plants varied from 2.6 to 3.6 cm whereas the selected control plants had floret rays length of 3.4 cm. Number of floret whorls varied from 2-4 in selected M_1V_3 plants whereas the control had 3 whorls. Wide variation in flower colour was observed in selected M_1V_3 plants varying from dark-purple in the control (Figure 1) to red, red-purple and yellow-orange in the treated plants and to red-purple in selected control (Figure 2).

2. Chronic irradiation

One plant was selected from the control, nine plants were selected from the M_1V_3 plants irradiated with 112 Gy and sixteen plants were selected from those irradiated with 140 Gy (Table 3; figures 3 & 4). The selected control plant had flower diameter of 7.3 cm, floret-disc diameter of 1.8, length of ray floret of 3.5, floret whorls of 2. The flower colour was pale-purple. The flower selected from 112 Gy-treated plants ranged in flower diameter from 6.0 to 7.5 cm while that of 140 Gy-treated plants were from 5.7 to 8.7 cm. The floret-disc diameter of 112 Gy-treated plants varied from 1.4 to 2.0 cm while that of the 140 Gy-treated plants was 1.2 to 2.5 cm, the length of ray florets varied from 2.7 to 3.8 cm and 2.7 to 4.4 cm in plants treated with 112 Gy and 140 Gy respectively. The selected control had 2 floret whorls whereas the irradiated flower had 2 to 4 floret whorls. Flower colour of plants treated with 112 Gy varied from red, red-purple, purple and yellow orange whereas these treated with 140 Gy ranged from red-purple, purple, orange-red, yellow orange and yellow.

In our experiment, we obtained flower colour mutation frequency of 8.2%, 9.3% and 15.3% in M_1V_3 plants treated with 30 Gy acute gamma rays, 112 Gy-and 140 Gy-chronic gamma rays respectively. The variants seemed to be very uniform and stable. Our selected variants from treated with acute gamma rays showed more variation in flower colour than those irradiated with chronic gamma rays. Yellow flowers were obtained only in M_1V_3 plants treated with 140 Gy chronic gamma rays. Therefore, different mutation spectrum for flower colour could be possibly obtained by application of two methods of irradiation treatment ie. acute and chronic. Our results confirm the report by Broertjes and Van Hartens (1988) that alteration of flower characteristics in ornamentals arising from mutagenesis resulted in 55% change in flower colour and 15% change in flower morphology. To change flower colour only, but at the same time retains most of original phenotype will facilitate the release of new varieties along with their original parents.

We do believe that the purple genotype 'Reagan Dark Splendid' which we used as starting material for colour mutation is an appropriate parent genotype for induced mutation. Schum and Preil (1998) suggested that selection of appropriate genotype is important in creating flower variability by mutation induction. In this experiment, our thirty six variants were selected by floriculturists and chrysanthemum growers based on their preference for colour, shape, size and morphology. We believe these variants have the potential to be new varieties.

Conclusion

The use of acute and chronic irradiation of *in vitro* culture of the chrysanthemum variety 'Reagan Dark' gave rise to several promising variants with desirable colour, shape and size. These thirty six variants will be investigated in field tests for performance, stability and uniformity as well as for consumer acceptance and market profitability.

Acknowledgment

The authors appreciate the research grant from Kasetsart University Research and Development Institute (KURDI) and the expert service from the International Atomic Energy Agency (IAEA) under TC-project (THA/5/045).

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Table 1. Phenotypic characters of chrysanthemum variety 'Reagan Dark' in M_1V_3 plants derived from *in vitro* acute and chronic irradiation with gamma rays.

Radiation dose	Method of irradiation	No. of plants studied	Average height at flowering time	Average no. of nodes/plant	Colour mutation frequency (%)
0	-	100	64.9 ± 1.4	31.3 ± 1.0	2
30	acute	687	61.7 ± 0.8	29.5 ± 0.9	8.2
112	chronic	848	64.7 ± 1.1	24.7 ± 1.3	9.3
140	chronic	564	64.3 ± 1.0	24.2 ± 1.0	15.3

Table 2. Characteristics of selected M_1V_3 variants derived from *in vitro* acute irradiation of chrysanthemum variety 'Reagan Dark'.

