Benefits and Risks of Genetically Modified Food Crops in Asia

Edited by *C. George Kuo*
Y. Yogo
Y. Yoshimura

Benefits and Risks of Genetically Modified Food Crops in Asia

Edited by
C. George Kuo
Food and Fertilizer Technology Center for Asian and Pacific Region
Taipei, Taiwan
Yasuhiro Yogo
Institute for Agro-Environmental Sciences, NARO
Tsukuba, Japan
Yasuyuki Yoshimura
Institute for Agro-Environmental Sciences, NARO
Tsukuba, Japan

Cover photo : Mt. Tsukuba taken by Y. Yoshimura

Benefit and Risk of Genetically Modified Food Crops in Asia

Published by
National Institute for Agro-Environmental Sciences
3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604, Japan
TEL: +81-29-838-8148
FAX: +81-29-838-8199
E-mail:marco2013gm@affrc.go.jp
Web:http://www.niaes.affrc.go.jp/sinfo/sympo/h25/20131009e.html

Copy right © 2016 by NIAES – Institute for Agro-Environmental Sciences, NARO
ISBN 978-4-908914-00-3
Issue Date September 1, 2016

Foreword

According to the Food and Agriculture Organization (FAO) of the United Nations, the world's population is expected to reach 9 billion by 2050, driven by growth in developing countries, including Asian region. The FAO also projects that it will require raising global food production by some 70% between 2005 and 2050 to feed such huge population, although arable land area and the fresh water resources are about to reach the limit.

The first green revolution during the 1960s and 1970s was accomplished with the array of programs and policies that introduced high-yield seeds along with petrochemical fertilizers, intensive irrigation techniques, pesticides, etc. Now the next green revolution is on-going. It is not the same as the first one, since it will depend not only on a few miracle varieties but also on tailoring existing varieties to biotic stresses such as emerging infectious diseases, pests and weeds, as well as abiotic stresses such as heat, drought, flooding, and saltiness. In this connection, advanced breeding techniques and genetically modified (GM) crops for stress resistance/tolerance hold promise to achieve high and stable yields under stress environments.

GM crops with herbicide resistant, insect resistant, or both traits are grown on approximately 3.7% of the world's total agricultural land and are expanding. The major crops are maize, soybean, canola and cotton, comprising 98% of global GM acreage (181 million hectares) in 2014, not only in developed but also developing countries. And the new GM crops with other traits such as drought tolerance, diseases resistance, plant-made pharmaceuticals (such as allergy vaccine), higher yield, and enhanced levels of vitamins and nutrients have been developed and likely will be launched soon.

However, insect-resistant GM crops may be toxic to harmless non-target species and beneficial insects, and have the potential to cause allergenic reactions in humans. And herbicide-resistant GM crops may be toxic to mammals. Furthermore, transgenes engineered into GM crops could be unintentionally introduced into the genomes of their free-living wild relatives, which then cause negative impacts on biodiversity. Thus, taking into account risks to human health and the environment, the Cartagena Protocol on Biodiversity was adopted and entered into force in 2000

Towards this direction, the National Institute for Agro-Environmental Sciences (NIAES) in collaboration with the Monsoon Asia Agro-Environmental Research Consortium (MARCO) and the Food and Fertilizer Technology Center (FFTC) organized the workshop on “Benefit and Risk of Genetically Modified Food Crops in Asia” on October 9-10, 2013 in Tsukuba, Japan. The workshop provided an opportunity for the concerned scientists to exchange the knowledge of a state-of-the-art on the development and safe use of GM food crops, to identify and discuss the main points of uncertainty regarding benefits and potential risks, and to establish a platform for technical cooperation and capacity building among Asian countries.

This book covers the part of the workshop. I hope that this book will support and contribute to advance research and development for beneficial and safety use of GM crops for the future.

Kiyotaka Miyashita
President
National Institute for Agro-Environmental Sciences

Preface

Genetically modified (GM) crops have vital importance and give huge benefits not only in food but also pharmaceutical or industrial purposes for human and biotic and/or abiotic stress tolerance for environment. Therefore, their cultivation areas are increasing year by year and grown in worldwide. On the other hand, GM crops have potential risks on human health and environmental conservation, such as biodiversity.

This book is a collection of 15 selected research papers presented at the MARCO-FFTC Joint International Workshop held in Tsukuba, October 2013, entitled “Benefits and Risks of Genetically Modified Food Crops in Asia”. This Workshop was organized by Food and Fertilizer Technology Center (FFTC) for the Asian and Pacific Region and National Institute for Agro-Environmental Sciences (NIAES) in Japan, in collaboration with the Monsoon Asia Agro-Environmental Research Consortium (MARCO). And it was also supported by College of Agriculture, Ibaraki University and National Institute of Agrobiological Sciences (NIAS).

We extend our deep appreciation to Japan’s Agriculture, Forestry and Fisheries Research Council and Taiwan's Council of Agriculture for financial support to the workshop.

Contents

Foreword

Preface

Symposium Outline

Symposium Background

Objectives

GM Crops for Food Security **1**
Josette Lewis

Targeted Modification of Plant Genomes **3**
Seiichi Toki

Development of Disease Resistant Rice Using the Transcription Factor WRKY45 **8**
Hiroshi Takatsuji

Transgenic Papaya for Ring Spot Virus Resistance **13**
Parichart Bruns, Namthip Pironrit, Bencharong Phungrat, Anjana Boonchod, Nuchard Warin, Simek Chowpongpang, Supat Atthatham and Wichai Kositsatana

Herbicide Resistance in Canola: An Essential Tool for Weed Management in Australia **17**
Christopher Preston and Sarah Morran

Field Trials of Insect Resistant Maize Expressing Bt Gene in Viet Nam **24**
Nguyen Van Liem

Maize Stacked with Genetically Modified Events in the Philippines **28**
Saturnina C. Halos

Development of Marker-Free Transgenic Plants **33**
Ayako Nishizawa-Yokoi and Seiichi Toki

Cloning and Expression Analysis of Genes and Field Evaluation of Salt Tolerant Genetically Modified Wheat (*Triticum aestivum*) **40**
Nasir Ahmad Saeed, Imran Habib, Moddassir Ahmad, Muhammad Rauf, Zahid Abbas Malik, Khurram Shahzad, Khadija Rahman, Zahid Mukhtar and Shahid Mansoor

Expression of MIPS and PAP Genes in Mungbean **51**
Sutkhet Nakasathien, Arunee Wongkaew, Giuseppe Dionisio and Vichan Vichukit

Development of Rice Seed-Based Allergy Vaccine for Treatment of Japanese Cedar Pollen Allergy **60**
Fumio Takaiwa

Oral Immunogenicity of Porcine Reproductive and Respiratory Syndrome Virus Antigen Expressed in Transgenic Plants **65**
Pung-Ling Huang, b*, Hui-Ting Chan, Yi-Yin Do,Min-Yuan Chia, Victor Fei Pang and Chian-Ren Jeng

Compositional Assessment of β-Carotene Biofortified Rice **70**
Jae Kwang Kim, Soo-Yun Park, and Yunsoo Yeo

General View of Environmental Impact Assessment of Genetically Modified Crops **73**
Yasuhiro Yogo

Environmental Impacts and Management Issues of GM Crops: Science/Regulatory Interaction in Comparative Perspective **80**
Masashi Tachikawa

Symposium Outline

Date: 8-10 October 2013
Venue: Tsukuba International Congress Hall (Epochal Tsukuba)
Tsukuba, Japan
Participation Fee: Free with advanced registration
Language: English

Organized by:
National Institute for Agro-Environmental Sciences (NIAES)
Food and Fertilizer Technology Center (FFTC)
Supported by:
College of Agriculture, Ibaraki University
National Institute of Agrobiological Sciences (NIAS)

Symposium Background

The UN Food and Agricultural Organization (FAO) estimates that global food production will need to increase by 40% by 2030 to support much of the population growth that will take place in the developing world as people move from rural to urban areas. Currently, two-thirds of the world's poor reside in Asia and the Pacific region and a recent sharp rise in food prices is bringing food shortages to the region's poor. Following the world unprecedented rise in cereal prices in 2007 to 2008, the global food prices have hit a new peak in the first quarter of 2011. It is a matter of urgency, therefore, to establish sustainable food security systems in Asia and the Pacific region. Compounded with a dwindling natural resource base during a time of global warming and climate change, food productivity increases with genetically modified (GM) crops in global agriculture becomes one of vitally important means to ensure sufficient availability of food and other raw materials for the growing population.

In this case, GM crop is a plant used for the agricultural purposes into which one or several genes coding for desirable traits have been inserted into the recipient plant through the process of recombinant DNA technology. These genes may originate not only from the same or other plant species, but also from organisms unrelated to the recipient crop. The first GM crops became available in the mid-1990s. Since then, the adoption and commercial planting of GM food crops are on a rising trend globally, making an important contribution to the development of crop production systems that reduces the risk of crop losses due to insects and weeds, requires fewer pesticide applications, and increases the yields for all types of farmers in developed and developing countries. GM crops now occupy over 10% of the world's arable land.

The crop traits targeted in genetic engineering are not completely different from those pursued by conventional breeding. Nonetheless, because genetic engineering allows for the direct gene transfer across species boundaries, some traits that were previously difficult or impossible to introduce through introgression in conventional breeding can now be developed with relative ease. World-wide on-going research and development for the coming generations of GM crops include improved quality traits such as higher nutrient contents of food products to help improve the health status of consumers, crops modified to produce special substances for pharmaceutical or industrial purposes, and crops designed to be heat, drought or salt tolerance for adapting to climate change, or toward no-till farming methods, reducing fuel use and CO_2 emissions so as to help mitigate climate change and bring about environmental integrity. Some of them are in the pipeline for commercial production.

In spite of manifold potentials, the development and use of GM crops have raised some concerns about their potential adverse effects on human health and environmental integrity that resulted in the complex socioeconomic and political issues. Nonetheless, as GM crops become increasingly prevalent on the global market, it is imperative for producers, consumers, regulators and other stakeholders in Asian countries to understand the potential and regulatory implications of this new trend. In
order to fill in the information gap on these issues, especially in view of risks and benefits, the Food and Fertilizer Technology Center (FFTC) for the Asian and Pacific Region and the National Institute for Agro-Environmental Sciences (NIAES) in Japan join hands to collaborate with the Monsoon Asia Agro-Environmental Research Consortium (MARCO) and other international/regional partners to organize the proposed workshop.

Objectives

- To exchange the knowledge of a state-of-the-art on the development and safe use of GM food crops
- To identify and discuss the main points of uncertainty regarding benefits and potential risks
- To establish a platform for technical cooperation and capacity building among Asian countries.

GM Crops for Food Security

Josette Lewis
World Food Center, University of California, Davis
Davis, CA 95616, USA

GM crops provide one more tool to improving agricultural productivity and, in combination with broader development interventions, to improving food security. Assessment of the impacts of the few GM crops being grown on broad scale in developing countries, along with progress of technologies in the pipeline, demonstrate that GM crops can increase incomes of small farmers. While the private sector has been the primary vehicle to develop and deliver GM crops to date, the broader application for food security will require public sector leadership spanning research, development, and policy.

The controversy over GM crops has clearly slowed the application of this technology. Still, several developing countries have large-scale production of GM crops that provide clear evidence of benefits to small farmers and, indeed, the rapid growth of acreage planted in these countries speaks to the value proposition for small-scale producers. The leading example, with both the broadest acreage and geographic production in developing countries is insect resistant or Bt cotton. Peer reviewed publications of impact assessments calculate an average yield increase in India of 24% for Bt cotton over conventional varieties, translating into a 50% increase in profit by smallholders. In China, where pesticide use is more prevalent, the yield gains are lower, on average 10%, but the lower production costs due to savings in labor and inputs, nets a $500 per hectare increase in incomes on average (Kathage and Qaim, 2012). The widespread adoption of Bt cotton in other developing countries – South Africa, Burkina Faso, Pakistan, among those in Asia – is likely due to the fact that cotton is not perceived as a food crop, thus not raising as many concerns about food safety, combined with cotton's higher value as a cash crop. GM maize, both as a food crop and animal feed, has been adopted in several developing countries, notably the Philippines and South Africa. Sixty percent of maize now grown in the Philippines is estimated to be GM varieties (Mutuc et al., 2012). A number of other developing countries – Indonesia, Vietnam, China, Kenya, among others – have advanced field trails and regulatory submissions for approval of GM maize.

Beyond these few examples under wide-scale production, experience supporting GM crop collaborations in developing countries through the U.S. Agency for International Development and the private sector over twenty years provides additional examples of significant progress and lessons for the path forward. It is important to focus on the goal: to facilitate equitable access to a greater range of improved varieties, not to win a debate about GM crops. Ultimately, countries must decide the paths for themselves. However, without access to hands-on experience with GM technology and, most critically to a range of GM traits and crops that target food security needs, the impact of this technology will be limited to where commercial opportunities are the highest.

A starting point for achieving broader impact is a vibrant level of research on GM crop development in the public sector. A leading role for the public sector will both broaden the political dialog on GM crops and broaden the range of crops and traits beyond what multinational companies currently provide. The private sector does have a role to play. As discussed earlier, several examples of technologies developed for high value markets such as the U.S. also have delivered benefits in developing countries. Commercial investments in field trials indicate that a number of Asian countries present commercial opportunities: India, China, Indonesia, Vietnam, and the Philippines. In most of these cases, however, the technologies were not developed specifically for these markets. We also see companies willing to engage in public-private collaborations to reduce risks or pursue opportunities where the commercial market is limited. Examples such as Bt brinjal in India with Mahyco and development of salt-tolerant rice in Bangladesh by Arcadia Biosciences illustrate the value of harnessing private sector investment for food security. However, stoking the pipeline with more specific applications of GM technology will increase the likelihood of success. This requires public funding, not just by donor nations, but funding by developing countries themselves. There are a number of promising examples and some successes to date.

In developing GM crops, the public sector needs to move beyond research toward product development. Often, public research ends with a publication of greenhouse data on a new transgenic plant. As industry knows well, much of this data is not a good prediction of efficacy in the field. Universities and national research institutions need to make the commitment and gain the skills to move GM crops into field trials and beyond. The major milestones in product development include demonstration of efficacy in the field, identification of a commercial event, regulatory submission and then approval, crossing of the GM event in multiple varietal backgrounds, and then seed multiplication and distribution. For conventional varieties in developing countries, these steps are either all done by the public sector, through a blend of public sector research and then private seed company development and marketing. Given the added cost and complexity of regulatory requirements for GM crops, the market opportunity for large seed companies, and the capacity of local seed companies, is more limited. The public sector will need to

fill some of the gaps and reduce the risk to move more types of GM crops and move them further towards commercialization. We see progress in this direction, and some successes where the public sector has taken new varieties to the market. The most successful of these is Bt cotton in China, which was developed and de-regulated through public sector efforts. In the U.S., the Department of Agriculture has successfully commercialized both virus-resistant papaya and plums. Recently, EMBRAPA in Brazil has also commercialized a virus-resistant bean for food security. Within Asia, we see progress through to the point of regulatory submissions for Bt brinjal in Bangladesh and late-blight resistant potato field trials in Indonesia. These illustrate that with government commitment and public funding, public researchers can both broaden and move more GM crops further toward impact.

As has been written in a number of fora, the policy environment for GM crops is a significant challenge. The cost and complexity of regulations decreases the diversity of GM crop-trait products and the diversity of technology developers reaching the market. The most pressing challenge is, however, more political than technical: the slow pace or unpredictable process of government decision-making that can stretch the timeline for developing GM crops past the patience of both public or private sector funding. While the political process will continue to evolve, a new generation of genetic tools becoming available present an opportunity to incorporate more than fifteen years of commercial production of GM crops along with greater scientific precision into the evolution of regulatory policies. One class of these new genetic tools, genome-editing technologies such as zinc-fingers, TALENS, and CRISPER/Cas, allow very targeted and specific changes to endogenous DNA rather than introducing sequences from other species as in conventional genetic engineering (Gaj et al. 2013). Cisgenics is an approach that involves introducing DNA sequences but ones that are derived from the same crop. As such, these "cisgenes" are not expected to produce unintended traits or properties in the crop. Lastly, new hybrid technologies such as reversible dominant male sterile and double haploids may involve one transgenic parent, but the transgenes are not passed on to the offspring. The recent regulatory decisions and discussions in the U.S. and Europe suggest that technologies such as these may not fall under current regulatory procedures for GM crops, but rather may be considered as posing less risk and thus requiring less regulation (USDA, 2013 & 2012; Waltz, 2011; Schouten et al., 2006; EASAC, 2013).

Such progress, in terms of GM crop technology development and opportunities to examine regulatory procedures for newly emerging technologies, suggest broader opportunity to leverage modern biotechnology for food security. To realize this opportunity will leadership from within developing countries – public sector researchers to broaden and take technologies further down the pipeline and to engage and inform local policy discussions.

References

EASAC Policy Report, 2013. Planting the future: opportunities and challenges for using genetic improvement technologies for sustainable agriculture. *EASAC Policy Report* ,21.

Gaj, T., Gersbach, C.A., and Barbas, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, *Trends in Biotechnology*, 31:397-405.

Kathage, J. and Qaim, M., 2012. Economic impacts and impact dynamics of Bt cotton in India, *Proceedings of the National Academy of Sciences, U.S.A.*, 109:11652-11656.

Mutuc, M.E.M., Rejesus, R.M., Pan, P., and Youobe Jr., J.M., 2012. Impact Assessment of Bt Corn Adoptoin in the Philippines, *Journal of Agricultural and Applied Economics*, 44:117-135.

Schouten, H.J., Krens, F.A., Jacobsen, E., 2006. Cisgenic plants are similar to tranditionally bred plants: International regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports*, 7:750-753.

USDA response letters to Scotts Miracle-Grow company and BioGlow LLC on cisgenics in 2012 and 2013, respectively, www.aphis.usda.gov/biotechnology/reg_loi.shtml.

Waltz, E., 2011. Cisgenic crop exemption. *Nature Biotechnology*, 29:667.

Targeted Modification of Plant Genomes

Seiichi Toki
Institute of Agrobiological Sciences, NARO, Tsukuba, Japan

Abstract

Targeted modification of plant genomes provides powerful tools for understanding detailed gene function and genetic pathways. Furthermore, pinpoint modification of endogenous plant genes is also an attractive method in plant molecular breeding.

One of the best studied technology currently available to induce specific DNA sequence changes into plant genomes is homologous recombination (HR) mediated gene targeting (GT) . GT can induce a variety of mutations, including substitution of a single or multiple nucleotides as well as insertions and deletions. We have successfully produced herbicide tolerant rice and tryptophan hyper-accumulating rice via GT mediated induction of point mutations. However, the frequency of HR mediated GT has been shown to be low in the order of 10^{-3} to 10^{-4} relative to random integration of the GT vector via non-homologous end joining (NHEJ). Therefore, an enhancement of HR pathway and a step to select GT cells are important for the establishment of an efficient GT system.

Of the various approaches taken to improve HR efficiency, utilization of engineered sequence-specific endonucleases to create targeted DNA double-strand breaks (DSBs) that stimulate HR at breaking sites is one of the most effective. We focused on optimizing expression of engineered nucleases and enhanced resection of DSBs sites as well as delivery of template DNA molecules to plant cells to achieve high frequency GT.

As a universal selection system for GT cells, a target-gene-independent selection system such as positive-negative selection with subsequent elimination of the positive selection marker leads to retention of the desired mutation. We demonstrate the use of piggyBac transposon system for the precise excision of marker gene.

We can also use engineered nucleases for targeted mutagenesis via NHEJ in plants. Plants with mutations in genes involved in NHEJ pathway have been tested to alter the mutation spectrum.

Keywords: gene targeting; engineered nuclease; positive-negative selection; piggyBac transposon; targeted mutagenesis

Introduction

Many valuable phenotypes in crops have been shown to be caused by point, or only a few, mutations. Therefore, targeted modification of plant genomes seem to be the cleanest and most direct gene manipulation technique for future molecular breeding. In addition, the precise modification of gene of interest (GOI) in plants via targeted modification is an ultimate technique in functional studies of GOI.

Recent developments of the engineered sequence-specific nucleases shed light on the field of targeted modification of the plant genome. Until now three classes of sequence-specific nucleases have been extensively used: zinc-finger nucleases (ZFNs); transcription activator-like effector nucleases (TALENs); and CRISPR-Cas RNA guided nucleases.

For sequence of a target gene by sequence-specific nucleases, double-strand breaks (DSBs) are repaired either by non-homologous end joining (NHEJ) pathway or homologous recombination (HR) (Fig. 1). Gene targeting (GT) is a transformation technology that can modify target gene using HR. In the case of GT, exogenously supplied vector sequences are used as template sequences for HR repair.

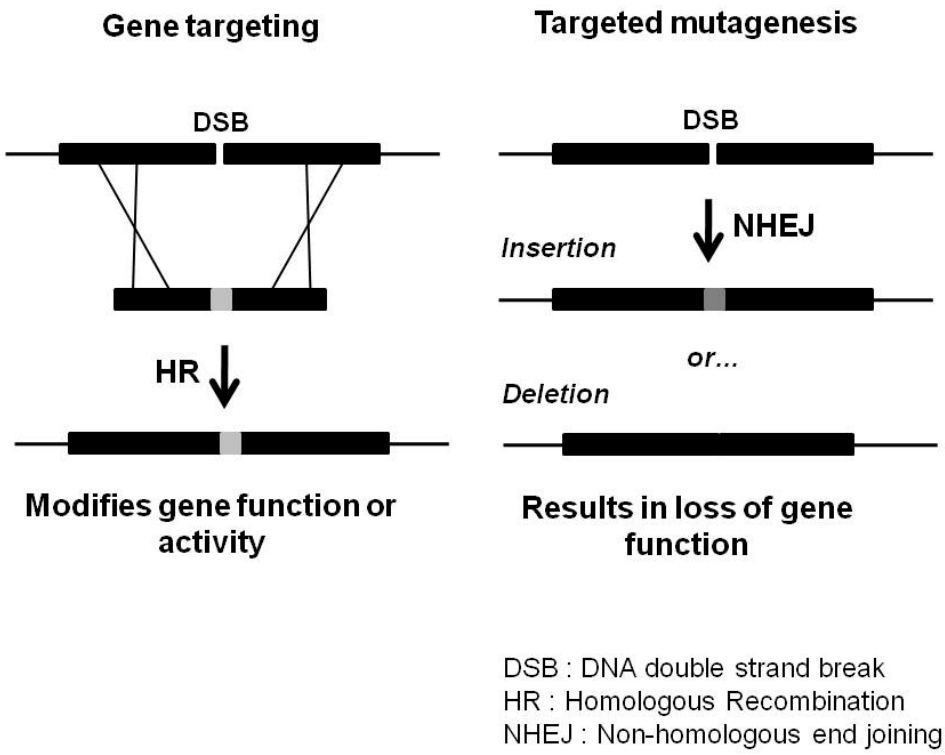


Fig. 1. Engineered sequence-specific nucleases mediated genome modification

DNA double stranded breaks (DSBs) are important for sequence-specific mutagenesis.
DSBs stimulate DNA repair system, homologous recombination (HR) or non-homologous end joining (NHEJ).
HR pathway is involved in gene targeting. NHEJ pathway is involved in targeted mutagenesis.

There is no doubt that induction of DNA double stranded breaks (DSBs) at the site of target gene is effective in improving GT efficiency in plants. However, HR is a minor pathway for DSBs repair in plants, and GT comprises several processes: transformation of a GT vector, the induction of a DSB at the target site, and HR at the DSB site using the GT vector as a template. Furthermore, a step to select GT cells is also important because; in most cases, GT-mediated modification of an endogenous gene does not confer a selectable phenotype such as drug resistance. Whereas the HR pathway is not efficient in plants, direct error-prone rejoining of the DSB ends by NHEJ induces mutations, which can be used for targeted mutagenesis.

This paper reviews four topics as key strategies for establishing an efficient and versatile GT system and targeted mutagenesis system in higher plants: 1) use of sequence-specific nucleases for targeted mutagenesis; 2) induction of DSBs and subsequent resection of DSB ends for improved GT; 3) positive-negative selection of GT cells and subsequent elimination of the positive selection maker gene for seamless GT; and 4) *in planta* GT, addressing the low transformation efficiency of GT vector.

Sequence-specific Nucleases-mediated Site-directed Mutagenesis

ZFNs are synthetic sequence specific nucleases with zinc finger (ZF) domains that recognize a specific DNA sequence, fused to nucleases domain of the restriction enzyme *Fok*I (Fig. 2).

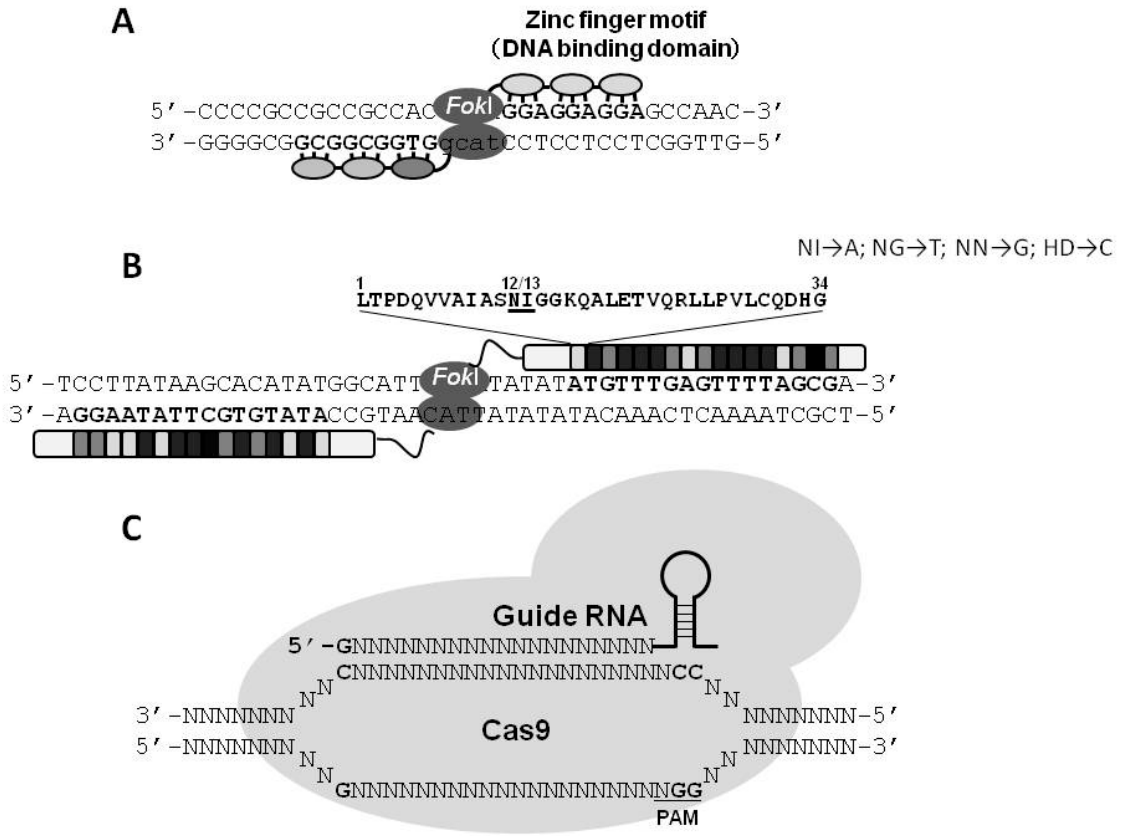


Fig. 2. Three classes of sequence specific nucleases

A . Two ZFNs (zinc finger nucleases) bound to their target sequences. Three ZFs (zinc fingers) recognize 9 bp sequence of DNA. A DNA cleavage domain comprised of the nuclease domain of restriction enzyme *Fok*I. Dimerization creates a DNA double stranded breaks (DSB).

B. Two TALENs (transcription activator-like effector nucleases) bound to their target sequences. RVDs (repeat variable di-residues) in each repeat in the TAL effector array make a base specific binding with the target DNA. *Fok*I nuclease domain is also used for DNA cleavage domain of TALENs.

C. CRISPR-Cas9 RNA guided nucleases. This system uses a nuclease, Cas9 (CRISPR-associated 9), that complex with small guide RNA to cleave DNA in a sequence specific manner at upstream of the PAM (protospacer adaptor motif).

Arabidopsis gene *ABI4* was targeted; mutations in this gene are expected to show a strong phenotype. The expression of the ZFNs was activated by a temperature-inducible system and candidate for mutated abi4 were screened by the Cel I Nuclease Assay. A frequency of 3% was obtained in somatic tissue. Mutation was transmitted to the offspring and expected phenotype was observed in mutants. Furthermore, using *ku80* mutant plants as targets for the ZFNs led to extent of sequence degradation at the junction sites was observed (Osakabe et al., 2010). Ku80 is a key factor of the NHEJ pathway. This result suggested that the mutation spectrum at the site of induced-DSBs is changeable by the regulation of genes involved in DNA repair. Similarly, Zhang et al. (2010) targeted two *Arabidopsis* genes *ADH1* and *TT4* with ZFNs.

TALENs are fusion of the *Fok*I cleavage domain and DNA-binding domains derived from TALE proteins derived from the plant pathogenic bacteria genus *Xanthomonas* (Cermak et al., 2011). Rice *Waxy* gene with TALENs was targeted. In this case TALENs construct was driven with strong constitutive promoter and transformed into rice callus. Mutated cells were detected at the stage of callus. Mutant plants were regenerated from mutated callus and grown into mature plants. Although TALENs are physically larger than ZFNs recognizing the same number of nucleotides, the singe-base recognition of TALE DNA binding domains affords greater design flexibility than triplet-restricted ZF domains.

Most recently, a new entrant—the CRISPR-Cas9 RNA guided nucleases—have been emerged. Compared with ZFNs and TALENs, CRISPR-Cas9 offers much simpler means of attaining specificity. CRISPR-Cas9 system uses RNA as its homing mechanism, while ZFNs and TALENs technologies both depend on custom-making DNA

binding proteins for each DNA target. On the other hand, high off-target effects of the CRISPR-Cas9 system have been reported in human cells. Until now, more than six successful plants site-directed mutagenesis papers were published. Now, it is a time of the CRISPR craze (Pennisi, 2013).

Induction of DSBs via Sequence-specific Nucleases

Engineered sequence-specific nucleases have been exploited widely in higher eukaryotes for the improvement of GT frequency. In higher plants Shukla et al. (2009) and Townsend et al. (2009) reported ZFNs-mediated GT modifications of the maize *IPK1* gene and the tobacco *ALS* gene, respectively. While, Kikuchi et al. (2009) reported that the frequency of DSB-induced GT was enhanced drastically by over-expression of the key factor of resection step namely either Blm or ExoI in chicken DT40 B lymphocyte cells. The counterparts of BLM and ExoI in rice can also enhance break-induced HR, as evaluated by the recombination monitoring system in rice (Kwon et al., 2012). In addition, ExoI over-expression enhanced frequency of ZFNs-mediated GT of rice endogenous gene (Osakabe et al. unpublished result).

Positive-negative Selection of GT Cells for Maker Genes

A GT system using a positive negative selection marker was originally developed to enrich knockout mutants in mice and successfully applied to rice (Terada et al., 2003). In this system, positive selection markers act to select transformed cells and negative selection markers act to remove random integration events. Although this system is a reliable one, accurate and effective excision of the positive marker is indispensable for exclusive introduction of only the desired mutation in to plant genome via GT. To remove a positive selection marker, the use of piggyBac transposon is currently expected. The PpiggyBac transposon is inserted into the host genome at TTAA elements and excised without leaving a footprint at the excised site. We have demonstrated that piggyBac transposon is capable of accurate transposase-mediated tarnsposition in rice, suggesting that exclusive integration of desired mutation could be performed by GT in rice. Although this strategy can be deployed without using a sequence specific nuclease, the approach should be effective in the combination of DSBs induction.

In Planta GT to Improve Transformation Efficiency

GT frequency must be improved using sequence specific nucleases. However with a conventional GT strategy, coordinating formation of a DSB with delivery of the repair template is essential. However set up of this situation is very difficult for plants which are recalcitrant for transformation. A novel GT method called *in planta* GT promises to solve this problem (Fauser et al., 2012). In this system, a GT donor vector flanked by two sequence-specific nuclease recognition sites is first stably integrated into plant genome. Expression of a sequence-specific nuclease cuts within target gene and also excises the integrated donor, and finally results in HR-mediated GT. We have also developed a comparable GT system using inducible sequence-specific nucleases.

Conclusion

Now, CRISPR-Cas9 systems are coming in the field of targeted modification of plant genome. The successful reports of targeted modification in plants will grow in the coming years. The information of comparative genomics and protein structure could lead to the design of improved proteins. These engineered proteins could be expressed in new cultivars which are produced by targeted modification.

References

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C,. Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Research*, 39:e82.

Fauser, F., Roth, N., Pacher, M., Ilg, G., Sánchez-Fernández, R., Biesgen C., and Puchta, H. 2012. In planta gene targeting. *Proceedings of the National Academy of Sciences, U.S.A.*, 109:7535-7540.

Kikuchi, K., Abdel-Aziz, H.I., Taniguchi, Y., Yamazoe, M., Takeda, S., and Hirota, K. 2009. Bloom DNA helicase facilitates homologous recombination between diverged homologous sequences. *Journal of Biological Chemistry*, 284:26360-26367.

Kwon, Y.I., Abe, K., Osakabe, K., Endo, M., Nishizawa-Yokoi, A., Saika, H., Shimada, H., and Toki, S. 2012. Overexpression of OsRecQl4 and/or OsExo1 enhances DSB-induced homologous recombination in rice. *Plant and Cell Physiology*, 53:2142-2152.

Osakabe, K., Osakabe, Y., and Toki, S. 2010. Site-directed mutagenesis in *Arabidopsis* using custom-

designed zinc finger nucleases. *Proceedings of the National Academy of Sciences, U.S.A.*, 107:12034-12039.

Pennisi, E. 2013. The CRISPR Craze. *Science*, 341:833-836.

Shukla, V.K., Doyon, Y., Miller, J.C., DeKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C., Arnold, N.L., Gopalan, S., Meng, X., Choi, V.M., Rock, J.M., Wu, Y.Y., Katibah, G.E., Zhifang, G., McCaskill, D., Simpson, M.A., Blakeslee, B., Greenwalt, S.A., Butler, H.J., Hinkley, S.J., Zhang, L., Rebar, E.J., Gregory, P.D., and Urnov, F.D. 2009. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature*, 459:437-441.

Terada, R., Urawa, H., Inagaki, Y., Tsugane, K., and Iida, S. 2002. Efficient gene targeting by homologous recombination in rice. *Nature Biotechnology*, 20:1030-1034.

Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K., and Voytas, D.F. 2009. High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*, 459:442-445.

Zhan, F., Maeder, M.L., Unger-Wallace, E., Hoshaw, J.P., Reyon, D., Christian, M., Li, X., Pierick, C.J., Dobbs, D., Peterson, T., Joung, J.K., and Voytas, D.F. 2010. High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases. *Proceedings of the National Academy of Sciences, U.S.A.*, 107:12028-33.

Development of Disease Resistant Rice Using the Transcription Factor WRKY45

Hiroshi Takatsuji
Institute of Agrobiological Sciences, NARO, Tsukuba, Japan

Abstract

To explore a cost-effective strategy for protecting rice from infectious diseases, the action mechanism of the chemical defense inducers in rice was investigated. This led to the finding of WRKY45, a key transcription factor in the rice SA pathway that plays a central role in the action of the chemical defense inducers. High-level expression of *WRKY45* (*WRKY45*-ox) rendered rice plants resistant to both rice blast caused by *Magnaporthe oryzae* and bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In addition, *WRKY45*-ox showed fairly strong resistance to brown-spot disease due to fungal pathogen *Cochliobolus miyabeanus*.

WRKY45 was found to be continuously degraded by ubiquitin-proteasome system, which presumably contributes to suppressing unnecessary defense activation in the absence of pathogen infection. A synergistic interaction of the crosstalk between cytokinin and salicylic acid (SA) signaling pathway was also found to play a role in reducing the cost of the defense system on plant growth. Nevertheless, *WRKY45*-ox rice plants exhibited somewhat impaired growth and yield, which were exacerbated by low temperature and high salinity.

To overcome the problems that hamper practical utilization of WRKY45, the expression of *WRKY45* transgene was optimized by using various constitutive promoters from rice, which successfully alleviated the growth/yield problem even under stress conditions. A new strategy for *WRKY45* expression was also developed, in which pathogen-inducible promoters in combination with translational enhancer sequence was employed. This strategy conferred rice with even stronger resistance to rice blast and leaf blight diseases with minimal adverse effects on plant growth/yield. These improved constructs have been introduced into a forage rice cultivar, in which field trials of the transformants are under way.

Keywords: rice blast, leaf blight, brown spot, chemical inducer, forage

Introduction

Rice (*Oryza sativa*) is the main staple food for a large segment of the world population. However, infectious diseases cause huge crop losses annually. Rice blast, caused by the fungus *Magnaporthe oryzae*, and bacterial leaf-blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), are among the most devastating diseases of cultivated rice worldwide. The severity of these diseases necessitates the development of effective control strategies.

In response to pathogen attacks, plants activate defense systems that are mediated through various signaling pathways. One such system is the salicylic acid (SA) signaling pathway, which is mediated by the endogenous signaling molecule SA that rapidly increases upon pathogen infection. Chemical defense inducers such as benzothiadiazole (BTH) (Lawton et al., 1996), tiadinil (Yasuda et al., 2006), and probenazole (Iwata et al., 1980) induce SA-pathway-mediated defense responses in plants, leading to disease resistance. The action of the chemical inducers is characterized by the term 'priming'; treatment of plants with the chemical inducers precondition plants for faster and stronger defense responses upon pathogen infection (Katz et al., 1998; Conrath et al., 2006). Recent studies have shown that the SA pathway also plays a crucial role also in rice (Shimono et al., 2007; Sugano et al., 2010; Takatsuji et al., 2010; Shimono et al., 2012). Several signaling components, such as transcription factors and protein kinases, involved in the rice SA pathway have been identified (Chern et al., 2005; Shimono et al., 2007; Sugano et al., 2010; Takatsuji et al., 2010).

Artificial activation of defense signaling by constitutive overexpression of signaling components can lead to strong enhancement of disease resistance. On the other hand, this approach often causes adverse effects on plant growth and crop yield due to tradeoff of energy and resources between defense responses and plant growth (Heil and Baldwin, 2002). Plants have regulatory mechanisms to avoid unnecessary defense activation. However, excessive activation of defense signaling often overrides such mechanisms. Therefore, careful tuning of the expression of introduced signaling molecules is necessary when we utilize the signaling components to develop practically useful disease resistant crops. In addition, constitutive overexpression of such signaling molecules can result in hypersensitivity to environmental factors. For example, overexpression of NPR1, a key regulator of the SA pathway in dicots, made plants hypersensitive to salt and drought stresses (Quilis et al., 2008). Overexpression of OsNPR1/NH1, a rice ortholog of NPR1, renders rice hypersensitive to light (Chern et al.,

2005). These responses are due to plants' strategy to survive changing environment in nature. However, such unfavorable phenotypes have to be avoided to effectively utilize the functions of signaling molecules for developing disease resistant crops.

We have identified a rice transcription factor WRKY45 that plays a central role in the SA-signaling pathway and BTH-induced disease resistance to various infectious diseases in rice. Strong disease resistance by overexpression of *WRKY45* was accompanied by growth inhibition and yield loss due to the tradeoff, in spite of intrinsic mechanisms to avoid such negative effects. Therefore, fine tuning of its expression was required for transgenic utilization of this gene. This paper reviews identification of WRKY45, its functional characterization, and optimization of its expression to develop practically useful disease resistant rice.

Role of WRKY45 in Rice Blast Resistance

To identify a key transcription factor involved in BTH-induced disease resistance, we characterized global gene expression patterns in wild-type rice (*Oryza sativa* cv. Nipponbare) in response to BTH by using an Agilent Rice Oligo Microarray. This analysis identified 326 BTH-responsive genes expressed at statistically significant levels and they included four genes encoding WRKY-family transcription factors, which had been implicated in the defense mechanisms in various plant species. To functionally characterize these genes, we generated rice transformants that overexpressed their cDNAs under the control of the maize ubiquitin promoter with a strong constitutive promoter activity. Blast resistance tests of the transformants showed that only those transformants that expressed the *WRKY45* cDNA exhibited greatly enhanced resistance to compatible races of blast fungus (races 007 and 003). We also generated *WRKY45*-knockdown (*WRKY45*-kd) rice plants, in which *WRKY45* expression is reduced, and tested them for blast resistance. Without any pretreatment, there was only small difference in the blast resistance between *WRKY45*-kd and control Nipponbare (NB) rice plants. However, obvious difference was observed in BTH-inducible blast resistance. BTH treatment induced strong blast resistance in wild-type rice. However, BTH induced only low level of blast resistance in *WRKY45*-kd rice. *WRKY45* knockdown also reduced the blast resistance induced by probenazole and tiadinil, which are also commercially available chemical defense inducers that act on the SA pathway (Shimono et al., 2007). These results indicate that WRKY45 plays an essential role in the blast resistance induced by this category of chemical defense inducers.

Comparison of the blast resistance with five blast resistant rice cultivars showed that *WRKY45*-ox rice plants were far more resistant than them (Shimono et al., 2012). In addition, *WRKY45*-ox rice plants were also highly resistant to panicle blast, which is more difficult to control than leaf blast because chemical defense inducers are less effective against panicle blast than leaf blast (Shimono et al., 2012).

WRKY45 Overexpression Confers Multiple Disease Resistance

Rice production suffers from damages by not only rice blast but also several other diseases. Leaf blight disease caused by *Xoo* is the most devastating besides rice blast. Thus, we examined the function of WRKY45 in leaf-blight resistance. Results showed that BTH-inducible leaf-blight resistance was reduced in *WRKY45*-kd rice (Shimono et al., 2012). In addition, overexpression of *WRKY45* strongly reduced the lesion by *Xoo* infection (Shimono et al., 2012). These results indicate that WRKY45-dependent disease resistance is also effective against *Xoo*. Moreover, we have shown that *WRKY45*-ox rice plants were also resistant to brown spot disease due to *Cochliobolus miyabeanus*. *Xoo* is a bacterial pathogen while *M. oryzae* and *Cochliobolus miyabeanus* are fungal pathogens; thus, WRKY45-induced disease resistance is effective against two different types of pathogens. Plant pathogens are classified by their life styles. Biotrophs are the pathogens that establish a long-term feeding relationship with living cells of the host, rather than killing the host cells. In contrast, necrotrophs kill host cells and feed on them. *M. oryzae* and *Xoo* are hemibiotrophs that are characterized by an initial biotrophic phase before switching to a necrotrophic growth stage. It is generally accepted that disease resistance by SA-signaling pathway is effective against biotrophs. The effectiveness of the overexpression of WRKY45 to the hemibiotrophs is consistent with this general rule, considering the presence of biotrophic phase during the infectious process of these hemibiotrophic pathogens. *C. miyabeanus* is thought to be a necrotroph. Considering our results, however, this pathogen could also have a short biotrophic phase. *WRKY45*-ox rice plants did not show any resistance to sheath blight disease, which is caused by a necrotrophic pathogen, *Rhizoctonia solani*; thus, the general rule for the effectiveness of SA-pathway-dependent defense appears to hold for the *WRKY45*-ox-dependent disease resistance.

