PRE-BREEDING AND GENE DISCOVERY
FOR FOOD AND RENEWABLE ENERGY SECURITY

Plant breeding has been significantly contributed to agricultural productivity through developing new superior crop varieties. The success of plant breeding depend, among others, on the availability of genetic resources, discoveries of genes that control important traits within such genetic resources and pre-breeding. Pre-breeding refers to all activities designed to identify desirable characteristics and/or genes from unadapted materials that cannot be used directly in breeding populations and to transfer these traits to an intermediate set of materials that breeders can use further in producing new varieties for farmers. It is a necessary first step in the use of diversity arising from wild relatives and other unimproved materials.

In additon to crop improvements, elucidating important genes in microbes is the key aspect for further application in industrial agriculture, such as biofuel, bioactive compounds, enzyme production, or as a gene source for biotechnology process such as marker genes or in developing superior plant such as high yield, resistant to pest and diseases, and adapting in marginal climate such as dry, submerged, saline, high temperature etc. Genome sequences of potential microbes using NGS technology reveal potential genes for industrial and plant engineering purposes.

This book contains selected papers on pre-breeding and discoveries in plants and microbes presented at the International Conference on Pre-breeding and Gene Discovery (ICPGD), held in Bogor, Indonesia, August 13–15, 2014. It consists of four chapters providing current status of pre-breeding and gene discovery in plant and microbes in Indonesia and some other countries. Chapter 1 provides a policy and supporting activities to pre-breeding and gene discoveries, which includes genetic resources management, public-private partnership and program on genetic resources utilization, in particular through the application of advance techniques. Chapter 2 consists of six papers related to pre-breeding and gene discoveries in plant. The content of this chapter is primarily on the emerging concept and understanding of advanced technology of pre-breeding and gene discovery in plants, such as the progress of genome sequences and molecular markers development and their application in plant breeding program, and strategies for gene discoveries as important for sustainable agriculture. Chapter 3 consists of six papers related to gene discoveries in microbes, primarily addressed current status of gene discovery in microbes in Indonesia, strategies of gene discovery for renewable energy development and crops productivity, and environmental metagenomic and microbial genomics. Annex contains selected abstract of papers presented at the conference in order to enrich readers' information on the topics.

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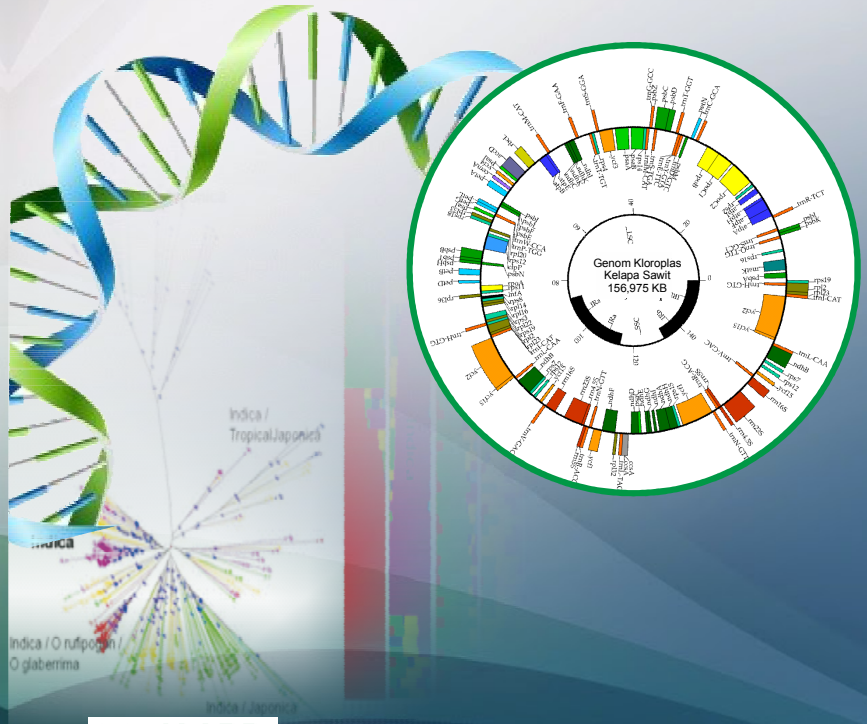

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PRE-BREEDING AND GENE DISCOVERY FOR FOOD AND RENEWABLE ENERGY SECURITY

PRE-BREEDING AND GENE DISCOVERY FOR FOOD AND RENEWABLE ENERGY SECURITY

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INDONESIAN AGENCY FOR AGRICULTURAL RESEARCH AND DEVELOPMENT (IAARD) PRESS
2016

PRE-BREEDING AND GENE DISCOVERY
FOR FOOD AND RENEWABLE ENERGY SECURITY

Printed in 2016

Cataloguing in publication

INDONESIAN AGENCY FOR AGRICULTURAL RESEARCH AND DEVELOPMENT
Proceedings pre-breeding and gene discovery for food and renewable energy security/Editors, Muhamad Sabran ... [*et al*.].--Jakarta: IAARD Press, 2016
xix, 195 hlm.: ill.; 24,5 cm

ISBN 978-602-344-134-1

1. Plant Breeding 2. Microbes
I. Title II. Sabran, Muhamad

631.52

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Members IKAPI No. 445/DKI/2012

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FOREWORD

Agricultural development in Indonesia is still faced with many challenges: most farmers are poorly resourced with land-ownership less than 0.5 ha and lives in poverty at rural areas; the production of some basic food sources barely keep pace with the rapid population growth; the unpredictable climate change threatens the effort to increase food production and might endanger food security. Fortunately, Indonesia is rich in biodiversity, which can be exploited to fulfill its necessity.

For the next 30 years, Indonesia gradually focuses its agricultural development toward a sustainable bio-industry agriculture system which treats agricultural land and other production factors as an industrial unit to produce main products for food security and other products for energy security and industry based on zero-waste principle, i.e. reduce, reuse and recycle. As a tropical country, Indonesia has the advantage of having high opportunity to harvest solar energy and transform it to biomass as the basis of bio-industry agriculture.

In order to face the above challenges and make use of those advantages, we need to optimally and sustainably use our rich genetic resources through pre-breeding and gene discovery. Our experties in this area are limited, not to mention the multidisciplinary nature of this subject. This calls for international collaboration among scientists of different background.

As the largest research organization in the country, particularly in agriculture and related sciences, Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, is now entering the second phase of its development by strengthening international networks and applications of advance sciences, such as biotechnology, bioinformatics, and bioprocesses. The shift in our prioritizing is reflected in recent investment, particularly in human resources and facilities, and expressed in our tagline: *Science*, *Innovation*, *Networks*. We took this big step in order to set the foundation for our research system to meet the challenges of agriculture in the coming years.

It is my sincere hope that this book will provide valuable information for agricultural scientists, plant breeders, gene bank managers, and research managers as well as policy makers in expanding their horizon in agriculture development, and trigger new bright ideas.

Dr. Muhammad Syakir
Director General of IAARD

PREFACE

This book contains selected papers presented at the International Conference on Pre-breeding and Gene Discovery for Food and Renewable Energy Security (ICPGD), held in Bogor, Indonesia, August 13–15, 2014, at the occasion of the 40th commemoration of the Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture. It consists of four chapters providing current status of pre-breeding and gene discovery in plant and microbes in Indonesia and participating countries in the ICPGD 2014.

Chapter 1 provides a policy and supporting activities to pre-breeding and gene discoveries, which include genetic resources management, public-private partnership, and program on genetic resources utilization, in particular, through the application of advance techniques. Chapter 2 consists of six papers related to pre-breeding and gene discoveries in plant. The content of this chapter is primarily on the emerging concept and understanding of advanced technology of pre-breeding and gene discovery in plants, such as the progress of genome sequences and molecular markers development and their application in plant breeding program, and strategies for gene discoveries which are important for sustainable agriculture. Chapter 3 consists of six papers related to gene discoveries in microbes, primarily addressed current status of gene discovery in microbes in Indonesia, strategies of gene discovery for renewable energy development and crops productivity, and environmental metagenomic and microbial genomics. In addition, Annex contains selected abstract of papers presented at the conference in order to enrich readers' information on the topics.

We wish to thank many parties that have contributed to the preparation of this book, particularly the Director General of IAARD and the Director of ICABIOGRAD that has provided us with tasks and resources for editing and printing. We wish all readers to enjoy and take advantages of this book.

Thank you.

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ACKNOWLEDGEMENTS

The editors would like to acknowledge the Indonesian Agency for Agricultural Research and Development's (IAARD) management for the permission to publish this book entitled "PRE-BREEDING AND GENE DISCOVERY FOR FOOD AND RENEWABLE ENERGY SECURITY". Special thanks to the Director General of IAARD, Director of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), speakers, and reviewers related to writing and publishing this book. Budget from national funding of ICABIOGRAD of fiscal year 2016 for the publication is greatly acknowledged.

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CHAPTER 1
PRE-BREEDING, CONSERVATION, AND CROP IMPROVEMENTS

Crop improvement is the earliest technology in the history of agriculture. For centuries, farmers domesticated and selected crops for their basic need. They also conserved the crops, saved and transferred the seeds and knowledge to the next generation. The advancement in science is then made possible to transfer genes that control the desired trait from one crop to the other through crossing or molecular ways. Modern crop varieties, on which our present and future food security depend, are the result of years of careful crossing and selection of highly refined, in general, genetically uniform material. Some of these varieties have been so successful that they have been adopted over large areas. This might have left the crop vulnerable to new pests, diseases, and climatic conditions, not to mention changes in consumer preferences. So, we need to continously look-out for new sources of useful traits for new challenges.

To bridge the gap between wild genetic resources and those plant breeders working with advanced and elite cultivars, broadening the base of gene banks and pre-breeding that link to the conservation and uses of plant genetic resources (PGR) are needed. Prerequisite for pre-breeding are proper management of gene bank, both *ex situ* and *in situ*, and identification of genes in the gene bank through gene discovery.

This chapter begins with an overview of genetic resources management in Indonesia, in term of conservation, uses and exchanges, and then be followed by a paper on current status of pre-breeding research involving wild species in Bogor and Cibodas Botanic Gardens and a paper on public-private partnership for pre-breeding, the Philippine public initiative and experiences. The last part in this chapter is the paper on genomic-based crops improvement to support Grand Strategy of Indonesian Agricultural Development (GSIAD) 2015–2045. It contains the program of IAARD through ICABIOGRAD on genomic research by providing genomic information on its collection of genetic resources and their use for genomic-based plant breeding.

PLANT GENETIC RESOURCES MANAGEMENT IN INDONESIA: CONSERVATION, USES, AND POLICY

Muhamad Sabran

ABSTRACT

As megadiversity country, Indonesia is rich in genetic resources but poor in genetic resources collection, data, and information. This review paper described the current states of genetic resources management in Indonesia, including *in situ* and *ex situ* conservation, information system, utilization of plant genetic resources (PGR), in particular for crop improvement, and regulation in harmony with international convention/ treaty. Recommendation is provided in term of delineation of task among institutions in PGR management, strengthening *ex situ* conservation, strengthening the information system, and supporting plant breeding and sustainable use of PGR for food and agriculture.

Keywords: genetic resources, conservation, sustainable use.

INTRODUCTION

Indonesia is a megadiversity country, which harbors about 110,483 species of plants, consists of 91,251 species of sporophyte and 19,232 species of spermatophyte (IBSAP, 2016). Among the 110,483 plant species that have been identified, the Ministry of Agriculture have determined 484 crop species under the ministry's supervision, of which 32 crop species will be highly prioritized which consist of 7 food crops (rice, soybean, maize, cassava, sweet potato, mungbean, and peanut), 10 horticultural species (chilli pepper, paprika, shallot, potato, mango, citrus, banana, durian, mangosteen, and salacca), and 15 estate crop species (rubber, coconut, oil palm, coffee, cocoa, cashew nut, pepper, clove, tea, jatropha, pecan, sugarcane, cotton, tobacco, and patcheoli) (Kementan, 2015).

Within plants species, there is genetic diversity that contributes to genetic resources. These genetic resources has been exploited for crops' improvement through breeding and domestication. The success of breeding depends heavily on the genetic diversity as the sources of genes. Therefore, proper management of genetic resources is very important for supporting the breeding program and others uses. Genetic resources management consists of exploration, conservation, characterization, evaluation, and uses. It is also supported by the database system and the policy and regulation for access, exchanges, and benefit sharing of the utilization of plant genetic resources (PGR).

PGR are under considerable threat of erosion due to climate changes, replacement of farmers varieties, land clearing, overexploitation, reduced water availability, population pressure, changing dietary, habits, environmental

degradation, changing agricultral systems, overgrazing, legislation and policy, pest, diseases, and weed. Conservation of genetic resources is therefore needed to maintain genetic diversity and avoid its loss or extinction.

Characterization and evaluation are neccessary in order for breeders and other users of plant genetic resources for food and agriculture (PGRFA) to make the most effective use of gene bank collection. They must be able to identify which accessions are likely to have the traits they need. Characterization data record a plant variety's distinct and heritable features. Evaluation data record the traits that are promising for crops improvement. This information can also help gene bank managers to organize subset collection based on particular traits or that feature maximum diversity. These subsets have been shown significantly improve the use of gene banks.

PLANT GENETIC RESOURCES CONSERVATION

In Situ and On-farm Conservation

In situ conservation is conservation of genetic resources at their original habitat. It may include on-farm conservation, i.e. conservation of plant and animal genetic resources at farmers field with traditional farming system. In crops, on-farm conservation is done when the crops are spread over in large areas with many farmers. Some examples of *in situ* conservation are:

1. The Tado community at Sano Nggoang Subdistrict, West Manggarai District, Flores Island, East Nusa Tenggara Province, traditionally conserved upland rice (mavo) varieties on-farm by indigenous knowledge. Traditional timing of mavo planting was determined by celestial observations to ensure optimal harvests. Mavo is planted by Tado farmers during the months of October–December (at the start of the rainy season) using traditional methods, and intercropped with maize, tubers, and leafy vegetables for household consumption, or with perennial crops, such as teak, to supplement household income. There are patterns of regional flux and inter- and intra-community dissemination of both ‘‘old’’ and ‘‘new’’ landraces over the past three generations. Of the 16 landraces available, there are nine old varieties and seven new ones. Farmers recounted frequently variety switching for a wide range of reasons, including dissatisfaction with a given variety's performance during a ‘‘bad’’ year, improved taste or heat tolerance of a new variety, and spontaneous experimentation with crop lines that yielded well on other farmers' fields. Older varieties have been replaced by newer varieties (Pfeiffer *et al*., 2006).
2. Farmers in South Kalimantan conserved some local varieties of *Mangifera*, *in situ* or on-farm (source: Indonesian Center for Horticultural Crops Research and Development/ICHORD). For example, kasturi (*Mangifera casturi* Delmiana) was conserved *in situ* because of its high price during the

harvest season. This fruit tree was also conserved because of its valuable services to environment. In Telaga Langsat Village of Hulu Sungai Selatan District of South Kalimantan, people revered many of trees, including kasturi and kuini (*M. odorata*), in their village and surrounding buffer forest (Sthapit *et al*., 2013). The informal rules and beliefs protected those trees from felling. The district government and the community have developed a new informal village regulation that is enforced by village protection group that limits the felling of the trees, since the old beliefs and tradition have been eroded due to the value of the timber that motivates to fell the trees. This is an example where a community regulation will motivate the *in situ* conservation of fruit tree.

3. The Kintamani Bali arabica coffee, is grown by small-holders farmers with organic practice at volcanic Kintamani region. Its bean size is generally larger than that of the arabica coffee beans grown in other region in Indonesia and has specific taste. Coffee farms suffer a water deficit during the dry season from July to November when soil water availability is not sufficient to meet the needs of coffee. However, the farmers apply organic manure routinely in order to maintain the soil water content, also use sufficient shade trees to keep the temperature down during the dry season and reduce transpiration. Relative humidity at mid-day is fairly high, more than 80%. A certificate of geographical indication (GI) protection for Kintamani Bali arabica coffee has been issued by the Directorate General for Intellectual Property Rights, Ministry of Law and Human Rights, on December 5, 2008. This is the first GI protected product in Indonesia (Mawardi, 2009).

The three examples of *in situ* conservation mentioned above represent the three reasons that motivate farmers to conserve PGR, i.e. to maintain culture and tradition, potential value of genetic resources, and the real market value of genetic resources. There might be other reason that motivate farmers to conserve genetic resources *in situ*. Unfortunately, there are no record on number, location, and type of crops that have been conserved *in situ* or on-farm in Indonesia.

In general, *in situ* conservation is the effort to protect species, genetic variations, and habitats in the indigenous ecosystem. The *in situ* approach comprises of the setting and management of protective areas, such as nature reserves, wildlife sanctuaries, national parks, natural recreation parks, protected forests, river boundaries, germplasm areas, and peat areas. In practice, the *in situ* approach is also used as a strategy to manage and protect wild animals outside the protective areas. In forestry and agriculture sectors, the *in situ* approach is also used to protect the genetic diversity of plants in the original habitat and in determination of protected species without specifying their habitats. Until 2005, there are 534 units of conservation areas covering more or

less 28 million hectares in Indonesia, where terrestrial conservation areas are 495 units (80.33%), while the marine conservation areas reached 39 units (19.67%). About 57.94% of the total conservation areas are national parks (FAO, 2013).

Within the Ministry of Agriculture, *in situ* and on-farm conservation are supervised by the Assessment Institute for Agricultural Technology (AIAT) in each province in Indonesia. A consortium for the inventory, conservation and characterization, and database management of local genetic resources have been established since 2012 involving several research centers and research institutes within the Indonesian Agency for Agricultural Research and Development (IAARD). The main players of this consortium are the AIATs, which do most of the fieldwork, and the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) coordinates the activities and gives technical supervision to the AIATs. The activities also include supervising the *in situ* conservation of local PGR as well as inventorying institutions, organizations, and communities' group that conduct genetic resources conservation.

Inventoring local PGRFA in all provinces in Indonesia showed their variation spreading over various agroecosystem. Database for this inventory including its mapping all over the country can be accessed online at www.bbsdlp.litbang.pertanian.go.id/sdgp/. The states and distribution of genetic resources are regularly monitored through this website.

Ex Situ Conservation

Ex situ conservation is conservation of genetic resources outside their natural habitat. It may consist of a series of activities, such as collection, characterization, and evaluation as well as maintenance and database management. The purposes of *ex situ* conservation are for security, exhanges and future use of the genetic resurces. It requires a good storage facilities and therefore more expensive than the *in situ* conservation. Considering the high cost and the technologies needed, the PGR conserved *ex situ* should be limited to those that endangered of lost or potentially contain valuable traits such as local variety or wild relatives of crops.

Within the Ministry of Agriculture, *ex situ* conservation of genetic resources is managed by the ICABIOGRAD. In addition, commodity-based research centers within IAARD have also their own collection for the commodity covered within their mandate. Table 1 presents the list of crops and the number of accession collected by research centers within IAARD.

The *ex situ* conservation methods varied among institutions according to the physiological characters of the crops. Crops with orthodox seeds are conserved in short- and medium-term storage, while those with recalcitrant

Table 1. Number of PGRFA collection at research centers within IAARD.

Crops	Research Centers/Institutes							
	ICABIOGRAD	ICCR	ILETRI	IMRI	IVEGRI	IOCRI	IFRI	ICRI
Rice	4116	2939						
Wild rice	94							
Wheat	83			441				
Maize	1052			441				
Sorghum	246			441				
Soybean	888		1072					
Peanut	821		179					
Mungbean	915		1052					
Minor legumes	179		0					
Cassava	556		310					
Sweet potato	1364		218					
Taro	257		0					
Minor tubers	276		0					
Vegetables					286			
Ornamental						373		
Fruits							1040	
Citrus and subtropical fruits								163

Sources: ICABIOGRAD (2015); ICFORD (2016); ICHORD (2016).

seeds and vegetatively propagated need to be conserved in field or *in vitro*. *In vitro* and cryopreservation are alternative methods for conserving crops with recalcitrant seeds or vegetatively propagated. These methods of conservation need large investation and protocol for each crop. The cryopreservation gene bank is now under construction at the ICABIOGRAD.

There are several other governmental organizations that also have activities for *ex situ* conservation of PGR within their respective mandates. The Ministry of Forestry and Environment maintains their plant collections, especially for economically important timber trees. The Forestry Research Center has 33 forests for research which in total covers 37,000 hectares area that conserve 234 species of trees (136 genera, 50 families) of which 167 species are local and 67 are exotic species (FAO, 2013; FORDA, 2016). Ministry of Health has 13 hectares area for *ex situ* conservation of 850 species of medicinal plants in Tawangmangu Garden, Central Java. Research Center for Science and Technology (Puspiptek), Serpong, Banten has 350 hectares for conserving 160,020 number of specimens from 37 families, 378 genera, and 602 species (FAO, 2013).

The Indonesian Institute of Sciences (IIS) manages four botanic gardens in Bogor, Cibodas, Purwodadi, and Bali (Eka Karya). The Bogor Botanic Garden conserves plants from lowland humid areas, the Cibodas Botanic Garden conserves plants from the highland humid areas, the Purwodadi Botanic Garden conserves plants from lowland dry areas, and the Eka Karya Bali Botanic Garden conserves plants from highland areas of the eastern part of Indonesia. The number of collection of these four botanic gardens are listed in

Table 2. In addition, the Research Center for Biology of IIS has also established the Wamena Biological Garden (WBiG) in 1995 (Rahmansyah and Latupapua, 2003), which became the only ex *situ* plant collection in Papua. About 150 local species were planted in more than 200 hectares land area. This garden also maintains cultivars of sweet potato, especially from the highland of Papua. Twenty hectares of germplasm collection are managed by the Research Center for Biotechnology, which conserves 2,250 accessions from 108 cultivars of 16 species fruit trees. This garden also has 360 accessions of cassava (120 genotypes), 710 accessions of taro (182 genotypes), 693 accessions of multipurpose trees from 10 species, and 43 accessions of *Jatropha curcas*.

Some local governments, under the supervision of the IIS, have also constructed botanic garden based on specific theme or priority of plants. Sungai Wain Botanic Garden in Balikpapan, East Kalimantan focuses on timber trees, nepenthes, and orchids, and Enrekang Botanic Garden in South Sulawesi acts of concentrating interest of Wallace plants (Hadiah, 2011). Bukit Sari Botanic Garden in Jambi is the remaining lowland forest among oil palm plantations in that area. This garden was established in 2003 with area of 300 hectares and about 400 species were found in that garden. Batu Raden Botanic Garden in Central Java conserves Javanese flora. This garden was established in 2004, covering 142.2 hectares and 107 species. Kuningan Botanic Garden represents Ciremai mountain flora and plants adaptable to stony areas. Liwa Botanic Garden in West Lampung focuses on ornamental plants and plants from South Bukit Barisan. Meanwhile, Katingan Botanic Garden in Central Kalimantan pays particular attention on fruit trees, while Puca Botanic Garden in Maros, South Sulawesi focuses on economical plants collections.

Table 2. Number of collection at botanical gardens within the Indonesian Institute of Sciences.

Botanical Garden	Families	Genera	Species	Specimen	Major plants
Bogor	218	1,227	3,301	13,061	Palms, orchids, medicinal plants, ornamental plants, shrubs and trees, aquatic plants, climbers, fruit trees
Cibodas	243	886	2,044	9,814	Fern, bryophytes, cacti, orchids, rhododendrons, gymnosperms, cinchona
Purwodadi	178	965	2,014	11,720	Succulents, orchids, mango, banana, medicinal plants
Eka Karya, Bali	242	1,078	2,403	21,502	Cacti, begonias, bamboo, medicinal plants, orchids

Sources: www.krbogor.lipi.go.id; www.krcibodas.lipi.go.id; www.krpurwodadi.lipi.go.id; www.krbali.lipi.go.id; FAO (2013).

REGULATION AND POLICY ON PGR

Interdependence on PGR

An in-depth analysis on countries interdependence on PGRFA demonstrates (Khoury *et al*., 2014) that national food supplies and production systems are highly interdependent worldwide in regard to PGR. Countries strongly depend on crops whose genetic diversity largely originating from outside their borders, both in their food supplies (65.8% dependence on nonindigenous crops for calories, 66.6% for protein, 73.7% for fat, and 68.6% for food weight as an average across countries worldwide) and in their production systems (71.0% for production quantity, 64.0% for harvested area, and 72.9% for production value). The global average of the degree of countries' dependence on crop genetic diversity originating from outside their borders is 68.7% across food supply variables, 69.3% across production variables, and 68.9% across all variables for all countries.

Acknowledging the interdependence on PGR, Indonesia engages in many international agreements on exchanges of genetic resources and the benefit arising from their uses. Although Indonesia is a genetic resources-rich country, it still needs genetic resources of nonindigenous crops from other countries. National dependence on nonindigenous crops have increased over the past 50 years as countries' food systems have become more diverse and at the same time more homogeneous worldwide (Khoury *et al*., 2014). At the other side, Indonesia needs to use its genetic resources sustainably as much as possible for people welfare, as stated in its constitution. This utilization of genetic resources, in some cases, requires collaboration with the technology-rich countries, which inevitably will involve exchanges of genetic resources.

Two international agreements that Indonesia intensively involved are the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA, the Treaty) and the Nagoya Protocol of the United Nations (UN) Convention on Biological Diversity (CBD). The ITPGRFA mostly deals with multilateral system (MLS) of access and benefit sharing of PGR, while the Nagoya Protocol provides rule for bilateral system of access and benefit sharing for all genetic resources, except human genetic resources.

The ITPGRFA

The ITPGRFA was adopted by the FAO conference in 2006 after 7 years of negotiation. The Secretariate of the Treaty hosted by the FAO. Currently, there are 140 contracting parties of the Treaty. Indonesia ratified the Treaty in 2006 through the National Law No. 4/2016. The Director of ICABIOGRAD, ex officio, is the National Focal Point of the Treaty in Indonesia. The main objective of the Treaty is conservation and sustainable use of PGRFA (ITPGRFA, 2013).

The Treaty recognizes the sovereign rights of contracting parties over their own PGRFA. In the exercise of such rights, the Contracting Parties agreed to establish a MLS of access and benefit sharing of PGR, which is efficient, effective, and transparent. Both are to facilitate access to PGRFA, and to share, in a fair and equitable way, the benefits arising from the utilization of these resources.

The MLS covers the PGRFA listed in Annex I of the Treaty that was established according to criteria of food security and interdependence. This MLS shall include all PGRFA in the list that are under the management and control of the Contracting Parties and in the public domain. The MLS shall also include the PGRFA in Annex I is held in the *ex situ* collections of the International Agricultural Research Centers of the Consultative Group on International Agricultural Research (CGIAR) and in other international institutions. Multilateral in this context means that a global pool of PGRFA is shared and managed jointly by all Contracting Parties of the Treaty. The Standard Material Transfer Agreement (SMTA) that has been multilaterally agreed as the standard private law contract to be used for the exchange of material from the MLS which reconciles the global nature of PGR.

All exchanges of genetic resources in the MLS are done according to the provisions of the SMTA, which is a standardized private law contract between a provider and a recipient (user) of material. This standard contract was adopted by the first session of the Governing Body in 2006. While the providers of material are usually public or international gene banks, both providers and recipients as users can be organizations, private entities, or individuals. More than 3.2 million crop accessions have been transferred and reported since January 2007 with SMTA. It also contains Dispute Resolution Procedures for the gene pool of the Treaty. FAO has accepted in principle to represent the interests of the Third Party Beneficiary under the SMTA and to initiate dispute resolution procedures to protect those interests. FAO and the Treaty have developed and documented useful experiences for other MEA conventions or other UN agencies to resolve genetic resource disputes.

The ITPGRFA was one of the first international legal instruments to put into practice the principle of benefit sharing when accessing plant genetic material and resources. In 2010, it started to provide financial support to farmers and local and indigenous communities conserving valuable plant genetic material for plant breeding and agriculture. The Treaty's benefit sharing fund utilizes four types of benefit sharing mechanisms, namely information exchange, technology transfer, capacity building, and monetary benefit sharing.

The Treaty calls for technology transfer as a form of nonmonetary benefit sharing (Article 13.2.b), backed by information exchange (Article 13.2.a), and capacity-building (Article 13.2.c). The Treaty provides that "Transfer of

technology to countries ... shall be carried out through ... all types of partnership in research and development" (Article 13.2.b.iii). Priority is given to "The implementation of agreed plans and programs for farmers in developing countries ... who conserve and sustainably utilize plant genetic resources for food and agriculture" (Article 18.5). The Treaty's Governing Body, in all its sessions, has called for Contracting Parties and other relevant stakeholders to explore innovative ways to realize effective technology transfer (Resolution 4/2011), emphasizing that technology transfer is required to enhance the capacity to use PGRFA through plant breeding, including the utilization of modern tools, traditional varieties, and the participation of farmers.

At the UN Conference on Sustainable Development (Rio de Janeiro, Brazil, June 21, 2012), a high-level round table convened by the Governments of Brazil, Indonesia, and Norway, adopted the Rio Six-Point Action Plan for the International Treaty. It recommended, as a priority, that stakeholders in the Treaty "establish a platform for the codevelopment and transfer of technologies, within the context of nonmonetary benefit sharing under the Treaty". Following the Rio de Janeiro Meeting in 2012, Indonesia and Brazil, through their respective national agriculture research organization established the Platform and convened its first meeting in Brasília on August 7–8, 2012. The meeting agreed on the objective and mission statement of the Platform. The second partners meeting of the Platform was held in Bandung, Indonesia on June 30–July 1, 2013, and agreed on institutional structure and the work plan of the Platform. The third meeting of the Platform in September 7, 2015 extended the scope and revised the work plan of the Platform (ITPGRFA, 2015).

The Nagoya Protocol

The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) is a supplementary agreement to the CBD. It provides a transparent legal framework for the effective implementation of one of the three objectives of the CBD "The fair and equitable sharing of benefits arising out of the utilization of genetic resources".

The Nagoya Protocol on ABS was adopted on October 29, 2010 in Nagoya, Japan and entered into force on October 12, 2014, 90 days after the deposit of the fiftieth instrument of ratification. Its objective is the fair and equitable sharing of benefits arising from the utilization of genetic resources, thereby contributing to the conservation and sustainable use of biodiversity. Indonesia then ratified the Protocol through National Legislation No. 11/2013.

The Nagoya Protocol will create greater legal certainty and transparency for both providers and users of genetic resources by establishing more predictable conditions for accessing to genetic resources, and helping to ensure benefit sharing when genetic resources leave the country providing the genetic

resources. It creates incentives to conserve and sustainably use of genetic resources and, therefore, enhances the contribution of biodiversity to development and human well-being.

The Nagoya Protocol applies to genetic resources that are covered by the CBD, and to the benefits arising from their utilization. It also covers traditional knowledge (TK) associated with genetic resources that are covered by the CBD and the benefits arising from its utilization. The Nagoya Protocol sets out core obligations for its Contracting Parties to take measures in relation to access to genetic resources, benefit sharing, and compliance.

National Legislation on Genetic Resources

There is no national law governing genetic resources, other than the ratification of the ITPGRFA and the Nagoya Protocol. There was a proposal to revise Law No. 5/1995 on Ecosystem and Biological Resources Protection by inserting provisions on genetic resources. This proposal, however, invites many criticism, particularly on the very broad coverage of the draft law and domination of the school of taught that the genetic resources need to be protected rather than being used sustainably. Discussion is now still underway on the proposed revision before being submitted to the parliament.

The lack of national law governing genetic resources creates difficulties in the implementation of the Nagoya Protocol on ABS which requires a competent authority as the entry point for negotiating the agreement on ABS for all available genetic resources. At lower legal hirarchy, several ministries establish ministerial regulation that generated from the two ratifications or other law within their respective mandates. For examples in the Ministry of Agriculture are Ministerial Regulation No. 37/2011, which regulates the permit to export and import of genetic resources for researches and the permit to explore genetic resources in the country, and Ministerial Regulation No. 15/2010, that provides guidance for genetic material transfer agreements. These regulations are only valid for PGRFA as it is within the mandates of the Ministry of Agriculture. Reference for these ministerial regulations are the Law on ITPGRFA Ratification (Law No. 4/2016) and the National Seed Law and the Law on Plant Variety Protection (Law No. 29/2000).

UTILIZATION OF PGR

Crop improvement is the earliest technology in the history of agriculture. For centuries, farmers domesticated and selected crops for their basic need. They also conserved the crops, saved and transferred the seeds and knowledge to the next generation. The advancement in science is then made possible to transfer genes that control the desired trait from one crop to the other through crossing or molecular ways. Modern crop varieties, on which our

present and future food security depend, are the result of years of careful crossing and selection of highly refined, generally genetically uniform material. Some of these varieties have been so successful that they have been adopted over large areas. This probably has left the crop vulnerable to new pests, diseases, and climatic conditions, not to mention changes in consumer preferences. So, we need to continously look-out for new sources of useful traits for new challenges.

The increasing demand of agricultural products, both in quantity and quality, and the challenges in agricultural production due to the climate changes, prompted the need for more effective and efficient crops breeding. Conventional methods of breeding by means of selection based on phenotypic traits neither efficient nor effective. Development in molecular biology and biotechnology gives the opportunity to apply the more efficient and effective breeding methods.

Application of biotechnology in crops improvement could be done by broading of genetic variation through *in vitro* techniques, such as somaclonal variation, anthere cultures, and somatic hybridization, genes or its promoter inclusion, and nucleic acid sequence editing in the genes. It will also shorthened the breeding cycles by combining the crossing methods and tissue culture techniques, both for rescuing the embryo and for haploidization in order to speed up the process of obtaining homozygous plant. Marker-assisted selection (MAS) will increase the accuracy and the efficiency of selection.

Breeding activities in Indonesia mostly done by recearch centers within the IAARD. Some universities and other research centers, such as the IIS and National Atomic Energy Agency of Indonesia (Batan), also have breeding activities, but their scopes and intensities are limited. Within the IAARD, crop-based research centers such as Indonesian Center for Food Crops Research and Development (ICFORD), ICHORD, and Indonesian Center for Estate Crops Research and Development (ICECRD) have their own breeding program to support the program of the Ministry Agriculture on crops within their respective mandates. The ICABIOGRAD develops the tools and methodology for improving the breeding methods as well as conducting the nonconventional breeding program in collaboration with the crop-based research centers.

The objective of genetic resources utilization through breeding are new varieties of crops. In the period of 2010–2013, 72 new varieties of food crops have been released and consist of 42 varieties of rice, 18 varieties of maize, 5 varieties of soybean, 5 varieties of peanut, 1 variety of sweet potato, and 1 variety of cassava. For horticultural crops, 91 new varieties have been released and consist of potato (3), shallot (4), chili pepper (3), kangkung (1), sallacca (1), papaya (1), and ornamental crops (4). For estate crops, 35 varieties have been released and consist of coconut (6), clover (4), sago (1), aren (1), patchouli (2),

tobacco (4), sunan pecan (2), vegetable pecan (1), rosella (4), sesame (2), pegagan (2), menta (10), fragrant root (2), turmeric (1), and purwoceng (1). These new varieties of crops have significant impact on agriculture production and their quality (Renstra Litbangtan, 2015).

The breeding program for food crops in the period of 2015–2019 will be focused on developing rice, maize, and soybean varieties that are high yield, short maturity, tolerant to biotic and abiotic stresses, and adaptive to suboptimal land, in particular those are affected by the climate changes. In horticulture, the breeding focus is for developing varieties with short maturity, better eating quality, seedless, and better performance. With those qualities, it is expected that the horticultural crops will reduce the price volatilities and improve distribution, in particular for bulky and voluminous horticultural crops.

Estate crops consist of medicinal crops, spicy crops, palm, sweetener crops, refresher crops, and other industrial crops. Breeding for perennial industrial crops will be focused on developing varieties/clones with shorth maturity, long productive period, tolerance to pest and diseases, tolerance to abiotic stresses, and high productivity. Other desired traits are: high oil content for some crops that will produce biofuel, patcheoli oil, and palm oil; high sugar content for sweetener crops; good quality of fiber for fiber crops.

INFORMATION SYSTEM

Information system will add value to the genetic resources. The global information system on PGRFA has been developed by many initiatives and conventions. The IAARD established gene bank for core collection at the ICABIOGRAD, which is designed to be the national gene bank for PGRFA, while the crop specific collection as well as the working collection are managed by crop-based research centers (sub-gene banks). In order to manage the agricultural genetic resources data, a database system has been developed since 2000 namely the Agricultural Genetic Resources Information System (AGRIS). It is a Microsoft- based stand alone database. This database system is used for internal purposes in order to manage data related to the gene bank operational management. The data managed in the database are: passport data, such as provenance; management of germplasm (storage) data, such as seed viability test, seed health, storage location, and seed stock; phenotypic characterization data; evaluation data, including resistance to pests and diseases, tolerance to biotic and abiotic stresses, as well as nutritional quality data; distribution, such as users who requested the accessions, accessions requested/distributed, purposes of uses, etc.

The other version of AGRIS is the web-based database, which is built using PHP-MySQL. It is used for coordinating and monitoring of agricultural genetic resources collection within sub-gene bank and central gene bank

networking. This system only provides the basic collection data of agricultural genetic resources, while the complete characterization data is managed by each sub-genebank.

The IIS is now developing the Indonesian Biodiversity Information Facility (InaBIF) which is the subsystem of the Global Biodiversity Information Facility (GBIF). The InaBIF will be linked to the database system in the Ministry of Agriculture (AGRIS), Ministry of Forestry and Environment, Ministry of Marine and Fisheries, and the provincial governments, in particular local botanic garden. The InaBIF will have wider scope than the AGRIS since it covers the species and ecosystem as well as the genetic resources (IBSAP, 2016).

RECOMMENDATIONS

Delineation of Task in Genetic Resources Management

There are many institutions, universities, and organizations that manage genetic resources for their own purposes. To avoid duplication or overlap of activities, there is a need to delineate the task or activities among those institutions, universities, and organizations. In the absence of the national law governing genetic resources, this delineation might be difficult to be agreed upon. However, delineation of task of institutions within a ministry is possible. Within the IAARD, we proposed the following delineation of task.

ICABIOGRAD will become the coordinator of the PGR management. It will host the national gene bank which has medium- and long-term conservation. It will also manage the database and information system on PGR. Any exchange of genetic resources should go through this institution. In utilization of genetic resources, ICABIOGRAD will do molecular breeding and develop marker for MAS.

The crop-based research centers within the IAARD, such ICHORD, ICECRD, and ICFORD, will manage genetic resources of crops within their respective mandates. They primary deal with working collection, i.e. materials underdevelopment, since they main mandate is to develop varieties of crops for specific purposes.

The provincial AIAT will conduct survey and inventorying local genetic resources within their respective province, characterize and conserve them on field gene bank or nursery garden. They also supervise farmers via *in situ* and on-farm conservation. All these activities shall be technically supervised and coordinated by ICABIOGRAD.

Strengthening *Ex Situ* Conservation

While Indonesia is a megadiversity country which is rich in genetic resources, it is poor in genetic resources collection. Its gene banks are poorly

managed and below international standard. There is no national gene banks and no network among gene banks. Delineation of task among institutions and organization is important steps to create and strengthen the networks among gene banks and also improve the capacity of the gene banks to meet international standard.

Some crops, such as underutilized crops, wild food species, forages, and crop wild relatives, are generally more difficult to conserve in gene banks than major food or forage crops. They require a sustainable system of *ex situ* conservation and use for both seed and vegetatively propagated species. Conserved materials should be replicated and stored in long-term facilities that meet international standards, in accordance with applicable international agreements.

To reduce unnecessary redundancy of germplasm accessions in current programmes, holders of crop diversity, including national gene banks, breeders, and non-governmental organizations, should coordinate and promote the exchange of information about PGRFA in line with national priorities and laws and relevant regional and international agreements. The information includes characterization and evaluation data on the genetic resources. This activity aims to enhance the use and management of PGR held in gene banks, to fill data gaps and make it easier for users to access the characterization and evaluation information, so that these resources can be deployed directly in the field or used in research and crop improvement. It will be particularly important to assess gene bank accessions and breeding materials for traits associated with mitigation and adaptation to climate change. The goal is to make gene bank collections as useful as possible. This activity may involve developing and adapting molecular techniques, like high-throughput evaluation methods for gathering characterization and evaluation data, creating core and trait-specific collections, particularly for crops of global importance, and improving the exchange of characterization and evaluation data.

Strengthening Informations System on PGRFA

Driven by the information technology revolution, there have been massive improvements in the availability and user-friendliness of PGRFA information over the past two decades. Nonetheless, there are still major gaps in the information available, including incomplete documentation of gene bank holdings worldwide, which represent a serious obstacle to efficient planning and use of PGRFA in research and crop improvement. Much existing data is still not accessible electronically and little information is available about on-farm genetic resources and crop wild relatives. A significant imbalance exists among regions and even among countries within regions with regard to their ability to access, manage, and disseminate information. This priority activity aims to strengthen the capacity of countries to manage PGRFA data and to support their

participation in regional and global information systems. It also aims to enhance the use of regional and global information systems and to strengthen the exchange and use of information and the sustainability of current information systems. The effectiveness of information systems should be monitored and differences between systems adequately addressed to facilitate interoperability and promote use. Existing data need to be verified and additional baseline data need to be collected for monitoring progress on sustainability and food security. The information held in national systems should be readily available to all actors with an interest in PGRFA.

Supporting Plant Breeding and Sustainable Use of PGRFA

Plant breeding programmes are still often ill-equipped to meet the demands placed upon them. Breeding programmes do not take enough advantage of the diversity available in gene banks nor do they often seek out the perspectives of farmers and other users when setting priorities. Besides, breeders make little use of techniques such as pre-breeding or genetic enhancement, which make collections more useable by furnishing breeding materials with traits to increase yields and resist pests and diseases and by increasing the amount of diversity available. Breeders should pay greater attention to underresearched crops and should make greater use of crop wild relatives as a source of genes for adapting crops to climate change.

To ensure that the breeding program meets the demands of users, particularly farmers, participatory breeding should be promoted. In participatory breeding, the breeders and users together set the breeding target. The crossing and preliminary selection are done by profesional breeders while advanced testing are done by farmers in farmers field. There are several variation on this delineation of task between breeders and farmers.

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CURRENT STATUS OF PRE-BREEDING RESEARCH INVOLVING WILD SPECIES IN BOGOR AND CIBODAS BOTANICAL GARDENS

Reni Lestari[1]* and Muhammad Imam Surya[2]

ABSTRACT

Bogor and Cibodas Botanic Gardens are government institutions established to conserve plant *ex situ*. Through the activities of conservation biology, up to date, Bogor and Cibodas Botanic Gardens have already had 21,203 numbers of the plant collection, of which around 1,700 of them are fruit plants. The process of plant breeding is begun by exploration of the plants in their natural habitat. The plants were collected, continued with the characterization and utilization of plant species. Furthermore, the technology to develop and utilize the collections is depending on the character and breeding system of the species. Fruit breeding is the purposeful genetic improvement of fruit crops through various techniques including selection, hybridization, mutation induction, and molecular techniques. At this time, Bogor and Cibodas Botanic Gardens are still trying to focus on this fruit species in order to support the future fruit breeding program. An ongoing fruit selection and breeding program are those of the genus and species of *Persea*, *Baccaurea*, *Garcinia*, *Nephelium*, *Durio*, *Mangifera*, *Artocarpus*, *Willughbeia*, *Rubus*, *Rhodomyrtus tomentosa*, and *Sorbus corymbifera*. The lack of progress of fruits breeding was due to two reasons, i.e. (1) vegetative propagation permits the genetic fixation of naturally occurring variation and (2) the difficulties and expense inherent in fruit breeding have inhibited long-term breeding efforts. Breeding program in the botanic gardens faces several problems associated with *ex situ* collection, including small population sizes, genetic drift, spontaneous hybridization, and inbreeding depression. In order to address some problems, we have to increase our botanic gardens network, either with research center, industry, university, or institute for *ex situ*/*in situ* conservation.

