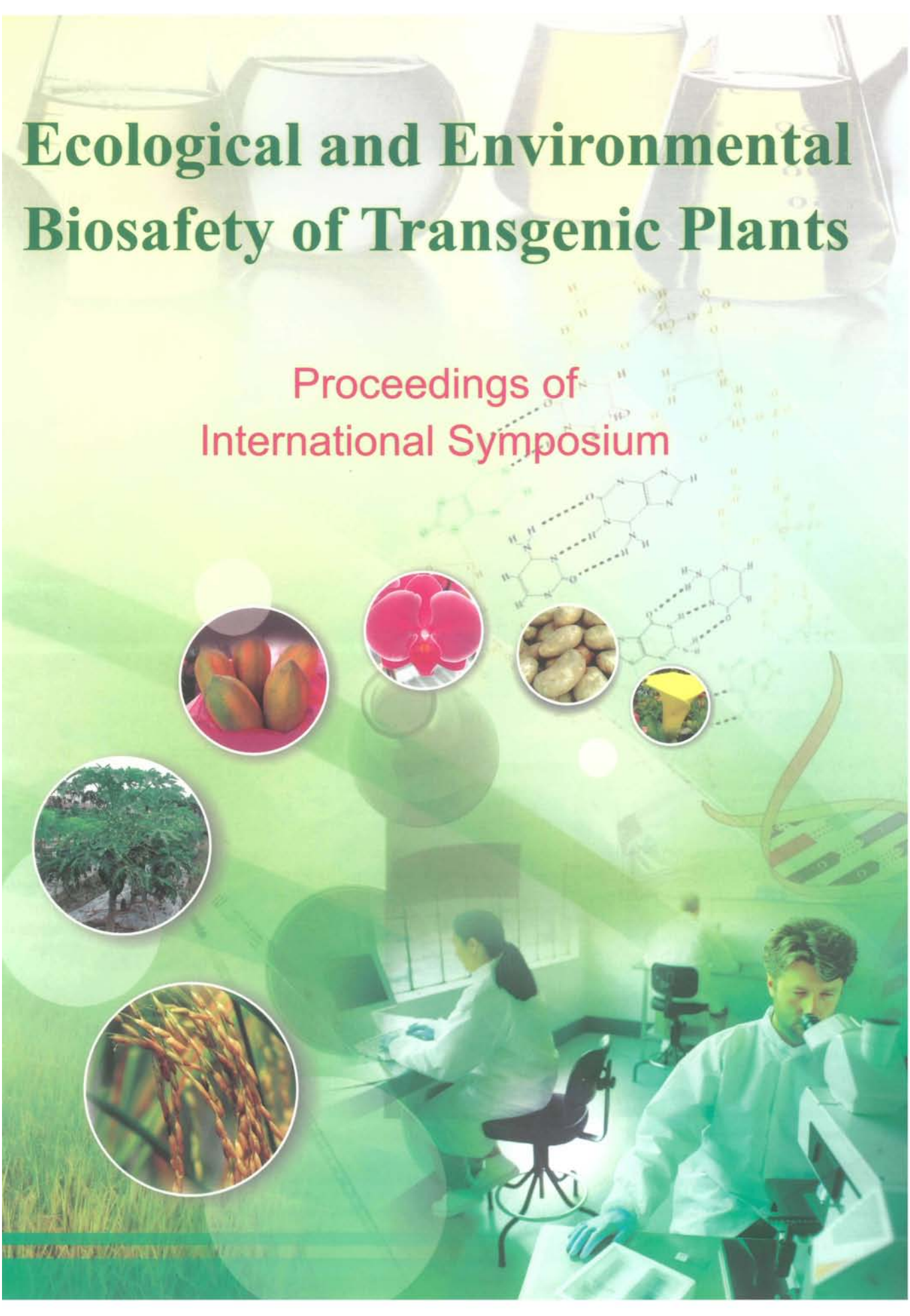
Ecological and Environmental Biosafety of Transgenic Plants

Proceedings of
International Symposium



International Symposium on

Ecological and Environmental Biosafety of Transgenic Plants



December 7- 8, 2006
TARI, Taichung, Taiwan

Special Publication of TARI No.126
ISBN-13:978-986-00-7734-6
ISBN-10:986-00-7734-7

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ISBN-13 : 978-986-00-7734-6

ISBN-10 : 986-00-7734-7

GPN : 1009503692

Taiwan Agricultural Research Institute, Spec. Publ. No.126

Printed in Taiwan, ROC, by Asia United Advertising Ltd.
December 2006

Ecological and Environmental Biosafety of Transgenic Plants

Proceedings of International Symposium

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Published by
Taiwan Agricultural Research Institute
Council of Agriculture
December, 2006

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Preface

Welcome to the "International Symposium on the Ecological and Environmental Biosafety of Transgenic Plants" hosted by the Taiwan Agricultural Research Institute (TARI) in Wufeng, Taichung County, Taiwan.

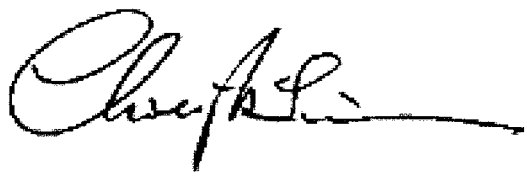
The rapid development of biotechnology has brought new concepts and techniques to agricultural research and production that human beings were unable to imagine just a few decades ago. Many new and novel plant varieties have been successfully developed through genetic manipulation; and many of them have already entered commercial production. However, more people are thinking about the impacts that genetically modified organisms (GMOs) might exert on the ecology and environment as more transgenic plants are being commercialized in many countries. The importance of strict biosafety assessments under a well-regulated system is therefore becoming a global issue.

As the people and government of Taiwan recognize the necessity and consequences of this issue, attention has been paid and efforts have been made to ensure the biosafety of GMOs for a long period of time. The Council of Agriculture (COA) and the National Science Council (NSC) of Taiwan took the initiative several years ago of establishing a core facility; and subsequently the new Agricultural Biotechnology Research Center was established within the well-known Taiwan Agricultural Research Institute (TARI) in 2006. The Center is equipped with a main research building, net houses, delicate greenhouses, and isolated trial fields suitable for research on biosafety assessments of transgenic plants. To mark the opening of the Center, TARI has organized the "International Symposium on the Ecological and Environmental Biosafety of Transgenic Plants", which is being held on December 7 and 8, 2006. More than 120 scientists and experts have been invited to attend, including 14 distinguished speakers from abroad as well as from local organizations and institutions.

The Symposium predominantly focuses on issues related to risk assessment and the ecological and environmental biosafety of transgenic plants. The major topics of the Symposium are: (1) biosafety assessments in principle and practice; (2) the building of facilities and capabilities for biosafety assessment of transgenic plants in field trials; (3) the scientific evaluation of GMOs' potential effects on the ecology and environment; (4) the ecological and environmental risk assessment/management leading to transparent and science-based approaches for regulating transgenic plants; and (5) technical cooperation and information exchanges, in particular on the experiences of how to create a more-transparent environment for regulating GMOs. The proceedings cover all of the scientific papers presented by the invited speakers, which should be of significant value to agricultural scientists dealing with implementation plans for regulating GMOs.

I would like to take this opportunity to express my gratitude to all participants whose attendance contributes to the very success of the Symposium. Special thanks are given to all authors for their prompt preparation of manuscripts and presentation materials. The Symposium and proceedings would not have been possible without the financial support from the Agriculture and Food Agency, COA, NSC, and the excellent assistance of the staff members of TARI.

Chien-Yih Lin,Ph.D.



Director General
Taiwan Agricultural Research Institute
Council of Agriculture

Opening Ceremony

Welcome Address

Dr. Chien-Yih Lin
Director General
Taiwan Agriculture Research Institute
Council of Agriculture

Dr. Lee, Deputy Minister, Council of Agriculture, Dr. Shaw, President, National Chung Hsing University, Dr. Li, the Executive Secretariat, National Science and Technology Program for Agricultural Biotechnology, Dr. Huang, Director General, Agriculture and Food Agency, Dear Honorable Guest, Invited Speakers, Participants, Ladies and Gentlemen:

As the Director General of the Agriculture Research Institute, it is my greatest honor to be the host of this very meaningful symposium. I would like to express my heartiest welcome to all of you attending this International Symposium on Ecological and Environmental Biosafety of Transgenic Plants. I am also grateful to the sponsors including the Council of Agriculture, the National Science Council, and the Agriculture and Food Agency.

The biosafety of the genetically modified organisms is an important issue being concerned with worldwide. In Taiwan, under the guidance and inspection of the Council of Agriculture, the Agriculture Research Institute is the major organization responsible for conducting the biosafety assessment of plant materials. All of the novel crop developed with transgenic technique must pass through the biosafety assessment before being released for commercial purposes. This is why the

Agricultural Biotechnology Research Center with all the necessary facilities, including isolated field and greenhouses, is set up in this Institute. To inaugurate the establishment of the new center and to accentuate our determination in guarding the biosafety of GMOs for the people, as well as to exchange information and practical experiences among scientists from different countries, this international symposium is organized and being held at this moment.

We are honored and pleased to invite 14 distinguished speakers from Canada, Japan, New Zealand, USA, and Taiwan to present their specialties in biosafety assessment. In addition, many experts in biotechnology and agricultural fields participate in this symposium to offer their experiences and expectations to a new and more advanced agricultural production system. On this special occasion, I foresee an opportunity of international cooperation in the continuous development of making GMOs more safe, applicable, and acceptable by not only the people in different countries but also the Mother Nature as well.