WRKY45-regulated Defense Genes

Microscopic analysis showed that most of *M. oryzae* hyphae were blocked before invading into rice cells in *WRKY45*-ox rice (Shimono et al., 2012). Small number of *M. oryzae* hyphae entered into rice cells; however, hypersensitive-reaction-like cell death induced in the invaded cells restricted further extension of fungal hyphae (Shimono et al., 2012). Microarray analysis revealed that 260 genes are regulated by WRKY45 in response to BTH (Nakayama et al., unpublished). In addition, several genes for the biosynthesis of diterpenoid phytoalexins, which are known to contribute to restriction of *M. oryzae* infection, were induced in *WRKY45*-ox rice after *M. oryzae* infection (Akagi et al., unpublished).

Alleviating Negative Effects of WRKY45-dependent Defense

Defense reactions against pathogens are usually accompanied by negative impacts on plant growth and crop yield as a consequence of tradeoff of energy and resources. We found that WRKY45 is continuously degraded by ubiquitin-proteasome system (Matsushita et al., 2013). This presumably contributes to suppressing unnecessary defense activation in the absence of pathogen infection. We also found that synergistic interaction of SA and cytokinin signaling crosstalk plays a role in reducing the cost of the defense system on plant growth (Jiang et al., 2013; Akagi et al., unpublished).

Cold and Drought Sensitivity Related to *WRKY45* Overexpression

The growth of rice transformants that express *WRKY45* at high levels varied with different growth conditions (Shimono et al., 2007). Results of field trials described below suggested low temperature is one of the factors that negatively affect the growth of *WRKY45*-ox plants. To test low temperature sensitivity of *WRKY45*-ox plants driven by the maize ubiquitin promoter (P_{Zmubi} lines), they were incubated at 8°C for 7 days in a growth chamber along with NB plants and then allowed to recover in a greenhouse for 7 days. As a result, only 2 and 3 plants out of 11 plants each of two independent P_{Zmubi} lines survived, whereas all the control NB plants survived.

High-salinity condition also had negative effects on the viability of P_{Zmubi} lines. After irrigation of plants with 250 mM NaCl for 10 days and then with water for 7 days, only 0, 2, 0 plants out of 16 plants each of three independent P_{Zmubi} lines survived, whereas 12 out of 16 control NB plants survived.

Defense genes such as *PR1a* and *PR1b* were highly induced by both the cold and high-salinity treatments in the P_{Zmubi} lines, while these genes were induced only slightly in control NB plants. These results suggest that the induction of defense genes by the environmental stresses is responsible for the negative effects of high-level *WRKY45* expression on the tolerance of these plants to those stresses. Our interpretation of these results is that the unnecessary defense gene induction by the stresses in the absence of pathogen infection probably wastes plants' energy and resources, which should otherwise be used for cold tolerance, leading to insufficient adaptation to the abiotic stress.

Optimization of WRKY45 Expression

As one of the strategies to optimize *WRKY45* expression, we attempted to drive *WRKY45* with new rice promoters. To this end, we isolated 2-kb upstream sequences of 16 rice genes, which we chose by referring to the levels and patterns of their expression, and fused them upstream of *WRKY45* cDNA. Then, we introduced the resulting 16 constructs into NB rice by *Agrobacterium*-mediated transformation and selected 4-30 transformed lines each containing an intact transgene of a single copy. Initial screening for enhanced disease resistance in T_0 and T_1 generations revealed that many transformants for several constructs had stronger blast resistance than those of control NB plants. Many of them also showed substantial growth retardation in a greenhouse; however, the growth of some of the lines that express *WRKY45* under the control of rice *OsUbi7* promoter (P_{OsUbi7} lines) was nearly compatible with those of NB plants. Expression levels of *WRKY45* in the P_{OsUbi7} lines were approximately 20% those of the P_{Zmubi} lines. In a histochemical analysis of P_{OsUbi7}:*GUS* rice transformants, GUS activities were detected in all the tissues of young seedlings, verifying that the *OsUbi7* promoter is a constitutive promoter.

Blast resistance test using T_3 homozygous lines showed that the P_{OsUbi7} lines were obviously more resistant to blast disease than NB, although they were less resistant than the P_{Zmubi} lines. Comparison of their blast resistance with those of previously known blast resistant rice varieties showed that the P_{OsUbi7} lines were more resistant than Koganenishiki and Mutsuhikari, and nearly as resistant as Chubu 32 and Sensho. These T_3 lines also showed strong leaf-blight resistance against three virulent strains of *Xoo*. Thus, despite the reduced *WRKY45* expression levels, the P_{OsUbi7} lines showed fairly high levels of resistance against both the diseases.

To evaluate agronomic traits of the P_{OsUbi7} lines in field, their T_2 homozygote lines were cultivated in a confined field in Korea (Busan), along with NB and P_{Zmubi} lines. In this condition, the growth of control P_{Zmubi}

line was severely impaired compared with NB. The bad growth of P_{Zmubi} line is presumably at least partly due to low temperature during early stage of rice growth in this field trial. By contrast, the growth of the P_{OsUbi7} lines was only slightly reduced even under this condition. In particular, some lines (#6, 8, 12, and 15) were comparable with or even better than NB except for plant heights. Both P_{Zmubi} and P_{OsUbi7} lines headed earlier than NB.

We also performed field trials of the transformants in a confined field in Colombia (Cali) for two seasons. Day lengths were short throughout the year (11-12 h) in Colombia, and this induced early heading of rice plants, resulting in less biomass compared with that in Korea. Interestingly, the P_{Zmubi} and P_{OsUbi7} lines headed later than NB under this condition, unlike in the confined field in Korea. Presumably due to this effect, relative growth traits of P_{Zmubi} lines vs. NB in Colombia were better than those in Korea. Importantly, agronomic traits of the P_{OsUbi7} lines were compatible or even better than those NB. Presumably, the positive effects of delayed heading compensated for or even overrode the negative effect of *WRKY45* expression on plant growth in the genetic background of NB.

We also tested whether the low temperature and high salinity sensitivities of rice plants due to *WRKY45* expression were improved in P_{OsUbi7} lines. In the low-temperature sensitivity test, all the plants and 11 out of 15 plants of P_{OsUbi7} lines #12 and #15, respectively, survived the low-temperature treatment. In the high-salinity treatment, 12 out of 16 plants each for both lines survived, similar to the NB plants. Thus, the P_{OsUbi7} lines were comparable with NB in the abiotic stress sensitivities.

Considering all these results, we conclude that the P_{OsUbi7} lines (#12 and #15) are optimized for the balance between disease resistance and agronomic traits.

WRKY45 Expression Using Pathogen-responsive Promoters

As an alternative strategy to optimize *WRKY45* expression, we attempted to express *WRKY45* under the control of pathogen-inducible promoters. Based on our microarray analysis of gene expression in response to blast fungus infection, we chose 8 blast inducible genes and used their 2-kb upstream sequences to drive *WRKY45* expression in rice. We also employed a 5'-untranslated region of rice alcohol dehydrogenase (*ADH*-5'UTR) (Sugio et al., 2008) that has a translational enhancer activity. Of the eight promoters, *PR1b* promoter was found to show the best performance. *PR1b*-promoter-driven *WRKY45* construct without the *ADH*-5'UTR conferred only weak disease resistance; however, an improved construct with *ADH*-5'UTR instead of *WRKY45*-5'UTR conferred very strong resistance to both blast and leaf-blight diseases to rice. In addition, the transformants, especially the line #5, showed fairly good agronomic traits in a greenhouse and the confined field in Korea. These results indicate that the use of *PR1b* promoter in combination with the *ADH*-5'UTR is an excellent strategy to express *WRKY45* in a pathogen-inducible manner.

Conclusion

We have developed two strategies to optimize the expression of WRKY45 to create rice lines that are resistant to multiple diseases. Two transformants overexpressing *WRKY45* driven by the constitutive *OsUbi7* promoter showed fairly strong blast and leaf-blight resistance with agronomical traits nearly comparable with NB rice in field and without negative effects of environment. The use of pathogen-responsive promoter *PR1b* in combination with the translational enhancer was also successful. As one of practical applications using this technology, we have introduced the optimized constructs to a forage rice cultivar "Tachisugata"; selection of lines in a confined field is in process. These constructs are potentially applicable to any other rice varieties.

References

Chern, M., Fitzgerald, H.A., Canlas, P.E., Navarre, D.A., and Ronald, P.C. 2005. Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. *Molecular Plant–Microbe Interacteractions*, 18:511-520.

Conrath, U., Beckers, G.J., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M.A., Pieterse, C.M., Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., and Mauch-Mani, B. 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions*, 19:1062-1071.

Heil, M. and Baldwin, I.T. 2002. Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in Plant Science*, 7:61-67.

Iwata, M., Suzuki, Y., Watanabe, T., Mase, S., and Sekikawa, Y. 1980. Effect of probenazole on the activities related to the resistant reaction in rice plant. *Annals ofl Phytopathology Society of Japan*, 46:297-306.

Jiang, C.J., Shimono, M., Sugano, S., Kojima, M., Liu, X., Inoue, H., Sakakibara, H., and Takatsuji, H. 2013. Cytokinins act synergistically with salicylic Acid to activate defense gene expression in rice. *Molecular Plant–Microbe Interacteractions*, 26:287-296.

Katz, V.A., Thulke, O.U., and Conrath, U. 1998. A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. *Plant Physiology*, 117:1333-1339.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal*, 10:71-82.

Matsushita, A., Inoue, H., Goto, S., Nakayama, A., Sugano, S., Hayashi, N., and Takatsuji, H. 2013. The nuclear ubiquitin proteasome degradation affects WRKY45 function in the rice defense program. *Plant Journal*, 73:302-313.

Quilis, J., Penas, G., Messeguer, J., Brugidou, C., and Segundo, B.S. 2008. The *Arabidopsis* AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. *Molecular Plant-Microbe Interactions*, 21:1215-1231.

Shimono, M., Koga, H., Akagi, A., Hayashi, N., Goto, S., Sawada, M., Kurihara, T., Matsushita, A., Sugano, S., Jiang, C.J., Kaku, H., Inoue, H., and Takatsuji, H. 2012. Rice WRKY45 plays important roles in fungal and bacterial disease resistance. *Molecular Plant Pathology*, 13:83-94.

Shimono, M., Sugano, S., Nakayama, A., Jiang, C.J., Ono, K., Toki, S., and Takatsuji, H. 2007. Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell*, 19:2064-2076.

Sugano, S., Jiang, C.J., Miyazawa, S., Masumoto, C., Yazawa, K., Hayashi, N., Shimono, M., Nakayama, A., Miyao, M., and Takatsuji, H. 2010. Role of OsNPR1 in rice defense program as revealed by genome-wide expression analysis. *Plant Molecular Biology*, 74:549-562.

Sugio, T., Satoh, J., Matsuura, H., Shinmyo, A., and Kato, A. 2008. The 5'-untranslated region of the Oryza sativa alcohol dehydrogenase gene functions as a translational enhancer in monocotyledonous plant cells. *Journal of Bioscience and Bioengineering*, 105:300-302.

Takatsuji, H., Jiang, C.J., and Sugano, S. 2010. Salicylic acid signaling pathway in rice and the potential applications of its regulators. *Japan Agricultural Research Quarterly*, 44:217−223.

Yasuda, M., Kusajima, M., Nakajima, M., Akutsu, K., Kudo, T., Yoshida, S., and Nakashita, H. 2006. Thiadiazole carboxylic acid moiety of tiadinil, SV-03, induces systemic acquired resistance in tobacco without salicylic acid accumulation. *Journal of Pesticide Science*, 31:329-334.

Transgenic Papaya for Ring Spot Virus Resistance

Parichart Bruns[1], Namthip Pironrit[1], Bencharong Phungrat[1], Anjana Boonchod[1], Nuchard Warin[1], Simek Chowpongpang[2], Supat Atthatham[3] and Wichai Kositsatana[3]
[1]Plant Research Laboratory, National Center for Genetic Engineering and Biotechnology, Kasetsart University, Kamphangsaen Campus, Nakhon Pathom, Thailand 73140
[2]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand 12120
[3]Department of Plant Pathology, Faculty of Agriculture Kamphangsaen, Kasetsart University, Kamphangsaen Campus, Nakhon Pathom Thailand 73140

Abstract

Papaya (*Carica papaya* L.) is an important fruit crop in Thailand. Despite the increasing demand for papaya fruit, production has not increased significantly. There are several factors limiting availability the major being papaya ringspot disease. Payapa ringspot disease caused by Papaya ringspot virus (PRSV) not only results in yield loss but also lowers the quality of fruit. Disease control using the practice of rogueing of diseased plants to reduce the spread of the disease is an option, but is not widely practiced. Genetic modification of papaya is a sensible alternative method for producing PRSV resistant papaya. PRSV was collected from diseased plants, the genome cloned, sequenced and the sequence analyzed. By introducing sequences from the viral genome into the papaya genome, transgenic papaya plants resistant to PRSV were developed, and shown to be a viable alternative in the control of the disease under screenhouse and small field conditions. Horticultural characters of GM papaya were found to be identical to that of non-GM papaya. Currently the cultivation of GM papaya is strictly regulated, being restricted to screenhouses. The impact of transgenic papaya on soil bacteria and beneficial insects were investigated. Although soil bacteria populations varied throughout the year for both GM and non-GM, the populations of both papaya types were identical. The pollen biology of papaya was also investigated and found to be identical for both GM and non-GM papaya. Non-GM papaya was therefore used as a model to study transgene transfer via pollen movement. In addition to directly trapping pollen and counting fertilized seeds, other techniques, such as computer modeling and Geographic Information System (GIS), are being employed to study transgene movement and their applicability for the monitoring and management of transgenic crops.

Keywords: beneficial insects, biosafety, environmental impact, papaya, papaya ringspot disease, transgenic papaya

Introduction

In 2008, the total production area of papaya (*Carica papaya* L.) in Thailand was 108,940 Rai (approx. 17,430 ha), located mainly in Northeastern and Eastern parts of the country. The total production was 201,099 tons with more than 98% for local consumption. Approximately 3,458 tons were exported as fresh fruit and canned fruit with the combined value of 111.9 million Baht (US$357,520).

In Thailand, papaya is only grown for 1-2 years and the location of papaya often is moved due to the severity of papaya ringspot disease, which was first reported in 1975 in Northeastern area. The disease is quickly spread to other area and cause severe loss of yield and dramatically reduces the fruit quality. In 2005, the disease was so severe that the production was reduced to 11% of previous year (Office of Agricultural Economics, 2010).

The disease is caused by Papaya Ringspot Virus (PRSV), which is a member of Potyviridae. There are two biotypes of PRSV: PRSV-P and PRSV-W. PRSV-P infected papaya and cucurbits while PRSV-W infects only cucurbits. PRSV-P can be transmitted by several aphid species. A wide host range in cucurbits for aphids makes easy for the spread of the virus. The disease management is ranging from removal of infected material and constant field survey. Several weeds commonly found around papaya plantation are cucurbits hence they should be eliminated. Mild strain of PRSV has also been used (Chiang et al., 2007). The effectiveness of these measures is variable. The disease is still prevalent.

In order to control the virus effectively, transgenic papaya was developed using pathogen derived resistance strategy. The concept is to introduce pathogen gene/DNA in plant genome and initiate plant resistance mechanism either via protein or RNA level (Baulcombe, 1996). Transgenic papaya was first developed in USA following the devastation loss by the disease (Lius et al., 1997). By introducing PRSV coat protein gene into papaya genome, several transgenic lines were developed. Transgenic papaya with papaya ringspot disease resistance has been proven to be successful in controlling the disease in Hawaii (Gonsalves, 1998). Although transgenic papaya developed in USA was found successful in disease management, studies into viral genome indicated sequence variation among PRSV found in different geographic locations (Tripathi et al, 2008; Olarte Castillo et al, 2011). In addition, each country has preference for papaya varieties due to their size, taste and flavor. It is therefore important to develop

transgenic papaya regionally. This paper is to summarize research and development of transgenic papaya in Thailand. In addition, relative techniques including GIS and modeling will be discussed.

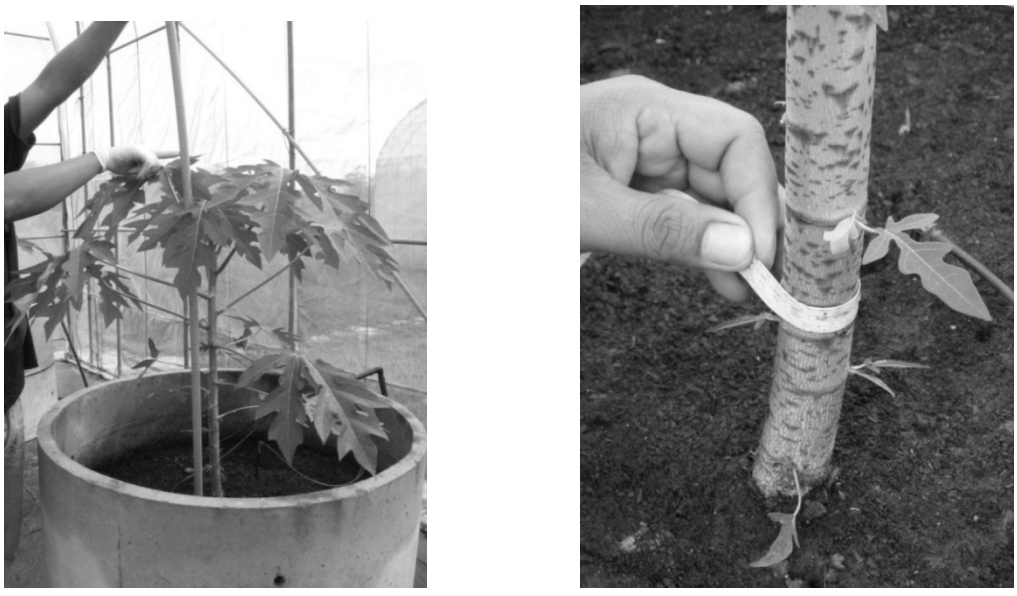
Current Status

Transgenic papaya was generated from Thai variety papaya “Khak Nual” and “Khak Dum” by introducing coat protein gene from PRSV isolated from Thailand using particle bombardment. Transgenic lines were tested for their molecular characters and their resistance to PRSV. A transgenic line with 2 PRSV-CP copies and PRSV resistant character were further tested for its agronomic characters.

Due to the regulation restriction, transgenic papaya was tested for their agronomic characters in the screen house condition (Fig. 1). The vegetative growth of transgenic papaya and its counterpart were compared and the data were statistically analyzed. They showed no statistically difference (Fig. 2) (Pironrit et al., 2011). The fruits from both non-transgenic and transgenic papaya were also compared. They are similar in size, shape and flesh color (Fig. 3). The cultivation was done for 2 consecutive seasons.

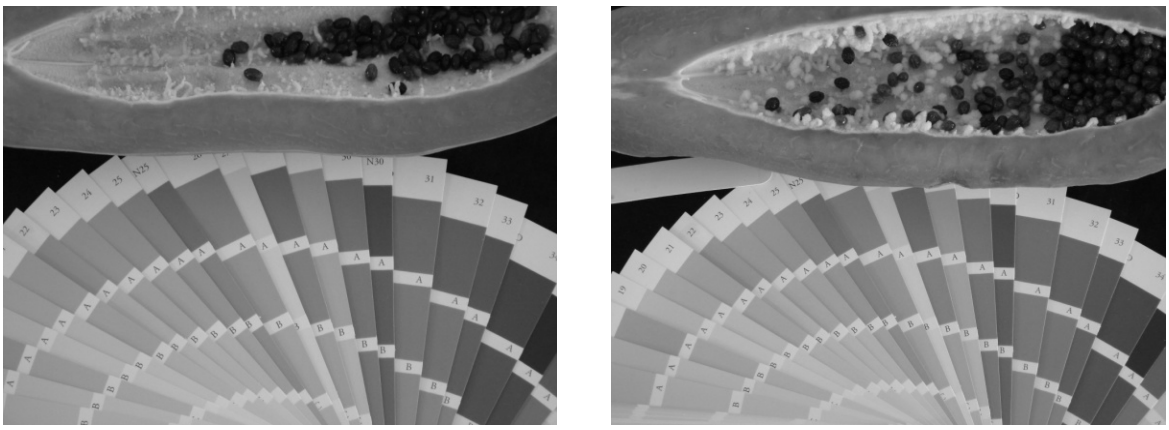
(a) **(b)**

Fig. 1. Screen house condition for Transgenic and non-transgenic papaya. The first season (a) growing in roles and second season (b) randomly



(a) **(b)**

Fig. 2. Measurement of vegetative growth in height (a) and diameter (b)

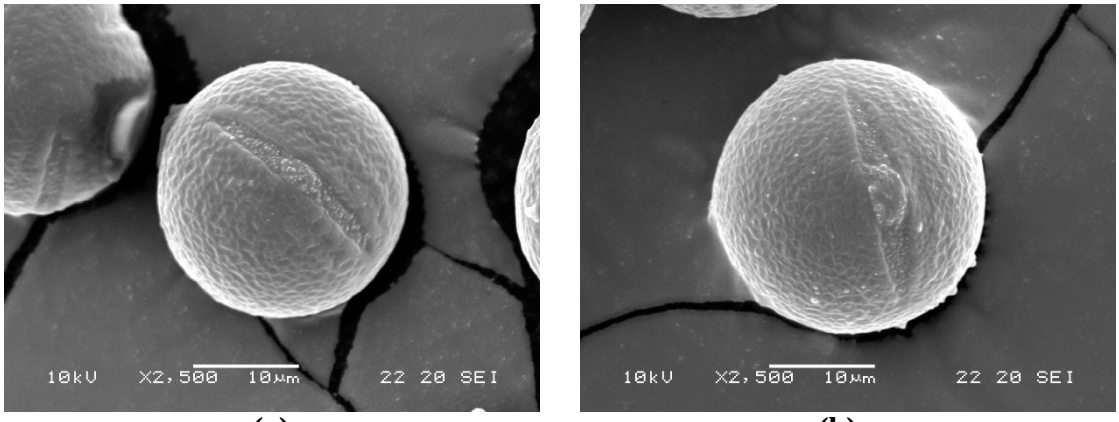


(a) **(b)**

Fig. 3. Flesh colour of transgenic papaya (a) and non-transgenic papaya (b)

The pollen biology of both non-transgenic and transgenic papaya was also studied. The pollen size, shape and its surface were investigated by scanning electron microscopy. The results showed that they are identical (Fig. 4). In addition, the viability and germination rate of both non-transgenic and transgenic papaya were statistically identical.

Pollen from three types of papaya flowers; staminate, elongata and reduced elongata were also investigated for its development, number, viability and germination rate. They showed no significant difference from each other (Phuangrat et al., 2013).



(a) **(b)**

Fig. 4. Scanning electron micrograph of transgenic papaya pollen (a) and non-transgenic papaya pollen (b). Both of them have finely recticulate surface and approximately 25 micron

Soil bacteria were investigated using Community Level Population Profiles (CLPP), total plate count and PCR method. The results showed that there was no significant difference in soil bacteria population and there was no horizontal gene transfer from transgenic papaya to soil bacteria.

Red spider mite (Eutranychus africanus) is a common pest found on papaya leaves in summer season. It is eaten by a beneficial predaceous mite (Neoseiulus lingisponosus). Leaves of non-transgenic and transgenic papaya were fed to the red spider mite. The mite was then fed to the predaceous mite and its biological life table was determined. The results showed that there was no significant difference in egg numbers, the ratio of male/female in hatched eggs and life span of those fed on non-transgenic and transgenic papaya leaves.

Although transgenic papaya is yet to be tested in the field condition due to the regulation, our studies have proven that non-transgenic and transgenic papaya showed identical in agronomic characters as well as pollen biology. Therefore the pollen movement under field condition was investigated using non-transgenic papaya. Pollen trap were set in the radius of 900 meters. Using Geographic Information System (GIS) based on wind speed and pollen size, the direction and pollen number can be successfully predicted (Sritake et al., 2011).

Conclusion

Transgenic papaya is still in needed to fight the severity of papaya ringspot disease. Transgenic papaya has been proven to be environmental safety. Several technologies such as GIS and computer modeling can be integrated into the farm management in order to monitoring and better planning of cultivation of non-transgenic and transgenic papaya.

References

Chiang, C.H., Lee, C.Y., Wang, C.H., Jan, F.J., Lin, S.S., Chen, T.C., Raja, J.A.J. and Yeh, S.D. 2007. Genetic analysis of an attenuated papaya ringspot virus strain applied for cross protection. *European Journal of Plant Pathology*, 118:333-348.

Baulcombe, D.C. 1996. Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell*, 8:1833-1844.

Gonsalves, D. 1998. Control of papaya ringspot virus in papaya: A case study. *Annual Review of Phytopathology*, 36:415-437.

Lius, S., Manshardt, R.M., Fitch, M.M.M., Slightom, J.L, Sanford, J.C. and Gonsalves, D. 1997. Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus. *Molecular Breeding*, 3:161-168.

Office of Agricultural Economics (2010).

Olarte Castillo, X.A., Fermin, G., Tabima, J., Rojas, Y., Tennant, P.F., Fuchs, M., Sierra, R., Bernal, A.J., and Restrepo, S. 2011. Phylogeography and molecular epidemiology of Papaya ringspot virus *Virus Research*, 159:132-140.

Parris, G.K. 1938. A new disease of papaya in Hawaii. *Proceedings of American Society for Horticultural Science*, 36:263–265.

Phironrit, N., Phuangrat, B., Burns, P. and Koritratana, W. 2010. Resistance of coat protein transgenic papaya and development of homologous transgenic papaya 116/5 resistant to papaya ringspot virus (PRSV) under screenhouse conditions in Thailand. Transgenic plant J 2010. Global Science Books.

Phuangrat, B., Phironrit, N., Son-ong, A., Puangchon, P., Meechai, A., Wasee, S., Kositratana, W. and Burns. P. 2013. Histological and morphological studies of pollen grains from elongata, reduced elongata and staminate flowers in *Carica papaya* L. Tropical Plant Biol. DOI 10.1007/s12042-013-9118-0.

Sritakae, A., Praseartkul, P., Cheunban, W., Miphokasap, P., Eiumnoh, A., Burns, P., Phironrit, N., Phuangrat, B., Kitsubun, P. and Meechai, A. 2011. Mapping airborne pollen of papaya (*Carica papaya* L.) and its distribution related to land use using GIS and remote sensing. *Aerobiologia*, 27:291-300.

Tripathi, S., Suzuki, J.N.Y., Ferreira, S.A. and Gonsalves, D. 2008. Papaya ringspot virus-P: Characteristics, pathogenicity, sequence variability and control. *Molecular Plant Pathology*, 9:269–280.

Herbicide Resistance in Canola: An Essential Tool for Weed Management in Australia

Christopher Preston and Sarah Morran
School of Agriculture, Food & Wine, University of Adelaide
PMB 1, Glen Osmond SA 5064, Australia

Abstract

Canola has become a major winter grain-producing crop in Australia with 2.97 million ha planted in 2012 producing 3.23 million ton of grain. Canola production increased from a minor crop in the early 1990s to the 3rd most widely grown crop today. Two major breeding innovations aided the expansion of canola area in Australia: resistance to blackleg (*Leptosphaeria maculans*) and resistance to herbicides. Several weed species cause major problems in canola production in Australia including wild radish (*Raphanus raphanistrum*), Indian hedge mustard (*Sisymbrium orientale*), shepherds purse (*Capsella bursa-pastoris*) and annual ryegrass (*Lolium rigidum*). Brassica weeds can be particularly problematic due to a lack of control options in conventional canola.

Canola resistant to the triazine herbicides was released in Australia in 1993. Triazine-resistant canola was readily adopted, despite the yield penalty, because it allowed ready control of Brassicaceae weeds. Canola resistant to imidazolinone herbicides was released in Australia in 2000. Imidazolinone-resistant canola took longer to be widely adopted. Imidazolinone herbicides are residual in the Australian environment and restrict the crops that can be planted in the next season. The release of hybrid cultivars with imidazolinone resistance led to an increase in planting of these varieties. Glyphosate-resistant canola was finally released in three states of Australia in 2008 and 2009 and it now represents more than 11% of the canola production area, mostly in Western Australia.

There currently exist three herbicide resistance types in Australian canola production. The choice of resistance type depends on environment, cropping rotation and weed burden. Triazine-resistant canola retains the largest market share and has been particularly favoured where Brassica weeds are a problem. Imidazolinone-resistant canola is preferred in higher yielding regions and where troublesome weeds, such as *Bromus* spp., occur. Glyphosate-resistant canola has suffered from the lack of residual activity of glyphosate, which has seen a reduction in plantings in high rainfall environments. Selection for herbicide resistant weeds is a concern for all canola types and appropriate herbicide rotations are recommended. This is the greatest risk associated with glyphosate-resistant canola given the importance of glyphosate in crop production.

Keywords: Triazine-resistant, imidazoninone-resistant, glyphosate-resistant, weed management, herbicide-resistant weeds

Introduction

Canola (known also as oilseed rape) is a group of *Brassica napus* cultivars developed from rapeseed with low erucic acid and low glucosinolates in the grain (Downey, 1988). Rapeseed oil is unsuitable for human consumption due to high levels of erucic acid (Abdellatif and Vles, 1970). The meal of rapeseed contains high concentrations of glucosinolates that make it unsuitable for animal feed. Breeding programs have reduced the content of both allowed the product to be used for human consumption and animal production. Low erucic acid rapeseed was first grown in Australia in 1969 and canola was introduced to Australia in 1979 (Colton and Potter, 1999).

The area of canola grown in Australia remained low during the 1980s, despite the introduction of varieties in Australia that had better blackleg tolerance, better oil quality and better adaptation to Australian conditions. The major impediment to canola production in Australia during this period was the failure to control Brassicaceae weeds, such as *Raphanus raphanistrum*, *Rapistrum rugosum*, *Sisymbrium orientale*, *Sinapis arvensis* and *Capsella bursa-pastoris* within the crop (Sutherland, 1999). Should seed from these weed species contaminate the grain sample, they would increase the erucic acid and glucosinolate content and the product could be downgraded. The problems with controlling Brassicaceae weeds tended to restrict canola production to areas where these weed species were less common (Colton and Potter, 1999).

The area sown for canola production in Australia started to increase during the 1990s and climbed dramatically to 1999 when more than 1.9 million ha of canola was grown (Fig. 1). Several factors contributed to the rise in canola plantings in Australia including: moves to continuous cropping requiring more break crops between cereals, strong prices for canola making it more profitable in lower rainfall areas and increased promotion of the benefits of break crops to cereal rotations. However, a key enabling factor in the adoption of canola was the introduction of triazine-tolerant canola varieties in 1993 (Colton and Potter, 1993). For the first time, control of Brassicaceae weeds in crop was possible.

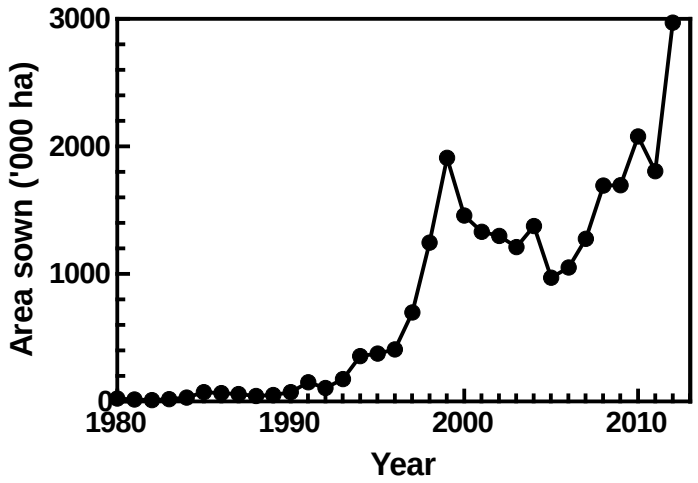


Fig. 1. Area sown to canola in Australia from 1980 to 2012. Collated from ABARE (1986-1994), ABARE (1995-2008), ABARES (2012) and ABARES (2013)

The area sown to canola decreased during the decade from 2000 to 2009, largely as a result of a series of major droughts in southern Australia. These droughts had a major impact on canola production in Australia as well (Fig. 2). There were reduced plantings in the lower rainfall regions during this period. The area sown to canola has increased again since 2009 with a total of 2.97 million ha planted producing 3.23 million t of grain in 2012 (ABARES 2013).

The area sown to canola in Australia has grown dramatically over the past two decades and it is now the third most widely grown crop in Australia after wheat and barley. The increase in area sown to canola since the early 1990s has not seen an increase in average yields of canola in Australia, which tend to average about 1.2 t/ha (Riffkin et al., 2012). Reasons for this are the expansion of canola into lower rainfall environments, droughts during the decade from 1999 to 2009 and the widespread adoption of triazine-tolerant canola for weed management. The lack of significant improvement in canola yield and increased risk associated with canola production has not limited the adoption of the crop. The benefits associated with management of cereal diseases and weeds through canola production far outweigh the risks.

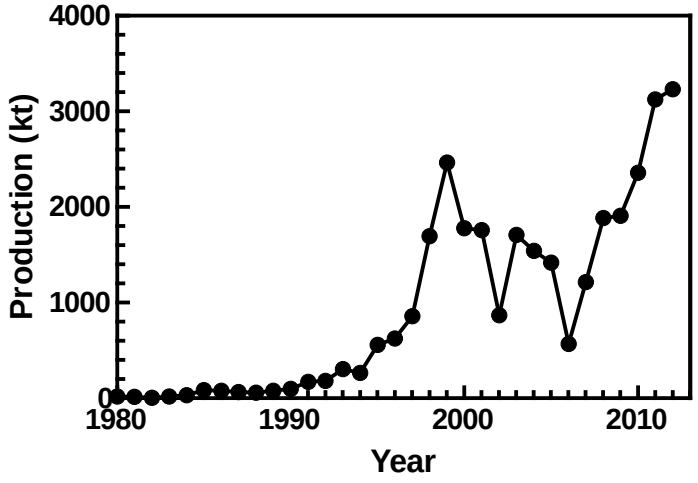


Fig. 2. Total production of canola in Australia from 1980 to 2012. Collated from ABARE (1986-1994), ABARE (1995-2008), ABARES (2012) and ABARES (2013)

Extent of Herbicide Resistant Weeds

Australian grain production occurs in extensive areas where farms are large and there is limited labour. The average grain farm size is more than 1000 ha in eastern Australia and over 2500 ha in Western Australia (Valle et al., 2013). In addition, there has been widespread adoption of no-till seeding systems where the crop is sown without any prior tillage and with minimal soil disturbance in the seeding operation. These two trends have resulted in a strong reliance on herbicides for weed control in grain production systems of Australia.

A consequence of the intense reliance on herbicides for weed control in Australia has been the evolution of herbicide resistant weeds. The first reported example of herbicide resistance in Australia was the evolution of annual ryegrass (*Lolium rigidum*) with resistance to diclofop-methyl in 1982 (Heap and Knight, 1982). Since that time, the number of weed species reported with resistance has increased to 40 weed species with resistance to numerous herbicides.

In extent and seriousness, the major herbicide resistance problem is with annual ryegrass. Resistance to seven different herbicide modes-of-action occurs in populations of annual ryegrass, with some populations resistant to several modes-of-action (Preston et al., 1996). Surveys in Western Australia (Owen et al., 2007), South Australia, Victoria (Boutsalis et al., 2012) and southern NSW (Broster et al., 2011) demonstrate that nearly all grain farms have herbicide resistant annual ryegrass on their land. In some areas, widespread multiple resistant to all herbicides that could be used in cereal production was present (Owen et al., 2007; Boutsalis et al., 2012). Other major grass weeds where resistance is causing difficulties include wild oats (*Avena* spp.), great brome (*Bromus diandrus*), rigid brome (*Bromus rigidus*) and barley grass (*Hordeum* spp.).

In addition to annual ryegrass, there are increasing resistance problems with broad leaf weeds. Two species in particular: wild radish (*Raphanus raphanistrum*) and Indian hedge mustard (*Sisymbrium orientale*), members of the Brassicaceae, have evolved resistance to four different herbicide modes of action. Multiple resistance in wild radish is widespread in Western Australia (Walsh et al., 2007) and is increasing in South Australia and Victoria (P. Boutsalis and C. Preston, unpublished data).

The greater difficulty of controlling herbicide resistant grass weeds in cereals in particular has led to canola becoming an important crop for managing weeds in the rotation. Canola also allowed the use of clethodim to control resistant grass weeds. Annual ryegrass populations that had evolved resistance to other grass herbicides were mostly still susceptible to clethodim (Boutsalis et al., 2012). In addition, the availability of herbicide tolerant types of canola has expanded the number of herbicides available for weed control.

Triazine Tolerant Canola

Triazine tolerant canola was originally developed in Canada by crossing a resistant individual of *B. rapa*, that was selected by triazine herbicide use in a corn field in Manitoba, with canola and then back-crossing to canola (Beversdorf et al., 1980). Triazine tolerant canola was grown for a short period in Canada, but was phased out with the introduction of other herbicide resistant traits (Devine, 2005). Resistance to the triazine herbicides results in a fitness penalty from reduced photosynthetic efficiency and reduced yields (Beversdorf et al., 1988; Robertson et al., 2002). The fitness penalty associated with triazine-tolerant canola was a significant factor in the disadoption of triazine-tolerant canola in Canada (Devine, 2005).

Triazine tolerant canola was first introduced into Australia in 1993 by public and private breeders (Colton and Potter, 1999). It was rapidly adopted due to its ability to manage Brassicaceae weeds and was a key enabling technology in the increase in area of canola in Australia. By 2003, more than 80% of canola grown in Australia was triazine tolerant (OGTR, 2003). The triazine herbicides used, atrazine and simazine, are not particularly efficacious against grass weeds, but helped reduce grass weed populations when clethodim was applied afterwards.

The yield penalty associated with triazine tolerant canola was not as much an impediment to adoption in Australia as it was in Canada. There are a couple of key reasons for this. Firstly, weed control in canola was essential in Australian farming systems; therefore using conventional canola was not an option. Secondly, Australian canola producers were not achieving their yield potential, even with conventional cultivars (Riffkin et al., 2012). The persistence of atrazine used post-emergent in canola caused problems for rotational crops in some drought years; however, the advantages of a cereal disease break and better weed control were greater. The main issue with triazine tolerant canola was poorer competition against grass weeds leading to greater pressure on clethodim.

Imidazolinone Tolerant Canola

Canola tolerant to the imidazolinone herbicides was developed through microspore mutagenesis and selection (Swanson et al., 1989). It was introduced into Canada in 1995 and was an immediate success (Tan et al.,

2005). Imidazolinone-tolerant canola (marketed in Australia under the Clearfield brand) was first introduced to Australia in 2000. This product offered an alternative to the triazine-tolerant technology for control of Brassicaceae weeds. However, adoption of imidazolinone-tolerant canola was much slower than triazine-tolerant canola, despite the lack of a fitness penalty.

A major disincentive to the adoption of imidazolinone-tolerant canola was the persistence of the herbicides in the environment, particularly in acid soils (Loux and Rees, 1993). The lack of breakdown of imidazolinine herbicides leads to an inability to plant wheat or barley after canola. In Western Australia, where acid soils dominate, there was very low adoption of imidazolinone tolerant canola.

A series of factors led to an increase in the sowing of imidazolinine tolerant canola in south-eastern Australia. The release of hybrid cultivars carrying the herbicide resistance trait that were more competitive against weed species provided additional advantages. At about the same time, there was a change in the imidazolinone herbicide mixture used to less residual compounds. A third factor was the rising importance of *Bromus* spp., where imidazolinone herbicides are particularly effective.

Glyphosate Tolerant Canola

Glyphosate tolerant canola (marketed in Australia under the Roundup Ready brand) is the only genetically-modified (GM) canola grown in Australia. It was developed through the introduction of two genes from soil bacteria; *EPSPS* from *Agrobacterium tumefaciens* CP4 that was resistant to glyphosate and *goxv247* gene from *Ochrobactrum anthropi* strain LBAA that degraded the herbicide (Barry et al,. 1992). Glyphosate tolerant canola was first marketed in Canada in 1996 and quickly became the dominant herbicide tolerant canola system in Canada (Dill, 2005). Glyphosate-tolerant canola was approved for introduction into Australia in 2003, but the canola growing states immediately placed a moratorium on its planting for market reasons (Foster, 2006). A limited release of glyphosate-tolerant canola was made in 2008 in NSW and Victoria following the lifting of the moratorium in those states. Western Australia followed suit in 2009 (McCauley et al., 2012).

Glyphosate has the advantage over triazine and imidazolinone herbicides in being broad spectrum and having no residual that interferes with cropping rotations. Despite these advantages, the adoption of glyphosate tolerant canola has not been rapid, with only 11% of the area planted to the crop. The greatest adoption has been in Western Australia where imidazolinine-tolerant canola is a less attractive option.

A major weakness of the glyphosate-tolerant canola system in Australia is the requirement to apply the last application of glyphosate at the 6-leaf stage and prior to bud formation. Due to the lack of any residual activity of glyphosate, any weeds that germinate after this time are not controlled or will require an additional herbicide. In higher rainfall regions with longer seasons, annual ryegrass control in particular can be compromised by late germinating weeds. This has led to some disadoption of glyphosate tolerant canola.

Issues Surrounding the Adoption of GM Canola

Despite permission to plant GM canola being provided by the regulator, State Governments imposed moratoria on the planting of the crop for market reasons (Foster, 2006). Market sensitivities to public perception of GM canola have previously impacted global trade of canola. The EU currently does not accept GM canola. Market acceptance of GM canola varies between countries and public perception has been shown to affect global trade flows (Foster, 2006). Major markets for Australian canola include Japan, EU and Pakistan (ABARES, 2012). The variation in acceptance of GM canola between these markets required segregation of GM and non-GM canola to be adopted.