Keywords: botanic garden, fruit crops, *ex situ*.

INTRODUCTION

Botanic gardens are institutions holding documented collections of living plants for the purposes of scientific research, conservation, display, and education (BGCI, 2014). The establishment of botanic garden in Indonesia was begun in 1817 by Casper Georg Carl Reinwardt, the first director of botanic garden in Bogor, which was called Buitenzorg at that time. Bogor Botanic Garden was named as *'s Lands Plantentuin te Buitenzorg*. Cibodas Botanic Garden or *Bergtuin te Tjibodas* was established on April 11, 1852 and was originally a part of the Bogor Botanic Gardens. It was located in an upland area, i.e. 1425 m above sea level that serves as an acclimatization area for exotic plants. The first exotic plant was planted in 1852 by Johannes Elias Teysmann, a Dutch gardener who became curator of Bogor Botanic Garden. He brought an important plant (*Cinchona calisaya*) to Java, an extract used for treating malaria (Suryana and Widyatmoko, 2013). Both of the gardens were parts of National

Biological Institute since 1962 and then under the Indonesian Institute of Sciences (IIS/LIPI) in 1986.

The Botanic Gardens declared their function as an institution for plant conservation, research, environmental education, recreation, and ecosystem services. Research activities at the Bogor and Cibodas Botanic Gardens could not be separated from the garden history. It was initially designed as a place for conducting research into economic plant introduction. Furthermore, botanic gardens were used to research and develop plants and seeds from other parts of Indonesia for cultivation during the 19th century. This is a tradition that continues today and contributes to the garden's reputation as a center of botanic research in Indonesia. During its developments, Bogor Botanic Garden also provided an opportunity to collect plants and seeds from other parts of the Archipelago and the Botanic Garden.

EX SITU CONSERVATION

Ex situ conservation is the conservation of biological diversity outside their natural habitats, such as botanic garden, seed bank, or zoo (CBD, 1992, 2002). Bogor and Cibodas Botanic Gardens are government institutions established to conserve plant *ex situ*. Based on International Agenda for Botanic Gardens in Conservation (Wyse Jackson and Sutherland, 2000), some of major activities of botanic gardens include conservation biology, environmental education programs, ethnobiological research, herbarium studies and plant taxonomy, horticultural research and training, laboratory research including *in vitro* plant cultivation, seed store and tissue banking, tourism, and many more. Many activities related to International Agenda for Botanic Gardens in Conservation have been implemented in Bogor and Cibodas Botanic Gardens. Through the activities of conservation biology, up to date, Bogor and Cibodas Botanic Gardens already have 21,203 number of plant collections (Table 1).

Botanic gardens are well placed to undertake many activities in integrated conservation. Moreover, we utilize our plant collections for breeding and development program. The process of plant breeding is begun by exploration. The plants that were collected continued with characterization and utilization of

Table 1. Collection of Bogor and Cibodas Botanical Gardens.

Location	Number of family	Number of genus	Number of species	Total collection	Number of fruit collection
Bogor Botanical Garden	213	1,248	3,406	16,272	More than 1200 from around 300 species
Cibodas Botanical Garden	164	673	1,279	7,191	around 500

Source: Subdivision Registration and Propagation of Bogor Botanic Garden (September 2016); Unit Registration of Cibodas Botanic Garden (September 2016).

Note: The collections are not included orchids and other ornamental plants in the greenhouses.

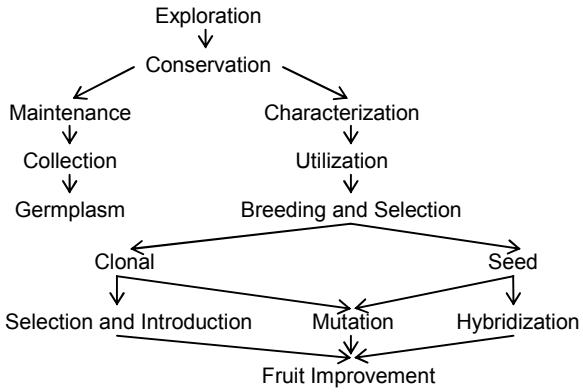

Figure 1. Strategy of fruit breeding program in botanical gardens.

plant species. Furthermore, the technology to develop and utilize the collections is depending on the character and breeding system of the species. The strategy of fruit breeding program in Bogor and Cibodas Botanic Gardens is presented in Figure 1.

CURRENT STATUS OF PRE-BREEDING RESEARCH

Fruit breeding is the purposeful genetic improvement of fruit crops through various techniques, including selection, hybridization, mutation induction, and molecular techniques. Botanic garden, as an *ex situ* conservation, has an important role in crop improvement. Moreover, in the domestication process, various useful species were chosen, cultivated, and improved by continuous selection. Through that process, the genetic improvement of those species have been achieved by selection, first from natural seedling population and then from the field that continued unique genotype fixed by vegetative propagation.

At this time, beside classical and modern taxonomy of plant collections as primary activities at Bogor Botanic Garden, there are some other research activities related with pre- and breeding researches of some important commodities, i.e. fruit, medicinal, and carbohydrate-producing plants. This paper clarifies research on fruit plants that focuses on identification of useful characters of local fruit germplasm and plant breeding program. At least eight genera, namely *Persea* (*P. americana*), *Baccaurea* spp., *Garcinia* spp., *Nephelium* spp., *Durio* spp., *Mangifera* spp., *Artocarpus* spp., and *Willughbeia* spp., have been studied.

The goals of research on *P. americana* or avocado were to evaluate and select avocado germplasm, and provide lowland and midland avocado varieties which are prominent and have period of harvesting distributed from January to December. The research was started on January 2011 in several cities in West Java Province, which are central for avocado fruit production in Indonesia. This

species is originated from lowland until highland of tropical America and during the 18th century it was spreading to Indonesian region (Anonymous, 2000). Evaluation was conducted on field and on laboratory observations according to IPGRI (1995) that presented at Table 2. The other activity conducted was analyzing nutrition content of avocado fruit, especially the content of unsaturated fat. Furthermore, selection was done based on fruit external and internal characteristics using scoring system. External characters included shape and size of fruit: condition, color, thickness, and texture of fruit skin. Internal character included easiness to peel: color, texture, taste, fibre, and thickness of fruit flesh, portion of edible part: size of fruit seed and attachment of cotyledon. Until the end of 2012, there were approximately 184 samples of avocado and 31 samples were chosen based on evaluation and selection on their fruit quality with harvest period distributed from January to December. The selected avocado plants then have been propagated by shoot-tip grafting and cultivated in the garden. Evaluation on the quality of fruit as well as the harvesting period of those avocado plants is still conducted at the moment. The hybridization then is going to be conducted to increase more quality of the fruits.

On the other hand, the researches for other fruit taxa, namely *Baccaurea* spp., *Garcinia* spp., *Nephelium* spp., *Durio* spp., *Mangifera* spp., *Artocarpus* spp., and *Willughbeia* spp. mostly originated from Kalimantan, were starting from 2007, including those from collection of Bogor Botanic Garden. Exploration, collection, and evaluation activities were conducted during fruit harvesting period in Kalimantan or fruit harvesting period of the plant collection at the garden. Kalimantan was chosen as location of the study, since this island is very rich with tropical fruit plant species that most of them are endemic (Airy Shaw, 1975; Bombard and Kostermans, 1985; Djufry and Jumberi, 2005; Jarret, 1959, 1960; Kostermans, 1958; Krismawati and Sarwani, 2005; Leenhouts, 1986; Subekti *et al.*, 2005). The evaluation of fruits character and variation for the taxa studied are presented in Table 3.

Table 2. The character of avocado plants evaluated during the exploration activities.

Character	Character
Size, shape, type, condition/vigor, age, and color of plants (tree, canopy, and trunk)	Fruit production
Length, diameter, and color of branches and twigs	Color, thickness, type, and weight of fruit skin
Flowering intensity	Color, fibre, thickness, weight, and taste of fruit flesh
Flowering and fruiting period	Attachment of cotyledon and length of seed cavity
Size, shape, type, smell, and color of leaves	Weight, shape, color, and size of seed
Length, diameter, color, and type of fruit	The content of unsaturated fat and other important nutrition of fruit (only selected sample)
Weight, type, and size of fruit	Degree and color of discoloration of open fruit after 4 hours)
Fruit size and shape uniformity	Shelf life of fruit

Source: IPGRI (1995).

From the evaluation result, it could be selected the potential species ones from each genus as a promising fruit product. Then, the potential fruit plants are going to be propagated and introduced to the society. The other activities were to improve more on the fruit quality through hybridization of those potential fruit plants. The potential species from the genus studied is presented in Table 4.

Currently, fruit breeding program in Cibodas Botanic Garden are focus on five genera, i.e. *Rubus* spp., *Rhodomyrtus* spp., *Sorbus* spp., *Persea* spp., and *Durio* spp. In the program of domestication and exploration of *Rubus* spp. from Indonesia mountain forest, we have done several studies. The study was begun by doing an exploration in the Mount Gede Pangrango. The exploration was aimed to study the ecology growth in the nature and also to identify the diversity of *Rubus* spp. Furthermore, several collections from the mountain forest have been used in many research activities, i.e. taxonomy, fruit breeding, selection and cultivation, and propagation. Taxonomy is the primary activity in the botanic gardens. Classical and modern taxonomy are used to study the genetic variation and high phenotypic plasticity in *Rubus* spp. Improvement of fruit quality are conducted by three steps, i.e. selection, mutation breeding, and cultivation. At present, we are using two species of *Rubus* (*R. fraxinifolius* and

Table 3. The evaluation of location and character of each species of fruit plants during the exploration and collection activities or during fruit harvesting period.

Location and character	Character
Date and time of evaluation	Weight and size of fruit
Local name of the plant species	Color, taste, thickness, smell, and water content of fruit flesh
Location and condition of plant habitat (altitude, latitude, slope, soil RH, and pH)	Number, size, weight, shape, and color of seed
Shape and vigor of plant canopy	Soluble solid content of fruit flesh
Branching pattern	Easiness to peel
The lowest branch position	Fruit harvesting period
Color and texture of trunk	Fruit production per tree
Color, shape, size, and position of leaves	Period during transport and fruit shelf life
Color and shape of flower	Price of fruit in the local market
Texture, thickness, color, and type of fruit skin	Other usage than fresh fruit

Table 4. The potential fruit species from each genus after evaluation and selection.

Genus	Species
Baccaurea	*Baccaurea reticulata*, *B. angulata*, *B. dulcis*, *B. racemosa*, *B. motleyana*
Garcinia	*Garcinia mangostana*, *G. atroviridis*, *G. bancana*, *G. celebica*, *G. dulcis*, *G. forbesii*, *G. parvifolia*
Nephelium	*Nephelium lappaceum*, *N. cuspidatum*, *N. juglandifolium*, *N. maingayi*, *N. ramboutanake*, *N. reticulata*, *N. uncinatum*
Durio	*Durio zibethinus*, *D. dulcis*, *D. graveolens*, *D. griffithii*, *D. kutejensis*, *D. oxleyamus*
Mangifera	*Mangifera indica*, *M. caesa*, *M. foetida*, *M. gedebe*, *M. kemanga*, *M. laurina*, *M. longipes*, *M. odorata*, *M. pajang*
Artocarpus	*Artocarpus heterophyllus*, *A. altilis*, *A. kemando*, *A. rotundus*, *A. integer*, *A. anysophillus*, *A. dadah*, *A. elasticus*
Willughbeia	*Willughbeia coreacea*, *W. angustifolia*

R. rosifolius) in the fruit breeding, selection, and cultivation program. Currently, we have nine candidates of mutants. Hybridization and polyploidy are prevalent in *Rubus*. Hybridization in *Rubus* occurs mostly between closely related species and in some instances between subgenera. Therefore, sometimes an improvement of *Rubus* used interspecific hybridization. *Rhodomyrtus tomentosa*, collection from Mount Tandikat, West Sumatera, is another species that using the technology of mutation breeding to increase fruit size and polyploidy. Fruit breeding program in *R. tomentosa* was begun in 2013 and, up till now, six candidates of mutants have been obtained.

Many of fruit species improvement are still based on grower-selected clones. The lack of progress of fruits breeding was due to two reasons. First, vegetative propagation permits the genetic fixation of naturally occurring variation. Second, the difficulties and expense inherent in fruit breeding have inhibited long-term breeding efforts (Janick, 2012). In Cibodas Botanic Garden, improvement of fruit species, such as *Persea* spp. and *Durio* spp., were conducted by vegetative propagation and grafting. Phenotypic variation of the population plays an important role in the future improvement. The activities of fruit improvement began by collecting several seeds and seedling from the local market. The preliminary selection is often based on qualitative characters, such as fruit appearance, flesh, color, and rootstock strength. Currently, we were using *P. rimosa* as a rootstock and *P. americana* at the upper stem. The primary aim is to obtain a single tree or seedling with outstanding quality. The other fruit breeding program in Cibodas Botanic Garden is *Sorbus corymbifera.* The domestication program of *S. corymbifera* began in 2012. The collection of *S. corymbifera* was obtained from the exploration in Mount Dempo, South Sumatera. At this time, Cibodas Botanic Garden is still trying to this species in order to support the future fruit breeding program. Furthermore, an ongoing fruit selection and breeding program at Bogor and Cibodas Botanic Gardens are presented in Table 5.

OPPORTUNITIES AND FUTURE CHALLENGES/INITIATIVE

Breeding program in the botanic gardens face several problems associated with *ex situ* collection, including small population sizes, genetic drift, spontaneous hybridization, and inbreeding depression (Volis and Blecher, 2010). Botanic gardens working in *ex situ* conservation and hoping to reestablish wild populations must ensure that *ex situ* collections contain as much genetic diversity as possible (Donaldson, 2009). The other main way to preserve genetic diversity is to collect plant material from as wide a variety of wild sources as possible and then grow it in the living collections of a number of different institutions that can then share germplasm for breeding and reintroduction programs. Plants in living collections can furnish genetic material as seeds, with unique genotypes, or as tissue, which produces clones (Rae, 2010). In order to address some problems, we have to increase our botanic gardens network, either with research center, industry, university, or institute for

Table 5. Ongoing fruit selection and breeding program at Bogor and Cibodas Botanical Gardens.

Plant names	Number of species	Number of collection	Collection location*	Sources	Technology	Target
Persea spp.	3	3049	BBG, CBG, and CSC	Indonesia (especially West Java, local market and bulk)	Selection, grafting, hybridization	Increased fruit quality Fruit harvesting throughout the year Increased seedling quality Registered plant variety (PVT)
Baccaurea spp.	17	155	BBG	Indonesia (especially West Java and Kalimantan)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality Registered plant variety (PVT)
Garcinia spp.	34	244	BBG	Indonesia	Selection, vegetative propagation	Increased seedling quality
Nephelium spp.	8	33	BBG	Indonesia (especially Kalimantan)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality
Durio spp.	8	55	BBG and CBG	Indonesia (especially Kalimantan, local market and bulk)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality
Mangifera spp.	21	83	BBG	Indonesia (especially Kalimantan)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality
Artocarpus spp.	19	86	BBG	Indonesia (especially Kalimantan)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality
Willughbeia spp.	3	17	BBG	Indonesia (especially Kalimantan)	Selection, vegetative propagation	Increased fruit quality Increased seedling quality
Rubus spp.	12	42	CBG	Indonesia mountain forest	Mutation breeding, hybridization, selection	Increased fruit quality Increased berry size Interspecific hybridization
Rhodomyrtus tomentosa	1	6	CBG	Mount Tandikat	Mutation breeding	Increased fruit size Polyploidy
Sorbus corymbifera	1	-	CBG	Mount Dempo	Vegetative propagation, mutation breeding	Increased fruit quality Polyploidy

*BBG = Bogor Botanical Gardens, CBG = Cibodas Botanical Garden, CSC = Cibinong Science Center.

ex situ/*in situ* conservation, for example, collaboration between Cibodas Botanic Garden and academy of chemical analysis in the domestication program of *Rubus*.

CONCLUDING REMARKS

Botanic gardens, one of centers for fruit collection and germplasms, provide breeders with the basic resources, i.e. genetic variation for fruit improvement. Moreover, characterization and evaluation are important in providing an inventory of genetic variation. The fruit collection and germplasms have an important function to assemble a range of accessions of fruit species to provide the genetic variation for the breeding program. Some accessions in germplasms were obtained at random from natural populations and, in this respect a representation of the genepool is emphasized.

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PUBLIC-PRIVATE PARTNERSHIP FOR PRE-BREEDING: THE PHILIPPINE PUBLIC INITIATIVE AND EXPERIENCES

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ABSTRACT

To accelerate new variety development, both the private and the public or government undertake in plant breeding in the Philippines. Furthermore, since plant genetic resources (PGR) play an eminent role in pre-breeding and crop improvement programs, agricultural development should be promoted by providing the policy framework, public investment, and support services for business enterprises. Philippine has experienced on public-private partnership for pre-breeding, breeding, and conservation of several commodities, such as rice, maize, papaya, eggplant, potato, and other agricultural crops. A number of crops varieties with desired traits have been improved through national and international collaborations. Therefore, to protect, conserve the PGR, and increase crop production, a number of relevant agricultural legislations and policies were also put into place in the Philippine.

Keywords: genetic resources, public-private partnership, policy.

INTRODUCTION

The development and commercialization of new crop varieties is primordial in agricultural development and crop farming productivity. The beginning of crop farming will always be dependent on the use of a good seed and its characteristics, which are highly attributable to the variety.

In the Philippines, plant breeding is an activity which both the private and the public or government undertake. Among others, there are private seed companies who are actively breeding and introducing new plant varieties. The government is also vigorously involved in breeding crops. Specifically, there are agencies under the Department of Agriculture that are involved in plant breeding, and the crops are rice, tobacco, cotton, sugarcane, coconut, and field legumes.

As a general rule, plant breeders develop varieties based on the problems in the field or what the farmers and consumers desire. Basically, farmers' choice of a variety is dependent on yield, resistant to pests and diseases, can grow under abiotic stresses, and other agronomic or horticultural traits. The consumers in general want agricultural product to be inexpensive, better tasting, more nutritious, longer shelf-life, aesthetically appearing, and others. Finding the balance what the farmers and consumers desire is a great challenge among breeders. One option in achieving the farmer-consumer balance is linking both these stakeholders even before plant breeding starts.

The Philippines has actively and successfully participated in public-private partnership programs on agriculture in the field of research and development,

capacity building, extension, and infrastructures over the past several years. Collaboration among various government agencies, private sectors, and universities is designed to bolster agricultural innovation systems, which are indispensable to combat the global social problems, such as poverty, hunger, malnutrition, climate change, and plant genetic erosion. FAO in 2013 stated that it has been estimated that 15.6 million Filipino people are suffering from chronic hunger.

Plant genetic resources (PGR) play an eminent role in food security. These have been used in pre-breeding and crop improvement programs. At present, the Philippine's Department of Agriculture is the government leading agency to operationalize for collecting, documentation, multiplication, regeneration, characterization, evaluation, conservation, and utilization of plant genetic resources for food and agriculture (PGRFA). Furthermore, it is mainly responsible for the promotion of agricultural development by providing the policy framework, public investment, and support services for business enterprises. Over the past few years, Philippine governments had pre-breeding research collaboration with international organizations, universities, and private sectors, particularly on rice, maize, papaya, potato, eggplant, coconut, and ornamentals.

PHILIPPINE EXPERIENCES ON PUBLIC-PRIVATE PARTNERSHIP FOR PRE-BREEDING

Rice

The pre-breeding activities in Golden Rice are in progress in collaboration with the International Rice Research Institute (IRRI), Philippine Rice Research Institute (PhilRice), and Syngenta. In spite of large collection of rice varieties in the Philippines, PSB Rc82 and IR64 were selected in Golden Rice Project because they were reported to have very high level of resistance/tolerance to biotic and abiotic stresses, high yield, with good eating quality and with high market demand. Scientists from these research institutions have been working further to develop a healthier beta-carotene-enriched rice variety to address the vitamin A deficiency problem. Based on the Food and Nutrition Research Institute's survey in 2008, Vitamin A deficiency affects 15.2% of children aged 6 months to 5 years. Using the genetically modified techniques, Golden Rice was developed with carotenoid genes (phytoene synthase and phytoene desaturase) from maize and a common soil organisms that together naturally produce beta-carotene in the rice grain. To date, Golden Rice was assessed in the field to determine if these developed varieties retain the same yield, pest resistance/tolerance, and grain quality.

Golden Rice was first developed by scientists from the Institute for Plant Sciences of the Swiss Federal Institute of Technology (Switzerland) and the Center for Applied Biosciences of University of Freiburg (Germany). They

teamed up with Syngenta to further improve their invention. Syngenta donated the gene materials to members of Golden Rice Networks, including the Philippines. Research on Golden Rice continues to be motivated due to its extensive contribution in solving the vitamin deficiency.

Moreover, Dr. Antonio Alfonso, a plant breeder from PhilRice also currently is developing a 3-in-1 rice that will contain various important traits, such as beta carotene, rice tungro disease, and bacterial leaf blight resistance.

Maize, Papaya, Eggplant, Potato, and Other Agricultural Crops

In 1997, the Department of Agriculture through the Bureau of Plant Industry (BPI) and 15 regions of Regional Integrated Agricultural Research Centers (RIARC), the University of the Philippine Los Baños-Institute of Plant Breeding (UPLB-IPB), Visayas State University (VSU), Central Mindanao University (CMU), and University of Southern Mindanao (USM) are public institutions mainly involved in R&D program on maize. The UPLB-IPB's main concerned on the selection and development of potential maize OPVs' and had tie-ups (in exchange of research fund) with Ayala Genetic Research Incorporated breeding and production. The said private seed company is always focused hybrid maize R&D and commercialization.

Aside from maize crops, UPLB-IPB also involved in pre-breeding, breeding, and conservation activities on other crops, such as papaya, eggplant, potato, ornamental plants, and other agricultural crops. In the past, they collaborated with international organizations, universities, and private sectors in exchange of research grants.

RELEVANT AGRICULTURAL LEGISLATIONS

The International Treaty for Plant Genetic Resources for Food and Agriculture (ITPGRFA) is promoting a public-private partnership for pre-breeding to support the development in plant breeding satisfying all necessities of the agricultural industries, adaptation to climate change, food security, market and consumer demands, germplasm availability, and many others. In the Philippines, a number of agricultural legislations and policies were put into place to protect and conserve our biological resources and increase crop production as well. This is in response to national priorities and international commitments on the conservation and use of PGRFA. First, the Philippine Seed Board was formed by the Department of Agriculture and Natural Resources through Special Order No. 50, Series of 1955 where it aimed to expand the breeding, selection, multiplication, and distribution of pure seeds on high yielding varieties of rice and corn. The Seed Industry Development Act of 1972 (Republic Act 7308) was enacted to replace the Philippine Seed Board. The policy promotes

and accelerates the development of the seed industry with greater intensity and strengthened capabilities.

The National Integrated Protected Areas System Act of 1992 (Republic Act 7856) was enacted to protect and maintain the natural biological and physical diversities of the environment notably on areas with biologically unique features to sustain human life and development as well as plant and animal life. The Administrative Order No. 3, Series of 1994, which laid down the Implementing Rules and Regulations of the Seed Industry Development Act of 1992 that the government shall conserve, preserve, and develop the PGR of the nation. The Bureau of Plant Industry (Executive Order 338) shall conduct upstream research and development of various crops, such as tropical fruits, legumes, potatoes, and semi-temperate vegetables, and conserve the Philippine PGR for continued development of new crop varieties of superior horticultural crops and agronomical characteristics. Also, the Plant Variety Protection Act of 2002 (RA 9168), was enacted to grant intellectual property rights to plant breeders with respect to their new plant variety.

CONCLUDING REMARKS

Partnership among public institutions, universities, private sectors, non-governmental organizations will strengthen the agricultural programs specifically on PGR particularly on pre-breeding and breeding R&D activities. Networks have a vital role in implementing the ITPGRFA and national programs. Thus, partnerships and networking will play a significant role to advance conservation and use of PGRFA.

ANNEX

Institutions and Germplasm Holdings in the Philippines

Institution	Germplasm Collection
Department of Agriculture	
Bureau of Plant Industry	Tropical and subtropical fruits, plantation crops, ornamentals, medicinal plants, herbs and spices, field and vegetable legumes, indigenous and semi-temperate vegetables, root crops
Philippine Rice Research Institute	Rice
Philippine Coconut Authority	Coconut
National Tobacco Administration	Tobacco
Sugar Regulatory Administration	Sugarcane
Fiber Industry Development Administration	Fiber crops
Southern Tagalog Integrated Agricultural Research Center	Citrus
Cotton Development Administrations	Cotton
Palawan Agricultural Experiment Station	Root crops
Eastern Visayas Integrated Agricultural Research Center	Fruits
Department of Environment and Natural Resources	
Ecosystems Research and Development Bureau	Bamboo, rattan, medicinal plants
Academy	
University of the Philippines Los Baños	Rice, fruits, plantation crops, spices, medicinal plants, ornamentals
Benguet State University	Fruits, root crops
Cavite State University	Coffee
Central Luzon State University	Tomato, sunflower
Central Mindanao University	Coffee, rubber
Leyte State University	Abaca, coconut, root crops
University of Southern Mindanao	Industrial crops
Ramon Magsaysay Technological University	Mango, cashew
Western Philippines University	Mango, cashew

Institution	Germplasm Collection
Non-Governmental Organization	
MASIPAG	Rice
SEARICE	Rice, root crops

GENOMIC-BASED CROPS IMPROVEMENT TO SUPPORT GSIAD 2015–2045

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ABSTRACT

The negative impact of climate change, the scarcity of fossil energy, and the depletion of natural resources become the limiting factors of agricultural production in Indonesia which has the fourth largest population in the world. Genomic strategies with related sciences may be relevant for assisting crop improvement to feed the growing population while preserving natural resources. Considering the importance of local plant genetic resources (PGR) in Indonesia for breeding program, therefore the PGR should be conserved, utilized and well managed for sustainable agriculture. The PGR has been conserved by research centers based on their national mandates, and under coordination of the Indonesian Agency for Agricultural Research and Development (IAARD). The IAARD initiated research program of genomic analysis to support breeding activities by providing information for breeder to choose a suitable parent or develop appropriate molecular markers, with a long-term goal of providing genomic-based selection systems for strategic crops. Furthermore, to anticipate the impact of climate changes, several efforts on crops improvement via gene discovery have successively been conducted in many ways. In the future, the successes achieved by plant breeding will be determined in term of sustainable improvements in agricultural production of food, feed, fiber, biofuels and other desirable crop products, which meet the society demand which is in good agreement with the Grand Strategy for Indonesian Agriculture Development (GSIAD) 2015–2045 of the IAARD. This chapter provides updated information of how genomics can assist crops improvement on the basis of local genetic resources in breeding programs to cope national challenges on agriculture.

Keywords: genomics, crop improvement, Indonesia, PGR.

INTRODUCTION

Advances in plant genomics offer breeders with new tools that allow the study of the whole genome, which can bridge classical plant breeding to a new revolution in plant breeding at the beginning of the 21th century. The rapid development of both conventional breeding techniques and genomic approaches lead to a new genomic-based plant breeding (Pérez de Castro *et al.*, 2012; Varshney and Tuberosa, 2007). Nowadays, genomic strategies are focused on the development of climate resilient crop varieties to ensure food security (Henry, 2014). Next-generation sequencing (NGS) technology may be directly relevant for accelerating breeding approaches to develop environmentally adapted crops from local resources.

Genomics will be important to develop more efficient new crop varieties needed to feed the fast growing population and climate change while preserving natural resources. Since genomic breeding is based on technical revolutions to

provide genome-wide molecular tools with large collections of markers and high-throughput genotyping strategies, these tools will accelerate the breeding process. In particular, genomics approaches are useful for dealing with complex traits, as these kinds of traits usually have a multigenic nature and a major important environmental influence (Collard and Mckill, 2008; Lorenz *et al.*, 2011; Varshney *et al.*, 2010). This chapter provides updated information of how genomics can effectively improve crops in local resources breeding programs to cope national challenges on agriculture in Indonesia.

NATIONAL CHALLENGE ON AGRICULTURE

As the world's population continues to grow rapidly, more food and other agricultural products are required to meet people's needs. In particular, the Indonesia population growth rate is approximately 1.49, which is estimated to be a higher population than that of Asia and in the global world. In 2050, the population of Indonesia is predicted to increase 68% of the population in 1995 and will impact to increase national food demand nearly two-folds. In addition to the food quantity from agricultural production, the need for more equitable life, which is characterized by higher the Human Development Index, also increases with demanding of more healthy and nutritious food. Population growth in Indonesia has also led to the conversion of agricultural land to nonagricultural land, which reaches 100,000 hectares per year, particularly in irrigated land. Consequently, the agricultural extension should be shifted to a suboptimal land area accompanied by intensification. Thus, a new technology that can solve the problem of adaptation in suboptimal land and increased productivity as stated above is required to meet the food shortfall in the future.

Renewable energy has been given a higher attention since last decade because of its potency to replace fossil fuel. Especially at present, the scarcity of fossil energy and increase in energy price become a main consideration of many countries to switch to renewable energy. Global biodiesel and biofuel supply are planned to be double over the 2010–2020 time frame to accommodate the world's demand requirements. In line with the world policy and Grand Strategy for Indonesian Agriculture Development (GSIAD) 2015–2045, the Indonesian government puts a strong interest in bioenergy development because it enables to enhance economic growth, job opportunities, and mitigation of CO_2 emission. Therefore, to implement well the national strategy of developing and using bioenergy is an urgent challenge.

The other challenge, the changing of climate as one of the important factors influencing agricultural production, would affect to reduce potential yields of many crops, especially in developing countries (Nelson *et al*., 2004). The negative impact of climate change on the agricultural sector in Indonesia is categorized in three parts, which involves continuous, discontinuous, and

permanent impacts. The first, continuous impact of climate change includes increased temperatures, changes in precipitation patterns and an increase in salinity of the coastal area. This impact will cause disrupted agricultural productivity and the cropping intensity and pattern. For example, the delayed planting time in 2013 caused declining rice production about 6.5% in West Java and Central Java and about 11% in Bali. Secondly, discontinuous impact of climate change comprises of climate anomalies (El Niño/La Niña) and climate extremes causing floods, droughts, and pest explosion that could potentially reduce productivity and total losses of various crops. The third part, the permanent impact of climate change may be the rise in sea levels, which can reduce the availability of land for agriculture. Furthermore, the severity and scopes of soil degradation have been observed in many regions of the world and Indonesia due to unauthorized cultivation of marginal land and deforestation to certain extent. The land degradation would decrease agricultural productivity, population migration, food insecurity, the destruction of basic resources and ecological system, and erosion of biodiversity (Las, 2007).

The negative impact of climate change on agricultural production, the scarcity of fossil energy, and the depletion of natural resources become the limiting factors worldwide. Moreover, the increased energy demand, climate change and environment destruction by human activity along with the increasing population could be a huge challenge for future agricultural production in Indonesia. Competing demands on agricultural land for food production and for the bioenergy provision must be reconciled with a rising recognition of the importance of environment and ecosystem services for adaptation, and climate change mitigation. Therefore, agriculture in the future requires new technologies to meet the related challenges of global food security and climate change. Such technology should be able to actualize the potential of genetic resources (GR) and the peculiarities of the local environment.

Research is taking place in multidiscipline that has accelerated crops improvement rate. Particularly, strong bioscience proves its diverse involvement in many areas, such as animal and plant breeding, health, diagnostics, and nutrition. While, bioinformatics supported by genomic sources can be a pivotal tool for molecular breeding, including in areas of crop improvement, plant protection, and enhancement of quality. Notably, bioscience and bioinformatics have a very important role in orient plant breeding programs based on local resources to boost the national economy, now and in the future.

LOCAL GENETIC RESOURCES MANAGEMENT

Indonesia is unquestionably one of the world's top biological diversity rich country. Because of the highly diverse plant genetic resources (PGR), their global conservation should be put on the top priority. The loss of the GR, which

sustain people, is now widely recognized as being critical as well. Several main factors, such as habitat degradation and fragmentation, landscape changes, overexploitation, and climate change, can cause biodiversity loss (Moeljopawiro and Suprahtomo, 2007; Sutoro, 2008). The local PGR in Indonesia are valuable materials for breeding program. Therefore, the PGR should be optimally utilized and well managed for sustainable agriculture.

Local resources breeding are oriented on five points. Firstly, broadened local genetic diversity of crops is to avoid risk on food security. Source of the novel alleles that confer enhanced productivities must be explored from *ex situ* and *in situ*, and the heritable diversity available on-farm including landraces. Therefore, pre-breeding is very critical to achieve this broadened genetic base of crops. Secondly, it is to define the breeding objectives which emphasize on yield, resistances to biotic and abiotic stresses, enhanced nutritional quality traits, and the multipurpose use of crop biomass. The third is innovating for result-oriented plant breeding including adoption of the powerful biotechnologies. For example, marker-assisted selection (MAS), supported by genomics, and the other omics, and information technology platforms, permits high throughput evaluation for breeding materials. The fourth is policy and strategic interventions. This consideration is the 'where' in the agricultural research and development for situating plant breeding. A result-oriented plant breeding must have access to the widest possible of heritable variation just as it needs an effective mechanism to deliver high quality seeds and planting materials to the growers. The fifth, partnerships for the reinvigorated crop improvement are important (Lorenz *et al.*, 2011; Varshney and Tuberosa, 2007).

PGR in Indonesia have been conserved by research centers based on their national mandates, and under coordination of the Indonesian Agency for Agricultural Research and Development (IAARD). The collection, exploration, characterization, and evaluation of PGR have been consistently conducted. IAARD continues to expand the diversity of local GR. Until 2012, the *ex situ* collections of PGR reached more than 20,000 accessions, including food crops, horticulture, and estate crops (Table 1). Among the crops, food crops seem predominantly in the collection. The PGR collection is managed by designated agricultural research centers under the coordination of IAARD, including Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD).

Since 2013, the local GR have been enriched by joint research between centers and assessment institute of IAARD under the coordination of ICABIOGRAD. This research program reveals that based on reinventory of local GR in home gardens owned by farmers, the majority crops cultivated are horticultural crops (fruits and vegetables). This reinventory is continued to characterization of the GR during the evaluation phase of 2014–2015 and 2016–2017.

To support all research programs in respect to PGR, the IAARD has four research and development centers for commodities and six research centers dealing with specific tasks, such as research centers for land resources, post-harvest, and biotechnology and genetic resources. In addition, a center for agricultural technology assessment is also involved in the program. Under the coordination of research and development center of commodities, there are commodity-based research institutes with specific mandate. While under the center for agricultural technology assessment, there are thirty-three assessment institutes for agricultural technology located in each province across Indonesia. In term of GR management, all of the research centers and research institutes are involved with specific activities. Research institute for commodities and institute for technology assessment conduct local GR exploration. Research institutes for commodities conduct phenotypic evaluation, ICABIOGRAD performes molecular evaluation, while nutrition aspect is evaluated by center of research and development for post harvest technology.

GENOME RESEARCH PROJECT TO ENHANCE BREEDING PROGRAM AT IAARD

Information of genome should be translated into a comprehensive understanding of valuable aspects of economically important plants. Plant genomics expectedly accelerate the discovery in interest crops to improve their agricultural production adaptive to climate change. Therefore, plant genomic research is believed to play a critical role in addressing the needs of increasing population and the climate change effect (Garg *et al*., 2014; Henry, 2014). In Indonesia, the challenges of producing sufficient food and a sustainable supply of bioenergy depend on the research outcomes, both basic and applied researches.

The genomic resources and tools allow association study for particular traits in breeding program. NGS technologies facilitate to produce a vast array of genomic information. Bioinformatics and NGS facilitate discovering genes and large number of molecular markers. Genomic approach, therefore, helps to screen germplasm collections for allelic variants. Resequencing allows for the genome-wide discovery of markers useful for high-throughput genotyping. Abundant genome-based markers, such as simple sequence repeat (SSR) and

Table 1. Plant genetic resources collection under the management of IAARD, Ministry of Agriculture, Indonesia.

Crop group	Number of accession/species
Food crop	11,435/20 species
Horticulture crop	2,065/32 species
Estate and industrial crops	7,714/20 species

Source: ICABIOGRAD (2015).

single nucleotide polymorphism (SNP), are important for germplasm management, enhancement, and use. These also enabled us to the identification of markers linked to genes and QTLs, MAS, and genomic selection (Pérez de Castro *et al*., 2012).

Starting in 2011, the IAARD initiated research program of genomic analysis to support breeding activities by providing information for breeder to choose a suitable parent or develop appropriate molecular markers. In addition, genomic analysis opens a new space in developing new breeding techniques. Genome analysis activities focused on strategic crops by considering the condition of the availability of genome sequence information. If the genome information is available and accessible, the activity leads directly to resequencing which facilitate genomic selection (GS) and genome-wide association studies (GWAS). If no genome references of the corresponding crops are available, then *de novo* sequencing analyses are performed.

The genomic analyses are conducted by joint research between ICABIOGRAD and other national or international institutes. In the case of food crops, GWAS on rice for productivity and early maturity is performed in collaboration with International Rice Research Institute (IRRI) scientists. Resequencing and SNP chip development for soybean focusing on productivity and acidic tolerance have been done in collaboration with Seoul National University (SNU), South Korea. In respect to bioenergy, jatropha genomic analysis is conducted through consortium with Kasetsart University (Thailand) and SNU.

To anticipate the impact of climate changes, several efforts on crops improvement have successively been carried out in many ways, especially identification of useful genes which are further their construction and validation. ICABIOGRAD has used this approach in a number of crops, such as rice, soybean, and maize. ICABIOGRAD focuses to develop varieties conferring *CsNitrL* gene for nitrogen use-efficiency and *CONSTANT* genes for early maturity in rice. In addition, several useful genes related to biotic and abiotic stresses are also identified and utilized for the purpose of climate adaptation as the impact of climate changes. Moreover, useful genes related to grain yield characters are also highly explored to increase crop productivity, such as *GS3* for grain size, *CKX* for grain number, and *DEP* genes for improving erect panicles. The example of research activities on gene discovery conducted in ICABIOGRAD-IAARD is presented in Table 2.

FUTURE PROGRAM ON MOLECULAR GENETICS TO SUPPORT GSIAD 2015–2045

As mentioned in the basic framework of GSIAD 2015–2045, in the future, Indonesian agriculture should be dignified, independent, advanced, equitable,

Table 2. Research activities on gene discovery in ICABIOGRAD-IAARD.

Anticipation	Improved target characters	Constructed useful genes	Crops
Climate mitigation	Nitrogen use-efficiency	*CsNitrL*	Rice
	Early maturity	*CONSTANT*	Rice
	Nodulation stimulation	*GmNFR*	Soybean
Climate adaptation:			
Abiotic stresses	Tolerance to drought	*OsDREB1*, *OsERA*, *csp*, *OsCCPK*, *HVA1*, *SNAC1*	Maize, rice
	Tolerance to salinity	*OsDREB1*, *HVA1*	Rice
Biotic stresses	Resistance to stem borer	*Cry1A*, *Cry1Ac*	Maize, rice
	Diseases resistance	WRKY and ERF transcription factors	Rice
	Resistance to brown planthopper (BPH)	*Tca*, *Tcd*	Rice
Crop productivity	Grain yield	*GS3* (grain size), *CKX* (grain number), *DEP* (erect panicle)	Rice

and prosperity. With the support of good policy, the sustainable agriculture is addressed on the basis of local GR and society agricultural-oriented effort. Based on the vision of realizing sustainable bioindustry agricultural systems—that produce healthy food and a variety of high value-added products from renewable tropical agricultural resources—human and natural resources, bioscience and bioengineering innovation systems along with the other pillars should be integrated. With the climate change threat, lessening of natural resources, and increasing population (Garg *et al*., 2014), therefore, other omics branches integrated with genomics and molecular genetics should be a part of future research for sustainable agricultural production through breeding program in Indonesia.

In the future, efforts to increase an effective breeding program will focus on not only immediate, but also long-term needs of people. The genetic knowledge would span scale of biology, which is extending from molecular genetics to multitrait phenotypes embedded with genotype-environment systems. The successes achieved by plant breeding will be determined with term of sustainable improvements in agricultural production of food, feed, fiber, biofuels, and other desirable crop products which meet the society demand (Cooper *et al*., 2014) which is in good agreement with the GSIAD 2015–2045 of the IAARD, Ministry of Agriculture, Indonesia. Consequently, in an effort to increase capacity in the local genetic resource-based breeding, the IAARD continues to develop and conduct collaborative research network in term of (a) Developing genomic database facilities of prioritized commodities in order to strengthen breeding program and related applied technology, (b) Maintaining facilities for genomic technology development, (c) Improving human power competency on bioinformatic, breeding, and genomics, (d) Strengthening the scientists networking and their capability on molecular breeding with emphasizing on

bioinformatic analysis, and (e) Mainstreaming the genomic and genetic research, development, and application/technology in agricultural technology development for strengthening national economy and food sovereignty.

CONCLUDING REMARKS

The rising demanding for food driven by population growth and unpredictable climate creates enormous challenges in developing country like Indonesia. To reduce energy use or renewable energy, we should send a signal to all people in the country to take these issues seriously. Thus, research from multidiscipline involving bioinformatics and genomics have a very important role to orient plant breeding programs based on local GR to boost the national economy. GRs are maximally used to support pre-breeding to broaden the genetic base, so GRs-oriented breeding is important. The IAARD, a governmental agency to coordinate all GRs, has four research and development centers for commodities and six research centers dealing with specific tasks. Since 2013, the local GRs have been enriched by a joint research between centers and assessment institute of IAARD. The IAARD initiated research program of genomic analysis to support breeding activities and new breeding techniques via national and international collaboration since 2011. Generally, resequencing of whole genome and high throughput genotyping are performed to develop molecular markers useful for selection in breeding. Efforts on crop improvement by means of gene discovery have also successively been done. Therefore, effective breeding program is schemed for long-term need of people and sustainable agriculture in Indonesia.

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CHAPTER 2
GENE DISCOVERIES IN PLANT

Plant breeding has significantly contributed to agricultural productivity through developing new superior crop varieties. These efforts have been supported by the availability of PGR or germplasms including land races and wild relatives that may contain important traits, such as high yield, resistant to pests or diseases, tolerant to abiotic stresses, or nutritional quality needed in breeding programs to improve existing elite varieties. Current progresses in plant genetic engineering allows effective transfer of important genes across species/taxa for their genetic improvement. Gene discovery contributes to the development of a trait or phenotype aiming at identification of the structure and function of genes.

This chapter begins with an overview of the current status of plant gene discovery research in ICABIOGRAD. As a research center within the IAARD, ICABIOGRAD has the mandate to evaluate the *ex situ* collection of PGR as well as search the genes that control important traits. Research in gene discoveries included gene identification, isolation, cloning, and characterization. Several techniques have been applied to identify genes, which can be used to develop transgenic plants. Based on these techniques, eighteen genes related to biotic stress, abiotic stress, productivity, N fixation, N fertilizer efficiency, and early maturity have been identified.

As molecular marker genotyping becomes more established and routine, research and breeding groups can focus more effort into population development and precise phenotyping—which should allow more rapid progress in gene discovery and pre-breeding efforts for crop improvement. One paper discussed the molecular marker technologies for pre-breeding applications and another paper revealed the mungbean genome sequence and its application in breeding program. Two papers gave examples on gene discovery research, i.e. discovery of sucrose metabolizing and related genes to enhance sugarcane productivity and genes discovery related to vegetative and transition from vegetative to reproductive stage in plant development. This chapter is concluded by a paper on elongation factor-1 alpha (EF-1 alpha) research and its possible contribution to agriculture development.