Finally, I wish the very success of this international symposium. I also wish to extend my sincerest welcome and blessings to all of you. To our foreign guests, I hope you have a pleasant stay in this lovely and friendly country.

Thank you very much.

Opening Remarks

Dr. Jen-Chyuan Lee
Deputy Minister
Council of Agriculture

Dr. Jei-Fu Shaw, President, National Chung Hsing University, Dr. Gwo-Chen Li, the Executive Secretariat, National Science and Technology Program for Agricultural Biotechnology, Dr. Huang, Director General of the Agriculture and Food Agency, Dr. Lin, Director General of Agricultural Research Institute, Distinguished speakers, Dear participants, Ladies and Gentlemen:

On behalf of the Council of Agriculture, I sincerely welcome all of you attending the "International Symposium on the Ecological and Environmental Biosafety of Transgenic Plants", especially those foreign friends coming from a long way to Taiwan.

The importance of biotechnology has been well recognized worldwide during the past decades. The impacts of its development are revealed in almost every aspect of our life, including the improvement of yield and quality of agricultural products. With a vast investment of manpower and budget into biotechnology, Taiwan has also made significant progresses in promoting the continuous development of agricultural production. For examples, transgenic papaya, rice, potato, broccoli, chrysanthemum, callalily, along with other important crops, have been developed and some are being evaluated in the field for the possibility of commercialization.

Of course, we do concern the possible long-term effects of GMOs on the environment, ecology, and human health as a whole. Therefore, the biosafety of

GMOs has always been an important issue when agricultural scientists are seeking breakthroughs in the technological parts. In a national framework to ensure the biosafety of GMOs, the Council of Agriculture is responsible for the assessment and management of GMOs to avoid any possible adverse effect. The Agricultural Biotechnology Research Center in the Taiwan Agricultural Research Institute will play a very important role in the risk assessment of GMOs before the transgenic plants are allowed to grow in the open fields for research or commercial purpose.

How to initiate and carry on a good program of the biosafety of GMOs is not simple and easy. Although we have already accumulated considerable information and experiences in this field, there are still much more for our agricultural scientists to learn and follow. The establishment of this new Agricultural Biotechnology Research Center is just one step forward; we have to do more to ensure the public the safety of our GMO products. I wish that our scientists will do their bests to build up their capacity in risk assessment of transgenic plants for individual crop within the shortest period of time, and field trials will then be followed. With this aim on mind, the holding of this Symposium becomes more meaningful and important. I deeply believe the outcome of this Symposium will render much help to our efforts in establishing a sound system of biosafety assessment.

Finally, I would like to express my sincere appreciation to Dr. Lin and his colleagues for holding this timely and important International Symposium. I would also like to thank the Organizing Committee for their hard working and excellent arrangements. I believe that this symposium will be a very successful one, and I hope all the participants will have a great time here. To our foreign friends, may you have a very pleasant stay here in Taiwan.

Thank you.

Special Remarks

Dr. Gwo-Chen Li
Chief Operation Officer
National Science and Technology Program / Agricultural Biotechnology Office

Dr. Lin, Director General of TARI, Dr. Lee, Deputy Chairmen of COA, Dr. Dai, Deputy Chairmen of NSC, distinguished guests and participants.

It is a great honor for me to have this opportunity to address you as part of the Opening ceremony for the International Symposium on Ecological an Environmental Bio-safety of Transgenic Plant.

On behalf of the Director General, Dr. Tuan-hua David Ho, and the office members of the NSTP/AB, Permit me to congratulate TARI for the completion of the construction and facilities for the environmental bio-safety assessment of transgenic plant. These new facilities not only open a new era of the development of GM crops in this country but also will bridge the gap in the path from R&D to commercialization.

As scientists, we sincerely believe that biotechnology will offer us more food, better food and better for environment. In the foresee future, GM crops with different agronomic traits will be grown and will dominated the food markets. For the passed eight years, the NSTP/AB has successfully built up the research capacity of the development of GM crops. Many primarily products, such as rice, tomato, potato, papaya, cauliflower and ornamental crops with various functional traits, have been developed. Unfortunately not single of them is on the market yet.

Because of its man made nature, the public concerns very much about hazards of GM crops to human health and the impacts to environment. Many countries have

set up the rregulatory system to evaluate the bio-safety of GM crops. Those crops can not release to the environment and consumed as food unless necessary scientific data have been worked out to prove that they are safe. The regulatory requirement is a barrier that we have to overcome in order to get our products to the market. The biotechnology products grown in Taiwan are definitely not just for domestic consumption. To get into the global trade market is our goal. For this goal Taiwan urgently needs to build its own capacity in the science and technology of bio-safety research. The capacity for bio-safety research in turn could provide policy and regulation based on sound science that would allow Taiwan to cope with the ever-evolving complex international regulatory environment.

The "International Symposium on Ecological an Environmental Bio-safety of Transgenic Plant" to be held today is a milestone of the development of GM crops in Taiwan, The symposium covers many important aspects of the regulatory systems I am sure it will give us more insight about the contents of bio-safety assessment of GM crops . I am looking forward to see the bright future of our bio-industry from now on. It is our sincere hope that the symposium will contribute significantly towards enhanced systems for the assessment and management of risks from GM crops.

Finally, I will like to express my respect to TARI for their many years' hard works to build up the facilities of bio-safety evaluation. I also hope that the symposium will not only provide interesting lectures and fruitful discussions, but will also bring about the broader results we all expect. I would like once again to wish all of you a very nice stay in Taiwan and very pleasant and successful meeting.

Presentation Papers

International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
1~19

Assessment of Emerging Virus Threats for Application of Transgenic Papaya Resistant to Papaya Ringspot Virus

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ABSTRACT

The transgenic resistance conferred by the coat protein (CP) gene of a virus has become the most effective method to prevent papaya from infection by the noxious *Papaya ringspot virus* (PRSV). In May 1998, PRSV-CP gene transgenic papaya Rainbow and SunUp were deregulated and granted approval for commercialization, representing the first successful application of a transgenic fruit tree in the world. Although the transgenic varieties are not resistant to most other PRSV strains from different geographic areas, breakdown of the resistance in Hawaii has not been recorded. Apart from Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation. The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity. However, during the 4-year-long field trials, the highly resistant PRSV CP-transgenic papaya lines were found to be susceptible to an unrelated potyvirus *Papaya leaf-distortion mosaic virus* (PLDMV) which has been identified in many places in Taiwan. To

overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene, including parts of the CP genes of PRSV and PLDMV, and conferring resistance against both PRSV and PLDMV, were developed. These transgenic papaya lines with double resistance are considered to have great potential for the control of PRSV and PLDMV in Taiwan. During the field test period, a super strain, PRSV 519, able to break down the resistance of YK-CP transgenic lines, was also noticed. The breakdown of the transgenic resistance by 519 is controlled by a strong gene-silencing suppressor, HC-Pro gene, of the virus in a transgene-homology-independent manner. We suggest that a chimeric construct targeting multiple viral genes, such as the genes of the CP, the replicase and the gene silencing suppressor HC-Pro of a potyvirus, may minimize the chances of the emergence of a super virus strain that can overcome the transgenic resistance, and provides more durable resistance against different viruses.

INTRODUCTION

There are many common names for papaya (*Carica papaya* L.), including papaw or paw paw (Australia), mamao (Brazil) and tree melon (China). The species is believed to be native to southern Mexico and neighboring Central America and to have been taken to Caribbean countries and South-east Asia during the Spanish exploration in the sixteenth century (Storey 1969). It then spread rapidly to India and Africa. Today, it is distributed widely throughout tropical and subtropical regions of the world. A papaya plant has a single, erect, tree-like herbaceous stem, with a crown of large, palmately and deeply lobed leaves. The main stem is cylindrical and hollow, with prominent leaf scars and spongy-fibrous tissue. Leaves are arranged spirally, with petioles extending horizontally up to 1 m long. Trees contain white latex in all their parts. Flowers are male, female, or hermaphrodite, are found on separate trees and

are borne in the axils of the leaves. The modified cymose inflorescence allows the flowers to be pollinated easily by wind and insects. The type of flowers produced may change on the same tree, depending on age and environmental factors, such as drought and temperature fluctuations. Hermaphroditic trees consistently produce male flowers, but only with few female flowers that produce fruits during warmer or cooler seasons, whereas female trees are more stable and always produce pistillate flowers under these conditions.

Papaya fruits are fleshy berries and superficially resemble melons. Fruits from female trees are spherical, whereas those from hermaphroditic trees are pyriform, oval or cylindrical with a grooved surface. Since the female fruits contain thinner flesh and more seeds in the central cavity, the hermaphrodite fruits are in more demand by consumers. The fruit is a good source of vitamin A and C (Manshardt 1992). Ripe fruits are largely used as a fresh dessert, while green fruits are often used in salads and pickled or cooked as a vegetable. Papain, a proteolytic enzyme present in the latex, collected mainly from green fruits, has various uses in the beverage, food and pharmaceutical industries, e.g. chill-proofing beer, tenderizing meat and in drug preparations for digestive ailments (Chan and Tang 1978). It is also used in bathing hides, softening wool and as soap for washing cloth.