When GM canola was first introduced to Australia, labeling rules for GM food products were in place, however no common terms for trade and grain specifications existed. Both government and industry organizations recognised the need for the development of protocols to manage the introduction of GM crops and the requirement for a clear definition of non-GM canola (Alcock, 2005). After the discovery of adventitious GM traits in non-GM canola in 2005 a limit was set at a maximum of 0.9% for delivered canola crops and 0.1% for commercial seed for planting (Primary Industries Ministerial Council, 2005).

The cropping of GM canola also required management at the farm level to maintain co-existence. A condition of access to GM seed by growers is the implementation of specific stewardship strategies. These strategies cover issues determined to be important in the management of GM and non-GM canola on farm with the objective of no more than 0.9% adventitious presence of GM traits in non-GM canola at delivery (Neilsen, 2010). Minimization of cross pollination is a fundamental requirement for the management of GM and non-GM canola on farm. Canola is predominantly a self-pollinated crop with a small amount of outcrossing which varies between plants, cultivar and environment (Beckie et al., 2003). Studies on the gene flow in canola have shown that it is usually less than 1% and falls dramatically with distance (Reiger et al., 2002). Modelling the effect of multiple donor fields on gene flow to non-GM fields predicted that it would remain on average below 0.13% with up to 80% of fields in the region being GM Canola (Baker and Preston, 2003). Therefore, growers are

required to implement a 5 m buffer between GM and non-GM crops to achieve the standards for non-GM certification.

Future of Herbicide Tolerant Canola

Herbicide tolerance remains a crucial component of canola production in Australia. The overwhelming majority of the area of canola production in Australia contains one of the three herbicide tolerance traits. Despite this dominance, there are threats to herbicide tolerance use. Atrazine is under regulatory pressure in Australia due to the appearance of this herbicide in water bodies. Tebuthylazine has been recently registered for use in triazine tolerant canola and will find use as a replacement for atrazine; however, it has limited activity on grass weeds.

The major on-farm risk associated with the use of herbicide tolerant canola types is the risk of resistance evolving to the herbicide in weed species. This is particularly problematic for imidazolinone tolerant canola, where many weed species in Australia already have resistance to this mode of action. The current expectation is that the major weeds being targeted with imidazolinone herbicides will have evolved resistance within a few years, given the high natural frequency of resistance (Preston and Powles, 2002). Perhaps the biggest current threat to canola production in Australia is the increase in clethodim resistance in annual ryegrass (Boutsalis et al., 2012). Clethodim has proved vital in managing annual ryegrass in canola and its loss due to resistance will make canola production more difficult. At present there is no obvious replacement for clethodim.

Almost as concerning is the evolution of resistance to glyphosate. Glyphosate is the most important herbicide of crop production systems. The widespread use of this herbicide has already resulted in resistance evolving in several weed species in Australia (Preston et al., 2009; Preston 2010; Malone et al., 2012). The additional use of glyphosate in glyphosate tolerant canola is likely to increase the risk of resistance. Surveys of fields where glyphosate tolerant canola was grown in 2008 failed to identify any evidence of glyphosate resistant weeds; however, other fields on the same farm did have resistant weeds (S. Morran, J. Malone, P. Boutsalis, R. Roush and C. Preston, unpublished data). In order to preserve the herbicides for as long as possible, producers of canola are encouraged to rotate the types of herbicide tolerance they grow.

Despite the successful release of GM canola in Australia, community and market concern remains surrounding GM crops. This was manifest as a price penalty for GM canola in 2010. The amount of price penalty for GM canola has decreased over the past year or so, suggesting less concern about the product in the market. There are three GM canola products with herbicide resistance in development in Australia. Monsanto is testing a canola containing two copies of the *EPSPS* gene from *A. tumefaciens* CP4 that will be tolerant to higher rates of glyphosate (OGTR, 2010). Bayer CropScience is testing canola containing both the *EPSPS* gene from *A. tumefaciens* CP4, the *goxv247* gene from *O. anthropi* strain LBAA and either the *pat* gene from *Streptomyces viridochromogenes* or the *bar* gene from *Streptomyces hygroscopicus* that is tolerant to both glyphosate and glufosinate (OGTR, 2011). Pioneer is testing canola containing the *gat4621* gene, modified from several gat genes in *Bacillus licheniformis*, which provides resistance to glyphosate (OGTR, 2012).

Conclusion

The disease and weed management advantages of canola along with strong markets for canola seed have resulted in canola being the third most widely grown crop in Australia. Herbicide tolerance in canola has been vital to its growth in Australia. Non-GM herbicide tolerant canola types still dominate canola production in Australia despite the successful introduction of glyphosate tolerant canola. Australia introduced a series of practices to ensure co-existence of GM and non-GM supply chains for canola. The successful application of co-existence measures has eased market concerns over GM canola. The main threat to expansion of canola production in Australia is the evolution of herbicide resistance in weeds. This will require the adoption of additional management strategies including the development of additional types of herbicide resistance in canola.

References

ABARE. 1986-1994. Commodity Statistical Bulletin. Australian Government Publishing Service, Canberra.

ABARE. 1995-2008. Australian Commodity Statistics. Australian Bureau of Agricultural and Resource Economics, Canberra.

ABARES. 2012. Agricultural commodity statistics 2012. Australian Bureau of Agricultural and Resource Economics and Sciences, Canberra.

ABARES. 2013. Australian crop report, September. Australian Bureau of Agricultural and Resource Economics and Sciences, Canberra.

Abdellatif, A.M M. and Vles, R.O. 1970. Pathological effects of dietary rapeseed oil in rats. *Annals of Nutrition and Metabolism*, 12: 285-295.

Alcock, K.T. 2005. Co-existence: an Australian perspective. Pp. 45-50 in Messéan, A. (ed.). Second International Conference on Co-existence Between GM and Non-GM Based Agricultural Supply Chains. Agropilis Publications, Montpellier.

Baker, J. and Preston, C. 2003. Predicting the spread of herbicide resistance in Australian canola fields. *Transgenic Research*, 12: 731-737.

Barry, G., Kishore, G., Padgette, M., Kolacz, K., Weldon, M., Re, D., Eichholtz, D., Fincher, K. and Hallas, L. 1992. Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. Pp. 139-145 in Singh, B.K., Flores, E. and Shannon, J.C. (eds.). Biosynthesis and molecular regulation of amino acids in plants. American Society of Plant Physiologists, Rockville, MD.

Beckie, H.J., Warwick, S.I., Nair, H. and Séguin-Swartz, G. 2003. Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). *Ecological Applications*, 13: 1276-1294.

Beversdorf, W.D., Hume, D.J. and Donnelly-Vanderloo, M.J. 1988. Agronomic performance of triazine-resistant and susceptible reciprocal spring canola hybrids. *Crop Science*, 28: 932-934.

Beversdorf, W.D., Weiss-Lerman, J., Erickson, L.R. and Souza Machado, V. 1980. Transfer of cytoplasmically inherited triazine resistance from bird's rape to cultivated oilseed rape (*Brassica campestris* and *B. napus*). *Canadian Journal of Genetics and Cytology*, 22: 167–172.

Boutsalis, P., Gill, G.S. and Preston, C. 2012. Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across south-eastern Australia. *Weed Technology*, 26: 391-398.

Broster, J.C., Koetz, E.A., and Wu, H. 2011. Herbicide resistance levels in annual ryegrass (*Lolium rigidum* Gaud.) in southern New South Wales. *Plant Protection Quarterly*, 26: 22-28.

Colton, B. and Potter, T. 1999. History. Pp. 1-4 in Salisbury, P.A., Potter, T.D., McDonald, G. and Green A.G. (eds). Canola in Australia, the First Thirty Years. Organising Committee of the 10th International Rapeseed Congress, Canberra.

Devine, M.D. 1995. Why are there not more herbicide-tolerant crops? *Pest Management Science*, 61: 312-317.

Dill, G.M. 2005. Glyphosate-resistant crops: history, status and future. *Pest Management Science*, 61: 219–224.

Downey, R.K. 1988. Canola: A Quality Brassica Oilseed. Pp. 211–15 in Janick, J. and Simon, J.E. (eds.), Advances in New Crops: Proceedings of the 1st National Symposium on New Crops. Portland: Timber Press.

Foster, M. 2006. GM Grains in Australia: Identity Preservation. Australian Bureau of Agricultural and Resource Economics, Canberra.

Heap, J., and Knight, R. 1982. A population of ryegrass tolerant to the herbicide diclofop-methyl. *Journal of the Australian Institute of Agricultural Science*, 48: 156-157.

Loux, M.M. and Reese, K.D. 1993. Effect of soil type and pH on persistence and carryover of imidazolinone herbicides. *Weed Technology*, 7: 452-458.

Malone, J., Cook, A., Wu, H., Hashem, A. and Preston, C. 2012. Management of glyphosate resistant weeds in non-agricultural areas. Pp. 184-186 in Eldershaw, V., ed. Proceedings of the 18th Australasian Weeds Conference. Weed Society of Victoria, Inc., Melbourne.

McCauley, R., Davies, M. and Wyntje, A. 2012. The step-wise approach to adoption of genetically modified (GM) canola in Western Australia. *AgBioForum*, 15: 61-69.

Nielsen, J.E. and Taylor I.N. 2010. Managing herbicide resistance in Southern Australian farming systems using Roundup Ready® canola. Pp. 254-257 in Zydenbos, S.M., ed. Proceedings of the 17th Australasian Weeds Conference. New Zealand Plant Protection Society, Christchurch, New Zealand.

OGTR. 2003. General release of Roundup Ready canola *Brassica napus* in Australia. Risk assessment and risk management plan. Available from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/ir-1

OGTR. 2010. Risk Assessment and Risk Management Plan for DIR 105 - Limited and controlled release of canola genetically modified for herbicide tolerance. Available from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/ir-1

OGTR. 2011.. Risk Assessment and Risk Management Plan for DIR 108. Commercial release of canola genetically modified for herbicide tolerance and a hybrid breeding system (GM InVigor® x Roundup Ready® canola). Available from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/ir-1

OGTR. 2012. Risk Assessment and Risk Management Plan for DIR 114 - Limited and controlled release of canola genetically modified for herbicide tolerance. Available from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/ir-1

Owen, M.J., Walsh, M.J., Llewellyn, R.S., and Powles, S.B. 2007. Widespread occurrence of multiple herbicide resistance in Western Australian annual ryegrass (*Lolium rigidum*) populations. *Crop and Pasture Science*, 58: 711-718.

Preston, C. 2010. Managing glyphosate resistant weeds in Australia. Pp. 250-253 in Zydenbos, S.M., ed. Proceedings of the 17th Australasian Weeds Conference. New Zealand Plant Protection Society, Christchurch, New Zealand.

Preston, C. and Powles, S.B. 2002. Evolution of herbicide resistance in weeds: initial frequency of target site-based resistance to acetolactate synthase-inhibiting herbicides in *Lolium rigidum*. *Heredity*, 88: 8-13.

Preston, C., Tardif, F.J., Christopher, J.T. and Powles, S.B. 1996. Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. *Pesticide Biochemistry and Physiology*, 54: 123-134.

Preston, C., Wakelin, A.M., Dolman, F.C., Bostamam, Y. and Boutsalis, P. 2009. A decade of glyphosate-resistant *Lolium* around the World: mechanisms, genes, fitness, and agronomic management. *Weed Science*, 57: 435-441.

Primary Industries Ministerial Council. 2005, Communique, Ninth Meeting, 26 October 2005, Canberra (www.mincos.gov.au/pdf/pimc_09.pdf).

Rieger, M.A., Lamond, M., Preston, C., Powles, S.B. and Roush, R.T. 2002. Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science*, 296: 2386-2388.

Riffkin, P., Potter, T. and Kearney, G. 2012. Yield performance of late-maturing winter canola (*Brassica napus* L.) types in the high rainfall zone of southern Australia. *Crop and Pasture Science*, 63: 17-32.

Robertson, M.J., Holland, J.F., Cawley, S., Potter, T.D., Burton, W., Walton, G.H. and Thomas, G. 2002. Growth and yield differences between triazine-tolerant and non-triazine-tolerant cultivars of canola. *Australian Journal of Agricultural Research*, 53: 643-651.

Sutherland, S. 1999. Weed management. Pp. 59-65 in Salisbury, P.A., Potter, T.D., McDonald, G. and Green A.G. (eds.). Canola in Australia, the First Thirty Years. Organising Committee of the 10th International Rapeseed Congress, Canberra.

Swanson, E.B., Herrgesell, M.J., Arnoldo, M., Sippell, D.W. and Wong, R.S.C. 1989. Microspore mutagenesis and selection: canola plants with field tolerance to the imidazolinones. *Theoretical and Applied Genetics*, 78: 525-530.

Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K. and Shaner, D. 2005. Imidazolinone-tolerant crops: history, current status and future. *Pest Management Science*, 61: 246–257.

Valle, H., Lubulwa, M., Shafron, W. and Dahl, A. 2013. Australian grains: financial performance of grain producing farms 2010–11 to 2012–13. ABARES research report 13.10 prepared for the Grains Research and Development Corporation, Canberra.

Walsh, M.J., Owen, M.J. and Powles, S.B. 2007. Frequency and distribution of herbicide resistance in *Raphanus raphanistrum* populations randomly collected across the Western Australian wheatbelt. *Weed Research*, 47: 542-550.

Field Trials of Insect Resistant Maize Expressing Bt Gene in Viet Nam

Nguyen Van Liem
Plant Protection Research Institute (PPRI), Ha Noi, Vietnam

Abstract

Results of confined and large-scale trials in different seasons and sites of Bt11 maize (*Zea mays* L.) that contains the Cry1Ab protein indicate that this GM maize did not appear to have any negative effects on arthropod diversity, community structure, abundance and population dynamics of non-target arthropod, including natural enemies (predatory and parasite arthropods), neutral insects, non-target herbivores, pollinators and soil fauna (springtails). These findings suggest that Bt11 maize does not disrupt non-target organism community structure. Results also indicate that Bt11 maize increased productivity and grower's income in terms of higher yield (increased 18.7%) and improved profitability (increased 18.9%), respectively. Results of field trials of Bt11 maize have been approved by the Ministry of Agriculture and Rural Development for applying the Bio-safety Certificate.

Keywords: Bt maize, field trials, environmental risk assessment, insect diversity, non-target organisms, corn borer

Introduction

Vietnam is one of the SE Asian countries which have a fast growth rate in maize production. However, its average yield is low, about 81% of the world average (FAOSTAT, 2011). Like other maize growing countries, insect pests are a key constraint in Vietnam, of which Asian corn borer (ACB), *Ostrinia furnacalis*, is considered as the major pest for all maize growing regions in Vietnam. Depending on season and region, its infestation ranges from 40% to 100% (Nguyen Quy Hung et al., 1978; Nguyen Đuc Khiem, 1995; Nguyen Van Thanh et al., 2006). Under the limitation of planting area in the coming years, improving yield by using high-yielding varieties is most likely the way to achieve the government's objectives of increasing maize production to supply the feed sector. And one of the strategies is to introduce insect resistant and/or herbicide tolerant GM maize varieties. Following the government's roadmap of approaching GM crops production, GM maize is the first crop which has been approved for field trials. Confined and large-scale field trials of GM maize were conducted for risk assessment on bio-diversity and environment in Vietnam in 2010 and 2011.

Seven GM maize varieties (three of Syngenta Vietnam Co. Ltd., three Dekalb Vietnam Co. Ltd., and one Pioneer Hi-Bred Vietnam Co. Ltd.) have been approved for field trials by the Ministry of Agriculture and Rural Development (MARD). Among them, five expressed a single event (insect resistance or herbicide tolerance) and two stack events (insect resistance and herbicide tolerance). Three out of five single events for testing contained Bt genes: Bt11 (Cry1Ab), MON89034 (Cry1A.105 and Cry2Ab2), and TC1507 (Cry 1F). Procedures for conducting all confined and large scale field trials were carried out in compliance with the Government's regulation of the Biosafety Management and Risk Assessment of GM Crops. Results from these trials showed that all Bt genes tested did not have any adverse effects on arthropod communities in trial fields. The insect diversity, insect community structure, and their population densities were not affected by presence of Bt gene in maize plant. The Shannon index (H') to measure species diversity indicated that arthropod diversity is generally the same in Bt maize and non-Bt maize fields. Moreover, there was increased productivity improved profitability. With these promising results, the concerned companies subsequently have submitted their applications for Biosafety Certificate for GM Crop to the Ministry of Natural Resources and Environment.

This paper describes the trials of Syngenta's Bt11 maize that contains the Cry1Ab protein under Vietnam environmental conditions. Bt11 maize has been used widely in USA, Canada, Argentina and several other countries. Protein Cry1Ab has highly specific insecticidal effects on Lepidoptera (Koziel et al., 1993) including ACB, European corn borer, *O. nubilalis*, and Mediterranean corn borer, *Sesamia nonagrioides*. Bt11 maize was also shown to be highly resistant to ACB in the Philippines (Ebora et al., 2005; Rasco et al., 2008). Non-target organisms such insect predators, insect parasitoids and other bio-functioning organisms were not affected in the Bt maize field (Mendelsohn et al., 2003; Candolfi et al., 2004; Romeis et al., 2008; Carpenter, 2011).

Materials and Methods

Bt maize and the respective isogenic non-Bt varieties were grown in parallel. Confined field trials were comprised of CT1 (NK66Bt11, without insecticides), CT2 (NK66, without insecticides), CT3 (NK66, with insecticides) and CT4 (C919, with insecticides). Each treatment had 3 replicates and each replicate was a plot of 21 m^2. To ensure adequate testing of the efficacy of the Bt corn, natural infestation of corn borer was augmented by artificially infesting 02 young larvae to

each plant when plant at the V5 stage (Abendroth et al., 2011). Trials were conducted for 3 consecutive planting seasons in 2010 and 2011 in two locations. Large-scale field trials were comprised of CT1 (NK66Bt11, without insecticides) and CT2 (NK66, without insecticides). Each treatment was a plot of 600 m^2 without replication under natural infestation of corn borer. They were conducted for one planting season in 2011 at four locations. Agronomic traits, insect profile, and soil fauna (springtails) were investigated or monitored, and plant damages rated by the method of Guthrie et al. (1960).

Results and Discussion

Agronomic traits

There were no differences between Bt11 maize (NK66Bt11) and non Bt maize (NK66) in reproduction, dissemination and survivability in large-scale field trial conditions. NK66Bt11 seeds did not have a greater likelihood of dormancy, ability to self disperse and persist in the natural environment than conventional maize, indicating that NK66Bt11 is not able to become a weed.

Impacts on non-target organisms

Results indicated that Bt11 maize had no effects on the number of arthropod orders and the number of species in each of the specific order such as Coleoptera (e.g., ladybird beetles), Homoptera (e.g., aphids), Hymenoptera (e.g. bees) and Araneae (e.g. woft spider). Using the Shannon index (H') to measure species diversity, results showed that in Bt11 and non-Bt maize fields insect diversity was generally the same.

Population dynamics of aphids (*Rhopalosiphum maidis*), one of the five major aphids especially in winter season (Nguyen Đuc Khiem, 1995), were same for all planting seasons and types of maize, displaying a progressive increase in number, reaching maximum when plant at tasseling stage (Abendroth et al., 2011) then declining by the end of planting season. No significant differences between tested maize entries were also observed in the damage indices, despite differences in location and stage of plant development.

Predatory ladybird beetles (PLBs), *Micrapis discolor*, *Menochilus sexmaculatus*, *Coccinella transversalis*, *Harmoniaoctomaculata*, *Harmonia axyridis*, and *Oenopia sauzeti* are natural enemies of ACB (Reyes et al., 2004; Alcantara, 2004) and maize aphids (Pham Van Lam, 2002). They were present in the maize field throughout the growth duration of maize plants. And their population dynamics were same for all types of maize tested within a specific trial site and planting season. Their population densities increased progressively and reached a peak when maize plants at pre-mature stage (Abendroth et al., 2011) with density of 32 to 43 ladybird beetles/20 plants in confined trials and of 25 to 27 ladybird beetles/20 plant in large scale trials. Observations indicated that PLB had a clear numerical response to maize aphids. In all trial locations and on both types of maize tested, there was a lag between the appearance of aphid and their PLB, with the peaks of PLB usually occurring about one week later than that of aphids.

Predatory spiders (PSs) are the most common invertebrate predators on maize. PSs commonly found in field trials were Woft spider (*Lycosidae* sp.), Lynx spiders (*Oxiopes* sp.), *Araneus* sp. and *Atypena* sp. Spider abundances and their population dynamics varied depending on trial site and trial types but were same on both Bt11 and non-Bt11 maize. These findings suggest that Bt11 did not have negative impacts on the appearance, diversity and population dynamics of PSs.

Collembola (springtails) is an arthropod whose high abundance of individuals and high species diversity make them good indicators of any quantitative or qualitative change in the soil and nutrient supply, which may result in changes in their taxonomic community structure (Bauchhenss, 1989). Field trials indicated that the number of *Collembola* species, their population densities and their diversity varied depending on trial site and planting season but were not different between Bt11 and non-Bt maize within a trial site. These findings suggested that Bt11 did not have negative impacts on the presence, diversity and population densities of *Collembola*.

All the aforementioned suggest that Bt11 maize did not have negative effects on the abundance, population dynamics of key arthropod predators, herbivores, soil fauna, and arthropod diversity. Our findings are in conformity with those reported by Alcantara (2004) and Reyes et al. (2004).

Efficacy on control of ACB

Under confined trial conditions, Bt11 maize showed high resistance against ACB. ACB larvae failed to establish in Bt11 plants, thus the leaves, stalks and ears were undamaged, except a minor damage on leaves recorded at Ba Ria – Vung Tau site. Non-Bt corn plants, however, were severely damaged by ACB on stalks and ears (Table 1). Under large scale field trial conditions, again, Bt11 maize showed high resistance against ACB at all trial sites (Table 2). With more healthy ears being harvested, Bt11 maize plants had a higher yield than that of the conventional hybrids. Our results were in conformity with findings reported by Ebora et al. (2005) and Rasco et al. (2008). These promising results clearly showed the potential benefits of the Bt maize through increased yield, production of higher quality and cleaner grains and the possible elimination of the use of chemical insecticides to control the ACB.

Table 1. Comparative the percentage (%) of damage caused by ACB at two confined field trial sites conducted in 2010

Damaged part	Hung Yen		Ba Ria-Vung Tau	
	NK66Bt11	NK66	NK66Bt11	NK66
Leaf	0.0	100.0	1.7	96.7
Tassel	0.0	4.1	0.0	7.4
Stalk	0.0	35.0	0.0	35.0
Ear	0.0	38.3	0.0	66.7

Table 2. Comparative the percentage (%) of damage caused by ACB at four large scale trial sites conducted in 2011

Damaged part	Hung Yen		Son la		Ba Ria – Vung Tau		DakLak	
	NK66Bt11	NK66	NK66Bt11	NK66	NK66Bt11	NK66	NK66Bt11	NK66
Leaves	0.0	27.0±1.22	0.0	4.0±1.00	0.0	38.0± 0.5	0.0	43.0± 1.2
Tassels	0.0	60.67± 3.72	0.0	15.0±2.24	0.0	31.0 ±1.0	0.0	35.0± 1.2
Stalks	0.0	100.0± 0	0.0	95.0±2.24	0.0	44.0± 0.7	0.0	84.0± 0.5
Ears	0.0	95.0± 2.24	0.0	100.0 ± 0	0.0	30.0± 0.6	0.0	70.0± 0.8

Economic profits

Under confined trial conditions, the yield difference between the Bt11 and non-Bt maize ranged from 1.57 to 1.81 t/ha, and average yield of Bt11 maize was 9.51 t/ha, an increase of 22.4% compared to 7.77 t/ha of non-Bt maize. Under large scale field trial conditions, the yield difference between Bt11 and non-Bt maize ranged from 1.21 to 2.14 t/ha, and the average yield of Bt11 maize was 9.56 t/ha, an increase of 18.7% compared to 8.06 t/ha of non-Bt maize (Fig. 1).

Although the total costs for production per hectare of Bt11 maize (ranged from 21.83 to 22.68 million VND) was higher than those of non-Bt maize (ranged from 21.15 to 21.97 million VND), the average gross income of Bt11 maize was 62.27 million VND and was 9.8 million VND (or 18.9% increase) higher than those of 52.39 million VND of non-Bt maize (Fig. 2). The profit of Bt11 maize ranged from 28.98 to 47.47 million VND/ha and was 9.2 million VND higher than the non-Bt maize which ranged from 22.90 to 35.96 million VND/ha or equal to 30% profit increase.

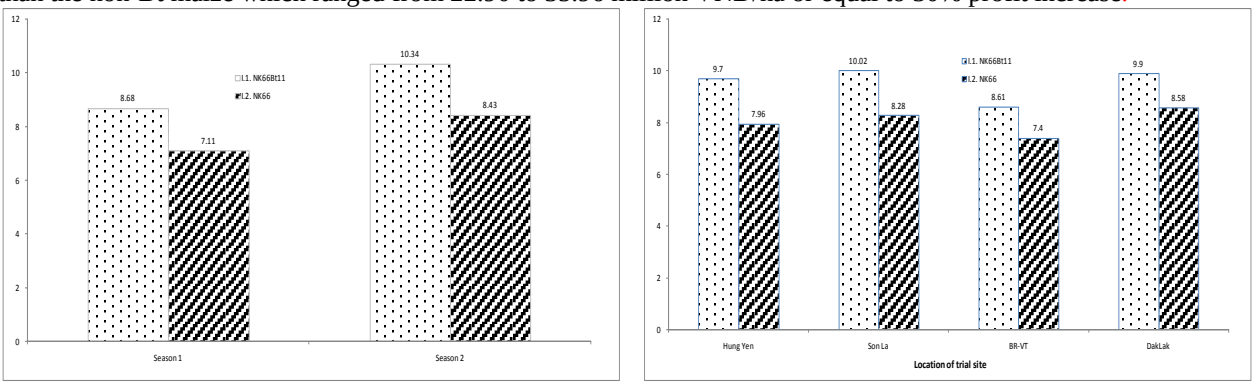


Fig. 1. Comparative yield of Bt11 maize and non-Bt maize in confined field trials (left) and large scale field trials (right)

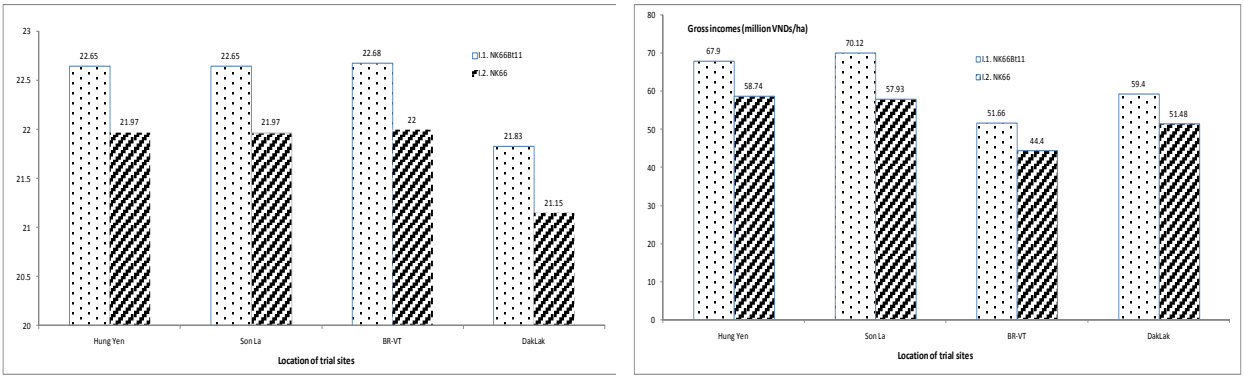


Fig. 2. Comparative total costs (left) and gross income (right) of Bt11 maize and non-Bt maize at four large scale field trial sites

Conclusion

Five events of GM maize were tested successfully for risk assessment on bio-diversity and environment under confined and large scale field trials in Vietnam conditions. The promising outcomes of these trials support the plan for GM crops commercialization in Vietnam in the coming years. And NK66Bt11 offers Vietnamese maize growers an advantage edge to avoid ACB with higher yields and income returns without affecting the agro-environment.

References

Abendroth, L.J., Elmore, R.W., Boyer, M.J., and Marlay, S.K. 2011. Corn growth and development. Iowa State Univ. Extension publication PMR1009.

Alcantara, E. 2004. Monitoring insect abundance and diversity in Bt corn. In: Impact assessment of Bt corn in the Philippines, Terminal Report. International Service for the Acquisition of Agribiotech Applications, 93 pp.

Bauchhenss, J. 1989. Vergleichende Untersuchungen der Collembolen- und Oribatidenbesiedlung von Weinbergen und naturnahen Flachen auf vier Weinberglagen Unterfrankens. Bayerisches Landwirtschaftliches Jahrbuch, 66:985-1010.

Candolfi, M.P., Brown, K., Grimm, C., Reber B., and Schimidli, H. 2004. A faunistic approach to assess potential side-effects of genetically modified Bt-corn on non-target arthropods under field conditions. Biocontrol Science and Technology, 14:129-170.

Carpenter, J.E. 2011. Impacts of GM crops on biodiversity. GM Crops, 2:7-23.

Ebora, R.V., Ampil, A.C., Placacpac, M.B., and Custodio Jr., C.G. 2005. Commercialization of Bt corn in the Philippine – A status report. Asia-Pacific consortium on agricultural biotechnology.C/o ICRISAT, NASC Complex, DevPrakashShastriMarg, Pusa CampusNew Delhi-110 012, India.

Guthrie, W.D., Dicke, F.F., and Neiswander, C.R. 1960. Leaf and sheath feeding resistance to the European com borer in eight inbred lines of dent com. Ohio Agric. Exp. Stn. Res. Bull. 860. 38 pp.

Mendelsohn, M., Kough, J., Vaituzis, Z., and Matthews, K. 2003. Are Bt crops safe? Nature Biotechnology, 21:1003-1009.

Ministry of Agriculture and Rural development (Crop Department). 2011. Report on maize production status and solutions for development in Northern provinces. Workshop document, 11pp. (in Vietnamese).

Nguyen Đuc Khiem 1995. Some study results on corn stem bore at Hanoi University No1. Plant Protection Journal, 5:3-6. (in Vietnamese).

Nguyen Quy Hung et al. 1978. Results of studying on corn stembore during 1972 – 1975. In "Scientific research on plant protection, p 126-142. (in Vietnamese)

Nguyen Van Thanh et al. 2006. Results of insect pests survey in the South in 1977-1979. In: Unpublished results of basic survey, diagnostic and identification of agricultural pests: 30 years of basic survey on agricultural crop pests (1976-2006). p 24-57 (in Vietnamese).

Pham Van Lam 2002. Results of identification of pests'natural enemies associated on some major crops during 1981-2002. Natural enemies resource: Research and application. Book 1, p 7-57 (in Vietnamese).

Reyes, S.G., Jovillano-Mostales, M.D.A., and Sicat, L.L. 2002. Biodiversity, community structure and population abundance of arthropods in Bt corn agroecosystems. Terminal Report, UPLB, 22 pp.

Rasco, Jr. E.T. 2008. Efficacy of insect protected maize (Bt11) against Asiatic corn borer (Ostrinia furnacalis Guenee). Philippine Journal of Crop Science, 33:82-89.

Romeis, J.R., Bartsch, D., Bigler, F., Candolfi, M.P., Gielkens, M.M.C., Hartley, S.E., Hellmich, R.L., Huesing, J.E., Jepson, P.C., Layton, R., Quemada, H., Raybould, A., Rose, R.I., Schiemann, J., Sears, M.K., Shelton, A.M., Sweet, J., Vaituzis, Z., and Wolt, J.D. 2008. Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. Natural Biotechnology, 26:203-208.

Maize Stacked with Genetically Modified Events in the Philippines

Saturnina C. Halos
Bureau of Plant Industry, Department of Agriculture
San Andres St. Malate, Manila, Philippines

Abstract

A crop stacked with genetically modified (GM) events is a crop carrying two or more transformation events resulting from the hybridization of two or more parents containing the different GM or transformation events. Philippine regulators have identified that in addition to risks identified for the individual transformation events, the possible interaction of the products of the individual transformation events in the crop is an added risk. The method of assessing this risk is addressed by two regulatory issuances: one for products with stacked GM events imported for direct use for food and feed or for processing and the other for products with stacked GM events intended for release into the environment. Risk assessment of products with stacked GM events where the individual events have already been assessed as safe, comprise of two steps. The first step is basically a review of the literature that provides information on the possibility that interaction between and among the products of the transgenes may occur. Should this be a possibility, a detailed risk assessment of the product with stacked GM events is required. If no possibility of interaction occurs, the applicant is notified that the stacked GM product may be used as applied for. If the individual GM events have not been previously assessed, a detailed risk assessment of the product with stacked GM event is undertaken and its assessment also applies to all products that may carry the individual events or the lesser combination of the events stacked in the product.

Since 2002, 25 maize products stacked with different GM events have been approved for import for direct use and three for propagation. The stacked events of maize approved for import for direct use contain 2-8 transgenes and stacked 2- 5 transformation events. The three stacked events approved for propagation contain the *Bacillus thuringiensis* gene for insect resistance and contain the glyphosate tolerant EPSPS gene and are designated Bt/RR. More than 85% of GM corn areas in the Philippines are planted with Bt/RR yellow corn. The rapid adoption of GM corn in the Philippines among hybrid yellow corn growers is due to a number of reasons notably the increase in income at about US$322/ha. The extensive adoption of GM corn has increased national corn production, national corn productivity and has stabilized the supply of feed corn for the livestock industry. Since 2006, the aggregated additional income from GM corn farming is more than US$101 million per year. No risk has been associated with stacking of different transformation events. Risks associated with extensively planted stacked transformation products are those associated with the single events conferring insect resistance, Bt and herbicide tolerance, RR. The risk with insect resistance is the spread of target insects resistant to Bt. Risks associated with RR corn is the spread of weeds tolerant to glyphosate and shifts in the weed species populations. The risk associated with Bt corn is managed by an insect resistance management strategy. Another risk management measure is a continuing monitoring program for insect resistance, glyphosate tolerance and weed population shifts is in place. The monitoring program has not identified any adverse environmental effect from the GM corn events themselves since 2003. The extensive planting of GM corn in sloping areas without the adoption of soil and water conservation measures is a cause of concern which the Department of Agriculture is trying to address.

Keywords: adoption, economic benefits, Philippine regulation, risks, stacked GM events

Introduction

In 2002, the Department of Agriculture (DA) of the Philippines issued Administrative Order No. 8 series of 2002 (DA AO8) which is the regulation of genetically modified crops and other products of modern biotechnology. This issuance states that authorizations from the Bureau of Plant Industry (BPI) must be sought for genetically modified crops for import for contained use, for import for direct use for food and feed or for processing, for field trials and for propagation. The authorization is based on the conduct of a satisfactory risk assessment. As per DA AO8, risk assessment among other things should be carried out on a case by case and by transformation event. With the advent of stacked GM events, BPI and the DA decided that stacking GM events poses another risk in addition to risks identified with a transformation event. This paper describes the regulation of stacked GM event, stacked GM events in maize approved in the Philippines, post-approval monitoring of approved stacked products, adoption of GM maize including stacked GM events and socio-economic and environmental notes on GM maize planting in the Philippines. Data on regulation and documents from the Department of Agriculture and the Bureau of Plant Industry are downloaded from www.biotech.da.gov.ph. Sources of other data are as indicated.

Health and Environmental Risks of GM Crops

The risks or possible dangers to human health and to the environment of GM crops have been the subject of agreements arrived at by the Codex Alimentarius and the Panel of Experts of the OECD. These risks or possible dangers were derived of what we know about plants and all the dangers that plants can pose. The risks or possible dangers that may

affect human and livestock health include: a) levels of natural toxicants, allergens and anti-nutrients may be increased in the GM crop; b) possible change in consumption patterns because of the new trait in the GM crop; c) the donor of the transgenes and DNA sequences may be toxic or allergenic and may transfer these unfavorable traits into the GM crop; d) the transformation system used may result in a genetically unstable GM crop and thus, it has inconsistent expression of its properties; e) the DNA insert/s may result in genetic instability; f) the position of DNA insert may result in genetic instability or other important genes may be adversely affected; g) the level of expression of the gene product may vary and therefore inconsistent in its effectiveness or it may be too high and cause allergenicity or toxicity; h) the possible toxicity, anti-nutrient activity and allergenicity of the products of the transgenes; and i) possible change in the nutritional content if it is a food crop.

Risks to the environment come from the possible changed interactions of the GM crop with its environment. It is possible for a GM crop to become more susceptible to its known pests; or to become invasive due to its new traits; or by hybridizing with its wild relative may result in a difficult to control weed or the extinction of the wild relative; or if it contains a new pesticide product, the GM plant may adversely affect beneficial and other non-target organisms; or that the GM crop may require a new agricultural practice and this practice may pose risk to the environment. Other risks may be identified depending on the GM crop and the traits it has acquired. One risk identified with the continuous use of the herbicide, glyphosate, with the continuous planting of herbicide tolerant (HT) crops is the possible reduction in weed diversity and the change in population structure resulting in the loss of beneficial arthropods dependent on lost weed species. It should be noted that not all the above mentioned risk applies to all GM crops since GM crops may differ with their new traits.

On the other hand, there are risks that have 100% probabilities of happening. For example, insect resistant crops such as Bt crops do select for insects that are resistant to the Bt protein. Extensive planting of a Bt crop eventually promote the survival and spread of resistant insects thereby posing the danger of reducing the effective life of the insect resistance crop or the Bt technology. Similarly, herbicide tolerant (HT) crops pose the danger of selecting for and spreading herbicide tolerant weeds. These dangers are bound to happen given the natural evolution of organisms.

Addressing Risks of GM Crops: Philippine Regulation

Risks are possible dangers that one can imagine pertaining a particular object or scenario. The Cartagena Protocol on Biosafety requires the conduct of risk assessment as basis for decision on the handling of products of modern biotechnology like GM plants. Risk assessment is a science-based series of procedures comprised of identifying risks or possible dangers, evaluating each individual risk or possible danger on the probability of it happening, the extent of the damage the danger can cause and the possible measures to minimize, avoid or reduce the damage.

In the Philippines, BPI has been mandated to regulate GM crops. It grants four types of biosafety permits: one, for the import of a GM crop of particular transformation event/stacked events for contained use; two, for the import of a GM crop of particular transformation event/stacked events for direct use as food, feed or for processing; three, for the conduct of field trials of GM crop of a particular transformation event/stacked events; and four, for the propagation of GM crop of a particular transformation event/stacked events. These biosafety permits are given only after a satisfactory risk assessment has been conducted indicating that the GM crop with a particular transformation event/stacked events is as safe as its non-GM counterpart for an indicated use.

Risk assessment proceeds based on a document called BPI risk assessment report form which requires technology developers to undertake the risk assessment of their GM product with a specific transformation event. The risk assessment comprise of a review of the scientific literature and the conduct of experiments. When an application is filed the applicant submits all the scientific documents supporting the statements they gave on the risk assessment report form. Two sets of review panels examine these documents to ascertain that the science was rigorous, the methods robust and the results support the contentions of safety of the product. When the results are vague and non-conclusive, additional studies are required to resolve the issue. In risk assessment, the properties of the GM crop are compared with its non-GM counterpart. For example, the level of the native toxicant, solanine in the GM eggplant is compared with the level of solanine in the non-GM eggplant. The risk assessment is specific to a particular product of a specific transformation event or stacked events regardless whether the same gene/s is involved e.g. corn MON810 and corn Bt11 have been transformed with the same *cry1Ab* gene but their risks are individually assessed. As we come to know more about risk assessment, new information may be generated concerning an authorized transformation event. In this case, the authorization is reviewed in light of the new information.

The BPI maintains an Approval Registry of GM crops with specific transformation event and stacked events for an intended use: for import for direct use for food, feed or for processing, for field trials and for propagation. This information is publicly available at www.biotech.da.gov.ph. Those listed in the registry are declared as safe as their non-GM counterpart and can safely be used for their intended use. In essence, those listed in the Approval Registry do not pose any risk other than those posed by their non-GM counterpart or if there is an identified risk, this risk is manageable and any possible damage minimized.

For risks that are certain to occur such as the selection and spread of insects resistant to the Bt protein by Bt crops and the selection and spread of weeds tolerant to the herbicide by herbicide tolerant crops. The regulators in the Philippines

require risk management procedures to delay the spread of Bt-resistant insects and resistant weeds. The rapid selection and spread of insects resistant to the Bt corn is managed by the adoption of an Insect Resistance Management (IRM) strategy. This strategy referred to as bag-in-a-bag strategy, comprise of the sale of seed bags containing separately packaged insect resistant (Bt) seeds and insect susceptible seeds at a ratio of 80:20 and requires farmers to plant the insect resistant (Bt) seeds and insect susceptible seeds within the same area to maintain the ratio of 80 (IR seeds):20 (insect susceptible seeds). This strategy reduces the selection pressure for the resistant insect and thus lengthens the useful life of the Bt technology. For herbicide tolerant GM crops, practices are recommended to the farmers to reduce the possible spread of herbicide resistant weeds. Monitoring plots have been established to determine change in weed diversity, weed species population shifts and herbicide tolerance of weeds in areas continuously planted with glyphosate tolerant crops.

Regulation of GM Crops with Stacked Events

A crop stacked with GM events is a crop containing two or more transformation events resulting from the hybridization of two or more parents containing the different GM events. That is, the parents in the breeding process are both genetically modified plants carrying different transgenes. These transgenes may confer similar traits e.g. insect resistance or different ones e.g. insect resistance and herbicide tolerance. A reasonable assumption is that the products of the transgenes are different and for the first time they are going to be present together in the same plant. Consequently, these gene products may interact and their possible interactions present a risk. It is possible for the protein products to exchange subunits and form a nasty protein. It is possible that the protein products may cancel each other's effect. To address these risks, the Department of Agriculture has issued two memorandum circulars, Memorandum Circular No. 8 October 11, 2004 Risk Assessment for Stacked Gene Products Imported for Direct Use as Food and Feed or Processing, and Memorandum Circular No. 6 September 16, 2004 Risk Assessment of Plants Carrying Stacked Genes for Release Into the Environment. All approved stacked products for propagation in the Philippines comprise of combinations of single events that were assessed individually and have been declared as safe as their non-GM counterparts. The stacked products were assessed for the possible interaction of the products of the single events. All approved products do not pose any danger from the interaction of the products of the transferred genes and do not pose additional risks beyond those identified for the single events.

Maize with Stacked GM Events Authorized in the Philippines

There are 25 maize products with stacked GM events authorized for import for direct use for food and feed and for processing in the Philippines (Table 1). These products differ in the number of transformation events that have been stacked from 2-5 and the number of transgenes transferred from 2-8. Despite these differences, almost all except one express only two combined traits – insect resistance and herbicide tolerance. The one exception carries the amylase gene in addition to insect resistance and herbicide tolerance. There are three corn products with stacked GM events approved for propagation. Two of these carry two transgenes and one has three transgenes. These stacked GM events express only two traits, insect resistance and herbicide tolerance. One product contains pyramided genes for insect resistance.