CURRENT STATUS OF PLANT GENE DISCOVERY RESEARCH IN INDONESIAN CENTER FOR AGRICULTURAL BIOTECHNOLOGY AND GENETIC RESOURCES RESEARCH AND DEVELOPMENT (ICABIOGRAD)

Tri Joko Santoso* and Kurniawan Rudi Trijatmiko

ABSTRACT

Future agriculture will be challenged by the need to provide food for the ever increasing human population and to provide crop-based materials for renewable energy such as biofuel. The efforts to meet such challenges, however, will be faced with resource scarcity, in particular water and arable land, and the more adverse effect of biotic and abiotic stresses due to climate changes. To help meet these future challenges, research into new crops and varieties suitable for Indonesian agricultural environment is needed to develop new plant varieties. Gene discovery is the first step in the genetic engineering pipeline. To discover genes that are associated with biological functions/processes or traits, a causal relationship has to be made between DNA sequence and observed phenotype. Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) has primary duties and functions on performing research related to cell biotechnology, tissue biotechnology, genetic engineering, and bioprospection of genetic resources. One of laboratories in ICABIOGRAD, i.e. Laboratory of Molecular Biology, conducts gene discovery activities, including gene identification, isolation, cloning, and characterization. Several techniques have been applied to identify genes, which can be used to develop transgenic plants. Based on the techniques applied in ICABIOGRAD, eighteen genes related to biotic stress, abiotic stress, productivity, N fixation, N fertilizer efficiency, and early maturity have been identified.

Keywords: gene discovery, genetic engineering, abiotic stress, productivity.

INTRODUCTION

Future agriculture will be challenged by the need to provide food for the ever increasing human population and to provide crop-based materials for renewable energy such as biofuel. The efforts to meet such challenges, however, will be faced with resource scarcity, in particular water and arable land, and the more adverse effect of biotic and abiotic stresses due to climate changes. To help meet these future challenges, research into new crops and varieties suitable for Indonesian agricultural environment is needed to develop new varieties, both by traditional breeding and genetic modification.

Gene discovery is the first step in the genetic engineering pipeline. To discover genes that are associated with biological functions/processes or traits, a causal relationship has to be made between DNA sequence and observed phenotype. Genes hold the information to build and maintain an organism's cells and pass genetic traits to offspring. All organisms have genes

corresponding to various biological traits, some of which are instantly visible. Many research have been conducted to determine how genes function in the intact organism. The most effective approach to determine how a gene functions in a cell or organism involves studying mutants that either lack the gene or express an altered protein of it. Because mutations can interrupt cellular processes, mutants often hold the key to understanding gene function. Working backward from the phenotype one then determines the organism's genotype, the form of the gene is responsible for that characteristic.

A modern working definition of a gene is a locatable region of genomic sequence corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and or other functional sequence regions. Recently, with numerous information of nucleotide sequences in the public databases, the exploration and identification of gene function often begins with a DNA sequence. One approach is to search databases for well-characterized proteins that have similar amino acid sequences to the protein encoded by a new gene, and from there the gene function can be explored further. Determining which cellular processes have been disrupted or compromised in such mutants, will then frequently provide a window to a gene's biological role. Genetic analysis of mutants or other genetic variants that are localized on the chromosome can be used to clone the corresponding genes by methods such as map-based cloning and transposon tagging, which are the two most common methods for cloning genes by a forward genetics strategy centered around the phenotype of interest.

CURRENT STATUS OF PLANT GENE DISCOVERY RESEARCH

The development of transgenic plants, from initial concept to commercialization phase, should pass through five stages and will take about 8–10 years. The five stages include (1) identification of genes, (2) proof of concept, (3) early development, (4) continued development, and (5) application for deregulation (Trijatmiko, 2011). At the stage of gene identification, cloning and screening of dozens of genes are conducted in accordance to the desired characters by testing on a model plant to identify which genes provide the expected efficacy. In ICABIOGRAD, there are several approaches to identify and isolate genes important to agriculture.

Two approaches are usually applied in gene cloning and analysis of gene function, forward genetics and reverse genetics (Bouches and Höfte, 1998). In forward genetics approach, mutant phenotypes are known long before their corresponding genes have been identified. Therefore, this approach works starting from mutants to gene sequences. The phenotype differences indicated by the mutant are compared to wild type and then identified sequences of the gene responsible to these differences. Examples for this approach are analysis

of mutants, either natural or man-made mutant, map-based cloning, and linkage mapping.

A significant limitation of classical loss-of-function screens—designed to dissect genetic pathways is that they rarely uncover genes that function redundantly. They are compensated by alternative metabolic or regulatory circuits, or those which have an additional role in early embryo or gametophyte development. Activation tagging using T-DNA is one approach that has emerged in plants to help circumvent these potential problems. This technique utilizes a T-DNA sequence that contains four tandem copies of the *Cauliflower mosaic virus* (CaMV) 35S enhancer sequence. This element enhances the expression of neighbouring genes either side of the randomly integrated T-DNA tag, resulting in gain-of-function phenotypes. Activation tagging has identified a number of genes fundamental to plant development, metabolism, and disease resistance in *Arabidopsis* (Tani *et al*., 2004). This technique has been applied to discover genes in ICABIOGRAD and two genes related to drought tolerance have been identified (Table 1).

Reverse genetics is an approach to discover the function of a gene by analyzing the phenotypic effects of specific gene sequences obtained by DNA sequencing. While forward genetics seeks to find the genetic basis of a phenotype or trait, reverse genetics seeks to find what phenotypes arise as a result of particular genetic sequences. Therefore, reverse genetic works start from known sequences of a gene to mutant and function (Bouches and Höfte, 1998). In rice, reverse genetics approach is widely used. By using protein sequence similarity of a gene, whose functions and involvement in biological processes in the organism are already known, a candidate gene can be identified and studied. Next step, the target sequence of gene is introduced to create a mutant plants (knockout or overexpression mutant). Phenotypic observation of the mutant will lead to the interpretation of the function of the genes.

Another technique for gene discovery in ICABIOGRAD is map-based or positional cloning. The first stage of map-based cloning is to identify molecular markers located close to the gene of interest. This procedure is usually done by selecting markers around the gene, followed by the selection of markers to obtain markers that cosegregated with the gene interest. Amplicon of DNA fragments flanking markers is then cloned in the cloning vector for the

Table 1. Genes identified by using activation tagging method in ICABIOGRAD.

Name of gene	Product of gene	Putative function	Target of trait
OSJNBa0004I20.14	Putative uridylate kinase	Essensial for ATP binding and/or enzyme catalist	Drought tolerance
OsPPCK2L	Phosphoenolpyruvate carboxylase kinase	One of genes that is induced particularly by drought	Drought tolerance

identification of further genes (Peters *et al*., 2003). Piao *et al*. (2009) have used a cloning approach to identify *ERECT PANICLE 3* gene in rice.

Steps of map-based cloning can be summarized as follows.

- Identify a marker tightly linked to your gene in a mapping population.
- Find a YAC or BAC clone to which the marker probe hybridizes.
- Create new markers from the large-insert clone and determine if they cosegregate with the gene.
- Identify a candidate gene from large-inset clone whose markers cosegregate with the gene.
- Perform genetic transformation to rescue the wild-type phenotype.
- Sequence the gene and determine if the function is known.

Based on map-based cloning approach, three genes which are related to productivity and early maturity traits target have been identified (Table 2).

Cloning by sequence homology uses the evolutionary conservation of the sequence to isolate homologous genes from different species (Michels, 2001). Often, gene products may serve the same functional role in different species, but sequence homology may be limited to short highly conserved domains or functional motifs. In such cases, PCR-based methods can be used to identify functional homologues. When two conserved motifs are identified within the same protein, PCR-based methods can be used to amplify the region contained between them. Genomic DNA or cDNA can be used as the PCR target. In ICABIOGRAD, thirteen genes have been identified by using homology sequence-based cloning with various characters target, i.e. biotic tolerance, abiotic tolerance, productivity, N fixation, N fertilizer efficiency, and early maturity (Table 3). Some of the genes have been subjected to preliminary proof of concept.

FUTURE PROGRAM IN GENE DISCOVERY

Several activities or programs related to gene discovery research in ICABIOGRAD can be summarized as follow.

Table 2. Genes identified by using map-based cloning method in ICABIOGRAD.

Name of gene	Product of gene	Putative function	Target of trait
LOC1g10110	Cytokinin dehydrogenase precursor	Ethylene response factor that plays a role in panicle and internode elongations as well grain filling	Productivity
LOC_Os01g15910	UTP-glucose-1-phosphate uridylyltransferase, putative	Involved in pathway of sugar or carbohydrate metabolism	Productivity
LOC_Os07g48260 (*OsWRKY47*)	Trancription factor protein	Superfamily of TFs having WRKY and zinc finger domains	Early maturity

- Utilizing a new technology such as RNA sequencing to strengthen gene discovery program.
- Maintaining facilities for gene discovery technology development.
- Improving human power competency on gene discovery and genetic engineering.
- Strengthening the scientists networking and their capability on gene discovery.

CONCLUDING REMARKS

ICABIOGRAD has primary duties and functions on performing research related to cell biotechnology, tissue biotechnology, genetic engineering, and bioprospection of genetic resources. One of laboratories in ICABIOGRAD, i.e. Laboratory of Molecular Biology, conducts gene discovery activities, including gene identification, isolation, cloning, and characterization. Several techniques have been applied to identify genes which can be used to develop transgenic plants. Based on the techniques applied in ICABIOGRAD, eighteen genes related to biotic stress, abiotic stress, productivity, N fixation, N fertilizer efficiency, and early maturity have been identified.

Table 3. Genes identified by using homology sequence-based cloning in ICABIOGRAD.

Gene identified			Preliminary of concept proofing		
Name of gene	Source of gene	Target of trait	Transformation	Function study	Organism model
OsERA1	*Oryza sativa*	Drought tolerance	√	–	Rice
BsCsp	*Bacillus subtilis*	Drought tolerance	√	–	Rice
OsDREB1A	*O. sativa*	Abiotic stress tolerance	√	√	Rice
OsWRKY76	*O. sativa*	Biotic stress tolerance	√	–	Rice
OsERF1	*O. sativa*	Biotic/abiotic stress tolerance	√	√	Rice
AV1	*Begomovirus*	Resistant to *Geminivirus*	√	√	Tobacco
OsGS3	*O. sativa*	Productivity	√	√	Rice
OsCKX1	*O. sativa*	Productivity	–	–	Rice
OsDep1	*O. sativa*	Productivity	√	–	Rice
CsNitrl1	*Cucumis sativus*	N fertilizer efficiency	√	–	Rice
GmNFR1a	*Glycine max*	N fixation efficiency	√	–	Soybean
OsMADS	*O. sativa*	Early maturity	–	–	Rice
AtCO	*Arabidopsis thaliana*	Early maturity	√	√	Rice, soybean

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MOLECULAR MARKER TECHNOLOGIES FOR PRE-BREEDING APPLICATIONS

Michael J. Thomson

ABSTRACT

Next-generation sequencing (NGS) technologies have been gaining widespread acceptability in the field of crop breeding. NGS technology enables to rapidly develop molecular markers that can be applied for pre-breeding applications. NGS has led to explore genetic variants for most agricultural plant and animal species, particularly single nucleotide polymorphisms (SNPs) which can be promising high throughput markers applied with automation. A large number of genotyping platform are now available that cater to broad range of SNPs density implying to practical application cost for pre-breeding. Low density SNPs are useful on sample throughput. In contrast, the high range spectrum of SNPs, called as SNP chip provides efficient genotyping for low number of samples. To complement to fixed arrays, the approach of using NGS for low-cost genotyping, called genotyping by sequencing (GBS) is also powerful to develop molecular markers to assist breeding program. IRRI has set up a Genotyping Services Laboratory (GSL) to accelerate rice breeding program with low cost-effective services which are supported by an efficient sample processing workflow with an integrated laboratory information management system (LIMS).

Keywords: single nucleotide polymorphisms, high-throughput genotyping, next-generation sequencing, pre-breeding.

INTRODUCTION

Recent advances in DNA sequencing and molecular marker technologies have the potential to accelerate research for gene discovery and pre-breeding applications. Next-generation sequencing (NGS) technologies have now enabled rapid whole genome sequencing, creating a wealth of data on genetic variants such as insertion/deletions (indels) and single nucleotide polymorphisms (SNPs). These provide sample polymorphisms for selecting molecular markers associated with important traits for crop improvement. At the same time, high-throughput genotyping platforms enable rapid processing of large numbers of DNA samples with sets of SNP markers. These technologies promise to accelerate the mapping of quantitative trait loci (QTLs), identification of candidate genes, and development of specialized genetic stocks that can be used for pre-breeding applications.

NEXT-GENERATION SEQUENCING (NGS) TO EXPLORE GENETIC VARIATION

The rise of NGS has led to a flood of sequence data for most agriculturally relevant plant and animal species. Massively parallel short read technologies from Illumina and Ion Torrent have enabled routine resequencing of genomes,

while long-read technologies such as from Pacific Biosciences are now making it possible to more rapidly develop high quality reference genomes. While it has been increasingly easy to obtain whole genome resequence data, the need for high quality *de novo* assembly reference genomes is essential for accurate annotation of gene sequences and to enable rapid and accurate alignment of resequence data. As the cost per base of sequencing rapidly declines, the bottleneck has now shifted to the need for bioinformatics expertise to analyze large amounts of sequence data. The difficulty is how to extract useful information from NGS data—whether in the form of informative SNPs that can be used to develop improved molecular marker assays or to speed up QTL cloning and identification of key genes and alleles for crop improvement.

HIGH-THROUGHPUT MOLECULAR MARKER TECHNOLOGIES

With NGS data, researchers are no longer limited by the number of available markers in any specific genome region, but instead are limited only by the cost of genotyping—and therefore must carefully choose the most relevant and cost-effective genotyping technology for their specific application. A large number of genotyping platforms are now available that cater to a broad range, from assaying a single SNP to over 1 million SNP markers at a time. For upstream research having a high-density SNP chip enables powerful new approaches, such as using genome-wide association studies (GWAS) to identify important loci for traits of interest, albeit at a relatively high cost. However, for practical applications for pre-breeding and deploying routine marker-assisted selection (MAS) in breeding programs, the large population sizes require a much lower cost per sample. For these applications, having a smaller number of high value SNP markers that are predictive for the desired trait characters is of greater importance. In this case, the DNA sample throughput is key: being able to extract DNA and genotype larger numbers of samples per day.

At the low range of the spectrum, there are a number of genotyping methods for running relatively small numbers of SNP markers on large populations. Fluorescently labeled SNP markers, such as TaqMan and KASPar markers, can be run one marker at a time on real-time PCR machines or fluorescent plate readers. For these methods, the cost is determined by the size of the PCR reaction volume—since fewer reagents are needed for smaller volumes. Thus, a 5 µl reaction in a 384-well PCR plate is more cost-effective than running 15 µl reactions in 96-well PCR plates. Moving to 1,536-well PCR plates can further reduce the cost, but at this point automation becomes necessary. The concept of miniaturizing the reaction chambers has also been successfully employed in the Array Tape system by Douglas Scientific and with the Dynamic Array Integrated Fluidic Circuit (IFC) from Fluidigm. Although these can greatly reduce the reaction volume, in some cases down to 0.01 µl, they

require high capital investment to purchase the equipment to run these highly specialized genotyping platforms.

At the higher range, genotyping with fixed microarrays, also called SNP chips, can provide efficient genotyping of several thousand up to several million SNP markers. For example, the Illumina Infinium technology provides efficient genotyping in the range of 3K up to several million markers per sample. Likewise the Affymetrix Axiom system can also provide efficient genotyping of fixed SNP sets. The disadvantage of fixed arrays, however, is that they cannot be changed very often and need a large initial commitment in order to get the volume discount needed to make them cost-effective. For this reason, they are best used when a "universal" design can be employed to make them widely usable across a broad range of germplasm—thus allowing the development cost to be spread across a large number of users. In addition to fixed arrays, the approach of using NGS for low-cost genotyping, called genotyping by sequencing (GBS), has recently become popular. This technique employs barcoded DNA adapters to enable highly multiplexed sequencing, in most cases allowing 96 up to 384 samples to be run in a single lane of a NGS machine, which brings the cost per sample much lower than standard NGS methods. As with other NGS techniques, however, there is considerable overhead needed for bioinformatics support to analyze the massive amounts of data obtained from running GBS on large populations.

In many cases, the new genotyping technologies require a relatively large capital investment in order to take advantage of the very low costs per sample. These platforms are most efficient when they run very number numbers of samples. Thus, there has been a shift for smaller labs to outsource their genotyping needs to commercial service providers or for core facilities or "genotyping hubs" to be set up to serve the needs of local or regional communities of researchers and breeders. This has been most readily apparent with NGS services—most labs find it more convenient to outsource their sequence needs, since it is difficult to have enough resources in a single group to keep a machine such as the IlluminaHiSeq running at full capacity. Some of the high-throughput SNP platforms, however, are more accessible to medium-sized labs or core facilities.

A HIGH-THROUGHPUT GENOTYPING FACILITY AT IRRI

The Genotyping Services Laboratory (GSL) has recently been set up at IRRI to accelerate progress in rice breeding by providing rapid and cost-effective marker services to rice research and breeding groups at IRRI and our research partners. SNP marker development and validation are being performed based on cloned genes and QTLs, GWAS results, and whole genome sequence data to identify predictive SNP markers at important genes for key traits for rice breeding programs. Multiple sets of 24 SNPs and 96 SNPs on a Fluidigm EP1 system are being used for rapid genotyping of trait-specific

SNP markers, including functional and gene-based SNPs, top GWAS hits, and flanking markers selected from 44K SNP data. Legacy SSRs and functional indel markers are also being genotyped on a 96-capillary Fragment Analyzer. At the same time, Golden Gate 384 SNP sets and an Infinium 6K SNP chip developed by Susan McCouch at Cornell University are being used for higher density genome scans on an Illumina system. The genome-wide scans are being used for diversity analysis, SNP fingerprinting, QTL studies, and characterizing donor introgressions in specialized genetic stocks. GBS approaches with 96 and 384 barcoded samples per sequence lane are also being evaluated in comparison to SNP array technology based on the number of loci, call rates, turnaround times, and cost per sample.

An efficient sample processing workflow with an integrated laboratory information management system (LIMS) is being optimized for more accurate sample tracking to minimize errors. Barcoded leaf tissue samples are harvested using a Brooks PlantTrak Hx, and then the DNA is extracted with a KingFisher 96 Flex magnetic bead system. A web-based, cloud-hosted LIMS based on Ocimum's Biotracker 5.0 LIMS is being configured to handle GSL's customer requests, sample tracking, inventory management, and workflow and task management. Moreover, web-based SNP data analysis tools have been deployed through the IRRI Galaxy workbench to speed up SNP data analysis. Future efforts will focus on large-scale deployment of GBS across breeding materials to enable QC genotyping, tracking of donor introgressions, and integration of genome-wide prediction into the variety development pipelines. The large-scale application of high-density markers will help transform IRRI's rice breeding programs and increase the rate of genetic gain towards developing high-yielding, stress-tolerant varieties for target environments and market segments.

CONCLUDING REMARKS

Recent advances in molecular marker technology have now enabled rapid high-throughput genotyping of DNA samples for pre-breeding applications, including diversity analysis, QTL mapping, near-isogenic line (NIL) development, fine-mapping, and marker-assisted selection. Research and breeding groups now have a large number of options from outsourcing to genotyping service providers to setting up an in-house high-throughput system based on one of the many genotyping platforms. With the rapid decrease in NGS costs, GBS will become increasingly attractive to handle high-density genome-wide marker scans, as long as adequate bioinformatics support and infrastructure is available. As molecular marker genotyping becomes more established and routine, research and breeding groups can focus more effort into population development and precise phenotyping, which should allow more rapid progress in gene discovery and pre-breeding efforts for crop improvement.

MUNGBEAN GENOME SEQUENCE AND ITS APPLICATION IN BREEDING PROGRAM

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ABSTRACT

Mungbean (*Vigna radiata* [L.] R. Wilczek) belongs to the subgenus *Ceratotropis* and is a fast-growing, warm-season legume crop that is primarily cultivated in developing countries of Asia. We constructed a draft genome sequence of mungbean to facilitate genome research into the subgenus *Ceratotropis* and to enable a better understanding of the evolution of leguminous species. The draft genome sequence covers 80% of the estimated genome size. In total, 22,427 high confidence protein-coding genes were predicted. Based on the *de novo* genome assembly of additional wild mungbean species, the divergence of domesticated and the sampled wild mungbean species appears to have predated the estimated domestication time. Moreover, the *de novo* assembly of a tetraploid *Vigna* species (*Vigna reflexo-pilosa*) provided genomic evidence of a recent allopolyploid event. To further study speciation, we compared *de novo* RNA-seq assemblies of 22 accessions from 18 *Vigna* species and identified speciation-related genes. With our whole genome sequencing and gene content data, a synteny relationship can be revealed via comparative analysis with well-characterized *Glycine max* quantitative trait loci (QTLs) to provide important clues for the identification of mungbean QTLs. The synteny blocks of seed size/germination and bruchid resistance QTL regions matched to the soybean synteny blocks with simple sequence repeat (SSR) markers linked to seed weight and nematode resistance. Hence, our resequencing-derived wild-genotype specific SNPs of mungbean and the comparative genomic information can further facilitate a variety of molecular breeding activities and will ultimately assist the identification of causal genes for the corresponding traits.

Keywords: mungbean (*Vigna radiata*), genome sequence, plant breeding.

INTRODUCTION

Mungbean (*Vigna radiata* [L.] R. Wilczek) is a diploid (2n = 2x = 22), warm-season legume species belonging to *Ceratotropis* subgenus. It is widely cultivated in South, East, and Southeast Asia for its edible seeds and sprouts. Mungbean is also a good intercropping crop since it grows quickly and it could improve soil fertility and texture via root rhizobial symbiosis that fixes atmospheric nitrogen (Zhang *et al*., 2008). Genetic diversity and archeological studies indicated that mungbean was domesticated in India (Fuller, 2007), which is currently the world's largest producer of mungbean. More than 50% of global annual production (about 6 million tons) of mungbeans comes from India, with China and Myanmar being the next largest producers (Nair *et al*., 2013). In spite of its economic importance, genomic research in mungbean is lagging behind other papilionoid legumes. Genome sequence assembly has been reported in *Medicago truncatula* (Young *et al*., 2011)*, Cicer arietinum* (Varshney *et al*., 2013)*, Lotus japonicas* (Sato *et al*., 2008)*, Glycine max* (Schmutz *et al*.,

2010), and *Cajanus cajan* (Varshney *et al*., 2012), but none of the species within the *Vigna* genus has been sequenced.

The *Vigna* genus contains several agriculturally important legumes, such as lentil (*V. mungo*), cowpea (*V. umbellata*), moth bean (*V. acontifolia*), and red bean (*V. angularis*). Wild *Vigna* species are also widespread in nature; some of the examples include *V. radiata* var. *sublobata*, *V. mungo* var. *silvestris*, and *V. reflexo-pilosa* var. *reflexo-pilosa*. The genome size of these species varies, ranging from 465 Mb to 1,394 Mb. Polyploidy can also be observed in this genus, as *V. reflexo-pilosa* is an allotetraploid (2n = 2x = 44). The genome of this allotetraploid species was also sequenced to gain further insights into the mechanism of the polyploidy formation in this genus. Genome expansion and polyploidization are considered as some of the major mechanisms of plant speciation, but the effects of polyploidy on species evolution still remain unclear (Soltis *et al*., 2010).

Here, we will present a summary of the features found in the assembled mungbean genome and demonstrate some of the potential uses of the sequence data for future mungbean improvement program. Further details and the actual data generated from the mungbean sequencing project can be accessed in the published paper (Kang *et al*., 2014).

MUNGBEAN GENOME ASSEMBLY

The individual selected for genome sequencing is an inbred line of *V. radiata* accession 'VC1973A'. This accession was developed at the World Vegetable Center (AVRDC) in 1982, and has been widely grown in Thailand, Korea, Taiwan, Canada, and China. The entire genome size is estimated to be 579 Mb, based on a flow cytometry analysis (Arumuganathan and Earle, 1991). The sequence data was generated using Illumina Hiseq 2000 from sequencing libraries with different insert sizes ranging from 180 bp, 500 bp, 5 kb, 10 kb, and 40 kb to produce 320-fold base pair coverage of the estimated genome size. In addition, GS-FLX+ was also used to produce long reads with approximately 5-fold genome coverage. Assembly of the short reads data using ALLPATHS-LG software (Gnerre *et al*., 2011) produced 2,800 scaffolds with an N50 length of 1,507 kb. The total length of scaffolds was about 431 Mb. The number of contigs assembled from the GS-FLX+ data using Newbler 2.5.3 software (Roche) was 180,372, and 144,213 of those contigs were consistent with the scaffolds assembled from the Hiseq 2000 data. Nonmatching GS-FLX+ contigs were chopped into pseudo 5k mate-pair reads and inputted to ALLPATHS-LG in order to improve the quality of the genome assembly. This resulted in the assembly of 2,748 scaffolds with an improved N50 length of 1,516 kb. The total length of the scaffolds remained at 431 Mb, representing 80% of the predicted

mungbean genome size of 543 Mb (based on 25-base k-mer frequency distribution.

GENETIC MAPPING AND MARKER DEVELOPMENT FOR CHROMOSOME ASSEMBLY

A genetic map is required to organize the assembled scaffolds into full chromosomes. The map was constructed using single nucleotide polymorphism (SNP) markers developed using genotyping by sequencing (GBS) on a mapping population derived from a cross between VC1973A and a Korean local landrace V2984. GBS was performed on 190 F_6 lines created using single seed descent, and resulted in the identification of 1,993 SNPs. Among those, 1,321 SNPs could be mapped to form 11 linkage groups.

These mapped SNPs were used to anchor 239 scaffolds to the genetic map. However, 86 scaffolds remained unoriented since they only carried a single SNP for anchoring. The anchored scaffolds produced pseudo chromosomes representing the 11 linkage groups with N50 length of 35.4 Mb and total combined length of 314 Mb, or approximately 73% of the total assembled scaffolds.

More DNA markers were developed for future use in marker-assisted breeding of mungbean by sequencing the genome of the male parent V2984 using Illumina HiSeq 2000 at 26× coverage. Successful alignment of 81.26% of the total generated short reads to the assembled reference sequence resulted in the identification of 603,143 SNPs and 83,691 indels with high quality score. SSR markers could also be developed from the sequence data using MISA script (http://pgrc.ipk-gatersleben.de/misa/misa.html). A total of 200,808 SSR were identified from 1,544 scaffolds and the number of tri-repeat unit SSRs that are preferred for genotyping was 17,898. SSR marker validation was performed on 46 of the SSR markers and the marker data were integrated into the genetic map. The marker data could be a very useful source of DNA markers for future genetic studies and breeding on mungbean.

GENE PREDICTION AND ANNOTATION

Genes were predicted and annotated from the repeat-masked mungbean genome sequence using *de novo* and homology-based gene predictions algorithms in Maker pipeline (Cantarel *et al*., 2008). The pipeline uses inputs from RNA-seq assemblies from mungbean leaf, flower, pod, and root tissues. It outputted predicted mungbean proteins that represent 97% match to the set of 248 eukaryotic core proteins, which could be used to assess the completeness of the genome sequence (Parra *et al*., 2007). Sequence length distribution of the genes, exons, coding DNA sequences (CDS), and introns from *Arabidopsis thaliana*, soybean, and mungbean were highly consistent, indicating that the

gene predictions were highly reliable. In total, 22,427 genes were identified with high confidence, and 18,378 of the genes were located on the pseudochromosomes. Comparison of 22,427 protein sets from *V. radiata* var. *radiata* and the protein sequences of *A. thaliana, M. truncatula, O. sativa,* and *G. max* in OrthoMCL software (Li *et al*., 2003) indicated that there were a total of 6,799 gene clusters that are shared among all five species, while 160 clusters were exclusive to *V. radiata* var. *radiata* proteins. Annotations were assigned to the predicted proteins using Interproscan (Mulder *et al.,* 2002) and BLAST against *Arabidopsis* proteins, in order to deduce the function and identity of the proteins. In addition to protein coding genes, the prediction pipeline also identified 2,310 noncoding genes, including 629 tRNAs, 280 rRNAs, 537 microRNAs, 717 small nucleolar RNAs, 110 small nuclear RNAs, and 37 other regulatory RNAs.

EVOLUTION AND DOMESTICATION OF MUNGBEAN

Comparison of 2,917 pairs of paralogous genes residing in duplicated collinear blocks within the mungbean genome indicated that the mungbean had experienced only one ancient whole genome duplication (WGD) at approximately 59 million years ago (MYA), which is near the origin of the Papilionoideae subfamily. This subfamily, which contains most of legume crops such as pigeon pea (*C. cajan*), common bean (*Phaseolus vulgaris*), soybean (*G. max*), and the *Vigna* genus, underwent an ancient WGD event at approximately 58 MYA (Sato *et al*., 2008; Schmutz *et al*., 2010; Varshney *et al*., 2012, 2013; Young *et al*., 2011). A smaller scale duplication event might have occurred in the mungbean genome at around 7–13 MYA, producing 252 tandem gene clusters that are enriched for GO terms such as “defense response”, “cell wall modification”, “secondary metabolic process”, “sulfate transport”, “recognition of pollen”, “transmembrane transport”, and “protein amino acid phosphorylation”.

The tetraploid *V. reflexo-pilosa* var. *glabra* is an allotetraploid and it was estimated that the donor species of the allopolyploid genome diverged at around 6.8 MYA. Based on transcriptome sequence data from 22 accessions of 18 *Vigna* species, it was established that one of the homoeologous genomes of *V. reflexo-pilosa* is closely related to diploid *V. trinervia.* The other genome was found to be more related to the angulares group and it also had a much older divergence time (2.7 MYA). This suggests that the progenitor of this genome has not been sampled or even extinct. In the case of mungbean, the closest relative among the twenty-two accessions was *V. subramaniana.*

Mungbean domestication is estimated to occur around 4,000–6,000 years ago, based on geographical distribution of wild mungbean and archaeological records from India (Fuller, 2007). Using sequence data from one wild relative of

mungbean (*V. radiata* var. *sublobata*) and both of the parental lines used for constructing the genetic map, the time of divergence between the wild and domesticated mungbean samples was estimated to be 1 MYA. This is much earlier than the estimated domestication time and highly similar to the domestication pattern of rice and soybean (Gross and Olsen, 2010; Kim *et al*., 2010).

COMPARATIVE GENOMICS FOR IDENTIFICATION OF USEFUL GENES

The development of molecular markers that are linked to important agricultural traits is critical for crop improvement programs. Despite the limited molecular marker resources in mungbean, there have been several efforts to identify the genomic regions related to domestication-related traits such as seed size and seed germination (Isemura *et al*., 2012). Molecular markers could also be designed to integrate important and useful alleles from wild mungbean, such as bruchid resistance, into domesticated mungbean. Using the assembled whole-genome sequence and gene content data, a comparative analysis with the well-characterized *G. max* genome and QTLs could be performed to look for a syntenic relationship and conservation of similar QTLs in mungbean. The synteny blocks containing seed size/germination and bruchid resistance QTL in mungbean matched with soybean synteny blocks containing SSR markers linked to seed weight and nematode resistance. This demonstrates the potential of comparative genomic studies based on genome sequence information in assisting the identification of the responsible genes for useful agricultural traits.

Genomic sequence data is also very useful in mining conserved sequence motifs that code for certain phenotypes. It has been reported that most resistance genes encode proteins with two core domains commonly referred as nucleotide-binding site (NBS) and leucine-rich repeat (LRR) (Meyers *et al*., 2003). These domains are conserved and can be searched using Pfam database (Bateman *et al.,* 2004) and built-up hidden markov model (HMM). Using a mungbean specific HMM of NB-ARC and LRR, 73 NBS-LRR genes were found along with 464 additional LRR genes lacking NB-ARC domain. All NBS-LRR genes had homology with known disease resistance genes in UniProt database (UniProt Consortium, 2012), while 19 of the 464 LRR genes without the NB-ARC domain were identified to be disease resistance and damage repair related genes.

Although further studies will need to be performed to prove the effect of those mined genes on phenotype, this study illustrated the potential of the exploitation of whole genome sequence data for practical breeding application. As richer omics databases are developed and knowledge in sequence to phenotype relationship improves, genome sequence data will become more

valuable tools for future breeders in developing new plant varieties with improved agricultural traits.

FUTURE PROSPECTS

Currently, mungbean is mostly cultivated in developing countries, which partly explain the relative scarcity of genomic research for this crop. This is unfortunate since mungbean could be an affordable source of dietary protein, folate, and iron (Keatinge *et al.*, 2011). The application of molecular techniques in mungbean breeding could be used to speed up the progress and gain of selection in mungbean breeding program, and this would be assisted by the availability of a reference genome sequence and DNA marker data that could be used in simple laboratory set up.

Mapping the chromosomal regions that code for a trait should become easier with the availability of numerous marker candidates and a reference sequence as a template for the construction of the map. Fine mapping and candidate gene identification will be aided by the genome annotation since potential gene function can sometimes be inferred from the coded protein sequence data, as in the case of NBS-LRR proteins. The complete sequence data will assist cloning and the design of functional DNA markers that will never be uncoupled from the target phenotype. Combined with marker-based breeding techniques, such as marker-assisted backcrossing (MABC) or genomic selection, future breeding program should become faster, more efficient, and also no longer resemble a black box process. Future breeding programs will also be able to utilize more information about the genetic components that could be incorporated in certain combinations to give rise to the desired traits for agricultural cultivation.

CONCLUDING REMARKS

The draft genome sequence of mungbean was successfully assembled and annotated with high-confidence predicted genes. Useful information based on the *de novo* genome assembly and RNA-sequences of cultivated and wild mungbean species was revealed, such as their divergence, genomic evidence of a recent allopolyploid event, the speciation-related genes identified in twenty-two *Vigna* accessions from different species and a synteny relationship for specific QTL regions. Future breeding programs can utilize these information to design markers or identify desirable genes to make mungbean breeding faster, more efficient, and also no longer resemble a black box process.

ACKNOWLEDGEMENT

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET) through Agri-Bio Industry Technology Development Program Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (114054-3).

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ELONGATION FACTOR-1 ALPHA (EF-1 ALPHA) RESEARCH RELATED TO AGRICULTURE DEVELOPMENT

Sony Suhandono

ABSTRACT

Elongation factor-1 alpha (EF-1 alpha) is abundant protein found in the cytoplasma of the cell. The main function of the protein is transporting tRNA to the translation machinery in the cell, the ribosome. The protein is also functioning as a molecular chaperon that is important for survival in saline media. The protein is encoded by six genes, which form a family of EF-1 alpha gene. In this paper, we also described our study on the promoter of the gene family. One of the promoters is a constitutive promoter, the others are differentially expressed in various tissues. We have also described two elongation factor thermo unstable (EF-Tu), which are prokaryotic type of the EF-1 alpha gene. One *tuf* gene (encoding the EF-Tu protein) was isolated from sugarcane and the other one was isolated from cassava. Both genes could increase the survival of recombinant *Escherichia coli* above 50ºC. The study of the genes expression and function may need certain knowledge in the advancement of plant biotechnology that is currently developing very fast. Scientist in Indonesia is encouraged to develop tools to improve our agricultural production. On the other hand, the development of plant biotechnology gives farmer opportunity to produce high value proteins to be used in health care industry.

Keywords: EF-1 alpha, EF-Tu, *tuf* gene, promoter, gene expression.

INTRODUCTION

The elongation factor-1 alpha (EF-1 alpha) was first recognized as mediated protein transport of tRNA into the translation machinery in the cell, the ribosome (Riis *et al.*, 1990). The protein, that found to interact with cytoskeletal protein as actin bundling protein, has roles in nuclear export of tRNA, proteolysis of misfolded proteins, modulating apoptosis, response to amino acid starvation, and viral replication (Sasikumar *et al*., 2012; Visweswaraiah *et al*., 2011). In many eukaryotic cells, the protein was encoded by more than one gene, called a gene family. *Saccharomyces cerevisiae* has two genes encoding EF-1 alpha protein. *Arabidopsis* and *Drosophila* were reported to have four copies and two copies, respectively (Axelos *et al*., 1989; Hovemann *et al*., 1988). Plant, such as sugarcane, may contain at least twenty copies of the gene (Vijaykumar *et al*., 2002).

Since 1990's, it was known that each of the genes in the family was expressed in different manner. Of two elongation factor genes from rabbit, one was expressed in skeletal muscle, heart, brain, and aorta, while transcripts are not detected in liver, kidney, spleen, and lung. Another one was expressed in all tissues examined, except skeletal muscle (Kahns *et al.*, 1998). Using quatitative PCR, Ransom-Hodgkins (2009) showed that all of four EF-1 alpha genes from *Arabidopsis* were expressed in all tissues sampled. However, the abundance of each transcript varied spatially.

The idea of differentially expressed of each gene in the family lead to promoter research. In early studies, it was found that EF-1 alpha promoter from human was very potent. Gopalkhrisnan (1999) showed that the EF-1 alpha promoter was superior than *Cytomegalovirus* (CMV) promoter when used in the cancer cell line. The CMV promoter was known unstable. Spontaneous extinction of CMV promoter activity in the cells has been observed in a temporal and cell type-dependent manner. This is not the case with the EF-1 alpha promoter (Gopalkhrisnan, 1999). In 2007, researcher from Korea showed that one of the EF-1 alpha promoter from human was stronger than *Cytomegalovirus* derived promoter when expressed in stem cell (Kim *et al*., 2007). The plant elongation factors promoter was first patented by Calgene (USA). The company was granted a world patent in 1998 for an EF-1 alpha from maize. They claimed that the promoter was driving expression in actively dividing cell in plant tissue (WO 1990002172 A1).

The need of new plant promoter to drive expression in plants other than viral derived promoter is becoming more concern. The viral gene may be translated in both directions or may be overlapped with other genes. Therefore, the promoter may contain unwanted proteins expressed in transgenic plant. In response to this concern, bioinformatic study was made on CaMV 35S promoter, which is widely used in plant biotechnology. Eventhough most of CaMV 35S promoter does not produce any allergen protein known to date, but still risk carrying unwanted protein still remains for any viral derived promoter (Podevin and Jardin, 2012).

We started research on EF-1 alpha promoter from cassava (*Manihot esculenta*) in 1998 under supervision of Prof. Monica Hughes at the Department of Biochemistry and Genetics, the University of Newcastle upon Tyne, England. Cassava was studied because the plant is one of major food staple in the world. In dryer parts of Africa, they eat raw cassava, which caused acute cassava poisoning, iodine deficiency disorders (IDD), tropical ataxic neuropathy (TAN), and konzo diseases. The diseases were known to be the impact of high cyanogenic glycoside in cassava root. Cyanogenic glycoside, such as linamarin, is a substrate for linamarase, which occured naturally in many species belong to the Euphorbiacea. The biochemical reaction produces cyanide gas, which caused sudden death and cell respiration failure. The molecular biology of linamarase from cassava was studied at the Newcastle University as well (Hughes *et al*., 1992). Scientist from over the world believed that there is no cassava variety without cyanogenic glycoside. This was confirmed by scientist from Embrapa, major genetic bank of cassava, located right at the center of biodiversity in Brazil as well as Centro Internacional de Agricultura Tropical (CIAT), Columbia. Cyanogenesis is one of major problem in cassava as food staple and industrial scale processing. Others are problems related to agriculture point of view.

At the same year, CIAT in Columbia initiated a Cassava Biotechnology Network to tackle problems in cassava, including cyanogenesis in cassava root,

which is a major crop for 500 million people in the world. Since there is no cassava variety without cynogenic glycoside, the only way to tackle the problem is by transgenic technology. More laboratories in the world studying trangenesis in cassava, including tissue culture, transformation efficiency, and also tools for gene expression. Reader can consult the proceedings of the 1st until the 6th International Scientific Meeting of the Cassava Biotechnology Network. Since cassava is very difficult to be transformed, until recently, many papers are still addressing cassava efficient transformation technique (Chetty *et al*., 2013; Zainuddin *et al*., 2012).

Besides the promoter studies, the EF-1 alpha may be important to enhance the quality of agricultural product. In general, maize was lack of amino acid lysine, which is abundant in EF-1 alpha protein. Comparative studies on eighty-three lines, from crosses between high lysine content varieties and low lysine content varieties, showed that the microsatellite marker, which was related to high lysine trait, was found to be associated with EF-1 alpha protein. Therefore, the quantities of EF-1 alpha protein indicated the high lysine content of maize varieties (Wang *et al*., 2001).

EF-1 alpha gene can also be used as an indicator for salt tolerant plant as well as applied to create plant variety tolerant to salt. Shin *et al*. (2009) reported that EF-1 alpha gene knock out could decrease *Arabidopsis* plant tolerant to salt. Furthermore, his group found that transgenic *Arabidopsis* created by overexpression of EF-1 alpha would increase its tolerance to salt. *In vitro* study using transgenic yeast also showed that the *Arabidopsis* EF-1 alpha gene (*AtEF-1 alpha*) displayed a chaperon activity in a dose-dependent manner. In term of molecular chaperon, the prokaryotic type of EF-1 alpha, which is called elongation factor thermo unstable (EF-Tu), was found to be important to maintain performance of photosynthesis during heat shock. It was shown that heat tolerant wheat accumulates more EF-Tu than susceptible variety (Ristic *et al*., 2008).

The coding sequence of the gene is also very conserved. In 1999, a group of scientist from USA and France was analyzing the use of the gene for systematic study. The EF-1 alpha tree is congruent with some other molecular phylogenies in identifying both the deepest branches and some recent relationships in the eukaryotic line of descent. The gene can be used for phylogenetic analysis of Metazoa, Fungi, Magnoliophyta, Euglenozoa, and insect (Danforth and Ji, 1998; Roger *et al*., 1999). Recently, the EF-1 alpha marker is commonly used as one of markers to study the systematics of insect pest or fungal disease in agriculture context. For example, identification of first reported dry bean (*Phaseolus vulgaris*) disease caused by *Fusarium cuneirostrum* in Canada was made using EF-1 alpha sequence (Henriquez *et al*., 2014). The EF-1 alpha gene was also used in the systematics of Hymenoptera, especially for those interested in studying the relationship of wasp, used for biological control or bees for pollinating agent (Klopfstein and Ronquist, 2013).

CURRENT RESEARCH STATUS ON EUKARYOTIC ELONGATION FACTOR-1 ALPHA

The EF-1 alpha gene from cassava was first cloned using genomic library in 1999. A *MeEF1-α1* gene or *Manihot esculenta* EF-1 alpha was characterized (Suhandono *et al*., 2001). The architectural of the gene is almost similar to the EF-1 alpha family from *Arabidopsis*. It consisted of two exons dan two introns. The first intron resides at the 5' UTR. Another one resides between first exon and the second exon. Some *Arabidopsis* transgenic lines were made by transforming the plants with GUS reporter gene driven by MeEF1-α1 promoter. The result showed that the promoter was induced by wounding in all plant organs, including flower, trichomes basal, stem, cotyledon, leaves, and roots (Suhandono, 2000). However, we used pCAMBIA vector that consisted of two bidirectionally CaMV promoters, which were placed at very close distance to each other. We have changed one of them using MeEF1-α1 promoter. This could interfere the result, because later study showed that position of the two promoters might interfere each another, eventhough they placed in opposite direction (Hampf and Gossen, 2007; Singer *et al*., 2011).

The second family of EF-1 alpha from cassava is *MeEF1-α2*. The gene was cloned using a genome walking technique. The promoter was fused to GUS reporter gene, also in pCAMBIA 1303 vector. The promoter had been transformed an orchid species using *in planta* transformation technique. The plant shows weak espression of GUS reporter gene in main vascular system of the orchid (Lidya, 2007; Semiarti *et al*., 2014). However, the promoter expression pattern has not been described in detail, hope this will be elucidate in the near future.