Lines	Flower diameter (cm)	Floret-disc diameter (cm)	Length of floret rays (cm)	Floret whorls	Flower colour
RGB Selected Control–1a	7.2 ± 0.2	1.8 ± 0.1	3.4 ± 0.1	3	Red-purple 74 C
RGB 3kr-1a	6.9 ± 0.3	1.6 ± 0.1	3.3 ± 0.3	3	Red-purple 72 B
RGB 3kr-2a	7.0 ± 0.1	1.7 ± 0.1	3.6 ± 0.2	3	Red-purple 70 B
RGB 3kr-3a	5.6 ± 0.2	1.5 ± 0.2	2.6 ± 0.1	3-4	Red 46 A
RGB 3kr-4a	6.0 ± 0.6	1.5 ± 0.2	3.0 ± 0.1	2	Red-purple 71 A
RGB 3kr-5a	5.7 ± 0.2	1.5 ± 0.2	2.6 ± 0.1	3	Red 46 A
RGB 3kr-6a	7.3 ± 0.5	2.0 ± 0.1	3.3 ± 0.3	2	Yellow-orange 22 D
RGB 3kr-7a	6.9 ± 0.3	1.6 ± 0.1	3.3 ± 0.3	3	Red-purple 72 B
RGB 3kr-8a	6.6 ± 0.1	1.5 ± 0.2	3.3 ± 0.3	3	Red-purple 74 A
RGB 3kr-9a	6.7 ± 0.1	1.5 ± 0.2	3.4 ± 0.3	2	Red-purple 74 A
RGB 3kr-10a	7.0 ± 0.1	1.6 ± 0.1	3.5 ± 0.1	2-3	Red-purple 72 B
RGB 3kr-11a	6.2 ± 0.4	1.7 ± 0.1	3.0 ± 0.1	2-3	Red-purple 60 A

Table 3. Colour and size of selected M_1V_3 variants derived from *in vitro* chronic irradiation of chrysanthemum variety 'Reagan Dark' with gamma rays of 112 and 140 Gy.

Lines	Flower diameter (cm)	Floret-disc diameter (cm)	Length of floret rays (cm)	Flow erwh orls	Flower colour
Selected Control	7.3 ± 0.2	1.8 ± 0.1	3.5 ± 0.1	2	Purple 75 D
V_1 11.2kr-1a	6.9 ± 0.4	1.6 ± 0.3	3.4 ± 0.4	2-3	Purple 75 C
V_1 11.2kr-2a	6.6 ± 0.8	1.7 ± 0.6	3.3 ± 0.2	3	Yellow-orange 18 C
V_1 11.2kr-3a	6.7 ± 1.0	1.7 ± 0.6	3.5 ± 0.3	3	Purple 75 C
V_1 11.2kr-4a	6.5 ± 0.5	1.5 ± 0.4	3.2 ± 0.3	2-3	Yellow-orange 18 D
V_1 11.2kr-6a	6.0 ± 0.8	1.4 ± 0.4	2.7 ± 0.4	3-4	Red-purple 74 C
V_1 11.2kr-7a	6.1 ± 1.1	1.5 ± 0.2	3.1 ± 0.2	3	Red 51 C
V_1 11.2kr-8a	7.5 ± 0.4	1.5 ± 0.1	3.8 ± 0.2	3	Red-purple 74 D
V_1 11.2kr-9a	7.3 ± 0.1	2.0 ± 0.2	3.5 ± 0.1	3	Purple 75 B
V_1 11.2kr-10a	6.6 ± 0.4	1.6 ± 0.3	3.4 ± 0.2	3-4	Purple 75 C
V_2 14kr-1a	6.8 ± 0.3	1.6 ± 0.1	3.1 ± 0.1	3	Orange-red 35 D
V_2 14kr-2a	6.1 ± 0.2	1.8 ± 0.2	2.4 ± 0.2	3	Purple 75 B
V_2 14kr-3a	7.0 ± 0.5	1.9 ± 0.2	3.6 ± 0.1	2	Yellow 11 C
V_2 14kr-4a	7.0 ± 0.0	1.9 ± 0.1	3.0 ± 0.0	3	Red-purple 74 D
V_2 14kr-5a	8.7 ± 0.3	2.5 ± 0.1	4.4 ± 0.1	2	Red-purple 69 B
V_2 14kr-6a	5.7 ± 0.3	1.4 ± 0.2	2.5 ± 0.1	3	Red-purple 74 C
V_2 14kr-7a	6.4 ± 0.1	1.7 ± 0.1	3.1 ± 0.1	3	Red-purple 74 C
V_2 14kr-8a	6.8 ± 0.6	1.3 ± 0.2	3.2 ± 0.2	2-3	Yellow-orange 20 A
V_2 14kr-9a	7.3 ± 0.3	1.6 ± 0.1	3.4 ± 0.1	2-3	Yellow-orange 20 a
V_2 14kr-10a	6.0 ± 0.1	1.4 ± 0.1	3.0 ± 0.1	3	Red-purple 74 C
V_2 14kr-11a	7.1 ± 0.2	1.8 ± 0.2	3.7 ± 0.1	3	Red-purple 69 B
V_2 14kr-12a	7.2 ± 0.2	1.8 ± 0.2	3.4 ± 0.1	3	Orange-red 31 D
V_2 14kr-13a	5.8 ± 0.5	1.2 ± 0.1	2.9 ± 0.2	3	Purple 75 B
V_2 14kr-14a	7.3 ± 0.3	1.7 ± 0.1	3.4 ± 0.2	2	Yellow 4 C
V_2 14kr-15a	6.0 ± 0.4	1.7 ± 0.1	2.7 ± 0.2	3	Red-purple 74 D
V_2 14kr-16a	6.4 ± 0.5	1.6 ± 0.3	2.9 ± 0.2	3-4	Red-purple 74 D