Table 1. The number and nature of stacked GM events in maize permitted for import for direct use for food, feed and processing and for propagation in the Philippines (2002- Feb, 2013)

A. Stacked GM events in maize authorized for import for direct use for food, feed and processing			
No of transformation events in stacked product	No of genes transferred	Traits endowed	No. of approved stacked events
2	2	Insect resistance, herbicide tolerance	11
	3	Insect resistance, herbicide tolerance	2
	4	Insect resistance, herbicide tolerance	1
3	3	Insect resistance, herbicide tolerance	4
	5	Insect resistance, herbicide tolerance	2
4	4	Insect resistance, herbicide tolerance	2
	4	Insect resistance, herbicide tolerance, amylase	1
	8	Insect resistance herbicide tolerance	1
5	8	Insect resistance, herbicide tolerance	1
Total			25

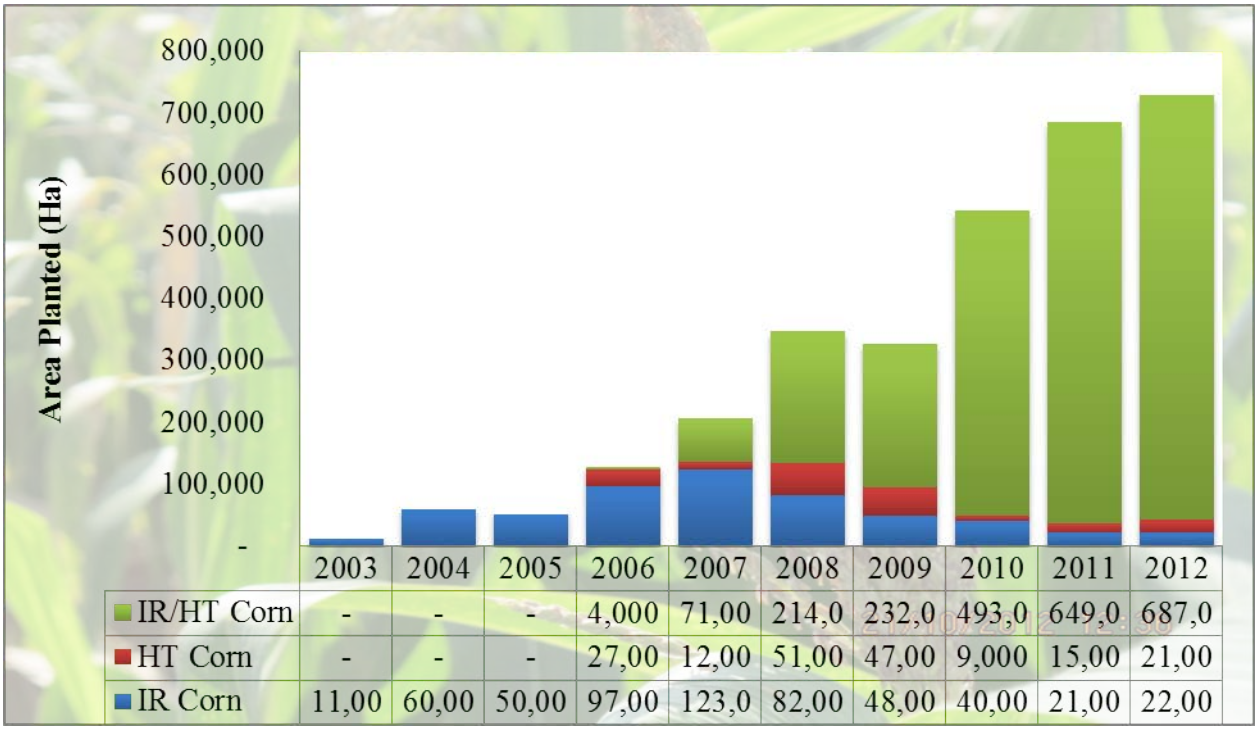
B. Stacked GM events in maize authorized for propagation			
No of events in stacked product	No of genes transferred	Traits endowed	No. of approved stacked events
2	2	Insect resistance, herbicide tolerance	2
2	3	Insect resistance, herbicide tolerance	1
Total			3

Adoption of GM Maize

Similar to other countries, GM corn was rapidly adopted by corn farmers in the Philippines. Ten years after its introduction, GM corn was planted in 730,000 ha in 2012 more than the area previously occupied by conventional hybrid corn of about 700,000 ha (Halos and Soriano, 2013). This area may further expand in the coming years because of the increasing demand of a growing livestock industry (Umengan, 2013 Pers. comm.). The first introduced varieties of GM corn carry only a single Bt transformation event conferring insect resistance (IR) and these varieties dominated the plantings of GM corn from 2003-2007 (Fig. 1). Another single event, conferring a different trait, tolerance to the herbicide (HT), glyphosate commercially referred to as RR and the stacked product IR/HT were introduced in 2006. Three years after their introduction, the stacked insect resistant and herbicide tolerant (IR/HT) varieties started to dominate GM corn plantings. As of 2012, more than 90% of the GM corn area was planted with stacked IR/HT varieties.

Post-Approval Regulation of Corn with Stacked GM Events

In compliance with the bag-in a-bag IRM strategy for IR corn, stacked IR/HT varieties are sold in 18 kg-seed bags containing separately packaged insect resistant herbicide tolerant (IR/HT) GM seeds and herbicide tolerant (HT) GM seeds at a proportion of 80:20 (bag-in-a-bag). Both IR/HT and HT seeds must be planted within a one-hectare area. The BPI monitors the contents of the seed bags and randomly checks the planting by farmers. Also part of the post-approval monitoring program is a continuing study on the susceptibility of the target insect collected from the field and F_2 populations to the IR protein by an independent scientist. The monitoring program for the HT trait comprise of experimental plots established to determine changes in weed species composition and diversity and identify glyphosate tolerant weeds. The technology developers have a continuing training program for farmers to monitor for insect resistance and to use practices that delay the spread of weeds resistant to the herbicide. So far no report of Asiatic corn borer (ACB) resistant to the Bt protein has been received since 2003. No shifts in weed species populating RR corn fields have been found since 2006.



	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
IR/HT Corn	-	-	-	4,000	71,00	214,0	232,0	493,0	649,0	687,0
HT Corn	-	-	-	27,00	12,00	51,00	47,00	9,000	15,00	21,00
IR Corn	11,00	60,00	50,00	97,00	123,0	82,00	48,00	40,00	21,00	22,00

Fig 1. GM corn adoption (x1000 hectares) in the Philippines (2003-2012)

Socio-economic and Environmental Benefits and Risk of GM Maize Planting

The rapid adoption of GM corn in the Philippines is promoted by several factors (Halos, 2012). One, the technology is highly effective and farmers are confident in using the technology. Bt corn appears to have fully eliminated the need to control ACB. HT corn which is primarily glyphosate tolerant corn has eliminated the need to hire and manage manual labor to control weeds. The use of HT corn appears to have solved the problem of dwindling labor supply in many areas. Two, farmers perceive the Bt corn as insurance against ACB damage and find it safer than pesticides. Three, majority of farmers have high yields and give them additional incomes over previous years of planting conventional hybrids. The higher yield of GM corn is due to the GM traits and the improved background genotypes. Four, another factor farmers cite for preference of GM corn is ease of management. They no longer worry about the proper time of applying the insecticide that controls the ACB and of managing recalcitrant human labor. Five, farmers have easy access to credit supplied by private corn financiers. The rural areas gain from the additional incomes accrued to farmers, an aggregate of about US$117 million in the first10 years. Last year, 2012, the aggregate added income from GM corn farming in 730,000 hectares was about ~US$450.8 million. In addition, the environmental benefit observed with Bt corn are the abundance of friendly arthropods and reduced risk from pesticide poisoning to farmers and farm animals. However, the risk of spreading insects resistant to the Bt protein remains as well as the spread of weeds tolerant to the herbicide glyphosate. Also, farmers find GM corn farming lucrative and have expanded into planting in sloping areas without appropriate adopting soil and water conservation measures. This is a problem the Department of Agriculture is trying to address.

References

Halos, S.C. 2012. Ten years of GM crop regulation in the Philippines. Paper presented at the Workshop on Biotechnology for food security and farmer prosperity, Nov 28-29, 2012, The Westin, Dhaka, Bangladesh

Halos, S.C. and Soriano, T.L. 2013. Ten years of GM crop regulation in the Philippines. *Philippine Journal of Crop Science*, In Press.

Development of Marker-Free Transgenic Plants

Ayako Nishizawa-Yokoi[1] and Seiichi Toki[1, 2]
[1]Plant Genome Engineering Research Unit, Division of Applied Genetics,
Institute of Agrobiological Sciences, NARO, Tsukuba, Japan
[2]Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan

Abstract

Plant transformation technology is widely utilized for molecular breeding and basic research in various plant species. Following introduction of the transgene into the plant genome, the use of selectable marker genes is necessary to isolate transformed cells from among non-transformed cells. However, the presence of selectable marker genes is dispensable for growing transgenic plants in the field, and indeed can lead to environmental problems and increased public concern about the safety of transgenic plants. Therefore, a suite of strategies has been developed to eliminate selectable marker genes from plant genomes after selection. To date, several methods, such as co-transformation, site-specific recombination, intra-chromosomal recombination and transposition, have been employed successfully to remove selectable marker genes from transgenic plants. Among them, marker excision using site-specific recombination and transposition is widely used in several plant species. However, a major problem using these methods is the presence of residual dispensable sequences such as the recognition sequences of site specific recombinase and footprints at the excised site, and the low efficiency of marker-free transgenic plant generation.

The *piggyBac* transposon derived from the lepidopteran cabbage looper moth *Trichoplusia ni* integrates into the host genome at TTAA elements and excises without leaving a footprint at the excised site TTAA elements. In addition, the *piggyBac* transposon system has been exploited successfully to eliminate a selectable marker without leaving a footprint in a variety of organisms. However, so far there was no evidence that the *piggyBac* transposon functions in higher plant cells. We have designed a transposition assay system that allows *piggyBac* transposition to be visualized as emerald luciferase (Eluc) luminescence in rice cells. The Eluc signal derived from *piggyBac* excision was observed in *piggyBac* transposase-expressing rice calli. PCR, Southern blot analyses and sequencing revealed efficient and precise transposition of *piggyBac* in these calli. Furthermore, we have demonstrated *piggyBac* excision from a reporter locus in T_0 plants without concomitant re-integration of the transposon and at high frequency.

These findings demonstrate that the *piggyBac* transposon is capable of accurate and effective transposase-mediated transposition in plant cells, indicating that a high frequency marker excision system for plant genomes could be established using this system.

Keywords : Selectable marker genes, Marker excision, *piggyBac* transposon

Introduction

Agrobacterium-mediated plant transformation has been used widely in the fields of both molecular breeding and basic research in various plant species. To identify transgenic plants conveniently, selectable markers are routinely used in the process of plant transformation. Marker genes that confer resistance to antibiotics [e.g., kanamycin for *neomycin phosphotransferase II* (*nptII*) and hygromycin for *hygromycin B phosphotransferase* (*hpt*)], or herbicides [e.g., bispyribac-sodium for *acetolactate synthase* (*ALS*) and bialaphos for *phosphinothricin acetyltransferase* (*bar*)], have been used in most transgenic research and crop development techniques. In addition, non-toxic metabolic intermediates, such as D-mannose for the *phosphomannose isomerase* (*PMI*) gene have also been employed recently as selectable markers for the production of transgenic plants (Miki and McHugh, 2004). Transgenic cells are selected following co-transformation of target cells with the gene of interest (GOI) and selectable marker genes on either a single T-DNA on separate T-DNAs. Transformed cells with the GOI and selectable marker gene can survive and proliferate on medium containing the appropriate antibiotics or herbicides. However, the presence of a selectable marker gene is dispensable for growing transgenic plants in fields and can in itself raise environmental problems and lead to public concern about the safety of transgenic plants. Therefore, a suite of strategies has been developed to eliminate selectable marker genes from plant genomes following the selection step. Several methods, including co-transformation, transposition, and site-specific recombination, have been employed successfully to date for removal of selectable marker genes from transgenic plants (reviewed in Hohn et al., 2001; Darbani et al., 2007; Woo et al., 2011). This review gives an overview of each of these strategies, and summarizes the state of the art in this field.

Co-transformation

The principle of the co-transformation strategy to remove a selectable marker gene from transgenic plants is that the selectable marker gene and the GOI are derived separately from different T-DNAs within the same or a different *Agrobacterium* strain. If the selectable marker gene and GOI cassettes are integrated into different loci of the host genome, subsequent crossing can separate the GOI from the selectable marker gene and allow the production of marker-free transgenic plants in T_1 progeny (Fig. 1A). However, this strategy is not suitable for plant species for which *Agrobacterium*-mediated transformation is difficult, because in such cases it is hard to introduce two different T-DNAs into the same cell. In addition, the two different T-DNAs occasionally integrate into linked loci in the host genome. Consequently, marker-free transgenic plants are obtained at only low frequency using this method, even in plant species for which an efficient *Agrobacterium*-mediated transformation system exists.

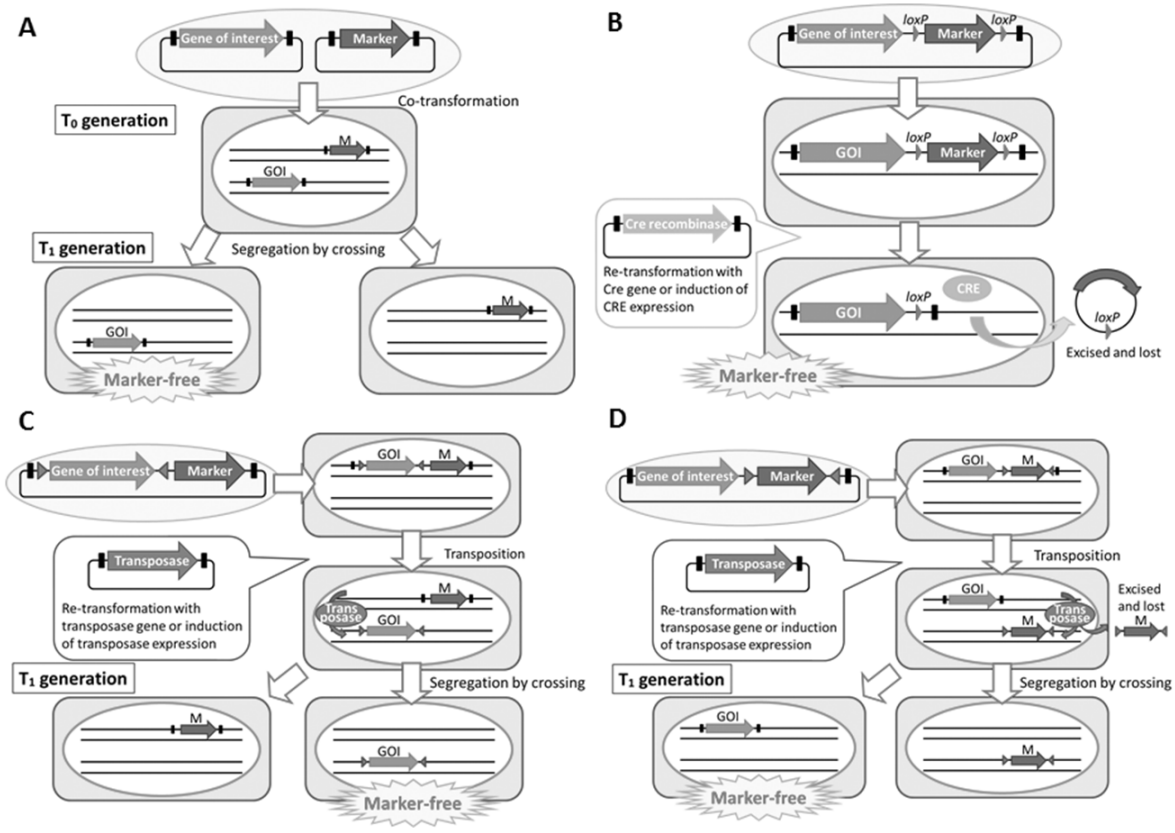


Fig. 1. Strategies for selectable marker gene removal using co-transformation (A), site-specific recombination (Cre/loxP system, B), and transposons (C, D)
Solid boxes show the border sequence of T-DNA. GOI, gene of interest; M, selectable marker gene.

Site-specific recombination

To produce marker-free transgenic plants using site-specific recombination methods, three well-described site-specific recombination systems have proved useful in several plant species: the Cre/*loxP* system from bacteriophage P1 (Sternberg and Hamilton, 1981), the FLP/*FRT* system from *Saccharomyces cerevisiae* (Senecoff et al., 1985) and the R/*RS* system from *Zygosaccharomyces rouxii* (Araki et al., 1985). The steps involved in generating marker-free plants via the Cre/*loxP* recombination system that have been used most extensively in model and crop plants are shown in Fig. 1B. The first step is the introduction, from a single T-DNA, of a GOI and selectable marker gene flanked by two Cre recombinase recognition sites (*loxP*). In the second step, retransformation with the Cre recombinase gene or induction of Cre recombinase expression in the transgenic plants harboring the GOI and selectable marker gene lead to excision of the marker gene. Finally, marker-free transgenic progeny not carrying the Cre recombinase gene cassette can be obtained via an additional breeding step. A major problem with using site-specific recombination methods is that it requires the presence of site-specific recombinase recognition sequences at the excised site.

Transposition

Transposons can be exploited to remove marker genes from transgenic plants in at least two ways (Fig. 1C, D). In the first, the GOI is placed on a transposable element so that it can relocate by transposition to a new locus distinct from the selectable marker gene at the original transgene locus (Fig. 1C). Alternatively, the transposon, which includes the selectable marker gene, is lost after transposition (Fig. 1D). In both cases, a segregation step via self-pollination or out-crossing are required to achieve marker-free transgenic plants lacking the transposase expression cassette.

Among several transposable elements adapted for use in the above techniques, the maize *Ac*/*Ds* transposon system is the most widely used, and has led to the successful production of marker-free transgenic plants in several studies (Goldsbrough et al., 1993; Ebinuma et al., 1997; Cotsaftis et al., 2002). However, a major problem with using a transposable element system for eliminating a marker gene is the residual footprint at the excised site, and the low efficiency of marker-free transgenic plant generation, because of the tendency for the transposon to reinsert elsewhere in the genome. Furthermore, it has been reported that these small dispensable sequences have the potential to interfere with transcriptional regulatory elements of surrounding genes in mammalian cells.

Precise marker excision using *piggyBac* transposon

The *piggyBac* transposon derived from the lepidopteran cabbage looper moth *Trichoplusia ni* (Cary et al., 1989) integrates into the host genome at TTAA elements, and has been used for transgenesis and insertional mutagenesis in a variety of organisms. Additionally, the *piggyBac* transposon excises without leaving a footprint at the excised site TTAA elements (Cary et al., 1989). However, until recently there was no evidence that this animal-derived transposon is capable of transposition in plant cells.

To test the use of the *piggyBac* transposon for effective marker excision in plants, we designed a transposition assay system in rice cells (Fig. 2). This system allows transposition of *piggyBac* transposon to be visualized as luminescence derived from reconstituted luciferase expression cassettes. The reporter construct carries an emerald luciferase (*Eluc*) gene expression cassette containing a *piggyBac* transposon harboring a mutant cytosine deaminase (*codA*) gene expression cassette as a negative selection marker in the *Eluc* gene. If the *piggyBac* transposon is excised precisely by the activation of transposase, the *Eluc* gene is restored and the calli become Eluc-positive. However, if the *piggyBac* excision leaves a footprint, the *Eluc* gene remains inactivated and the calli are Eluc-negative. CodA protein converts non-toxic 5-fluorocytosine (5-FC) into the toxic 5-fluorouracil (5-FU) (Perera et al., 1993), providing a useful negative selection marker in rice. Therefore, the growth of codA-expressing rice calli is expected to be suppressed on medium with 5-FC.

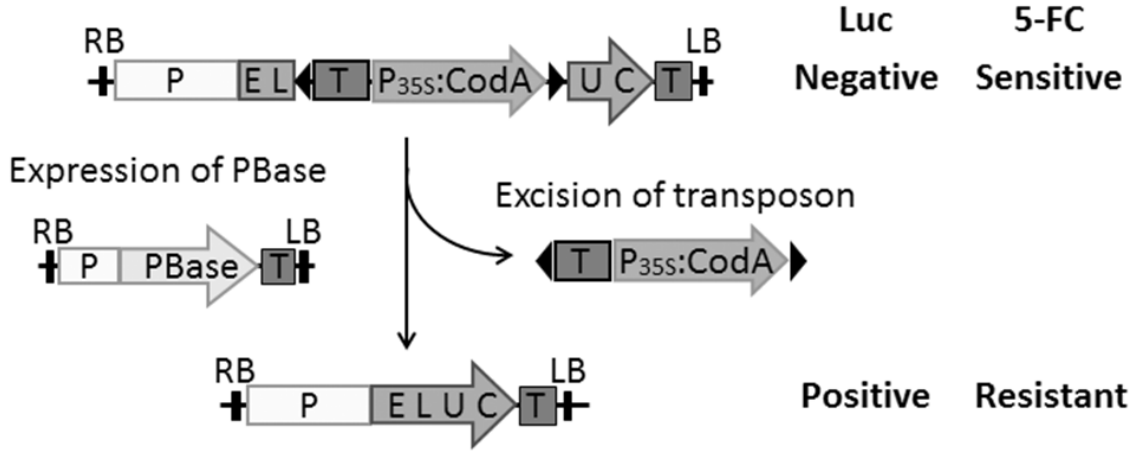


Fig. 2. Schematic representation of excision assay to detect transposition of *piggyBac* transposon as luciferase luminescence in rice calli

The reporter constructs carry a *Eluc* expression cassette containing *piggyBac* transposon harboring a *codA* expression cassette as a negative selection marker in the *Eluc* gene. Transgenic rice calli are Eluc-negative and 5-FC-sensitive. Upon precise transposon excision from the reporter locus, transgenic calli become Eluc-positive and 5-FC-resistance. However, if the *piggyBac* excision leaves a footprint, the *Eluc* gene is inactivated and the calli remain Eluc-negative. LB, left border; RB, right border.

Seven-day-old rice calli were infected with *Agrobacterium* harboring reporter constructs containing *piggyBac* transposon. Transgenic lines with a single copy of reporter constructs were selected by Southern blot analysis, and were transformed with *Agrobacterium* to introduce the control vector or expression vector of transposase PBase, and selected with or without 5-FC. After a 4-week selection period, Eluc luminescence was detected on PBase transgenic rice calli but not on control calli (Fig. 3). However, no significant differences in Eluc luminescence were seen between transgenic rice calli grown on N6D with and without 5-FC (Fig. 3). These results suggest that the *piggyBac* transposon was excised precisely from the *Eluc* expression cassette via the activation of PBase.

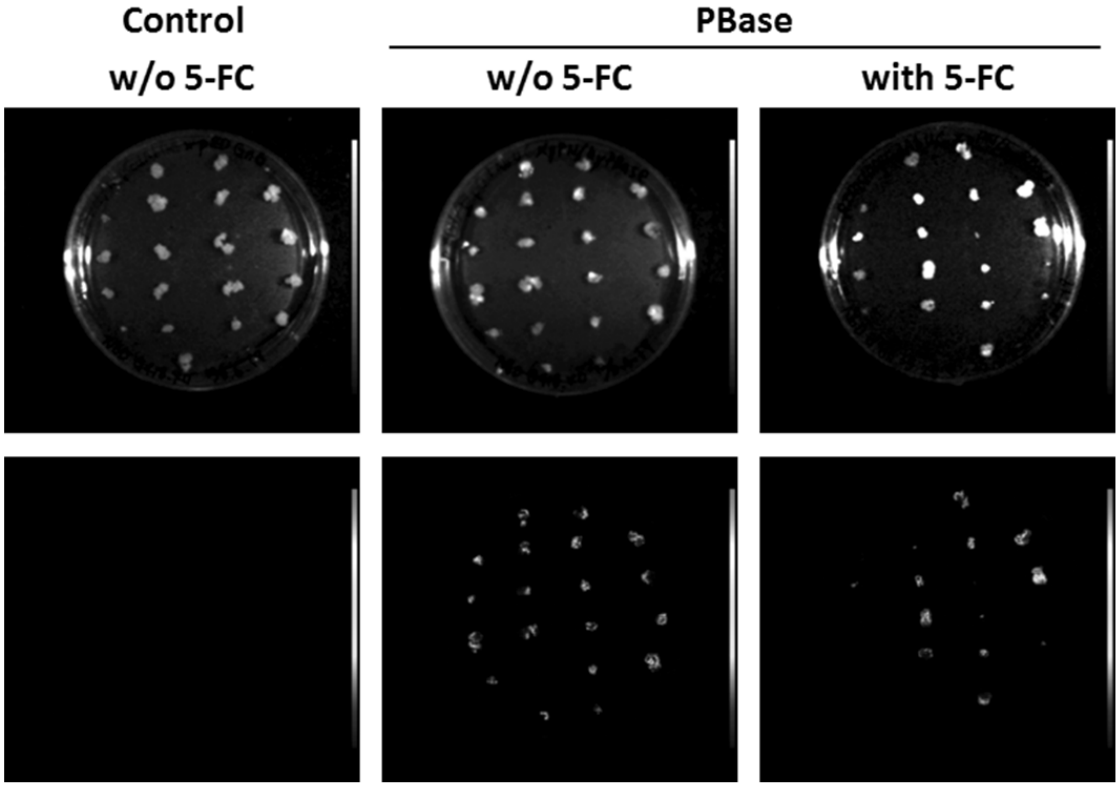


Fig. 3. Analysis of PBase-induced *piggyBac* excision in rice calli
Bright-field (upper panels) and LUC luminescence (lower panels) images of control (left panels) and PBase-expressing (middle and right panels) rice calli. LUC luminescence derived from an Eluc gene restored by *piggyBac* transposition was detected after a 4-week selection period with or without (w/o) 5-FC.

To confirm the Eluc expression results, Southern blot analysis were performed with genomic DNA extracted from three independent chimeric calli with Eluc-negative and -positive PBase transgenic calli with or without 5-FC treatment. Southern blot analysis with a specific *codA* probe illustrated in Fig. 4A revealed 8.2-kb fragments derived from the full-length reporter construct in all transgenic lines (Fig. 4B). In addition to 8.2-kb fragments, 3.9-kb fragments expected from the *piggyBac*-excised reporter cassette were detected using the 3'-flanking region of the *Eluc* gene probes in PBase transgenic calli treated with and without 5-FC (Fig. 4B). Furthermore, the *piggyBac* transposition rate was estimated to be 52.3–70.0 % and 28.1–66.8 % in three independent lines of PBase transgenic calli treated with or without 5-FC, respectively. These results indicate that negative selection with *codA* had no apparent effect on the frequency of *piggyBac* transposition. In addition, we analyzed the sequence of the *piggyBac*-excised PCR products; 20 clones from each transgenic line were sequenced; a TTAA element was restored after transposon removal in all cases (Fig. 4C). These results indicate that rice cells are just as conducive as animal cells to *piggyBac* transposition.

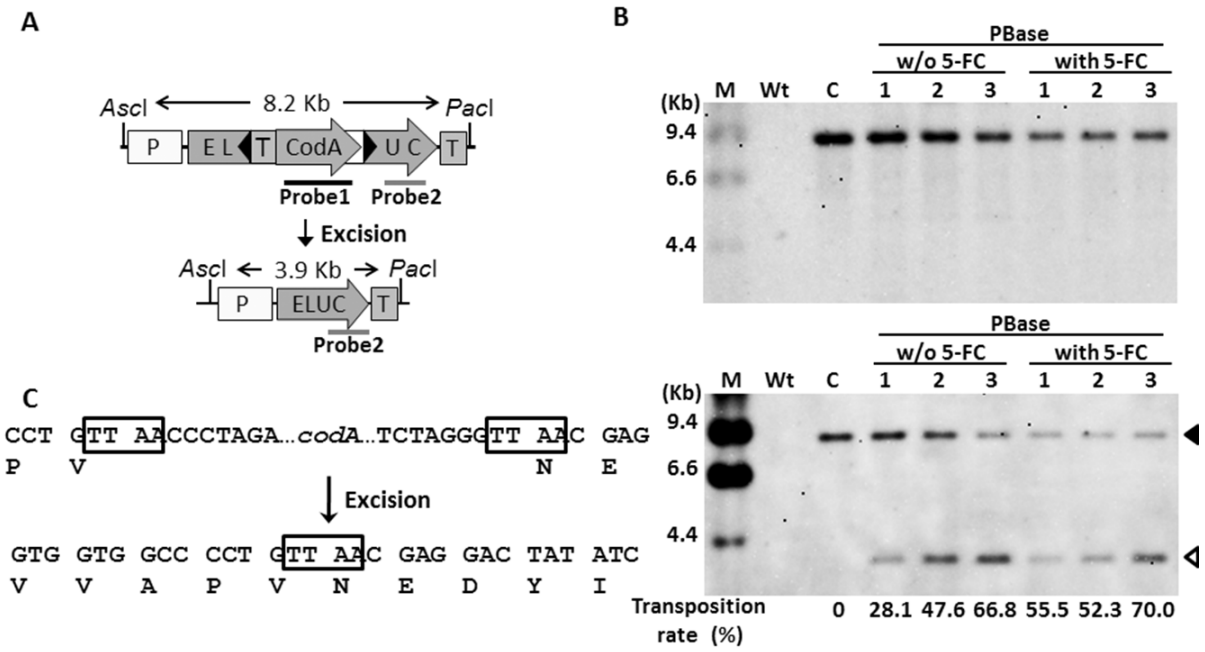


Fig. 4. Molecular analysis of PBase-induced *piggyBac* excision in rice calli
(A) Structure of the full-length reporter vector and *piggyBac*-excised reporter locus. Bars represent DNA probe fragments used for Southern blot analysis. (B) Southern blot analysis using a specific *codA* (upper panels) or 3' flanking region of *Eluc* gene probe (lower panels) in wild-type (Wt), control (C) and PBase transgenic calli treated with or without (w/o) 5-FC. When digested with *Asc*I/*Pac*I, bands of 3.9 kb (open arrowhead) derived from the *Eluc* gene restored by *piggyBac* transposition were detected in addition to the original 8.2 kb bands (filled arrowhead). (C) Nucleotide sequences of the full-length reporter vector (top) and *piggyBac* excision site in the *Eluc* reporter cassette (bottom) in PBase transgenic rice calli. Open boxes highlight the duplicated TTAA sequence flanking the *piggyBac* transposon.

Eluc-positive calli derived from PBase transgenic calli were transferred to shoot regeneration medium with or without 5-FC. PCR analysis showed that the *piggyBac*-excised fragment, but not the full-length reporter construct-specific fragment, was detected in all 5-FC-resistant PBase regenerated plants (Table 1). A higher proportion of T_0 plantlets (on average, 71.7%; Table 2) contained the *piggyBac*-excised band, and lacked the full-length reporter construct-specific fragment without 5-FC selection. Our findings indicate that PBase-mediated *piggyBac* excision occurs at the callus stage. While 40.0 and 41.0% of regenerated plants treated with or without 5-FC, respectively, were shown to harbor a re-integrated *piggyBac* transposon, a *codA*-specific fragment was detected by PCR analysis. These results suggest that 5-FC negative selection did not enrich *piggyBac* excised transgenic rice cells and did not fully suppress re-integration of the *piggyBac*. Our findings suggest that the *piggyBac* transposon system could be used to eliminate selectable markers in plants without the need for negative selection by codA.

Table 1. PCR analysis of *piggyBac* excision events with 5-FC treatment in PBase expressing T_0 plants

Line no.	No. of T_0 plants analyzed	*piggyBac* excision from reporter locus			Frequency of *piggyBac* excision		
		without *CodA*	with *CodA*	Total	without re-integration	with re-integration	Total
3	10	6	4	10	60.0	40.0	100

Nishizawa-Yokoi et al. 2014.

Table 2. PCR analysis of *piggyBac* excision events without 5-FC treatment in PBase expressing T_0 plants

Line no.	No. of T_0 plants analyzed	*piggyBac* excision from reporter locus			Frequency of *piggyBac* excision		
		without *CodA*	with *CodA*	Total	without re-integration	with re-integration	Total
1	36	4	12	16	11.1	33.3	44.4
2	40	12	22	34	30.0	55.0	85.0
3	20	3	14	17	15.0	70.0	85.0
4	30	24	3	27	80.0	10.0	90.0
5	21	4	10	14	19.0	47.6	66.7
6	20	5	6	11	25.0	30.0	55.0
7	29	10	12	22	34.5	41.4	75.9
Ave.					30.7	41.0	71.7

Nishizawa-Yokoi *et al., Plant J.* in press.

Conclusion

To date, transposable elements have been thought not to be able to transpose effectively in heterologous species. However, we have shown that the animal-derived *piggyBac* transposon can transpose effectively and accurately in rice cells (Nishizawa-Yokoi et al., in press). Our results suggest that the *piggyBac* transposon system has several advantages over other marker excision systems currently utilized in plants, and that it could be applied universally, not only to model plants but also to agriculturally important crops. Therefore, *piggyBac* transposon might be an effective tool not only in marker excision but also in the reversible plant transgenesis systems in which transgene delivery from T-DNA and sequential excision of the transgene from the genome are both mediated by *piggyBac* transposon. Li et al. (2013) have reported recently the generation of an excision competent / integration defective *piggyBac* transposase. Thus, we are progressing towards the establishment of more effective marker excision strategies using modified PBase.

References

Araki, H., Jearnpipatkul, A., Tatsumi, H., Sakurai, T., Ushio, K., Muta, T. and and Oshima, Y. 1985 Molecular and functional organization of yeast plasmid pSR1. *Journal of Molecular Biology,* 182:191-203.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E. and Fraser, M.J. 1989 Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology,* 172:156-169.

Cotsaftis, O., Sallaud, C., Breitler, J.C., Meynard, D., Greco, R., Pereira, A., and Guiderdoni, E. 2002 Transposon-mediated generation of T-DNA- and marker-free rice plants expressing a Bt endotoxin gene. *Molecular Breeding,* 10:165-180.

Darbani, B., Eimanifar, A., Stewart, C.N. and Camargo, W.N. 2007 Methods to produce marker-free transgenic plants. *Biotechnology Journal*, 2:83-90.

Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M. 1997 Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proceedings of National Academy of Sciences U.S.A.,* 94:2117-2121.

Goldsbrough, A.P., Lastrella, C.N. and Yoder, J.I. 1993 Transposition mediated repositioning and subsequent elimination of marker genes from transgenic tomato. *Biotechnology,* 11:1286-1292.

Hohn, B., Levy, A.A. and Puchta, H. 2001 Elimination of selection markers from transgenic plants. *Current Opinion in Biotechnology,* 12:139-143.

Li, X., Burnight, E.R., Cooney, A.L., Malani, N., Brady, T., Sander, J.D., Staber, J., Wheelan, S.J., Joung, J.K., McCray Jr., P.B., Bushman, F.D., Sinn, P.L. and Craig, N.L. 2013 *piggyBac* transposase tools for genome engineering. *Proceedings of National Academy of Sciences U.S.A.*, 110:E2279-2287.

Miki, B. and McHugh, S. 2004 Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *Journal of Biotechnology,* 107:193-232.

Nishizawa-Yokoi, A., Endo, M., Osakabe, K., Saika, H. and Toki, S. 2014. Precise marker excision system using an animal-derived piggyBac transposon in plants. *Plant Journal*, 77:454-463.

Perera, R.J., Linard, C.G., and Signer, E.R. 1993 Cytosine deaminase as a negative selective marker for Arabidopsis. *Plant Molecular Biology,* 23:793-799.

Senecoff, J.F., Bruckner, R.C. and Cox, M.M. 1985 The FLP recombinase of the yeast 2-micron plasmid: characterization of its recombination site. *Proceedings of National Academy of Sciences U.S.A.*, 82:7270-7274.

Sternberg, N. and Hamilton, D. 1981 Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *Journal of Molecular Biology*, 150:467-486.

Woo, H.J., Suh, S.C. and Cho, Y.G. 2011 Strategies for developing marker-free transgenic plants. *Biotechnology and Bioprocess Engineering*, 16:1053-1064.

Cloning and Expression Analysis of Genes and Field Evaluation of Salt Tolerant Genetically Modified Wheat (*Triticum aestivum*)

Nasir Ahmad Saeed, Imran Habib, Moddassir Ahmad, Muhammad Rauf, Zahid Abbas Malik, Khurram Shahzad, Khadija Rahman, Zahid Mukhtar and Shahid Mansoor
National Institute for Biotechnology and Genetic Engineering (NIBGE),
P.O Box 577, Jhang Road, Faisalabad, Pakistan

Abstract

Wheat is the major staple food in Pakistan. It is grown on an area of 8.6 million hectares with an annual production of 24 million tons. Although Pakistan is the 6th largest producer of wheat but the average 2.7 t/h yield is quite low as compared to other developed countries. Salinity and drought are the two major limiting factors. Currently, 6.4 million hectares of cultivable land is salt affected and this area is increasing at the rate of 40,000 hectares per year. The major salinity affected areas are located in Punjab and Sindh provinces. In addition to breeding tools, biotechnology offers new tools for genetic improvement of this important food crop. Tissue culture and transformation procedures were developed for local elite wheat varieties. A number of salt and drought tolerance enhancing genes were cloned under different promoters. These genes were amplified from different sources including barley, kallar grass (*Leptochloa fusca*, a highly salt tolerant grass) and *Arabidopsis* etc. Salinity tolerance enhancing genes include *AtNHX1, LfVP1, LfNHX1, HVA1* and *LfHKT1* homologs were isolated from barley and kallar grass. For proof of concept, initially *LfHKT1, LfNHX1* and *LfVP1* genes were tested in yeast hybrid system and then transformed into model tobacco plant. Further, all these genes were transformed into different wheat varieties like Seher-2006, Punjab-2011, Ufaq and Bobwhite and confirmed by different molecular methods. Transgenic wheat lines were tested at lab, pot and field conditions under salinity stress (at different EC and SAR levels).

Results indicated that *NHX1, LfVP1*, *HKT1* and *HVA1* transgenic plants showed better tolerance to salinity stress and gave higher grain yield than control. Physiological and agronomic data for various traits were collected. In year 2012-13 cropping seasons, a total of 500 transgenic wheat lines were planted in the field and pots at 8 locations across Pakistan. From these studies, 10 salt tolerant transgenic lines were selected. National Biosafety Committee (NBC), Ministry of Climate Change, Government of Pakistan has approved these cases for biosafety studies, risk analysis and limited release in the open environment. The materials are being tested in the field at NIBGE; NIAB; WRI, AARI; Soil Salinity Research Institute, Pindi Bhattian, Bio-Saline Research Station, Pakka Anna, NIFA, Peshawar, KPK and NIA, Tandojam, Sindh. These plants could perform better under salinity stress conditions and contribute to food security in the region

Keywords: Abiotic stress, gene isolation, genetically modified wheat, salinity

Introduction

Wheat is the major staple food and leading grain around the world. Worldwide, it is grown on an area of 227 million hectares with a total production of 690 million tones. In Pakistan, it is grown on an area of 8.6 million hectares with an annual production of 24 million tons (Year 2011-12). Wheat production is dependent on weather and rate of rain fall during wheat season. The current production of wheat grain is not sufficient to meet the growing demand of the country.

In Pakistan, salinity and sodicity are the most important factors limiting the productivity of wheat and other crops. This problem is serious in southern Punjab and upper Sindh provinces. Currently, 6.4 million hectares of land is salinity affected and this area is increasing at the rate of 40,000 hectares per annum (Alam and Khan, 2006). The impact of salinity on agriculture production reflect that soil salinity causes a reduction of about 25 percent in the production of major crops (Kahlown et al., 2003).

According to the USDA Salinity Laboratory, saline soil can be defined as soil having an electrical conductivity (EC) of the saturated paste extract of 4 dSm^{-1} (4 dS m^{-1} = 40 mM NaCl) or more, and sodium adsorption ratio of less than 15. Plants sense salt stress through both ionic (Na^+) and osmotic stress signals. Osmotic homeostasis is established either by Na^+ compartmentation into the vacuole or by biosynthesis and accumulation of compatible solutes or by selective uptake of Na^+ at the expense of K^+. Under salinity and sodicity, sodium gains entry into root cell cytosol through cation channels or transporters or into the root xylem stream via an apoplastic pathway depending on the plant species. Sodium proton antiporters (Na^+/H^+) are present on plasma membrane and tonoplast. Membrane bound Na^+/H^+ antiporter (*SOS1*) is mainly involved in sodium exclusion from root epidermal cells under salinity while *NHX1* is located on the tonoplast (vacuolar membrane). *NHX1* is involved in compartmentation of sodium in the vacuole.

For osmotic adjustment, plants use inorganic ions such as Na^+ and K^+ and/or synthesize organic compatible solutes like sugars and protective proteins (LEA). Vacuolar sequestration of Na^+ is an important and cost-effective strategy for

osmotic adjustment that also reduces the Na^+ concentration in the cytosol. Na^+ sequestration into the vacuole depends on expression and activity of Na^+/H^+ anti-porters as well as on V-type H^+-ATPase and H^+-PPase. The cytosolic Na^+ concentration is maintained at non-toxic levels by halophytes and glycophytes in spite of the considerable influx of Na^+ into the cytosole. The compartmentalization of Na^+ into the vacuoles averts the deleterious effects of Na^+ in the cytosole. Moreover, the compartmentalization of Na^+ (and Cl^-) into the vacuole allows the plants to use NaCl as an osmoticum, maintaining an osmotic potential that drives water into the cell. *NHX1* family of genes was originally discovered in yeast (*Saccahromyces cerevisiae*) genetic system. *Arabidopsis* genome sequencing projects has identified genes with significant similarity to the yeast *NHX* gene product. *AtNHX1* homologues from many other plant species have been reported and are mostly based on their sequence homology to the *Arabidopsis* gene. It seems *NHX1* system is highly conserved between many different plant species and genetic manipulation of this system in crops like wheat will likely result in improved salt tolerance (Zhang et al., 2004). Transgenic plants overexpressing *AtNHX1* gene have shown significantly higher salt (200 mM NaCl) tolerance than wild-type plants. Similarly, accumulation of LEA proteins (late embryogenesis proteins) in the cytosol has improved the tolerance against salinity. High affinity potassium transporter (HKT) genes allow selective entry of potassium (K^+) into the cell and keeps Na^+ out of the cell.