In 2012, the full genome data was published freely in the Phytozome (Prochnik *et al*., 2012). We found complete six genes of EF-1 alpha family in the Phytozome database. Eventhough some of coding region was not completed, we managed to extract all of six promoters from the gene family. Further three promoters, namely MeEF1-α3, MeEF1-α5, and MeEF1-α6 were characterized transiently in tobacco seedlings, tomato fruit, and banana fruit (Suhandono *et al*., 2013). This time, we had been using a pBI-121 as an *Agrobacterium*-mediated vector. In this vector, the CaMV promoters located upstream of the tested promoter, intermitent with a kanamicyn resistant gene for selection marker (Figure 1). Hope this distance will diminish interference effect of the tested promoter as indicate in Hampf and Gossen (2007) and Singer *et al*. (2011). The promoters were found unique in term of spatially expression pattern, except the MeEF1-α6 was found constitutive in all organs observed of the plants. Quantitative analysis of transient expression of the MeEF1-α6 promoter in the fruits showed that the promoter was stronger than CaMV 35S promoter. In banana fruit, the expression of MeEF1-α6 was twice stronger than CaMV 35S promoter. Another promoter, MeEF1-α4, is still under construction. A preliminary study has also been made on EF-1 alpha from sugarcane

(*Saccharum officinarum*). We found low transient expression in vascular tissue of early seedlings and seeds (Farah, 2009).

CURRENT RESEARCH STATUS ON PROKARYOTIC ELONGATION FACTOR-1 ALPHA

In response to climate change, we have some research on EF-Tu performance as molecular chaperon from various species, which is relatively tolerant to heat shock. We know that the cassava and sugarcane are two crops that are very tolerant to heat stress. EF-Tu from sugarcane cDNA was cloned after the plant was heat stressed at 45ºC for a few hours (Amanda and Suhandono, 2013). The gene encodes EF-Tu protein is usually called *tuf* gene. The gene sequence is very similar to other chloroplast EF-Tu from plants of the same family. This is probably an indication that the gene may be useful as a barcoding gene.

In vivo analysis was performed by cloning the gene into a bacterial expression vector, pET32b. Viability assay (immediate heat shock) was shown that at 50ºC the bacterial survival was considerably higher in IPTG-induced culture than noninduced culture. Hundreds of colonies were still surviving at 50ºC in IPTG-induced culture, while no colonies was grown in the control plate (noninduced culture). However, when the culture was induced for a few hours before heat shock, the induced culture still produced several colonies at 60ºC, while in the noninduced culture showed no colonies at all. Other result showed that the cassava EF-Tu also caused significant growth at 54ºC on induced culture. This showed that the EF-Tu from both plants could increase the ability of *E. coli* recombinant to survive higher heat shock (Amanda, 2011; Baadriah, 2012).

The EF-Tu is actually encoded for chloroplast and mitochondria. From genome sequence of cassava, we found four *tuf* genes. Two genes were very similar to *tuf* gene from chloroplast and another two were very similar to mitochondrial *tuf* gene. Research on the EF-Tu gene family of cassava is ongoing effort in our lab, in order to further characterize the promoter and the function of the protein. The EF-Tu may function as molecular chaperon in the plastid as well as in prokaryotic cells, therefore the promoter are potentially inducible to environmental factor, such as light or heat intensity.

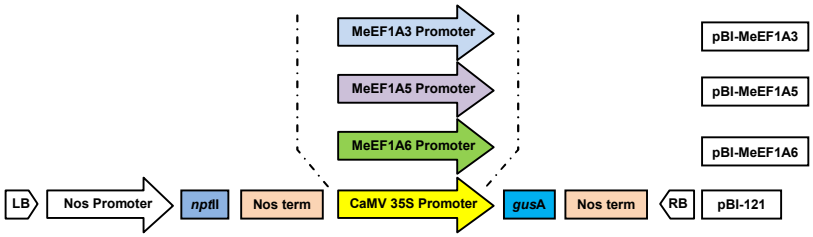

Figure 1. Genes construct to study the MeEF1-α3, MeEF1-α5, and MeEF1-α3 (Suhandono *et al*., 2013).

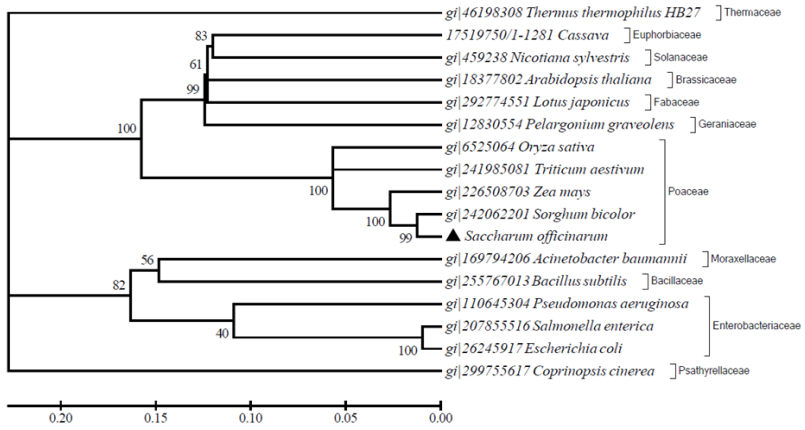

Figure 2. Phylogenetic tree of the EF-Tu cDNA sequence from *Saccharum officinarum* and other EF-Tu gene sequences (Amanda and Suhandono, 2013).

TECHNOLOGY CHALLENGES

Eventhough the production of corn and soybean has been boosted with transgenic technology, scared to the side effect of the transgenic product exists in the society. On the other hand, the knowledge in transgenic technologies is still developing. Development of promoter as gene expression regulator is very few. Scientists are developing the promoter for they own interest, very few ended up in the patent document. Browsing Indonesian database patent shows that all patented promoters owned by biotech companies overseas, some are already obsolete. Recent publication on transgenic rice from Indonesian scientist is still using classical CaMV 35S promoter (Rachmat *et al*., 2014). It is true that new plant promoters are described and patented around the globe, but not many laboratories in Indonesia are interested in developing promoter to be used in research or industry.

The development of transgenic technology also includes other expression regulators, such as enhancer, insulator, silencer, etc. Special attention is currently highlighting how to isolate the transgene from silencing or interference from other gene regulator in the genome (Heger and Wiehe, 2014; Schoborg and Labrador, 2014; Singer *et al*., 2011). Despite of many insulator alternatives, one insulator was discovered from *Arabidopsis* with only less than twenty base pair long (Patent US 2006/0174370 A1). New vectors are currently discovered in order to reduce alien DNA inserted into plant genome (Xin and Guo, 2012) or facilitating multiple gene expression (Binder *et al*., 2014) or vectors with nonantibiotic selection marker such as using antiapoptosis gene (Khanna *et al*., 2007).

On the other hand, easy and practical transformation technology has been developed for each plant species. Within 2014, many papers published under subject *in planta* transformation, which means transformation methods avoiding

plant tissue culture technique. *In planta* transformation has been developed recently in orchids (Semiarti *et al*., 2014), in biodiesel plant *Jatropha curcas* (Jaganath *et al*., 2014), in rice (Naseri *et al*., 2012), and in bell pepper (Arthikala *et al*., 2014). Gene transformation does not always mean inserting alien gene to an organism or—in this context—a plant species. This technology can also be used to mutate the existing gene by recombination, called gene targeting (Endo and Tok, 2014) or genome editing technique called TALEN (Bogdanove, 2014).

Transient transformation technology is also developing. The technology aims to directly assay the gene product without production of transgenic plant. Instead, the transient technology used plants' tissue as a bioreactor. This is particularly useful to study metabolic pathway, or for fast production of gene product and also for promoter characterization. Transient technology, in combination with statistics approach, has been used for promoter study of the EF-1 alpha promoter from cassava (Suhandono *et al*., 2013). At the same year, a method for efficient transient expression using *Agrobacterium* has been uncovered by manipulating the *Agrobacterium* media before transformation. This manipulation will activate *vir* genes to be more infectious. So far, the method was applied in *Agrobacterium*, but it is not impossible to be developed in other plant species (Wu *et al*., 2014). Furthermore, transient expression has been used to developed synthetic biology in plant, such as producing secondary metabolite for drugs, therapeutic proteins, or virus like particle (Sainsbury and Lomonossoff, 2014).

CONCLUDING REMARKS

Studying the EF-1 alpha gene, either from prokaryotic or eukaryotic, may contribute to scientific achievement. The study is an opening window in order to enhance our building capacity in plant biotechnology. The study is also contributing to the development of agriculture as well as adapting the advancement of biotechnology in the area. Understanding and applying biotechnology are not only improving our farmer products, but also could be used to produce valuable proteins or secondary metabolite to be used in health care industry. Plant-made recombinant protein is also another venue to increase the value of agriculture, shifting from only producing food to producing pharmaceutical product, 'molecular pharming' (Elfahmi *et al*., 2014; Rachmawati *et al*., 2014; Suhandono *et al*., 2013).

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DISCOVERY OF SUCROSE METABOLIZING AND RELATED GENES TO ENHANCE SUGARCANE PRODUCTIVITY

Bambang Sugiharto

ABSTRACT

Phosynthetic carbon asimilation is a major determinant that limits growth and productivity in plants. Study on the activities of the carbon assimilating enzymes revealed that among the enzymes activity of sucrose-phosphate synthase (SPS, EC. 22.4.1.14) is fluctuated in parallel with sucrose content and growth of sugarcane. Cloning of the genes encoding for SPS found the presence of two cDNA clones, *SoSPS1* and *SoSPS2*, in sugarcane and the transcript of *SoSPS1* to be predominant in leaves, but that of *SoSPS2* to be distributed conservatively in all tissues. To increase sucrose accumulation, the *SoSPS1* gene was overexpressed in transgenic tomato and sugarcane. As the consequences, the activity of SPS and sucrose content was significantly increased in leaves of the transgenic plants, but not concomitant followed by significant increase of sucrose contents in the sink tissues of both transgenic plants. This discrepancy of sucrose accumulation might because have not accompanied by sucrose loading mechanism between leave as a source and sink tissue. Thus, the *SoSUT1*-cDNA encoding for sucrose transporter protein was cloned from sugarcane and double overexpression of *SoSPS1* and *SoSUT1* increased sharply sucrose content in sugarcane stem and fruit production in tomato. With regard to sucrose content, it is well reported that sucrose acts as a potent osmoprotectant that might induce drought stress tolerant. Whether the increased of sucrose content in transgenic sugarcane induce the drought tolerant is still remain to be elucidated. In addition, identification of a gene responsible for drought tolerance found a *SoDip22*-cDNA encoding for a hydrophilic protein with molecular mass of 15.9 kDa and the function might to adapt to drought stress. However, the drought stress tolerant sugarcane recently was achieved by genetic engineering of glycine betaine content.

Keywords: sucrose-phosphate synthase, sucrose transporter protein, sucrose, genetic engineering, sugarcane.

INTRODUCTION

Photosynthesis involves many processes, starting for harvesting the light energy from sun in photochemistry to CO_2 fixation and the subsequent carbon metabolism. In C4 plants like sugarcane, the major carbon assimilating enzymes are phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), and ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco). These enzymes can be considered as essensial limiting growth and productivity in sugarcane. However, carbon partitioning into sucrose, a major carbon mobile from source to sink tissue, plays a pivotal role in governing the plant productivity.

Sucrose and starch are primary products of the photosynthetic carbon assimilation in plants. The starch is synthesized in chloroplast and serves mainly as an intermediate deposit for photoassimilate, whereas sucrose is a carbon mobile synthesized in cytosol and plays a central role in exporting photoassimilates throughout the plant cells. Most of the photosynthetically assimilated carbon can be allocated, either to sucrose or to starch depending on several factors. This partitioning is an important to plant growth since sucrose synthesis is a prime determinant of carbon export from photosynthetically active cells (Gifford *et al.*, 1984).

Sucrose-phosphate synthase (SPS) is believed to be the key enzyme controlling biosynthesis of sucrose in plants (Huber and Huber, 1996). The SPS catalyzes a formation of sucrose-6 phosphate from fructose-6 phosphate and UDP-glucose in cytosol, then is converted into sucrose and is subjected for export from source to sink tissue. The activity of SPS can be a limiting factor for *de novo* sucrose synthesis in plants. Studies in sugarcane leave showed that SPS activity determines sucrose production and the growth rate (Sugiharto *et al*., 2005).

In most plants, sucrose synthesized in leaves is transported long distance in the vein to support the growth and development of sink tissue, such as roots, flowers, fruits, and seed. The transportation of sucrose is facilitated by sucrose-transporter proteins (SUT) which have an important role in symplasmic and apoplastic phloem sucrose loading mechanism. Many studies have indicated that reduction of the expression of sucrose transporter has deleterious effects on plant growth and development and enhancing the expression increased growth and development of plant (Kuhn and Grof, 2010).

GENETIC ENGINEERING OF SUCROSE METABOLIZING GENES

Recently, molecular tool have become available allowing for a more precise *in vivo* manipulation of postulated biochemical pathway. Genes encoding of the enzymes involved in biochemical pathway can be used either to inhibit or enhance of the enzymatic activities. Thus, it permits to change metabolism activity and increase growth and productivity of plants.

Genes encoding for SPS have been isolated from many plants, including from sugarcane (Sugiharto *et al*., 1997). We had identified the presence of two genes encoding for SPS protein, *SoSPS1* and *SoSPS2*, encoded for photosynthetic active SPS1 and constitutive SPS2 proteins, respectively. It was reported that overexpression of gene encoding for maize SPS elevated SPS activity and increased fruit sugar contents in transgenic tomato (Laporte *et al*., 1997). Moreover, the overexpression of *Arabidopsis* SPS gene elevated

sucrose pool in sink tissue and significantly increased stem height and dry biomass to the control in transgenic cotton (Park *et al*., 2008). We had also demonstrated that overexpression of sugarcane *SoSPS1* gene increased sugar content in transgenic tomato (Dewanti *et al*., unpublished results) and sugarcane (Miswar *et al*., 2007).

Sucrose synthesized in leaves as the source tissue is translocated to sink tissue and that the translocation is facilitated by SUT. There is a gene family encoding for SUT protein and among them SUT1 protein has a high affinity for sucrose translocation (Kuhn and Grof, 2010). Although many studies have indicated that reduction of expression of sucrose transporter genes have deleterious effects on plant growth and development, enhancing the expression of SUT1 protein increase sugar content in transgenic potato (Leggewie *et al*., 2003) and growth rate of pea cotyledon (Rosche *et al*., 2002). Thus, cloning of genes encoding for sucrose transporter were successfully conducted from sugarcane (Sugiharto, unpublished results) and that the overexpression of the gene increased sucrose content in transgenic sugarcane stem (Harjo, unpublished results).

The SPS is a key enzyme for sucrose synthesis in leave and the SUT is a responsible protein for the sucrose translocation from leave to the sink tissue. Thus, overexpression of the genes for SPS and SUT will enhance sucrose synthesis and translocation that lead to more significant sucrose content in sink tissue. We have found that double overexpression for the genes encoding for sugarcane SPS1 and SUT1 proteins significantly increase fruit production in transgenic tomato and sugar content in stem of transgenic sugarcane. This trait will be considered as an important target to increase growth and production in plants.

INTER-RELATION BETWEEN SUCROSE ACCUMULATION AND DROUGHT-TOLERANCE

Plants accumulate a set of proteins and low molecular weight compounds called compatible solutes under stress conditions. Compatible solutes are compounds that accumulate in stress-tolerant plant under water stress. They are water-soluble and do not disturb plant cell metabolism, such as sugars, amino acids, and polyols. They are involved in osmoregulation and stabilization of protein structure to maintain protein stability during water stress. Thus, introduction of the genes for biosynthesis of compatible solutes might useful to improve water tolerance of plants.

We have developed transgenic sugarcane either overexpression of the genes for sucrose biosynthesis or sucrose transporter. Overexpression of the

genes increased sucrose contents and that of double overexpression of the genes for sucrose synthesis and sucrose transporter increased more pronounced sucrose contents in stem of sugarcane. Thus, it is important to hypothesize that the increase of sucrose content due to the overexpression of those genes might lead to increase water tolerance in sugarcane.

Sucrose accumulation is a complex process and the protection against any stress caused by sucrose accumulation appears to use different mechanism to those used to protect from stress induced by water stress (Iskandar *et al.*, 2011). Molecular studies during water stress in sugarcane revealed that the expression of drought-inducible gene named *SoDip22* encoded for a small peptide is increased (Sugiharto *et al.*, 2002). This finding suggests that the protein functions to adapt to drought stress in leaves tissue, but detail study on this protein remains to be elucidated. Recently, drought-tolerant sugarcane has been developed by transformation of *betA* gene encoding for choline dehydrogenase from *Rhizobium meliloti*. The transformation resulted in increased glycine betaine contents that act as osmoprotectant and protect sugarcane against drought-stress. However, characterization of sucrose accumulation and water stress tolerance in sugarcane is still an important research topic to be elucidated in near future.

CONCLUDING REMARKS

Genetic engineering of sucrose metabolizing genes have achieved by cloning gene of SPS and SUT. Cloning have been done in the genes encoding for SPS present in two cDNA clones, *SoSPS1* and *SoSPS2* as well as *SUT1* in sugarcane and overexpressed. Overexpression of the genes for SPS and SUT will enhance sucrose synthesis and translocation in sink tissue, while the double overexpression for the genes significantly increase fruit production in transgenic tomato and sugar content in stem of transgenic sugarcane. The increase of sucrose content due to the overexpression of those genes might lead to increase water tolerance in sugarcane. A number of findings of molecular studies in respect to sucrose accumulation to protect water/drought stress were reported. However, characterization of sucrose accumulation and water stress tolerance in sugarcane need to be further elucidated in near future.

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GENE DISCOVERY RELATED TO VEGETATIVE AND TRANSITION FROM VEGETATIVE TO REPRODUCTIVE STAGE IN PLANT DEVELOPMENT

Endang Semiarti

ABSTRACT

Biotechnology of plants is one of the most powerful tools for cultivar improvement as well as for studying gene function in plants. In the past two decades, isolation and characterization of functional gene(s) and genetic transformation procedures for some of the commercially important plants have been established. In our laboratory, we have focused our efforts to isolate KNOX homeobox homologous gene, a key gene for activating shoot apical meristem to induce vegetative growth of *Phalaenopsis* orchids, namely *Phalaenopsis Orchid Homeobox1* (*POH1*) gene and develop the genetic transformation methods for various orchids, especially for commercially important orchids, such as *P. amabilis*, *Vanda tricolor*, *Spathoglottis plicata*, and *Coelogyne pandurata*. Furthermore, we are also intensively doing research on the isolation of *Phalaenopsis aphrodite Flowering Locus-T* (*PaFT*) homologous from Indonesian orchid species *P. amabilis* based on the PaFT cDNA sequence from Taiwan and generated transgenic plants of *P. amabilis* orchid, in order to understand the function of the gene and accelerating flower initiation of the orchids. Our results showed that a 1,086 bp POH1 cDNA (40.18 kDa) has been synthesized from leaves of early/juvenile vegetative stage and a 700 bp PaFT cDNA (27.75 kDa) has been synthesized from late/adult vegetative stage and were cloned by Lambda ZAP II cloning system. The structure of POH1 protein consisted of three protein domains, namely KNOX2 family, ELK family, and homeodomain. Homeodomain of POH1 shared 100% identity with DOH1. PaFT protein consisted of Phosphatidyl Ethanolamine-Binding Protein (PEBP) in interval base 73–483 and CETS family protein at sequence 7–519. Based on these orchid studies, some approaches for plant breeding can be utilized for other horticultural plants.

Keywords: orchids, vegetative, *POH1*, reproductive, *PaFT.*

INTRODUCTION

Plant development consists of three phases: embryo, vegetative, and reproductive (Howell, 1998). During plant life cycle, there are some groups of genes involved in each phase, namely embryo gene family, vegetative gene family, and reproductive gene family. These three phases of development is continuous. Experimental plant biologists, physiologists, and molecular geneticists contributed extensively to our understanding of plant processes. At present, almost every developmental process in the life cycle of plants is being scrutinized with molecular genetic tools. It is well known that the genome of a model plant *Arabiodpsis thaliana* has been completely sequenced at the year of 2000, generating a catalogue of the 25,000 genes required for plant life. It can be assumed that all physiological process in *Arabidopsis* plant are also occurred in other plants with some different character specific to each plant

species. It is enabled us to analyze the counterpart gene of *Arabidopsis* in other plants based on sequence homologous analyses. In *Arabidopsis*, *KNOTTED1* (*KN1*)*-like homeobox* (*KNAT1*) gene is expressed in the vegetative meristem (Lincoln *et al.*, 1994). As a member of homeobox gene family, KNAT1 protein functions to maintain the stem-like cells of shoot apical meristem (SAM) and activate the SAM to produce leaf primordia in the shoot apex. Flower initiation in *Arabidopsis* is regulated by a group of *Flowering Locus-T* (*FT*) (Koornneef *et al*., 1991; Ratcliffe and Riechmann, 2002) and Kojima *et al*. (2002) isolated *Heading date3* (*Hd3a*) gene from rice and found that *Hd3a* was orthologous gene of *FT* and it also controlled flower initiation in rice.

In this paper, we reported the isolation and characterization of functional genes that control early phase of vegetative phase and transition from vegetative to reproductive phase from cDNA library of Indonesian wild orchid *Phalaenopsis amabilis* (L.) Blume. To understand the function of the cloned genes, we were inserted the genes into genomes of some orchid species. Understanding the function of the gene will help us to looking for a superior gene from interest plants and use it for plant breeding. Therefore, the work in *Arabidopsis* have broad significance in flowering plants and may help in increasing the yield of important crops (Coupland, 2004), including orchids and other important crops. Sim *et al*. (2007) and Hee *et al*. (2009) successfully induced *in vitro* flowering of hybrid *Dendrobium*.

CURRENT STATUS, MAIN CHALLENGES, AND PRINCIPAL TRENDS

At present, some genes related to vegetative and reproductive development were isolated and characterized from various orchids by many researchers wordwide. Orchid is a model plant for mass propagation by tissue culture (Arditti and Ernst, 1993), but the problem is the growth rate of orchid is too long. It is worth to look for the best way to accelerate the growth rate of *in vitro* propagation by tissue culture. Yu *et al.* (2000) reported that a *KNOTTED1* homologous gene that isolated from *Dendrobium* 'Madame Thong-In' in Singapore, namely *Dendrobium Orchid Homebox1* (*DOH1*), played an important role to create shoot architecture in orchid. Overexpressed *DOH1* in transgenic *Dendrobium* produced multiple shoots. Due to the function of *DOH1* as homebox gene, it might be use for accelerate the growth rate of plant cell in shoot formation that will improve the technique of micropropagation. Based on KNAT1 cDNA sequence, we screened cDNA library from early stage of vegetative phase and finally isolated a 897 bp in length of cDNA, then hereinafter we named *Phalaenopsis Orchid Homeobox1* (*POH1*). The structure of POH1 protein consists of three protein domains, namely KNOX2 family, ELK family, and homeodomain (Figure 1). Homeodomain of POH1 shares 100% identity with DOH1. Since *Phalaenopsis* and *Dendrobium* orchids have different pattern in shoot development, namely sympodial and monopodial, this data

suggests that the homeodomain of POH1 and DOH1 function for shoot formation in very early stage of orchid development.

Predicted KNOX2 domain functions for homodimerization. POH1, like any other KNOX homeodomain proteins, works in homodimer stage. Homeodomain is needed for binding the substrate or DNA of target sequence. ELK superfamily, alongside with KNOX1, functions to suppress gen target expression to keep plant development in correct way (Nagasaki *et al.*, 2001). Based on characteristic prediction of POH1, it is predicted that POH1 works in dimer stage, which suppress the expression of target gene that induces abnormal form in plant development.

Even though POH1 has same functions like any other KNOX homeodomain proteins to maintain plant development, it has specific characters in protein structures. POH1 is predicted has additional 33 amino acids chain between ELK superfamily and homeodomain. This characteristic only occurs in POH1 that means a special character of POH1, which can be distinguished to other KNOX homeodomain proteins.

POH1 protein is closely related to *Dactylorhiza fuchsia* and *Orchis anthropophora* KNOX homeodomain proteins. Phylogenetic analysis using two different methods maximum likelihood (Figure 2) and maximum parsimony (Figure 3) showed a similar result. The protein domain for every species was almost identical, the different occured in sequence that connected one domain with other domain. This phenomenon indicated that the protein was very conserved in every species and might have an important role in plant development.

Four DEF-like MADS-box genes homologous family were isolated and characterized from *Oncidium* 'Gower Ramsey' orchids (Tsai *et al.*, 2004) and in

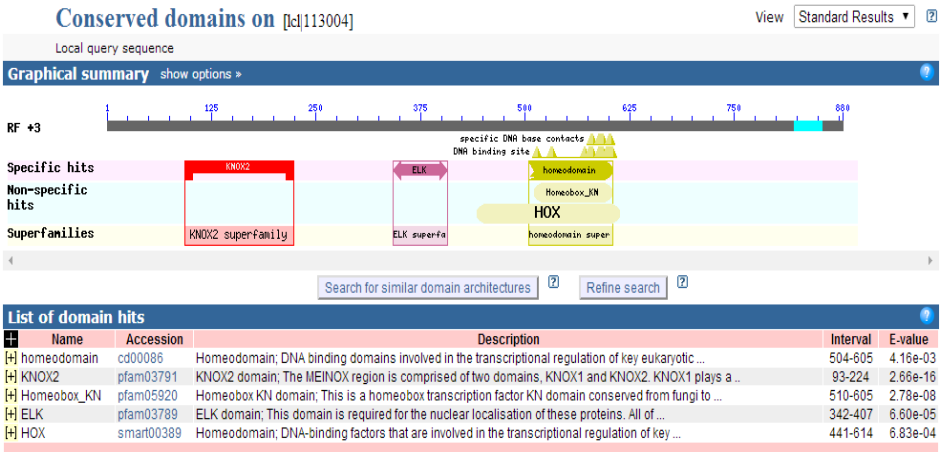

Figure 1. POH1 predicted protein structure using BlastX.

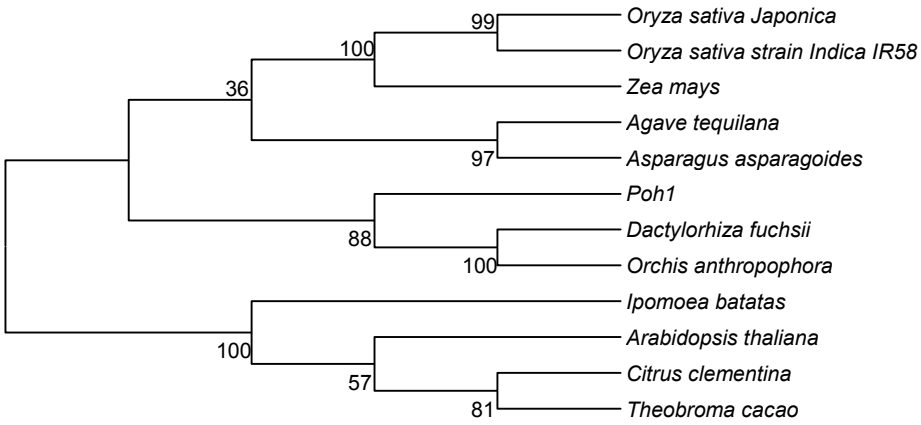

Figure 2. Phylogenetic analysis of POH1 protein using maximum likelihood method.

turn these genes regulate other flower genes for producing flowers. If we could modify and maximize the role of the MADS-box gene, we will get early flowering cultivar plants or other good traits. A homologous *FT* gene was isolated by Jang *et al*. (2015) from *P. aphrodite* from Taiwan. Under research collaboration, we analyzed the cDNA library from *P. amabilis* from Indonesia using rapid amplified cDNA-ends (RACE)-PCR. We got a 700 bp in length of cDNAs that differed from the reference of *P. aphrodite* from Taiwan, although 600 bp of it was identical each others. Then, we inserted independently the cDNA into protocorm (developing orchid embryo) of *P. amabilis* by *Agrobacterium*-mediated genetic transformation to observe what is the difference between them. Synthesized cDNAs were sequenced and then the length of cDNA was converted into the molecular weight (kDa) using the link: http://www.molbiol.ru/eng/scripts/01_06.html. In order to understand where and when the PaFT protein initially produced and function in plant, we were analysing the protein production of these two cDNAs.

Predicted protein characteristic of PaFT showed that PaFT has Phosphatidyl Ethanolamine-Binding Protein (PEBP) in interval base 73–483, which acted as substrate binding site (Figure 4). PEBP substrate-binding site could allow protein to adhere to the inner leaf of bilayer membranes and could relay signals from the membrane to the cytoplasm (Banfield *et al.*, 1998). CETS family protein at sequence 7–519 was specific sequence for *FT* gene, which was induced rapidly upon activation of *CONSTANS*. *CONSTANS* activation was light dependent, higher in long day period than short day period (Yamaguchi *et al.*, 2005). Based on predicted protein structure, PaFT was predicted located at cell membrane and took part in signal transductions for flowering mechanism.

Phylogenetic analysis using both maximum likelihood (Figure 5) and maximum parsimony methods (Figure 6) showed that Indonesian *Phalaenopsis*

PaFT was very similar to Taiwan's PaFT compared to that of *Cymbidium* and *Oncidium. Phalaenopsis* PaFT was closer to *Oncidium* than to *Cymbidium*. Due to the logic assumption that *Phalaenopsis* and *Oncidium* have similar environment properties to flowering, it might caused by the similar structure of *FT* gene in *Phalaenopsis* and *Oncidium*. Database of *FT* gene in Orchid is still very limited, so it likely becomes a good field to be developed. We are analyzing *P. amabilis* transgenic plants that harbour Ubipro::PaFT T-DNA and the experiment is now in progress.

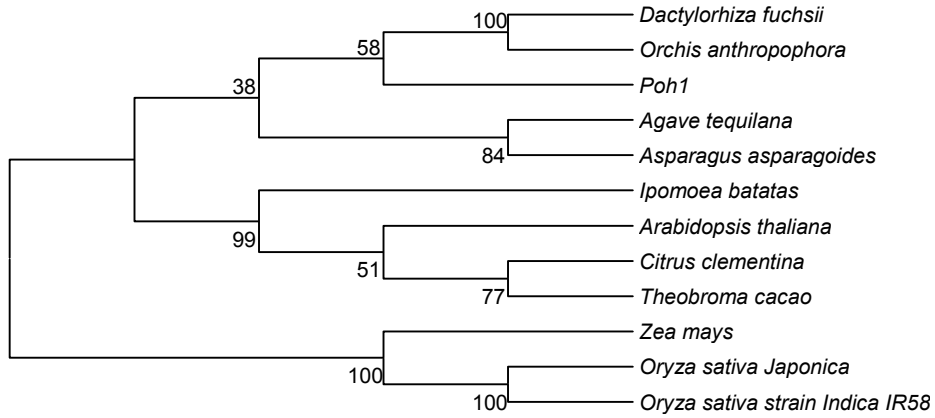

Figure 3. Phylogenetic analysis of *POH1* using maximum-parsimony method.

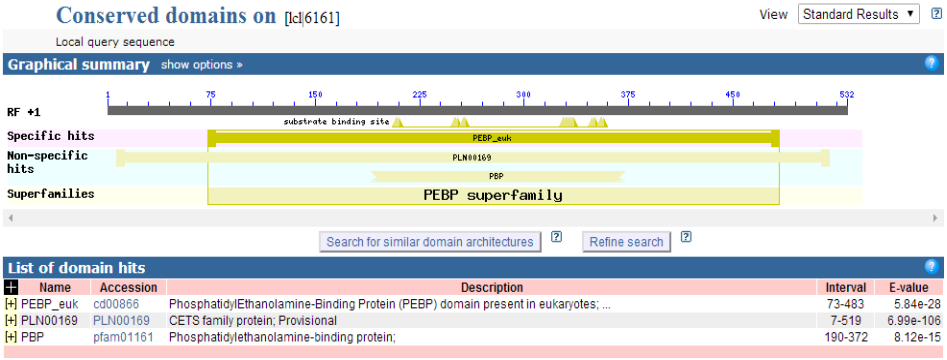

Figure 4. Predicted protein characteristic of PaFT using Blastx.

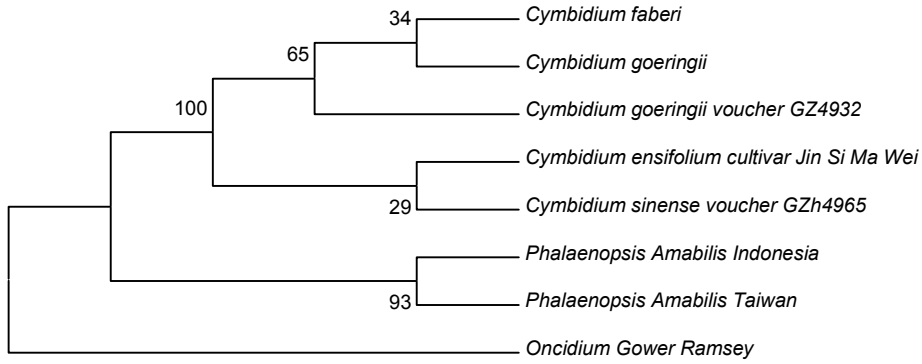

Figure 5. Phylogenetic analysis of PaFT using maximum likelihood method.

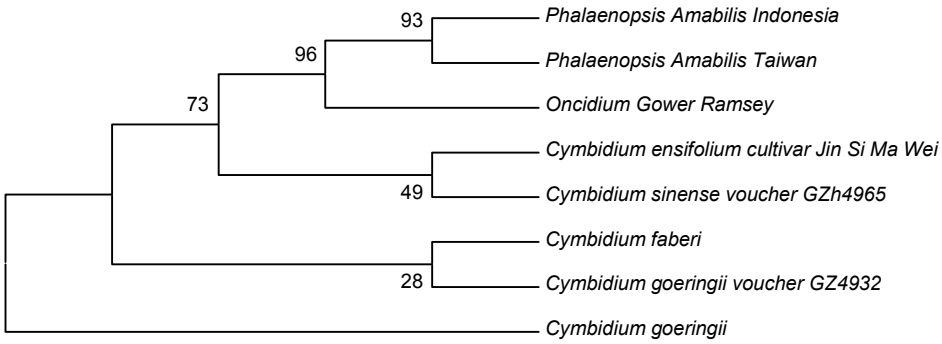

Figure 6. Phylogenetic analysis of PaFT using maximum parsimony method.

FUTURE INITIATIVES AND REGION/COUNTRY-SPECIFIC DEVELOPMENT FOCUS WITH POSSIBLE CONTRIBUTIONS

Indonesia is tropical country that serves a good habitat for orchid cultivation. It is worth to try to improve the cultivation of national orchids for seedlings and flower production to meet the need of the country for both national and international needs through improvement of plant breeding. As biotechnological approach, some genes can be used as molecular and genetic markers to screen a superior plants from siblings of out cross pollinated plants seedlings. Beside that, a key gene for vegetative development and transition of vegetative to reproductive development can be used to create superior transgenic plants, that in turn it can be mass propagated by tissue culture.

DEVELOPMENT SCOPE AND OPPORTUNITIES

It is important to develop a new tools on biotechnology to help people for creating high yield and quality of crop plants flowers. It is a good opportunity to

create a new trait of crops, that easily mass propagated to produce large quantity of plants in relatively short time through plant tissue culture of transgenic plants harbor a superior gene for inducing multiple shoots formation that meet the industry and community needs. The other benefit to use the superior genes are people will be able to select good plants easily from second-generation of classical/conventional breeding based on the sequence of superior functional gene in plant genome using SNPs or RFLP method. The most precise method, we can create superior and stable transgenic plants those express desired trait as expected.

The results showed that the method of *Agrobacterium*-mediated transformation in three orchid species, *P. amabilis*, *V. tricolor*, and *C. pandurata*, using three-week-old intact protocorm (Semiarti *et al*., 2011), is convenience and can be applied for creating another transgenic of important crops.

CONCLUDING REMARKS

Two key genes of orchid development have been isolated and characterized from Indonesian moth orchids *P. amabilis* (L.) Blume, namely *POH1* gene for activating shoot apical meristem during shoot initiation and *PaFT* gene for transition from vegetative to reproductive phase in flower initiation. The function of these genes can be explored more deeply to elaborate the genetic network with other potential genes throughout plant development, in which the activity and the function of each gene will help us to solve some problems to meet our need and the large scale of production as industry need.

ACKNOWLEDGMENTS

The authors thank to the Directorate of Higher Education (DGHE), Ministry of Education and Culture of Republic Indonesia for giving STRANAS Research Grant for 3 years (2012–2014) in Contract No. 001/SP2H/PL/DIT.LITABMAS/ III/2012, 089/SP2H/PL/DIT.LITABMAS/V/2013, and Hibah Bersaing Research Grant for 2 years in Contract No. LPPM-UGM/1045/2010. We also thank to the Ministry of Research and Technology of Republic Indonesia for giving Riset Unggulan Terpadu (RUT) Research Grant for 3 years (2002–2004) in Contract No. 14.15/SK/RUT/2004. We thank to all students in the Laboratory of Biotechnology, who were involved in this research, also thank to Royal Orchids Nursery (Prigen, East Java) and Titi Orchid Nursery (Yogyakarta) for giving plant materials to this research as collaborators.

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CHAPTER 3
GENE DISCOVERIES IN MICROBES

Microbes make up about 60% of the earth's biomass, yet less than 1% of microbial species have been identified. Paradigm of 99% unculturable microbes suggests there is abundance source of genes products to provide food and renewable energy for evergrowing population. Gene discovery and elucidating important gene in microbes is a key aspect for further application in industrial and sustainable agriculture.

Microbial biotechnologi research and their applications in agriculture as biofertilizers, phytohormones, biocontrols, biofuel, bioindustry, and plant virus and diseases has been conducted in ICABIOGRAD. Scientists at the Gadjah Mada University, Yogyakarta, have also conducted some research on microbiology. Through genes cloning and analyses of bacterial genomes some interesting findings have been obtained. They are also exploring the possibility for the activation of cryptic clusters that may encode novel secondary metabolites, especially for *Streptomyces.* Two papers in this chapter reflected the current states of microbial biotechnology research at this two institutions.

One paper in this chapter described the importance of environmental metagenomic DNA and abundant available microbial genomes information as sources for mining new enzymes. Combining metagenomic approach and available microbial genomic information enabled us to produce industrially novel enzymes efficiently. One paper summarizes some important recent developments in the understanding of functional genetics of key microbial and enzyme biocatalysts for the production of biofuels for the future. Another paper described a research result on methane oxidizing community in soil rhizosphere of rice which responsible for methane oxidation and their unique physiological properties, such as nitrogen fixation and trichloroethylene degradation. This chapter is concluded by a paper on discovering glycoside hydrolase (GH) genes from marine microorganisms. This paper describes the discovery of genes encoding for GHs with α-amylase, chitosanase, and β-glucosidase activities from marine microorganisms.

CURRENT STATUS ON MICROBIAL GENE DISCOVERY IN ICABIOGRAD

Eny Ida Riyanti*, Edy Listanto, and Kusumawaty Kusumanegara

ABSTRACT

Agricultural biotechnology deals with the manipulation through genetic engineering of living organisms or their components to produce useful products for various applications in biological sciences. Research on microbial biotechnology has been conducted in Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) since 1990 in order to support agricultural practices in Indonesia. This review contains microbial biotechnology research and their applications in agriculture, such as biofertilizers, phytohormones, biocontrols, biofuel, bioindustry, plant virus and diseases.

Keywords: ICABIOGRAD, microbial research.

INTRODUCTION

Development of agriculture biotechnology will require new genes, enzymes, or compounds to meet human needs. Explorations on new genes are potentially come from microbes, as microbes are highly diverse compared to other organisms in the world such as animals or plants. Microbes grow and adapt in all of part of our planet. Only less than 1% was identified and a small portion of it can already be utilized for human life (Curtis and Sloan, 2004; Torsvik and Ovreas, 2002; Torsvik *et al*., 2002). They are incredible role to ensure the survival of other organisms, due to their role in the biochemical cycles in nature.

Microorganism is a microscopic unicellular or multicellular organisms, consists of very diverse organisms, including bacteria, fungus, protozoa, archaea, and viruses. Beneficial microorganisms have a long history for human life, such as for traditional food and beverage preparation. Indonesia has a huge microbe diversity to be utilized. Indonesia has also traditional fermented food and beverage, such as “tempe” (fermented soybean), “tape” (fermented cassava), “bekasam” (fermented fish), etc. This diversity can be utilized to support the development of the country as in the case of food security, environmental sustainability, medical and industrial purposes.

CULTURE COLLECTION IN ICABIOGRAD FOR GENE SOURCES

Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) culture collection was named as “Biogen Culture Collection/BiogenCC” and have more than 3,000 microbial collection, which have potential application for plant phytohormone, biofertilizer,

decomposer, bioremidiation, biocontrol for plant pathogen and pest, and also for industrial purposes, such as enzymes or fine chemicals. This potential microbes may also have potential genes for medical purposes or development of crop plant.

MICROBIAL GENE FOR PROMOTING PLANT GROWTH

Modern agriculture relies heavily on chemical fertilizers, pesticides, and plant growth promoter. However, the negative impact of the use of chemical fertilizers, pesticides, and synthetic plant growth promoter often overlooked. Potential soil and endophytic microbes as bio-fertilizer, biocontrol of plant growth regulator and producer will be able to substitute the use of chemicals in agriculture.

Plant growth-promoting bacteria (PGPB) are soil/rhizosphere bacteria, which can benefit to plant growth by different mechanisms (Glick, 1995; Khan *et al*., 2009; Rodriguez *et al*., 2006). The potential use of PGPR for substituting or replacing chemical fertilizer is promising, as they have no negative impact on environment and sustainable agriculture. The utilization of microbial genes responsible for plant growth promoting compounds will give opportunity for constructing superior agriculture plants.

A range of diazotrophic plant growth-promoting rhizobacteria (PGPR) participates in interactions with agricultural plants, significantly increase their vegetative growth and grain yield. Free-living bacteria of the genus *Azospirillum* live in close association with rice roots. These bacteria produced phytohormone indole acetic acid (IAA) to the environment (Ji *et al*., 2014; Susilowati *et al*., 2015). IAA was isolated from *Azospirillum* strains isolated in ICABIOGRAD were investigated for its effect on root development and plant height of rice variety IR64 *in vitro*. Rice cultures of variety IR64 were grown *in vitro* and inoculated with *Azospirilllum*. Production of IAA by the bacterium during its growth period in rice plant culture medium containing different levels of nitrogen was observed. *Azospirillum* strains, Az15 and Az44, had a high ability to produce IAA, i.e. 57.93 µg/ml at 12 days after incubation (DAI) and 40.42 µg/ml at 7 DAI, respectively. The IAA production patterns of *Azospirillum* Az15 and Az44 in the liquid medium were fluctuative until the end of the incubation period, while that of the strain Az7 was linear. Strain Az7 gave a better effect on the root development and plant height than strains Az15 and Az44. Inoculation of rice culture with *Azospirillum* is expected to reduce nitrogen application on rice IR64 by the IAA production as indicated by significant changes in the root growth and development (Lestari *et al*., 2007).

Multifunctional *Azospirillum* spp. have been isolated in ICABIOGRAD (Riyanti *et al*., 2012; Susilowati *et al*., 2015). Genetically improvement of the multifunctional *Azospirillum* have been performed using chemical mutagenesis

ethyl methanesulfonate (EMS) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and using transposon EZ-Tn5<kan-2>Tnp produce range of mutants with different properties of nitrogen fixation, IAA, and P solubilizing improvement. Nine selected mutants with increasing capability to solubilize P (determined by clear-zone formation on Pikovskaya's medium) have been characterized for nitrogenase activities and IAA production compared to wild type Aj Bandung 6.4.1.2. The effect of mutagenesis on IAA production and nitrogenase activities varied among the mutants. Two mutants, AzM 3.7.1.16 and AzM 1.7.2.12, showed superiority in the production of IAA, while two mutants, AzM 1.5.1.14 and AzM 3.7.1.15, were superior in nitrogenase activities. The EMS mutagenesis of *Azospirillum* sp. showed enhanced dissolving capability of insoluble phosphate (tricalcium phosphate) and increased IAA production and nitrogenase activity. *Azospirillum* was mutated using transposon EZ-Tn5<kan-2>Tnp. The electrotransformation resulted in 20 out of 22 transformants tested contained the marker gene (*npt*). Ten, six, and four mutants have increased, decreased, and lost phosphate-solubilizing function, respectively. Mutant with elevated phosphate solubilizing ability may be selected further to be utilized as biofertilizer while others may be useful for identification of genes responsible for phosphate solubilization (Hadiarto *et al.*, 2012; Riyanti *et al.*, 2012).