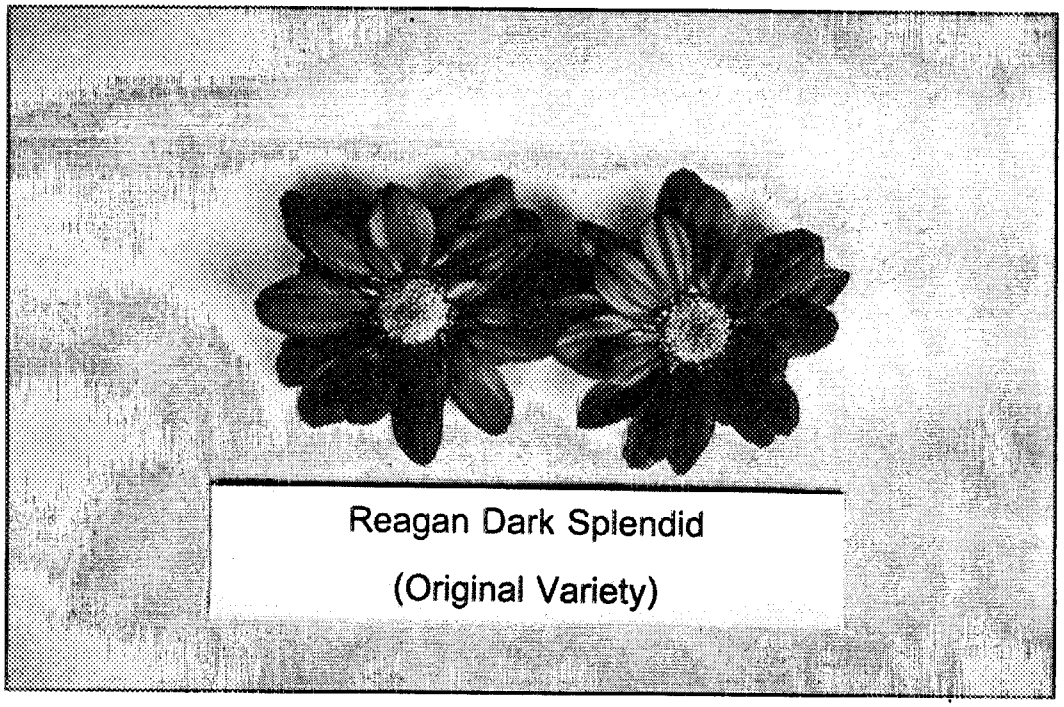

Figure 1. Purple colour of the original variety 'Reagan Dark Splendid'.