In this study, three sodium proton antiporter genes (*AtNHX1*, *LfNHX1* and *HvNHX1*), one LEA protein (*HVA1*) and one *LfHKT1* (High Affinity Potassium transporter) were cloned and transformed into wheat and tobacco (model plant) and tested for their tolerance to salinity. These genes alone or in combination may be used for developing salinity tolerant wheat and may help in getting more food from marginal and degraded salt affected lands.

Materials and Methods

Cloning of *NHX1s, HVA1* and *HKT* genes

In this study, three *NHX* homologues, LfVP1, *HVA1* and *LfHKT1* gene were cloned. The detail is given in Table 1. *AtNHX1* gene was amplified from cDNA of *Arabidopsis thaliana* and cloned under *CaMV35S* promoter in pGreen0029 plant expression vector containing *bar* gene as plant selection marker. *LfNHX1* gene was amplified from *Leptochloa fusca* (local name: Kallar grass) cDNA library (Accession No. JF933902.1). This gene was initially cloned in TA cloning vector but later sub-cloned into Gateway expression vector under different promoters (*CaM35V, 2xCAMV35S, OsActin* and *ZmUbiquitin*). *HvNHX1* cDNA clone (Accession No. AB089197) was obtained from Atsunori Fukuda, NIAS, Japan, and sub-cloned under three promoters (2xCAMV35S, OsActin and ZmUbiquitin). Sub-cloning of *LfNHX1* and *HvNHX1* genes were done in collaboration with Gerald Berkowitz, Univ. of Connecticut, USA. *LfVP1* gene was amplified from *Leptochloa fusca* (local name: Kallar grass) cDNA library (Accession No. GQ387485.1). This gene was initially cloned in TA cloning vector and sequenced. *LfVP1* gene was then cloned into Gateway expression vector under different promoters (CAM35V, 2xCAMV35S, OsActin and ZmUbiquitin). *HVA1* is late embryogeneis abundance (LEA) protein identified in many plants. cDNA of this gene was amplified from barley (*Hordeum vulgare*) and cloned under *rd29* and *FMV* promoters in CGT-6400A vector. The selection marker was *bar* gene (glufosinate ammonium). This work was done in collaboration with Donald Danforth, Plant Science Center, USA. Lf*HKT1* was amplified from cDNA library of *Leptochloa fusca* (HKT Accession No. JN547411.1) and cloned in Gateway cloning vectors under three promoters (2xCAMV35S, OsActin and ZmUbiquitin). HKT genes (*HvHKT, LfHKT, OsHKT*) were cloned in pYES-DEST52 vector under Galactose inducible promoter in yeast expression system for functional complementation analysis.

Table 1. List of salt stress responsive genes and constructs being used for tobacco and wheat transformation

Gene name	Construct	Vector	Promoter	Plant selection marker
AtNHX1	*AtNHX1*	pGreen0029	CAMV35	Bar
LfNHX1	*LfNHX1*-pB	pBWG2D,1	CAMV35S	Basta
	LfNHX1-002 *LfNHX1*-003 *LfNHX1*-004	pIKPb002 pIKPb003 pIKPb004	ZmUbi OsActin 2xCAMV35	Hyg
LfVP1	*LfVP1*-pB	pBWG2D,1	CAMV35S	Basta
	LfVP1-002 *LfVP1*-003 *LfVP1*-004	pIKPb002 pIKPb003 pIKPb004	ZmUbi OsActin 2xCAMV35	Hyg
HvNHX1	*HvNHX1*-002 *HvNHX1*-003 *HvNHX1*-004	pIKPb002 pIKPb003 pIKPb004	ZmUbi OsActin 2x CAMV35	Hyg
HVA1	*HVA1*	FMV-HVA-tnos-CGT 6400A rd29p-HVA-tnos-CGT 6400A	FMV Rd29	Basta
HKT1	HKT-002 HKT-003 HKT-004	pIKPb002 pIKPb003 pIKPb004	ZmUbi OsActin 2x CAMV35	Hyg
	HvHKT2;1-PMDC	PMDC84	2x CAMV35	Hyg
	HvHKT2;1-PSPDK1677	PSPDK1677	2x CAMV35	Hyg
	HvHKT2;1-pMBb7	pMBb7Fm21GW-UBIL	Ubi	Bar

Transformation of wheat

Wheat plants grown in the field were tagged at anthesis. After 14-16 days, post anthesis spikes were cut from the tillers and immature seeds were removed from spikelets. For initiation of callus from immature embryos MS basal medium (Murashige and Skoog, 1962) was used. For callus induction 2 mgL^{-1} 2, 4-D was added to nutrient media and embryos were placed scutellum side up whereas axis side was kept towards the media surface. Hundred embryos per genotype in three replicates were cultured and placed at a controlled temperature of 25 ± 2C° in the dark. A hyper virulent *Agrobacterium* strain AGL-1 harboring the desired plant expression vector was utilized for *Agrobacterium*-mediated transformation wheat cultivars through co-cultivation of immature embryos on media supplemented with 400 µM (w/v) acetosyringone (Phytotechnology Labs A104). After co-cultivation the inoculated embryos were subjected to two sub culturing cycles of 15 days each on callus induction media supplemented with 160 mgL^{-1} Timentin (Ticarcillin disodium salt) for the suppression of *Agrobacterium*. Tissues indicating morphogenesis were shifted to IK4 regeneration media (1 mgL^{-1} IAA + 1 mgL^{-1} kinetin) fortified with appropriate antibiotic selection herbicide. Plantlets with 2.0-5.0 cm long roots were extracted from media, rinsed with distilled water and shifted to small plastic pots containing coco peat and compost in 3:1 ratio. To acclimatize these pots were first kept under growth room conditions for seven days and then transferred to glasshouse for further hardening.

Transformation of tobacco

Leaf discs were cut from top 2-3 leaves under aseptic conditions and placed on inoculation medium. The leaf discs were placed upside down. Plates were sealed with parafilm and incubated at 16/8 light and dark photoperiod at 25 ± 1°C. Explants were pre-incubated for 48 hours on inoculation medium containing 0.1 mg L^{-1} NAA + 1 mg L^{-1} BAP. *Agrobacterium* suspension was poured into sterile petri-plate and all leaf discs were placed in the bacterial suspension for 25-30 minutes with gentle shaking after regular interval. The leaf discs were removed from suspension and blotted dry on sterile filter paper and transferred to co-cultivation medium. Plates were sealed with parafilm. Co-culture was performed for 48-72 hours at 25 ± 1ºC (depending on the growth of bacteria) under light conditions. Leaf discs were removed from co-culture medium and placed on selection medium. Leaf discs were placed on regeneration medium and incubated under 16/8 hours photoperiod at 25 ± 1°C for two weeks. Regenerated leaf discs were transferred to fresh selection medium. When shoots became larger, these were shifted into magenta containers. After washing roots with tap water, plants were transferred to sterile soil pots and kept at 25 ± 2°C under 16/8 hours photoperiod for hardening. Control experiment was also performed. Non-transformed leaf discs were placed on two different media, one having antibiotic and other without antibiotic, to observe the effect of selection agent (Spectinomycin, 100 mg L^{-1}).

Testing of *AtNHX1, LfVP1, LfNHX1* and *rd29-HVA1* transgenic wheat and tobacco plants

Transgenic *AtNHX1* wheat plants were evaluated on 10dSm^{-1} salinity stress in pots. These plants including wild-type were taken to maturity and data of grain weight per plant was recorded. To test salt stress tolerance of *LfVP1* and *LfNHX1* overexpressing transgenic tobacco plants, T_0 seed of transgenic and WT plants were germinated on MS medium supplemented with 100, 200 and 250 mM NaCl. After10 days resistant and WT seedlings were transferred to MS medium supplemented with 250 mM NaCl. Salt stress of 200 mM was also applied to transgenic and WT plants in pots. Germination test was performed to screen out the best performing wheat plants transformed with *HvNHX1* gene under *ZmUbi, OsAct* and *2XCaMV35S* promoters were tested on different salinity levels with comparison of control plants. T_0 seed of transgene and control were germinated on MS medium containing the different salt concentrations of 200mM NaCl and 250mM NaCl. After that, transgenic plants with better tolerance were shifted to pots. Similarly transgenic wheat plants having *rd29-HVA1* gene were grown on saline soil at EC 10 dSm^{-1} and data was recorded. Trials of these transgenes were performed in pots and field (plot size: 3.6 m^2) having CRD, RCBD layout designs, respectively.

Results and Discussion

Testing of *AtNHX1* transgenic wheat plants

Thirteen *AtNHX1* transgenic wheat events (T_6 generation) were tested for salinity tolerance in the field. Transgenic plants performed much better than the non-transgenic plants and exhibited 35.52 to 82.98 percent increase in grain weight per plant with respect to their wild-type. Similarly these same transgenic *AtNHX1* events were planted on salt affected lands at Biosaline Research Station, Pakka Anna and Soil Salinity Research Institute, Pindi Bathian on soil having 13dSm^{-1} salinity. Six transgenic lines showed higher yield than wild-type that ranged from1561 to 1870 kg/ha whereas, non-transgenic plants managed to offer only1417 kg/ha grain yield which is about 32 % less than the best performing transgenic line 2b3-1. On the other hand, at SSRI, *AtNHX1* transgenic events again performed much better than the corresponding wild-type under salinity stress. Grain yield of transgenic plants ranged from 2473 to 3713 kg/ha however non-transgenic control showed a lower yield of 2060 kg/ha under same salinity stress. Transgene 4b12-1 was the highest performer with 80% higher yield potential than the wild-type under severe field based salt stress. Two transgenic events 2b3-3 and 4b12-4 consistently performed better under both field and pot based salinity regimes and will be investigated further at multiple stress levels for reproducible and stable performance (Fig. 1). Initially this gene was transformed into tomato and tobacco model system. Transgenic tobacco plants showed better performance in saline condition in petri plates and soil pots. This gene has many other reports where plants showed salinity tolerance. So this gene was trasnformed into wheat. This material is now in field trials.

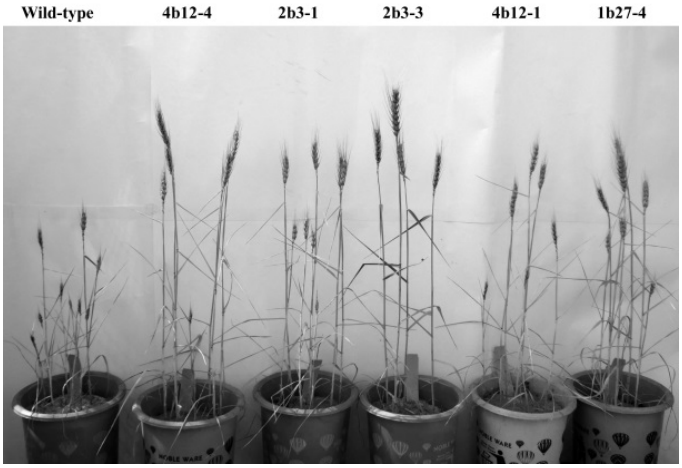


Fig. 1. Testing of *AtNHX1* wheat transgenics and wild type plants for salt tolerance

Testing of *LfVP1* and *LfNHX1* transgenic tobacco plants

Transgenic seeds of *LfVP1*-P4, *LfNHX1*-PB3 and control plants were germinated on MS_0, 100, 200 and 250 mM NaCl. At 100 mM NaCl difference were observed in WT plant and transgene. Transgenic plants showed better growth at 200 and 250 mM. At 200 mM NaCl T_1 seeds of *LfVP1* and *LfNHX1* transgenes showed segregation and some of plants (null) behaved like WT and were unable to maintain their normal growth (Fig. 2a; Fig. 3a). The WT plants become yellowish and de-shaped after few days of germination (Fig. 2b; Fig. 3b). Chi square test confirmed single gene segregation pattern in *LfVP1*-P4 and *LfNHX1*-PB3 transgenic T_1 seeds germinated on 200 mM NaCl. Putative seedlings of

LfVP1 and *LfNHX1* transgenes were also shifted to MS_0 with 250 mM NaCl. Transgenic seedlings of *LfVP1* (Fig. 2c) and *LfNHX1* (Fig. 3c) remained green while WT plant showed loss of chlorophyll (Fig. 2 d) and (Fig. 3 d). Previous studies showed that overexpression of vacuolar *AtNHX1* conferred tolerance to both salt and drought stresses in transgenic petunia plants (Xu et al., 2009). Reduced leaf area and leaf water potential was observed in wild type plants than in transgenic plants when subjected to drought and salt stresses which could be due to an enhanced osmotic adjustment in the transgenic plants (Brini et al., 2007). It was concluded that the vacuolar H^+-PPase plays important roles in maintaining the proton gradient across the vacuolar membrane and vacuolar compartmentation of Na^+. More accumulation of Na^+ in the vacuoles of transgenic cells was under salt stress as compared to wild type (Duan et al., 2007). H^+-pyrophosphatase and Na^+/H^+ antiporter gene from a halophyte grass *Leptochloa fusca* enhanced salt and drought tolerance in the *LfVP1* and *LfNHX1* containing transgenic tobacco plants. Results suggested that candidate genes can contribute in engineering salt tolerance in crop plants.

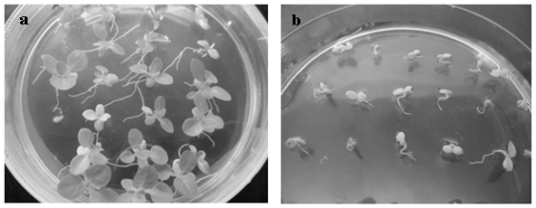


Fig. 2. a) Germination of *LfVP1*-PB4 transgenic seeds on MS_0 with 200 mM NaCl for salt tolerance b) germination of WT seeds on 200 mM NaCl c) growth of transgenic seedlings on MS_0 with 250 mM NaCl d) growth of WT seedlings on 250 mM NaCl

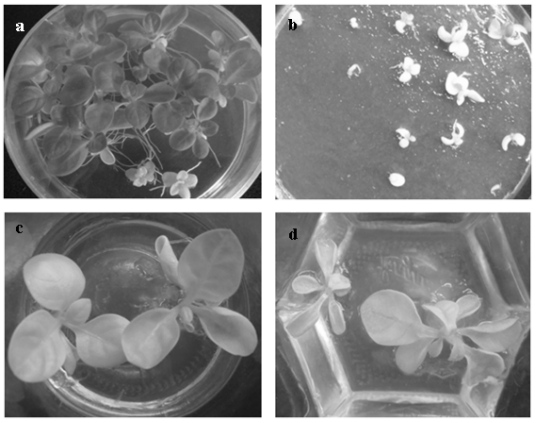


Fig. 3. a) Germination of *LfNHX1*-PB3 Transgenic seeds on MS_0 with 200 mM NaCl for salt tolerance b) germination of WT seeds on 200 mM NaCl c) growth of transgenic seedlings on MS_0 with 250 mM NaCl d) growth of WT seedlings on 250 mM NaCl

Testing of *HvNHX1* transgenic wheat plants

Germination test was performed to screen out the best performing wheat lines transformed with *HvNHX1* gene under *ZmUbi*, *OsAct* and *2XCaMV35S* promoters on different salinity levels in comparison with control plants. T_0 seed of transgene and control were germinated on MS medium containing different salt concentrations of 200mM NaCl and 250mM NaCl (Fig. 4). Transgenic plants with better tolerance were shifted to pots. Significant differences were observed between germination of transgenes and wild type plants. In case of both salt concentrations, maximum germination and shoot root length was observed in transgenic plant transformed with *2XCaMV35S-HvNHX1* gene construct followed by *ZmUbi-HvNHX1* gene constructs. Comparatively low germination was observed in case of *OsAct-HvNHX1*- transgenic plants. Wild type plant showed poorest germination response and no germination was observed on 200mM and 250mM NaCl. These results suggested that promoter play an important role in gene expression. For future prospect, comparative expression of transgenic plants will also be estimated.

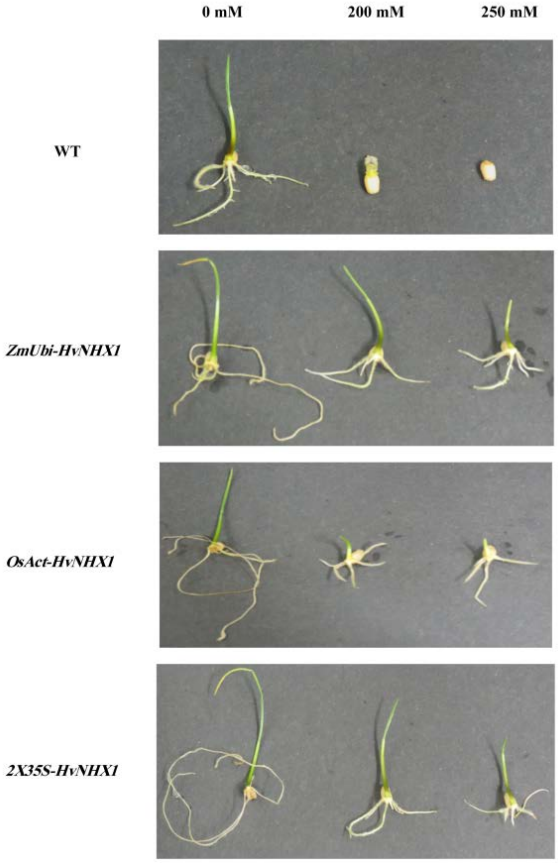


Fig. 4. Germination analysis of *HvNHX1* transgenic plants on 0, 200 and 250 mM NaCl

Testing of *rd29-HVA1* transgenic wheat

To check the stress inducible expression of barley *HVA1* gene in T_1 transgenic wheat, the effect of salinity stresses on seed germination and seedling development was evaluated at various concentrations of sodium chloride (Fig. 5). It was observed that at higher NaCl concentration of 200 mM, transgenic lines ST1 and ST5 (71.7%), ST2 and ST4 (68.8 %) and ST6 (75.0 %) managed to retain highest mean germination rate whereas wild-type exhibited much reduced (43.3 %) germination at the same salt level (Fig. 6A). *HVA1* transgenic events demonstrated lower NaCl sensitivity for primary root development than wild-type at highest NaCl concentration (Fig. 5). The relative root length was reduced to 66.2 % in wild-type however less reduction of 51.4, 45.1 and 42.6% was observed for three transgenic events ST3, ST4 and ST6, respectively (Fig. 6B). At 200 mM, wild-type showed average shoot length reduction of 81 % when compared to non-NaCl treatment. At the same NaCl level, the transgenic events were relatively less affected where plants ST4, ST1, ST2 and ST6 managed to retain 46.3, 43.0, 40.7 and 40.0% of shoot lengths in comparison to non-NaCl conditions (Fig. 6C). These results indicated that stress inducible expressions of *HVA1* gene in wheat enable the transgenic plants to better withstand the exogenously introduced NaCl stress.

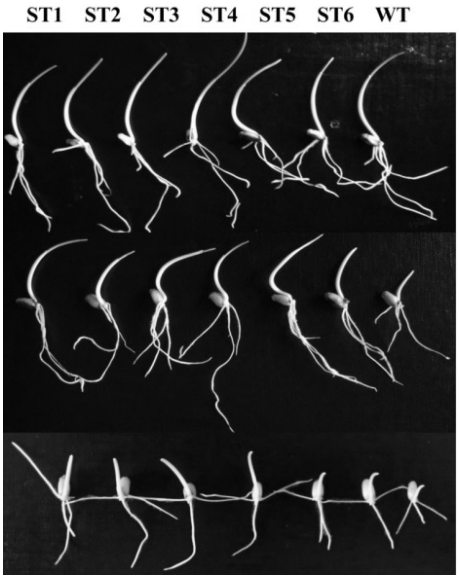


Fig. 5. Germination rate, root and shoot development assay of six *HvNHX1* transgenic plants with corresponding wild-type at three NaCl treatments of 0, 100 and 200 mM

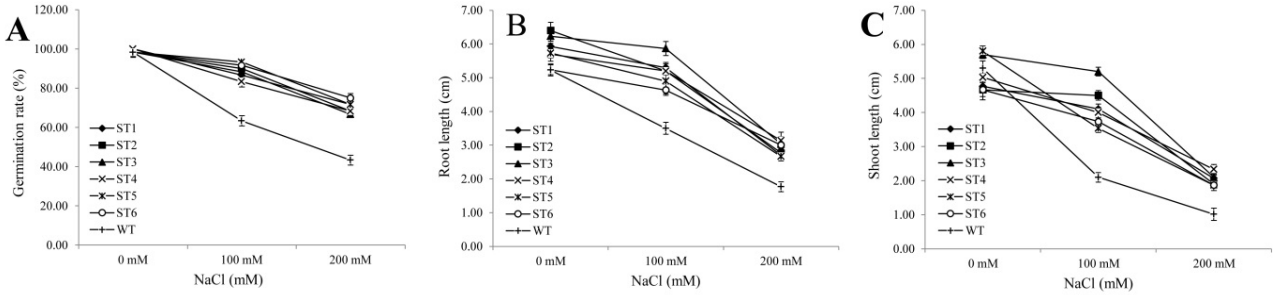


Fig. 6. Performance of six *rd29-HVA1* transgenic plants with corresponding wild-type under NaCl induced salt stress of 0, 100 and 200 mM for: A) Germination rate (%), B) Root length (cm), C) Shoot length (cm)

Membrane Stability Index (MSI) and Electrolyte leakage (EL) of transgenic wheat under salt stress

To check the osmo-protective and membrane stabilizer role of *rd29A-HVA1* gene, membrane stability of transgenic plants including wild-type was evaluated after exposing these plants to both NaCl and water deficit stress. It was observed that the membrane of all transgenic plants was more thermodynamically stable than the wild-type. At 200 mM NaCl concentration almost similar pattern of MSI was observed with ST6 having the most stable membrane with a MSI value of 43.3% followed by 40.1, 35.8, 33.3, 30.7 and 26.7% in transgenes ST5, ST4, ST3, ST1 and ST2 respectively. The lowest MSI of 21.8 % was demonstrated by wild-type (Fig. 7A).

At elevated NaCl concentration of 200 mM, enhanced electrolyte leakage was noted in all the evaluated wheat genotypes however this increase in EL was again much prominent in wild-type (83.8%) than in transgenic plants. The minimum EL of 61.6% was again exhibited by transgene ST6 whereas ST5, ST1, ST4 and ST3 were the closest performers with 64.7, 66.9, 67.2 and 69.0% EL respectively (Fig. 7B).

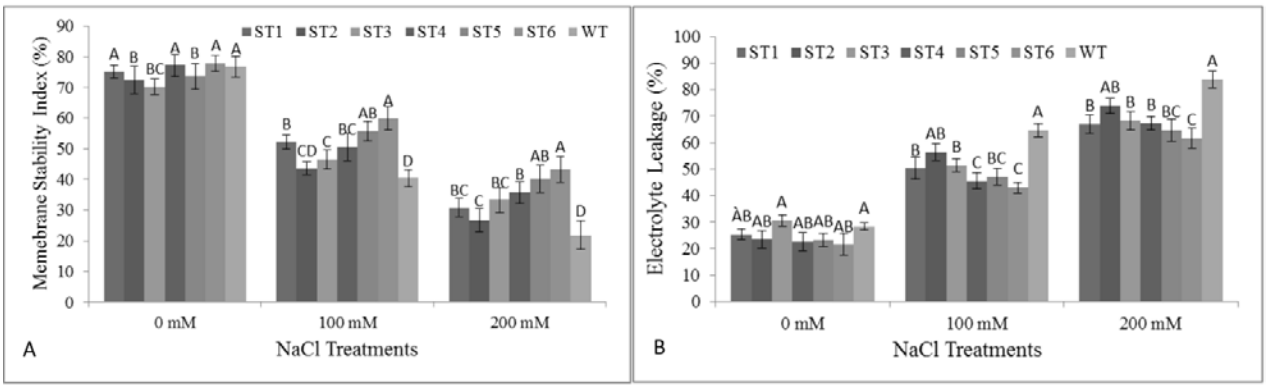


Fig. 7. Membrane stability index (A) and electrolyte leakage (B) of transgenic wheat line at 0, 100 and 200 mM NaCl

Determination of Na^+ and K^+ accumulation and Na^+/K^+ ratio in *rd29-HVA1* transgenic plants

At excessive soil salinity of 20 dSm^{-1}, a decrease in leaf K^+ contents was visible for all the evaluated genotypes. The potassium contents ranged from 46.9 to 66.8 $mg.g^{-1}$ of dw with maximum decrease in K^+ contents was observed in wild-type (55.2%) followed by ST3 (51.6%), ST4 (45.8%), ST2 (44.3%) and ST5 (42.7%) whereas minimum decrease of 39.3 and 40.% was noted in transgenic events ST6 and ST1 respectively (Table 2). A quite notable increase in leaf Na^+ contents was observed for all the genotypes growing at salinity level of 20 dSm^{-1}. This surge in leaf sodium ion constituents was relatively minimum in transgenic events ST6, ST1 and ST5 that showed 49.51, 52.54 and 53.34 $mg.g^{-1}$ of dw Na^+. In contrast, the maximum leaf Na^+ contents were observed in the wild-type (72.38 $mg.g^{-1}$ of dw) followed by 63.9 and 58.36 $mg.g^{-1}$ of dw in ST3 and ST4, respectively (Table 2). A visible reduction in K^+/Na^+ ratio of all the wheat lines was observed at 20 dSm^{-1} salinity treatment. Maximum ratio of 1.36 was demonstrated by transgenic line ST6 closely followed by transgenes ST1, ST5 and ST2 with 1.23, 1.14 and 1.043 K^+/Na^+ ratio, respectively. In contrast wild-type was found to be the least K^+ accumulator with a minimum K^+ to Na^+ ration of 0.65 followed by ST3 (0.80) and ST4 (1.00) (Table 2).

Table 2. Potassium ion (K^+), sodium ion (Na^+), K^+/Na^+ ratio and proline contents of *rd29-HVA1* transgenic and wild-type wheat plants under three NaCl treatments

Genotype Name	K^+ contents			Na^+ contents			K^+/Na^+ ratio			Proline contents		
	0.4mM	10dSm^{-1}	20dSm^{-1}	0.4mM	10dSm^{-1}	20dSm^{-1}	0.4mM	10dSm^{-1}	20dSm^{-1}	0.4mM	10dSm^{-1}	20dSm^{-1}
ST1	108.3ab	88.5c	64.8fg	3.58ij	7.78fg	52.54de	30.36b	11.39cd	1.230h	0.31h	4.08e	5.15b
ST2	103.5b	78.4de	57.6h	3.40ij	8.11fg	55.53cd	30.54b	9.70def	1.043h	0.24h	3.43f	4.83bc
ST3	105.5ab	69.1f	51.1i	3.65hij	9.13fg	63.94b	29.05b	7.59fg	0.80h	0.26h	4.28de	4.76c
ST4	107.3ab	75.5e	58.1h	3.20ij	8.42fg	58.36c	33.57a	8.99ef	1.00h	0.29h	4.49cd	6.35a
ST5	105.5ab	82.6d	60.4gh	3.05j	7.36fgh	53.34d	34.74a	11.2cde	1.14h	0.29h	4.15e	6.14a
ST6	110.1a	81.7d	66.8f	3.16ij	6.78ghi	49.51e	34.93a	12.07c	1.36h	0.34h	4.33de	6.13a
Wild-type	104.7b	68.1f	46.9i	3.44ij	10.58f	72.38a	30.50b	6.49g	0.65h	0.28h	2.87g	4.08e
Mean	106.4A	77.7B	57.97c	3.35C	8.31B	57.94A	31.96A	9.64B	1.032C	0.287C	3.947B	5.348A
CV %	3.79			9.71			9.72			6.51		

Mean in each column and in the last row sharing the same letter did not differ significantly at probability level of P≤0.05 according to LSD test.

Estimation of proline contents of *rd29-HVA1* transgenic plants under salt stress

In response to increasing salinity stress a gradual increase in plant proline contents was noted. At extreme salinity level (20 dSm^{-1}), quite elevated leaf proline contents were noticeable. ST4 was found to be the highest proline accumulator (6.35 $mg.g^{-1}$ dw) closely followed by ST5 and ST6 with 6.14 and 6.13 $mg.g^{-1}$ proline contents. Contrary to that minimum proline accumulation (4.08 $mg.g^{-1}$ dw) was observed in wild-type followed by 4.83 and 5.15 $mg.g^{-1}$ proline in case of ST2 and ST1, respectively (Table 2).

Semi-quantitative RT-PCR of salt stressed genotype

Semi-quantitative RT-PCR analysis was conducted to check the transcript level of *rd29A-HVA1* gene after exposing the seedlings of transgenic and wild-type seedlings to salinity stress of 100 and 200 mM NaCl for five days after germination on the same stress inductive media (Fig. 8). The expression of *HVA1* gene was clearly up-regulated in transgenes ST4, ST5 and ST6 at 100 mM NaCl treatment whereas, relatively lower gene expression was observed in case of ST1, ST2 and ST3 while, no transcript was visible in wild-type at the same stress level. AT 200 mM NaCl stress, enhancement induction of *HVA1* gene was showed by all the tested transgene. Highest *HVA1* transcript was observed in transgene ST5 followed by ST6 and ST3 however no amplification was detected in wild-type.

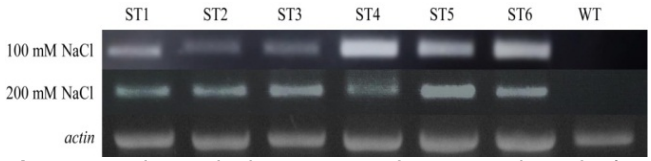


Fig. 8. Semi-quantitative RT-PCR for expression of *rd29A-HVA1* gene in 14 day old transgenic and wild-type wheat seedlings challenged by NaCl (100 and 200 mM). Wheat actin gene was used as internal control

Expression patterns of *HvHKT2;1* in transformed tobacco plants

Tobacco leaf disc were transformed with *HvHKT2; 1-PSPDK1677* construct using GV3101 *Agrobacterium* strain. Out of 70 transgenic events, 30 events were selected on the basis of better growth and performance for relative quantification. Plants were selected on dual antibiotics (Streptomycin and Spectinomycin) on MS media for 4–6 weeks.

Transgenic tobacco plants were shifted from MS medium to soil pots when they have attained good vigor (3–4 leaves). Transgenic plants were initially tested for *HKT* transcript analysis by irrigating with distilled water. Some of the plants were also watered containing 50–100 mM NaCl salt. Leaf yellowing was observed as symptoms of salt stress in transgenic tobacco while control plants completely turned yellow and dried up after 3–4 weeks. Water was withheld for 10-15 days to see the difference in growth response of transgenic and control plants (Fig. 9). RNA was isolated from transgenic plants and 2µg RNA was used for reverse transcription. Reverse transcription product (100 ng) was used in all the cases as standard comparison of transcripts using 18S rRNA as internal control. Normalization of RNA and cDNA was done for relative quantification of *HKT* transcript analysis on NanoDrop. Fig. 10 shows the result of quantitative analysis of *HKT* transcripts from transgenic tobacco plants.

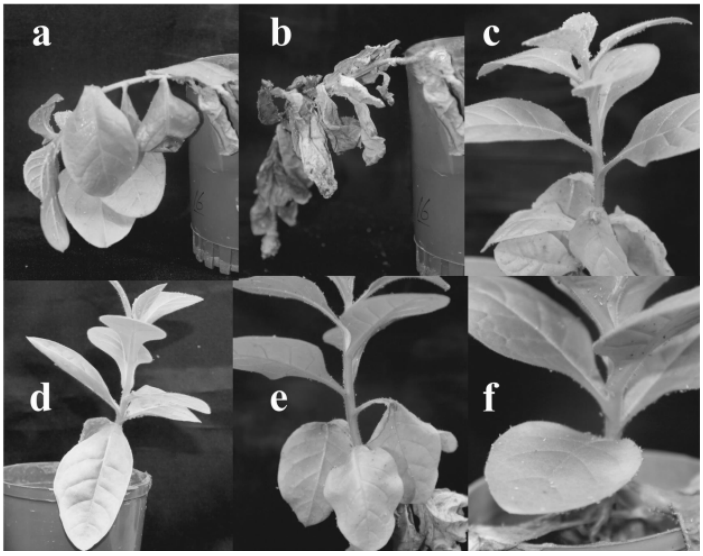


Fig. 9. *HvHKT2;1* transgenic tobacco plants irrigated with water containing 100mM NaCl
Salt stress symptoms can be seen as yellowing of leaves. a – b) Control plant before and after salt water treatment respectively while c – f are different transgenic events after salt water treatment.

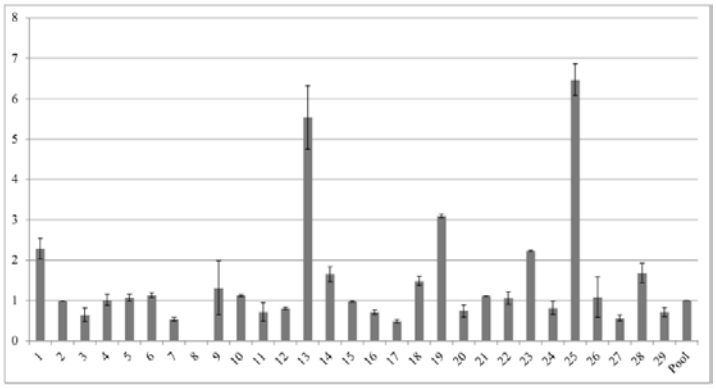


Fig. 10. Transcript analysis of wild type *Arabidopsis* (Columbia) transformed with *HvHKT2;1-PMDC* construct. X-axis shows transformation events (transgenic plants) while Y-axis represents transcript levels

Tobacco plants were used to transiently express the GFP fusion protein in leaf tissue by *Agrobacterium* infiltration method. Confocal microscopic studies revealed GFP signals on plasma-membrane which provided strong evidence that some transcripts have spliced out introns properly hence producing a complete functional protein (Fig. 11). Transient gene expression is emerging as a potential tool for cell specific transient gene expression studies for elucidating gene regulation and expression (Kajiyamaa et al., 2008). GFP fusion constructs were also used for subcellular localization of protein in yeast cells (Rajagopal et al., 2007) of aquaporins in *Arabidopsis* (Boursiac et al., 2005). Transient expression of a target gene infused with green fluorescent protein (GFP) is a reliable method to study expression of fusion protein and cellular and sub cellular compartmentalization by confocal microscopy (Banuelos et al., 2002; Levy et al., 2005). *HvHKT2;1* GFP fusion constructs were also used for stable transformation of wheat and *Arabidopsis* for future studies to further elaborate the validation of results. The same constructs for GFP fusion protein studies were used for transient gene expression for GFP constructs. Localization of *HvHKT-GFP* fusion protein was found by transient expression analysis at the plasma membrane of plant cell. The plasma membrane is also the site of expression of other *HKT* proteins including *PutHKT2;1* (Ardie et al., 2009), Mc*HKT1*;1 (Su et al., 2003), *AtHKT1;1* (Sunarpi et al., 2005), *OsHKT2;4* (Lan et al., 2010) and *OsHKT2;1* (Horie et al., 2007).

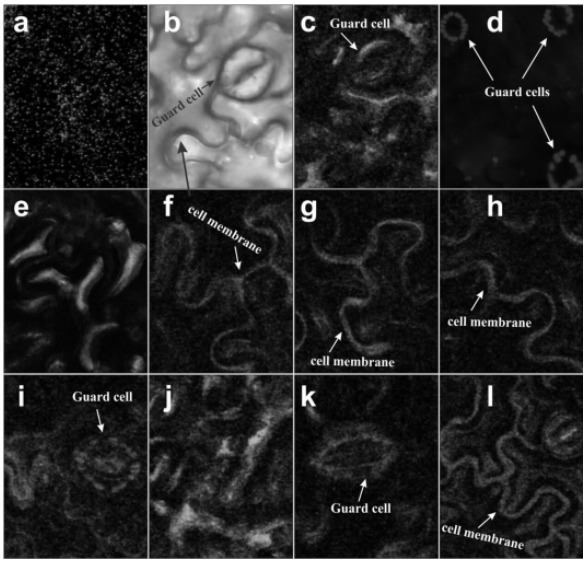


Fig. 11. Transient HKT gene expression studies in tobacco. Confocal microscopic studies showing HKT protein localization on plasma membrane from leaf samples of transiently expressed HKT-FGP fusion protein. a) Leaf section viewed from control plant showing auto-fluorescence. b) Under bright field c) same leaf with GFP fluorescence. d) Red fluorescence showing guard cells from the same site as B. e to l show different leaves and infiltration sites with different constructs; *HvHKT2;1-PMDC* (b, c, d, e, f and g) and *HvHKT2;1-pMBb7* (h, i, j, k and l).

Transformation of *HvHKT2* gene into wheat

Wheat variety Punjab-2011 was transformed with *HvHKT2;1-PMDC* and *HvHKT2;1-pMBb7* genes. *HvHKT2;1* was cloned without stop codon in *pENTR DEST-52* Gateway entry vector. Entry clone was used to sub-clone *HvHKT2;1* (in frame cloning) with GFP in both expression vectors . Transgenic plants were selected on Hygromycin (PMDC construct) and Basta (*pMBb7* construct) and confirmed by PCR amplification of *HKT* gene specific primers. T_0 and T_1 generation of these plants were raised in the field. Homozygous T_2 plants will be subjected to salinity analysis in coming wheat season.

Conclusion

Salinity poses a constant threat to world's agriculture. In this study, we attempted to clone and test salinity tolerance enhancing genes in tobacco (model system) and wheat. Transgenic wheat plants containing *AtNHX1* (10 events) and *rd29-HVA1* (6 events) performed better under salt stress conditions. *LfVP1*and *LfNHX1* genes were characterized in tobacco. *HKT* genes were initially characterized in yeast system and then transformed into wheat. *HvNHX1* gene was transformed in wheat and tested at lab scale in perti plates. These genes have the potential to perform better under saline conditions and contribute to higher grain yield.

Acknowledgements

This work was supported by Punjab Agricultural Research Board (PARB), Government of Punjab, Pakistan Research Grant No. 337 and 103. *HVA1* gene was cloned in Pak-US collaboration project "Identification and cloning of drought related genes in wheat". This work was also supported by Higher Education Commission (HEC) by awarding PhD fellowships to Muhammad Rauf, Khurram Shehzad and Zahid Abbas Malik.

References

Alam, S.M. and Khan, M.A. 2006. Managing salinity with soil conditioners. Article published in Dawn Economic and Business Review. P III. May 1-7.

Ardie, S.W., Xie, L., Takahashi, R., Liu, S. and Takano, T. 2009. Cloning of a high-affinity K+ transporter gene PutHKT2;1 from Puccinellia tenuiflora and its functional comparison with OsHKT2;1 from rice in yeast and Arabidopsis. Journal Experimental Botany, 60:3491-3502.

Banuelos M. A., Garciadeblas, B., Cubero, B., and Rodriguez-Navarro, A. 2002. Inventory and functional characterization of the HAK potassium transporters of rice. Plant Physiology, 130:784-795.

Boursiac, Y., Chen, S., Lu, D.T., Sorieul., M., van den Dries, N, and Maurel, C. 2005. Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. Plant Physiology, 139:790-805

Brini, F., Hanin, M., Mezghan, I., Berkowitz, G.A., and Masmoudi, K. 2007. Overexpression of wheat Na+/H+ antiporter TNHX1 and H+ pyrophosphatase TVP1 improve salt and drought-stress tolerance in Arabidopsis thaliana plants. Journal of Experimental Botany, 58:301-308.

Duan, X.G., Yang, A.F., Gao, F., Zhang, S.L., and Zhang, J.R. 2007. Heterologous expression of vacuolar H+-PPase enhances the electrochemical gradient across the vacuolar membrane and improves tobacco cell salt tolerance. Protoplasma, 232:87-95.

Horie, T., Costa, A., Kim, T.H., Han., M.J, Horie, R., Leung, H.Y, Miyao, A., Hirochika, H.,. An, G., and Schroeder, J.I. 2007. Rice OsHKT2;1 transporter mediates large Na+ influx component into K+-starved roots for growth. EMBO Journal, 26:3003-3014.

Kahlown, M.A., Chang, M.H., Ashraf, M and Hassan, M.S. 2003. Research Report-4: Salt affected soils and their reclamation. Pakistan Council of Research in Water Resources, Islamabad.

Rajagopal, D., Agarwal, P., Tyagi, W., Singla-Pareek, S.L., Reddy, M.K., and Sopory, S. 2007. Pennisetum glaucum Na+/H+ antiporter confers high level of salinity tolerance in transgenic Brassica juncea. Molecular Breeding, 19:137-151.

Kajiyama, S., Joseph, B., Inoue, F., Shimamura, M., Fukusaki, E., Tomizawa, K., and Kobayashi, A. 2008. Transient gene expression in guard cell chloroplasts of tobacco using ArF excimer laser microablation. Journal of Bioscience and Bioengineering, 106:194-198

Levy, M., Wang, Q., Kaspi, R., Parrella, M.P. and Abel S. 2005. IQDl. A novel nuclear calmodulin-binding proicin modulates glucosinolates accumulation and plant defense responses in Arabidopsis. Plant Journal, 43: 79-96.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tissue culture. Physiological Plantarium, 15: 473-476.

Su, H., Balderas, E., Vera-Estrella, R., Golldack, D., Quigley, F., Zhao, C., Pantoja, O., and Bohnert, H.J. 2003. Expression of the cation transporter McHKT1 in halophyte. Plant Molecular Biology, 52:967-980.

Sunarpi, Horie., T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., Chan, W.Y., Leung, H.Y., Hattori, K., Konomi, M., Osumi, M., Yamagami, M., Schroeder, J.I., and Uozumi, N. 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na+ unloading from xylem vessels to xylem parenchyma cells. Plant Journal, 44:928-938.

Xu, K., Nong, P., Luo, L., and Xia, T. 2009. Overexpression of AtNHX1, a vacuolar Na+/H+ antiporter from Arabidopsis thaliana, in Petunia hybrida enhances salt and drought tolerance. Journal of Plant Biology, 52:453-461.

Zhang, J.Z., Creelman, R.A., and Zhu, J.K., 2004. From laboratory to field using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiology, 135: 615-621.

Zhu, J. K., 2002. Salt and drought stress signal transduction in plants. Annual Review of Plant Biology, 53:247-273.