Detection of key functional genes involved in nutrient cycling, such as for nitrogen fixation (*nifDHK* and *amoABC*), producing phytohormone (*Tmo* and *IPDC*), producing ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase gene/*Acds*), are also being investigated in ICABIOGRAD.

MICROBIAL GENE FOR PLANT DEFENSE TO DISEASES AND PESTS

The concept of pathogen-derived resistance (PDR) has been applied in engineering virus resistant crops (Prins *et al.*, 2008). Elucidating the molecular mechanisms that underlie the host-pathogen interaction is one of the key aspects for successful application of PDR. The role of p27 in *Red clover necrotic mosaic virus* (RCNMV) replication has been studied. The study showed that domains and critical amino acids in p27 of RCNMV required for its association with and targeting of ER membranes in *Nicotiana benthamiana* plants have been determined using a C-terminally GFP-fused and biologically functional p27. Confocal microscopy and membrane-flotation assays using an *Agrobacterium*-mediated expression system showed that a stretch of 20 amino acids in the N-terminal region of p27 is essential for the p27-plant ER membranes association. This approach was done using alanine-scanning mutagenesis. The domain contains amino acids required for the formation of viral RNA replication complexes and negative-strand RNA synthesis (Kusumanegara *et al.*, 2012).

About 214 endophytic bacteria have been isolated from potato plant cultivar Granola and Atlantic from Lembang and Garut, West Java. Two isolate have been identified as *Micrococcus endophyticus* and *Paracoccus halophylus* which have capability for enhancing plant resistance to *Phytophtora infestans* as well as producing compounds like phytohormone, such as IAA, gibrelic acid, zeatin, and ABA like (Akhdiya, 2014). These endophytes might have potential genes for plant growth benefit.

Entomophathogen fungi and bacteria have been isolated and characterized, such as *Paecylomyces* sp., *Beauveria bassiana*, *Metharizium anisopliae*, *Hirsutella citriformis*, *Cordicep* sp., and *Serratia marcescens*. The latter bacterium has been demonstrated to cause 70% mortality of brown planthopper insects within 10 days in a glasshouse experiment.

B. bassiana BB200109 is one of entomopathogenic fungi potential for insect biocontrol. One of its modes of action is by producing chitinase when infecting its host. The extracellular chitinase (60.25 kDa) characterization has been done at various conditions, i.e. temperature, pH, metal ion, and incubation time. It had an optimum activity at pH 4, temperature 50ºC, and optimum incubation time of 90 minutes. Metal ions (60 mM) Mn^{2+} served as activator, while EDTA, K^{+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Na^{+} acted as inhibitors. The chitinase was demonstrated to have lower affinity to chitin substrate as indicated by high K_M value of 0.266 mg/l and a V_{max} of 0.067 mg/l sec (Suryadi *et al*., 2013).

The potential use of *Photorhabdus* spp. to control the population insect pests, especially in rice plant, has been investigated. *Photorhabdus* spp. toxin of HJ isolates has been isolated and purified. The bioassay using *Tenebrio molitor* larvae-3 indicated that after 48 h application, the percentage of larvae mortality by crude antigen was lower (73%) than by fraction antigen (93%). The PAb fraction of the protein reacted with *Photorhabdus* spp. antigen and could differentiate between *Photorhabdus* spp. and other bacterial filtrate, such as *Xanthomonas oryzae* pv*. oryzae* (Xoo), *X. campestris* pv*. glycinea*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *glycinea*, or *P. fluorescens*, however it showed cross reaction with *Escherichia coli*. The effective concentration for detection of *Photorhabdus spp*. toxin as well as specificity test against other bacterial antigens should be further investigated (Suryadi *et al*., 2006).

Fast identification method is crucial for plant pathogen identification, especially for *Xoo*, the causal agent of rice bacterial leaf blight (BLB) disease. Pathovar specific primers for *Xoo*, i.e. *Xoo*2976F, *Xoo*2976R, and *Xoo*80F (Lang *et al*., 2010), have been successfully applied for pathogenic determination of isolates originated from five locations in Indonesia and showed that *xa5*, *Xa7*, and *Xa21* resistance genes were still effective against the prevalent *Xoo* in those regions (Tasliah *et al*., 2013).

Antibacteria

Streptomyces producing antibacterial compound for enteropathogenic *E. coli* K1.1, *P. pseudomallei* 02 05, and *Listeria monocytogenes* 5407, have also been isolated. The antibacterial compound extracted from the isolate A 3.5 had a similar effectiveness to antibiotics bacithracyn (10 units) and neomycin (30 g). Meanwhile, the antibacterial compound extracted from isolate F6.1 had a similar effectiveness to antibiotics colistin (10 g) and doxyciclin (30 g) (Susilowati *et al*., 2007).

Industrial Enzyme

Thermostable enzymes are potential enzymes for industrial purposes. Indonesia has diverse high temperature environment, such as volcanoes or hot water spring, as source for thermophilic bacteria. *Bacillus stearothermophiles* has been isolated and a thermostable endo-α-amylase have been isolated and characterized using several methods. The endo-α-amylase (192.932,8 Dalton) had a K_M of 1.06 mg/ml and V_{max} of 1.21 mol/min, with optimal activity at pH 7 and 90ºC. Its activity was inhibited by the divalent cation chelator, such as EDTA and CuSO4, but activated by calcium ion. This enzyme converted cassava starch into glucose, dextrin, maltose, and oligosaccharides. After 24 hours of hydrolysis, the concentration of glucose and maltose reached 51.970 ppm and 10.090 ppm, respectively. This enzyme is promising to be studied further for industrial purposes (Lestari *et al*., 2010).

Bioethanol

Rising concern on fossil fuel depletion and environmental issue increase the demand on environmentally friendly renewable bioenergy such as bioethanol. Corn-based and sugar-based bioethanol are promising to substitute for fossil fuel demand. However, this approach competes with the need on food and feed demand. Bioethanol from lignocellulosic agriculture wastes has high level of interest compared to other biofuel based on the superiority on its properties, low price, and environmental concerns also sustainable availability (Riyanti, 2009). However, lignocelulosic waste is a recalcitrant material, which needs high capital costs for degradation. Research on this area will open up the potential use for bioethanol production.

The advantage of ethanol fermentation at high temperatures encourages the study of thermophilic bacteria ethanologenic assembly. In addition, the ability of thermophilic bacteria in the use of biomass pentose sugar degradation products derived from lignocellulosic degradation provides an opportunity to reduce the cost of bioethanol production. Thermophilic bacteria *Geobacillus thermoglucosidasius* M10EXG have been isolated (Jeon *et al*., 2008) and the kinetic growth has been evaluated (Riyanti and Rogers, 2009a). Two important

microbial genes, *pyruvate decarboxylase* (*pdc*) from mesophilic bacteria *Zymomonas mobilis* and alcohol dehydrogenase (*adhT*) from thermophilic bacteria *G. thermoglucosidasius* M10EXG (Jeon *et al*., 2008) have been isolated and cloned into vector for ethanol production. The expression of *pet* operon to produce ethanol in both thermophilic and mesophilic bacteria using the shuttle vector pMK18 have been evaluated. *Pet* operon is a gene arrangement consisting of ethanol production genes (*pdc* and *adhT*). Construction of *pet* operon using these genes was performed using mesophilic-thermophilic pMK18 shuttle vector. *Pet* operon expression in *E. coli* mesophilic bacteria can produce 0.3 g/l of ethanol with *adhT* activity around 0.02 U/mg protein and *pdc* activity around 0.004 U/mg protein. Construction and expression of the *pet* operon using *pdc* and *adhT* from thermophilic and mesophilic bacteria and the use of mesophilic-thermophilic shuttle vector as its backbone were reported for the first time. However, further research for system construction for the *pet* operon on *Thermus thermophilus* HB27 thermophilic needs to be done for optimum construct. These results are expected to initiate the development of genetic manipulation techniques in thermophilic bacteria, in particular the development of manipulation techniques for thermophilic ethanologenic (Riyanti and Rogers, 2009b).

FUTURE CHALLENGE

ICABIOGRAD have diverse microbial culture collection containing beneficial soil bacteria producing phytohormone, fixing nitrogen, solubilizing phosphate; endophytic bacteria and fungus; plant pathogens including plant viruses. This collection needs to be maintained and utilized optimally for exploration of potential genes for agricultural and industrial purposes. Many of them have been characterized and explored for several purposes, such as antibacterial, anti-plant pathogenic activity, entomopathogenic activity, lignocelluloses biodegradation, plant phytohormones, and biofertilizers.

Studies on several protein and genes, such as p27 for virus replication, *pdc* and *adh* for bioethanol production IAA production, and genes for phytohormones and lignocellulosic degradation useful for agricultural and industrial purposes as well as for crop improvement have been conducted.

Genome sequencing of potential microbes using NGS technology is challenging to be conducted in ICABIOGRAD to reveal potential genes for industrial and plant engineering purposes. Elucidating important genes in microbes is the key aspect for further application in industrial agriculture, such as biofuel, bioactive compounds, enzymes production, or as a gene source for biotechnology process, such as marker genes, or in developing superior plant with high-yielding ability, tolerant to pest and diseases, and good adaptability to abiotic stresses, such as dry, submerged, saline, and high temperature.

CONCLUDING REMARKS

Research on microbial biotechnology has long been conducted in ICABIOGRAD with earlier focuses on *in situ* application of microbes for agricultural purposes, such as biofertilizers, biodecomposers, bioremediation, and biocontrols, whereas microbial research for industrial enzymes and fine chemicals production have a less portion in the past. Recently, research on microbial genes for the development of biofuels and other purposes has been initiated in ICABIOGRAD in respond to increasing demand for industrial agriculture purposes.

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CURRENT STATUS OF MICROBIAL GENE DISCOVERY RESEARCH IN GADJAH MADA UNIVERSITY

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ABSTRACT

Microbes have an important role in human life and offer new solutions to longstanding challenges in agricultural and industrial sectors. This leads many scientists at the Gadjah Mada University to elucidate the microbes contribution to earth's biosphere and life by conducting several researches, such as gene cloning and genomic analysis, and exploring novel secondary metabolites produced by specific microbe species. Thus, this review describes research activities and the progress achieved by several working scientist groups at the Gadjah Mada University. These groups focused on gene discoveries for varied products like for bacterial pigments production, for drugs development, for vaccines development in fish, and from extreme environment for industrial applications.

Keywords: genome sequencing, carbazole dioxygenase, secondary metabolite, serine protease, fish vaccine.

INTRODUCTION

Microbes have a very vital role in our life and accounts for more than 60% of the total biomass on this earth. Identifying and harnessing their unique capabilities will offer us new solutions to longstanding challenges in environmental and waste cleanup, energy production and use, medicine, industrial processes, agriculture, and other areas. Scientists at the Gadjah Mada University, Yogyakarta, also are starting to appreciate the role played by microbes in our life and the contributions of microbes to earth's biosphere. Through genes cloning and analyses of bacterial genomes have been obtained some interesting findings. Especially for *Streptomyces*, we are also interested in exploring the possibility for the activation of cryptic clusters that may encode novel secondary metabolites.

GENE DISCOVERY FOR BACTERIAL PIGMENTS PRODUCTION (DR. JAKA WIDADA GROUP)

Indigo is one of the largest selling textile dyes, used on cotton and wool fabrics. Indigo can be microbiologically produced from indole by enzymatic formation of a dihydrodiol in a reaction catalyzed by enzymatic complexes with dioxygenase, which are involved in degradation of aromatic compounds (Ensley *et al*., 1983; O'Connor *et al*., 1997). The resulting dihydrodiol is chemically dehydrated to form indoxyl, which in turn dimerizes to indigo. Cloning and expression of the genes coding for the dioxygenase and those for the specific electron transport system in *Escherichia coli* is able to transform indole to

indigo. In nature, carbazole (CAR) is degraded by the CAR operon that consisting dioxygenase system. This CAR dioxygenase system is unique because only consist one gene dioxygenase and specific electron transport system. Ten bacteria that could use CAR as a sole source of carbon were isolated from environmental samples. Southern hybridization analysis of the genomes from the newly isolated ten CAR-utilizing bacteria revealed that eight of the isolates carried gene clusters homologous to the CAR-catabolic car operon of *Pseudomonas resinovorans* strain CA10. Cloning and sequencing analysis showed that two car operons and the neighboring regions of *Pseudomonas* sp. strain K23 are nearly identical to that of strain CA10. In contrast to strains CA10 and K23, *car*EF genes did not exist downstream of the car gene cluster of *Janthinobacterium* sp. strain J3. In the car gene clusters, strains CA10, K23, and J3 have Rieske-type ferredoxin as a component of carbazoledioxygenase. In the upstream regions of the carJ3 and carKA1 gene clusters, ORFs whose deduced amino acid sequences showed homology to *Gnt*R-family transcriptional regulators were identified. Interestingly five isolates (J3, J8, J11, J14, and J27) produced high concentration of indigo when induced by CAR or anthranilic acid. These results suggested that CAR dioxygenase systems from isolates J3, J8, J11, J14, and J27 could be developed as indigo production expressed in *E. coli*.

GENE DISCOVERY FOR DRUGS DEVELOPMENT (LABORATORY OF ACTINOBACTERIA AND DR. JAKA WIDADA GROUP)

Secondary metabolites of *Streptomyces* offer great potential for the development of new medicines. We determined the complete genome sequence of *Streptomyces* sp. GMR22, a soil bacterium, producing an

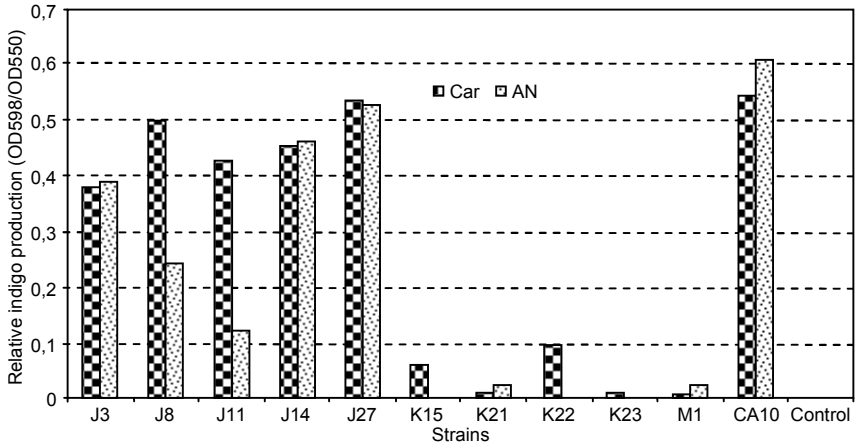

Figure 1. Relative indigo production of twelve carbazole-degrading strains harboring *car*Aa-like gene.

antifungal agent, isolated from Cajuput plantation, Wanagama, Yogyakarta, Indonesia. Here, we report the 11,420,050 base pair linear chromosome of this organism, containing the largest number of genes so far discovered in a bacterium. The 9,480 predicted genes include more than 59 clusters coding for known or predicted secondary metabolites and 17 numbers of RNAs. The genome contains an unprecedented proportion of regulatory genes, predominantly those likely to be involved in biosynthesis of cofactors, vitamins, protestic groups, and pigment. The genome sequence of *Streptomyces* sp. GMR22 will greatly increase our understanding of this bacterium as well as aiding the generation of new drug candidates by "heterologous expression of pleiotropic regulators". From this genome sequence analysis, we also found that GMR22 harbor two novel ACC deaminase genes and three novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase that potential to be used to develop abiotic stress and glyphosate-resistant crops.

In general, the selection of antibiotic-producing *Streptomyces* were performed using antagonists to test a number of microbial pathogens, so that only strains that have the ability to inhibit forwarded for further investigation, while strains that do not have the ability discarded. For us, it was also interesting to explore further *Streptomyces* strains that do not produce an antifungal compound, in producing new bioactive compounds such as anticancer and anti-inflammatory. Our hypothesis is the bioactive compounds produced from these strains may be safe when the compounds are developed as a drug, because of the low cytotoxic activity against nontarget cells. *Streptomyces* sp. GMY01 is a strain that does not produce antifungal compound and has a rapid growth. Cytotoxicity assay of secondary metabolites of GMY01 against breast cancer cells (T47D and MCF7) showed that this strain produces bioactive compounds that have very high cytotoxic activity against those breast cancer cells, but very low cytoxic activity to normal cells (NIH3T3). Whole genome sequence analysis of GMY01 showed that 51 biosynthetic gene clusters for secondary metabolites were identified by antiSMASH, namely 2 siderophores, 4 terpenes, 1 bacteriocins, 2 lantibiotic, 3 type-1 polyketide synthases (T1-PKS), 1 T2-PKS, 1 T3-PKS, 8 nonribosomal peptide synthetase (NRPS), 2 butyrolactones, 1 ectoine, 1 T1-PKS-butyrolactones-NRPS hybrid, 1 HglKS/T1-PKS hybrid, 1 lantipeptide-NRPS hybrid, 1 NRPS/T1-PKS hybrid, and 24 of other gene clusters that may encode new secondary metabolites (Herdini *et al*., unpublished results).

GENE DISCOVERY FROM EXTREME ENVIRONMENT FOR INDUSTRIAL APPLICATIONS (DR. M. SAIFUR ROCHMAN GROUP)

Halophilic microorganism is a group of microorganism that is able to live in the environment with high concentration of salt. Usually, the salt concentration can reach 20% to 30%. Halophilic microorganisms are widely distributed across

all the kingdom of life: Archaea, Bacteria, and Eucarya. In some ecosystems, salt-loving microorganisms live in such large numbers that their presence can be recognized without the need for a microscope. Some saltern crystallizer ponds are colored pink-red by Archaea (*Haloquadratum* and some others representative of *Halobacteriales*), Bacteria (*Salinibacter*), and Eucarya (*Dunaleilasalina*). The interesting thing from the halophilic microorganisms is that they usually can produce enzymes/proteins, which are active and stable in the extreme environment. These enzymes/proteins usually active in the conditions in high concentration of salt, organic solvent, and metal ions are present. Therefore, it can be used for the industrial application purposes. Therefore, the objectives of this research were to explore the serine protease gene from halophilic microorganisms and to study their application for industrial activities.

In this research, three types of serine protease genes were successfully cloned into expression vector: two serine protease genes from extreme halophilic archaea (*Halobacterium* sp. NRC-1) and one serine protease gene from moderate halophilic bacteria (*Chromohalobacter salexigene*-BKL-5). From the expression check analysis, only serine protease gene from moderate halophilic bacterium could be well overexpressed.

GENE DISCOVERY FOR VACCINES DEVELOPMENT IN FISH (DR. MURWANTOKO GROUP)

Iridovirus was known as agents that caused serious systemic disease in freshwater and marine fishes. The mortality up to 100% of orange-spotted grouper (*Epinephelus coioides*) due to *Iridovirus* infection has been reported in Indonesia. The gene encoding capsid protein of *Iridovirus* is supposed to be conserved and has the potency for the development of control methods. The objectives of this study were to clone the genes encoding capsid protein *Iridovirus* and to analyze their sequences. The spleen tissues of orange-spotted grouper were collected and their DNA extracted. The DNA fragment of capsid protein of *Iridovirus* genes were amplified and cloned in pBSKSII. The Jepara clone (IJP03) contained complete open reading frame (ORF) of the gene composed by 1,362 bp nucleotides, which encoded 453 amino acids. Those Jepara and Bali (IGD01) clones shared 99.8% similarity in nucleotide level and 99.4% at amino acid level. Based on those sequences, Indonesian *Iridovirus* was belonged to genus *Megalocystivirus* and shared 99.6–99.9% similarity on nucleotide level with DGIV, ISKNV, MCIV, and ALIV (Murwantoko *et al*., 2009).

Koi herpesvirus (KHV), which also known as *Cyprinid herpesvirus* 3 (CyHV-3), *Koi herpes-like virus*, and *Carp interstitial nephritis gill necrosis virus* (CNGV) caused significant morbidity and mortality in koi and common carp (*Cyprinuscarpio*). The case fatality rate of this disease is 80–100%.

Glycoprotein has been used for vaccine development as subunit vaccine against viruses. The aim of this research was to clone and express membrane glycoprotein of ORF124 KHV and ORF25 KHV as candidate of recombinant vaccines. Result showed that ORF124 KHV (isolate from Indonesia) had 100% similarity with CyHV-3 strain TUMST1 (from Japan), 99% similarity with KHV strain KHV-U (from USA) and KHV strain KHV-I (from Israel). Prediction analysis of T-cell and B-cell epitopes showed that ORF124 KHV protein had 14 and 11 T-cell epitopes of IAd and Rothbard/Taylor patterns, respectively, and had 10 B-cell epitopes, suggested that the protein could be used as a vaccine candidate. ORF124 gene has been expressed in *E. coli* under pET32-a(+) vector. In addition, Indonesian KHV's ORF25 isolate has high similarity with CyHV-3 DNA strains Japan, USA, and Israel by 98%. T-cell epitope prediction using GENETYX showed that ORF25 has 9 and 8 epitopes of IAd and Rothbard/Taylor patterns, respectively. B-cell epitope prediction using "B-Cell Epitope Prediction Tools" from IEDB showed ORF25 has 3 epitopes by Emini surface accessibility scale, 23 epitopes by Karplus and Schulz flexibility scale, 10 epitopes by Kolaskar and Tongaonkar antigenicity scale, 4 discontinue epitopes, and 4 linear epitopes based on Ellipro software. This result suggests that KHV's ORF25 is predicted to be immunogenic. KHV ORF25 recombinant protein has been successfully produced. Production was achieved in *E. coli* BL21cd (DE3). The protein was produced in insoluble form and optimal when IPTG induction at OD 1, with a concentration of 1 mM and incubated for 18 hours. Protein with a size of about 53 kDa was successfully purified using Ni-NTA (Murwantoko *et al*., 2012; Murwantoko *et al*., unpublished results).

CONCLUDING REMARKS

Indole was successfully transformed into indigo, one of the largest selling textile dyes. CAR dioxygenase systems from five selected isolates could be developed as indigo production expressed in *E. coli.* In regard to gene discovery for drugs development, *Streptomyces* sp. GMR22 has been sequenced. Its complete genome and some predicted genes that may greatly aid the generation of new drugs candidates were identified. Novel genes related to resistance to abiotic stress and glyphosate were found. Another strain, GMY01, produced bioactive compounds with high cytotoxicity against breast cancer cells. To cope with extreme environment, serine protease gene from halophilic microorganisms was targeted for their industrial application. Three types of serine protease genes were successfully cloned, but only serine protease gene from moderately halophilic bacterium could be well overexpressed. For vaccine development, cloning and sequence analysis of the gene encoding capsid protein *Iridovirus*, as well cloning and expression of the membrane glycoprotein of KHV's ORF124 and ORF25 as candidate of recombinant vaccines have been done.

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ENVIRONMENTAL METAGENOMIC DNA AND MICROBIAL GENOMICS INFORMATION AS SOURCES FOR NEW ENZYME GENES DISCOVERY

Is Helianti

ABSTRACT

The novel enzyme gene discovery is very essential from scientific and industrial aspects. Environmental metagenomic DNA and abundant available microbial genomes information can be considered as potential sources for mining new enzymes. Currently, microbial enzymes have contributed significantly in various applications, such as industrial application, pharmaceutical application, and others. There are two major challenges related to enzymes production to be applied in the mentioned fields. The first challenge is how to find novel enzymes with excellent properties for industries, such as high thermostability, high stability at alkaline pH, etc. Advanced approach such as metagenomics offers a shortcut method to obtain new enzyme genes directly from the extreme environment as untapped sources. However, this approach has technical obstacle to be solved such as the difficulty in obtaining a sufficient number of DNA samples from extreme environments and the need for sophisticated equipment to perform sampling and activities assay by high-throughput screening. The second one is how to produce the enzymes more efficiently by using other microbes as a host producer. The availability of microbial genomic bioinformatics can be useful abundant sources for mining new function of unknown sequences or for identifying the enzymes genes that useful for bacterial strain improvement. For instance, microbial strain producer host for extracellular enzymes can be engineered by eliminating other competitive enzyme genes to reduce the secretion burden, so that the target enzyme genes taken from the metagenomic library can be secreted more efficiently.

Keywords: enzyme gene discovery, bacterial strain improvement, metagenomics, microbial genomic information.

INTRODUCTION

The ancient people had been applying enzymes in their daily lives without knowing the concept of the enzyme for a long time. For instance, Egyptians had made the bread for thousands of years. Similarly, Japanese have known to make Japanese wine and fermented food since hundreds of years ago. All these stuffs require enzymes in the processes. Nowadays, enzymes are produced by some enzymes manufactures through fermentation and applied in our everyday life. Amylases are applied in the food, beverages, and the textiles industry. Similarly, xylanases are applied in the paper and pulp industry. Asparaginases and digestive enzymes are applied as therapeutic agents/drug.

Enzymes have also a high economic value. According to data from market research (http://www.iforp.in/2016/07/advanced-enzyme-technologies-ltd-aetl.html) the global demand of industrial enzymes was USD 3,630 million in

2012, and will be increased to USD 4,760 million in 2017, with a growth rate of 6% during the forecast period of 5 years. Of the percentage, food and beverage enzymes shared USD 1,320 million in 2012 and predicted to increase to USD 1,865 million in 2017, with a growth rate of 8%. While, the demand of specialty enzymes was USD 1,500 million in 2012, it was predicted to increase to USD 2,190 million in 2017, with a growth rate of almost 10%. It is worthy to note, these commercial enzymes are mostly derived from microorganisms.

Microorganisms are the valuable enzymes producer; they are the oldest and most diverse population of creatures on this planet and living in almost the entire biosphere. We can find 40 million microorganisms in one gram of soil, 10 millions of microbes in 1 ml of seawater. Even in the human body, microbes occupy the largest population in the gut flora or gastrointestinal tract, namely 100 trillions (Cowan *et al*., 2005). Microbes also play significant role in the process of biogeochemical cycles on this planet and bioremediation of polluted environment. In short, human being cannot live without the presence of microbes.

However, of microbes existing on earth, only an extremely small portion can be cultured and cultivated under artificial laboratory conditions, while the remaining, more than 99% cannot be cultured (Cowan *et al*., 2005). From this remarkably limited number of culturable microbes, scientist sequenced thousands of them that have interesting features, industrially important, or even pathogen for humans. This abundant digital database is currently still need to be explored and exploited. Since the vast majority of microbes have not been explored yet; the extreme environment is very potential untapped sources for novel enzymes gene discovery. In this short review, we will describe how to obtain the new genes from these sources, environment, and available microbial genomes information and how to apply the results in the process of enzymes production.

TWO MAJOR CHALLENGES IN THE PRODUCTION OF ENZYMES

There are two major challenges related to issues of enzymes production for industrial scale from scientific and industrial perspectives. First, how to obtain an enzyme with properties that suit the needs of the industry, such as high thermostability, high stability at alkaline pH, and others. This is the classic challenge for many years since scientists applied enzymes in many fields. The discovery of extremophiles since three decades ago has opened the potential of the extreme environment as sources for industrially valuable enzymes. Extremophilic enzyme is the most potential enzyme for industry. However, to isolate and culture these extremophilic microorganisms is not easy. There are some interesting species of extremophiles, such as hyperthermophilic *Pyrococcus*, methane-producing *Methanococcus*, hyperthermophilic but

obligate aerobic *Aeropyrum*, have been isolated and sequenced, and their genetic and enzymatic differences are well documented. However, extremophilic microorganisms are still the least well understood because our ability to study and understand their metabolic potential has been hampered by our inability to isolate pure cultures. In spite of the recent development of new culturing techniques, most extremophiles cannot be cultured using traditional culturing technologies. For this obstacle, we can use the advanced approach called metagenomic for obtaining new enzymes. However, if we access directly to the environmental DNA, the problem of the very low biomass densities may occur under the conditions hostile for life, which often do not yield sufficient DNA and decreases the effectiveness of cloning (Ferrer *et al*., 2007).

The second challenge is how to produce the enzymes gene that has been isolated more efficiently in the original producer or another (surrogate) microbial host. The current advancement of genomic has drastically decreased our laborious work, but requires new knowledge such as bioinformatics. The availability of microbial genomics can facilitate us to improve microbial strain as the host for the process of enzymes gene cloning as well as the precious sources for novel gene mining using bioinformatics approach. For example, this data is beneficial for us to identify the competitive enzymes gene of the surrogate producer host and eliminate it in order to reduce the enzymes secretion burden, so that the target enzyme genes taken from the metagenomic library—for instance—can be produced more efficiently.

UTILIZATION OF MICROBIAL GENOMIC INFORMATION TO FIND NEW ENZYMES AND TO IMPROVE BACTERIAL PRODUCER STRAIN FOR ENZYMES PRODUCTION

Since the first new completed sequence of *Haemophilus influenzae* determined in 1995, in 2013, there are at least 3,943 microbes whose whole genomes have been sequenced including bacteria, archaea, and fungi (compiled from various sources). This abundant data is still the large untapped sources for novel genes finding. For example, a very popular industrial bacterium *Bacillus licheniformis* that have been completely sequenced in 2004 (Rey *et al*., 2004), has 4,208 predicted protein-coding genes with an average size of 873 bp, seven rRNA operons, and 72 tRNA genes; about 20% of the genes are uniques for this strain. The analysis is suggesting that there is still much novelty yet to be identified in this fully sequenced bacterial strain and still have to be studied and explored.

Annotation is the first step required for the assignment of a putative function to a novel gene (Quinn-sanger *et al*., 2002), and it is available in big data for many users. The genes in this context can be defined as a locus containing a promoter, open reading frame (prokaryotes), introns and exons (eukaryotes), paralogs, etc. How can we obtain novel gene from these

abundant databases? Basically, there are several mining techniques including homology motif matching, signal sequence matching, gene prediction models, and query sequence matching (Quinn-sanger *et al*., 2002). Using this *in silico* or bioinformatics approach, the predicted gene will be retrieved and we can carry out further validation and determine biological by cloning and expression. Then we can apply the result in "knocking out or knocking in" the gene in chromosomal DNA of bacterial producer host for bacterial for strain improvement useful in industries.

A whole transcriptome RNA-Seq analyses has been conducted to study the gene expression at five selected growth stages of an industrial-oriented protease production process employing a germination deficient derivative of *B. licheniformis* DSM13. The study identified genes involved in the adaptations to changing environmental conditions during the fermentation process, especially genes contributing to central carbon metabolism, amino acid transport and metabolism, starvation and stress responses, and protein secretion (Wiegand *et al*., 2013). Identification of a novel two-peptide lantibiotic, lichenicidin based on rational genome mining for LanM proteins has been done, and in this study *B.licheniformis* was used as a model (Begley *et al*., 2009). Novel nitrilase have also been identified from microbial genomic information using bioinformatics and experimental methods (Seffernick *et al*., 2009). The utilization of genomic information have also been conducted to improve the properties of bacterial strain *B. licheniformis* F11 to improve the secretion of protease for efficient deproteinization of shrimp shells and production of high-molecular-mass chitin and chitosan (Hoffmann *et al*., 2010). Based on this improved strain and *B. licheniformis* genomic information, currently, we are also developing the mutant bacterial strain that can produce xylanase more efficiently (not published yet).

CULTIVATION VERSUS THE METAGENOMICS METHOD

Before metagenomic era, cultivation methods followed by directed evolution and rational protein design using computational tools is already established and utilized to improve enzymes. These developments until now have helped application of microbial enzymes in industry. However, metagenomics opened a way to find hitherto unknown protein from environmental sampling. Without isolation of the microbes or cultivation of the microbes with desirable traits, the genes could be obtained and directly engineered by the same protein engineering process. Compared to conventional culture-dependent method, metagenomic promises faster way and the discovery the hitherto unknown enzymes gene from unculturable microorganisms. In this discovered new enzymes production process, of course, cultivation is still needed. The difference here is that cultivation refers to that of a surrogate organism—the microbial host exploited for archiving and expressing the harvested genes obtained as mentioned above.

Metagenomics is basically similar to the conventional genome library construction and screening, with the difference that the 'genome' cloned is not from a single well characterized laboratory clone, but came from the entire microbial community present in an environmental sample (Ferrer *et al*., 2009). Principally, there are two major strategies conducted in metagenomics approach, namely sequence homology-based and activity-based strategy.

Sequence homology-based strategy or PCR-based approach is simpler procedure. It is based on the identification, by sequence comparisons of members of the enzyme class of interest, of highly conserved functional motifs for the design of primers for PCR amplification in, and gene probing of DNA libraries (Ferrer *et al*., 2009). We have cloned and expressed functional xylanase gene directly from moderate hot spring using this PCR approach (Helianti, 2007). In other reports, this strategy led to the identification of new enzymes from a variety of environments, such as lactamases, esterase, and carboxypeptidases (Fang *et al*., 2014; Ferrer *et al*., 2007). However, this strategy has the major limitation; it depends on known protein structures and thus cannot enable discovery of new protein classes—either with the same catalytic activity or with different catalytic activities for the offered substrate—with sequence elements that differ from the conserved sequences of the primers (Ferrer *et al*., 2009).

The second strategy, activity-based screening of metagenome libraries, though often more laborious than PCR-based procedures is not dependent on previous knowledge and can yield fundamentally new knowledge or enzymes. The examples of this approach are the finding of new esterase and xylanase (Hayashi *et al*., 2005; Park *et al*., 2007). However, the problem of the very low biomass densities often occurs under the conditions hostile for life that caused low yields DNA and reduces the effectiveness of cloning. We found that, the more hostile environment, the more difficult to extract DNA (Helianti and Nurhayati, 2007).

CONCLUDING REMARKS

This short review described the importance of environmental metagenomic DNA and abundant available microbial genomes information as sources for mining new enzymes. Combining metagenomic approach and available microbial genomic information enabled us to produce industrially novel enzymes efficiently. Metagenomic approach enabled us to obtain a new class enzymes or completely new enzymes. Furthermore, utilization of microbial genomes information supported us to perform bacterial strain improvement and use it as a host for enzymes production.

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GENE DISCOVERY IN MICROBES FOR RENEWABLE ENERGY DEVELOPMENT

Christopher Marquis

ABSTRACT

Concern over future supply of conventional energy resources and the effects of rising atmospheric carbon dioxide levels arising from fossil fuel consumption have led to a rapid increase in research and development in renewable energy. Amongst the suite of renewable energy options, bioenergy has emerged as an important contributor to, in particular, providing new options for transportation fuels. Microorganisms play an important role as producers of biofuels through fermentation, generators of enzymes for key bioconversions and as providers of key synthetic substrates. With new tools available to interrogate cellular genetics as well as cellular control mechanisms, there is enhanced potential to improve the robustness and productivity of these biocatalysts. This paper summarizes some important recent developments in the understanding of functional genetics of key microbial and enzyme biocatalysts for the production of biofuels for the future.

Keywords: microorganism, biofuels, biocatalysts, metabolic engineering.

INTRODUCTION

Total world energy consumption in 2014 is estimated to be over 600 EJ and approximately 40% of this energy is met by oil (BP Statistical Review). Transportation uses represent approximately 62% of this oil consumption. Since 2000, the most significant growth in global energy consumption has been largely in non-OECD countries and, following the global financial crisis in 2008, total energy use in the non-OECD countries has exceeded that in the OECD. Projected growth suggests global demand for liquid fuels will reach 115 million barrels (oil equivalent) per day by 2040 with projected prices ranging between USD75 and USD237 (2011) per barrel (US Energy Administration, 2013). This projected growth coupled with concerns over the future availability of low cost oil reserves and the concerns over net carbon emissions from fossil fuel use have led to the search for alternative energy sources.

Renewable energy describes a suite of potential solutions to assist in meeting the world's future energy needs and at the same time reducing the reliance on fossil fuel resources (IEA, 2014). The solutions that fall under this umbrella are at various stages of maturity with respect to their development and implementation. Renewable energy options for power generation are becoming well established with solar and wind energy demonstrating the fastest rates of growth over the last decade as well as biomass in some regions.

Bioenergy may be defined as the utilization of bioresources for the production of useful energy for power, heating, and fuels for vehicle

transportation. The bioresources that are involved in bioenergy production include plant and algal resources that can provide substrates for energy generation when utilized by appropriate microorganisms that facilitate bioconversion of these substrates. Therefore, microorganisms have an important role to play in this new energy future. Traditionally, this has been by way of fermentation, which is a relatively simple bioconversion of sugars to produce combustible solvents such as ethanol. Other roles include the production of various hydrolytic enzymes that may assist in conversion of non-fermentable and recalcitrant feedstocks into fermentable products and the generation of new enzymes for biodiesel production. Furthermore, with developments in our understanding of cell metabolism and regulation through the "omics" sciences (genomics, metabolomics, fluxomics, and proteomics), there is much greater capacity to manipulate microorganisms in a targeted way to, for example, improve microorganism performance.

THE CASE FOR AND AGAINST INVESTMENT IN RENEWABLE ENERGY

Many arguments for and against the development of renewable energy options are made, from both scientific and socio-political perspectives. The arguments for the development of renewable fuel energy options include the; lowering of dependence on fossil fuels, reduced noxious emissions, improved energy security, increased local employment, and partial remittance of net carbon released when compared with fossil fuels alone. Arguments against the use of renewable energy options include the high relative cost of biofuel production, use of agricultural resources for energy over food and fuel, environmental issues, such as deforestation, associated with expanding agriculture, and concerns over performance when used in petroleum/diesel blends.

Since 2000, there has been a rapid growth in the production of biofuels across the globe, expanding from 16 billion litres per annum (p.a.) in 2000 to 115 billion litres p.a. in 2013. In 2013, biofuels accounted for 3.5% of all transportation fuels globally and global biofuels output is expected to be 135 billion litres by 2019 (Platts, 2014). In terms of biofuel options for transport fuels

Table 1. 2013 Data on the use of renewable energy resources globally.

Source or type of energy	Amount	Growth in 2013 (%)	Notes
Wind	628 TWh	20.7	5.3% world power generation
Solar	124.8 TWh	33.0	
Other (geothermal, biomass, waste)	481 TWh	7.7	
Biofuels (ethanol and biodiesel)	65.3 Mtoe	6.1	2.8% world liquid fuels
Total Renewables (excl. hydro)	279 Mtoe	16.3	
Hydro	856 Mtoe	2.9	

Source: BP Statistical Review of World Energy (2014).

(some listed in Table 2) a key factor in the techno-economic feasibility of future growth of these industries is improvements in the microorganisms responsible for fermentation, the development of new enzymes and, for biodiesel production, realizing the potential of microalgal-based oil substrates.

GENETIC TOOLS FOR GENERATION OF IMPROVED BIOCATALYSTS

A range of tools is available to deepen our understanding of microbial processes and create modifications to microorganisms that improve performance for a particular application. Starting from wild-type, modified strains can be created by cycles of mutation followed by screening, cross-breeding of haploid strains with desired features for cells with a sexual cycle (e.g. yeasts), genetic modification via plasmid transfection or genome editing using a range of tools to knock in/out particular genes. With relevance to renewable energy, this might include strain development for improved fermentation performance through to the generation of new enzymes (hydrolyases, lipases) through directed evolution strategies (Turner, 2009), that improve reaction rates and recyclability, or microalgae development to enhance specific oil content as well as growth capacity in bioreactor systems.

Table 2. Properties of liquid biofuels when compared to standard petroleum and diesel.

Fuel	Density ($kg.m^{-3}$)	Energy density ($MJ.l^{-1}$)	Octane[5]/ Cetane No.	Dynamic viscosity (cP)[3]	Examples of bioproduction methods
Ethanol	789[1]	21.2	109/-	1.2	Fermentation of molasses, starch hydrolysates, and hydrolyzed lignocellulosics
Biodiesel	860–890[2]	26.7	-/>51	4.0–5.7[4]	Chemical or enzymic transesterification of oils and fats
n-Butanol	810[2]	29.2	94–96/-	2.9[3]	Anaerobic fermentation on molasses and starch hydrolysates (ABE process)
Methanol	795[3]	17.9	109/-	0.6	Hydrolysis of biomethane derived from anaerobic waste treatment or Syngas
Green aviation fuel	~800	33.5	100/130	-	Fractionated hydrogenated oils from plants and algae. Production of Fischer-Tropsch paraffins via Syngas
Dimethyl ether	668[3]	19	-/60	-	Chemical synthesis from Syngas derived from biomass gasification
Standard gasoline	715–765	34.8	>91/8	0.55–0.9	Not applicable
Standard diesel	800–840	38.6	-/>45	3.0–4.8	Not applicable

[1]At 25 ºC, [2]At 15 ºC, [3]At 20 ºC, [4]At 40 ºC, [5]RON.

This review will now focus on recent and future developments for microorganisms in established biofuel production (bioethanol and biodiesel) as well as developments for some of the potential future bioenergy products, such as butanol, and the potential for generating entirely new biocatalysts for biofuel production.

Biobutanol

Butanol has been produced industrially by fermentation for nearly 100 years. The clostridial acetone-butanol-ethanol (ABE) fermentation process was originally developed by Weizman and industrial development was driven initially by a shortage of acetone in World War I for explosives manufacture. The most common species used is the Gram-positive anaerobic *Clostridium acetobutylicium*. The solvent production is most strongly associated with the onset of sporulation and typically the relative molar yields of the solvents are 3 : 6 : 1 (acetone : butanol : ethanol). The metabolism of glucose to these products is depicted in Figure 1.

The recent renewed interest in butanol as a renewable transport fuel component has reignited research endeavors in strain and bioprocess development as well as industrial interest (for example, Butamax, a joint venture between BP and DuPont). In another example, Gevo operates a 70 million litres p.a. plant in Luverne, Minnesota, utilizing corn starch (McMillan *et al*., 2014). Butanol presents some advantages over ethanol as a biofuel to blend with

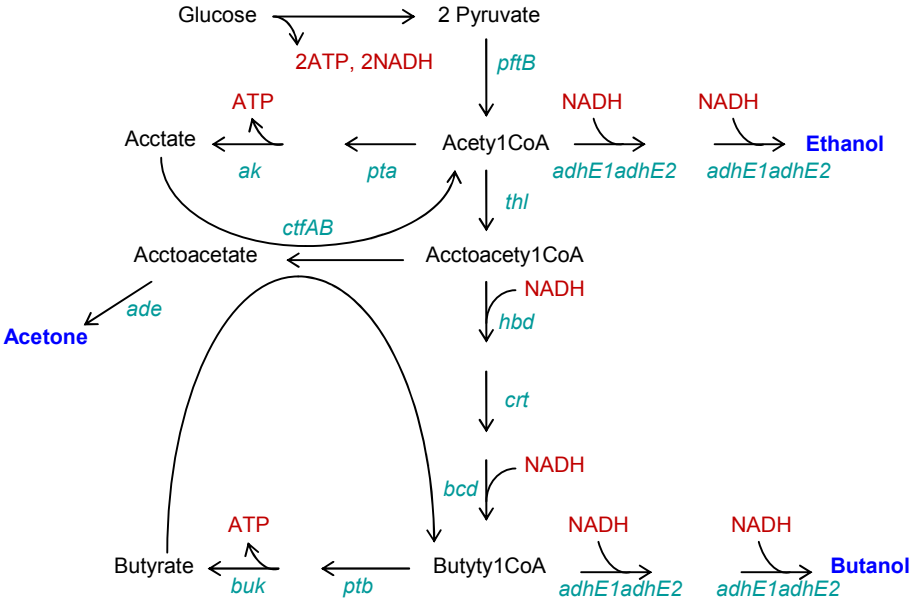

Figure 1. Metabolism of glucose to acetone, butanol, and ethanol (ABE).

petroleum. Firstly, it can be used as a “drop-in” fuel at higher blending ratios of up to 16% v/v. It has a significantly higher energy density compared to ethanol, is less volatile and is not hygroscopic. In terms of production, there is capacity to make use of existing bioethanol plant infrastructure and, with some retrofitting, a bioethanol plant can be used as a biobutanol manufacturing facility (McMillan *et al*., 2014; Schiel-Bengelsdorf *et al*., 2013). Furthermore, the same first and second-generation feedstocks used for ethanol fermentation can be utilized.