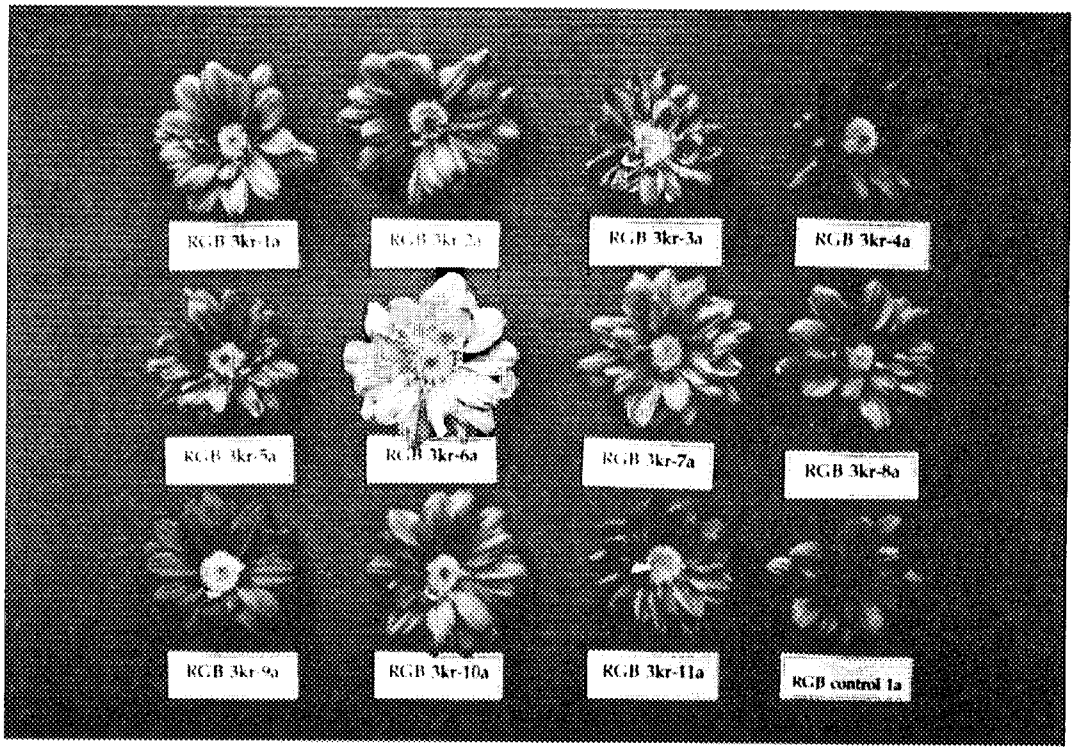

Figure 2. Selected variants from M_1V_3 plants irradiated with acute gamma rays of 30 Gy.