Papaya grows relatively easily and quickly from seeds and can reach up to 10 or 12 ft in height. Fruits are ready to be harvested 9--12 months after planting and a tree can continue producing fruits for about 2--3 years, when the height of the plant is too tall for efficient harvesting. Since plant sex cannot be distinguished before flowering, three to five seedlings are normally planted together and only the most vigorous hermaphrodite ones at flowering are selected and cultivated. In 2005, the FAO

estimated that about 3.9 hundred thousand hectares and about 6.8 million metric tons of fruit were harvested (Table 1). Brazil, Mexico, Nigeria, India and Indonesia yield more than 70% of the total world production. The extensive adaptation of this plant and wide acceptance of the fruit offer considerable promise for papaya as a commercial crop for local and export purposes. Like banana, pineapple and mango, papaya is one of the important cash crops in the tropics and subtropics. However, the production of this economically important fruit crop is limited by the destructive disease caused by *Papaya ringspot virus* (PRSV), and the fragile and perishable nature of the fruit limit large-scale exportation, with the result that papaya lags behind banana and pineapple in world markets.

Table 1. World papaya production in 2005. Data from the Food and Agriculture Organization (FAO), Statistical Division, 2005. (http://faostat.fao.org/faostat/)

Country	Hectares (×1,000)	Metric tons (×1,000)
Brazil	36.5	1650
Mexico	26.3	956
Nigeria	91.0	755
India	80.0	700
Indonesia	24.0	647
Ethiopia	11.0	230
Congo	12.5	210
Peru	12.5	180
China	5.8	160
Venezuela	7.5	140
Colombia	7.0	137
Philippines	5.5	132
Thailand	9.0	131
Cuba	11.0	120
Kenya	8.0	86
Others	41.6	514
Total	389.2	6753

Worldwide Threat by PRSV Infection

Production of papaya has been limited in many areas of the world due to the disease caused by *Papaya ringspot virus* (PRSV) (Purcifull et al. 1984). Papaya ringspot disease is the major obstacle to large-scale commercial production of papaya (Yeh and Gonsalves 1984). PRSV was first reported in Hawaii in the 1940s (Jensen 1949a), and then became prevalent in Florida (Conover 1964), Caribbean countries (Adsuar 1946; Jensen 1949b), South America (Herold and Weibel 1962), Africa (Lana 1980), India (Capoor and Varma 1948; Singh 1969), the Far East (Wang et al. 1978) and Australia (Thomas and Dodman 1993). To date, most of the major papaya plantation areas of the world suffer from devastation by this virus.

PRSV is a member of the genus *Potyvirus* (Purcifull et al. 1984; Murphy et al. 1995), is transmitted non-persistently by aphids and is sap-transmissible in nature. The PRSV genome contains a single-stranded positive sense RNA of about 40 S (De La Rosa and Lastra 1983; Yeh and Gonsalves 1985). Strains of PRSV from Hawaii (Yeh et al. 1992) and Taiwan (Wang and Yeh 1997) have been sequenced; both contain 10,326 nucleotides. The viral RNA encodes a polyprotein that is proteolytically cleaved to generate eight to nine final proteins, including the coat protein for encasidation of the viral genome (Yeh et al. 1992). The virus has a single type of coat protein (CP) of 36 kDa (Purcifull and Hiebert 1979; Gonsalves and Ishii 1980). It induces cylindrical inclusion (CI) (Purcifull and Edwardson 1967) and amorphous inclusion (AI) (Martelli and Russo 1976) in the cytoplasm of host cells. The former consists of a protein of 70 kDa (cylindrical inclusion protein, CIP; Yeh and Gonsalves 1984) and the latter of a protein of 51 kDa (amorphous inclusion protein, AIP; De Mejia et al. 1985a, b). In papaya, PRSV causes severe mosaic and distortion of leaves, ringspots on fruits and water-soaking oily streaks on the upper stems and

petioles. It stunts the plant and drastically reduces the fruit size and quality.

No Effective Control Measures

Although tolerant varieties of papaya have been described (Cook and Zettler 1970; Conover 1976; Conover et al. 1986), resistance to PRSV does not exist in *C. papaya*, making conventional breeding difficult (Cook and Zettler 1970; Wang et al. 1978). Tolerance to PRSV has been found in some papaya lines and has been introduced into commercial varieties, but their horticultural properties, such as sweetness, hardness, shape, and shelf-life, are still not commercially desirable (Mekako and Nakasone 1975; Conover and Litz 1978). Other control methods for PRSV, including agricultural practices such as rouging, quarantine, intercropping with corn as a barrier crop and protecting transplanted seedlings with plastic bags, provide only temporary or partial solutions to the problem (Wang et al. 1987; Yeh and Gonsalves 1994).

PRSV HA 5-1, a cross-protecting mild mutant strain of PRSV that was selected following nitrous-acid treatment of a severe strain (HA) from Hawaii (Yeh and Gonsalves 1984), was tested extensively in the field and has been used commercially in Taiwan and Hawaii since 1985 to permit an economic return from papaya production (Wang et al. 1987; Yeh et al. 1988; Yeh and Gonsalves 1994). However, using the approach involving the deliberate infection of a crop with a mild virus strain to prevent economic damage by more virulent strains has several drawbacks, including the requirement for a large-scale inoculation program, reduction in crop yield and losses of cross-protected plants due to super-infection by virulent strains (Stubbs 1964; Gonsalves and Garnsey 1989; Yeh and Gonsalves 1994).

A Transgenic Approach for Control of PRSV

The concept of 'pathogen-derived resistance' (Sanford and Johnston 1985) proposes that transforming plants with a pathogen's gene would generate resistance to the infection of the corresponding pathogen. By this concept, Powell-Abel et al. (1986) first demonstrated that transgenic tobacco plants expressing the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) conferred resistance to TMV infection. The CP gene-mediated transgenic resistance has been proven effective for protecting tobacco, tomato, potato and other crops from infection by many different viruses (Beachy 1990; Lomonossoff 1995; Goldbach et al. 2003). Thus, the transgenic approach has become the most effective method to protect crops from virus infection.

In order to solve the problems caused by PRSV, Gonsalves' group at Cornell University and Hawaii started a research project in the late 1980s to develop transgenic papaya. Ling et al. (1991) first demonstrated that the expression of the PRSV HA 5-1 CP gene in tobacco afforded a broad-spectrum of protection against different potyviruses. However, effective gene transfer systems require reliable and efficient procedures for plant regeneration from cells. Fitch and Manshardt (1990) reported that somatic embryogenesis from immature zygotic embryos of papaya could be integrated into a useful gene transfer technology. In the same year, Fitch et al. (1990) incorporated the CP gene of HA 5-1 into papaya via microprojectile bombardment and obtained plants resistant to infection by the severe Hawaii HA strain. Among their transgenic papaya lines, line 55-1 was virtually immune to infection by HA.

Successful Application of Transgenic Papaya in Hawaii

The plants of transgenic papaya line 55-1 are highly resistant to Hawaiian PRSV isolates under glasshouse and field conditions (Fitch et al. 1992; Lius et al. 1997). The resistance is triggered by post-transcriptional gene silencing (PTGS), an RNA-mediated specific degradation process of the innate nature of plants against pathogens (Baulcombe 1996, 1999; Hamilton and Baulcombe 1999; Gonsalves 2002). However, the resistance is affected by the sequence identity between the CP transgene and the CP coding region of the challenge virus (Tennant et al. 1994). For example, Rainbow (a CP-hemizygous line derived from SunUp crossed with non-transgenic Kapoho) is susceptible to PRSV isolates outside Hawaii, and SunUp (a CP-homozygous line of 55-1) is resistant to a wider range of isolates from Jamaica and Brazil, but is susceptible to isolates from Thailand and Taiwan (Gonsalves 1998, 2002; Tennant et al. 2001). This characteristic of sequence homology-dependent resistance limits the application of CP-transgenic papaya for controlling PRSV in geographic regions other than Hawaii (Gonsalves 2002).

The field trial of the homozygous line SunUp and hemizygous line Rainbow indicates that both of them offer a good solution to the PRSV problem in Hawaii (Ferreira et al. 2002). By May 1998, Rainbow and SunUp were deregulated by the US Animal and Plant Health Inspection Service and Environmental Protection Agency, and granted approval from the Food and Drug Administration for commercial application (Gonsalves 2002). This is the first successful case of a transgenic fruit tree being commercialized in the world.

New Threat by an Unrelated Virus for Transgenic Papaya in Taiwan

A CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation (Cheng et al. 1996). The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity (Bau et al. 2003). Several lines highly resistant to the homologous strain (PRSV YK) provide wide-spectrum resistance to three different geographic strains from Hawaii, Thailand and Mexico (Bau et al. 2003). During four repeats of field trials from 1996 to 1999, transgenic papaya exhibited high degrees of protection against PRSV in Taiwan (Bau et al. 2004). Unfortunately, 18 months after plantation in the fourth field trial, unexpected symptoms of severe distortion on fully expanded leaves, stunting of the apex, water-soaking on petioles, and stem and yellow ringspots on fruit were noticed on PRSV CP-transgenic papaya plants. The causal agent was distinguished from PRSV by host reactions and serological properties (Bau 2000) and later identified as *Papaya leaf-distortion mosaic virus* (PLDMV), a potyvirus originating from Okinawa, Japan, in 1954 (Maoka et al. 1996). All of the PRSV CP-transgenic papaya lines were susceptible to PLDMV infection when evaluated under glasshouse conditions. Therefore, in Taiwan, once PRSV CP-transgenic papaya is widely applied for the control of PRSV, PLDMV will become a serious threat to papaya production.