In order for bioderived butanol to be economically feasible as a fuel product, significant advances in cell line development need to be achieved. Targets for improvement include elevating butanol yields through altering carbon flux to favor butanol production over other solvents, improved stress tolerance to acid intermediates (acetate and butyrate) and solvents (most importantly butanol), decoupling of the onset of sporulation with solventogenesis (Xue *et al*., 2013; Zheng *et al*., 2009). Another issue to be overcome is the high requirement for reducing equivalents (NADH) when using less reduced carbon substrates, such as glucose, as the fermentation carbon source. Chemical mutagenesis followed by selection has been reported for achieving some of these goals. Some examples of targeted efforts (gene-specific) to modify *C. acetobutylicium* performance are summarized in Table 3.

In addition to developments in production cell lines, extractive fermentation processes to reduce solvent stress have been investigated to overcome the toxicity of the solvents produced. For industrial feasibility, process development will have to work hand-in-hand with strategies to improve cell line performance. In addition, the capacity for production strains to utilize carbon derived from second-generation lignocellulosic feedstocks will further improve the feasibility of biobutanol as a transport fuel.

Table 3. Some genetic targets in *C. acetobutyliceum* of interest for improved butanol production.

Target Gene	Gene function in relation to butanol production	Outcome
buk, *pta*	Carbon flux to butyrate, acetate	Inactivation/repression improves butanol production
adhE1	Carbon flux to butanol	Improved butanol production
solR	Inactivation of this gene (transcriptional repressor)	Elevated acetone, butanol levels
spoOA~P	Transcriptional regulator. Gene product downregulates *ptb* and upregulates *adc*	Overexpression improved butanol production
adc,ctfAB	Acetone formation genes	Overexpression elevated acetone and butanol production
Cellulosome complex	Aim to engineer functional cellulase capability in host	Functional cellulase activity shown
groES, *dnaK*	Aim to improve solvent tolerance	Improved tolerance to butanol
hup CBA	Downregulation of hydrogen uptake hydrogenase genes to lower demand on NADH	Improved butanol and acetone ratio

Sources: Jang *et al*. (2012a); Schiel-Bengelsdorf *et al*. (2013); Xue *et al*. (2013); Zheng *et al*. (2009).

Biodiesel

Biodiesel describes a heterogenous range of products derived from the transesterification of plant and animal triglycerides by reaction with a simple alcohol, usually methanol. The most commonly reported processes are catalyzed using caustic compounds (in the form of sodium methoxide). However, this process is not without problems, which include soap formation with poorer quality oils (high free fatty acid content), production of a less valuable glycerol by product and toxicity and explosion hazards handling sodium methoxide. Another problem is the source of the oil feedstock, which is largely food oils (soybean, canola, palm, and coconut oil, for example). Some biodiesel plants can access waste feedstocks, such as used cooking oils, *non comestible* seed oils and tallow. Biodiesel production capacity in the United States reached 2.9 billion gallons p.a. (195 plants) with actual production at 1.8 billion gallons in 2013 (McMillan *et al*., 2014) and Europe has seen substantial growth in biodiesel production over the last decade.

An alternative approach to catalyzing the transesterification reaction utilizes lipases instead of sodium hydroxide/sodium methoxide. Lipase catalysis produces a cleaner glycerol by-product and eliminates soap formation that can arise from caustic reactions using oils high in free fatty acids (Cesarini *et al*., 2014). Lipases for this purpose have been sourced from a number of bacterial and fungal species, including *Pseudozyma antarctica, Rhizopusoryzae, Mucormeihei*, and *Thermomyces lanuginosus*. Nozozymes has an immobilised *P. antarctica* lipase product (Novozym 435) that has been shown to successfully generate FAEE biodiesel in a straight batch reaction (Tupufia *et al*., 2013) and FAME using various methanol fed-batch protocols (for example; Price *et al*., 2014). The same company also markets a newer product, Callera Trans L (using a lipase from *T. lanuginosus*), that has been employed on an industrial scale by, for example Blue Sun Biodiesel (Missouri, USA) to produce 120 million litres p.a. of biodiesel (McMillan *et al*., 2014). Developments in enzymes for this purpose have included improved reaction rates at modest temperatures, resistance to inactivation by phospholipids and methanol, capacity to immobilize and extended activity to allow enzyme reuse. Cross-linked whole cell biocatalysts (a strategy to lower catalyst cost) have been shown to effectively produce biodiesel over repeated batch cycles (Sun, 2011).

Microalgae are being explored by the research community and industry to supply oils for biodiesel manufacture, to overcome the inherent problems of accessing “first-generation” food oils to make fuels. A range of microalgal species have been evaluated for this purpose, in particular marine or salt tolerant species, which can be cultivated in marginal or nonagricultural areas. The justification for using microalgal biooils includes higher specific oil productivity ($g/m^2/day$) and removing market competition for food-based oils. Genera that have been widely described include *Chlamydomonas*,

Nannochloropsis, and *Chlorella*. In terms of genetic modification to improve production strains, researchers have targeted improvements to photosynthetic efficiency and modification of triacylglycerol (TAG) profiles to generate biodiesels with optimum fuel parameters, such as viscosity and low gelling temperatures (Yu *et al*., 2011). A recent review (Misra *et al*., 2013) identified a range of genetic targets for improved bio-oil production in microalgae, through a thorough review of available genomic (and other "omic") datasets including a variety of desaturase, elongase, and acyltransferase genes. Yu and colleagues described targets for bio-oil accumulation based on success in oil seed plants (Yu *et al*., 2011) including acetyl CoA carboxylase (regulates fatty acid synthesis), elevating glycerol-3-phosphate levels by overexpression of glycerol-3-phosphate dehydrogenase (*gpd1*), downstream targets in fatty acid synthesis pathways and modifying metabolic flux to alter fatty acid composition such as downregulating fatty acid desaturases.

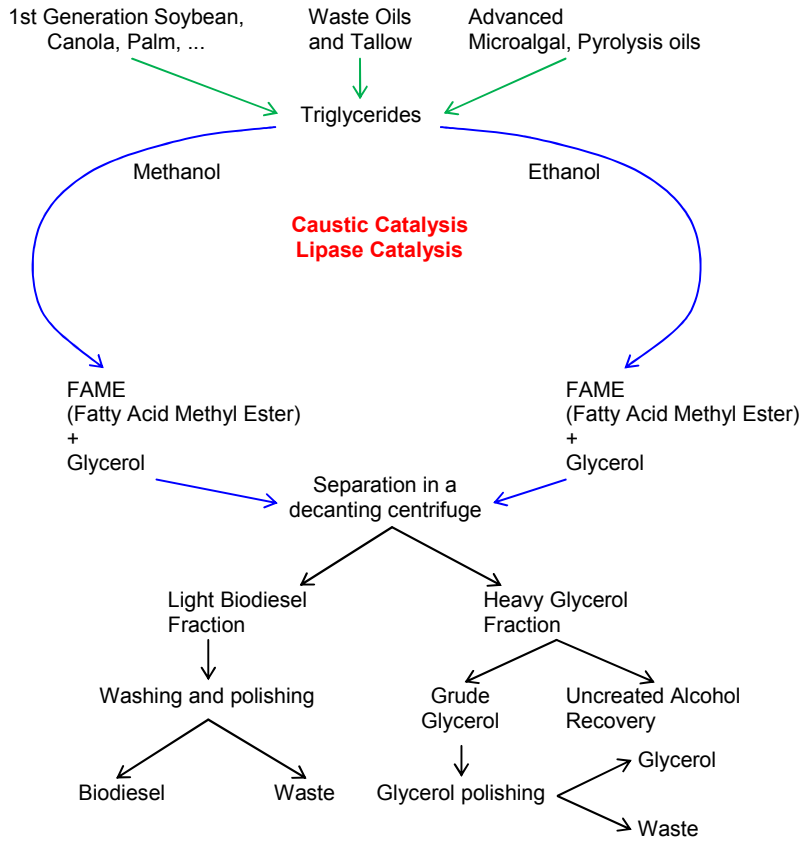

Figure 2. Biodiesel production.

Bioethanol

The United States is the largest global producer of biofuels and indeed bioethanol. However, production in the US has plateaued as usage has reached close to 10% of total transport fuels across the country, which equates to the upper limit of current fuel specifications ("blend wall"), and due to limitations in production and distribution infrastructure (Figure 3).

Brazil is also a major producer of bioethanol, having embarked on a national initiative to develop a bioethanol industry in the 1970s. For both these major producers, the feedstock for production are so-called "first-generation" resources, whereby the bioresource providing the fermentable substrate has an alternate use as a food source, with consequent effects on substrate price.

Fermentation of first-generation resources (simple sugars derived from sugarcane and sugar beet) and hydrolyzed starches (corn, wheat, and cassava) is largely undertaken by strains *of Saccharomyces cerevisiae*. Desirable features of strains used to make industrial ethanol from these first-generation resources include ethanol tolerance, high growth rates, controllable flocculation (Bauer *et al*., 2010), lower by-product (e.g. glycerol and acetate) formation and thermotolerance (Jang *et al*., 2012b; Nielsen *et al*., 2013) to allow application in simultaneous saccharification and fermentation (SSF) processes. This yeast (*S. cerevisiae*) possesses no amylotic enzymes, therefore starchy substrates need to be hydrolyzed using amylase/glucoamylase (EC 3.2.1.1) enzymes. These

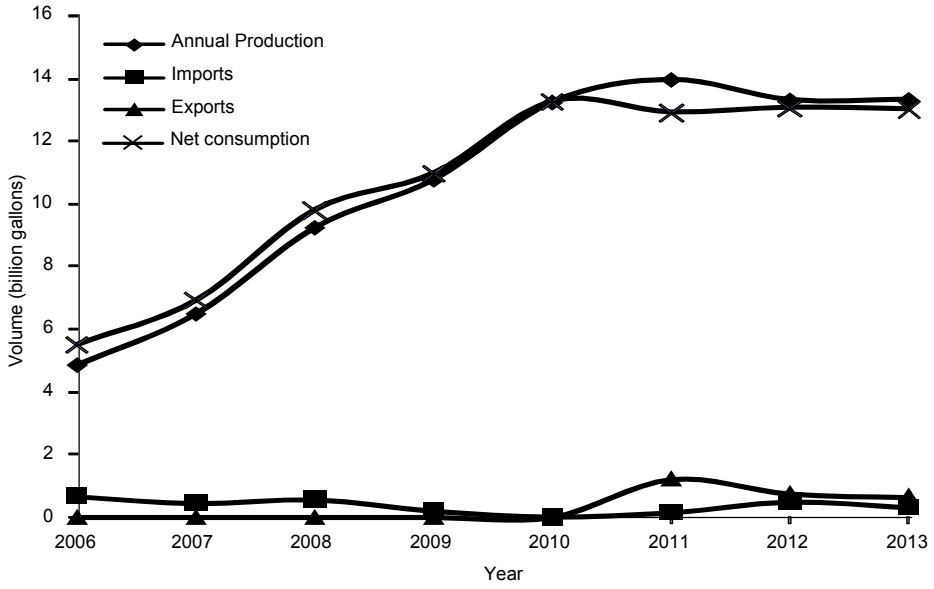

Figure 3. USA ethanol production and consumption statistics from 2006–2013 (IEA, 2013).
Source: IEA (2013).

enzymes are produced in native and recombinant strains of various fungi and bacillus and are applied either sequentially (in a separate reactor prior to fermentation) or simultaneously SSF processes (Li *et al*., 2014). Through processes of directed evolution and other techniques, desirable properties, such as stability and activity at high temperatures, have been achieved, more recently, for example, by screening enzymes from hyperthermophilic Archea.

Fermentation of second-generation resources (woody biomass including waste from forestry operations, agricultural residues and energy crops such as switchgrass) provides additional challenges. The issues include cost effective methods to hydrolyze cellulose to glucose, how to handle the substantial notionally “nonfermentable fraction” released from hemicellulose hydrolysis, the problem of the toxicity of furfurals and polyphenols generated and liberated through hydrolysis and the economic use of the lignin fraction. However, since 2012, lignocellulosic fermentation has realized its industrial potential, with the commissioning of the Beta Renewables Facility in Italy, which has the capacity to process 270,000 tons of lignocellulosic biomass (rice straw, wheat straw, and *Arundo donax*) to produce ethanol. In 2013–2014, a number of second-generation facilities were commissioned in the United States, including an 80 million litres p.a. plant in Emmetsburg Iowa (a joint venture between DSM and Poet LLC) using corn stover, the Abengoa plant in Hugoton Texas, utilizing agricultural and wood wastes and corn wastes, producing 100 million litres p.a. and 22 MW of electrical energy. Key to the success of second-generation processes is the development of low energy, low cost processes for lignocellulose hydrolysis (principally through enzyme development) and development of strains to effectively utilize the hexose and pentose sugar products in the resulting hydrolysates. In terms of enzyme development, many of the advances have been achieved in industry and for second-generation feedstocks, this has focused on; improved cellulases (Medie *et al*., 2012), development of microorganisms with enhanced cellulolytic ability (la Grange *et al*., 2010) and improved tolerance to toxins generated during lignocellulose processing (Liu, 2011).

A significant by-product of the ethanol process is production of yeast, which has value as a source of single-cell protein (SCP), to supplement livestock feeds. In most jurisdictions, the use of genetically modified (GM) yeasts would prevent this occurring. An Australian company, Microbiogen (Microbiogen, 2014), has developed an ethanologenic strain of *S. cerevisiae* over a 10 year breeding program that can utilize xylose for biomass growth, resulting in potential applications in second-generation bioethanol processes using lignocellulosic hydrolysates. Furthermore, this yeast biomass is not genetically modified and therefore can be processed to produce valuable protein by-products without regulatory issues.

FUTURE DEVELOPMENTS AND THE ROLE OF CELL ENGINEERING FOR RENEWABLE ENERGY

There remains ongoing opportunities for the use of biotechnological tools to contribute to improving bioprocesses to contribute to energy production, including combined thermochemical/biochemical treatment of biomass to yield Syngas and ethanol, the use of synthetic biology and recombinant DNA technology to create new microbial substrates for example, improving the performance of standard hosts and the development of engineered *E. coli* and Cyanobacteria, improving enzymes through bioprospecting, mutation, and evolutionary techniques. In some recent reviews, the capacity of these modern advanced tools to progress biocatalyst generation are described including high throughput robotic systems to evaluate cDNA libraries of relevant genes (Hughes *et al*., 2011) and systems biology approaches to cell line engineering (Jang *et al*., 2012b; Kim *et al*., 2013; Lee *et al*., 2013; Sakuragi *et al*., 2011; Singh *et al*., 2011). To conclude this paper, some of the areas related to improved microorganisms will be briefly reviewed.

ENGINEERING OF BACTERIA AND YEASTS FOR IMPROVED BIOFUELS PRODUCTION

There are numerous studies on the engineering of bacteria, such as *E. coli*, *Z. mobilis*, and *P. putida*, and yeasts (Neilsen *et al*., 2013) for applications in ethanol production. Compelling reasons to choose alternate hosts include the availability of a much wider range of genetic tools for modification, a deeper understanding of their respective genomes and intrinsic features such as solvent tolerance (for example in pseudomonads such as *P. putida*). By way of example, metabolic engineering of *E. coli* (adding six biosynthetic genes) resulted in an *E. coli* strain that produced modest levels of butanol. Further modification, with genes added to drive carbon flux to acetyl CoA and deleting genes associated with mixed acid fermentation to develop a NADH driving force, resulted in reported butanol yields of 15 g/l (Jang *et al*., 2012a).

ENGINEERING OF CYANOBACTERIA FOR BIOFUELS PRODUCTION

Cyanobacteria have proved compelling targets for genetic modification for biofuel generation because of their high photosynthetic rates—and consequent growth rates—in simple media and their ability to fix CO_2—and in some cases nitrogen—and potentially convert this directly to ethanol (photofermentative ethanol production; Angermayer *et al*., 2009), butanol, or bio-oils, obviating the cost of utilizing expensive sugar substrates. Furthermore, there are tools available to genetically modify a number of species (for example, *Synechocystis sp*. PCC 6803) as well as the availability of light-driven promoters, such as *psb*AIII (Qintana *et al*., 2011), opening up the prospect for creating strains that

can produce biofuels. For ethanol production, strains have been created that carry *pyruvate decarboxylase* (*pdc*) and *alcohol dehydrogenase II* (*adh*) genes with evidence of modest ethanol production. A strategy to generate strains that can produce isobutanol have included the addition of three genes, enzymes to generate 2-ketoisovalerate, ketoacid decarboxylase, and alcohol dehydrogenase (*YqhD*) from *E. coli*. Strategies to generate other energy carriers, such as hydrogen, biofuels, and biofuel precursors, have also been recently described (Ducat, 2011). A comprehensive review of cyanobacterial photosynthesis and carbon metabolism by Qinatana and colleagues describes a range of potential gene targets for developing *Cyanobacteria* for biofuel applications (Qintana *et al*., 2011). As with biofuel production in *E. coli* toxicity of the generated solvent, at relatively modest levels, remains a problem to be tackled, along with low levels of production.

CONCLUDING REMARKS

In the last 20 years, concerns over climate change, increased global demand for energy coupled with diminishing cheap oil resources, has driven a great deal of research and development in renewable energy, including biofuels. This has coincided with rapid developments in the “omics” sciences, permitting a much greater depth in the understanding of cellular function as well as the capacity to undertake more complex metabolic engineering tasks. From a genetics perspective this research has focused on both the sources of fermentable substrate (Arruda, 2012; Ko *et al*., 2011; Mizrachi *et al*., 2012; Sonneweld and Kossman, 2013) and as discussed here, the microbiology of biofuels production. Along with capacity to rapidly evolve and improve enzyme performance, there will be great advances in our ability to economically produce truly renewable transport fuels through the development of modified cellular and protein biocatalysts.

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STRATEGY ON PROMOTING MICROBIAL GROWTH OF METHANOTHROPHIC BACTERIA TO ENHANCE PADDY PRODUCTIVITY WHILE REDUCING METHANE EMISSION FOR CLIMATE ADAPTATION

I Made Sudiana[1]*, Dwi N. Susilowati[2], Shigeto Otsuka[3], Senlie Octaviana[1], Maman Rahmansyah[1], Arwan Sugiharto[1], and Atit Kanti[1]

ABSTRACT

Food security of Indonesia is problematic due for four reasons: land shortage, lacking of irrigation infrastructure, climate anomaly, and fertilizer and pesticide availability. Soil microorganism in general could offer an alternative solution for increasing crop productivity as well as maintaining ecosystem services. We particularly interested in methane oxidizing community in soil rhizosphere of rice which responsible for methane oxidation and their unique physiological properties, such as nitrogen fixation and trichloroethylene degradation. The challenge of this approach is how to stimulate the growth and activities of methanothrophic bacteria since obligate methanothroph are mostly slow growing Proteobacteria. The new concept is exploring facultative methanothrop bacteria that are able to use other carbon sources than methane that are widely available in soil. We isolated methanothroph bacteria from paddy root which are able to oxidize methane and use several carbon sources include degrading tricholoroethylene. These bacteria also harbor particulate methane monooxygenase and methanol dehydrogenase. Most of the isolated methanothrop are aerobic bacteria, therefore aerobic rice cultivation system will beneficial for oxidizing methane, but under aerobic condition, soluble phosphate could be limiting factor for P-availability, therefore augmentation of phosphate solubilizing microorganism would be necessary. Regional initiative of aerobic rice cultivation is already adopted in Asian countries. Further research on plant nutrient behavior under aerobic-anaerobic regime and microorganisms involve are important issue on optimizing sustainable rice cultivation for climate adaptation.

Keywords: methanothoph, phosphate solubilizing microorganism, paddy soil.

INTRODUCTION

Rice is important staple for most Asian, and Indonesia is biggest rice importer. On the other hand, flooded rice cultivation system is listed as one of important contributor of methane emission. Several technologies of rice cultivation with less methane emission is being explored (Liesack *et al*., 2000). Methane from rice field is mostly produced by methanogen (Chin *et al*.,1999). There are two important groups of methane producing bacteria from rice field: acetoclastic group which produce methane through fermentation of soluble organic substrate produce mainly acetic acids and further converted into methane (Bourne *et al*., 2001). The other group (hydrogenotrophic bacteria) produce methane through reduction of CO_2 and H_2 produce methane (Singh and Dubey, 2012). Both groups of bacteria produce methane under low redox

potential value (-200mV). Since 1970s, research on methane consuming bacteria verifying obligate methane oxidizing bacteria are important microbes which are able to utilize methane and other C1 as sole carbon sources (Wartiainen *et al*., 2006). In the case of methane emission reduction, there are three group of methanotroph involved. The use of enzymes known as methane monooxygenases to catalyze the oxidation of methane to methanol is a defining characteristic of methanotrophs. Type I methanotrophs, mostly belonged to gamma subclass of Proteobacteria, are indicated by soluble methane monooxygenase (sMMO) enzymes, type II having particulate methane monooxygenase (pMMO) in their cell wall, and type X have both sMMO and pMMO. Ability of methanotrophic bacteria to oxidize methane and form methanol are important biological process for reducing methane emission. Other important process that transform methanol into formaldehyde is methanol dehydrogenease encoded by methanol dehydrogenease enzyme. Our research presents reduction of methane emission from rice field, performing either SRI type or conventional paddy cultivation practices, and molecular analyses on the gene involved in the methane transformation.

Recently, Modin *et al*. (2010) recognize the presence of facultative methane oxidizing bacteria belong to Spingopyxis group. This group of bacteria does not only oxidize methane, but also degrade trichloroethylene and oxidize dimethyl sulfide. This shows the important of facultative oxidizing bacteria in bioremediation processes. This particularly important when intensive rice cultivation system greatly depends on pesticide and much nutrient input.

Most of methanothropic bacteria are aerobic microbes, indeed some microbes using NO_3 or SO_4 as terminal electron acceptor are reported (Ussler and Paull, 2008). Therefore, the aerobic soil condition will stimulate methane oxidation (Chin *et al*., 1999). This key concept is important for rice cultivation system with less methane emission.

CURRENT STATUS OF RICE CULTIVATION SYSTEM

Currently, there are several rice cultivation systems in Indonesia, namely water submerged irrigation cultivation system, intermittent irrigation, and upland rice. Traditionally submerge irrigation system are mostly practiced by farmer (Zou *et al*., 2005). This irrigation system has two benefit: reducing weed growth and keeping the soil under slightly anaerobic condition, with the redox value of around +50 mV in soil surface and about -50mV in soil about 10cm under soil surface (McDonald *et al*., 1995; Song *et al*., 2008). Besides that, submerged rice cultivation system has a mechanism to preserve fertility and sustainability of natural production. Erosion of the topsoil in paddy fields can be minimized because the flow of water retained and deposited by inundation due to dike or retained by the rice plant (Sumarno and Suyamto, 2009).

Upland rice cultivation system is classified into dryland that is a stretch that has never flooded or inundated with water on most of the time in a year. Although the dryland in Indonesia is quite extensive, with estimates of about 60.7 million hectares or 88.6% of the land area, while the area of paddy fields only 7.8 million hectares or 11.4% of the land area. Most of the dryland in Indonesia spread in lowland <700 m above sea level (60.65%) and plateau 700–2500 m.a.s.l. (39.35%) of the total area of dryland in Indonesia. Dryland farmers usually rely on rainfall to irrigate their land, because for this purpose requires almost no cost. In months, there was no rain or drought, agricultural land is often left fallow. Until now, the contribution upland rice production reached only 5–6% (ICFORD, 2008).

MAIN CHALLENGES

Our studies clearly show aerobic cultivation system (Figure 1B) emitted 80% less methane then flooded irrigation system (Figure 1A), but weed growth was faster in aerobic cultivation system. With a redox potential value of -50 mV and pH 6.2, under this condition most of the phosphate are in the form of $H_2PO_4^-$ and $HPO_4^=$ and thus available for rice growth (Figure 2). Other trace elements, such as ZN, Cu, and Fe, are also available at reduced environment (Chen *et al*., 2006; Ivezić *et al*., 2012; Liu *et al*., 2013). The dynamic of macro- and micronutrient in environment is illustrated in Figure 2.

The availability of macroelement, such as P and N, and microelement are greatly affected by pH and redox potential (Stumm and Morgan, 1996). The dynamic of such nutrient could be also affected by soil salinity (Sardinha *et al*., 2003; Zaini *et al*., 2009). Manipulating the dynamic and profile of plant nutrient is key important strategy to enhance plant productivity as well as maintaining their ecosystem service. Soil microorganisms have been a focus of long-term study to enable scientist to evaluate their contribution on promoting plant growth (Compant *et al*., 2010), and one important plant nutrient is phosphate (Hameeda *et al*., 2008).

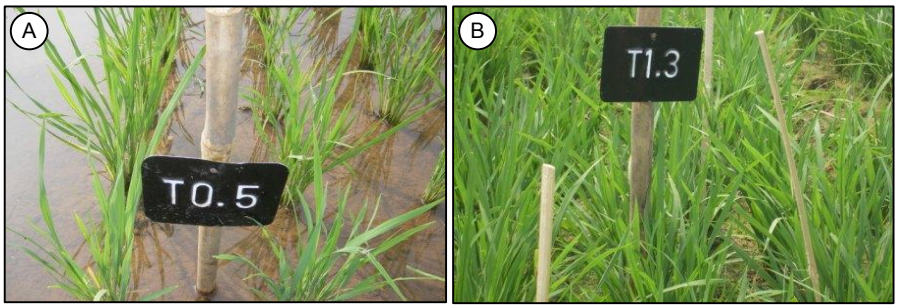

Figure 1. The phenological characters of paddy IR64. A = flooded rice cultivation system, B = intermittent irrigation system/aerobic cultivation system.

UPTAKE AND TRANSPORT

Phosphorus is one of seventy nutrients essential for plant growth. Its functions cannot be replaced by any other nutrient and an adequate supply of P is needed for optimum growth and reproduction. The total P concentration in

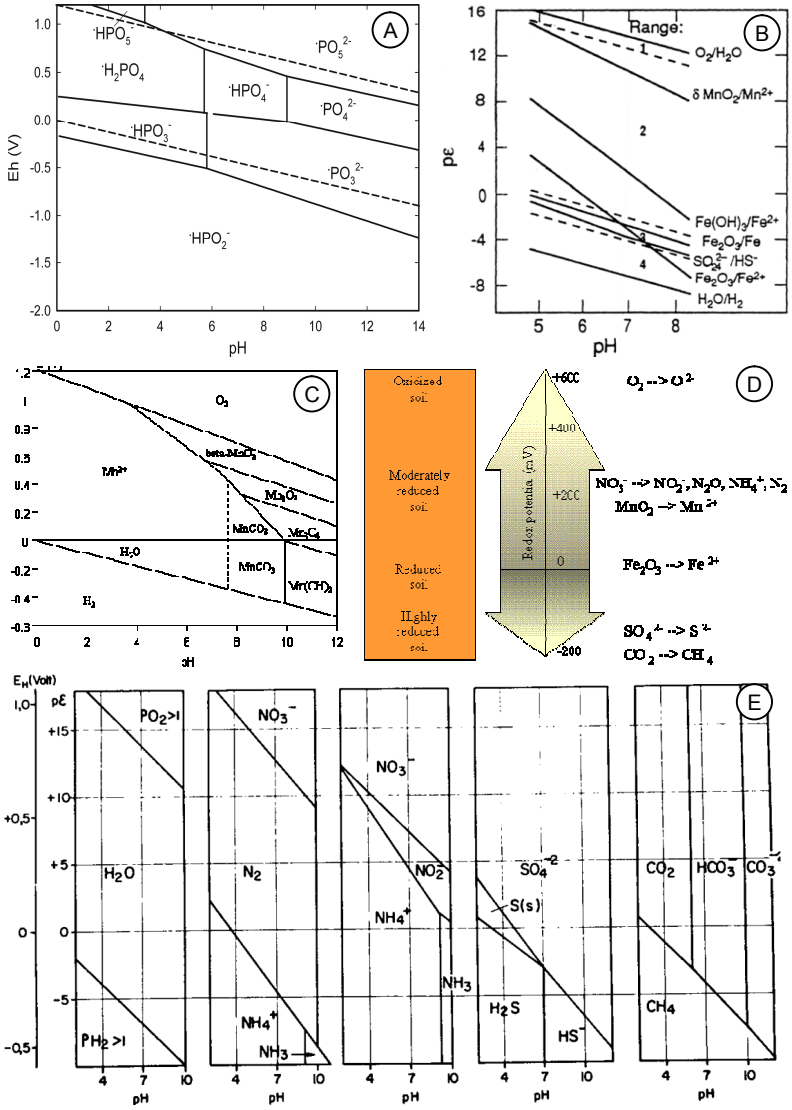

Figure 2. Dynamic of macronutrient (phosphorus) and micronutrient as affected by redox potential and pH. A = profile of phosphate species, B and C = profile of manganese and iron (Fe), D = redox potential profile in water column, E = profile of phosphate, nitrogen, sulphate, carbondioxide, and methane.

Source: Stumm and Morgan (1996).

agricultural crops generally varies from 0.1 to 0.5 percent (Laditi *et al*., 2012). Phosphorus is taken up mostly as the primary as orthophosphate ion ($H_2PO_4^-$) or, but some is also absorbed as secondary orthophosphate ($HPO_4^=$). Phosphorus is classified as a major nutrient, frequently deficient for crop production, and is required by crops in relatively large amounts (Hameeda *et al*., 2008). Therefore, optimizing phosphorus fertilization is key important for maximizing crop productivity.

FUTURE INITIATIVES AND REGION/COUNTRY-SPECIFIC DEVELOPMENT FOCUS WITH POSSIBLE CONTRIBUTIONS

Climate change has affect plant productivity and water shortage could be importance issue on agriculture productivity. Developing drought tolerance rice thorough breeding and genomic modifications are important research topic in Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) and Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture. They have been developing super rice cultivar with drought tolerance. When developing such drought tolerance rice for upland soil, we should consider nutrient absorption behavior of rice under more oxidized soil environment (Henckel *et al*., 2000). In this issue, considering optimal exploitation of plant associated microbes would be a key strategy to enhance nutrient availability, improve soil structure (Nechitaylo *et al*., 2010), increase plant adaptability and productivity (Okami *et al*., 2011). Several phosphate solubilizing microbes produce growth hormone were isolated from Merapi (Indonesia) after 6 months eruption and similar microbes were isolated from rice root (Table 1). They are important beneficial microbes since they were able to increase productivity of cucumber grown on volcanic materials (Senlie *et al*., 2012).

The microbial community structures, as shown by diversity of 16S rDNA, of volcanic materials after 6 months eruption and rice soil were also analyzed which showed great diversity of microbial community in volcanic materials after 6 months recovery. Such microorganism would be beneficial for stimulating plant growth. Microbial community structure of soil would give diversity and succession as affected by environmental disaster such as eruption in Mount Merapi (Indonesia). The microbial communities gradually grow to reach highest diversity. Such information would be important to see how microbial community affect recovery of soil function (Artz *et al*., 2006; Romantschuk *et al*., 2000).

Table 1. Isolated phosphate solubilizing bacteria from Merapi and growth hormone producing capacity.

Isolate code	Medium calcium phosphate	Aluminium phosphate	IAA production
BS.1.F	+	-	+
BS.2.K	+	+	+
BS.3.ML2	+	+	-
BS.4.ML4	+	-	+
BS.5.ML	+	-	-
BS.6.TM1	+	+	-
BS.7.TM2	+	+	+
BS.8.E01.2	+	-	-
BS.9.E02.2	+	-	-
BS.10.E01	+	-	+
BS.11.E30	+	-	-
BS.12.E31	+	-	-
BS.13.E30	+	-	-
BS.14.E60	+	-	+
BS.15.E03	+	-	+
BS.16.EGb	+	-	-
BS.17.EGb	+	-	-
BS.18.E02	+	-	-
BS.19.E04	+	-	-
BS.20.E01	+	-	-
BS.22.EC	+	-	-
BS.23.NEK	+	-	-

Source: Senlie *et al*. (2012).

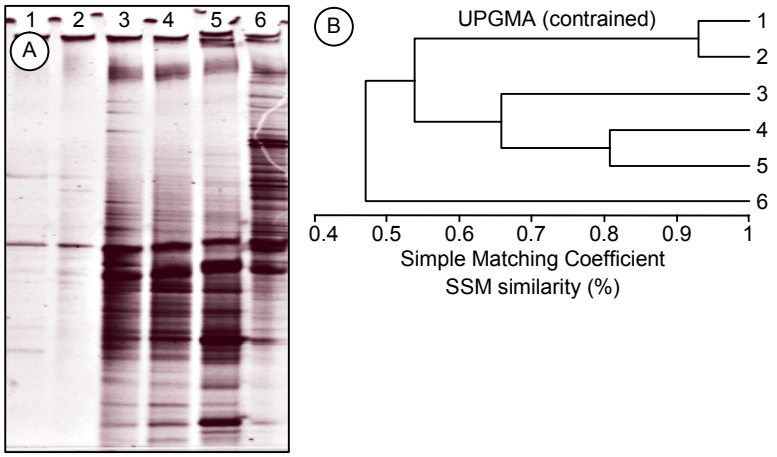

Figure 3. DGGE profile of 16s rRNA (A) and phylogeny analyses of their relatedness (B). 1 = 0 month after eruption, 1 month after eruption, 2 = 3 moths after eruption, 3 = six months after eruption, 4 = soil sample of rice field in Mount Salak, 5 = soil of rice field in Cibinong, 6 = marine sediment.

Source: Senlie *et al*. (2012).

DEVELOPMENT SCOPE AND OPPORTUNITY

Dynamic of microelement for plant growth is strongly affected by pH and redox potential of soil. The most important is how these micronutrients could be managed through irrigation and cropping system. Aerobic cultivation with many modifications on cropping system could offer solution on reducing methane emission rate but enhancing rice productivity. The important strategy is then understanding soil chemical and biological character of various rice cultivation system at various soil type (Chin *et al*., 1999; Jalota *et al*., 2009).

Upland rice production system, which was successful in Lombok, should be promoted in other region. Though East Nusa Tenggara suffered from water shortage, local farmers are very keen of cultivating rice (Figure 4). Selection of rice varieties, both local and super hybrid, is the important strategy to boost rice productivity and share social benefit to local community. Upland soil cropping system with alternate plantation provide better ecosystem service through creating better nutrient mobility and biodiversity creation (Zheng *et al*., 2010). With upland soil area of about 5 million hectares, development of cereal would offer potential productivity and better food security for Indonesia. Identifying optimal place for cereal production is other the strategic issue in ensuring food security in Indonesia.

Genotypic analyses of rice field clearly show that many functional gene for methane oxidation are existed in both aerobic soil and flooded soil (Figure 5 and 6), which implies functional genes are already there, then how to stimulate the gen works are very crucial for enhancing rice productivity with low greenhouse gas emission.

We isolated several methanotrophs from rice soil and they were able to degrade tricholoroethylene (JICA-JST Report, 2013). These groups of bacteria are further studied to verify their physiological uniqueness and their ecological role. These groups of microorganisms will be very useful in the future for methane emission reduction from rice field and for bioremediation of pesticide contaminated soil.

Figure 4. Upland rice in Oemasi, East Nusa Tenggara, Indonesia.

CONCLUDING REMARKS

Understanding the microbial community structure of soil microorganism in rice field and their ecology are important strategy to fully optimize their physiological uniqueness in mediating nutrient transformation and mobility for plant growth. Managing the redox potential and pH of soil together with organic nutrient mobility are key strategy to optimize plant growth and productivity. Exploring genetic diversity of rice plant which are able to grow under extreme condition are quite challenging to obtain most productive and adaptable rice in tropical areas facing impact of climate change.

ACKNOWLEDGEMENTS

This project was funded by JICA-JST SATREP project and Mitigation and Disaster Prevention Project (PN9-LIPI 2012-2014). The authors express sincere gratitude to local government and all local collaborators in East Nusa Tenggara, Bali, and West Java for their support in the implementation of this project.

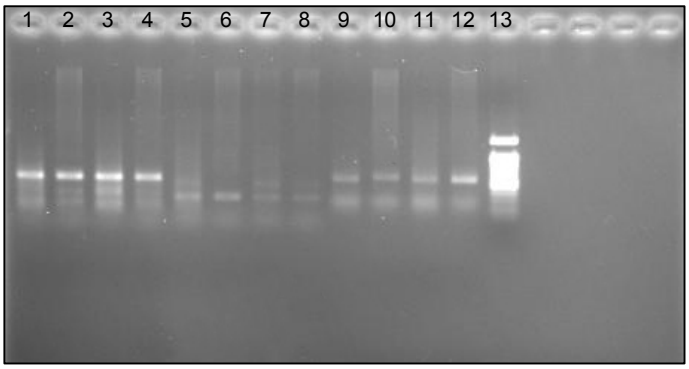

Figure 5. Detection of *pmO*, *smO*, and *moxF* genes in paddy soils. 1–4 = *moxF* gene, 5–9 = *mmoB* gene, 10–12 = *pmoA* gene.

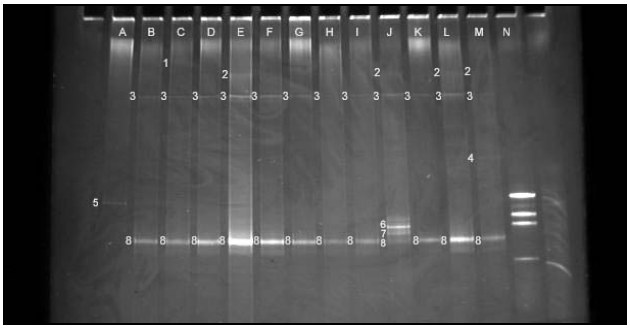

Figure 6. DGGE profile of *pmO* gene in soil with methane gas or with NMS medium addition.
Source: Sudiana *et al*. (2009).

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DISCOVERING GLYCOSIDE HYDROLASE GENES FROM MARINE MICROORGANISMS

Dessy Natalia

ABSTRACT

Glycoside hydrolases (GHs) are carbohydrate-active enzymes that are widely used in various industrial processes in food, pharmaceutical, textile, paper and pulp industries as well as detergent and production of biofuel. GH with wide range of both catalytic activity and specificity can be isolated from enormous microbial diversity in nature. Screening for microorganisms producing GH can be based on their ability to degrade polysaccharides. The corresponding genes can be isolated through genome mining in more than 2,000 microbial genomes. Alternatively, the corresponding genes can also be obtained through a direct phenotypic detection from a host cell transformed with a genomic library. Unfortunately, a vast majority (>99%) of microorganism cannot be cultured. Therefore, a metagenomic approach through activity-based or sequence homology-based screening can be employed to discover more new GH encoding genes. This paper describes the discovery of genes encoding for GHs with α-amylase, chitosanase, and β-glucosidase activities from marine microorganisms. The gene coding for α-amylase (*baqA*) from *Bacillus aquimaris* MKSC 6.2 was pulled out by a combination of standard PCR using degenerate primers based on α-amylase from closely related *Bacillus* genome and inverse PCR methods. *baqA* encodes a protein of 512 amino acids BaqA, which its sequence is homologous to that of proteins forming a $(\beta/\alpha)_8$ TIM barrel that is typical of amylase. A phylogenetic analysis revealed that BaqA belongs to GH13 family, but is grouped in a novel subfamily with other putative α-amylases. On the other hand, the gene encoding chitosanase from *B. amyloliquefaciens* ABBD (*csn1*) was amplified by PCR using primers designed from the published putative *B. amyloliquefaciens* chitosanase gene. The *csn1* gene encodes a protein of 278 amino acids long that its sequence shares 99% homology with putative chitosanases (GH46 family) of *B. amyloliquefaciens* subsp. *plantarum* CAU B946 and *B. amyloliquefaciens* Y2. Finally, employing similar genomic PCR approach, a gene coding for 465 amino acids long β-glucosidase (*bglZ*) was also discovered. The nucleotide sequence of *bglZ* shares 97% identity with that of from *B. amyloliquefaciens* FZB42. Further, the BglZ amino acid sequence analysis suggested the presence of $(\alpha/\beta)_8$ TIM barrel structure.

Keywords: glycoside hydrolase, α-amylase, β-glucosidase, chitosanase, marine microorganism.

INTRODUCTION

Glycoside hydrolase (GH) comprises of enzymes with capabilities to hydrolyzes various kind of glycosidic bonds, thus are carbohydrate acting enzymes. There are more than 150,000 sequences of GH families have been reported in 2013 (Lombard *et al.*, 2014). Members of GH family are varying in substrate specificity and activity, hence they are further divided into 133 families based on their amino acid sequences, at least 14 clans based on their typical

structural motifs, and well over 100 subclasses based on classification of mechanistic enzyme activity (http://www.cazy.org/Glycoside-Hydrolases.html). Expectedly more to come from extensive exploration of microbial diversity, as more than 2000 microbial genome is available in the NCBI database and metagenomic approach further harness genome diversity from uncultured microorganisms (Adrio and Demain, 2014). Enzymes of these families are widely used in various industrial processes or in direct applications. Therefore, search glycosyl hydrolase enzymes with desired properties, has always been exciting.

Marine life accounts for ~3.62×10^{30} prokaryotes and represents over 70% of prokaryotes on earth (Whitman *et al.*, 2010). So far, only few have been reported, of which more than 177 genomes of marine microorganisms were sequenced at J. Craig Venter Institute (http://www.jcvi.org/cms/research/past-projects/microgenome/overview/). Thus, microbial diversity is immense and challenging for further exploration. Nevertheless, the currently available marine microorganism genome database allows the search of genes encoding GHs.

Bacteria from various marine habitats in Indonesia have been exploited to screen for enzymes with activities to degrade starch (α-amylase), chitosan (chitosanase), or cellulose (cellulase). The corresponding genes are then isolated by genomic PCR using putative gene sequences available in the database. In this paper, the discovery of α-amylase gene from marine *Bacillus aquimaris* MKSC 6.2 as well as chitosanase and β-glucosidase genes from *B. amyloliquefaciens* ABBD is presented.

α-AMYLASE OF *Bacillus aquimaris* MKSC 6.2

α-Amylase (EC 3.2.1.1) catalyzes the hydrolysis of branched α-1,4-glycosidic bond of starch producing linier oligosaccharide and α-limit dextrins. The hydrolysis is the first step in starch processing thereby α-amylase is one of industrially important enzymes. The enzyme is found in various organisms ranging from archae, bacteria to eukaryote. α-Amylases share a common structural motif, that is $(\beta/\alpha)_8$ TIM barrel, and reaction mechanism (Janecek *et al.*, 2014). However, the properties of α-amylase from different source are varying in term of optimum conditions for the activity, kinetics, and specificity.

Bacillus aquimaris MKSC 6.2 was discovered upon isolation of bacteria from a soft coral *Sinularia* sp. in Merak Kecil Island, West Java. The bacterium was identified by a 16S rDNA analysis and demonstrated α-amylase activity by means of degradation of raw starches from corn, rice, sago, cassava, and potato (Puspasari *et al.*, 2011). The α-amylase gene fragment of marine *B. aquimaris* MKSC 6.2 was first obtained by PCR using degenerate primers based on several putative α-amylase amino acid sequences from closely

related *Bacillus* sp. SG1, *B. coahuilensis*, and *B. weihenstephanensis*. The complete α-amylase gene was then obtained by inverse PCR (Puspasari *et al.*, 2013). The open reading frame of *B. aquimaris* MKSC 6.2 α-amylase gene (*baqA*) consists of 1539 bp encoding for a 512 amino acids long protein (GenBank accession number: JN797599). BaqA contains amino acid sequence resembling that of having $(\beta/\alpha)_8$ fold, which is typical of α-amylase. This structural motif hosts the catalytic domain of α-amylase whilst the binding of substrates is facilitated by its C-terminal domain, which is the starch/ carbohydrate binding domain (SBD/CBD). However, amino acid sequence analysis suggested that the SBD/CBD is absent in BaqA. This is an interesting finding because BaqA is capable of degrading raw starches. Further, a phylogenetic analysis revealed that BaqA belongs to GH13 family and is grouped in a novel subfamily with some other putative α-amylases (Figure 1).