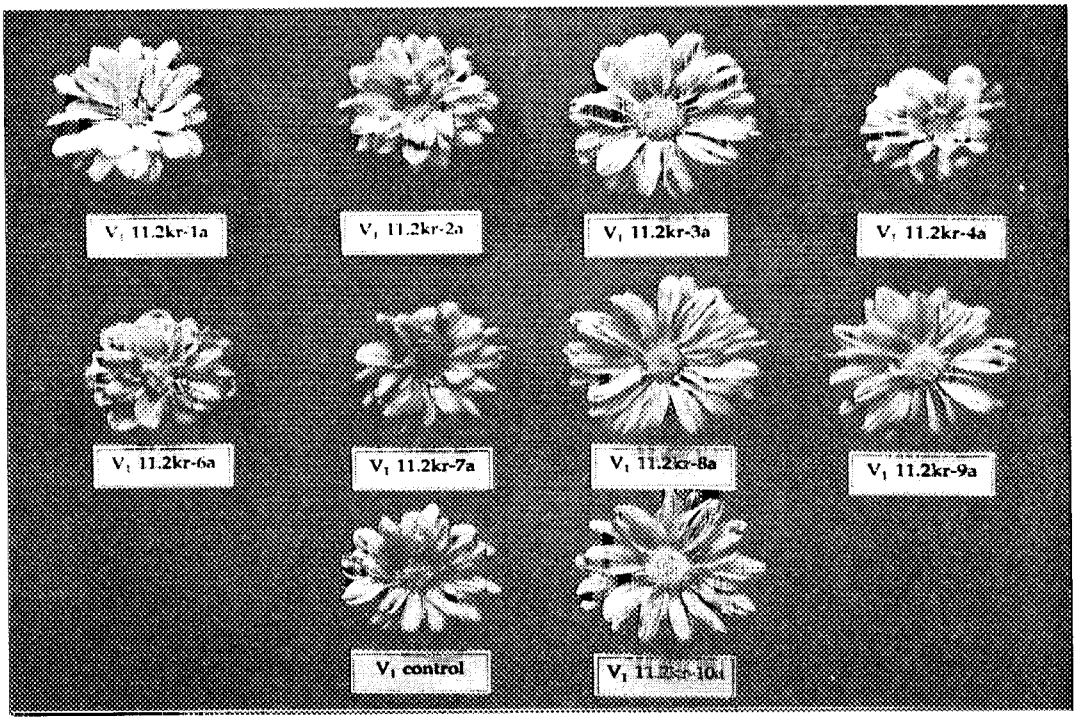

Figure 3. Selected variants from M_1V_3 plants irradiated with chronic gamma rays of 112 Gy.

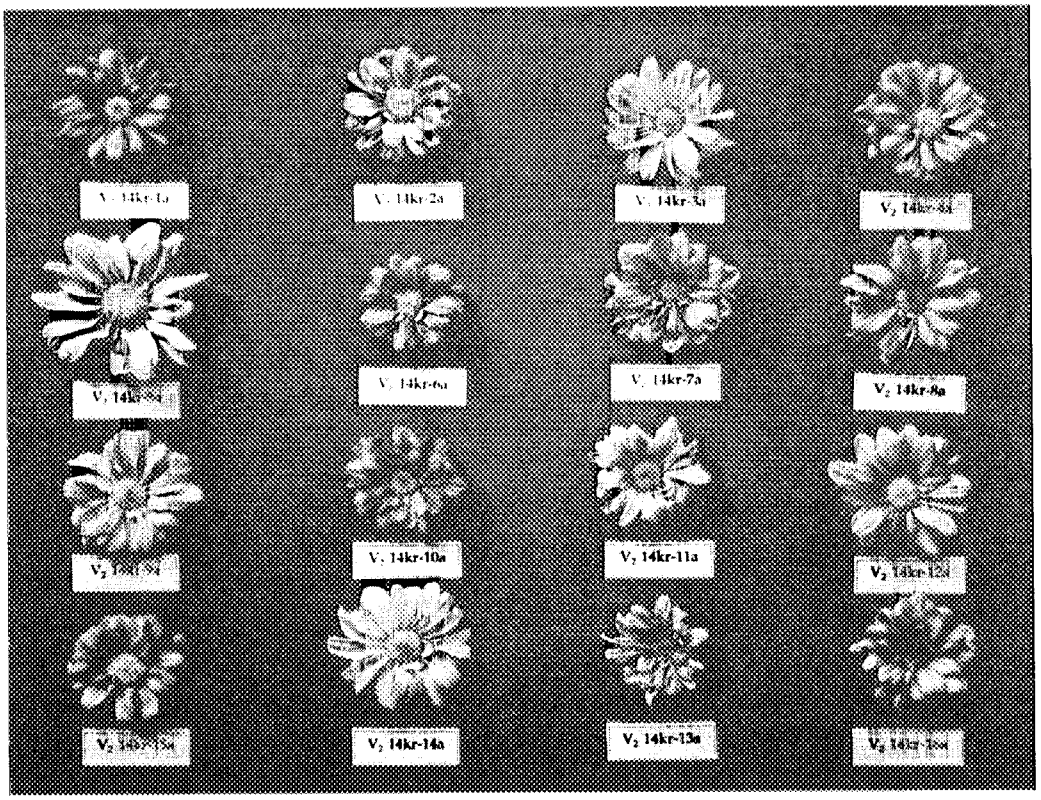

Figure 4. Selected variants from M_1V_3 plants irradiated with chronic gamma rays of 140 Gy.