A need of Multiple and Durable Resistance to Different Viruses

In order to control two or more viruses, transgenic plants with multiple resistances have been generated by combining the entire CP gene of more than one virus, with each gene driven by a promoter and a terminator (Fuchs and Gonsalves 1995). Transgenic lines expressing these chimeric CP constructs were resistant to the

corresponding viruses and protected from mixed infection, such as *Cucumber mosaic virus*, *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* (Fuchs and Gonsalves 1995; Tricoli et al. 1995; Fuchs et al. 1998). Furthermore, the novel approach proposed by Jan et al. (2000) indicated that transgenic plants with resistance to a potyvirus and a tospovirus could be obtained through the PTGS mechanism by fusing a segment of tospoviral N gene to a segment of potyviral CP gene. The same strategy was used to develop double resistance to both PRSV and PLDMV. An untranslatable chimeric construct that contained the truncated PRSV CP and PLDMV CP genes was then transferred into papaya. Through the PTGS mechanism, transgenic papaya plants carrying this chimeric transgene exhibited resistance to both PRSV and PLDMV under glasshouse conditions (S.D. Yeh, unpublished results). These transgenic papaya plants with a double resistance are believed to have considerable potential for the control of PRSV and PLDMV in Taiwan.

Resistance Breakdown by Super Strains of PRSV with a Strong Gene Silencing Suppressor

In 4-year field trials, super PRSV strain 5-19 infected transgenic papaya plants were found (Tripathi et al. 2004). The nucleotide identity between the transcript of the CP transgene and PRSV 5-19 RNA is less divergent than that between the CP transgene and other PRSV geographic strains that are not able to overcome the transgenic resistance (Tripathi et al. 2004), indicating that the breakdown of the transgenic resistance is not correlated to the sequence divergence between the infecting virus and the transgene. In order to analyze the role of the gene-silencing suppressor HC-Pro of this super strain, the viral recombinant was constructed by replacing an HC-Pro region of PRSV YK with that of 5-19 and the resistance against the

recombinant was evaluated in transgenic papaya. Results showed that the heterologous HC-Pro region of 5-19 alone was sufficient to break down the transgenic resistance in a transgene sequence-homology independent manner, even though the sequences of the transgene transcript shared 100% identity with the genome of the infecting virus (S.D. Yeh, unpublished results). The breakdown of the transgenic resistance by a strong gene-silencing suppressor of a super strain has strong implications for the application of transgenic crops in virus control. We suggest that a chimeric construct targeting multiple viral genes, including the gene determining viral virulence and gene silencing suppression, such as the HC-Pro gene of a potyvirus, may minimize the chance of the emergence of a supervirus that may overcome transgenic resistance.

Transgenic Papaya Generated in Other Geographic Areas

Because of the apparent homology dependence of PRSV CP transgene-associated resistance, the utilization of a CP gene of a local prevalent strain is a prerequisite in order to obtain effective PRSV resistance in transgenic papaya lines for a particular geographic region, as long as genetic variation among virus strains in that region is not a limiting factor (Gonsalves 2002). Using the CP genes of local PRSV isolates to transform local papaya cultivars has been reported in different countries. Lines et al. (2002) used an untranslatable PRSV CP coding region as a transgene to develop two Australian transgenic papaya cultivars which showed immunity to the local PRSV isolate in glasshouse and field tests. Fermin et al. (2004) constructed PRSV-resistant plants by transforming independently with the CP genes of PRSV isolates from two different areas of Venezuela. All the transgenic lines, including R_0 and inter-crossed or self-crossed progenies, revealed different levels of resistance to homologous and heterologous isolates from Hawaii and Thailand. In Florida, transgenic papaya lines

carrying the CP gene of the local strain were generated, and the transgenic resistance was introduced to elite papaya cultivars by conventional breeding (Davis and Ying 2004). In addition to the CP gene, the truncated replicase (RP) gene of PRSV was used as a transgene to generate transgenic papaya through *Agrobacterium*-mediated transformation (Chen et al. 2001). PRSV inoculation tests showed that the RP gene conferred resistance to PRSV in transgenic papaya.

CONCLUSIONS

The transgenic resistance conferred by the CP gene of a virus has become the most effective method to prevent papaya from infection by the noxious PRSV. In the late 1980s, Gonsalves' group at the Cornell University and University of Hawaii started a program to develop transgenic papaya resistant to PRSV, using the PRSV CP gene, through particle bombardment. In May 1998, PRSV-CP gene transgenic papaya Rainbow and SunUp were deregulated and granted approval for commercialization, representing the first successful application of a transgenic fruit tree in the world. Although the transgenic varieties are not resistant to most other PRSV strains from different geographic areas, breakdown of the resistance in Hawaii has not been recorded. Apart from Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation. The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity. However, during the 4-year-long field trials, the highly resistant PRSV CP-transgenic papaya lines were found to be susceptible to an unrelated potyvirus *Papaya leaf-distortion mosaic virus* (PLDMV) which has been identified in many places in Taiwan. To overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene, including parts of the CP genes of PRSV and PLDMV, and conferring resistance

against both PRSV and PLDMV, were developed. These transgenic papaya lines with double resistance are considered to have great potential for the control of PRSV and PLDMV in Taiwan. During the field test period, a super strain, PRSV 519, able to break down the resistance of YK-CP transgenic lines, was also noticed. The breakdown of the transgenic resistance by 519 is controlled by a strong gene-silencing suppressor, HC-Pro gene, of the virus in a transgene-homology-independent manner. We suggest that a chimeric construct targeting multiple viral genes, such as the genes of the CP, the replicase and the gene silencing suppressor HC-Pro of a potyvirus, may minimize the chances of the emergence of a super virus strain that can overcome the transgenic resistance, and provides more durable resistance against different viruses. Transgenic papaya resistant to pathogens other than PRSV have also been developed. It is expected that resistance to insects, tolerance to herbicides, and other important fruit traits will be the next targets of research.

ACKNOWLEDGEMENTS

We thank the financial supports from the Council of Agriculture of Taiwan, R.O.C. for the development and field evaluation of transgenic papaya lines resistant to *Papaya ringspot virus*

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
21~38

Environmental Risk Assessments and the Regulation of Genetically Engineered Plants in the United States

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It is a very great honor and pleasure to be able to share some thoughts with you during this symposium about how we evaluate the environmental safety of transgenic plants.

I would like to thank the organizers for providing this opportunity for all of us to hear a variety of perspectives, including those of research scientists and government regulatory scientists. As we have often seen over the years, the free exchange of ideas makes it possible to have a incorporate the best available scientific knowledge when using risk assessments (RAs) to make decisions into a dynamic regulatory system.

In the interest of clarity, let me to note at the outset that I will tend to use the term "genetically engineered" or GE rather than the term "transgenic." This field seems to have a proliferation of terms that are often used synonymously, such as recombinant, transgenic, GE, GM, GMO, and LMO. My use of the term GE is not intended to minimize any preference that some may have for some of these other terms.

For today's presentation, I would like to discuss each of the following:

1. The US regulatory system and when the regulatory agencies use environmental RA to inform the decision-making process
2. Main elements of the RA methodology
3. Examples of using the RA methodology in specific cases
4. RA and decision-making
5. Conclusions

1. The US regulatory system and when the agencies use environmental RA

Let me set the stage with a brief sketch of the regulatory structure in the United States that applies for transgenic plants and other products of biotechnology. As you may know, in the United States we did not pass a separate biotechnology law to regulate GE organisms, but instead operate under a Federal Coordinated Framework for the Regulation of Biotechnology. We have used the existing laws for comparable traditional organisms and products, and then applied these laws in each specific case of a GE organism.

Generally, three agencies are responsible for the regulation of GE plants and other GE organisms:

- The U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) is a regulatory agency responsible for the protection of agriculture and the environment against pests and diseases. Within APHIS, Biotechnology Regulatory Services (BRS) is responsible for implementing the APHIS biotechnology regulations that govern the importation, interstate movement, and environmental release of GE plants

and other GE organisms that may pose a risk of possible direct or indirect harm to plants.

- The Environmental Protection Agency (EPA) is responsible for the assuring the safe use of pesticidal substances, whether applied externally, like traditional herbicides and insecticides, or incorporated into the plant itself in the case of GE plants engineered to produce a pesticidal substance within the plant's tissues. EPA also sets the allowable levels, or "tolerances", of a pesticide that can be on or in food.

- The Food and Drug Administration (FDA) is responsible for assuring the safety of the U.S. food, drugs, and cosmetics. Under FDA's authority, animal feed falls under the heading of food.

Each of the agencies has highly trained staff members assigned to conduct safety reviews that will be used by decision makers in our agencies.

In light of the symposium's focus on environmental risk assessments, we won't hear more about FDA reviews, but those who are interested may want to look at the considerable information that FDA provides online about their regulatory approach (http://www.cfsan.fda.gov/~lrd/biotechm.html).

APHIS and EPA environmental RAs come into play at certain stages of research and development of GE plants:

- When permits are issued for field tests (controlled, environmental releases)
  - APHIS regulates all GE plants

- EPA regulates GE plants that produce a plant incorporated protectant or PIP (e.g., the Bacillus thurinigiensis protein)

∞ Just prior to commercialization.