Expression of *MIPS* and *PAP* Genes in Mungbean

Sutkhet Nakasathien[1], Arunee Wongkaew[1], Giuseppe Dionisio[2] and Vichan Vichukit[1]
[1]Department of Agronomy, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand
[2]Department of Molecular Biology and Genetics, Aarhus University, Slagelse, Denmark

Abstract

A comparative gene expression study was conducted on the biosynthesis and degradation of phytic acid in mungbean seeds and seedlings. The former was focused on the first and rate-limiting step enzyme, D-*myo*-inositol-3-phosphate synthase (MIPS) that catalyzes the reaction from D-glucose 6-phosphate to D-inositol 3-phosphate. The latter was on purple acid phosphatase (PAP) that hydrolyzes phosphate and phosphate ester to provide inorganic phosphate during germination and seedling development. We have characterized the expression of both *myo*-inositol 3-phosphate synthase (*MIPS*) gene and purple acid phosphatase (*PAP*) gene. The mungbean *MIPS* and *PAP* transcript levels were observed in the early stage of seed development and germination, respectively. In addition, the spatial expressions of these genes were found to be organ-specific. Results from these studies pave the way for the genetic engineering approach to improve the bioavailable phosphorus and minerals in leguminous seeds which will certainly contribute to increasing nutritional value to animals in the meantime reducing phosphorus contamination to the environment.

Keywords: mungbean, phytic acid, *myo*-inositol 3-phosphate synthase (MIPS), purple acid phosphatase (PAP)

Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate; $InsP_6$) is the major storage form of phosphorus in seeds (Loewus and Murthy, 2000). Its first step of synthesis is the conversion from glucose 6-phosphate to *myo*-inositol 3-phosphate by the enzyme, *myo*-inositol 3-phosphate synthase (MIPS). The accumulation and expression of *MIPS* mRNA has been reported occurring in the phytate bodies during rice seed development (Yoshida et al., 1999). In transgenic rice using RNAi technique showed that seed-specific silencing of MIPS can reduce the phytic acid content meanwhile increase the available inorganic phosphate level (Ali et al., 2013).

During seed development, phytic acid is deposited as globoids with storage proteins in protein bodies. The storage phytate is hydrolyzed by phytase during germination to provide inorganic phosphate and *myo*-inositol to the growing seedling. Phytase is the member of purple acid phosphatase (PAP), which have been characterized and reported in several plants. Holme et al. (2012) reported that the seed from barley cisgenic lines with the extra HvPAPhy_a copies showed that phytase activities increased 2.6- and 2.8-fold.

Although phytic acid is important for the growth of seedlings but it has negative charge in their molecule and can bind the positive charge mineral cations like magnesium, zinc or iron forming the mixed salt complexes known as phytate. Therefore, phytic acid has nutritional and environmental implications when seeds are used as food or feed. In the situation of nutrition, phytic acid inhibits the absorption of mineral nutrients in monogastric animals such as human, swine and poultry. They cannot digest phytate effectively due to lack of phytase in their gastrointestinal tract. Moreover, the phytate from the animal waste will be released to the environment causing phosphorus pollution.

In the present study, we show the potential of *MIPS* and *PAP* genes (Wongkaew et al., 2010; Wongkaew et al., 2013) to be the target genes for reducing phytic acid content in mungbean seeds.

Materials and Methods

Plant materials

For *MIPS* cDNA investigation, seeds of mungbean KPS1 (*Vigna radiata* (L.) Wilczek cv. KPS.1) were sowed and pot-grown under natural light conditions. At anthesis, each flower was tagged. And developing pods were harvested within 2-day intervals, starting from 5 days after flowering (DAF) until seed maturation. Other plant parts (young leaves, stems, and roots) were sampled at 15-day old planting.

To study the mungbean *PAP*, mungbean KPS1 seeds were germinated by using modified method of Mandal and Biswas (1970). Mungbean cotyledons were harvested at 12-h intervals until 120 h after germination. Leaves, hypocotyls and radicles were obtained 72 h after germination.

All plant materials were immediately frozen in liquid nitrogen and kept at -80°C until use.

Isolation and expression analysis of *MIPS* and *PAP* cDNA

Total RNA was extracted from sampled tissues using the method of Salzman et al. (1999). The *MIPS* and *PAP* cDNA were isolated by reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA end (RACE) kit (GeneRacer Kit, Invitrogen), and all PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced at Macro Gen Company, Seoul, Korea.

Sequence analyses were performed using the BLASTX program obtained from the National Center for Biotechnology Information (NCBI). The phylogenetic relationship from the deduced amino acid sequences were constructed by the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replicatesusing the MEGA version 5 software program (Tamura et al., 2011). The phylogenetic tree of plant PAPs was obtained by using PhyloWidget (Jordan and Piel, 2008). Comparisons of deduced amino acid sequences were performed using the GeneDoc program.

The transcript levels of *MIPS* and *PAP* were examined using RNA blot analysis during seed development as well as in other organs. The copy numbers of *MIPS* and *PAP* genes in the genome of mungbean were determined by DNA blot analyses.

Results and Discussion

Isolation and expression analysis of *MIPS* cDNA

The 1,779 bp of mungbean *MIPS* cDNA (*VrMIPS1*) contained a 1533 bp coding sequence that could encode a putative protein of 510 amino acids which a predicted molecular weight (MW) of 56.5 kDa. The nucleotide sequences of *VrMIPS1* gene was deposited in the GenBank database under accession number EU239689. The amino acid identity of VrMIPS1 comparison with the other plants MIPS showed a high degree of sequence identity of 90 to 96% and 87 to 89% when compared to dicots and monocots, respectively (Fig. 1). In addition, the phylogenic tree indicated that the amino acid sequence of monocot MIPS and dicot MIPS were in separated clusters (Fig. 1).

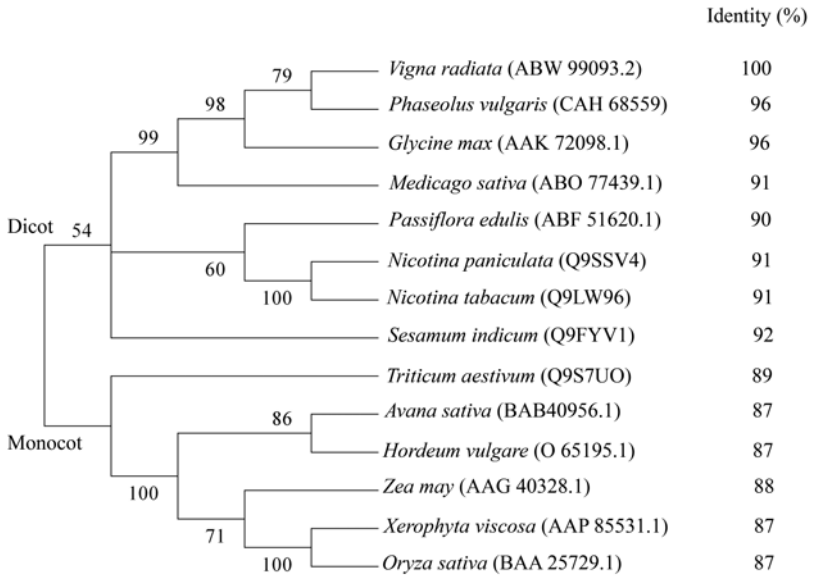


Fig. 1. Phylogenetic analysis of VrMIPS1and other MIPS predicted putative protein. A bootstrap was generated after 1000 cycles of bootstrap replicates theNeighbor-Joining method.

Alignment of VrMIPS amino acid sequences with other plant MIPS showed that VrMIPS1 contains four conserved domains, GWGGNNG (domain 1), LWTANTERY (domain 2), NGSPQNTFVPGL (domain 3) and SYNHLGNNDG (domain 4), which are important for MIPS functions, such that domain 1 is responsible for binding of co-factor NAD^+ and catalyzing the reaction (Fig. 2) (Majumder et al., 1997; Bachhawat and Mande, 1999).

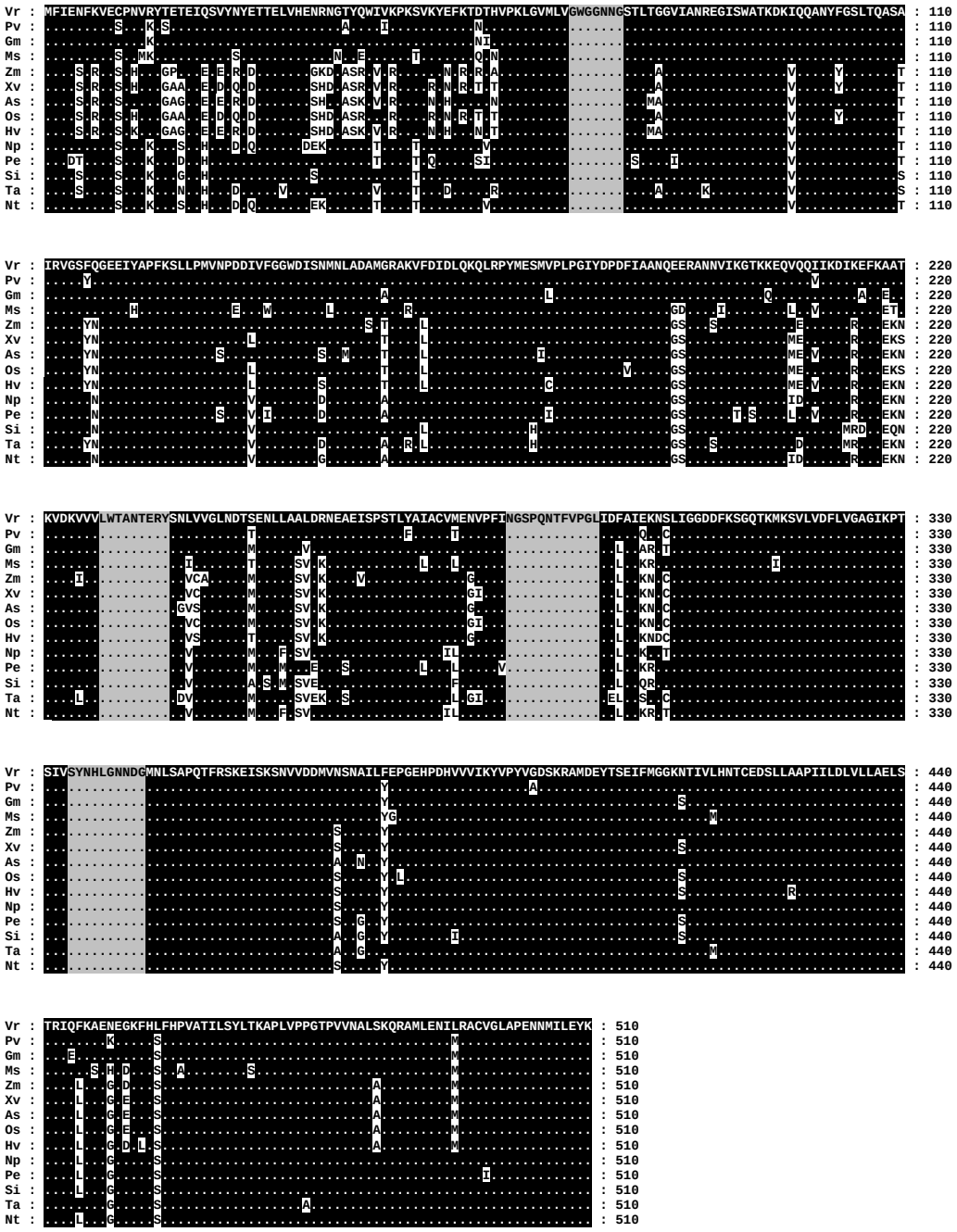


Fig. 2. Alignment of VrMIPS1 with other plant MIPS amino acid sequences. Conserved GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG in MIPS functional domains are highlighted by shaded gray. Amino acid identical to VrMIPS1 residues are indicated by dot and shaded black. Abbreviations of the species of each sequence are: Vr, *Vigna radiata* (ABW 99093.2) ; Pv, *Phaseolus vulgaris* (CAH 68559); Gm, *Glycine max* (AAK 72098.1); Ms, *Medicago sativa* (ABO 77439.1); Zm, *Zea mays* (AAG 40328.1); Xv, *Xerophyta viscose* (AAP 85531.1); As, *Avena sativa* (BAB 40956.1); Os, *Oryza sativa* (BAA 25729.1); Hv, *Hordeum vulgare* (O 65195.1); Np, *Nicotiana paniculata* (Q9SSV4); Pe, *Passiflora edulis* (ABF 51620.1); Si, *Sesamum indicum* (Q9FYV1); Ta, *Triticum aestivum* (Q9S7UO); Nt, *Nicotiana tabacum*.

The expression of *VrMIPS1* mRNA in different plant parts and during the embryogenesis showed that *VrMIPS1* accumulates in stem, root, and developing seed, whereas slight signal could be observed in leaves (Fig. 3a,b). The temporal expression of *VrMIPS1* mRNA during the embryogenesis showed that the highest *VrMIPS1* transcript levels can be detected between 7 and 9 DAF and gradually decreased after 13 DAF (Fig. 3a).

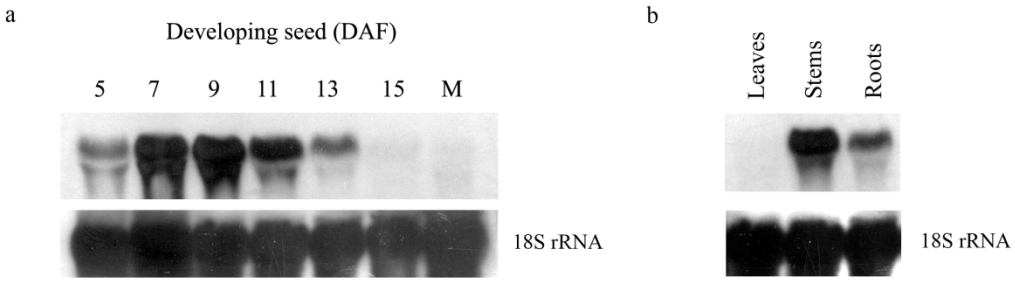


Fig. 3. Differential transcription of the *VrMIPS1* geneduring seed development and in various plant parts of mungbean. (a) Transcription patterns in developing mungbean seeds from 5 DAF to maturity(M). (b) Transcription patterns in plant parts (young leaves, stems and roots) at 15 days old planting.

In kidney bean, Abid et al. (2012) reported that the Pv_BAT93*MIPS* expresses early at the cotyledonary stage of seed development and reached a peak at 8 and 9 days after pollination (DAP) decreased after 12 DAP. The relationship between the expression of the *MIPS* mRNA and phytic acid synthesis in developing seed has been reported in rice. In situ hybridization of developing rice seeds showed that localization and accumulation of globoids or phytin-containing particles were corresponded with the increase pattern of *RINO1* transcripts (Yoshida et al., 1999). Therefore, the expression pattern of *VrMIPS1* may be correlated with the accumulation of phytic acid (Reddy et al., 1989; Loewus et al., 1990).

DNA blot analysis showed that *VrMIPS1* was produced single band when digested with *Bam*HI, *Sal*I and *Xbal*I but three band pattern with *Eco*RI. The results suggested that at least three copies of *VrMIPS1* gene are present in mungbean genome (Fig. 4). In kidney bean, DNA blot analysis suggested that there were at least three copies of *Pv_BAT93* gene in the genome (Abid et al., 2012). One sequence was found in rice (Yoshida et al., 1999) and yellow passion fruit (Abreu and Aragao, 2007), and at least four in soybean (Hegeman et al., 2001). Our results supported the idea that the multiple *MIPS* genes may be used to specific physiological function in different organs (Abreu et al., 2007).

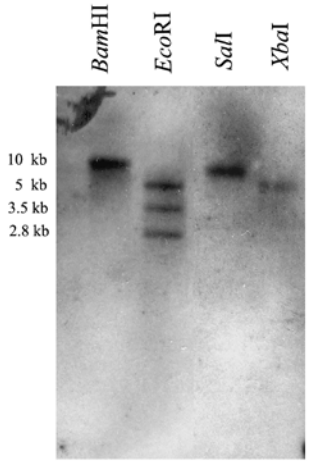


Fig. 4. DNA gel blot analysis of mungbean genomic DNA digested with *Bam*HI, *Eco*RI, *Sal*I and *Xbal*I.

Isolation and expression analysis of *PAP*cDNA

A full-length of munbean *PAP* cDNA (*VrPAP1*) was deposited in the GenBank database under accession number EU871632. The *VrPAP1* revealed to be 1864 bp nucleotides which has an open reading frame of 1644 bp. The putative protein encoded by *VrPAP1* has 547 amino acid residues with a predicted molecular weight of 62.07 kDa. Comparison between VrPAP1 and other plant PAP amino acid sequences showed 72, 68 and 65% identity with soybean phytase (GenBank acc. No. AAK49438), *Medicago truncatula* PAP (acc. No. AAX71115) and *Nicotiana tabacum* PAP (acc. No. ABP96799), respectively. In addition, VrPAP1 also showed identity with *Medicago truncatula* PAP (acc. No. XP_003618565), *Arabidopsis thaliana* PAP23 (acc. No. NP_193106) and *Ricinus*

communis PAP (acc. No. XP_002521081) with 70, 69 and 67% of identity, respectively. The phylogenetic tree of the VrPAP1 and other PAP homologues from various plant species showed that the PAP proteins were divided into three major groups: I - PAPs with known phytase activity, II - short plant PAPs and III - other PAPs and PAP-like proteins (Fig. 5).

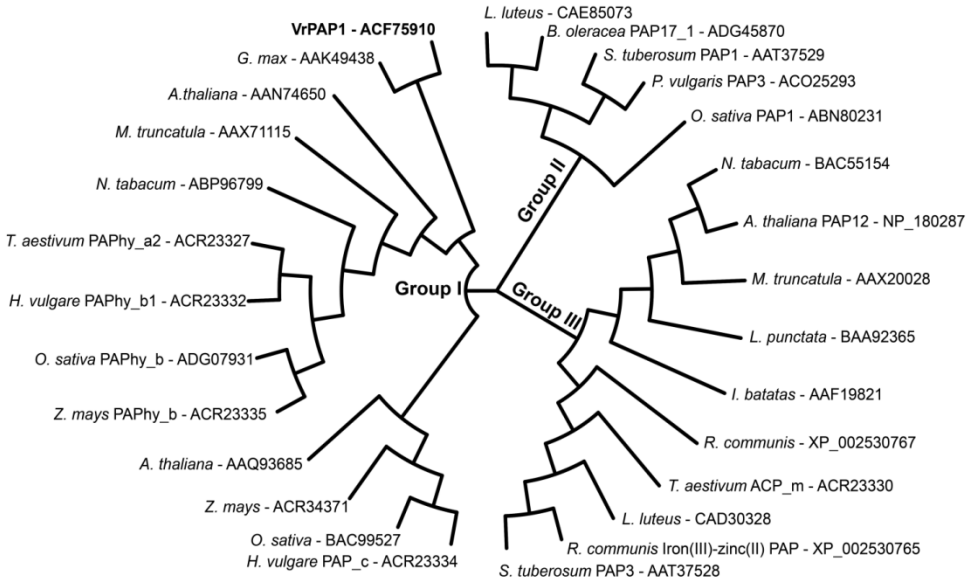


Fig. 5. Neighbour-joining phylogenetic tree of VrPAP1 (bold) with other homolog PAPs proteins in plant constructed using MEGA 5 and PhyloWidget.

Alignment of amino acid from VrPAP1 and other plants PAP indicated that, the VrPAP1 polypeptide contains purple acid phosphatase conserved motifs (D*X[G/H*] - (X_n) - GD*XX[Y/X] - (X_n) - GN*H[E/D], and VXXH* - (X_n) - GH*XH*) which were similarly reported in several plant PAPs such as soybean, wheat, barley, maize and rice (Hegeman and Grabau 2001, Dionisio et al., 2011). In addition, VrPAP1 amino acid sequence showed partial homology with four conserved motifs, 1) R-G-(H/V/Q/N)-A-(V/I)-D-(L/I)-P-(D/E)-T-D-P-(R/L)-V-Q-R-(R/N/T); 2) S-(V/I) V-(R/Q)-(Y/F)-G; 3) A-M-S-X-X-(H/Y)-(A/Y/H)-F(R/K) -T-M-P; and 4) D-C-Y-S-C-(S/A)-F-X-X-X-T-P-I-H, which were previously reported in wheat, barley, maize and rice PAP phytase (Dionisio et al., 2011) (Fig. 6).

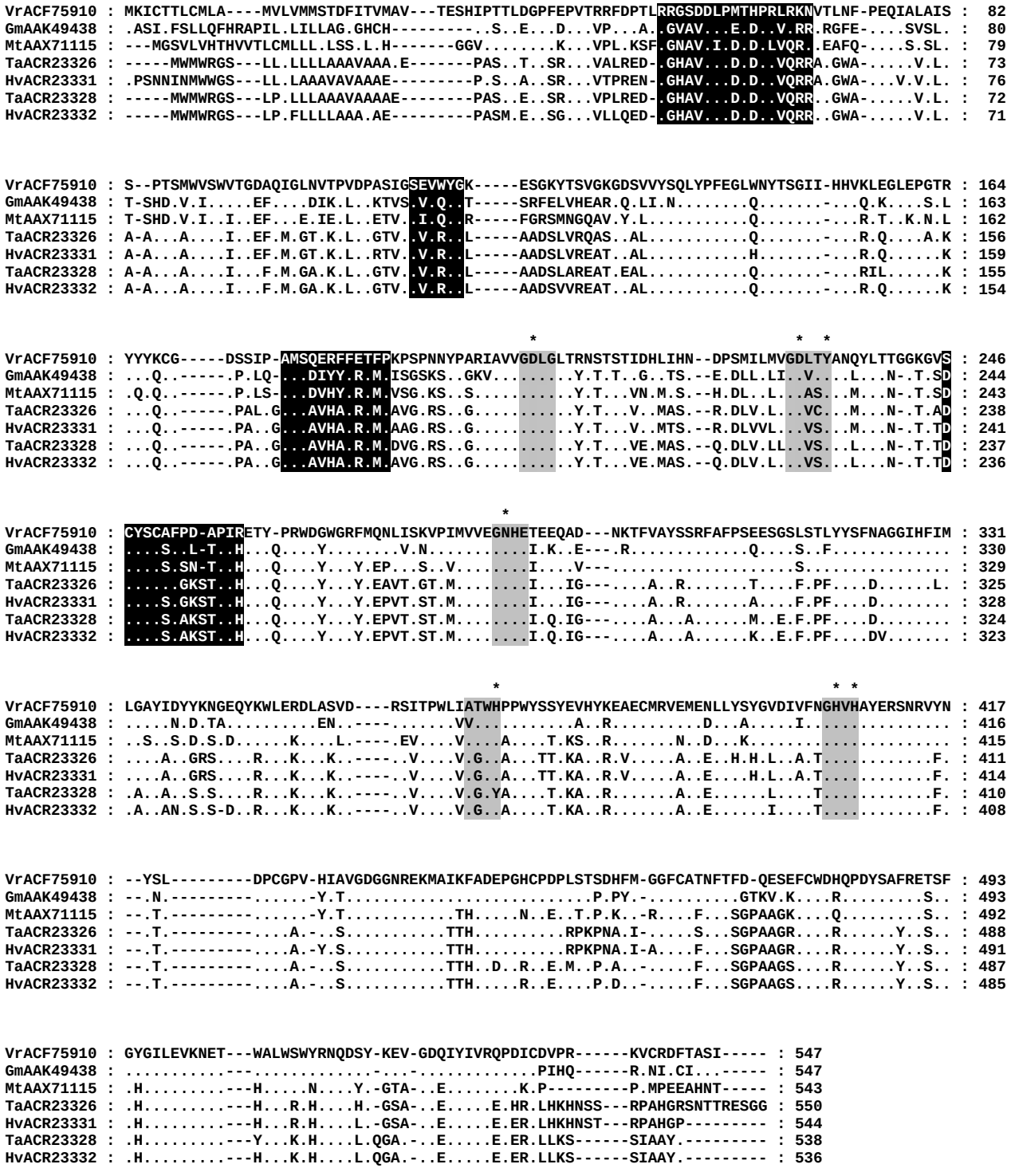


Fig. 6. Alignment of the deduced amino acid sequences of VrPAP1 with other PAPs from several plant species. Amino acid identical to VrPAP1 residues are indicated by dots. Gray shading, PAPs conserved regions; asterisk, the metal-binding residues; black shading, PAPhy motifs from PAPs with known phytase activity. The first two letters of each protein label represent the abbreviated species name followed by the GenBank accession number. Vr, *Vigna radiata*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Ta, *Triticum aestivum*; Hv, *Hordeum vulgare*.

The *VrPAP1* transcripts could be found in cotyledons and hypocotyls and very slightly in leaves, whereas undetected in dry seeds or radicles (Fig. 7a,b). The transcription of *VrPAP1* in cotyledons during germination was also observed. The *VrPAP1* expressed in the early stage of germination after 12 h of germination with the maximun

level after 24 h and gradually decreased until undetectable after 96 h (Fig. 7b). The qRT-PCR analysis of barley and wheat *PAPhy* isogene b showed high expression in the early germinating grain (Dionisio et al., 2011). Therefore, the early and seed-specific expression of *VrPAP1* can be hypothesized that the *VrPAP1* could involved in phosphorus mobilizationand supply inorganic phosphate to other parts of tissues during seedling growth.

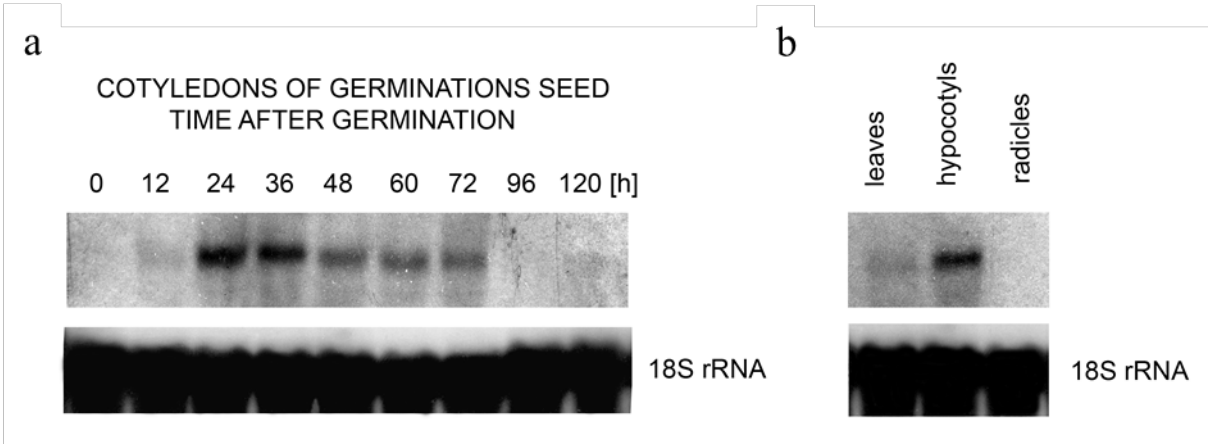


Fig. 7. RNA gel blots analysis of *VrPAP1* expression during seed germination and plant parts. (a) Pattern of the *VrPAP1* transcripts in cotyledons of germinating mungbean seeds from 0 to 120 hours after germination. (b) Pattern of the *VrPAP1* transcripts in plant parts of munngbean seedling at 72 hours after germination.

DNA blot analysis was performed to determine the complexity of the mungbean *PAP* gene. Genomic DNA was digested with *Bam*HI, *Eco*RI and *Hind*III and probed with partial sequence of *VrPAP1*. Only one strongly hybridized band in each digest was observed, indicating that a single gene encoding the *VrPAP1* is present in the mungbean genome (Fig. 8). In *Medicago truncatula* genome, two copies of *PAP* (Xiao et al., 2006a) and *PAP* phytase (Xiao et al., 2006b) genes exist whereas at least 2-3 copies of *NtPAP4*, *NtPAP12*, *NtPAP19* and *NtPAP21* genes (Kaida et al., 2003) are present in the *Nicotiana tabacum* genome.

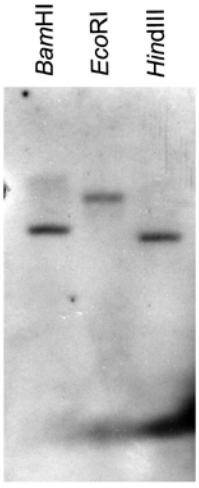


Fig. 8. DNA gel blots analysis. Mungbean genomic DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII.

Conclusion

We have examined expression patterns of *MIPS* and *PAP* genes in mungbean. Results showed that *VrMIPS1* is highly conserved at amino acid levels compared to other plants. Whereas, results from *VrPAP1* showed that *VrPAP1* belongs to the purple acid phosphatase family and only partial matching to the motifs site as known as PAP phytases

(Dionisio et al., 2011). Mungbean *MIPS* and *PAP* transcript levels were detected in the early stage of seed development and germination, respectively.

From our results, we propose the two transgenic approaches for reducing phytic acid content during seed development. First is the knock down *MIPS* gene in the first step of phytic acid synthesis and the second is engineering the overexpression of *PAP* to degrade the accumulation phytic acid during seed development. However, the level of phytic acid reduction in plant seed should be concerned because the *myo*-inositol intermediates in phytic acid synthesis are involved in key plant metabolic pathway. Therefore, the genetic modification of plant phytic acid reduction should focused on both seed phytic acid content which suitable for plant growth, especially at the early stage of germination, and increasing bioavailability of essential minerals in aminal feed with the target of improving the environment by a reduction of phosphorus contamination.

References

Abid, G., Sassi, K., Muhovski, Y., Jacquemin, J.M., Mingeot, D., Tarchoun, N., and Baudoin, J.P. 2012. Comparative expression and cellular localization of *myo*-inositol phosphate synthase (MIPS) in the wild type and in an EMS mutant during common bean (*Phaseolus vulgaris* L.) seed development. *Plant Molecular Biology Reporter*, 30:780-793.

Abreu, E.F. and Aragao, F.J. 2007. Isolation and characterization of amyoinositol-1-phosphate synthase gene from yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) expressed during seed development and environmental stress. *Annals of Botany*, 99:285-292.

Ali, N., Paul, S., Gayen, D., Sarkar, S.N., Datta, S.K., and Datta, K. 2013. RNAi mediated down regulation of myo-inositol-3-phosphate synthase to generate low phytate rice. *Rice* 6:12.

Dionisio, G., Madsen, C.K., Holm, P.B., Welinder, K.G., Jørgensen, M., Stoger, E., Arcalis, E., and Pedersen, H.B. 2011. Cloning and characterization of purple acid phosphatase phytases from wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea maize* L.) and rice (*Oryza sativa* L.). *Plant Physiology*, 156:1087-1100.

Jordan, G.E. and Piel, W.H. 2008. PhyloWidget: web-based visualizations for the tree of life. *Bioinformatics*, 24:1641-1642.

Hegeman, C.E., Good, L.L., and Grabau, E.A. 2001. Expression of D-myoinositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. *Plant Physiology*, 125:1941-1948.

Hegeman, C.E. and Grabau, E.A. 2001. A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. *Plant Physiology*, 126: 1598-1608.

Holme, I.B., Dionisio, G., Pedersen, H.B., Wendt, T., Madsen, C.K., Vincze, E., and Holm, P.B. 2012. Cisgenic barley with improved phytase activity. *Plant Biotechnology Journal*, 10:237-247.

Kaida, R., Ono, S.K., Kamada, H., Okuyama, H., Syono, K., and Kaneko, S.T. 2003. Isolation and characterization of four cell wall purple acid phosphatase genes from tobacco cells. *Biochimica et Biophysica Acta*, 1625:134-140.

Loewus, F., Everand, J., and Young, K. 1990. Inositol metabolism: precursor role and breakdown. In: Morre DJ, Boss WF, Loewus F (eds) Inositol metabolism in plants. Wiley, New York, pp 21-45.

Loewus, F.A. and Murthy, P.P.N. 2000. *myo*-inositol metabolism in plants. *Plant Science*, 150:1-19.

Majumder, A.L., Johnson, M.D., and Henry, S.A. 1997. 1 L-myo-Inositol 1-phosphate synthase. *Biochimica et Biophysica Acta*, 1348:245-256.

Mandal, N.C. and Biswas, B.B. 1970. Metabolism of inositol phosphates: I. Phytase synthesis during germination in cotyledons of mung beans, *Phaseolus aureus*. *Plant Physiology*, 45:4-7.

Reddy, N.R., Pierson, M.D., Sathe, S.K., and Salunkhe, D.K. 1989. Phytates in cereals and legumes. CRC, Boca Raton.

Saitou, N. and Nei, M. 1987. The neighbor - joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406-425.

Salzman, R.A., Fujita, T., Salzman, K.Z., Hasegawa, P.M., and Bressan, R.A. 1999. An improved RNA isolation method for plant tissue containing high of phenolic compounds or carbohydrates. *Plant Molecular Biology Reporter*, 17:11-17.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28:2731-2739.

Wongkaew, A., Nakasathien, S., and Srinives, P. 2010. Isolation and characterization of D-myo-inositol-3-phosphate synthase from mungbean (*Vigna radiata*). *Plant Molecular Biology Reporter*, 28:122-127.

Wongkaew, A., Srinives, P., and Nakasathien, S. 2013. Isolation and characterization of purple acid phosphatase gene during seedling development in mungbean (*Vigna radiata* cv. KPS1). *Biologia Plantarum*, 57:267-273.

Xiao, K., Harrison, M., and Wang, Z.Y. 2006a. Cloning and characterization of a novel purple acid phosphatase Gene (*MtPAP1*) from *Medicago truncatula* Barrel Medic. *Journal of Integrative Plant Biolology*, 48: 204-211.

Xiao, K., Zhang, J.H., Harrison, M., and Wang, Z.Y. 2006b. Ectopic expression of a phytase gene from *Medicago truncatula* Barrel Medic enhances phosphorus absorption in plants. *Journal of Integrative Plant Biolology*, 48:35-43.

Yoshida, K.T., Wada, T., Koyama, H., Mizobuchi-Fukuoka, R., and Naito, S. 1999. Temporal and spatial patterns of accumulation of the transcript of *myo*-inositol-1-phosphate synthase and phytin-containing particles during seed development in rice. *Plant Physiology*, 119:65-72.

Development of Rice Seed-Based Allergy Vaccine for Treatment of Japanese Cedar Pollen Allergy

Fumio Takaiwa
Institute of Agrobiological Sciences, Naro, Tsukuba Japan

Abstract

Japanese cedar pollen allergy is the predominant seasonal allergy disease in Japan. About 30% of Japanese is afflicted with this pollinosis. The main treatment of this allergy is pharmacotherapy which can not cure. Allergen-specific immunotherapy (SIT) is the only clinically available treatment that targets the cause of allergic disease and leads to long-lasting relief from allergic symptoms. Conventional treatment involves repeated subcutaneous injections with increasing doses of a crude allergen extract for at least three years, sometimes accompanied by adverse side effects, such as anaphylaxis. Therefore, convenient, safe, and effective allergen-specific immunotherapy has been sought after. Plant-based edible vaccine containing hypoallergenic antigens may be able to address this requirement, since it allows high doses of administration without side effects and antigens bioencapsulated within plant cells can be effectively delivered to immune cells in gut-associated lymphoid tissues (GALT) without severe degradation. Furthermore, seed-based immunization as a vaccination strategy has many advantages for the induction of immune tolerance in terms of high productivity and stability in addition to its simplicity, low cost of production and safety. In this study, rice seed-based allergy vaccines against Japanese cedar pollen allergens, Cry j 1 and Cry j 2, have been exploited and their efficacy was examined by oral administration of transgenic rice seeds containing destructed hypoallergenic Cry j 1 and Cry j 2 to model mice. Allergen-specific $CD4^+$ T cell proliferation and IgE and IgG levels were markedly reduced when compared with those in mice fed non-transgenic rice seeds. Not only production of Th2-type cytokines such as IL-4, IL-5 and IL-13, but also sneezing frequency and the infiltration of inflammatory cells in the nasal tissue as clinical symptom of pollinosis were significantly suppressed. Therefore, rice seed-based edible vaccines accumulating hypoallergenic allergens are expected to be effective and applicable for treatment of various type-I allergy diseases. Many pre-clinical experiments are done now toward clinical trial with this rice seed-based vaccine in patients.

Key words: Allergy vaccine, cedar pollen allergy, hypoallergen, oral vaccine, immunotherapy, transgenic rice

Introduction

In industrial countries, 30% to 40% of the population suffers from some kinds of allergic disease such as asthma, allergic rhinitis, conjunctivitis, atopic dermatitis, etc. Number of patients steadily increase over the last few decades and becomes an 'epidemic' problem. Treatment strategies for these allergic diseases generally involve pharmocotherapy using antihistamines, leucotient receptor antagosists or corticosteroids (Holgate and Polosa, 2008). However, although these approaches reduce the clinical symptoms; they are not curative. It is well-established that allergen-specific immunotherapy is the only way to modulate these immune reactions, with effects that persist for many years without further treatment (Larche et al., 2006). Conventional allergen-specific immunotherapy has been practiced for almost a century. Success can be achieved by repeated subcutaneous injections of increasing doses of native allergen extracts over a period of at least 3 to 5 years (Frew, 2010). However, this treatment is sometimes accompanied by severe side-effects, such as anaphylaxis that is caused by capture of allergen together with specific anti-allergen IgE on the surface of mast cells and basophils. The development of safer, more effective and convenient allergen-specific immunotherapy has therefore long been sought. To solve these problems, we developed seed-based oral allergy vaccines accumulating hypoallergenic tolerogen that permit to administrate high amounts of tolerogen without side-effect and to make treatment convenient, safe and easy.

Japanese cedar pollinosis and production of seed-based allergy vaccine

Japanese cedar pollen allergy is severe public health problem in Japan. Approximately 30% of the population is currently afflicted with this pollinosis between February and April each year. Peoples with circulating specific IgE against cedar pollen allergens (potential patients) account for up to 60% of the general population, thus patient number will steadily increase. The main allergens causing this pollinosis are Cry j 1 and Cry j 2, the major T cell epitopes of which have been well-characterized. Cry j 1 has pectate lyase activity and is specifically localized in the pollen cell wall. Cry j 2 has polygalacturonase activity and is localized in the amyloplasts of pollens.

To create safe tolerogen by reducing the allergenicity of these allergens (i.e. responsible for reactivity with IgE),

their tertiary structures have to be first altered by fragmentation, site-directed mutagenesis and molecular shuffling (reassembling), since exposed tertiary structure is generally recognized by specific IgE (Valenta et al., 2010). Peptide immunotherapy using dominant T-cell epitopes derived from allergens represents another approach to develop safer treatment, because regions involved in allergenicity can be completely removed while retaining immunogenicity (Larche, 2007). Second, the route of administration should be changed from subcutaneous injection to mucosal application (oral, sublingual, nasal delivery), providing safer and more convenient treatment.

Oral vaccination is especially convenient as a route of administration because it offers needle-free delivery. However, significant efficacy has not been reported in several previous studies due to degradation of crude allergen extracts or native allergens in the gastrointestinal tract before reaching gut-associated lymphoid tissue (GALT). Compared to subcutaneous injection (systemic administration), much higher doses of antigens (10- to 100-fold) are generally required to achieve the same level of efficacy via oral immunotherapy. When hypoallergenic allergen derivatives are produced in transgenic plants (seeds) and directly delivered as oral vaccine, it is more resistant to gastrointestinal enzymes than the purified one. This is because plant-based antigen is bio-encapsulated by double barriers characteristic of plant cells (cell wall and organelle) (Streatfield, 2006; Pelosi et al., 2012). This protects the antigen from digestive enzymes in the gastrointestinal tract. It is also important that not only mucosal but also parenteral immune reactions are modulated by oral administration. Furthermore, immune tolerance is inclined to be induced by oral administration rather than systemic injection like food proteins in diets.

When recombinant hypoallergenic tolerogens or T cell epitope peptides derived from major allergens are produced in transgenic plants, seed offers ideal production platform for these vaccines in terms of non-contamination of human pathogens, large scale production, high stability at ambient temperature (no need cold-chain), needle-free administration and low-cost production (Streatfield, 2007). To enhance accumulation levels in transgenic seeds without affecting vegetative growth, tolerogens have to be specifically expressed under control of the strong seed-specific promoter and to be efficiently transported to protein bodies, providing a wide deposition space as intracellular compartment (Takaiwa, 2011 & 2013). Gene sequences encoding tolerogens have to be synthesized using codons frequently used in many seed protein genes (codon optimization). Moreover, rare codons and destabilizing sequences have to be avoided. Production in the specific germplasm with low seed proteins results in improvement of yield of recombinant proteins. This is attributed to the rebalancing or compensatory effect of proteins to maintain the same nitrogen levels in seeds. Therefore, creation of vacant deposition space for produced recombinant proteins through mutation or suppression of storage proteins accumulated at the same target deposition site is crucial for enhancing the accumulation levels.

Principle of allergen-specific immunotherapy

The basic principle of allergen-specific immunotherapy is to induce immune tolerance to allergens through multiple cellular and molecular mechanisms leading to reduction in inflammatory cell recruitment and activation, and in mediator secretion released by degradation of mast cell and basophils (Akids et al., 200; Juntel et al., 2011). Peripheral T-cell tolerance is characterized mainly by the generation of allergen-specific regulatory T-cells (Tregs) ($Foxp3^+$ $CD4^+CD25^+$, induced $Foxp3^+CD4^+$, Tr1, and Th3) leading to suppressed T-cell proliferation and Th1 and Th2 cytokine responses against allergens. This is accompanied by a significant increase in allergen-specific IgG4 and IgG1 subclasses of IgG antibody and in IgA through IL-10 and TGF-β produced by Tregs, and a decrease in the specific IgE in the late stage of disease. IgG4 and IgA act as blocking antibodies by inhibiting the cross-linking of IgE on mast cells and basophils and the IgE-facilitated allergen presentation to T cells. Moreover, Treg cells are also involved in suppression of effector cells of allergic inflammation such as mast cells, basophils and eosinophils via anti-inflammatory cytokines (IL-10 and TGF-β) or cell-to-cell contacts.

Immune reaction is modulated by depending on dose, frequency, duration and formulation of antigens. Particulate formulation is more effective than soluble one through resistance to digestive enzymes. High dose of antigens are known to induce apoptosis, anergy of specific T cells, whereas continuous low dose administration results in active suppression through induction of regulatory cells such as $iFoxp3^+$ $CD4^+$, Tr1 and Th3. Immune tolerance is induced by complex mechanisms depending on case-by-case situation. Elucidation of underlining molecular mechanisms will be required.