5. Program of the FNCA Workshop on Plant Mutation Breeding 2001

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The FNCA Workshop on Plant Mutation Breeding 2001

-Molecular Biological Techniques-

August 20-24, 2001

Rama Gardens Hotel Bangkok, Thailand

Program

Sunday, August 19

Arrival of participants

Monday, August 20

8:30-9:00 **REGISTRATION**

OPENING SESSION: Conference Room

9:00-9:15 **WELCOME ADDRESS**
President of Kasetsart University (KU)
Prof. Dr. Thira Sutabutra

9:15-9:30 **REMARKS**
Director-General,
Research and Development Bureau, MEXT of Japan
Mr. Tsutomu Imamura
(Mr. Hamasaki will read a REMARKS for Mr.Imamura.)

9:30-9:45 **OPENING ADDRESS**
Secretary-General, OAEP
Mr. Kriengkorn Bejraputra

9:45-10:00 Break

Session 1 **PRESENTATION OF COUNTRY REPORT**
Molecular Biological Techniques for Mutation Breeding
(30 minutes presentation including question and answer)

<Chairperson : Vietnam>

10:00-10:30 **1-1 Country Report of China**
"Screening Rice Starrch Mutants via Endosperm Phenotypic maker, Physical/Chemical index, and Microsatellite maker"
Dr. Dianxing Wu
Assistant Professor of Zhejiang University

10:30-11:00 **1-2 Country Report of Indonesia**
"Biotechnological Approach in Crop Improvement by Mutation Breeding in Indonesia"

Dr.Soeranto Human
Center for Application of Isotopes and Radiation, National Atmic Energy Agency

10:00-11:30 **1-3** **Country Report of the Japan**
"Genetic and Molecular Dissection of Naturally Occurring Variations in Rice"
Dr. Masahiro Yano
Department of Molecular Genetics, National Institute of Agrobiological Sciences,

11:30-12:00 **1-4** **Country Report of Korea**
"Current Status of Molecular Biological Techniques for Plant Breeding in the Republic of Korea"
Dr.Seong Han Sohn
Biochemistry Division, National Institute of Agricultural Science and Technology, RDA, Korea

12:00-13:30 Lunch

Session 1 CONTINUE
<Chairperson: Korea>

13:30-14:00 **1-5** **Country Report of Malaysia**
"Molecular Techniques as Complementary and Additional Tools in Orchid Mutagenesis"
Dr. Mohd, Nazir Basiran and Mrs. Sakinah Ariffin
Malaysia Institute for Nuclear Technology Research(MINT)

14:00-14:30 **1-6** **Country Report of the Philippines**
"Status of Biotechnology with Emphasis on Molecular Techniques for Mutation Breeding in the Philippines"
Dr. Avelina G. Lapade
Philippine Nuclear Research Institute(PNRI)

14:30-15:00 **1-7** **Country Report of Vietnam**
"Application of molecular techniques to crop mutation breeding in Vietnam"
Prof. Dr. Tran Duy Quy (AGI)
Agricultural Genetics Institute (AGI)

15:00-15:30 **1-8** **Country Report of Thailand**
"Induced Mutations and Marker Assisted Breeding in Soybean"
Dr. Somsong Chotechuen
Department of Agriculture (DOA)

15:30-15:45 Break

Session 2 INVITED PAPERS

<Chairperson : The Philippines>

15:45-16:10 **2-1 Rice Gene Discovery**
Dr. Apichart Vanavichit
Kasetsart University (KU)

16:10-16:35 **2-2 Molecular Technique for Detection of Genetic Variation in Horticultural Crops**
Dr. Suchirat Sakuanurungsirikul
Department of Agriculture (DOA)

16:35-17:00 **2-3 Mutation Induction in Chrysanthemum through In Vitro Acute and Chronic Irradiation with Gamma Rays**
Prof. Dr. Siranut Lamseejan
Kasetsart University (KU)

18:00 **RECEPTION Hosted by OAEP**

Tuesday August 21

Session 3 ROUND TABLE DISCUSSION (1)
Conclusion of Phase II-Project

<Chairperson: Malaysia>

9:00-10:20 **3.1 Mutation Breeding Database**
Dr. Shigeki Nagatomi
National Institute of Agrobiological Resources