- APHIS reviews prior to granting nonregulated status
- EPA reviews prior to registering the PIP
- EPA reviews to set tolerance levels for PIP in food and feed (allowable, safe levels)

∞ In response to compliance infractions, such an unauthorized environmental release, the RA can be used to decide on the need for remedial actions.

2. Main elements of the methodology for RA

RA was developed as a formal discipline during the latter half of the 20th century to address questions related to chemical and radiation safety. Many of these RAs used quantitative approaches, although qualitative RAs have been used as well. The main elements of the RA are relatively simple, and they can be applied flexibly to address a wide range of situations. They are sometimes expressed simply as risk being a function of a hazard and the likelihood of exposure to the hazard.

Hazard x Exposure = Risk

When assessing the risk of a simple system, the RA can be fairly simple, but GE plants are usually not a simple system. Scientists and government regulators around the world have worked together to identify useful information and approaches that can be used in a practical way for conducting RA of GE plants.

Even before the creation of the first GE plant in 1983, scientists and regulators around the world were discussing the best ways to approach RA for these and other GE organisms. A great deal of international collaboration was done by countries working under the umbrella of the Organization for Economic Co-operation and Development (OECD). The first OECD report contained a number of recommendations, including that for the public to have confidence in the products of modern biotechnology, governments must have proper mechanisms in place to regulate their safety ("International Trends and Perspectives", OECD 1982).

In 1986, OECD published the first guidance document entitled "Recombinant DNA: Safety Considerations" (sometimes referred to as the 1986 OECD 'Blue Book'). This was the first OECD publication to respond to the 1982 recommendation for safe regulation. It put forward key safety concepts for development and commercialization of GE organisms, including GE plants. The Blue Book included advice on several topics including risk assessment, agriculture and the environment, and concepts for safely conducting small-scale field testing of GE plants. The advice in the Blue Book was developed with input from hundreds of experts from OECD countries, and has since been used as the basis of regulation of GE plants in many countries.

The Blue Book was followed by subsequent guidance documents echoed many of the same considerations that could be taken into account when evaluating the environmental safety of GE organisms. Other similar and complementary guidance was included in the UNEP International Technical Guidelines for Safety in Biotechnology (1995) and Annex III of the Cartagena Protocol on Biosafety (2000).

Representatives of the U.S. regulatory agencies were active participants in the international discussions that led to the development of these guides.

The United States has also worked closely with other countries to assemble information useful in the evaluation of GE plants, including information related to traditional agricultural practices. Two examples of this include the 1993 OECD work on "Traditional Crop Breeding Practices, A Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology" and the numerous plant species consensus documents on developed cooperatively by member countries working under the auspices of the OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology. For access to Consensus Documents for the Work on Harmonisation of Regulatory Oversight in Biotechnology, see the website links available at http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html.

These OECD documents highlight the widespread acceptance of a comparative approach when evaluating the safety of GE plants. This comparative approach is used not only in the United States, but also in many other countries around the world. This approach takes into account the great deal of prior experience we have accumulated in evaluating the traditional plants.

This is an outgrowth of the concept of familiarity, which was described in the 1993 OECD document on "Scale-up" (Safety Considerations for Biotechnology: Scale-up of Crop Plants, OECD, 1993). The familiarity concept "...is based on the fact that most genetically engineered organisms are developed from organisms such as crop plants whose biology is well understood". "Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of plants and

micro-organisms into the environment..." For plants, familiarity takes account of a wide-range of attributes including, for example, knowledge and experience with "the crop plant, including its flowering/reproductive characteristics, ecological requirements, and past breeding experiences". This provides risk assessors with a useful tool in reviewing information related to the biology of the host organism when conducting a RA.

The concept of familiarity is based on the fact that most GE organisms are developed from organisms such as crop plants whose biology is well understood. Familiarity is not a RA in itself, but the concept facilitates RA. Familiarity often means having enough information to be able to make a judgment of safety or risk (U.S. National Academy of Sciences, 1989). Familiarity can also be used to indicate appropriate management practices including whether standard agricultural practices are adequate or whether other management practices are needed to manage the risk. Familiarity allows the risk assessor to draw on previous knowledge and experience with the introduction of non-GE plants and micro-organisms into the environment, and this prior experience can indicate appropriate management practices for introductions of the GE-counterparts. As familiarity depends also on the knowledge about the environment and its interactions with introduced organisms, in some cases the RA in one country may not be applicable in another country. However, as more field tests are performed, it should be possible to derive additional information about the organisms involved, and their interactions with a number of environments.

Recognizing that there is a general agreement of the general approaches used when conducting environmental RA for GE plants and other GE organisms, a group of public sector researchers have developed a useful, step-wise guide for conducting an

environmental RA. This approach is summarized below, and has the advantage of being consistent with the approach used in the United States and most other countries that are conducting environmental RA of GE organisms. Further information on the guide developed under this initiative can be found at their website (http://pubresreg.org/). The guide is currently in its second draft, and the steering committee is accepting comments and suggestions before making revisions (see "Draft guide for Notifications and Risk Assessments for Releases of GMOs" online at http://pubresreg.org/Members/Kim/working%20groups/biosafety%20protocol/CPB/Notificationguide).

The methodology for RA typically follows the following steps:

- Hazard identification
- Likelihood estimation
- Consequence evaluation, including a baseline assessment
- Risk estimation
- Risk management
- Consideration of overall risk

A phased approach provides a systematic way of working through the RA:

Phase 1: Consideration of each of the inserted genes and sequences individually.

Phase 2: Consideration of the whole plant, including available empirical information on the resulting GMO in order to evaluate potential unintended effects or changes to the GE plant (e.g., synergistic effects, insertion effects).

Phase 3: Consideration of risk management and overall risk.

In the case of the first two phases of the RA, looking at the inserted genes then the whole plant, the risk assessor works through the first three steps of hazard identification, likelihood estimation, then considering the consequences (includes a consideration of a baseline assessment which is often a comparison with the non-GE counterpart of the GE plant).

Looking at the hazard identification, there are a several key considerations, especially considering that this will drive the other steps of the RA. Throughout the identification of potential hazards, it is important that there are plausible, scientifically sound reasons for suggesting that something may pose a hazard. Another key consideration is that the "hazard" is actually a hazard and not just something that occurs. Many risk assessors find that potential hazards are more easily clarified if the assessor writes down a clear description of the potential hazard. This makes it easier to see when the "hazard" is too vague to be readily evaluated. Clarity at this step makes it easier to identify which information in really relevant in not only ascertaining the nature of the hazard, but also estimating the likelihood of the hazard occurring and the resultant consequences. This written description of the potential hazard also makes it easier for the risk assessor to get additional input from people who may have greater knowledge necessary for the comparison to the non-GE plant or the existing agricultural practices.

Consideration of the inserted genes and sequences is often fairly straightforward if the risk assessor has firsthand knowledge or access to the scientific literature. Oftentimes, it is more difficult to decide how to evaluate the whole plant, i.e., all of the potential changes that may have occurred apart from the expected phenotype of the introduced genes and sequences.

There has been considerable interest in finding useful ways to evaluate the possible unintended changes that may have occurred in the GE plant. It is well known from non-GE plants that plants exhibit heritable, unintended genetic changes that may be caused by spontaneous mutations arising from chemicals, radiation, or other environmental factors. The pioneering work of Barbara McClintock on maize transposable elements has led to the discovery of similar transposable elements in virtually every plant species where researchers have sought them out, so it is clear that genetic changes may be mediated by endogenous transposable elements. Soma-clonal variation is another mechanism that has been frequently found in plants that have undergone tissue culture, as many GE plants have (as well as many non-GE plants). These are just a few examples that make clear the need to be able to address the potential for unintended changes to the GE plant, but how is this done in a practical manner?

Two ideas mentioned previously, the concept of familiarity and the comparative approach, have pointed the way for risk assessors to gather information that can be used to evaluate the likelihood, or unlikelihood, that there have been unintended changes made to the GE plant. Over the course of several years of technical discussions related to RAs done by USDA-APHIS and their counterparts in the Canadian Food Inspection Agency (CFIA) found that they used a similar approach that was useful in evaluating whether it was likely that unintended changes had occurred in a GE plant. These discussions arose from reviews that each agency had done when developers sought the final approvals before commercialization of GE plants. The risk assessors decided to develop a document that would be describe their experience in their RAs to date, and thereby more clearly describe the approach they had found useful in addressing these questions. (APHIS and CFIA refer to these appendices as "living documents" to be

periodically updated to reflect experience accumulated over time; for more information see http://www.inspection.gc.ca/english/plaveg/bio/harmone.shtml).

This "Appendix 2" describes the type of environmental information that is most commonly seen in the applications to CFIA and APHIS just prior to commercialization. One section of "Appendix 2" deals with "Phenotype of the transgenic plant" and lists a number of characteristics that may be useful in making inferences about whether unintended changes were likely or unlikely to have occurred in the GE plant. These are the characteristics of plants that we have a great deal of familiarity in evaluating, since they are the characteristics that are of interest to plant breeders, agronomists and horticulturalists. By looking at the information about the GE plant's phenotypic characteristics, it is possible to draw inferences about whether it is likely or unlikely that unintended changes have occurred to the GE plant. Each of these phenotypic characteristics is controlled by many genes, and a lack of change in the phenotype can lend support to the conclusion that these many genes have not been perturbed. This is the logic behind this approach. There is also good empirical evidence to support this approach, because this is the approach that humans have used for thousands of years in improving various plant species.