CHITOSANASE FROM *Bacillus amyloliquefaciens* ABBD

Chitosan is a polymer that has the D-glucosamine (GlcN) residue as a major monomer and small quantity of N-acetyl-D-glucosamine (GlcNAc) residue. Hydrolysis of β-1,4 glycosidic bond of chitosan by chitosanase (EC 3.2.1.1.32) results in chito-oligosaccharides, which have various potential

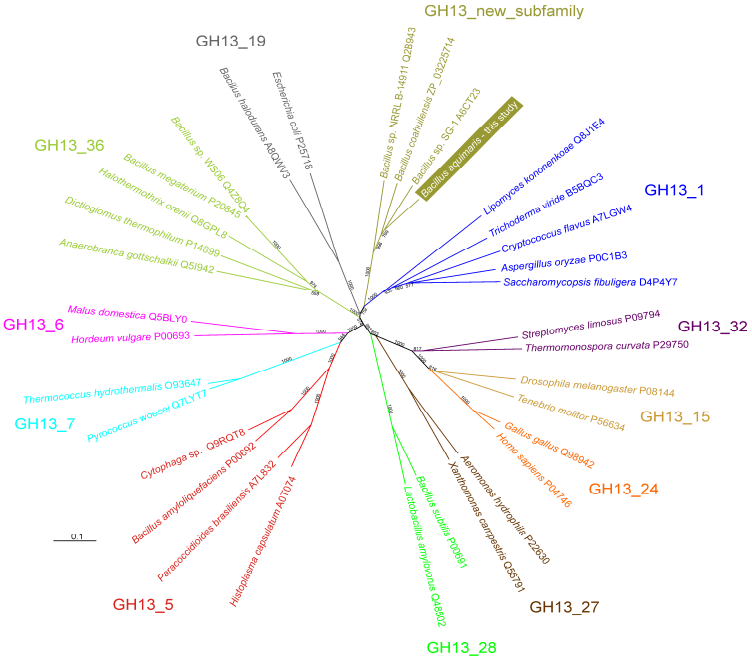

Figure 1. Phylogenetic tree of α-amylases (EC 3.2.1.1) from GH13 family.
Source: Puspasari *et al.* (2013).

medical applications as antibacterial, antifungal, antiinflammation, antitumor as well as antidiabetic agents (Somashekar and Joseph, 1996). Chitosan can be originated from partially deacetylated chitin, which can be isolated from shrimp shell waste. Thus, chitosanase can be useful to increase the commercial value of the waste for the production of chitooligosaccharides. Chitosanases have been found in bacteria, fungi, microorganisms, viruses, and plants, and they are are classified in five GH families: GH5, GH8, GH46, GH75, and GH80 (http://www.cazy.org/Glycoside-Hydrolases.html).

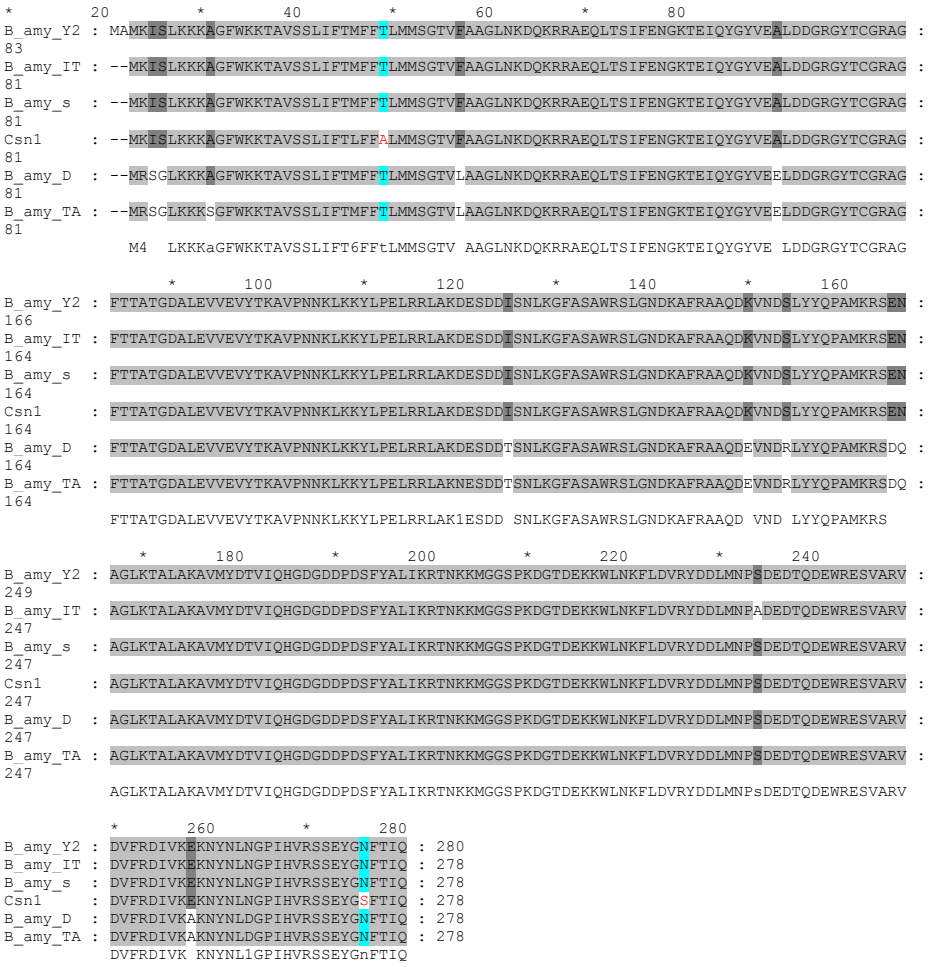

Figure 2. Alignment of sequences from chitosanases from *B. amyloliquefaciens*. B_amy_IT = *B. amyloliquefaciens* IT-45, B_amy_s = *B. amyloliquefaciens* subsp. *plantarum* CAU B946, Csn1 = *B. amyloliquefaciens* ABBD, B_amy_D = *B. amyloliquefaciens* DSM 7, B_amy_TA = *B. amyloliquefaciens* TA208. Unique residues A27 and S274 are shown in red.

Source: Muliasari (2013).

An open reading frame encoding a novel chitosanase (*csn1*) from *B. amyloliquefaciens* ABBD, a bacterium isolated from a hard coral *Acropora* sp. (Nurachman *et al.*, 2010), was amplified in PCR using primers based on the published sequence of putative chitosanases of *B. amyloliquefaciens*. The open reading frame of *csn1* (GenBank accession number: KC968227) is 837 nucleotides long and encodes 278 amino acids. Amino acid sequence analysis suggested that Csn1 belongs to superfamily of GH46 with catalytic residues of Glu55, Asp71, and Thr75, and it shares 99% homology with putative chitosanases from *B. amyloliquefaciens* subsp. *plantarum* CAU B946 and *B. amyloliquefaciens* Y2, and 87–89% homology with chitosanase from *B. subtilis* and *Bacillus* sp. Despite sharing high amino acid sequence identity with other *B. amyloliquefaciens* chitosanases, *B. amyloliquefaciens* ABBD has two unique amino acid residues, namely Ala27 and Ser274 (Figure 2), which might result in some interesting properties.

β-GLUCOSIDASE OF *Bacillus amyloliquefaciens* ABBD

β-Glucosidase (EC 3.2.1.21) hydrolyzes the β-1,4-glycosidic bond of oligosaccharides and is usually employed in the last step of conversion of cellulose into glucose, after processing by endoglucanase and/or exoglucanase. Interestingly, the enzyme is also capable of performing biosynthesis reaction hence it has a wide application in biotechnology (Wallecha and Mishra, 2003). Based on the amino amic sequence similarity, β-glucosidases have been grouped into GH families 1, 3, 9, 30, and 116 (http://www.cazy.org/Glycoside-Hydrolases.html).

A gene coding for β-glucosidase (*bglZ*) was successfully obtained from *B. amyloliquefaciens* ABBD, a bacterium isolated from hard coral (Kurniasih *et al.*, 2014). The full-length *bglZ* (GenBank accession number: JX422023) contains an open reading frame of 1398 bp that shares 97% identity with β-glucosidase gene from *B. amyloliquefaciens* FZB42 (GenBank accession number: ABS75964). BglZ is a protein of 465 amino acids and belongs to the GH1 family, which also has $(\beta/\alpha)_8$ barrel structure. Amino acid sequence alignment of BglZ to β-glucosidase from *Bacillus polymyxa* (BglA) and *Paenibacillus polymyxa* (BglB) suggests that Glu171 and Glu364 as the catalytic residues of BglZ, (Figure 3). It has been reported that residues of Gln20, His121, Tyr296, Glu405, and Trp406 play a role in substrate interaction in BglA (Sanz-Aparicio *et al*., 1998). Three out of five possible substrate interaction residues of BglZ are identical with those of BglA, however two other residues are substituted by serine and alanine at those in the corresponding positions of BglA, which might lead to different substrate specificity.

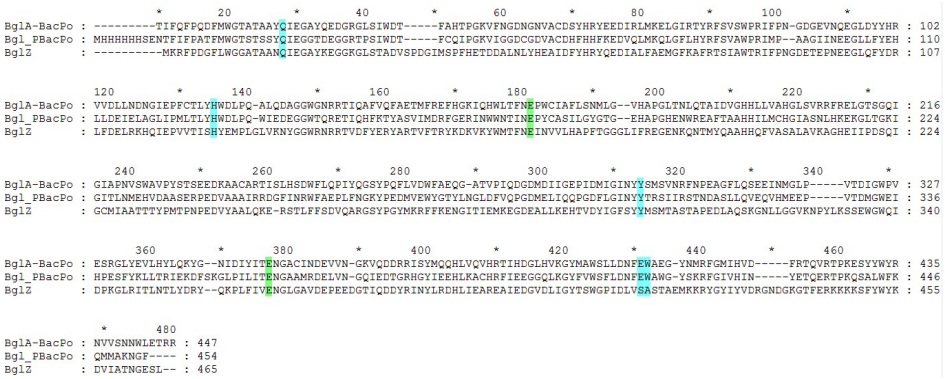

Figure 3. Amino acid sequence alignment of BglZ *B. amyloliquefaciens* ABBD, BglA of *B. polymyxa,* and BglB *Paenibacillus polymyxa*. Conserved catalytic residues are highlighted in green and conserved residues for substrate recognition are shown in blue.

Source: Kurniasih (2012).

FUTURE DIRECTION

Genes coding for some GHs (α-amylase, β-glucosidase, and chitosanase) were successfully fished out from *B. aquimaris* MKSC 6.2 and *B. amyloliquefaciens* ABBD through genomic approach. The *B. aquimaris* genome is unavailable, thus more GHs encoding genes could still be discovered upon its genome elucidation. The genome of *B. amyloliquefaciens* is available, providing good reference to unravel new GH encoding genes in the ABBD local strain. In the future, GH genes hunting using metagenomic approach from marine habitat, such as sea grass and soft coral, are also of a great interest.

A strong collaboration among university, research institution, and industry at national and international level is fundamental to facilitate and accelerate the discovery of more and novel GH enzymes, which are of importance for science and various biotechnology applications. Ultimately, government support for research in gene discovery from Indonesian microbial diversity is essential.

CONCLUDING REMARKS

B. aquimaris MKSC 6.2 was discovered and the α-amylase gene (*baqA*) was isolated and characterized to be capable of degrading raw starches. A gene encoding novel chitosanase (*csn1*) from *B. amyloliquefaciens* ABBD, a bacterium isolated from a hard coral *Acropora* sp., was also successfully identified and showed some interesting properties of amino acid. A gene encoding for β-glucosidase (*bglZ*) was obtained from *B. amyloliquefaciens* ABBD, which was isolated from hard coral, had specific properties for catalytic residues that might lead to different substrate specificity. Thus, genes encoding

some GHs (α-amylase, β-glucosidase, and chitosanase) were successfully fished out from *B. aquimaris* MKSC 6.2 and *B. amyloliquefaciens* ABBD through genomic approach. In the future, a strong collaboration at national and international level with government support is fundamental to accelerate the GH genes discovery from Indonesian microbial diversity.

ACKNOWDEGEMENTS

This research was financially supported by Hibah Desentralisasi, Directorate General of Higher Education, Ministry of Education and Culture, Indonesia and Riset Unggulan of Bandung Institute of Technology. I would like to thank Wangsa T. Ismaya for critical reading this paper.

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ANNEX

SELECTED ABSTRACTS

This chapter provides abstracts of research papers that have been presented at the ICPGD 2014. These abstracts more or less represented current status of gene discoveries and pre-breeding research conducted by various research institutes in Indonesia. There are 49 abstracts which covers various topics, such as evaluation of genetic resources of several crops to biotic and abiotic stresses, including molecular evaluation; gene identification and computational biology infrastructure. Crops plants covered in these abstracts include rice, soybean, mungbean, potato, sweet potato, citrus, chilli, saccharum, oil palm, jatropha, cocoa, medicinal crop, whereas microbial research presented include marine fungus.

Full papers of those abstracts can be requested from the authors whose addresses are indicated in the abstracts. Some of those abstracts might have been published elsewhere.

ABSTRACTS OF ORAL PRESENTATION

DISCOVERY OF YIELD-POTENTIAL GENE FROM INDONESIAN RICE LANDRACE AND ITS APPLICATION IN BREEDING PROGRAM

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ABSTRACT

Rice is one of the most important staple foods for nearly half of the world's population. Increasing the rice production is essential for food security in Asia and Africa, where both population and rice demand are rapidly increasing. Here, we report the discovery of a novel gene important in enhancing the grain yield of *indica* cultivars. Significant yield increase in modern *indica* cultivars was achieved through the identification and utilization of *SPIKELET NUMBER* (*SPIKE*), derived from Indonesian rice landrace. *SPIKE* increased grain yield of tropical *indica* cultivars, IR64 and IRRI146, by approximately 20% in the field, through the improvement of the plant architecture of both sink and source organs with no change in grain quality and growth duration. Breeding program introducing *SPIKE* into several *indica* cultivars (including 'Ciherang') through marker-assisted selection (MAS) is currently ongoing. Field trials and MAS breeding have initiated in the IRRI-Japan Collaborative Research Project, with the collaboration of Indonesian Agency for Agricultural Research and Development (IAARD). The application of *SPIKE* in rice breeding, to cope with crop intensification and climate change, is also discussed. The use of *SPIKE* in rice breeding could contribute to food security in *indica* rice-growing regions.

Keywords: rice, yield, total spikelet number.

IDENTIFICATION AND CONSERVATION BLAST RESISTANCE GENE FROM INDONESIAN RICE GERMPLASM INTO DOUBLE HAPLOID LINES

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ABSTRACT

Blast disease, caused by fungal *Pyricularia grisea* Sacc., is one of the most devastating diseases in rice. The use of blast-resistant varieties is one of the most efficient ways to control blast disease. Molecular marker application was available to assist selection process on the developing resistance varieties. Based on previous research, some alleles of blast resistance genes, *Pi1*, *P2*, *Pi9*, *Pi33*, and *Pib* were detected on IR54, while *Pir4* and *Pir7* were detected on Bio110. The double haploid (DH) lines were produced by cultured the anther of F_1 lines from the double-crossing IR54/Parekaligolara//Bio110/Markuti. The objectives of this research were to evaluate blast resistance response (leaf-blast and neck-blast) of DH lines in greenhouse and blast endemic field (Sukabumi) and to identify blast resistance genes on DH lines by using molecular markers. Forty-nine DH lines were selected based on the resistance response and the genotype evaluation by using molecular markers. The results showed that DH lines showed vary responses to races 123, 133, and 173. Among 49 lines tested, there were two genotype groups that showed best performance on blast resistance, i.e. BMIP-24-1-1 and BMIP-40. From the greenhouse and field selections, there were five promising lines that resistant to leaf-blast and neck-blast. The molecular markers analysis were showed *Pib* gene contributed the leaf-blast resistance to race 123, while *Pi1* and *Pir7* genes contributed the leaf-blast resistance to races 123 and 133. Primer PirSNP7 was the most precision to be used as selection markers for leaf-blast resistance.

Keywords: molecular markers, double haploid rice lines, blast resistance genes.

RESPONSIVENESS OF TWENTY-FIVE CULTIVARS OF RICE (*Oryza sativa* L.) TO ARBUSCULAR MYCORRHIZA FUNGI DETECTED BY MICROSATELLITES AND RAPD MARKERS

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ABSTRACT

Diversity of responsiveness intraspecies of rice (*Oryza sativa* L.) with arbuscular mycorrhizal fungi (AMF) is required to develop rice cultivars that are more responsive to AMF infection. Those rice plants are known to be drought-tolerant and able to take phosphate. Molecular characterization was conducted by using RAPD, SSR, and STS markers. The results showed that Arias (21.92%), Rojolele 20 (14.75%), and Situ Bangendit (14.23%) had a moderate responsiveness level. The highest three cultivars responsiveness Arias and Situ Bagendit have close genetic distance. There were polymorphisms based on RAPD and SSR markers. However, the responsiveness has not been identified.

Keywords: rice, mycorrhiza responsiveness, RAPD, SSR.

TOLERANCE OF RICE GERMPLASM ACCESSION TO ABIOTIC STRESSES

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ABSTRACT

Abiotic stresses become a major constraint especially in unfavorable environment. Indonesia has many abiotic stresses, such as drought, salinity, submergence, stagnant flooding, iron toxicity, and acid sulfate toxicity. Moreover, submergence and stagnant flooding not only affect to rainfed lowland, but also jeopardize irrigated areas. The over flows from the big river were risky to the paddy field. Those all constraints were affect to rice yield directly, therefore the tolerance to the abiotic stress will be important train to be improved. Tolerance of germplasm accession to abiotic stresses was conducted in Sukamandi, Indramayu, and Banyuasin during 2013. The aim of the research was to evaluate the tolerance of germplasm accession to salinity, acidic sulphate, stagnant flooding, and submergence stresses. Augmented design was used as a field design without replication. The results showed eight accessions were tolerant to 8 ds/m of salinity at screen house. The accessions with score 3–5 were Cingri, Beras Merah Sleman, Beronaja, Celebes, Maros, Cingri, Pare Pulu, Harum Manis, and Glabed. However, there was not accession tolerant to 12 ds/m of salinity. The screening in the saline area showed that Cingri (accession number: 1123) and Celebes Maros (accession number: 1135) were most tolerant than other accessions. The submergence screening resulted two tolerant accessions, i.e. Untup Rejeb (accession number: 7962) and Padi Cina (accession number: 1864). Those lines had a 100% of survival rate after 14 days of submergence. There were 160 accessions having a complete tolerance to acid sulphate (Fe toxicity, low pH). Ten accessions showed early maturity and medium of plant height, so it could be used directly as a donor for acid sulphate or Fe toxicity. Fifty-six accessions were tolerant to stagnant flooding during entire growth with score 3, but only nine accessions produced productive tiller and normal panicle and grain filling. Those lines were Mutiara, Pandan Harum, Talun Bagang, Si Bujang Banu, Serepet Tinggi, Padi Kuning, Puluk Bolong, Penyulu Jambu, and Popot.

Keywords: *Oryza sativa*, acidic sulfate, salinity, stagnant flooding, submergence.

QUANTITATIVE TRAIT LOCI CONTROLLING ALUMINUM TOLERANCE IN INDONESIAN LOCAL RICE VARIETY *Dupa*

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ABSTRACT

Aluminum (Al) toxicity is a major constraint to crop production in acidic soils worldwide. Widespread acidic soil areas make it is difficult to reclaim them and eliminate Al toxicity by liming. Developing Al-tolerant rice varieties remains the feasible way to overcome Al toxicity. Quantitative trait loci (QTL) analysis was carried out to identify genes controlling Al tolerance in Indonesian local variety, *Dupa*. An F_4 generation derived from *Dupa* (tropical *japonica*, tolerant) and ITA 131 (*indica*, susceptible) was used for mapping. Phenotypic screening was conducted on hydroponics using Magnavaca's solution, and 750 µM $AlCl_3$ was used as treatment. Rice tolerance was measured based on the growth inhibition of the total root length after five days of treatment. A set of 384-plex GoldenGate VeraCode oligo pool assay (OPA) on the Illumina BeadXpress platform was used for SNP genotyping. The inhibition of total root length under Al stress is a good parameter in determining Al tolerance in rice seedlings. QTL analysis revealed the presence of three QTLs associated with Al tolerance that were Alt1-1, Alt 1-2, and Alt3, with LOD of 3.5, 2.7, and 2.99, respectively. These three QTLs explained 48.5% of the total phenotypic variance in the population. Genes associated with Al tolerance were found colocalized with these QTLs region. QTL network analysis showed an epistasis effect between two chromosomes associated with the inhibition of total root growth. The existence of many small QTLs and epistasis indicate the complexity of the mechanism of tolerance to Al toxicity.

Keywords: aluminum tolerance, Magnavaca's solution, *Dupa*, QTL.

USE OF SSR MARKERS IN THE MAIZE PRE-BREEDING PROGRAM AT ICERI: GENETIC DIVERSITY IN MAIZE INBRED LINES

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ABSTRACT

Sticky waxy maize is very susceptible to downy mildew. Until recently, sticky waxy maize variety resistant to downy mildew is not available commercially for the consumers. Sixty-nine maize inbred lines collection of Indonesian Cereal Research Institute (ICERI), representing two groups of phenotypic characters, the downy mildew resistance and sticky waxy maize, were investigated using simple sequence repeats (SSRs) markers in order to determine the extent and distribution of its genetic diversity. Twenty-two SSRs markers distributed throughout the maize genome were chosen based on their level of polymorphism. A total of 168 alleles were detected with an average of 7.7 alleles per locus and a range of 4 to 11 alleles per locus across the 69 lines. The observed heterozygosity (HoP) among the lines ranged from 0 to 0.18 with an average of 0.07. Meanwhile, genetic distances were found ranging from 0 to 9.8. The cluster analysis based on SSRs distinguishes the lines according to the pedigree data as well as the resistance degree to downy mildew and sticky waxy. A far genetic distance between sticky waxy and downy mildew lines was detected among the lines. These findings support the development of sticky waxy maize resistance to downy mildew through the cross between lines of waxy group and lines of downy mildew resistant with far genetic distance.

Keywords: maize inbred, waxy, downy mildew, genetic diversity, SSR markers.

GENOME-WIDE SNP IDENTIFICATION IN INDONESIAN SOYBEAN USING ILLUMINA HiSeq

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ABSTRACT

Soybean (*Glycine max*) is an important legume crop that should be improved for its valuable benefit in Indonesia. To date, even though publicly accessed genomic and genetic databases of soybean are available, high-throughput sequencing in Indonesian-bred soybean is slowly progressed. With the advance of next-generation sequencing (NGS) technology, it is important to identify single nucleotide polymorphism (SNP) among soybean genotypes. Therefore, with the aims of identifying SNP could be useful as marker candidate, we resequenced three Indonesian soybean genotypes and detected SNPs generated by Illumina high-throughput sequencing technology. Shot sequencing with paired-end reads revealed that total produced bases of Anjasmoro, Tanggamus, and Wilis were around 1.02 Gb, 1.38 Gb, and 1.15 Gb, respectively. Based on mapping with genome reference William 81 ranging from 95.23% to 96.56%, abundant SNPs were identified. Based on individual alignment, Tanggamus gave the highest total SNP number (775,645 SNPs), whereas Anjasmoro (389,943 SNPs) and Wilis (344,202 SNPs) were comparable. Ratio of transitions and transversions with a range of 2.1–2.2 suggests the high precision of this sequencing data. Most of SNPs were located in intergenic regions, and approximately 4,586 SNPs in coding regions across 20 chromosomes need further analysis to identify their functional effect of protein changes. Thus, this sequencing technology made the whole genome SNP discovery was more effective. We demonstrated a large number of SNPs identified in soybean genomes and these potential SNP markers would serve a tool for genomic-assisted selection in soybean breeding program in Indonesia in the future.

Keywords: *Glycine max*, next-generation sequencing, SNP.

INVOLVEMENT OF HETEROTRIMERIC G PROTEIN ALPHA SUBUNIT OF SOYBEAN TO ALUMINUM TOXICITY

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ABSTRACT

Genetic improvement of tolerant plants to acid and aluminum stress is very important to increase the national agricultural production. Plant heterotrimeric G proteins alpha subunit (Gα) have been implicated in a number of signaling processes. The aim of this research was to examine the role of G protein alpha subunit under acid and aluminum toxicity in plant. Result indicated that aluminum treatment (pH 4 + 1.6 mM $AlCl_3$) for 24 h highly increased accumulation of aluminum in soybean susceptible plant cv. Lumut. The expressions of Gα and peroxidase genes were expressed higher under aluminum stress than pH 6 or pH 4 stresses. Both Gα and peroxidase genes were expressed earlier and highest at the 8 h, 24 h, and 48 h. Recently, by using mastoparan, an activator of Gα, we found that acid and aluminum stresses could increase expression of Gα in soybean tolerant plant cv. Slamet. To elucidate the mechanism, we analyzed the functional of Gα gene during abiotic stress by using mutant of Gα gene from tolerant plant cv. Slamet. Loss of function of Gα gene was induced accumulation of aluminum in root. Result of this study demonstrated that the heterotimeric G protein alpha subunit playing a role in the acid and aluminum toxicity.

Keywords: G alpha gene, aluminum stress, gene expression, soybean.

EVALUATION OF SWEET POTATO POLYCROSS F_1 GENOTYPES RESISTANCES AGAINST SCAB DISEASE (*Sphaceloma batatas*)

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ABSTRACT

West Java has been known for having wide varieties of sweet potato landraces. However, its quality and quantity production has been limited by the presence of scab disease caused by *Sphaceloma batatas* (anomorph of *Elsinoe batatas*). Therefore, resistance to scab disease has become character targeted for sweet potato resistant varieties development. Polycross among 69 local sweet potato landraces collected from West Java had produced 601 F_1 genotypes. Their resistance against scab disease was evaluated under field condition with artificial inoculation. The result demonstrated that the majority of the tested F_1 genotypes had high resistance against scab diseases. This was demonstrated by more than 50% of the assessed population were resistant to scab diseases as showed by low value of diseases severity. The resistance stability was reassessed on the following growing season. F_1 genotypes with high resistant and susceptible character to scab infection from both seasons were chosen for SSR marker selection associated with scab resistance. Bi-plot analysis demonstrated that the primers of IBCIP-13, IbR03, IbR12, IbR19, and IBSSR09 were correlated with genotypes that had high resistance character to scab disease. Therefore, those SSR markers have potential to be used further in the marker-assisted selection process for scab resistant varieties development program.

Keywords: resistance, sweet potato, scab, *Sphaceloma batatas*, SSR marker.

EXPRESSION STUDIES OF SKINNING INJURY AND CURING PROPERTIES OF TWO CULTIVARS OF SWEET POTATO

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ABSTRACT

Skinning injury in sweet potato is inevitable and associated with postharvest loss due to storage diseases and weight loss. The strategy is to screen for and breed for cultivars with a more durable skin, which can resist skinning, cosmetic desiccation at wound sites, and fresh weight loss. Genes involved in general abiotic stress (*ELIP3*), wound stress (pre-lignin biosynthesis gene *TAL*), lignin biosynthesis (*PAL*, *CCOMT*, and *CAD*), and suberin (*Ext*) were used to study for their transcript abundance in order to understand the tolerance to skinning injury mechanism associated with lignin and suberin formation. Two sweet potato cultivars (L10-70 = skinning injury resistant; L07-146 = skinning injury sensitive) storage roots were skinned and placed in a chamber to cure at 28–29.5ºC and at a relative humidity of 85–90% for 0, 2, 4, 8, 12, and 24 h. For uncured treatment, roots were held at ambient room temperature and ambient relative humidity for the same time course. The results showed that the highest mRNA transcripts of *TAL* and *PAL* were at 12 h followed by *CCOMT* and *CAD* at 24 h were only observed in L10-70 in the curing treatment. In L07-146, the highest *PAL* transcript was expressed early (4 h), followed by *CCOMT* (8 h), and *TAL* (24 h) and downregulation of *CAD*. Interestingly, *Ext* transcript was only observed in noncured treatment (8 h and 12 h) in both cultivars. Furthermore, these studies also revealed that these genes were regulated in opposite fashion in both cultivars with difference in timing of their induction under curing condition. These results showed clear evidence for a coordinated gene signaling cascade in pre- and lignin biosynthesis pathway. Taken together, these studies demonstrated that major differences in skinning tolerance between these two cultivars were due to the ability of the skinning resistant cultivar to maintain highly regulated order in its transcript activity in response to skinning injury. The genes up-regulated in response to curing due to skinning may be useful to identify expression markers for screening sweet potato cultivars resistant to skinning injury and better wound healing in breeding programs.

Keywords: *Ipomoea batatas*, skinning injury, curing, lignification, RT-PCR.

CROSS ABILITY OF *Ipomea trifida* AS WILD PROGENITOR TO *Ipomoea batatas*

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ABSTRACT

Ipomoea trifida, as wild relative to sweet potato *I. batatas* originated from Citatah, West Java, has been considered possessing genes for resistant to biotic and abiotic stresses. The objective of this study was to evaluate the cross ability of *I. trifida* as wild progenitor to *I. batatas*. Interspecific crosses between *I. batatas* × *I. trifida* had been made from January–Juni 2014 at field research station of Padjadjaran University in Jatinangor, Sumedang. North Caroline Design 2 was employed to hybridize three accessions of *I. batatas*, i.e. (1) 219x17, (2) F1(4)T1, and (3) Narutokintoki as female parents, to six accessions of *I. trifida*, i.e. (1) UP.IT.02, (2) UP.IT.05, (3) UP.IT.06, (4) UP.IT.07, (5) UP.IT.09, and (6) UP.IT.10 as male parents. Ten crosses had been made for each cross combination. Result showed that based on 300 crosses that had been made, the highest survival interspecific crosses were 40%, 40%, 50%, and 60% for cross combinations of 219x17 × UP.IT.07, Rancing × UP.IT.07, Narutokintoki × UP.IT.09, and F1(1.2)NAR × UP.IT.05, respectively. Nevertheless, 52 survival capsules of F1 hybrids had been produced and 82 seeds had been harvested. Eventhough the different responses from both sides of female and male parents have been recorded, UP.IT.07 performed as the most compatible male parent (wild progenitor). Therefore, the accession could be employed as male parent in breeding program of sweet potato, particularly for resistant to biotic and abiotic stresses objectives.

Keywords: *Ipomoea batatas*, *Ipomoea trifida*, wild progenitor, sweet potato.

GENETIC DIVERSITY AND ORIGIN OF INDONESIAN BAMBARA GROUNDNUT (*Vigna Subterranea* [L.] Verdcourt) USING MICROSATELLITE ANALYSIS

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ABSTRACT

Bambara groundnut (*Vigna subterranea* [L.] Verdcourt) or kacang bogor is not native crop of Indonesia. This crop has been well adapted in Indonesian agroecology since hundred years. West Java has been the first central planting area of bambara groundnut in Indonesia, while Gresik in East Java has been the second one. The legume crop contents 60% carbohydrate, 21% protein, and 6% fat. It has a high market price as fried snack, roughly three times of groundnut price. Further breeding program requested genetic diversity information of Indonesian bambara groundnut landraces as well as their origin in Africa. In controlled environment rooms in School of Biosciences, the University of Nottingham, United Kingdom, 56 individual genotype accessions of a collection of Indonesian landraces were planted and DNA extracted. Eleven codominant microsatellite markers with different size range of polymorphic SSR markers were generated from the previous study and were employed for amplification 56 DNAs of Indonesian landraces. Furthermore, 114 landrace accessions with pre-existing microsatellite data from a previous study were combined with 56 landraces analysed with the genetic diversity analysis through analysis of molecular variance (AMOVA), principle component analysis (PCA), and cluster analysis. The result showed that Indonesian bambara groundnut has high variability within individual landraces and it is most likely originated from the East or Southern African regions. Spices trading in the 15th century may be one route through which bambara seed reached West Java.

Keywords: bambara groundnut, genetic diversity, AMOVA, PCA, cluster analysis.

DNA FINGERPRINTING ANALYSIS OF INDONESIAN MUNGBEAN VARIETIES USING SSR MARKERS

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ABSTRACT

Mungbean (*Vigna radiata* L.), secondly after soybean, is an important legume crop for its nutrition and economical value in Indonesia. Although a number of mungbean varieties have been released in Indonesia, the genetic resources are not well managed yet, such as duplications, mixed seed, and mislabeling is possibly found. Varieties identification is still mainly based on morphological and physiological characters that cannot easily prove a uniqueness of a variety making its protection for commercial exploitation is less concerned. Therefore, DNA fingerprinting analysis is considered as a potential approach to prove a uniqueness of a variety from existing varieties by determination of its genetic identity. This study aimed to find a marker set for identification of Indonesian mungbean varieties that can be useful as DNA fingerprint profile references. A total of 22 Indonesian mungbean accessions consisting of 16 improved and six local varieties have been analyzed using 55 simple sequence repeats (SSRs) markers developed on the basis of genomic analysis of Korean mungbean accessions, Sunhwanokdu and Gyeonggijaere. SSR analysis revealed that eight multi-allelic markers had at least four alleles and high polymorphic information content (PIC) value (> 0.6) have been successfully selected for identification of varieties. The selected markers enabled to differentiate each variety according to their genetic with the lowest distance of 0.125. The phylogenetic analysis generated two main clusters showing that local varieties from Nusa Tenggara and Sulawesi were grouped with some improved varieties (cluster I) and had a far distance with local varieties from Java island (cluster II). This result demonstrated that each mungbean variety in Indonesia had a specific DNA fingerprint profile as identity, indicating the robustness of the selected marker set as a tool. The genetic identity of variety was shown by eight digital values, which represented a series of alleles produced by corresponding marker. The DNA fingerprint profile of each variety would be beneficial as identity reference of mungbean in Indonesia.

Keywords: mungbean, SSR markers, DNA fingerprint, variety identity, phylogenetic.

DEVELOPMENT OF MOLECULAR MARKERS RELATED TO HUANGLONGBING DISEASE FOR CITRUS AND ITS WILD RELATIVES

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ABSTRACT

Citrus spp. is the one of main fruit commodity and has been widely cultivated in Indonesia. However, development of this commodity hampered by the presence of Huanglongbing (HLB) disease that devastating citrus plantation in various regions in Indonesia. The study was conducted based on the results of previous studies on genetic markers associated with susceptibility to HLB, using twenty-five types of citrus and its wild relatives. Developments of genetic markers were performed using sequenced characterized amplified region (SCAR) method; it found a specific fragment associated with susceptibility to HLB. As the other approaches, five primers designed to see the genetic profile of citrus responses to HLB infection. The results showed variation in DNA fragments of twenty-five types of citrus generated by the three primers, i.e. WRKY40, CSD1, and EDS1. It has been confirmed that PA02 developed by SCAR method could not be used as a genetic marker of plant responses to HLB.

Keywords: *Citrus* spp., wild relatives, Huanglongbing (HLB), disease response, genetic markers.

IDENTIFICATION OF MALE STERILITY GENES IN CITRUS

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ABSTRACT

Seedlings from citrus species and cultivars show precocious flowering after autumn seed germination. HY16 is a monoembryonic and male sterile hybrid-derived from a cross of Hanayu (*Citrus hanaju*) × *Yuzu* (*C. junos*). 'Kiyomi' tangor (Satsuma × 'Trovita'), a male sterile and monoembryonic plant, was also used to determine the male sterile genotype of grapefruit. Both seed parents generated seedlings with precocious flowering ability. Seeds were collected in November of 2006–2011 in Fukuoka, Japan, and allowed to germinate in a greenhouse (>10°C). Male sterile anthers were observed in hybrid seedlings from several crosses, i.e. HY16 × grapefruit, (HY16 × grapefruit) × grapefruit, HY16 × 'Ruby Blood' orange, (HY16 × grapefruit) × 'Ruby Blood' orange, HY16 × 'Trovita' orange, and 'Kiyomi' × grapefruit. On the basis of segregation analysis for male sterility (MS) in these progenies and on the fact that HY16 and 'Kiyomi' did not have cytoplasmic restoration factor R for MS, male sterile genotype was estimated to be ms1ms1Ms2ms2ms3ms3 (R-) for HY16, Ms1ms1ms2ms2ms3ms3 (R-) for 'Kiyomi' and ms1ms1Ms2Ms2Ms3ms3 orMs1ms1Ms2Ms2ms3ms3 (R+) for grapefruit with male fertile anthers. Here, MS is a recessive character; ms1 and ms2 are complementary genes with upper stream expression of ms2, and ms3 is an epistatic gene to ms1 and ms2 genes. Male sterile seedlings appear when ms3 is recessive homozygous (ms3ms3) and either ms2 or ms1 is recessive homozygous (ms2ms2 or ms1ms1). A population of 101 individuals from the cross of HY16 × grapefruit was chosen for mapping of MS genes. Using bulked segregant analysis with 260 12-mer oligonucleotide random primers, seven RAPD markers linked to Ms1 or Ms3 allele of grapefruit were detected and mapped. Five markers flanked at the closest 9.9 cM with the dominant of Ms1 or Ms3 alleles and two markers flanked at the closest 18.6 cM with the recessive of ms1 or ms3 alleles.

Keywords: male sterility, citrus, complementary genes, recessive genes, mapping.

IDENTIFICATION AND CHARACTERIZATION OF P5CS GENES FAMILY FROM *Saccharum officinarum* L. UNDER DROUGHT STRESS

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ABSTRACT

Increasing world sugar demand might be fulfilled with land extensification, which includes the use of dry area. Development of drought tolerance and high productivity sugarcane variety could be achieved by plant genetic engineering. Under drought condition, proline will be accumulated and functioned as an osmoregulator in plant cells. Δ^1-Pyrroline-5-carboxylate synthase (P5CS) is a rate-limiting enzyme in proline biosynthesis. This enzyme is encoded by *P5CS* gene family. We cloned two homologous *P5CS* genes from sugarcane, *SoP5CS1* (accession number: KF178299) and *SoP5CS2* (accession number: KF178300), which encode 729 and 716 amino acid polypeptides, respectively. The identity between these two genes was 74% based on nucleotide sequences. The *SoP5CS1* gene had 98% identity with *SbP5CS1* (accession number: GQ377719.1) and *SoP5CS2* had 99% identity with *SαP5CS* (accession number: EF113257.1). In this experiment, sugarcane plantlets were exposed to medium containing PEG 6000 (40%) for 12, 24, 48, and 72 hours. Proline concentration was measured after treatment and genes expression were analyzed by real time-qPCR. The results showed that the proline concentration was increased 12 folds (9.8 μmol.g^{-1}) after 48-hour stress treatment. The highest expression of *SoP5CS1* occured at 24-hour treatment with approximately 16 times from plant without PEG (control plant) and decreased gradually at 48-hour and 72-hour treatments. The highest expression of *SoP5CS2* was occured at 24-hour drought stress with approximately 3.6 folds compared to control. In drought treatment, the expression level *SoP5CS1* was higher than *SoP5CS2* and has increased significantly at 12-hour treatment. It is suggested that the *SoP5CS1* gene contributes more significantly to the production of proline during drought stress than *SoP5CS2*. Hence, *SoP5CS1* could potentially be used as a marker to screen sugarcane variety for drought tolerance. The gene construct has been generated for further research in the development of transgenic plant tolerant to drought.

Keywords: cloning, drought, expression, *P5CS*, sugarcane.

ANALYSIS OF SEVERAL HORMONES, PHENOLIC COMPOUNDS, AND DNA OF NORMAL AND ABNORMAL GENOTYPES OF OIL PALM (*Elaeis guineensis* Jacq.) CLONES

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ABSTRACT

Tissue culture-derived oil palm plants can develop abnormal flowers, in which the stamen primordial is converted into carpel-like tissue or mantled fruit, and sterile male flower. This abnormality can be heritable and individual palm may show variation in mantling. The aim of this research was to study the differences between normal and abnormal genotype based on DNA and the content of several hormones and phenolic compounds. The research consisted of (1) selection of AFLP primer which can produce polymorphic bands, (2) genetic similarities and specific DNA bands, and (3) β-carotene, α-tocopherol, several hormones and phenolic compounds content of normal and abnormal plants. Polymorphic primers were selected from 20 pairs of AFLP primers amplifying DNA of ABN1 clone. Selected primers were used to amplify DNA of ABN1, ABN2, and ABN3 clones with normal and abnormal fruits to study the genetic similarities and to obtain specific DNA bands. Hormones (IAA, GA, and zeatine), β-carotene, α-tocopherol, and phenolic compounds content of all clones were analysed by UPLC. The results showed that from the 20 primers tested, 10 primer combinations *Eco*RI/*Mse*I could produce polymorphic bands. Only one or two DNA bands were able to show the differences between normal and abnormal plant in the same clone. However, there was no specific DNA band as marker for all clones. Plants from all clones had high genetic similarity (92–99%). β-carotene, α-tocopherol, IAA, and GA contents were higher in normal fruit compared to abnormal one. While, phenolic compounds, such as syringate, caffeate, 4-hydroxybenzoate, ferulate, chlorogenate, galat, and β-coumaric acids contents, were higher in mantled fruit compared to normal fruit. Different approaches will be required for early detection and identification of mantled fruit.

Keywords: oil palm, *Elaeis guineensis*, tissue culture, somaclonal variation, fruit abnormality, AFLP.

ANALYSIS OF BIOLOGICAL NETWORK OF GENES BY ClueGO IN NORMAL AND MANTLED OIL PALM FRUITS

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ABSTRACT

Advancement of DNA sequencing technologies has major impact on the development of molecular biology, especially in understanding genes interaction in certain times or conditions. With the large number of genes produced through this high-throughput experiment, a proper analysis tool was needed for data interpretation. On the other hand, functional analysis of a large number of genes, to capture biological information and systematically organize the wealth of data, is still a challenging and daunting task. ClueGO is one of the tools to analyze a large number of genes. ClueGO is an easy to use Cytoscape plug-in that strongly improves biological interpretation of a large number of genes' lists. It can analyze one or compare two lists of genes and comprehensively visualize functionally grouped terms. The aim of this study was to obtain biological interpretation of sequencing data from normal and mantled oil palm fruits so that the genes that influence the occurrence of mantled fruit can be determined. Some groups of gene were overexpressed and suppressed in mantled fruit, based on its molecular function under ClueGO analysis. Groups of genes involved in N-methyl transferase, biotin carboxylase, cytochrome-c oxidase, and myo-inositol transmembrane activities were overexpressed in mantled fruit. While, the groups of genes involved in inositol-tetrakisphosphate 1-kinase, triose-phosphate transmembrane transporter, gamma-glutamyl transferase, and MAP kinase activities were suppressed. Based on the results, ClueGO is considered as the proper analysis tool to interpret comparison of two large lists of genes with biological interpretation.

Keywords: oil palm, ClueGO, DNA sequencing, biological network.