3.2 Mutant Stock Repository
Dr. Etsuo Amano
Fukui Prefectural University

3.3 Mutation Breeding Manual
Dr. Florencio Isagani S. Medina III
The Philippines

10:20-10:40 Break

< Chairpersons : Philippines and Japan>

10:40-12:00 **3.4 Conclusion and Discussion of Phase II-Project**

12:00-13:30 Lunch

Session 4 ROUND TABLE DISCUSSION (2)
Proposal Phase III-Project

<Chairpersons : Indonesia and Japan>

13:30-15:00	**The Proposal from Each Country for Breeding of Seed and Vegetatively Propagated Plants**
15:00-15:20	Break
15:20-17:00	**Continue**
18:00	**RECEPTION Hosted by JAPAN**

Wednesday August 22

Technical Visit

7:30	Start from Hotel
8:00-9:30	Visit Gamma Irradiation Service and Nuclear Technology Research Center (GISC), KU
9:30-11:30	Visit Office of Biotechnology Research and Development and Nuclear Techniques, DOA
11:30-13:00	Lunch
13:00-13:45	Move
13:45-16:30	Visit Pathumthani Rice Research Center, Rice Research Institute, DOA
16:30-17:30	
18:15	Return to Hotel

Thursday August 23

Session 5 ROUND TABLE DISCUSSION (3)

How to Promote Further the Regional Cooperation in the Field of Agriculture

<Chairpersons: China and Japan>

9:00-10:30	**5.1 Discussion on future project on Mutation Breeding**
10:30-10:40	Break

10:40-12:00	**Continue**
12:00-13:30	Lunch
13.30-15:15	<Chairpersons: Thailand and Japan> **5.2 Discussion on the project of Mutation Breeding**
15:15-15:45	Break
15:45-17:00	<Chairpersons :Thailand and Japan> **5.3 Discussion on Future Plan of FNCA in the field of Agriculture**
19:00	**Dinner Hosted by Kasetsart University (KU)**

Friday August 24

Session 6 ROUND TABLE DISCUSSION (4)

Conclusion and General Discussion

9:00-11:00	<Chairpersons: Malaysia and Japan> 1. Mutation Breeding 2. Biofertilizer 3. Research Cooperation 4. Other Topics
11:00-11:30	**Draft and Deliberation on Minute**
11:30-12:00	**Adoption of Minute** **Closing remarks** Remarks with Gratitude Dr. Shigemitsu Tano (Japan)
12:00-13:30	LUNCH PARTICIPANTS DEPARTURE FOR HOME

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6. List of Participants in the FNCA Workshop on Plant Mutation Breeding 2001

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国際単位系（SI）と換算表

表1　SI基本単位および補助単位

量	名称	記号
長さ	メートル	m
質量	キログラム	kg
時間	秒	s
電流	アンペア	A
熱力学温度	ケルビン	K
物質量	モル	mol
光度	カンデラ	cd
平面角	ラジアン	rad
立体角	ステラジアン	sr

表2　SIと併用される単位

名称	記号
分，時，日	min, h, d
度，分，秒	°, ′, ″
リットル	l, L
トン	t
電子ボルト	eV
原子質量単位	u

1 eV=1.60218×10^{-19}J

1 u=1.66054×10^{-27}kg

表3　固有の名称をもつSI組立単位

量	名称	記号	他のSI単位による表現
周波数	ヘルツ	Hz	s^{-1}
力	ニュートン	N	$m\cdot kg/s^2$
圧力，応力	パスカル	Pa	N/m^2
エネルギー,仕事,熱量	ジュール	J	N·m
工率，放射束	ワット	W	J/s
電気量，電荷	クーロン	C	A·s
電位，電圧，起電力	ボルト	V	W/A
静電容量	ファラド	F	C/V
電気抵抗	オーム	Ω	V/A
コンダクタンス	ジーメンス	S	A/V
磁束	ウェーバ	Wb	V·s
磁束密度	テスラ	T	Wb/m^2
インダクタンス	ヘンリー	H	Wb/A
セルシウス温度	セルシウス度	℃	
光束	ルーメン	lm	cd·sr
照度	ルクス	lx	lm/m^2
放射能	ベクレル	Bq	s^{-1}
吸収線量	グレイ	Gy	J/kg
線量等量	シーベルト	Sv	J/kg