Information on the plant's phenotypic characteristics is also a way to provide supporting evidence that the plant is unlikely to pose a problem in being more invasive or environmentally persistent than the non-GE counterpart of the plant (i.e., be more of a problem as a weed). The list in the Appendix 2 section on "phenotype" suggests possible parameters to measure for the GE plant. The list is not mandatory or exclusive of other possible characteristics, but gives guidance on practical parameters with which plant scientists have a great deal of experience. Examples

include: growth habit, life-span, vegetative vigor, ability to overwinter or overseason), number of days to onset of flowering; number of days for flowering, number of days until maturity, seed parameters (e.g., seed, dormancy, seedling emergence), proportion of plants surviving from seedling to reproduction, outcrossing frequency, interactions with pollinator species, pollen parameters (physical, biochemical, biological), fertility, asexual reproduction, seed dispersal factors, interaction with symbionts (e.g., vesicular-arbuscular mycorrhizal fungi, rhizobia), and stress adaptations (e.g., to biotic or abiotic stressors).

This is just a brief overview of Appendix 2 and how it can be useful in conducting an environmental RA. The full text can be found online at http://www.inspection.gc.ca/english/plaveg/bio/usda/appenannex2e.shtml.

3. Examples of using the RA methodology in specific cases

Confined environmental releases – field tests

In the case of field tests, these are controlled environmental releases that are limited in geographic area and in duration. In a typical test, the GE plant is in the environment for several months, starting with planting and ending with harvest. In the season following harvest, the test site is monitored to detect any volunteer plants that may have survived from the test, and these plants are eradicated before they can disseminate and persist in the environment. Because the opportunities for environmental interaction are relatively limited in field testing, the key consideration for reducing any possible a risk is to confine the GE plants during the test and afterward. If we were to relate this to the RA equation from above, the confinement serves to reduce the exposure or likelihood parameter.

Decisions on confinement approaches take into account our familiarity with the plant biology, especially the reproductive biology. Confinement decisions also take into account the phenotype of the GE plant and whether the phenotype is similar in kind to the phenotypes that of non-GE plants. Many, if not most, GE plants field tested to date have been developed to express characteristics similar to the characteristics that have been bred into traditional plant varieties. These characteristics include resistance to pathogens and insect pests, improved quality traits, increased yield, and even tolerance to certain herbicides. Appropriate confinement approaches, then, take into account the gene(s) introduced, the resultant phenotype, and the similarity of the phenotype to plants with which we have experience from traditional plant improvement. Likewise, the confinement approach considers the experience gained in the production of specific plant phenotypes in the course of plant breeding practices and production of certified seed varieties.

Historically in the United States and elsewhere, the isolation distances for certified seed production have been commonly used as part of the confinement practices used for field testing GE plants that have been engineered with traits that are similar in kind to the varieties commonly developed through traditional plant breeding programs. These are the types of GE plants that have been most frequently field tested in the United States and other countries.

In recent years, there has been some limited field testing of GE plants that have been engineered to express traits that are not similar to the traits used in traditional plant variety improvement programs. In many instances, the goal is not to produce a new variety to go into general agricultural production, but rather to use the GE plants to produce a high-value protein that can be extracted from the plants after the test.

After extraction, the protein would be further processed or formulated into a product (e.g., medicinal or therapeutic compounds, food processing agents, enzymes for use in manufacturing processes).

Because these types of GE plants are so dissimilar from the plants we are most familiar with, APHIS and regulatory officials in other countries have chosen to use more stringent approaches to achieving high levels of confinement of these GE plants when grown in the controlled environmental releases (in many ways the term field test does not apply as well, since that term is more associated with typical agronomic field tests done for variety development). As one might expect, the rigor for these environmental releases takes considerable preparation, long before an application is submitted to APHIS for a field test. APHIS has provided copious guidance to prospective applicants, and the agency has recently reformatted this guidance in a consolidated User's Guide that is available from the website of APHIS' Biotechnology Regulatory Services (http://www.aphis.usda.gov/brs/pdf/Pharma_Guidance.pdf).

Unconfined environmental releases – determination of nonregulated status

Let me turn briefly to the environmental RA that APHIS does when someone petitions for the agency to grant a determination of nonregulated status. This would be analogous to the reviews done in some countries when developers seek authorization for placing on the market. In the course of the assessment APHIS considers the range of potential impacts on the environment arising from a decision to grant nonregulated status. With nonregulated status the GE plant is no longer subject to APHIS authorizations when importing, moving interstate, or releasing the GE plant into the environment. These are very extensive reviews, and encompass more information than needed for a RA prior to authorizing a field test.

When we discussed RA for field tests, we said that confinement of the GE during the controlled release was a key part of the evaluation. In the case of the RA when a developer petitions APHIS for nonregulated status for a GE plant, confinement doesn't have the same significance, since the nonregulated GE plant will be unconfined and used in the same manner as other non-GE plants. Therefore, the RA does not focus on ascertaining outcrossing rates, but rather focuses on determining what the biological consequence is likely to be if and when outcrossing occurs.

Let me share an example from several years ago when APHIS reviewed a petition to grant nonregulated status for rice lines that had been engineered to express phosphinothricin acetyltransferase (PAT), the enzyme that confers tolerance to the broad-spectrum herbicide glufosinate. There was some initial concern, because in the United States red rice is a close relative of cultivated rice, and red rice is a problem weed in rice production. Control of red rice is difficult in part because the herbicides that will kill red rice will also kill the rice crop. In light of this, the developer wanted to develop a cultivated rice variety that would be able to tolerate exposure to a broad spectrum herbicide like glufosinate, and thereby give the farmer an additional way to control red rice weeds in the rice fields.

Outcrossing of rice to red rice does occur, although at a very low frequency, but in this RA, we focused more on the consequences of the outcrossing rather than trying to make precise determinations of the outcrossing frequency. In doing so, we recognized that there were adequate management tools available to prevent any outcrossing from having a significant impact on the options available to growers or the environment itself. In essence, all of the existing weed control strategies then in

use for red rice in rice production would still be effective in controlling any red rice plants that might acquire the *pat* gene and glufosinate tolerance from the GE rice.

This petition review highlighted another important lesson when conducting an environmental RA. It's important for the reviewers to write down as much of the steps and thought process as possible and thereby lay out each scenario is detail. The detailed scenario helps to clarify which information is needed and which information is peripheral or not needed at all to address the potential hazard that has been identified during the steps of the RA.

In the course of conducting this RA, our reviewers had some very interesting discussions around a term that many of us have heard over the years. The term is "super weed", and it is one that seems to have captured the imagination of the popular press and some members of the general public. We heard fears that if the *pat* gene outcrossed from the GE rice to the red rice, the red rice would become a "super weed". We addressed this issue in our RA and reached the conclusion that if red rice did acquire the pat gene, the red rice would no longer be sensitive to the herbicide glufosinate. This would not affect its sensitivity to other herbicides, whether broad spectrum like glyphosate or narrow spectrum like the graminicides. Other weed control mechanisms, such as flooding, tillage, and crop rotation, would still be just as effective in controlling the red rice that had acquired the tolerance to glufosinate. So the myth of super weed is not warranted. In fact, our colleagues in weed science find this public concern about super weeds to be a notion that has more support from public perception than from scientific fact.

This example makes clear, once again, how important it is to not only work through the RA in a methodical, systematic manner, but also to clearly summarize how the RA was done and how the conclusions were reached. Sometimes scientists do not realize that the logic of their assessment is not clear if the reader does not have the same background to fill in the details or connections. In general, it is always possible to improve the clarity of the summaries of our RAs. This builds the confidence of the decision-makers, as well as the general public. This clarity is also beneficial for future petitioners, so they can better understand the nature of the RAs that are done.

In the United States, guidance to developers is an important ingredient in making a regulatory system work more smoothly. Each of the U.S. regulatory agencies provides guidance to developers that are relevant to different stages in the research and development of GE plants. In addition to written guidance (accessible through agency web sites) each of the agencies encourages early and frequent consultations to provide further information. The web sites also provide access to relevant resource materials, including materials that serve as frequent references when conducting RAs.

CONCLUSIONS

In this brief introduction, I hope I have provided a flavor of our experience in the United States over the years in conducting environmental RA on the use of GE plants. Our approaches have been developed and refined over many years in which we have continued to seek out and use the best available scientific information when doing our environmental RAs.

Although I have touched only briefly on the importance of clear documents to summarize the RAs to both technical experts and the general public, it is important to

mention that these documents make it possible to build and maintain the confidence of others in the quality and conclusions of our RAs. Likewise, these documents become useful in the bilateral and multilateral technical discussions we have with our counterparts in other countries who are engaged in assessing the environmental consequences of using GE plants.

SELECTED RESOURCES FOR ADDITIONAL INFORMATION

United States Regulatory Agencies Unified Biotechnology Web page for information on US regulation of GE organisms and role of US regulatory agencies: http://usbiotechreg.nbii.gov/

Recombinant DNA Safety Considerations. Safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques ("The Blue Book"), OECD, 1986.

Field Testing of Genetically Modified Organisms: Framework for Decisions. U.S. NAS - National Academy of Sciences, National Academy Press, Washington DC. USA 1989.

Safety Considerations for Biotechnology: Scale-up of Crop Plants, OECD. 1993a.

Traditional Crop Breeding Practices: A Historical Review to serve as a Baseline for Assessing the Role of Modern Biotechnology, OECD 1993b.