ANALYSIS OF THE DIFFERENCE BETWEEN EMBRYOGENIC AND NONEMBRYOGENIC CALLUS OF OIL PALM USING SCANNING ELECTRON MICROSCOPE

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ABSTRACT

Tissue culture technique has been used to produce elite oil palm clonal materials in commercial scale. The development of *in vitro* culture of oil palm can be done through callus, embryogenic callus, and somatic embryo formation. However, the percentages of embryo somatic production through callus embryogenesis are still low. The problems in embryogenesis phase are the difficulty to identify oil palm somatic embryo development stages and to differentiate embryogenic callus with nonembryogenic callus visually. The objectives of this research were to analyse the development of oil palm embryogenesis and to specify phenotypic characters of embryogenic and nonembryogenic callus by using scanning electron microscope (SEM). The explant source used in this experiment was 14-month-old embryoid-line A_1 and these embryos were cultured in solid medium every three months. The result showed that there were five development stages of somatic embryo, i.e nodular, globular, scutellar, coleoptilar, and shoot. Embryogenic calluses have specific characters with smooth and lighter cell surface, while nonembryogenic calluses have rough and darker cells surface. The smooth surface of embryogenic callus was related to extracellular matrix surface network for communication among embryogenic calluses. These matrices consist of pectin and arbinogalactan and also covered the clump of embryogenic callus.

Keywords: *Elaeis guieensis* Jaqc., embryogenic callus, somatic embryogenesis, scanning electron microscope, extracellular matrix

BioGRID DATABASE IMPLEMENTATION ON CYTOSCAPE 2.8 SOFTWARE TO DEVELOP OIL PALM GENES INTERACTION NETWORK

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ABSTRACT

BioGRID is an online interaction repository database that established through comprehensive curation efforts. Gene interaction network can help in identifying important genes in certain pathways. The objective of this study was to develop a gene interaction network of oil palm using BioGRID database and Cytoscape 2.8 software. Transcriptome data of mantled developing oil palm flower and fruit in public database in NCBI was used in this study. Gene interaction network of oil palm that related to the mantled was successfully created. *EgAG* and *EgAP3* were identified as mads-box protein cmb1 and mads-box transcription factor 16, respectively. *EgBRM* was identified as a chromatin structure-remodeling complex subunit snf21. It was related with *EgH4* that was identified as a histone h4. *EgCMT2*, which was related with SUMO-conjugating enzyme and small ubiquitin-related modifier genes, was identified as a DNA-methyltranferase 1. *EgSUVH3* was identified as a histone-lysine N-methyltransferase that has connection with *EgTPL*. *EgTPL* was identified as a topless-related protein that has the most interaction with other genes in this gene interaction network. In conclusion, BioGRID database and Cytoscape 2.8 software can be used to make oil palm genes interaction network.

Keywords: BioGRID, Cytoscape 2.8, oil palm, interaction network.

POLYPLOIDY INDUCTION OF *Artemisia cina* USING PLANT GROWTH REGULATOR 2,4-DICHLOROPHENOXYACETIC ACID AND BENZYLADENINE

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ABSTRACT

Artemisia cina is a medicinal plant that uses in anthelmintic, antibacterial, antifungal, antitumor, and antimalarial because of their secondary metabolites content. Content of secondary metabolites in *A. cina* is low. Polyploidization is the way to improve plant quality, includes increasing of secondary metabolites. Polyploidization can be done by adding chemical compounds, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA). The aim of this study was to induce polyploidy using plant growth regulator 2,4-D and BA in *A. cina* root culture. The comparison of the treatment consisted of 2,4-D and BA both in 1 ppm, 1.5 ppm, 2 ppm, and 3 ppm that were combined in 16 treatments. Root culture examined at 21 days after treatment. Chromosome examination was conducted by squashing method and chromosome numbers were calculated under microscope. The results showed chromosomes number was various in 2x, 3x, 4x, 5x, 6x, 7x, and 8x, with the modal number of chromosome x = 9. Diploid chromosome was found in all treatment, but the highest chromosome number 8x = 72 only found in treatment by combination of 1 ppm 2,4-D + 2 ppm BA and 1.5 ppm 2,4-D + 1 ppm BA. Tetraploid plant was found in treatment by combination of 2 ppm 2,4-D + 1 ppm BA, 2 ppm 2,4-D + 1.5 ppm BA, and 3 ppm 2,4-D + 2 ppm BA. Tetraploid plant was found more frequently in treatment by combination of 2 ppm 2,4-D + 1 ppm BA. This result shows that tetrapoid plant which is the target in improving plant quality at many studies of plant breeding can be created by inducing plant growth regulator by combination of 2 ppm 2,4-D + 1 ppm BA in *A. cina*.

Keywords: polyploidization, *Artemisia cina*, 2,4-dichlorophenoxyacetic acid, benzyladenine, tetraploid plant.

ISOLATION OF GENE ENCODING EXTRACELLULARLY SECRETED PROTEASE FROM MARINE ENDOPHYTIC FUNGUS *Xylaria psidii* KT30

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ABSTRACT

Marine fungus *Xylaria psidii* KT30, isolated from seaweed *Kappaphycus alvarezii* BRKA-1, is known to have cytotoxic activity against HeLa cervical cancer cells and protein pellet. The cytotoxin is believed to be the type of serine proteases and exhibit antibacterial activity against *Bacillus subtilis* and *Escherichia coli*. Until now, information about the gene encoding protease in *X. psidii* KT30 has not been known. The purpose of this research was to determine the gene encoding protease from marine-derived endophytic fungus *X. psiidi* KT30 through isolation and characterization by molecular techniques. Isolation of the gene fragment was begun with synthesis of cDNA from total RNA extracted from 15-day old culture of the fungal mycelium. Partial gene amplification through 3' RACE - RT-PCR obtained a band size approximately 900 bp. The partial gene was cloned into pGEM-T vector using *E. coli* TOP10 and DH5α as the host. Colony PCR results showed that the partial gene have inserted into the plasmid and the band size was similar to RT-PCR results. The obtained gene fragment will be useful for construction of bioinformatics library in an effort to develop anticancer drugs in medical field.

Keywords: *Xylaria psidii* KT30, antibacterial, anticancer, gene encoding, protease.

A SINGLE DIRECTED MUTAGENESIS OF PHYTASE GENE OF *Aspergillus niger* FOR CONSTRUCTION IN pCAMBIA1303

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ABSTRACT

Phosphorus (P) is taken up by plants as orthophosphate (Pi). However, in most cultivated soils for agriculture, organic P comprises 30–80% of the total P, and approximately 60–80% of organic P exists in the form of phytate and is not directly available to plants. Thus, improving phytate-P bioavailability is important for plant P nutrition and for sustainable agricultural development due to the exhausting P ore resources worldwide. Objective of the research was to construct the phytase gene of *Aspergillus niger* in pCAMBIA vector. However, phytase gene construction in pCAMBIA could not be conducted due to the presence of all pCAMBIA restriction enzyme sites in phytase gene. In order to enable phytase gene cloning to pCAMBIA1303, a single direct mutation of *Bst*EII restriction site of the gene was made. The mutation was done by using PCR method in which nitrogen base T was replaced with C or A. The single mutation was verified by *Bst*EII enzyme cutting and sequencing before and after mutation. The mutation has allowed phytase gene construction in pCAMBIA successfully conducted.

Keywords: phytase gene, mutagenesis, *Aspergillus niger*, PCR.

CONSTRUCTION OF EXPRESSION VECTORS OF PLANTARICIN W FROM *Lactobacillus plantarum* S34

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ABSTRACT

Plantaricin W, a bacteriocin from *Lactobacillus plantarum*, has potentially useful as alternative antibacterial such as against *Salmonella thypi*. In *L. plantarum*, plnW gene encoding plantaricin W is controlled by strict regulatory mechanism of bacteriocin system that has different condition from expression in *Escherichia coli*. In order to obtain compatible lactobacilli expression system, plnW was cloned using pSIP system vector with the bacteriocin regulon from regulatory element were derived by sakacin A or sakacin P. The plnW was synthesized by PCR and ligated into pSIP302 and pSIP409 vectors at multiple cloning site. The recombinant plasmid was transformed into *E. coli*. It was sequenced and the result showed that plnW was successfully cloned with 450 bp in size. This study further can be used to develop a system for inducible gene expression study in lactic acid bacteria.

Keywords: plantaricin W, pSIP system vector, *Lactobacillus plantarum* S34.

OPTIMIZATION OF RECOMBINANT PLANTARICIN F EXPRESSION FROM *Lactobacillus plantarum* S34 IN *Escherichia coli*

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ABSTRACT

Recombinant plantaricin F (plnF) with 52 amino acids in length is an antimicrobial protein isolated from bekasam-derived *Lactobacillus plantarum* S34. Previously, plnF harboring both leader and mature peptide fused with 6× histidine theriodoxin has been successfully expressed in *Escherichia coli* BL21 (DE3) pLysS by using pET32a as the expression vector. The objective of this study was to investigate the optimal conditions for plnF recombinant expression in *E. coli* BL21 (DE3) pLysS. Different incubation times (1 hours, 2 hours, 3 hours, 4 hours, and 5 hours after induction), IPTG concentrations (0,1 mM, 0,5 mM, and 1 mM) and induction temperatures (22ºC, 25ºC, and 37ºC) were compared for obtaining the optimal expression conditions. The expressed recombinant plnF has a molecular mass of 24 kDa when separated on SDS-PAGE. The production of recombinant plnF was temperature dependent. When the host was induced with 0.5 mM IPTG treatment under the incubation temperature of 25ºC, the level of recombinant plnF production could be enhanced. Its production was optimum when the host was incubated at 22ºC and was lowest at 37ºC. The recombinant plnF expression gradually increased following longer periode time of incubation and reached optimum level at 5 hours after incubation. In summary, recombinant plnF harboring both leader and mature peptide fused with 6× histidine theriodoxin has been successfully expressed in *E. coli* BL21 (DE3) pLysS by using pET32a as a vector expression.

Keywords: recombinant plantaricin F, antimicrobial protein, optimal expression.

RECOMBINANT PRODUCTION OF PLANTARICIN W FROM *Lactobacillus plantarum* S34: CLONING AND EXPRESSION IN *Lactobacillus acidophilus*

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ABSTRACT

Plantaricin W (plnW) is a bacteriocin from *Lactobacillus plantarum* that can inhibit a large number of pathogenic bacterium. In production of recombinant plantaricin with view from the food safety demands, lactic acid bacterium (LAB) would be better than *Escherichia coli* since most of them are "generally recognized as safe" (GRAS). The aim of this study was to obtain crude of bacteriocin plnW in gram-positive bacterium *L. acidophilus*. *plnW* gene encoding plnW from *L. plantarum* S34 that isolated from Indonesian native food fermentation 'Bekasam' was cloned into lactobacilli expression vector pSIP409 then transferred to *L. acidhophilus* strain C1-9 by electroporation method. The plantaricin gene has been expressed intracellularly. It showed 16.83 kDa protein in SDS-PAGE. The results lead the way to overexpression of plnW by inducing peptide.

Keywords: *L. plantarum* S34, *L. acidhophilus* C1-9, plantaricin W, pSIP409.

ABSTRACTS OF POSTER PRESENTATION

PARENTAL POLYMORPHISM AND BC_1F_1 GENOTYPING IN DEVELOPMENT OF RICE (*Oryza sativa* L.) CSSL BASED ON 'Ciherang'

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ABSTRACT

Increasing yield potential of modern *indica* cultivars is essential to meet the rising demand for rice production due to increasing human population, faced with the threats of climate change and dwindling natural resources. The use of chromosome segment substitution lines (CSSLs) should be more effective for identification of genes that will be useful for improvement of yield potential. The aims of this study were to: identify polymorphic microsatellite markers among parents, observe the morphological trait differences between them, verify the F_1 individuals, determine genotype of BC_1F_1 individuals, and identify introgression of chromosome segment from donor parent on genetic background. This study used 'Ciherang' as recipient parent and new plant type line 'B11143D' as donor parent. Out of 513 microsatellite markers surveyed, 155 (30.2%) markers distributed throughout the 12 chromosomes showed polymorphisms between 'B11143D' and 'Ciherang'. Both parents showed significant differences on flowering time, plant height, flag leaf length, flag leaf area, tiller number, productive tiller number, panicle length, spikelet number per panicle, and 1000-grain weight. Marker genotyping using three polymorphic markers showed that 26 of 27 plants tested were heterozygous. Eighteen of 40 BC_1F_1 individuals selected based on their genotype. Selected plants will be backcrossed with 'Ciherang' to produce BC2F1 population. These results provide a strong basis to develop a CSSL panel that will facilitate the mapping of genes controlling the yield potential.

Keywords: CSSL, 'Ciherang', new plant type, *Oryza sativa*, microsatellite markers.

IDENTIFICATION OF *Hd6* GENE IN RICE MUTANT VARIETIES (*Oryza sativa* L.)

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ABSTRACT

The nature of heading date trait of rice can be detected through *Hd6* gene. The objective of this research was to identify the heading date trait in rice mutant varieties based on their specific DNA fingerprint using microsatellite markers. Rice mutant varieties, such as cv. Kahayan, Merauke, Sulutran 1 and 2, and positive control 'Nipponbare', were used in this experiment. Several primers to days to heading used for identification of these mutant varieties using PCR-based method. Result showed that all of rice mutant varieties consist of a diverse variety of maturity classes identified with linked to *Hd6*, which were found in chromosome 3 of rice genome. Based on seven primers analysis, all mutant varieties including 'Nipponbare' did not possess some genes related to days to heading as demonstrated no amplicons from primer 1 and primer 6, respectively. Rice mutant varieties cv. Merauke, Kahayan, Sulutran 1 and 2 had compliment to primers 2, 3, 4, 5, and 7 with size of each primer were 780 bp, 450 bp, 600 bp, 660 bp, and 540 bp, respectively. This DNA fingerprint data is very useful to differentiate the heading date trait among the rice mutant varieties.

Keywords: DNA fingerprinting, SSR marker, *Hd6* genes, heading date.

PATHOTYPES EVALUATION OF INDONESIAN *Xanthomonas oryzae* pv. *oryzae* POPULATION CAUSING BACTERIAL LEAF BLIGHT ON DIFFERENTIAL RICE

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ABSTRACT

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most destructive bacterial diseases of rice in Indonesia. The deployment of resistant cultivars is thought to be the best approach to control BLB. The purpose of this work was to study virulence of fifteen *Xoo* isolates population collected from three different regions in Indonesia (North Sumatera, South Sumatera, and South Sulawesi) based on five Indonesian differential rice genotypes and ten near isogenic lines (NILs) that have been known for BLB resistance. In addition, this study also aimed to monitor response of NILs to BLB disease under field assessment. Based on the susceptibility of the NILs genotypes with single resistance gene, the fifteen isolates showed different virulence pattern indicating the pathogen diversity. Eight different pathotypes were present, as demonstrated by a particular virulence pattern of each isolate on the genotypes. Evaluation of *Xoo* using Indonesian differential rice genotypes (Kozaka system) revealed that BLB pathogen was dominated by unknown *Xoo* pathotypes followed by pathotype XII. However, the *Xoo* pathotypes showed differently on the reaction to NILs and Indonesian differential rice genotypes. The field assessment demonstrated the incidence and severity of BLB disease on rice genotypes ranging from 25% to 100% and 5.5% to 72.91%, respectively. While the mean disease index ranged from 1.15 to 72.9. The disease response varied among rice genotypes. Importantly, IRBB50 (*Xa4* + *xa5*), IRBB51 (*Xa4* + *xa13*), IRBB52 (*Xa4* + *Xa21*), IRBB53 (*Xa4* + *Xa21*), IRBB56 (*Xa4* + *xa5* + *xa13*), IRBB57 (*Xa4* + *xa5* + *Xa21*), IRBB59 (*Xa4* + *xa13* + *Xa21*), IRBB64 (*Xa4* + *xa5* + *Xa7* + *Xa21*), IRBB66 (*Xa4* + *xa5* + *Xa7* + *xa13* + *Xa21*), IRBB7 (*Xa7*), Angke (*Xa4* + *xa5*), and Code (*Xa4* + *Xa7*) showed highly resistant to the BLB pathogen. These genotypes could be potential as genetic materials by pyramiding of several resistance genes for improved rice resistance to BLB in Indonesia.

Keywords: rice, virulence, BLB, pathotype evaluation, *Xanthomonas oryzae* pv. *Oryzae*.

ISOLATION AND SEQUENCE ANAYSIS OF TUNGRO VIRUS ORF IV FROM INDONESIAN RICE CULTIVAR *Oryza sativa* L.: A WAY TO CREATE INDONESIAN TUNGRO TOLERANT RICE MUTANT

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ABSTRACT

Food security is one of the pillars of development. Thus, it is important to enhance its productivity. Of the several factors limiting rice production, several biotic factors operate against rice in which include mainly bacteria, fungi, and viruses. The tungro disease is caused by the joint infection with two dissimilar viruses, a bacil-form-DNA virus, the *Rice tungro bacilliform virus* (RTBV) and a spherical RNA virus, *Rice tungro spherical virus* (RTSV). The tungro viral complex (RTBV and RTSV) are transmitted from plant to plant by green leafhopper/GLH (*Nephotettix virescens*). RTBV is unable to be vectored by GLH alone; it requires the presence of RTSV. It indicates that RTSV is a helper for virus transmission. The symptom of disease is caused by the presence of RTBV. The genome of RTBV consists of four open reading frames (ORFs), which encode functional proteins. Of the four, ORF I, II, and IV are relatively small, potentially encoding proteins of molecular weights between 12 and 46 kDa, whereas ORF III is much larger, encoding a potential protein of molecular weight 194 kDa. ORF IV of RTBV is unique because it exists only in RTBV. We are improving rice tolerant to tungro by silencing ORF IV. Here, we report the isolation of ORF IV of RTBV from 'Ciherang' rice cultivar by PCR. The primers were designed using the sequence of ORF IV of RTBV (Acc. M 65026.1). The amplicons of ORF IV of RTBV were 1.17 kb from the total genomic DNA of the infected plants. The sequence alignment has confirmed that this ORF IV gene has been isolated and showed 92% similarity with the reference sequence. This research is still in early step to clone into pCAMBIA 1305.1 binary vector. Then, the amplified virus gene can be used further to generate tungro resistant rice mutant.

Keywords: tungro, ORF IV, sequence analysis, rice mutant.

GENETIC VARIABILITY AND HERITABILITY OF GREEN SUPER RICE (GSR) LINES TO *Rice ragged stunt virus* AND *Rice grassy stunt virus*

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ABSTRACT

Brown planthopper (BPH) is one of the main pest of rice in Indonesia and it occured every years with fluctuate acreage. BPH is also a vector of *Rice ragged stunt virus* (RRSV) and *Rice grassy stunt virus* (RGSV). Green Super Rice (GSR) was designed to have resistance to major pests and diseases so that it needs less pesticides and thus save to the environment. GSR was developed in IRRI and China, and it was tested in Indonesia since 2009. This research was aimed to study the genetic variability and heritability of twenty-six selected GSR lines to RRSV and RGSV along with four check varieties. The experiment was conducted during dry season (DS) of 2010 in ICRR Sukamandi and Pusakanagara Experimental Station. The experiment was arranged following randomized complete block design with three replications. Transplanting was done into 21 days old seedling into 25 cm $\times$ 25 cm planting space of 1 m $\times$ 1 m plot. BPH and virus infestations were occured naturally due to BPH outbreak along the season. Percentage of plant showing RRSV and RGSV symptom was measured as consideration the resistance of plant to the viruses. The results showed that the tested genotypes had high genetic variability and heritability classified as medium in the resistance to RGSV. The genotypes showed low genetic variability and heritability in the resistance to RRSV. ZHONGHUA 1, HUA 564, and HHZ 11-Y6-Y1-Y1 were consistently resistant to RGSV and thus could be used as donor of resistance to RGSV. It implies that breeder effort is feasible to develop resistant lines to RGSV.

Keywords: genetic variability, heritability, RRSV, RGSV.

ISOLATION OF A *SUMO E2 Conjugating Enzyme (SCE1)-like* GENE FROM RICE AND ITS FUNTIONAL ANALYSIS IN RESPONSE TO DROUGHT STRESS

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ABSTRACT

Small ubiquitin-like modifier (SUMO) conjugating enzyme is one of the enzymes involved in post-translational regulatory process in eukaryotes, named sumoylation. It has important role in improving plant tolerance to abiotic stresses, such as salt, drought, heat, cold, etc. In our previous analysis of a rice mutant line tolerant to PEG 6000, we identified an *SCE1-like* gene about 12.3 kb upstream of Ds transposon-based activation tagging insertion in the rice cv. Nipponbare genome. Here, we report the isolation of the *SCE1-like* gene from rice cv. Nipponbare and the establishment of transgenic rice over-expressing the gene. We successfully amplified a 483 bp fragment by RT-PCR and cloned it into pGEMT-Easy vector. BLASTX analysis of the amplicon showed that it has 100% identity to the *SCE1-like* gene. The isolated gene was then subcloned into expression vector pCAMBIA1305 and introduced into rice cv. Nipponbare by *Agrobacterium*-mediated transformation. Functional analysis of the identified *SCE1-like* gene is being performed to further understand the roles of the gene in plants during drought stress.

Keywords: *SCE1-like* gene, cloning, drought stress, functional analysis.

RESPONSE OF VERY EARLY MATURING RICE GENOTYPES TO VEGETATIVE AND GENERATIVE PHASE DROUGHT STRESS CONDITIONS

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ABSTRACT

Global climate change had caused the spread of drought prone areas. Right now, drought tolerant released variety is very limited. Meanwhile, there were plenty of germplasm collections had not been screened yet for drought tolerance, especially field screening. This research was aimed to know the response of 23 rice genotypes consisted of 2 released varieties, 10 local varieties, and 10 introduced lines to vegetative and generative phase drought stress conditions. The experiment was conducted in ICRR Experimental Station in Sukamandi during dry season of 2009. There was no replication and analysis was conducted by T test. The result showed that plant responded to drought through the following traits: plant height and tiller number of 1 month and 2 months after planting, chlorophyll content during exposed to drought stress and recovery, yield (g/clump), shoot length, straw weight, panicle length, shoot diameter, and filled and unfilled grain/panicle. There was almost no significant difference between vegetative and generative drought stress occurrence. It might due to severe drought stress happened during the season. Based on yield, drought sensitivity index (DSS), and relative values (RV), there were two genotypes identified to be relatively tolerant to vegetative and generative drought, i.e. OM1490 and Situ Patenggang. OM1490 was then to be released as Inpari 13. OM1490 had higher yield compared to Situ Patenggang at optimum (28.17 g/clump vs. 12.12 g/clump), vegetative (24.11 g/clump vs. 13.54 g/clump), and generative drought stress conditions (24.72 g/clump vs. 0.36 g/clump).

Keywords: rice, drought, yield, very early maturing.

FIELD GROWN NOVEL TRANSGENIC *INDICA* RICE WITH POLISHED GRAIN IRON LEVEL POTENTIALLY PROVIDES SUFFICIENT DIETARY IRON IN RICE-BASED DIETS

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ABSTRACT

Iron (Fe) and zinc (Zn) deficiencies are the most prevalent forms of micronutrient malnutrition globally. Rapid progress in biofortification through transgenic approaches demonstrates the feasibility to enhance Fe in polished rice by expressing iron storage, chelator or transporter genes or their combination. Nutritionists estimate that about 14 ppm of iron is needed in milled rice to provide sufficient dietary iron in rice-based diets (30% of the estimated average requirement). To date, there has not been any published report showing polished rice grain iron close to this target level under field condition. We evaluated seven different constructs containing soybean ferritin (*SferH-1*) and rice nicotianamine synthase (*OsNAS2*) genes under control of various promoters. We developed more than 1600 transgenic events of IR64 mega variety. We selected eleven promising events showing high intensity in the iron staining and having single T-DNA insertion. The homozygous lines and negative segregants of the selected events were evaluated in the confined field trials in Los Banos, Philippines, and in Cali, Colombia. A single insert transgenic event showing no yield penalty, with polished grain Fe concentration of 15 $\mu g.g^{-1}$ (6 fold increase) and Zn of 45.7 $\mu g.g^{-1}$ (3 fold increase) was identified. Synchrotron analysis showed the distribution of Fe and Zn in the starchy endosperm. Caco-2 cell-line assay showed increase in Fe bioavailability of the transgenic rice grain. Characterization of the integration site showed that no coding region of endogenous rice gene was disrupted. This novel transgenic line has a potential significant impact on the alleviation of Fe and Zn deficiencies.

Keywords: Fe biofortification, transgenic, rice, *indica*, confined field trial.

PHENOTYPIC EVALUATION AND PRELIMINARY STUDY ON GENE EXPRESSION OF GANYONG (*Canna indica*) FOR SHADE TOLERANCE TRAITS

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ABSTRACT

Ganyong (*Canna indica*), known as edible canna that is underutilized species, is one of the highly potential food crops and an alternative source of carbohydrates as rice substitutes that needs to be developed. Since the plant tolerates to shade and can grow on marginal land, the area of cultivation is not competing to other food crops. *PHYA* belongs to phytochrome gene family of photoreceptors that involves in light signaling. The purposes of our study were to evaluate the phenotypic differences as well as to measure the transcripts of the photoreceptor genes under high and low light intensities. For gene expression study, currently we focused on *PHYA*, *GAPDH*, and *ACT2* genes. *GAPDH* and *ACT2* are commonly used as reference genes for gene expression study. In this study, the red variant of *C. indica* rhizomes originating from West Java, Indonesia was planted under shaded and nonshaded environments. The phenotypic differences between these environments were measured to validate the effect of shade treatment. The total RNA was isolated from young leaves, converted into cDNA, and the phytochrome transcripts were measured using quantitative real-time PCR. As the results, phenotypically the shaded *C. indica* showed more leaves than the nonshaded ($p = 0.026$). The leaves were thinner ($t28 = 9{,}290$; $p < 0.001$) and longer ($t27 = 4.826$; $p < 0.0001$) than the nonshaded. Moreover, the shaded plants were slower in flowering than the nonshaded (12 days difference). The weight of rhizomes was 0.58 kg and 2.32 kg for the shaded and nonshaded plants, respectively. Additionally, in our preliminary gene expression study, we have designed the degenerate primers for the genes of interest. At the moment, the amplicon of each gene is subjected to the process of sequencing. As a conclusion, the phenotypic data as shown by the shaded *C. indica* was consistent with shade-avoidance response, with exception to flowering time.

Keywords: *Canna indica*, ganyong, phenotypic characters, shade tolerance.

IDENTIFICATION OF EARLY-TUBER INITIATION GENOTYPE OF *Ipomoea trifida* GERMPLASMS FROM CITATAH-WEST JAVA BASED ON HIGH-DENSITY SELECTION METHOD

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ABSTRACT

Sweet potato (*Ipomoea batatas*) is an autohexaploid species (2n = 6x = 90; genome = B), which evolved from diploid species of *I. trifida* (2n = 2x = 30; genome = B). The complexity of chromosome number, self-incompatibility and cross-incompability traits, and the low flowering rate on a number of sweet potato's clones constrained the progress in its breeding on hexaploid level. Genetic improvement on diploid level using *I. trifida* germplasms is a breakthrough to accelerate alleles fixation, particularly on quantitative traits. *I. trifida* germplasms from Citatah has been reported in carrying superior tuberous root traits. Eight millions seedlings (86 kg of seeds) were screened using high-density selection method to obtain *I. trifida* genotypes which have early-tuber initiation. Through this method, several *I. trifida* genotypes with early-tuber initiation (less than one month) had been identified. These genotypes could be used in developing early-tuber initiation and high yield genotypes.

Keywords: *Ipomoea trifida*, sweet potato breeding, allele fixation, high-density selection method, early tuber initiation.

MOLECULAR CHARACTERIZATION OF BLACK POTATO (*Plectranthus rotundifolius* [Poir.] Spreng.) MUTANTS INDUCED BY GAMMA-RAYS IRRADIATION TOLERANT TO DROUGHT AND SALINITY STRESS

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ABSTRACT

Black potato (*Plectranthus rotundifolius* [Poir.] Spreng.) is one of the alternative food for several parts in Indonesia. Genetic improvement for black potato is limited as low genetic variation. Plant breeding through mutation, e.g. irradiation of γ rays, can be assumed to improve genetic diversity. The aim of this study was to characterize mutants of black potato irradiated in γ rays that are tolerant to salinity and drought stress. Putative mutants irradiated in γ rays each were treated in 0,1–0,5% NaCl and 0,1–0,6% PEG to assess their tolerance to salinity and drought stress, respectively. Each of these treatments was subjected to molecular characterization using five primers of ISSR (UBC 807, UBC 811, UBC 812, UBC 834, and UBC 835) and five primers of RAPD (OPA-13, OPB-10, OPB-17, OPD-8, and OPN-14). PCR amplifications of ISSR and RAPD of putative mutants treated in salinity stress generated 94 fragments to which 86 (95%) bands were polymorphic and 27 were specific bands (ranging from 250–1700 bp). Meanwhile, amplification of ISSR and RAPD of putative mutants exposed to drought stress yielded 69 fragments to which 34 (49%) bands were polymorphic and three were specific bands (ranging from 400–800 bp). All of the results of ISSR and RAPD profiles were combined to perform principal component analysis (PCA). Result showed that mutants were divided into three groups based on specific band that may play role in the formation of groups of mutants. The most distinct group with control character indicates the mutant genotype. M343(1) and SC80(6) were genetically distinct with control plants. It was also suggested that the ISSR and RAPD could be reliable markers for characterization of mutants of black potato.

Keywords: black potato (*Plectranthus rotundifolius*), ISSR, RAPD, salinity tolerant, drought tolerant

DETECTION OF THE EXISTENCE OF ANTHOCYANIN STRUCTURAL GENES BY MORPHO-MOLECULAR ANALYSIS IN *Capsicum* spp.

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ABSTRACT

Anthocyanin, as a plant pigment, is commonly detected to have correlation with resistance level of plants in response to pathogen attack. In chilli peppers (*Capsicum* spp.), anthocyanin content is highly detected in ornamental peppers. Therefore, to increase the level of anthocyanin in an improved variety of pepper can be done by transferring anthocyanin biosynthesis genes to edible pepper in a breeding program. The aim of this study was to detect the existence of anthocyanin structural gene by morpho-molecular analysis as the basic method for selection in a breeding program. DNA was isolated from the leaf of parental pepper that pigmented (ornamental pepper) and unpigmented anthocyanin (edible pepper), also from F_1/F_1 reciprocal generation by CTAB Doyle and Doyle method with modification. Four key anthocyanin structural genes encoding dihydroflavonol 4-reductase (DFR), chalcone synthase (CHS), chalcone isomerase (CHI), and UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT) were detected by amplification with specific primers. Morphological characterization was observed at stem, leaf, flowers, and immature fruit in parental pepper and F_1/F_1 reciprocal generation (twenty plants each). The results showed that there was different intensity of anthocyanin pigmentation in stem nodes, edge of the petal, and immature fruit. *DFR* and *UFGT* genes were present in almost F_1/F_1 reciprocal, differed with *CHI* and *CHS* genes. Related to the existence of anthocyanin structural genes, this result provides new information about the selection program in the next generation.

Keywords: breeding, *Capsicum* spp., anthocyanin biosynthesis gene.

DETECTION OF POTENTIALLY SEEDLESS CITRUS DERIVED FROM PROTOPLAST FUSION BASED ON ISSR MARKERS

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ABSTRACT

Fruit product with quality-oriented and efficient in its production is absolutely necessary in order to compete in the domestic and the global market. Seedless orange (*Citrus* sp.) is one of the main parameters of a must-have in order to be able to compete in the global market. *C. nobilis* var. Simadu that is widely known in Indonesia as Medan orange competes in the global market, but it contains a large number of seeds within the fruit (15–23). The effort to improve the quality of the fruit was done by inserting a gene trait of seedless Satsuma mandarin orange through protoplast fusion technique. Acceleration revenue of seedless plant traits resulted from protoplast fusion is necessary with early detection of molecular basis. The molecular markers used were ISSR markers that compared plant derived from protoplast fusion to both parents. The results showed that the band pattern of plant derived from protoplast fusion showed similarity with the other types of seedless citrus (Satsuma mandarin, Santang, and Ponkam) pattern based on DNA amplification.

Keywords: oranges, protoplast fusion, seedless, ISSR.

SOMACLONAL VARIATION ANALYSIS OF OIL PALM AND ITS CLONES USING MICROSATELLITE MARKERS

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ABSTRACT

Oil palm ortets with high genetic stability can be used as a source of tissue culture explants, because they potentially produce identical clones (true-to-type). The objective of this research was to establish genetic similarity or genetic stability among ortets and their clones based on sixteen microsatellite markers. The oil palm material used for analysis includes oil palm ortet and its clones from ”trial clone evaluation”. Four different genotypes of ortets and twenty clones were used to evaluate genetic changes at the DNA level. Genomic DNA was extracted from young leaf samples and DNA quality was examined using both agarose gel electrophoresis and NanoDrop methods. PCR amplification products were visualized using QIAxcel system. Data analysis was performed using NTSYS-pc ver. 2.02. Genetic stability of clones was determined by comparing the size of clone and ortet alleles. Clones with low coefficient similarity were suspected of low genetic stability based on sixteen microsatellite markers. The differences of alleles between ortet and its clones were assumed to be caused by genetic changes. The result showed that the genetic stability of ortets as explants in the propagation of oil palm by *in vitro* techniques was different based on sixteen microsatellite markers. Ortet C has a high degree of genetic stability there is 0.97 and ortet A has the lowest degree of genetic stability there is 0.69. This was presumably due to the influence of tissue culture process, but further research on methylation and sequence of DNA is still needed.

Keywords: somaclonal variation, *Elaeis guineensis* Jacq., tissue culture, molecular marker.

ASSESSMENT OF GENETIC DIVERSITY IN CACAO COLLECTED FROM KOLAKA, SOUTH-EAST SULAWESI, USING SSR MARKERS

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ABSTRACT

Cacao (*Theobroma cacao* L.) is a tropical tree that mainly cultivated for its valuable seeds, which can be used for the production of chocolate and cosmetics. The important of cacao make this species becomes one of the primary export commodities in many countries including Indonesia. Generally, cacao plantation in Indonesia is being cultivated by small landholders. Kolaka, which is located in South-East Sulawesi province, has long been known as one of the cacao production center in Indonesia. Therefore, many different cacao germplasm can be found in this region. The objective of this study was to assess genetic diversity and relationships of twelve selected cacao clones collected from cacao plantation in Kolaka. Sixteen SSR markers were used to analyse genetic diversity of cacao. Of those markers, 14 markers exhibited polymorphism and generated a total of 70 alleles with an average of 5 alleles per locus. Average polymorphism information content (PIC) values resulted in this study is 0.59. The cluster analysis using UPGMA method based on the genetic similarity coefficient revealed that all cacao clones were separated into two major groups. The first group consisted of nine cacao clones that were further divided into two subgroups, whereas the second one hold three cacao clones. This result indicates that most of cacao clones derived from Kolaka have similar genotypes. Meanwhile, three clones that were clustered separately from the others could be good candidate for cacao breeding program. The information provided in this study would be useful for future cacao breeding, especially to release a new variety.

Keywords: *Theobroma cacao* L., Kolaka, genetic diversity.

EARLY DETECTION OF WHITE ROOT DISEASE ON RUBBER TREE (*Hevea brasiliensis*) BASED ON DNA ANALYSIS

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ABSTRACT

White root disease is caused by *Rigidoporus microporus*. The disease is almost found in all rubber planting areas throughout the world and it is considered to be one of the main pathogen. Traditionally diagnosis of plant disease is recognized based on characteristics of symptoms that caused by the disease and looking for the presence of the pathogen on the surface. However, pathogen may not be present on the surface, symptom can be obscured. The occurance and the prevalence of plant pathogen usually vary from season to season and symptoms can be misleading. We developed molecular biology method using metagenomic approach and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to investigate the presence of *R. microporus* on the soil to prevent the spread and control of the disease. Soil samples were collected from rhizosphere of rubber tree in Medan. Samples were classified by external symptoms of the plants to infested and noninfested. Meanwhile, *R. microporus* was isolated from infested rubber root to examine the presence of the pathogen. DNA of soil samples and *R. microporus* were extracted and 18S rDNA regions were amplified using primer NS-1 and GC-fung that containing GC clamp. PCR amplification products were separated on DGGE to determine the presence of *R. microporus*. The pathogen was visualized by the DNA bands and compared with DNA band of *R. microporus*. The results were showed that visualization on DGGE gel of infested soil indicated containing DNA of the pathogen. However, soil samples of PB330_6a, PB330_8a, and PB260_4a from noninfested soils were also indicated containing pathogen DNA. This was due to the presence of the pathogens in the soil, although it did not nor has not yet infected plants. The availability of PCR-DGGE as an early diagnosis was suggested.

Keywords: white root disease, rubber tree, *Rigidoporus microporus*, DGGE.

GENETIC VARIABILITY OF JATROPHA (*Jatropha curcas* L.) BASED ON MORPHOLOGICAL, AGRONOMICAL, AND RAPD MARKER ANALYSES

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ABSTRACT

Jatropha is a potential biodiesel plant to develop because it does not compete as a source of food or feed. This potential is supported by its tolerance to marginal land. The aim of this study was to characterize and to analyze the genetic variability, heritability, correlation, and resemblance among thirteen genotypes of jatropha collection of SBRC-IPB. The research was conducted using stem cuttings of thirteen jatropha genotypes originating from several regions in Indonesia, namely South Sulawesi, Papua, Parung Panjang (Bogor), Mount Salak (Tangerang), Sumba (East Nusa Tenggara/NTT), Bima, Lombok (West Nusa Tenggara/NTB), and Aceh Besar (NAD). The cuttings were planted in field with 2 m $\times$ 2 m row spacing. Result of the study showed that genotypes had narrow to large genetic variability based on all of the characters studied. The number of inflorescence per plant was chosen as selection criteria to develop high productivity jatropha genotypes. Based on the dendrogram from morphological and agronomical characters on 85% similarity, all genotypes were classified into four groups. Dendrogram based on RAPD markers with 11 primers used was not able to classify the 13 jatropha genotypes observed into the same group as the dendrogram based on morphological and agronomical characters. Based on morphological and agronomical characters and molecular markers, genotypes originating from adjacent areas do not always gather in a same group. Instead, genotypes originating from different areas and geographically may gather in one group. This result showed that there is a need to find new sources of genetic variability through the introduction of jatropha from other countries, hybridization or mutation to obtain new variety of jatropha.

Keywords: heritability, dendrogram, selection criteria, genotype resemblance.

MOLECULAR CHARACTERIZATION OF FOUR ACCESSIONS OF KELADI TIKUS (*Thyponium* spp.) BASED ON matK GENE SEQUENCE

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ABSTRACT

Identification of medicinal plants performed by phenotypic evaluation has limitations. DNA-based techniques have been applied to identify the important medicinal plants and crude drugs. The aims of the research were to identify and to know the relationship between four accessions of keladi tikus (*Thyponium* spp.) based on the matK gene sequence. The four accessions of keladi tikus were collected from Central Java (Matesih, Salatiga, and Ungaran) and Bali (Singaraja). All DNA accessions were isolated and amplified by specific primer of matK gene for keladi tikus. For each accession, PCR product was cloned in pGEM-T Easy vector and the recombinant DNA plasmid was isolated and amplified for sequencing preparation. Sixteen sequences of matK gene were submitted and analyzed for their homology in the GenBank. The phylogenetic tree based on genetic relationships analysis was done by Genetyx software with UPGMA program. The result showed that the accessions of keladi tikus were identified as *T. flageliform* and *T. roxburghii*. The phylogenetic tree showed that they were separated in three groups.

Keywords: *Thyponiuum* spp., matK gene, phylogenetic.

ANALYSIS OF ETHANOLIC NEEM (*Azadirachta indica* A. Juss) EXTRACTS AND THEIR TOXICITY TO MICE

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ABSTRACT

Neem (*Azadirachta indica* A. Juss), known as a multipurpose plant, contains high-level antioxidant. The phytochemical compound of neem is flavonoid. This research was aimed to determine contents of active compound, total flavonoid concentration in neem extracted from leaves and seeds using ethanol, and to conduct toxicological investigation of chronic treatment with ethanol of leaves and seeds extracts of *A. indica* A. Juss on liver tissue sections. The extract was then administered to mice at various doses by oral administration. Phytochemical analysis of neem plant was determined by Harborne methods and its total flavonoid concentration was carried out using spectrophotometry methods. This study showed that total flavonoid concentration obtained from ethanol neem leaves extract was 0.14% while that of from ethanol seeds extract was 2.60%. The antioxidant capacity of the plant extracts was measured by their ability to scavenge free radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl). Thus, leaves of *A. indica* A. Juss acted as an antioxidant agent. The IC50 values of the examined plant extracts were 1005.63 ppm (leaves) and 14.92 ppm (seeds). Based on Gleason classification, these extracts were categorized as nontoxic materials. Histological examination of liver revealed some abnormal, but not significant morphological characteristic. For acute toxicity, liver tissues showed presence of activated kupffer cells, sinusoidal dilatation, and cytoplasmic vacuolation. However, histopathology analysis showed that ethanol extract tends to destroy liver of mice. Moreover, microscopic histology picture showed tendency of heavier damage effect of administration of ethanol extract at dose more than 5000 mg/kg body weight.

Keywords: ethanolic, extract, neem, toxicity, mice.

CLONING AND EXPRESSION PLANTARICIN W FROM *Lactobacillus plantarum* U10 IN *Escherichia coli*

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ABSTRACT

Bacteriocins, referred to as lantibiotics (lantionine-containing antibiotic peptides) produced by lactic acid bacteria, may provide valuable alternative for nontoxic and safe treatment of human infection instead of classical antibiotics. In this study, plantaricin W gene (*plnW*) was originally amplified from indigenous *Lactobacillus plantarum* U10 by PCR. PCR product of plnW then clone into pGEMT Easy System by TA cloning as purpose to replicate. The recombinant pGEM containing *plnW* gene was amplified using *plnW* specific primers containing either *Bam*HI or *Hin*dIII site. Gene of *plnW* was ligated into pET32(a) Trx-fusion system as expression vector. Thus, vector cloned to *Escherichia coli* BL21 plysS. Fusion *plnW* expressed under IPTG induction. The results of this study known that the *plnW* gene *L. plantarum* U10 with 750 bp was successfully expressed in *E. coli* BL21 plysS with 43 kDa as recombinant fusion plantaricin W (Trx-HisTag-PlnW).

Keywords: plantaricin W, *Lactobacillus plantarum* U10, fusion, pET32(a).

BPPT COMPUTATIONAL CLOUD SERVICE FOR COMPUTATIONAL BIOLOGY

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ABSTRACT

High performance computation (HPC) infrastructure became an integral part of research and development in computational biology, especially in genomics, transcriptomics, and proteomics bioinformatics. The large size and the complexity of biological data require a massive resource of computational infrastructure. In order to meet the challenge, we developed computational infrastructure for scientific computation based on linux cluster and open source software. We have successfully deployed HPC facility based on linux cluster.

Keywords: bioinformatics, computational infrastructure, linux cluster.

EFFECTS OF DATA QC ON ILLUMINA DATA READS IN GENOME ASSEMBLY

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ABSTRACT

High-throughput next-generation sequencing (NGS) technologies produce a massive amount of sequence data. For example, the modest Illumina sequencing platform is able to produce 15 Gb of sequencing data. However, it is common that the data produced contains error, which can affect the downstream data analysis. In order to minimize the effects of errors in downstream analysis, data quality control of Illumina reads was performed. The effects of corrected reads and uncorrected reads in assembly process were compared using a real data set of sago genome produced by Illumina GAIIx. As a result, error correction of reads produced longer contigs compared to those of uncorrected reads.

Keywords: bioinformatics, data QC, next-generation sequencing (NGS), *Metroxylon* sago.

LIBRARY PREPARATION FOR NEXT-GENERATION SEQUENCING OF SAGO GENOME

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ABSTRACT

Preparation of high quality libraries is a critical first step in the next-generation sequencing (NGS) workflow, especially for Illumina platform. Since its stringent requirement of sample, then quality of the library is very important. Here, we described a procedure to prepare libraries of sago genome for Illumina platform. There are basically four steps in this procedure, i.e. genomic DNA extraction, library preparation, library QC, and library amplification. The goal of this procedure was to obtain a library of around 300 bp fragment of sago genome.

Keywords: next-generation sequencing, amplification, sago, genome.