表4　SIと共に暫定的に維持される単位

名称	記号
オングストローム	Å
バーン	b
バール	bar
ガル	Gal
キュリー	Ci
レントゲン	R
ラド	rad
レム	rem

1 Å=0.1nm=10^{-10}m

1 b=100fm^2=10^{-28}m^2

1 bar=0.1MPa=10^{5}Pa

1 Gal=1cm/s^2=10^{-2}m/s^2

1 Ci=3.7×10^{10}Bq

1 R=2.58×10^{-4}C/kg

1 rad=1cGy=10^{-2}Gy

1 rem=1cSv=10^{-2}Sv

表5　SI接頭語

倍数	接頭語	記号
10^{18}	エクサ	E
10^{15}	ペタ	P
10^{12}	テラ	T
10^{9}	ギガ	G
10^{6}	メガ	M
10^{3}	キロ	k
10^{2}	ヘクト	h
10^{1}	デカ	da
10^{-1}	デシ	d
10^{-2}	センチ	c
10^{-3}	ミリ	m
10^{-6}	マイクロ	μ
10^{-9}	ナノ	n
10^{-12}	ピコ	p
10^{-15}	フェムト	f
10^{-18}	アト	a

(注)

1．表1－5は「国際単位系」第5版，国際度量衡局1985年刊行による。ただし,1 eVおよび1 uの値はCODATAの1986年推奨値によった。

2．表4には海里，ノット，アール，ヘクタールも含まれているが日常の単位なのでここでは省略した。

3．barは，JISでは流体の圧力を表わす場合に限り表2のカテゴリーに分類されている。

4．EC閣僚理事会指令ではbar，barnおよび「血圧の単位」mmHgを表2のカテゴリーに入れている。

換算表

力	N(=10^{5}dyn)	kgf	lbf
	1	0.101972	0.224809
	9.80665	1	2.20462
	4.44822	0.453592	1

粘度　1 Pa·s(N·s/m^2)=10 P(ポアズ)(g/(cm·s))

動粘度　1 m^2/s=10^{4}St(ストークス)(cm^2/s)

圧力	MPa(=10bar)	kgf/cm^2	atm	mmHg(Torr)	lbf/in^2(psi)
	1	10.1972	9.86923	7.50062×10^3	145.038
	0.0980665	1	0.967841	735.559	14.2233
	0.101325	1.03323	1	760	14.6959
	1.33322×10^{-4}	1.35951×10^{-3}	1.31579×10^{-3}	1	1.93368×10^{-2}
	6.89476×10^{-3}	7.03070×10^{-2}	6.80460×10^{-2}	51.7149	1

エネルギー・仕事・熱量	J(=10^7 erg)	kgf·m	kW·h	cal(計量法)	Btu	ft·lbf	eV
	1	0.101972	2.77778×10^{-7}	0.238889	9.47813×10^{-4}	0.737562	6.24150×10^{18}
	9.80665	1	2.72407×10^{-6}	2.34270	9.29487×10^{-3}	7.23301	6.12082×10^{19}
	3.6×10^6	3.67098×10^5	1	8.59999×10^5	3412.13	2.65522×10^6	2.24694×10^{25}
	4.18605	0.426858	1.16279×10^{-6}	1	3.96759×10^{-3}	3.08747	2.61272×10^{19}
	1055.06	107.586	2.93072×10^{-4}	252.042	1	778.172	6.58515×10^{21}
	1.35582	0.138255	3.76616×10^{-7}	0.323890	1.28506×10^{-3}	1	8.46233×10^{18}
	1.60218×10^{-19}	1.63377×10^{-20}	4.45050×10^{-26}	3.82743×10^{-20}	1.51857×10^{-22}	1.18171×10^{-19}	1

1 cal= 4.18605J（計量法）

= 4.184J（熱化学）

= 4.1855J（15℃）

= 4.1868J（国際蒸気表）

仕事率　1 PS(仏馬力)

= 75 kgf·m/s

= 735.499W

放射能	Bq	Ci
	1	2.70270×10^{-11}
	3.7×10^{10}	1

吸収線量	Gy	rad
	1	100
	0.01	1

照射線量	C/kg	R
	1	3876
	2.58×10^{-4}	1

線量当量	Sv	rem
	1	100
	0.01	1

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