Rice seed-based peptide vaccine against Japanese cedar pollen allergy

Oral administration of the dominant T-cell epitope of Cry j 2 inhibited specific T-cell responses in Cry j 2 sensitized mice in a mouse model of Japanese cedar pollinosis. Sneezing frequency as a measurable clinical symptom was decreased not only by systemic injections, but also by oral administration of the dominant T cell epitope of Cry j 2 (Hirahara et al., 1998). To confirm the efficacy of rice seed-based oral vaccines for inducing immune tolerance against cedar pollen allergens by allergen-specific immunotherapy, major mouse T cell epitopes derived from Cry j 1 (p 277-290) and Cry j 2 (p245-259) were inserted into the C-terminal highly variable regions of the soybean storage protein glycinin A1bB1b acidic and basic subunits. They were then expressed as fusion proteins

in the endosperm of transgenic rice seed under the control of the endosperm-specific glutelin *GluB-1* promoter (Takagi et al., 2005a). Modified glycinin A1aB1b containing T cell epitopes specifically accumulated to 7 μg/grain in mature dry seeds. In a preclinical model, the proliferative responses of allergen-specific $CD4^+$ T cells, synthesis of specific IgE, and production of histamine were less in mice orally administered transgenic rice seeds (0.2g) daily for 4 weeks prior to a systemic challenge with crude pollen allergens than in mice fed with non-transgenic control rice seeds. The production of allergy-associated Th2-type cytokines such as IL-4, IL-5, and IL-13 was also inhibited by rice-based T cell epitope peptide feeding. Histamine release from mast cells was also suppressed. Furthermore, allergy symptoms such as sneezing were alleviated after exposure to cedar allergens. These results indicated that mucosal immunization with rice seeds containing T cell epitopes efficiently induced immune tolerance.

Based on this confirmation of the feasibility of an oral peptide immunotherapy program in this animal model, a human version of a rice-based peptide vaccine was developed against cedar pollen allergy. An artificial hybrid peptide 7Crp gene composed of seven linked dominant human T cell epitopes (96 amino acids) derived from the Cry j 1 and Cry j 2 allergens was synthesized using seed-optimized codons for each amino acid (Takagi et al., 2005b). The hybrid 7Crp peptide elicited a positive response in 92% of 48 volunteers with pollinosis without binding to specific IgE, which indicated that it could be used as a safe and effective tolerogen (Hirahara et al., 2001). Notably, the 7Crp peptide had a greater effect on T cell proliferation than that of a mixture of the seven individual T cell epitope peptides.

This 7Crp peptide was specifically expressed in transgenic rice seeds as a secretory protein under the control of several strong endosperm-specific promoters (Takaiwa et al., 2009). The GluB-1 signal peptide and KDEL ER retention signal were included to increase the accumulation of the 7Crp peptide. Accumulation of the 7Crp peptide was markedly higher at approximately 60 μg/grain, as determined in CBB-stained SDS PAGE gels, and accounted for 6% of total seed protein. It was mainly deposited in ER-derived PB-I in the endosperm.

Transgenic rice seeds containing 7Crp were orally administered to Cry j 1-sensitized B10.S mice, which recognize only one epitope derived from Cry j 1 as the major epitope. Mice were then nasally challenged with intact Cry j 1 allergen. Both the T cell proliferative response against Cry j 1 and specific serum IgE levels were lower than those in control mice fed non-transgenic rice seeds (Takagi et al., 2005b). T cell proliferative activity was retained even after boiling 7Crp transgenic rice seeds for 20 min at 100°C or autoclaving for 20 min, which indicates that oral immune tolerance may still be effective when used with steamed or cooked rice. A safety evaluation of 7Crp transgenic rice seeds showed that the amino acid, lipid, carbohydrate, protein, fatty acid, mineral, and vitamin composition of the transgenic seeds was essentially identical to the non-transgenic control counterpart (Takagi et al., 2006). An oral safety study was performed by administering high and low doses of steamed rice to cynomolgus macaques for 26 weeks (Domon et al., 2009). No adverse effects were observed.

Transgenic rice seed containing hypoallergenic Cry j 1 and 2 tolerogens

Since humans with different genetic backgrounds respond differently to various T cell epitopes, peptide immunotherapy using T cell epitopes may not be applicable to all Japanese cedar pollinosis patients in spite of its proven safety. Thus, to treat a broader range of allergy patients, the entire Cry j 1 and Cry j 2 molecules were destroyed by a molecular shuffling and fragmentation process that perturbed the tertiary structure to identify an allergen-specific IgE. The full length of mature Cry j 1 (1-353 aa) was divided into three overlapped fragments. These three fragments with lengths of 131 to 144 amino acids were inserted into highly variable regions of the acidic subunits of GluA-2, GluB-1 and GluC, and were then expressed as fusion proteins with these different types of glutelins under the control of the rice endosperm-specific glutelin *GluB-4*, 16kDa and 10 kDa prolamin promoters. On the other hand, the coding sequence of Cry j 2 was restructured in the form of a tail to top inverse orientation to disrupt the tertiary structure. This shuffled Cry j 2 was attached to the KDEL ER retention signal at the C terminus and expressed under the control of the glutelin *GluB-1* promoter with its signal peptide sequence. A binary vector harboring four expression cassettes (three Cry j 1/glutelin fusions and one shuffled Cry j 2) was introduced into the good-taste rice variety genome lacking three glutelin genes by *Agrobacterium*-mediated transformation (Fig. 1). Three chimeric glutelin-Cry j 1 fragments were detected as glutelin precursors with molecular masses from 56 to 60 kDa, but were not processed into mature acidic glutelins containing Cry j 1 subunits. The shuffled Cry j 2 accumulated as a visible CBB-stained band with a molecular mass of 38 kDa. Ten to 25 μg of four individual antigens accumulated in one dry grain (approximately 20 mg) and were deposited into ER-derived PB-I (Wakasa et al., 2013). Little or no allergenicity by fragmentation and shuffling was confirmed by binding capacity to specific IgE or the basophil degradation assay.

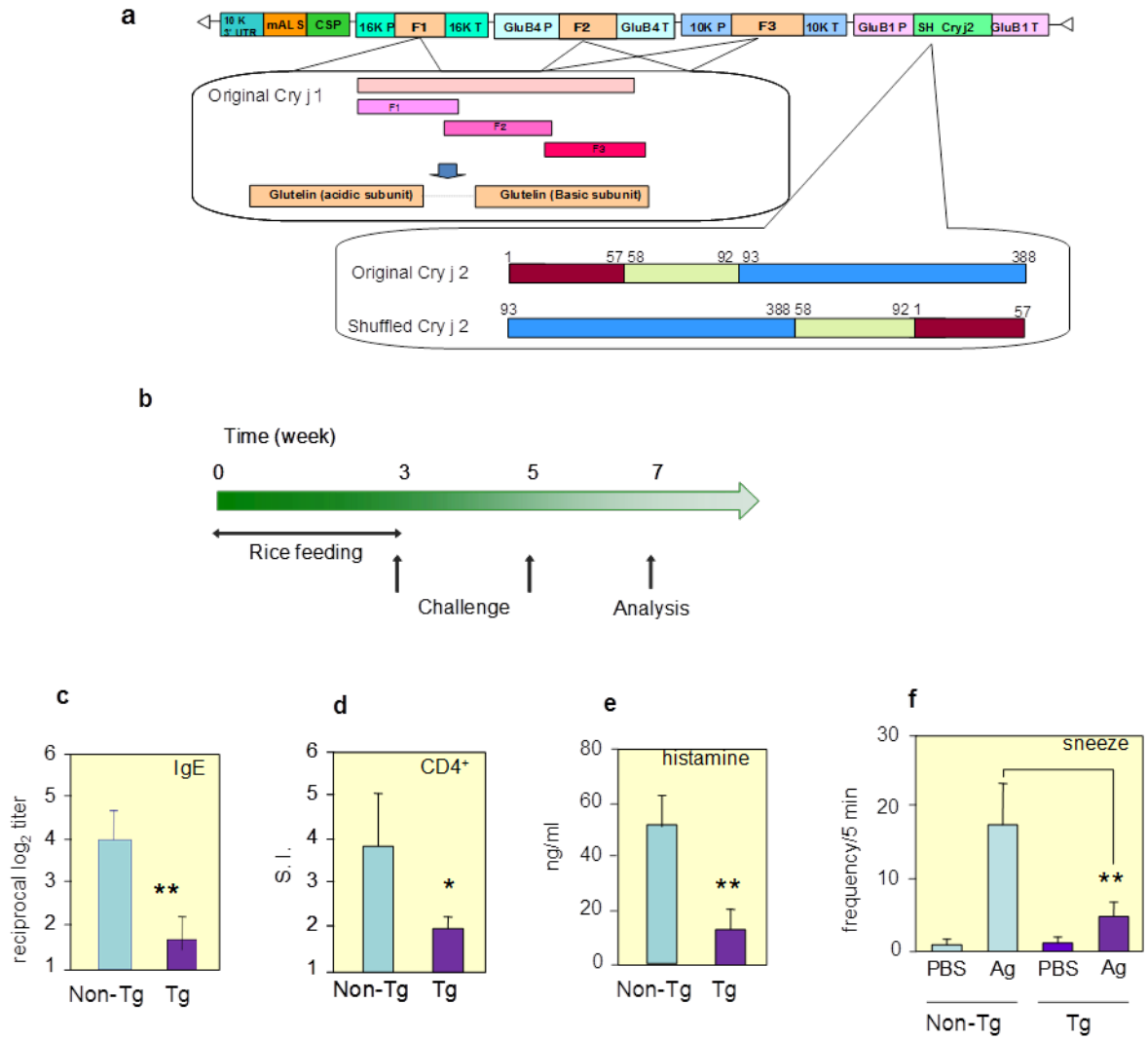


Fig.1. Vector construct used for the expression of hypoallergenic Cry j 1 and Cry j 2 in transgenic rice seeds and the induction of oral immune tolerance by the oral administration of transgenic rice seeds

a. The Cry j 1 allergen was divided into three overlapped fragments, each of which was inserted into the C terminal highly variable region of the acidic subunit of three different glutelins. The Cry j 2allergen was shuffled in the form of a tail to top orientation. The three glutelin/Cry j 1 fragment fusions and one shuffle Cry j 2 were linked to endosperm promoters and were then introduced into the binary vector. Four expression constructs were introduced into the rice genome via *Agrobacterium*-mediated transformation.
b. Experimental time line used for the induction of oral immune tolerance in mice administrated transgenic rice seeds.
c. Allergen-specific IgE levels
d. Allergen-specific splenic $CD4^+$ T cell proliferative responses
e. Serum histamine levels
f. Frequency of sneezes (sneeze number/5min after exposure to cedar pollen) sky blue column: mice fed with normal non-transgenic rice seeds magenta column: mice fed with transgenic seeds.

Transgenic rice seeds (0.6 g) containing accumulations of these destructed whole Cry j 1 and 2 molecules were fed daily to mice for 21 days, which were then challenged twice using a crude cedar pollen allergen. Allergen-specific $CD4^+$ T cell proliferation and IgE and IgG levels were markedly lower than those in mice fed non-transgenic rice seeds (Fig. 1). The production of Th2-type cytokines such as IL-4, IL-5, and IL-13 was decreased by the oral administration of transgenic rice grains. Sneezing frequency, which is a clinical symptom of pollinosis, and the infiltration of inflammatory cells in the nasal tissue, such as eosinophils and neutrophils, were also significantly reduced. These results suggest that the oral administration of transgenic rice seeds containing structurally disrupted Cry j 1 and Cry j 2 antigens is a more promising approach than with those containing the major T cell epitopes (7Crp) for the induction of immune tolerance against Japanese cedar pollinosis due to its applicability to a broader range of patients. At present, essential assessments such as chronic toxicity, phamacokinetics required for development of biological and establishment of GMP system required for production of rice seed-based allergy vaccine are carried out toward clinical trial using cedar pollen allergy patients.

Conclusion

Safer and more convenient allergen-specific immunotherapy has been desired as an alternative of the conventional subcutaneous one. Tolerogens have to be improved by removing allergenicity with retaining immunogenicity to elicit more effective desensitization and to lead longer lasting effects. Administration route is also desired to be changed from the subcutaneous to mucosal route to relieve from pains. Mucosal route has additional benefit, since not only systemic but also mucosal immune reactions are modulated. Transgenic rice has been generated by accumulating high amounts of hypoallergenic tolerogens against Japanese cedar pollen allergens in edible parts of rice grain. When this rice seed-based allergy vaccine was fed to model mice, immune tolerance was effectively induced and clinical symptom was also alleviated, confirming the efficacy of seed-based edible vaccine against allergy diseases.

References

Akdis, M and Akids, C.A. 2007. Mechanisms of allergen-specific immunotherapy. *Journal Allergy Clinical Immunology*, 119:780-789.

Domon, E., Takagi, H. Hirose, S. Sugita, K. Kasahara, S. Ebinuma, and Takaiwa, F. 2009. 26-week oral safety study in macaques for transgenic rice containing major human T-cell epitope peptides from Japanese cedar pollen allergens. *Journal Agricultural Food Chemistry*, 57:5633-5638.

Frew, A.J. 2010. Allergen immunotherapy. *Journal Allergy Clinical Immunology*, 125:S306-313.

Hirahara, K., T. Tatsuta, T. Takatori, M. Otsuka, H. Kirinaka, Kawaguchi, J., Serizawa, N., Taniguchi, Y., Saito, S., Sakaguchi, M., Inouye, S., and Shiraishi, A. 2001. Preclinical evaluation of an immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens. *Journal Allergy Clinical Immunology*, 108:94-100.

Hirahara, K., Saito, S. Serizawa, N. Sasaki, R. Sakaguchi, M. Inouye, S., Taniguchi, Y., Kaminogawa, S., and Shiraishi, A. 1998. Oral administration of a dominant T-cell determinant peptide inhibits allergen-specific TH1 and TH2 cell response in Cry j2-primed mice. *Journal Allergy Clinical Immunology*, 102:961-967.

Holgate, S and Polosa, R. 2008. Treatment strategies for allergy and asthma. *Nature Review Immunology*, 8: 218-230.

Juntel, M and Akdis, C.A. 2011. Immunological mechanisms of allergen-specific immunotherapy. *Allergy*, 66: 725–732.

Larche, M. 2007. Peptide immunotherapy for allergic diseases. *Allergy*, 62: 325-331.

Larche, M., Akdis, C.A., and Valenta, R. 2006. Immunological mechanisms of allergen-specific immunotherapy. *Nature Review Immunology*, 6:761–771.

Pelosi, A., Shepherd, R., and Walmsley, A.M. 2012. Delivery of plant-made vaccines and therapeutics. *Biotechnology Advances*, 30:440-448.

Streatfiled, S.J. 2006. Mucosal immunization using recombinant plant-based oral vaccines. *Methods*, 38:150-157.

Streatfield, S.J. 2007. Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnology Journal*, 5:2-15.

Takagi, H., Hiroi, T., Yang, L., Tada, Y., Yuki, Y.,Takamura, K., Ishimitsu, R., Kawauchi, H., Kiyono, H., and Takaiwa, F. 2005a. A rice-based edible vaccine expressing multiple epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proceeding of the National Academy of Sciences U.S.A.*, 102:17525-17530.

Takagi, H., Saito, S., Yang, L., Nagasaka, S., Nishizawa, N., and Takaiwa, F. 2005b. Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine. *Plant Biotechnology Journal*, 3:521-533.

Takagi, H., Hirose, S., Yasuda, H., and Takaiwa, F. 2006. Biochemical safety evaluation of transgenic rice seeds expressing T cell epitopes of Japanese cedar pollen allergens. *Journal Agricultural Food Chemistry*, 54:9901-9905.

Takaiwa F, Hirose, S., Takagi, S., Yang, L. and Wakasa, Y. 2009. Deposition of a recombinant peptide in ER-derived protein bodies by retention with cysteine-rich prolamins in transgenic rice seed. *Planta*, 229:1147-1158.

Takaiwa, F. 2011. Seed-based oral vaccines as allergen-specific immunotherapy. *Human Vaccine*, 7:357-366.

Takaiwa, F. 2013. Update on the use of transgenic rice seeds in oral immunotherapy. *Immunotherapy*, 5:301-312.

Wakasa, Y., Takagi, H., Hirose, S., Yang, L., Saeki, M., Nishimura, T. Kaminuma, O., Hiroi, T., and Takaiwa, T. 2013. Oral immunotherapy with transgenic rice seed containing destructed Japanese cedar pollinosis. *Plant Biotechnology Journal*, 11:66-76.

Valenta, R., Ferreira, F., Focke-Tejkl, M., Linhart, B., Niederberger, V., Swoboda, I., and Vrtala, S. 2010. From allergen genes to allergy vaccines. *Annual Review of Immunology*, 28:211-241.

Oral Immunogenicity of Porcine Reproductive and Respiratory Syndrome Virus Antigen Expressed in Transgenic Plants

Pung-Ling Huang[a, b*], Hui-Ting Chan[a], Yi-Yin Do[a],Min-Yuan Chia[c], Victor Fei Pang[c], and Chian-Ren Jeng[c]

[a]Department of Horticulture & Landscape Architecture, National Taiwan University, Taipei, Taiwan 10617

[b]Graduate Institute of Biotechnology, Chinese Culture University, Taipei, Taiwan 11114

[c]Graduate Institute of Veterinary Medicine, National Taiwan University, Taipei, Taiwan 10617

Abstract

Vaccines produced in transgenic plants constitute a promising alternative to conventional immunogens, presenting the possibility of stimulating humoral and cellular immunity against mucosal pathogens when administered orally. The advantages for transgenic plant vaccines in comparison with the traditional vaccines include low cost, easy for storage and administration. Porcine reproductive and respiratory syndrome virus (PRRSV) enters the host via the mucosa of gastrointestinal, respiratory, and reproductive tracts. Thus, it is deliberated by using transgenic plants as oral vaccine to activate common mucosal immunity for the prevention of PRRSV infection. The gene sequence of ORF5 corresponding to the neutralizing epitope of PRRSV was constructed in the plant expression vector and expressed as a subunit vaccine in both tobacco and banana. Pigs fed with these transgenic plant leaves developed specific mucosal and systemic immune responses, and these responses were enhanced significantly by subsequent oral boosting. In this paper, we describe transgenic plants expressed GP5 can be an effective system for potent and stable subunit vaccines.

Keywords: glycoprotein 5, oral immunization, oral immunogenicity, oral vaccine, subunit vaccine, transgenic banana.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease, is a viral disease characterized by reproductive impairment in breeding animals and respiratory tract illness in pigs of any age. PRRS is a highly infectious and the most significant disease in swine industry worldwide. The PRRS virus (PRRSV) is an enveloped RNA virus in the genus *Arterivirus*, belonging to the family of *Arteriviridae* and the order of *Nidovirales*. It contains a single-stranded, positive-sense, polyadenylated RNA genome. The viral genome is approximately 15 kb in size, composed of nine open-reading frames designated as ORFs 1a, 1b, 2a, 2b, and 3-7 (Conzelmann et al., 1993; Meulenburg et al., 1993). PRRSV strains are divided into two genotypes based on genetic and antigenic characteristics: genotype 1 (European subtype) and genotype 2 (North American subtype). Phylogenetic analyses of ORF5 fragments indicated that Taiwan PRRSV strains belong to the North American subtype and form at least 5 major genetic clusters distinctively distributed in Taiwan pig farms (Shen et al., 2010).

The ORF5-encoded major envelope glycoprotein GP5 has been demonstrated as the main target for virus-neutralizing antibodies in the course of a natural PRRSV infection in pigs (Gonin et al., 1999). The GP5 also forms a disulfide-linked heterodimer with the non-glycosylated membrane protein M, encoded by ORF6, and this association is assumed to be a prerequisite for virus assembly (Snijder et al., 2003). The formation of GP5/M heterodimers has been proposed to be involved in post-translational modification and transport of GP5 and may play a crucial role in immune responses against PRRSV infection (Jiang et al., 2006). In this article, we address the expression of either GP5 protein or GP5/M heterodimers could be used as a strategy to develop a new generation of vaccines against PRRSV.

Oral Administration of Plant-Expressed Subunit Vaccines in Pigs

The PRRSV infection has become endemic and widely spread in most pig-producing countries worldwide. There is currently no recommended drug therapy for PRRSV infection. The virus is mainly controlled by needle injection of vaccines including either killed or attenuated live vaccines (Wang et al., 2011). However, current live and killed PRRSV vaccines administered by a parenteral route are ineffective in inducing complete protection of PRRS (Renukaradhya et al., 2012). Thus, alternative approaches in the development of PRRSV vaccines are

necessary to reduce the disease incidence. Since pigs are infected by PRRSV via the mucosal route of reproductive and/or respiratory tracts, it is possible to activate common mucosal immunity by using orally administered vaccine to prevent PRRSV infection. As plants represent the promising bioreactor system for the production of safe subunit vaccines, we have investigated the application of tobacco and banana plants for the development of plant-based oral vaccines against PRRSV.

The gene sequence of ORF5, which encodes PRRSV envelope glycoprotein GP5, corresponding to the neutralizing epitopes of PRRSV MD-001 strain was driven by the cauliflower mosaic virus 35S promoter and constructed in the plant binary vector pGreen which contains β-glucuronidase (GUS) gene and kanamycin resistance gene (npt II) as the first version of our constructs used in this study. Furthermore, a plant-specific endoplasmic reticulum (ER) retention signal (HDEL) and a matrix-attachment region (MAR) from banana ubiquitin gene were added to the C-terminal of ORF5 gene for effectively increasing the stability of target proteins in plant cells. The resultant plant expression vector was transformed into tobacco and the amount of PRRSV GP5 synthesized in the transgenic tobacco leaves was estimated approximately 0.011% of total soluble proteins. A similar vaccination-dependent gradual increase of anti-PRRSV IgG and IgA in serum and saliva, respectively, and the levels of PRRSV-specific blastogenic response of peripheral blood mononuclear cells (PBMCs) were seen in pigs fed on transgenic tobacco leaves. It has been demonstrated that pigs fed on GP5-expressed tobacco could develop specific mucosal as well as systemic humoral and cellular immune responses against PRRSV (Chia et al., 2010a).

To enhance immune responses by transgenic tobacco in pigs to PRRSV, the second generation of constructs in this study contained the pentameric B subunit of heat-labile enterotoxin (LTB) of *Escherichia coli* that has a strong potential as a mucosal adjuvant was fused to ORF5 gene with Gly-Pro (GP) linker in between as a hinge to minimize the conformational changes in the tertiary structure of the target protein. After evaluated the feasibility of co-expression of LTB and PRRSV GP5 in transgenic tobacco plant and its immunogenicity in pigs, the LTB-GP5-coexpressed tobacco-treated group had relatively higher and longer immune responses than the GP5-expressed tobacco-treated group (Chia et al., 2011a).

Moreover, higher PRRSV-specific neutralizing antibody (NA) titers, serum IgG response, and lymphocyte proliferative responses were induced by DNA constructs co-expressing GP5 and M proteins of PRRSV conjugated by GPGP linker than that expressing GP5 or M alone not only in mice (Chia et al., 2011b) but also in pigs (Chia et al., 2010b). The transgenic tobacco harboring the third generation of constructs containing LTB co-expressed with ORF5 and ORF6 genes are currently under evaluation. On the other hand, recombinant GP5 protein levels in banana leaves regenerated from embryogenic cells transformed with the PRRSV ORF5 gene via *Agrobacterium*-mediated method (Fig. 1) were measured and ranged from 0.021%–0.037% of total soluble protein. A vaccination-dependent gradational increase in the elicitation of serum and saliva anti-PRRSV IgG and IgA was observed in pigs immunized with recombinant GP5 protein by orally feeding transgenic banana leaves for three consecutive doses at a 2-week interval and challenged with PRRSV at 7 weeks post-initial immunization (Chan et al., 2013).

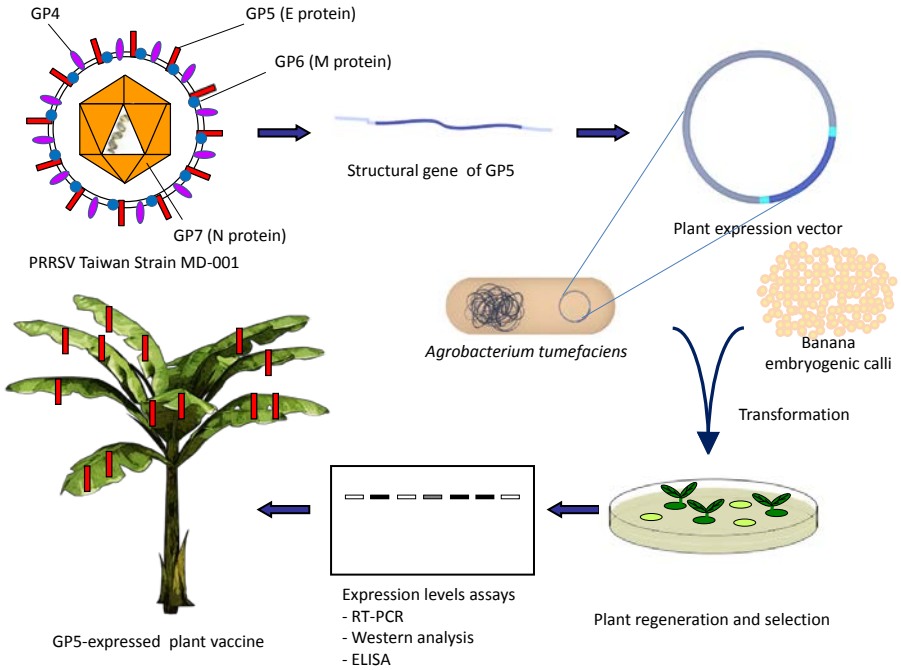


Fig. 1. Major experimental events taken place during genetic transformation to produce subunit vaccines in banana plants

Proposed Mechanism of Action of Oral Vaccines

Application of oral vaccines to induce specific mucosal antibody response may represent the most effective approach to preventing PRRSV infection. The plant-based oral vaccines provide a safe strategy for inducing protective immune responses without injection-related hazards. After oral administration, the plant-produced antigen proteins will access to the mucosal cells and induce antigen-specific immune responses in both systemic and mucosal compartments (Pogrebnyak et al., 2005). Schematic representation of proposed mechanism of action of plant-based oral vaccines is depicted in Fig. 2. Transgenic plants that express antigens in their edible tissue can elicit specific immune responses upon administration via the oral route. However, vaccines administered orally are subject to proteolysis in the gastrointestinal tract. Part of the vaccine antigens encapsulated naturally in the cell walls and other plant tissue structures may be protected from proteolysis and hence have capacity to induce an immune response. Alternatively, the antigens may be released during the plant cell degradation process in the stomach and intestines by the action of digestive or bacterial enzymes (Dhama et al., 2013). Efforts have focused on dose levels expressed in plant and efficient delivery of vaccine antigens to the mucosal lining of the gut that facilitate the protective mucosal immune responses.

Peyer's patches (PP) are lymphoid follicles located in the mucous membrane lining of the small intestine. They are part of the mucosal-associated lymphoid tissues (MALT) and an enriched source of secretory immunoglobulin (IgA) producing plasma cells. PP interact with antigens located in the intestine to produce antibodies and have potential to populate mucosal tissue and serve as mucosal immune effector sites (Streatfield, 2006; Hefferon, 2010; Takahashi et al., 2010). These organized lymphoid follicles contain germinal centers which represent the primary sites of mucosal B cell differentiation (Brandtzaeg and Johansen, 2005). Through these follicles vaccine antigen penetrates into epithelium of intestine and interacts with M-cells which are highly specialised antigen-transporting cells present within the epithelium of the small intestine. M-cells are characterized by the apical and basolateral surface being deeply invaginated to form a large intraepithelial pocket where the components of immune system like B-cells, T-cells and macrophages are accumulated (Nicoletti, 2000). Both B and T lymphocytes are the mediators of immunity under the control of dendritic cells (DCs). Antigens are captured by DCs in mucosal lymphoid tissues beneath M cells and in T-cell areas. DCs then migrate to lymphoid organs and secrete cytokines to initiate immune responses (Banchereau and Steinman, 1998). Both B and T lymphocytes can be efficiently activated by DCs within these lymphoid follicles (Banchereau and Steinman, 1998; Hefferon, 2010; Takahashi et al., 2010). B cells can directly recognize native antigen through their B-cell receptors whereas T-helper cells recognize fragments of antigens bound to molecules of the major histocompatibility complex (MHC) on the surface of an antigen-presenting cell (Banchereau and Steinman, 1998). $CD4^+$ T cells with the appropriate MHC-peptide specificity are thus activated and differentiate in effector T-cells that produce preferentially T helper 1 (Th1) or Th2 cytokines under the control of regulatory T (Tr) cells (Siegrist, 2013). Activated $CD4^+$ T cells presumably exert supportive functions for DCs resulting in further activation of B cells. Upon receiving additional signals from follicular T cells, B cells undergo massive clonal proliferation and differentiate into plasma cells for the secretion of large amounts of antigen-specific IgA and IgG antibodies (Siegrist, 2013). These IgA/IgG antibodies are transported across the epithelial cells into secretions of the lumen where they can interact with the antigen present in the lumen (Rudzik et al., 1975; Yuki and Kiono, 2003).

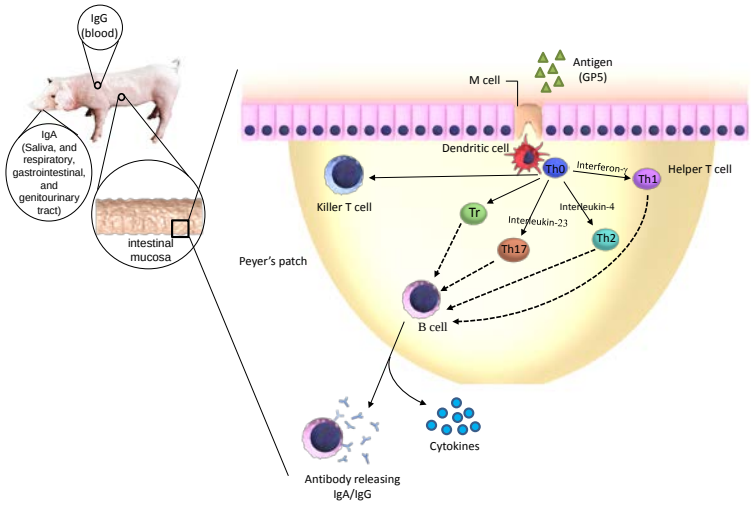


Fig. 2. Proposed mechanism of action of oral vaccines

B cell: specific type of white blood cell; IgA: immunoglobulin A; IgG: Immunoglobulin G; Th0: native T cell; Th1: type 1 helper T cell; Th2: type 2 helper T cell; Th17: type 17 helper T cell; Tr: regulatory T cell; M cell: microfold cell.

Conclusion

In this paper, we reported a new generation of PRRSV vaccines, the recombinant GP5 subunit vaccine produced by transgenic tobacco and banana. Pigs administered with these plant-based oral vaccines developed specific humoral and cell-mediated immune response against PRRSV. These results support the foreseeable potential of developing novel edible plant-based oral vaccines for prevention of epidemic diseases in the future. Our study also showed promising results for plant-based vaccine in animal systems, suggesting potential applications to human banana vaccines.

Plant-based vaccines offer several advantages over current conventional vaccines, including that storage and transportation are convenient after lyophilization, production costs are low, and the contamination of mammalian pathogens can be avoided. However, several unique aspects associated with this new technology must be considered. There are many technical challenges like degradation of vaccine antigen by enzymes of upper digestive tract, dosage regime, and oral tolerance. Nevertheless, the most challenging concern should be the regulatory and economic issues and public perception with the use of genetically modified plants. The use of bioreactor culture systems for mass propagation of antigen-containing plant cells may provide a possible solution to this concern. Special aspects with regard to potential bioreactor production include the antigen content and the immunogenicity of the cultured cells, and physical and chemical factors affecting cell growth.

References

Banchereau, J. and Steinman, R.M. 1998. Dendritic cells and the control of immunity. *Nature,* 392:245-252.

Brandtzaeg, P. and Johansen, F.E. 2005. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunological Reviews,* 206:32-63.

Chan, H.T., Chia, M.Y., Pang, V.F., Jeng, C.R., Do, Y.Y., and Huang, P.L. 2013. Oral immunogenicity of porcine reproductive and respiratory syndrome virus antigen expressed in transgenic banana. *Plant Biotechnology Journal,* 11:315-324.

Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., and Jeng, C.R. 2011a. Evaluation of the immunogenicity of a transgenic tobacco plant expressing the recombinant fusion protein of GP5 of porcine reproductive and respiratory syndrome virus and B subunit of *Escherichia coli* heat-labile enterotoxin in pigs. *Veterinary Immunology and Immunopathology,* 140:215-225.

Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Cheng, C.H., Pang, V.F., and Jeng, C.R. 2011b. Improved immunogenicity of DNA constructs co-expressing the GP5 and M proteins of porcine reproductive and respiratory syndrome virus by glycine-proline-glycine-proline (GPGP) linker in mice. *Taiwan Veterinary Journal* 37:12-23.

Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., and Jeng, C.R. 2010a. Immunogenicity of recombinant GP5 protein of porcine reproductive and respiratory syndrome virus expressed in tobacco plant. *Veterinary Immunology and Immunopathology,* 135:234-242.

Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F., and Jeng, C.R. 2010b. The immunogenicity of DNA constructs co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus conjugated by GPGP linker in pigs. *Veterinary Microbiology,* 146:189-99.

Conzelmann, K.K., Visser, N., Van Woensel, P. and Thiel, H.J. 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. *Virology,* 193:329-339.

Dhama, K., Wani, M.Y., Deb, R., Tiwari, K. K, R., Barathidasan, R., Kumar, A., Mahima, Verma, A.K., and Singh, S.D. 2013. Plant based oral vaccines for human and animal pathogens–A new era of prophylaxis: current and future perspectives. *Journal of Experimental Biology and Agricultural Sciences,* 1:1-10.

Gonin, P., Pirzadeh, B., Gagnon, C.A., and Dea, S. 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. *Journal of Veterinary Diagnostic Investigation,* 11:20-26.

Hefferon, K.L. 2010. The mucosal immune response to plant-derived vaccines. *Pharmaceutical Research,* 27:2040-2042.

Jiang, Y., Xiao, S., Fang, L., Yu, X., Song, Y., Niu, C., and Chen, H. 2006. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. *Vaccine,* 24:2869-2879.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moonen, P.L., den Besten, A., de Kluyver, E.P., Wensvoort, G., and Moormann, R.J. 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology,* 192:62-72.

Nicoletti, C. 2000. Unsolved mysteries of intestinal M cells. *Gut,* 47:735-739.

Pogrebnyak, N., Golovkin, M., Andrianov, V., Spitsin, S., Smirnov, Y., Egolf, R., and Koprowski, H. 2005. Severe acute respiratory syndrome (SARS) S protein production in plants: development of recombinant vaccine. *Proceedings of the National Academy of Sciences USA*, 102:9062-9067.

Renukaradhya, G.J., Dwivedi, V., Manickam, C., Binjawadagi, B., and Benfield, D. 2012. Mucosal vaccines to prevent porcine reproductive and respiratory syndrome: a new perspective. *Animal Health Research Reviews*, 13:21-37.

Rudzik, R., Clancy, R.L., Perey, D.Y.E., Day, R.P., and Bienenstock, J. 1975. Repopulation with IgA-containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patch and bronchial lymphocytes. *Journal of Immunology*, 114:1599-1604.

Shen, S.Y., Ma, W.J., Yu, C.Y., and Chang, C.C. 2010. Genetic variation of porcine reproductive and respiratory syndrome viruses in Taiwan. *Taiwan Veterinary Journal*, 36:305-314.

Siegrist, C.A. 2013. Vaccine immunology. In: Plotkin, S.A., Orenstein, W., and Offit, P.A. (eds) Vaccines. 6th ed. Elsevier Inc. p.17-36.

Snijder, E.J., Dobbe, J.C., and Spaan, W.J.M. 2003. Heterodimerization of the two major envelope proteins Is essential for Arterivirus infectivity. *Journal of Virology*, 77:97-104.

Streatfield, S.J. 2006. Mucosal immunization using recombinant plant-based oral vaccines. *Methods*, 38:150-157.

Takahashi, I., Nochi, T., Kunisawa, J., Yuki, Y., and Kiyono, H. 2010. The mucosal immune system for secretory IgA responses and mucosal vaccine development. *Inflammation and Regeneration*, 30:40-47.

Wang, A., Wang, D.J., Chowda-Reddy, R.V., Chen, H., and Ma, S. 2011. Development of a plant-based vaccine against Porcine reproductive and respiratory syndrome virus: research progress and future prospects. *American Journal of Plant Science and Biotechnology*, 5:127-131.

Yuki, Y. and Kiono, H. 2003. New generation of mucosal adjuvants for the induction of protective immunity. *Reviews in Medical Virology*, 13:292-310.

Compositional Assessment of β-Carotene Biofortified Rice

Jae Kwang Kim, Soo-Yun Park, and Yunsoo Yeo
National Academy of Agricultural Science,
Rural Development Administration,
Suwon 441-707, Republic of Korea

Abstract

One important aspect in assessing the safety of genetically modified (GM) crops for human consumption is characterizing their nutrient composition. A β-carotene-biofortified rice was generated by inserting phytoene synthase (*Psy*) and carotene desaturase (*Crtl*) genes isolated from *Capsicum* and *Pantoea* into the genome of a conventional variety of rice (Nakdongbyeo). Nutrients (proximates, minerals, and vitamins), anti-nutritive components (trypsin inhibitors and phytic acid), and ferulic acid in GM rice were compared with those in the parent line Nakdongbyeo. Statistical comparisons to test for equivalence showed that all of the analyzed components in the GM plants were equivalent to those in its non-transgenic counterpart, and most nutritional components fell within the range of values reported for other commercial lines, indicating the safety of the GM plant.

Key words: genetically modified crop, β-carotene, transgenic rice, nutrient, substantial equivalence

Introduction

Commercialization of GM rice has lagged behind that of other cereals such as maize. The reason could be that rice is cultivated in more than 100 countries around the world and is a staple for about a half of the world's population; thus its safety must be strictly evaluated prior to market availability (Jiao et al., 2010). Safety assessment of GM food is based on the concept of substantial equivalence, developed by the Organization for Economic Co-operation and Development (OECD) and further elaborated by the Food and Agriculture Organization/World Health Organization (FAO/WHO). This concept embraces a comparative approach to identify possible differences between GM food and its traditional counterpart. Therefore, substantial equivalence is not a safety assessment per se but is an important starting point for a safety assessment. Composition analysis is a major factor assessed when determining substantial equivalence.

To assess substantial equivalence, the OECD consensus document on rice has identified the key food and feed nutrients and anti-nutrients under consideration for assessment in new rice varieties (OECD, 2004). Comparative compositional analyses have been reported for glufosinate-tolerant rice in the USA (Oberdoerfer et al., 2005) and insect-resistant rice in China (Li et al., 2007). The statistical analyses used in those studies demonstrated the safety approach. Equivalence boundaries were set to 20% of the means, as recommended by the Nordic Council of Ministers (Nordic Council, 2000). This paper reports a series of chemical analyses following the guidelines of the OECD consensus document were conducted to assess the effects of the new gene insertion on the nutritional composition and anti-nutrient content of novel biotechnology-derived rice.

Development of Carotenoid-rich Rice

Carotenoids are important for human health as a source of provitamin A and components to reduce the incidence of several diseases (Landrum and Bone, 2001). To alleviate vitamin A deficiency through biofortifying crops, a carotenoid-biofortified rice was developed using a bicistronic system to coordinately express two carotenoid biosynthetic genes, phytoene synthase (*Psy*) from *Capsicum* and carotene desaturase (*Crtl*) from *Pantoea* (Ha et al., 2010). A synthetic 2A sequence optimized for the rice codon was used to generate the *PAC* (*Psy-2A-Crtl*) construct under the control of a single rice globulin promoter. In this study, the nutritional composition of the PAC rice was compared with that of a conventional counterpart (cv. Nakdongbyeo). Both were grown in the same field in Korea for comparision. Total carotenoid level was 0.04 μg/g in nontrangenic rice and 1.14 μg/g in PAC rice, respectively. The PAC rice accumulated lutein (0.3 μg/g), zeaxanthin (0.1 μg/g), α-carotene (0.1 μg/g), and β-carotene (0.6 μg/g) in its grain. In addition, the composition values of the PAC rice were compared with values obtained from previously published study (Ha et al., 2010).

Comparison of Transgenic and Non-transgenic Rice

The comparative analysis of the rice grain was completed using a statistical procedure to assess equivalence. Performing an ordinary t-test and inferring equivalence from the absence of a significant difference entails an

uncontrolled increase in the risk of false-positive conclusions; that is, the assumption of "equivalence." In other words, a "nonsignificant difference" is different from "significant equality." Statistical equivalence for a component was assumed if the mean values of the two treatments did not differ "too much"; that is, the difference in the mean values was within a certain interval. Thus, we followed the advice of the FDA and the Nordic Council for accepting equivalence limits.

The moisture levels in all samples were maintained consistently, and no significant differences were found for the mean values of protein, lipid, ash, fiber, and carbohydrate between the transgenic rice grain and its nontransgenic counterpart (Table 1).

Table 1. Comparison and analysis of equivalence of the proximates measured in brown rice

Component	*p*-value[a]	Non-transgenic	PAC	Analysis of equivalence[b]	OECD [c] (2004)
Moisture, % fw[d]	<0.05	10.95 ± 0.61[e]	10.56 ± 0.27	yes	14
Protein, % dw[f]	<0.05	7.04 ± 0.40	7.49 ± 0.17	yes	7.1–8.3
Lipid, % dw	<0.05	2.36 ± 0.10	2.07 ± 0.07	yes	1.6–2.8
Ash, % dw	<0.05	1.26 ± 0.10	1.33 ± 0.09	yes	1.0–1.5
Fiber, % dw	<0.05	1.19 ± 0.26	0.98 ± 0.08	yes	0.6–1.0
Carbohydrates[g], % dw	<0.05	89.56 ± 0.73	89.12 ± 0.20	yes	87.4–90.3

[a]Based on the two one-sided test, confidence intervals, 100 (1 – 2 × α)%, where α = 0.05, were calculated to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [b]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [c]Source: OECD data. [d]fw, fresh weight. [e]Each value is the mean ± standard deviation (n = 6). [f]dw, dry weight. [g]Carbohydrate levels were estimated by the formula: % carbohydrates = 100 – (% protein + % lipid + % ash).