Commercialization of Agricultural Products Derived through Modern Biotechnology: Survey Results, OECD 1995a.

Report of the OECD Workshop on the Commercialization of Agricultural Products Derived through Modern Biotechnology, OECD 1995b.

Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology, OECD 1995c.

"Draft guide for Notifications and Risk Assessments for Releases of GMOs" http://pubresreg.org/Members/Kim/working%20groups/biosafety%20protocol/CPB/Notificationguide

International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
39~59

Engineering Resistance and Disease Management in Ornamental Crops

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ABSTRACT

Viral diseases of ornamental plants cause major losses in productivity and quality. Host pl1ant resistance offers an effective means of controlling plant diseases caused by viruses. It minimizes the necessity for the application of pesticides. However, there are many ornamentals in which no natural disease resistance is available. Genetic engineering allows the introduction of specific, in some instances broad spectrum, disease resistance derived from other species, or even from the pathogen itself into plant genotypes that have been selected for desirable horticultural properties.

Key words: Agricultural biotechnology; Floriculture; Genetic enhacement; Molecular breeding; Virus resistance.

INTRODUCTION

Grower cash receipts for U.S. floriculture and environmental horticulture crops, as estimated by USDA's Economic Research Service (USDA-ERS, 2004), reached $14.3 billion in 2003, of which $5.6 billion represented floral crops. Grower cash receipts for all floriculture crops (cut flowers, cut greens, flowering and foliage

potted plants, and bedding and garden plants) have an average annual increase of 4-6 percent since 1991. The nursery and greenhouse industry comprise one of the fastest growing segments of US agriculture. Two-thirds of the value of U.S. floriculture production in 2003 consisted of bedding and garden plants and potted flowering plants, while woody landscape plant producers accounted for over 50% of the greenhouse and nursery crop value.

The Importance of Disease and Disease Resistance in Ornamental Crops

More than any other sector of agriculture and horticulture, the visual quality of the ornamental product at the retail level is critical. This is especially true for cut flowers and potted plants. Visible symptoms of disease caused by viruses, therefore, have a major impact on quality. Unlike diseases incited by other pathogens where chemical methods aimed at prevention of infection have been quite successful, control of viral diseases has been much more problematic. Direct and indirect effects of viral and infections include reduction in growth, reduction in vigor, costs of attempting to maintain crop health, and, of significance to the ornamental industry, reduction in quality and/or market value (Hadidi *et al.*, 1998).

Many crops are susceptible to multiple viruses, each of which may cause serious economic losses. In addition, infected plant material may not be acceptable for export (Loebenstein *et al.*, 1995). In practice, several different crops are grown in the same facility. At least 125 different viruses have been identified that infect and cause disease in ornamental plants (Cohen, 1995).

Control of viral diseases of floral crops usually focuses on use of clean propagation materials that have been indexed and shown to be free of known pathogens.

However, the use of virus-free propagation material is not in itself adequate, as many viruses can also be transmitted by an insect vector, such as aphids, whiteflies or thrips. In addition, several important viruses of ornamentals affect multiple crop genera, making it important that all crops grown in the facility are virus-free in order to prevent transmission between different crops. Current strategies of controlling viral diseases in floral crops include early detection and removal of infected material from production areas as well as preventative measures to control the insect vectors (Matthews, 1998; Powell and Lindquist, 1992). Screening of greenhouses, isolation of virus-tested clean propagation stock from production areas, elimination of weeds and non-production reservoir plants from greenhouses and surrounding areas, monitoring of insect populations and judicious use of pesticides are all needed for control.

The use of resistant varieties is by far the most recommended approach for control of disease in many crop species. Conventional breeding strategies require the identification of sources of disease resistance genes. This is a difficult task in ornamentals due to the diversity of floral and nursery crop species that are susceptible to such a large diverse group of pathogens. Also, the overriding importance of appearance and general horticultural traits, the large number of cultivars which are produced per crop, and the rapid turnover in cultivars, have made breeding for disease resistance extremely difficult in floral crops. Even where effective disease resistance can be identified in related germplasm, introgression of a single gene into horticulturally desirable plant lines requires multiple back-crossings which may result in reduced expression of the resistance compared to the source material. New tools and genes have been developed for use in the genetic engineering of plants to introduce effective resistance to plant diseases and to

understand the mechanisms of resistance. This approach should allow increases in both productivity and quality of ornamental plants in an environmentally friendly manner, thereby reducing the use of and reliance on chemical control of pests that vector the virus.

Progress and Uunique Issues in the Ttansformation of Ornamental Plants

Advances in biotechnology have provided new opportunities to solve practical horticultural problems. The development of technologies for gene identification and gene transfer in plants has provided the opportunity for genetically engineering disease resistance into horticulturally desirable cultivars without altering critical quality traits (Daub *et al.*, 1996; Hadidi *et al.* 1998; Hull, 2002). There are essentially three sources of transgenes for protecting plants against viruses: natural resistance genes, genes derived from viral sequences (pathogen-derived resistance), and genes from various other sources (Hull, 2002).

Transformation of ornamental plants has lagged behind efforts in the major field crops. This is further hampered by that fewer groups have worked on the regeneration systems that are necessary for an efficient transformation for a particular crop. In many crops, transformation or regeneration systems are cultivar-dependent. We are now at a point where transformation systems have been demonstrated for a significant number of ornamentals (Davies *et al.*, 2003; Deroles *et al.*, 1997, 2002; Griesbach, 1994; Hutchinson *et al.*, 1992; Robinson and Firoozabady, 1993; Schuerman and Dandekar, 1993, Zuker, 1998) allowing much less cultivar restriction in regeneration and transformation (Castillon and Kamo; 2002, Deroles *et al.*; 2002, Kamo, *et al.*, 1995a, b, 1997).

As technologies for regeneration and gene transfer in plants improve and broaden across diverse genera, transgenic approaches to develop disease tolerant ornamental crops become more promising. Transgenic approaches for breeding woody ornamentals are attractive. Less space is required to grow out and evaluate germplasm in identifying disease resistance traits. Also, long generation time of backcrossing of woody plants can be eliminated. The costs, potential benefits, and risks of using genetic engineering to create disease resistant ornamental plants must be carefully evaluated. Unlike many agronomic crops, woody and perennial ornamental crops remain in the field for years or decades. Thus, the expression of the transgene must be durable in all seasons that the plant could come under attack from the pathogen, and must also be stable enough to withstand multiple cycles of plant dormancy. The long-lived nature of these perennial ornamentals also poses unique problems for environmental risk assessment. The plant could serve as a reservoir, either for an escaping transgene or for a plant pest that has overcome the engineered resistance. On the other hand, few ornamental plants are cultivated in a typical monoculture. The selective pressure on pathogens to overcome the transgenic resistance is not as strong as it would be in monocultured agronomic crops. Because the fruit and seed of many ornamental plants serve as food for wildlife, the aspects of ingestion and seed dispersal by wildlife must also be considered. However, unlike most agronomic and other horticultural crops, FDA issues regarding human ingestion should not be an issue with ornamental crops. Issues regarding gene recombination in seed production are minimized in the many ornamental crops that are vegetatively propagated.

TRANSGENIC APPROACHES

Pathogen-derived resistance

Pathogen-derived resistance is mediated either by the protein encoded by the transgene (protein-mediated) or by the transcript produced from the transgene (RNA-mediated). Extensive research with genes from viruses and other sources has documented the efficacy of viral sense or antisense genes (coat protein, replicase, satellite RNAs, defective interfering RNAs) in protecting plants against virus infection following transfer and expression of these genes in plants (Goldbach *et al.*, 2003; Hadidi *et al.*, 1998; Hull 2002; Ziegler and Torrance, 2002).

Several lines of research indicate that the best approach for this pathogen-derived "virus-induced" resistance is one mediated by an RNA-based post-transcriptional gene silencing (PTGS) mechanism. This plant defense system, one aspect of RNA silencing, results in degradation of mRNA produced both by the transgene and the virus (Hammond *et al.*, 1999; Lu *et al.*, 2003; Vance and Vaucheret, 2001; Wassenegger, 2002). In general, protein-mediated resistance provides moderate protection against a broad range of related viruses while RNA-mediated resistance offers high levels of protection only against closely related strains of a virus (Dawson, 1996; Goldbach *et al.*, 2003; Lu *et al.*, 2003).

Using various coat protein (CP) sense, CP antisense, or replicase sense viral genes, several groups are working to introduce virus resistance into various ornamentals, including chrysanthemum (Sherman *et al.*, 1998; Yepes *et al.*, 1999), gladiolus (Hammond and Kamo, 1995a, 1995b; Kamo *et al.*, 2000a, 2000b; Kamo, Hsu, and Jordan, unpublished), lily (Langeveld *et al.*, 1997), and various orchids (Deroles *et al.* 2002).