Table 2. Comparison and analysis of equivalence of the minerals, vitamins, anti-nutrients, and ferulic acid measured in brown rice

Component	*p* value[a]	Non-transgenic	PAC	Analysis of equivalence[b]	OECD[c] (2004)
Copper, μg/g	<0.05	3.35 ± 0.75[d]	3.22 ± 0.64	yes	1–7
Iron, μg/g	<0.05	13.16 ± 2.35	12.40 ± 1.07	yes	2–60
Sodium, μg/g	<0.05	32.34 ± 1.84	34.91 ± 3.33	yes	20–400
Zinc, μg/g	<0.05	26.92 ± 1.20	26.34 ± 3.91	yes	7–33
Calcium, mg/g	<0.05	0.15 ± 0.01	0.14 ± 0.03	yes	0.1–0.6
Potassium, mg/g	<0.05	2.51 ± 0.11	2.77 ± 0.05	yes	0.7–3.2
Magnesium, mg/g	<0.05	1.17 ± 0.08	1.25 ± 0.10	yes	0.2–1.7
Vitamin B_1, μg/g	<0.05	4.29 ± 0.41	4.38 ± 0.32	yes	2.9–6.1
Vitamin B_2, μg/g	<0.05	0.20 ± 0.02	0.22 ± 0.04	yes	0.4–1.4
Vitamin E, μg/g	<0.05	14.0 ± 1.4	14.4 ± 0.6	yes	9–25
Trypsin inhibition, TIU/mg		<0.1	<0.1	yes	Not available
Phytic acid, %	<0.05	0.87 ± 0.12	0.98 ± 0.06	yes	Not available
Ferulic acid, μg/g	<0.05	60.17 ± 1.41	58.05 ± 2.78	yes	Not available

[a]Based on the two one-sided test, confidence intervals, 100 (1 – 2 × α)%, where α = 0.05, were calculated to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [b]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [c]Source is OECD data. Each value is the mean ± SD (n = 6).

Equivalence was demonstrated in the brown rice for all minerals, vitamins, anti-nutrients, and ferulic acid (Table 2). All mean values calculated in the GM rice samples, except for vitamin B2, were within the ranges reported

by the OECD (2004). Vitamin B2 levels were lower than those provided by the OECD, including in the nontransgenic control, indicating that this was not caused by GM. The inhibition activity by trypsin inhibitors was very low in both the PAC brown rice and the nontransgenic counterpart compared with 100–184 trypsin inhibitor units /mg detected in soybean (Li et al., 2007).

Conclusion

Key nutritional components measured in brown rice derived from GM rice (transgenic β-carotene biofortified PAC rice) were found to be substantially equivalent to those of the nontransgenic counterpart. Based on the principle of substantial equivalence, as articulated by the WHO and the OECD, these data support the conclusion that PAC transgenic rice is as safe as its traditional counterpart.

References

Ha, S.H., Liang, Y.S., Jung, H., Ahn, M.J., Suh, S.C., Kweon, S.J., Kim, D.H., Kim, Y.M., Kim, J.K. 2010. Application of two bicistronic systems involving 2A and IRES sequences to the biosynthesis of carotenoids in rice endosperm. *Plant Biotechnology Journal*, 8:928–938.

Jiao, Z., Si, X.X., Li, G.K., Zhang, Z.M., and Xu, X.P. 2010. Unintended compositional changes in transgenic rice seeds (*Oryza sativa* L.) studied by spectral and chromatographic analysis coupled with chemometrics methods. *Journal of Agricultural and Food Chemistry*, 58:1746–1754.

Landrum, J.T., and Bone, R.A. 2001. Lutein, zeaxanthin, and the macular pigment. *Archives of Biochemistry and Biophysics*, 385:28–40.

Li, X**.**, Huang, K**.**, He, X**.**, Zhu, B**.**, Liang, Z**.**, Li, H**.**, and Luo, Y**.** 2007. Comparison of nutritional quality between Chinese indica rice with *sck* and *cry1Ac* genes and its nontransgenic counterpart. *Journal of Food Science*, 72:420–424.

Nordic Council. 2000. Safety assessment of novel food plants. Chemical analytical approaches to the establishment of substantial equivalence (TemaNord 1998: 591). Nordic Council of Ministers, Copenhagen, Denmark.

Oberdoerfer, R.B., Shillito, R.D., De Beuckeleer, M., and Mitten, D.H. 2005. Rice (*Oryza sativa* L.) containing the *bar* gene is compositionally equivalent to the nontransgenic counterpart. *Journal of Agricultural and Food Chemistry*, 53:1457–1465.

OECD. 2004. Environment, health and safety publications series on the safety of novel foods and feeds. No. 10. Consensus document on compositional considerations for new varieties of rice (*Oryza sativa*): key food and feed nutrients and anti-nutrients. Organization for Economic Co-operation and Development, Paris, France.

General View of Environmental Impact Assessment of Genetically Modified Crops

Yasuhiro Yogo
Institute for Agro-Environmental Sciences, NARO, Tsukuba, Japan

Abstract

The Open-ended Ad Hoc Working Group was established in 1996. The meeting of Intergovernmental Committee for the Cartagena Protocol (ICCP) was held in 2000-2002. And the Conference of the Parties serving as the meeting of the Parties to the Protocol (COP-MOP) was first held in 2004 and the COP-MOP6 was held in 2012. Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (CBD) was adopted on 2000, and entered into force on 2003. The protocol is aimed at protecting biodiversity from the adverse impact of living (genetically) modified organism ("GMO" hereinafter) by using modern biotechnology. It covers not only domestic cultivation but also import/export. Then Advance Informed Agreement (AIA) concerning about "liability and redress" and "Access and Benefit Sharing (ABS)" will be necessary.

Biosafety Clearing-House (BCH) was established based on the Article 20 of CPB, in order to facilitate the information exchange and assist the Parties to implement CPB. The recent topic in the Open-ended On-line Forum in BCH is alignment of the guidance and training manual. The guidance was developed by the Ad Hoc Technical Expert Group (AHTEG) with input from the Forum in accordance with decisions BS-IV/11 and BS-V/12 in COP-MOP. The guidance consists of three parts, i.e. 1) the roadmap for risk assessment of GMO; 2) risk assessment of specific types of GMOs, such as stacked traits, abiotic stress, trees and mosquitos; and 3) the monitoring of GMOs released into the environment. And the training manual also consists of three parts, i.e. Module 1: Overview of Biosafety and the CPB, Module 2: Preparatory Work – Understanding the context in which a risk assessment will be carried out, and Module 3: Conducting the Risk Assessment.

In general, risk assessment is to determine the risk quantitatively and/or qualitatively in combination with hazard and exposure assessment. The risk assessment of GMOs on biodiversity follows this concept with four steps, such as hazard identification, exposure assessment, hazard characterization and risk characterization, once the event was treated as a GMO. Then each event (product) will be assessed the risk scientifically on a case by case basis with the concept of familiarity. And the progeny lines will be automatically approved, although the stacked events should be evaluated in view point of the interaction among the traits of parent events.

On the other hand, GM crop, for example, bring benefits depending on traits, e.g. plant health against biotic and abiotic stress, higher yield, better quality, and so forth. Regulatory science should be introduced for further discussion on the practical use of this technology.

Keywords: Advance Informed Agreement, Biosafety Clearing-House, biological diversity (biodiversity), Cartagena Protocol on Biosafety, genetically/living modified crop, risk assessment, regulatory science

Introduction

Genetically modified organisms (GMOs) have been utilized worldwide, for pharmaceuticals, industrial chemicals, crop improvement, and so forth. Commercially cultivated area and countries of genetically modified (GM) crops have been increasing year by year in the world, and the total area was ca. 170 million ha, and ca. 30 countries in 2012. The area in the developing counties, such as Brazil, Argentina, China, and India, was dramatically increased and the cumulative area caught up with the developed countries (James, 2012). And the situation in GM crop, i.e. import only to active cultivation, is different among countries, even within monsoon Asia countries.

The traits of GMO have been diversifying, starting from herbicide tolerance to abiotic stress tolerance, and so forth. GM crops with abiotic stress tolerance aim at better growth than non-GM crop. Therefore it is difficult to use the concept of "substantial equivalence" between GM and non-GM crops for impact/risk assessment on biodiversity.

This paper is to summarize environmental impact assessment of GM crop, based on Cartagena Protocol on Biodiversity (CPB), and to explain the "Guidance" and "Training manual" in view point of risk assessment and regulatory science.

Cartagena Protocol

The distinctive events happen in every decade. In the 1970s, GMO Test Guidelines were established for research and Asilomar Conference on Recombinant DNA was held in 1975. In the 1980s, Organization for Economic Co-operation and Development (OECD) Council published "Safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques" (OECD, 1986). In the 1990s, OECD made Good Developmental Principles (GDP) for field experiment (small/preliminary) of plant and microbe, and also established the concept on scale-up. The Convention on Biological Diversity (CBD) was opened for signature at the Earth Summit in Rio de Janeiro on 5 June 1992. The main goals in the Convention are 1) conservation of biodiversity, 2) sustainable use of its components, and 3) fair and equitable sharing of benefits arising from genetic resources, i.e. Access and Benefit Sharing (ABS). In the 2000s, CPB to CBD was adopted and entered into force (Mackenzie et al., 2003). The protocol is aimed at protecting biodiversity from the adverse impact of GMO by using modern biotechnology. It covers not only domestic cultivation but also import/export. Then Advance Informed Agreement (AIA) concerning about "liability and redress" and "Access and Benefit Sharing (ABS)" will be necessary. A total of 166 Parties (countries) ratified CPB now, although many major GM crop growing countries, such as USA, Canada, Argentina, and Australia, do not ratify until now. In the 2010s, UN declared the period from 2011 to 2020 as the UN-Decade on Biodiversity.

The Open-ended Ad Hoc Working Group was established in 1996. The meeting of Intergovernmental Committee for the Cartagena Protocol (ICCP) was held in 2000-2002. And Conference of the Parties serving as the meeting of the Parties to the Protocol (COP-MOP) was first held in 2004 and the COP-MOP6 was held in 2012. Biosafety Clearing-House (BCH) actively drives the international harmonization of CPB.

Basic Concept of CPB

CPB is aiming at conserving/protecting biodiversity from the potential risks of GMOs generated by modern biotechnology, but it does not consider the benefits of GMOs (Secretariat of CBD, 2000). There are several basic concepts in CPB, such as precautionary approach, transboundary movement, packaging and transportation, Advance Informed Agreement (AIA), independence to the agreements under the World Trade Organization (WTO), facilitation of information exchange.

Risk Assessment

The Health and Environmental Sciences Institute (HESI) is the nonprofit institution composed of a wide range of scientists from academia, government and industry, in order to identify and resolve the global human health and environmental issues. RISK21 is one of the projects of HESI, and the mission is to bring applicable, accurate and resource appropriate approaches to the evolving world human risk assessment. There are four principles, such as problem formulation based, exposure driven, prior knowledge, and enough precision to make decision. Their activities were conducted based on the "Risk Assessment Concept".

The environment has two characteristics, complex and uncertainty. Because it is composed of abiotic (soil, water, air) and biotic (plant, animal, microbe) constituents, and each constituent and their correlations vary between place and period, hence we have to do "decision making" under such conditions (Bradbury et al., 2004).

In general, risk assessment is to determine the risk quantitatively and/or qualitatively in combination with hazard and exposure assessment as shown in Fig. 1. Recently the problem formulation is the key issues to start the risk assessment (Nickson, 2008). The risk assessment of GMOs on biodiversity follows this concept with four steps, such as hazard identification (Identification of adverse characteristic of GMO), exposure assessment (Likelihood assessment), hazard characterization (Consequence assessment), and risk characterization (Risk estimation/evaluation), followed by and/or in relation to risk management and risk communication.

Once a certain event ("Product") with modern molecular technology was treated as a GMO, we start the above steps. Then each product will be assessed the risk scientifically on a case by case basis with the concept of familiarity. And the progeny lines will be automatically approved, although the stacked events should be evaluated in view point of the interaction among the traits of parent events.

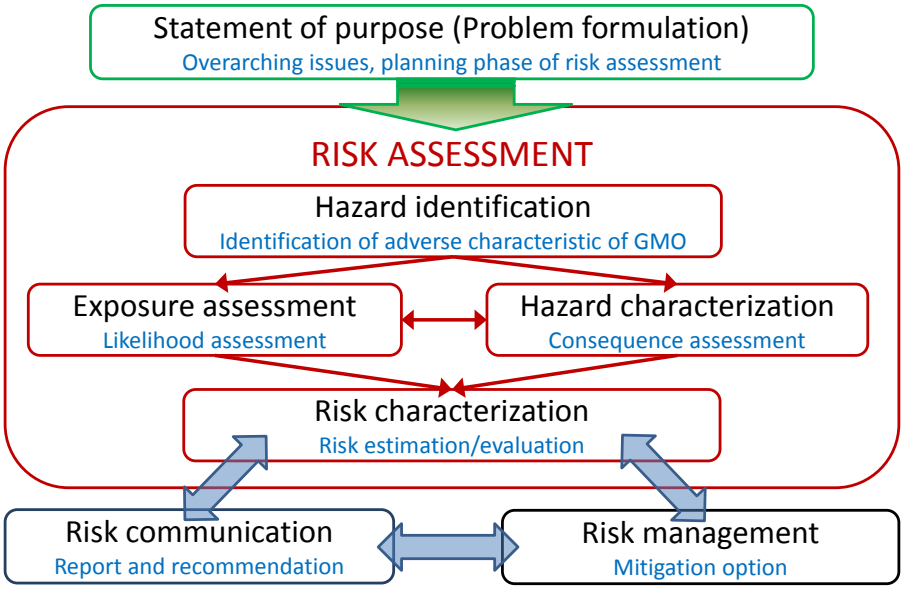


Fig. 1. General scheme of risk assessment in relation to GMO

Guidance

Biosafety Clearing-House (BCH) was established based on the Article 20 of CPB (Information exchange and the biosafety clearing-house), in order to facilitate the information exchange and assist the Parties to implement CPB. In response to the COP-MOP decision BS-IV/11 the Discussion Groups (from 2008) and Real Time Online Conferences (Open-ended On-line Forum, 2009) were organized. The recent topic in the Forum is alignment of the guidance and training manual.

The guidance was developed by the Ad Hoc Technical Expert Group (AHTEG) with input from the Forum in accordance with the decisions BS-IV/11 and BS-V/12 in COP-MOP (BCH, 2012). The guidance consists of three parts: 1) the roadmap for risk assessment of GMO; 2) risk assessment of specific types of GMOs, such as stacked traits, abiotic stress, trees and mosquitoes; and 3) the monitoring of GMOs released into the environment. Guidance covers wide range of living organisms, however, mainly focused on plants but not animals.

Table 1. Contents in "Guidance on risk assessment of living modified organisms"

Preface
Objective and scope of this guidance
PART I: Roadmap for risk assessment of living modified organisms
Background, Introduction, Overarching issues in the risk assessment process, Quality and relevance of information, Identification and consideration of uncertainty, Planning phase of the risk assessment, Establishing the context and scope, The choice of comparators, Conducting the risk assessment
Step 1: An identification of any novel genotypic and phenotypic characteristics associated with the living modified organism
Step 2: An evaluation of the likelihood of adverse effects being realized
Step 3: An evaluation of the consequences should these adverse effects be realized
Step 4: An estimation of the overall risk
Step 5: A recommendation as to whether or not the risks are acceptable or manageable
Related issues, Annex (Flowchart for the risk assessment process)
PART II: Specific types of LMOs and traits
A. Risk assessment of living modified plants with stacked genes or traits
Introduction, Objective and scope, Planning phase of the risk assessment, Conducting the risk assessment
B. Risk assessment of living modified plants with tolerance to abiotic stress
Introduction, Planning phase of the risk assessment, Conducting the risk assessment
C. Risk assessment of living modified trees
Background, Scope, Introduction, Planning phase of the risk assessment, Conducting the risk assessment
D. Risk assessment of living modified mosquitos
Introduction, Objective and scope, Planning phase of the risk assessment, Conducting the risk assessment, Related issues
PART III: Monitoring of living modified organisms related into the environment
Introduction, Objective and scope, Monitoring and its purposes, Development of a monitoring plan
Use of terms

Guidance was described in accordance with CPB, Article 1 to 40, and Annex I to III. Risk assessment is written in Article 15 and Annex III of CPB. In Article 15, Risk assessments biological diversity and human health should be conducted in a scientifically sound manner, in order to identify and evaluate the possible adverse effects of GMOs. And the key issues were written in Annex III, such as objective, use of risk assessment, general principles, methodology, and points to consider. And four general principles of risk assessment are specified in Annex III, i.e. A) scientifically sound and transparent manner, B) how to consider the lack of scientific knowledge or scientific consensus, C) likely potential receiving environment based on non-modified recipients, D) Case-by-case basis

Aforesaid C and D should be considered differently among the counties (“Parties” in CPB). Firstly, native (wild) species, which have potential to hybridize with GMOs, is different among countries. Environmental risk of GM rice and soybean should be carefully evaluated in Asian Parties, since their native crossable species exist, such as wild rice (*Oryza rufipogon*) and soybean (*Glycine soja*). Secondary, potential of weediness of crop itself varied under different receiving environment. Most of crop lost the weediness during the long history of breeding. However, some crop such as canola, creeping bentgrass, has certain level of dormancy and voluntarily emerge in the certain condition. Thirdly, the crop, cultivation area, size, rotation are different among the Parties. Rice is the major crop in monsoon-Asia, and the farm size is relatively small and crop rotation is complex, in comparison to developed countries in US and Europe. In addition, some Parties allow to import but not cultivate. Impact of GM crop to biodiversity between intended use (cultivation) and commodity (Food, Feed and Processing, FFP) is largely different in view point of probability.

The Roadmap in PART I provide guidance on assessing environmental risks of GMOs, and illustrated in the flowchart. As mentioned above, it is composed based on the general scheme of risk assessment, with some modifications. It may be useful for the Parties to conduct and/or review their own risk assessment system.

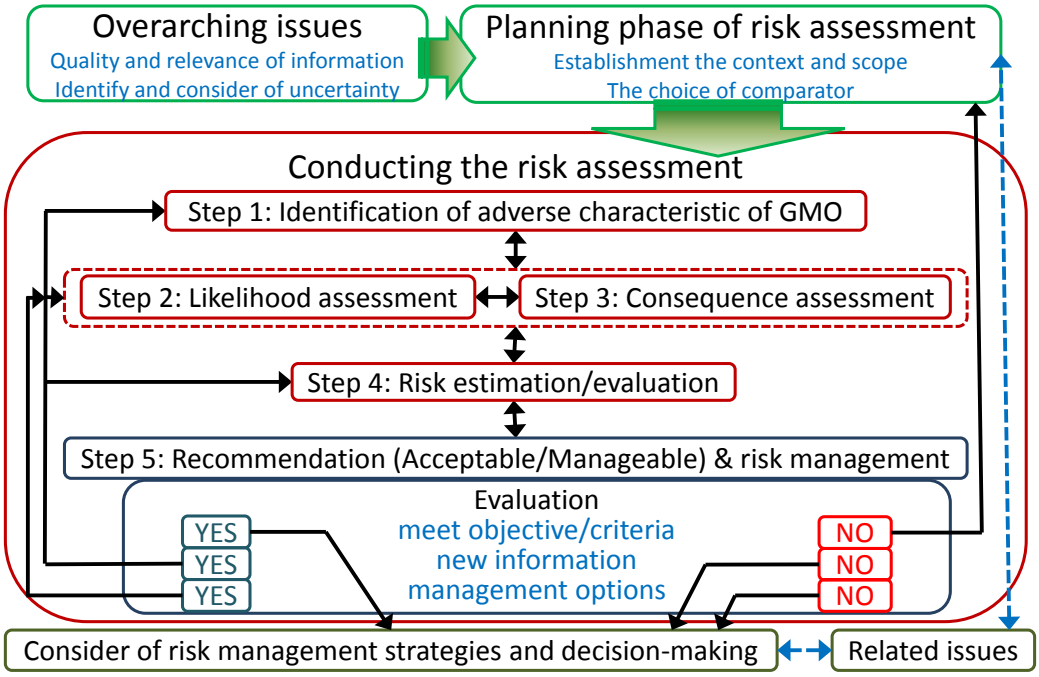


Fig. 2. Outline of the flowchart for the risk assessment

The GM traits are diversified currently, 1) biotic stress in plant protection: insects (Bt), disease and herbicide tolerance, 2) abiotic stress: draught/wet and heat/cold, 3) good growth: high yield, dwarf/erect, 4) for human: long storage, ingredient modified food, and pharmaceuticals (pollen allergy mitigation), 5) environment: phytoremediation of soil, and 6) others: flower color and industrial materials. And stacked events are also getting more and more commercially used and complex. Synergistic/antagonistic action may occur with or without activator/repressor, and the impact to biodiversity may change quantitatively and/or qualitatively. Therefore, risk assessors must carefully evaluate the risk of interaction among the events in product and science-based in each case.

Training Manual

The training manual consists of three parts, i.e. Module 1: Overview of Biosafety and the CPB; Module 2: Preparatory Work – Understanding the context in which a risk assessment will be carried out; and Module 3: Conducting the Risk Assessment.

Module 1 is introductory sections, which contains basic concepts in biosafety and an introduction to the Cartagena Protocol on Biosafety and other international biosafety-related bodies and organizations.

Module 2 is for national risk assessors to carry out risk assessment. It describes protection goals, assessment endpoints, biosafety framework, practices and principles and so forth. And it also includes the expert advice and the roles of the risk assessor.

Module 3 is for overview of the risk assessment methodology, such as overview of the risk assessment methodology, overarching issues, Planning phase, Conducting the risk assessment (Step 1 to 5), and Preparing a risk assessment report and recommendation. Finally, risk assessors judge whether or not the risks are acceptable or manageable.

Table 2. Contents in "Training manual on risk assessment of living modified organisms"

Module 1: Overview of Biosafety and the Cartagena Protocol on Biosafety
Introduction to biosafety and the Cartagena Protocol on Biosafety: history, biosafety, LMO, objective/scope, AIA, LMOs-FFP, Competent National Authorities, Risk Assessment (Article 15 and Annex III) , BCH
Other international biosafety-related bodies: IPPC, Codex, FAO, WOAH, OECD, WTO
References, Annex
Module 2: Preparatory Work – Understanding the context
Introduction
National context: protection goals and assessment endpoints, biosafety framework, authorities, practices and principles, other national and international obligations
Expert advice and the roles of the risk assessor: scientific advisory body, responsibilities, stakeholder participation
References
Module 3: Conducting the Risk Assessment
Introduction
Overview of the risk assessment methodology
Overarching issues: Information needed, Consideration of uncertainties
Planning phase: context and scope, assessment endpoints or representative species, Establishing the baseline, choice of comparator
Conducting the risk assessment: Step 1: Identification of any novel genotypic and phenotypic characteristics associated with the LMO that may have adverse effects Step 2: Evaluation of the likelihood Step 3: Evaluation of the consequences Step 4: Estimation of the overall risk Step 5: Identification of risk management and monitoring strategies
Preparing a risk assessment report and recommendation
References

Regulatory Science

GM crops bring wide range of benefits depending on traits, e.g. plant health against biotic and abiotic stress, higher yield, better quality, and so forth as described above. Regulatory science (RS) was first used by Alan Moghissi (US-EPA) in the 1970s. And RS targeted on pharmaceuticals followed by environmental sciences. Dr. Mitsuru Uchiyama, former director of National Institute of Health Science, first introduced in1987 in Japan. Pesticide Science of Japan first introduced RS concept to agricultural area, and start the Special Committee on Pesticide Regulatory Science in 1994. Japan's Ministry of Agriculture, Forestry and Fisheries and Ministry of Health, Labour, and Welfare also introduced RS that prioritized for risk management of food safety on chemicals and microbes.

On the other hand, GM crops have potential risks to environment and human health. And once the negative impact occurred, large effort and cost may be needed to recover and/or to manage. RS concept should be introduced for further discussion on the practical use of this technology (Falck-Zepeda, 2009; McLean et al., 2012; Secretariat of CBD 2003; Segger et al., 2012).

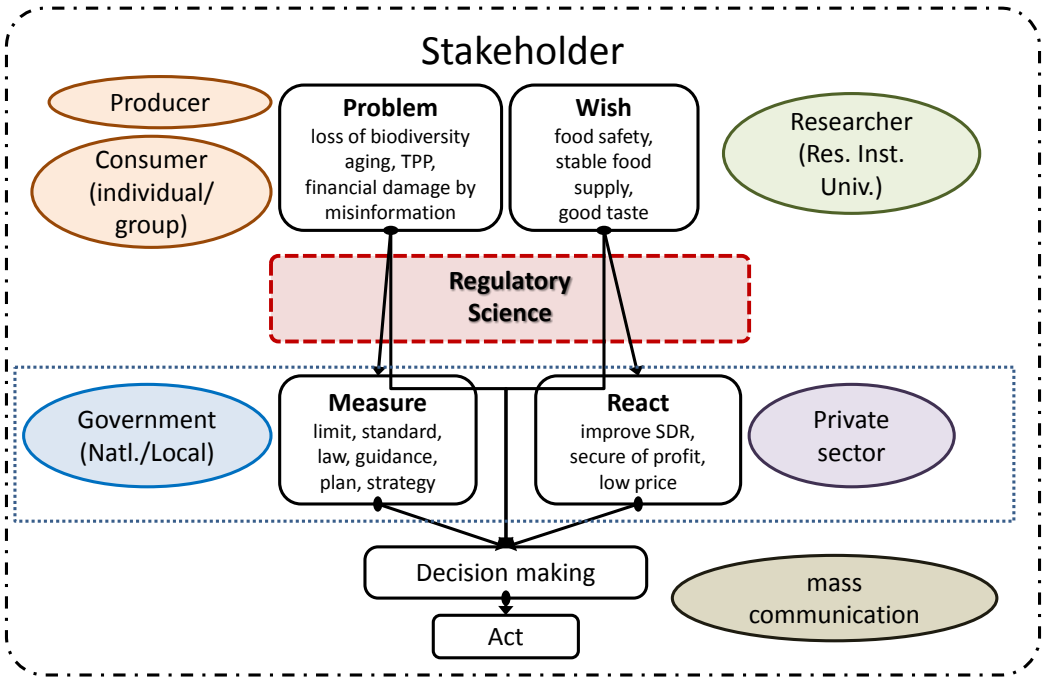


Fig. 3. Scheme of regulatory science

Conclusion

Commercially cultivated area of GM crops is increasing year by year, not only in developed countries but also in the developing countries, including monsoon Asia region. And even if a certain Party did not cultivate, an unignorable amount of GM crops could be imported, especially after TPP (Trans-Pacific Strategic Economic Partnership Agreement) issues are settled. In addition, international harmonization on GMO will progress more and more via several international bodies, such as BCH/CBD, OECD, and Codex. Therefore, it is important to share information and cooperate to move forward regulatory science, including risk and benefit analysis of GM crops in monsoon Asia countries, for preservation of biodiversity and food safety.

References

1. Bradbury, S.P., Feijtel, T.C.J., and Van Leeuwen, C.J. 2004. Meeting the scientific needs of ecological risk assessment in a regulatory context. Environmental Science and Technology, 463A-470A.
2. Falck-Zepeda, J.B. 2009. Socio-economic Considerations, Article 26.1 of the Cartagena Protocol on Biosafety: What are the Issues and What is at Stake? AgBioForum, 12: 90-107.
3. James, C. 2012. ISAAA Briefs, BRIEF 44: Global Status of Commercialized Biotech/GM Crops. pp315.
4. Mackenzie et al., 2003. An explanatory guide to the Cartagena Protocol on Biosafety. IUCN Environmental Law Centre, IUCN Environmental Policy and Law Paper No. 46. pp295.
5. McLean et al., 2012. The status and impact of biosafety regulation in developing economies since ratification of the Cartagena Protocol. Joint Departmental Discussion Paper 3, Agriculture and Rural Development & Environment Departments, The World Bank, pp25.
6. Nickson, T.E. 2008. Planning environmental risk assessment for genetically modified crops: Problem formulation for stress-tolerant crops. *Plant Physiology*, 147:494–502.
7. OECD, Organization for Economic Cooperation and Development, 1986. Recombinant DNA Safety Considerations. Safety Considerations for Industrial, Agricultural and Environmental Applications of Organisms derived by Recombinant DNA Techniques. pp74.
8. Secretariat of the Convention on Biological Diversity, 2003. Cartagena Protocol on Biosafety: From negotiation to implementation, CBD New Special Edition. pp40.
9. Secretariat of the Convention on Biological Diversity, 2000. Cartagena Protocol on Biosafety to the Convention on Biological Diversity: text and annexes. Montreal, Canada. pp30.
10. Segger, M.C.C., Perron-Welch, F., and Frison, C. 2012. Legal Aspects of Implementing the Cartagena Protocol on Biosafety. Cambridge University Press, ISBN: 9781107004382.

[Related URL]
11. BCH: Guidance on Risk Assessment of Living Modified Organisms (Revised on 19 July 2012). Available at: http://bch.cbd.int/onlineconferences/forum_ra.shtml.
12. BCH: Training Manual on Risk Assessment of Living Modified Organisms in the context of the Cartagena Protocol on Biosafety. Available at: http://bch.cbd.int/cpb_art15/training.shtml

Environmental Impacts and Management Issues of GM Crops: Science/Regulatory Interaction in Comparative Perspective

Masashi Tachikawa
Ibaraki University, Ami-town, Ibaraki, Japan

Abstract

Since the advent of commercialization of GM crops in 1990s, food safety and environmental safety are the two major areas of concerns. While there are various international mechanisms for scientific communication and international harmonization in the area of food safety, such as OECD and Codex, we can observe relatively limited effort in the area of international collaboration regarding environmental safety. Environmental conditions are different from country to country, and, therefore, we can observe much variation among jurisdictions regarding how environmental safety issues are addressed in each country's own policy framework. The cases of EU and US give us a very impressive contrast. This paper shows how each jurisdiction is facing with new challenges not only because of new scientific findings, but also because of their institutional settings which were set long before. This leads to us to take into account more carefully about science/regulatory interactions and path dependency of regulatory development in the area of environmental safety issues of GM crops.

Keywords: US and EU regulation, environmental safety, science/regulatory interactions

Introduction

There is a great divergence in regulatory approaches to secure food safety and environmental safety of genetically modified (GM) crops between the United States and the European Union. This paper discusses how each jurisdiction is facing with new challenges not only because of new scientific findings, but also because of their institutional settings which were set long before. This leads to take into account more carefully about science/regulatory interactions and path dependency of regulatory development in the area of environmental safety issues of GM crops. The paper analyzes two directions of influence, that is, the influence of scientific advancement on policy/regulation, and the influence of policy development on science/research in two jurisdictions, US and EU; and draw lessons from the contrasting situation of these two jurisdictions.

Impacts of Scientific Advancement on Policy

1) US

The US has been an epicenter of research and development of transgenic organisms. Not only transgenic crops, but also animal, microorganism are actively under development. Various traits have been developed in the US, and these developments demand new challenges for regulatory agencies.

As the US has never introduced a new bill regarding genetically modified organisms (GMOs0, and regulatory agencies have been applied existing regulations to GMOs. For example, the US Department of Agriculture (USDA) has regulatory authority over plant pest (Plant Protection Act, PPA), and USDA is applying this authority to GMOs since they are using genetic materials derived from plant pest, such as cauliflower mosaic virus.

Today we are witnessing various technical advances called as new breeding techniques (NBT) which consist of various techniques, such as genome editing, grafting, reverse breeding and so on. Some of the NBT would not be covered by the PPA because they are not involved with plant pest. As the novel plant breeding techniques are being developed and applied to crop improvement, the above mentioned regulatory authority are now being questioned by many experts (Kuzma and Kokotovich, 2011; Montgomery, 2012). One of the cases which vocally demonstrated this issue is the Kentucky bluegrass (KB) cases raised by Scotts Miracle-Gro Company in July 2011. The GM herbicide tolerant KB was created using vectors and sequences which are not regarded as plant pest. The Animal and Plant Health Inspection Service (APHIS) of USDA recognized that the modified KB is out of scope of the PPA, and therefore, not regulated from the viewpoint of the APHIS.

This kind of situation has urged USDA to revise their regulatory framework in order to properly catch up with technical advances. Actually the initiative was started and the draft Environmental Impact Statement was issued in

July 2007 by the USDA-APHIS (2007). However, facing with much criticism against the revised rule-making, the USDA has not succeeded to change their regulation to broaden their regulatory scope.

Originally, US regulatory policy was set when the Office of Science and Technology Policy (OSTP) under the US Administration has introduced the "Coordinated Framework for Regulation of Biotechnology" in 1986. As the new biotech products appears, OSTP has been playing an active role to indicate how various agencies should expand and reinterpret their authority to cover those new biotech products. For example, while regulatory policy for transgenic salmon was originally examined by the USDA, the OSTP finally assigned its regulatory authority to the Food and Drug Administration (FDA) by referring to the Federal Food, Drug, and Cosmetic Act (FFDCA) when the OSTP issued case studies for new type of transgenic organisms in 2000. However, this decision has posed various questions regarding the FDA-CVM's (Center for Veterinary Medicine) regulatory scope and possible impact of GM animal. For example, critics have raised the question whether the FDA-CVM can deal with the issues of environmental safety of GM animal in a proper way based on the existing FDA-CVM's expertise. Eventually, the FDA has introduced various measures to accommodate these questions, such as collaboration with other environment-related agencies, public hearing, issuing guidance document, and so on.

As there are so many field trials are being conducted at various stages, some unintended contamination of GM crops were found, which leads to disruption of domestic and international food and feed trade. Some of the examples of contamination by unauthorized GM crops include; Starlink, Bt10, LLRice601, Triffid flax, RR wheat, and so on.

In response to these unauthorized commingling, USDA and biotech industry have decided to strengthen oversight of total chain of crop research and development by introducing schemes such as USDA-led BQMS (Biotechnology Quality Management System) and industry-led ETS (Excellence Through Stewardship). These schemes are intended to improve quality control and avoid misbehavior throughout the entire stages of R&D. At the same time, three federal agencies, USDA, FDA, and the Environmental Protection Agency (EPA), have issued their guidance documents to inform agencies' policy on the low level presence (LLP) issue. For example, USDA has issued "APHIS Policy on Responding to the Low-Level Presence of Regulated Genetically Engineered Plant Materials" (2007). The bottom line of US LLP policy is that all federal agencies would take counter-measures for LLP based on scientific risk rather than just legal standpoint. In short, if the LLP would not pose substantial risk to environment or human health, regulatory agency does not demand recall of the product-in-question from the market while the company would be given criminal punishment. This sort of response shows a sharp contrast with other jurisdictions, such as EU and Japan, where unauthorized events need to be removed and zero-tolerance policy is applied here.

2) EU

In the EU, the scientific findings promptly resulted in regulatory responses which tend to induce more stringent rule-making. And now, the EU has one of the most stringent regulatory frameworks in the world regarding GMOs.

The original environmental release directive, 90/220/EEC, was revised as 2001/18/EC in 2001 and this revision was originally called for by some member states when politicians learned the impact of GM crops on non-target organisms, such as Monarch butterfly. The news have prompted the EU politicians to stop further authorization of the GMOs (lead to, so-called, de facto moratorium) and demanded a new regulatory framework. The new directive, 2001/18/EC, was created during the deep political/social distrust with food safety regime in the aftermath of the icident of bovine spongiform encephalopathy (BSE). Therefore, precautionary principle is one of the important bases to be referred to protect environment from the release of GMOs. Regulatory trigger to be covered in the directive is the rDNA techniques employed to create organisms, and therefore, regarded as process-based regulatory framework. As environmental concerns are one of the major issues in the GMO regulation, various stringent measures had been introduced, such as post-market environmental monitoring (PMEM) and reauthorization procedures at every 10 years. The European Food Safety Authority (EFSA) has published various documents to respond to these political requests based on environmental concerns. So far, the EFSA GMO Panel has published more than 15 guidance documents in order to take into consideration scientific issues related to environmental and food safety.

As in the US, NBT is also an issue of contention in the EU regarding how current regulatory framework can fit in and properly regulate their potential risk. The discussion was initiated by the Netherlands, and after the discussion by the expert group, political negotiation seems to going on inside and outside of the European Commission. As mentioned above, EU regulatory approach is based on the GM technique specified in the directive which is a trigger to make certain crops under the regulation. However, this process-based approach is facing with difficulty because certain types of NBT product do not contain transgene at the final stage even if they had at their initial stage. In short, EU's process-based approach might accommodate current situation better by taking into account a product-based judgment.

Impacts of Policy Development on Science

1) EU

In the EU, stringent regulatory framework along with destructive activities on field trials and public concerns has discouraged biotech companies to continue the development of transgenic crops in Europe. Also organic farming industry pushed the EU to take appropriate measures to avoid commingling with GM crops. In this situation the European Commission had issued recommendation on coexistence in 2003 just after de facto moratorium was lifted, and urged member states to introduce coexistence measures under their discretion. Coexistence issues have given great impetus on EU research community to develop various coexistence measures, ranging from gene flow modeling to biological containment strategy, such as cleistogamy. Under the 6th Framework Programme (FP), three major coexistence projects, SIGMEA, Co-Extra and Transcontainer, were conducted. Some of the components are continued in the succeeding project called PRICE under the FP7 which seeks to establish practical and feasible coexistence measures at farmers' field level. In order to discuss the result of these coexistence researches and share best practice information, GM coexistence conference (GMCC) has been organized and held biannually in the EU[1]. Coexistence researches have made various achievements in terms of the ways in which to avoid unintended commingling of GM products in non-GM supply chain. While some of the findings are very useful to policy-makers in member states when they try to introduce coexistence measures, actual coexistence measures tend to be based on political judgment rather than scientific data. For example, isolation distances for maize coexistence show a great difference, ranging from 600 m (Luxemburg) to 25 m (the Netherlands). This means that isolation distances are the outcome of political compromise rather than scientific judgment.

While coexistence research has been actively pursued in European research community, the EU policy bodies are still slow to process application dossier. And this situation finally made biotech companies to leave their business in EU. Recently Monsanto has withdrew all commercial planting applications to the European Commission except MON810, and also German-based biotech company BASF has moved their research base to the US.

2) US

Compared with the EU, research and development activities in the US have been enjoying greater degree of freedom since the advent of biotechnology. However, there are some policy-related factors US developers need to take into account when commercialize their GM products.

First, as GM alfalfa and GM sugar beet cases suggest, activist groups are proactively file lawsuits since USDA-APHIS's environmental assessment does not take into account broad issues, such as non-target impact, impact on conservation areas, socio-economic impact, organic market, and so on. In the above mentioned cases, the USDA was forced to draft full Environmental Impact Statements, and these lawsuits seems to have given USDA a more cautious position on deregulation of GMOs as far as the deregulation could have far-reaching impact on various business entities. It is observed that the average time for approval of GMOs tends to take longer time than before in the US[2].

Second, as transgenic crops become more popular in the US agriculture, more complex scientific issues appear and demand closer attention. For example, as stacked varieties are more popular and the number of events increase, it becomes more complex how to evaluate interaction among these events and environment. As of the case of plant incorporated protectants (PIPs), if the stacked variety has two or more PIPs, this combination is regarded as a new pesticide, and therefore, company is requested to submit data to the EPA, and register it under the FIFRA regulation. While this requirement is not applicable to non-PIPs events, scientists would need to consider complex interaction among various events and/or traits. Another example is weed problem in the US and other countries where glyphosate tolerant crops are widely adopted. In contrast to Bt crops, federal agencies have no authority over industry/farmers in terms of weed management. Therefore, USDA has no mandatory measures to avoid over-dependence on glyphosate among farmers. In other words, growing incidents of herbicide tolerant weeds can be regarded as outcome of regulatory limitation of US agencies, and need to find a creative solution outside of regulatory framework.

Conclusion

Regulatory frameworks have their profound impact on how things develop afterwards. It is called as path dependency, and we can find these impacts on both sides of the Atlantic. the US seemed to have enjoyed a larger degree of freedom, but this freedom are now posing complex issues on new situation surrounding stacked varieties and regulatory limitations (loopholes) in case there is no connection with plant pest. In the EU, the stringent regulatory framework has given impetus on research community to thoroughly review environmental safety issues and incorporate various measures to secure safety, such as post-market monitoring. Coexistence research has also

[1] Recently the conference is being held outside of the EU, and this means that coexistence issue becomes a global issue today.

[2] According to the Europabio report, the US needs 25 months for deregulation, in contrast to EU where 45 months are required for authorization.

become a research focus in the EU. However, this stringency urged industry to avoid GM crop development, and find a new path to employ existing biotechnology, which finally develop various new breeding techniques. Process-based regulation is now again under examination how this framework could be fitted in the NBT issues.

What could we learn from these experiences in the US and the EU? Both cases seem to give us various lessons from both positive and negative aspects. As regards of the EU, European scientific exercise is very comprehensive and cautious, typically found in the area of environmental safety research. But this situation hinders real experiences of cultivation of GM crops in a commercial scale. Put in short, too much scientific exercise leaves little room for real experiences. In contrast, the US case suggests that much experience (commercial plantings and scientific research) have been accumulated which would eventually beyond the regulatory framework. Because of the patchwork nature of US regulatory framework, scientific and commercial advancement are now going beyond their regulatory reach. Put in short, much experience might go beyond the reach of regulatory framework. This kind of regulatory gap needs to be fixed up in the future.

An ideal point to hit the balance would be located somewhere in-between. We need to find such a point by taking into account sound scientific development on the one hand, and environmental stewardship under appropriate regulatory framework on the other.

Table Science/Policy Interaction in the US and the EU

	Science → Policy		Policy → Science	
US	* new types of GMOs, such as fish * new breeding techniques (NBT) * large number of field trials and LLP issues	* questioning the USDA's authority over plant pest * quality control measures, such as BQMS and ETS	* lawsuits of GM alfalfa and sugar beet forced USDA to prepare EIS * greater degree of freedom regarding commercialization and research	* complex trait and stacked genes, weed management issue need more attention * more cautious scientific review takes more time for deregulation
	much commercial and scientific experience, which go beyond regulatory framework			
EU	* impact on non-target organisms, such as Monarch butterfly * new breeding techniques	* new regulation was introduced (2001/18/EC) with precautionary principle * post market environmental monitoring * process-based regulation can accommodate with NBT?	* coexistence guidance is proposed in 2003 * slow process of authorization	* coexistence research, such as gene flow modeling and biological containment, has been widely done * biotech company gave up commercialization of GM crops, and move to the US
	much scientific exercise, little real experience			

References

EuropaBio, 2011. Approvals of GMOs in the European Union. http://www.europabio.org/approvals-gmos-european-union.

Kuzma, J. and Kokotovich, A. 2011. Renegotiating GM crop regulation. Targeted gene-modification technology raises new issues for the oversight of genetically modified crops. *EMBO Report*, 12:883-888.

Montgomery, E. 2012. Genetically modified plants and regulatory loopholes and weakness under the Plant Protection Act. *Vermont Law Review*, 37:351-379.
USDA-APHIS. 2007. Introduction of genetically engineered organisms: Draft programmatic environmental impact statement. July. http://www.aphis.usda.gov/brs/pdf/complete_eis.pdf.

Acknowledgement

I would like to express my sincere appreciation to Dr. Yasuhiro Yogo for his insightful advices to prepare this paper.

ISBN 978-4-908914-00-3