Defective Interfering RNA and DNA

Defective interfering RNAs (DI-RNAs) or DNAs (DI-DNAs) are deletion mutations of the viral genome that are able to replicate in a parasitic fashion, utilizing the replicase complex of an active infection. DI-RNAs and DI-DNAs are not able to replicate on their own. In most cases DI-RNA reduces the replication level of the parental virus, resulting in reduced symptom expression, although at least one DI-RNA intensifies symptom expression (Li *et al.*, 1989). DI-DNAs have similar competitive effects on DNA viruses (Stanley *et al.*, 1990). Kollar *et al.* (1993) showed that a *Cymbidium ringspot virus* (CymRSV) DI-RNA protected transgenic *N. benthamiana* againt CymRSV infection, while Stanley *et al.* (1990) demonstrated protection against geminivirus infection from a DI-DNA. Stanley *et al.* (1997) have also shown the presence of a naturally-occurring DI-DNA in *Ageratum yellow vein virus* infections of *Ageratum conyzoides*. Rubio *et al.* (1999) developed a DI-RNA from *Tomato bushy stunt virus* (TBSV) that conferred broad-spectrum protection against related tombusviruses in *N. benthamiana*; the DI-RNA was expressed at low levels in healthy transgenic plants, but was amplified to very high levels following TBSV infection, and resulting in plant recovery.

Ribozymes

Ribozymes are RNA molecules that autocatalytically cleave sequences complementary to their binding site. Ribozymes have potential to confer resistance against viruses if expression levels and activity are sufficient. Some viroids and viral satellite RNAs self-process from multimeric replicative forms by ribozyme activity, and some success has been achieved in protecting transgenic plants against specific pathogens. One line of transgenic melon expressing a ribozyme against *Watermelon*

mosaic virus 2 (WMV2) was found to have immunity to WMV2 (Huttner *et al.*, 2001).

Anti-viral peptides

Another anti-viral approach that may have considerable applicability to ornamentals is the expression of dominant interfering peptides ('aptamers') that interact with essential viral proteins. The first example of this approach is the expression in *N. benthamiana* of a 29 amino acid peptide selected for interference with multiple tospovirus N-proteins (Rudolph *et al.*, 2003). The transgenic plants were highly resistant to *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), and *Chrysanthemum stem necrosis virus*, while a lower level of resistance was observed with *Tomato chlorotic spot virus*, and delayed disease development with *Impatiens necrotin spot virus* (INSV) (Rudolph *et al.*, 2003).

Ribonucleases

It may be possible to introduce broad spectrum resistance against RNA viruses and viroids, based on the expression of ribonucleases specific for double-stranded RNA (dsRNA). DsRNA is a feature of the replication of RNA viruses and viroids, but is not normally found in healthy plant cells. Two approaches that have been tested are the expression of the yeast dsRNA-specific RNase Pac1, and the mammalian interferon-induced 2',5'-oligoadenylate synthetase (2-5A)/RNase L system.

Ribosome-inactivating proteins

A number of ribosome-inactivating proteins have been described from a variety of plant species, and several have been expressed in transgenic plants (Stevens, 1981). Their activities have been reviewed by Tumer *et al.* (1999).

Antibodies for Viral Resistance

A recent scientific breakthrough that has presented another possibility for controlling plant diseases is the use of transgenic plants that produce antibodies to specific plant pathogens (During *et al.*, 1990). Complete antibodies or single-chain variable fragment (scFv) antibodies have been expressed in plants by transient expression using viral vectors, agroinfiltration, or biolistics, or after stable integration of a transgene directly into the plant genome (Schillberg *et al.*, 2001). Neutralization of one or more viral proteins (viral proteases, replicases, movement proteins) should interfere with viral infection, virus assembly, movement of virus within the host, symptom expression, aphid transmission, and/or virus replication. Resistance by simultaneously expressing several antibodies with different target specificities will increase the likelihood of developing long-lasting, broad-spectrum resistance.

Transgenic Plants in Virus Control

Examples of virus resistance that are particularly relevant to ornamentals include a number of viruses with wide host ranges that include several ornamentals, such as *Arabis mosaic virus* (ArMV), *Cucumber mosaic virus* (CMV), *Chrysanthemum virus B* (CVB), *Tobaco mosaic tobamovirus* (TMV) and other tobamoviruses, as well as tospoviruses including TSWV and many potyviruses. Spielmann *et al.* (2000) reported a delay in infection, and some escape from infection, in *N. benthamiana* expressing ArMV CP. Multiple constructs have conferred resistance to CMV in tobacco, tomato, and cucurbits expressing CMV CP or replicase genes (Gonsalves *et al.*, 1992; Anderson *et al.*, 1992), dsRNA (Kalantidis *et al.*, 2002), or satellite RNA (Harrison *et al.*, 1997). Chrysanthemum has been transformed with different forms of the CVB CP gene (Mitiouchkina *et al.*, 2006). Resistance to tobamoviruses has been reported in a variety of transformed plants, including expression in tobacco of

truncated replicase (Golemboski *et al.*, 1990), defective movement protein (Cooper *et al.*, 1995), CP (Powell-Abel *et al.*, 1986), and anti-TMV scFv (Zimmermann *et al.* 1998). Resistance to tospoviruses has been conferred by various N-protein constructs (Pang *et al.*, 1993), and an anti-viral peptide (Rudolph *et al.* 2003). Jan *et al.* (2000) showed that a composite tospovirus (TSWV partal N-gene)-potyvirus (*Turnip mosaic virus*; TuMV) CP construct resulted in PTGS and resistance against both TSWV and TuMV.

There are ornamentals that have already been transformed with viral or anti-viral genes in order to obtain virus resistance, although not all published studies include resistance data. Yepes *et al.* (1995) used biolistic transformation of leaf or stem explants to obtain a total of 82 transgenic lines from four cultivars. The construct used was the TSWV N-gene, and stem explants regenerated more efficiently than leaf pieces. In a subsequent paper, Yepes *et al.* (1999) compared the biolistic method to *Agrobacterium*-mediated transformation, and utilized the N-genes of TSWV, INSV, and GRSV. Sherman *et al.* (1998) used *Agrobacterium* to transform chrysanthemum cv. 'Polaris' with either a full-length (N+), a translationally-truncated (Nt), or an antisense (N-) copy of the N-gene of a dahlia isolate of TSWV; one Nt and two N- lines were fully resistant to challenge by viruliferous thrips carrying a virulent chrysanthemum isolate of TSWV. Several N+ and other lines were infected but showed significantly reduced symptoms of TSWV compared to non-transgenic controls (Sherman *et al.*, 1998). Resistance to mechanical inoculation with TSWV has also been demonstrated in four cultivars of *Gerbera* (Korbin *et al.*, 2002), and in *Osteospermum* (Allavena *et al.*, 2000). *Osteospermum* has also been transformed with several constructs derived from *Lettuce mosaic virus*, but no resistance assays were reported (Mörbel *et al.*, 2002). Borth *et al.* (2006) have

characterized partial resistance in transgenic *Dendrobium* orchids transformed with the *Cymbidium mosaic virus* CP or mutated movement protein gene. Kamo *et al.* (1997, and unpublished) have transformed gladiolus with *Bean yellow mosaic virus* (BYMV) CP and antisense RNA constructs, that were each effective in *N. benthamiana* (Hammond and Kamo, 1995a,b) as well as CMV CP and defective replicase constructs (Kamo, Hsu, and Jordan, unpublished). The BYMV CP and antisense gladiolus lines showed delayed virus accumulation, but not effective resistance (Kamo *et al.*, submitted), while the other lines are undergoing analysis. Lily has been transformed with a defective CMV replicase gene (Lipsky *et al.*, 2002), and De Villiers *et al.* (2000) attempted transformation of *Ornithogalum* with the *Ornithogalum mosaic virus* CP gene, but no information on resistance in either crop is available. Similarly, Berthomé *et al* (2000) report transforming geranium (*Pelargonium* × *hortorum*) with the *Pelargonium flower break virus* CP gene, or rat 2',5'-oligoadenylate synthetase (2-5A), or yeast Pac1 dsRNA-specific RNase, but without resistance assessment.

Transgenic plants expressing full-size antibody or antibody fragments against the coat proteins have been reported for several different plant viruses (Fecker *et al.*, 1997; Tavladoraki *et al.*, 1993; Voss *et al.*, 1995; Zimmerman *et al.*, 1998). High levels of resistance against TSWV were obtained in transgenic *N. benthamiana* expressing scFv antibodies targeting the TSWV N-gene product (Prins *et al.*, 2005). The recent development of transgenic *N. benthamiana* and gladiolus expressing scFv antibodies against CMV (Hsu *et al.*, 2006; Hsu and Kamo, *unpublished*) and *N. tabacum* expressing antibodies against TSWV NP protein showing various degrees of resistance to viral infection illustrates the potential of this technology as a means of controlling these wide-host range viruses in ornamental plants (Xu *et al.*, 2006). A recombinant antibody directed against an epitope of the TSWV G1 glycoprotein

conserved among a large number of tospoviruses has been expressed in plants (Franconi *et al.*, 1999). Antibodies directed against evolutionarily conserved functional domains, such as viral protease, movement protein and replicase should provide more potent, broad spectrum resistance against viruses (Schillberg *et al.*, 2001).

CONCLUDING COMMENTS

Results obtained to date suggest that there are multiple approaches to achieve resistance to viral diseases in transgenic plants. Resistance attained by expression of viral sequences, antiviral antibodies, ribozymes, antiviral peptides, or dsRNA-specific nucleases is likely to be durable. Such resistance mechanisms are not known to have previously imposed selection pressure in virus evolution. The durability of resistance against an individual pathogen in a single plant would be further increased by additive effect of combining different resistance mechanisms against the same pathogen.

Combining resistance against different diseases is also possible by transgenic approaches, as multiple genes can be introduced into a horticulturally-desirable genotype on a single construct. Introduction of multiple transgenes against different viruses has been demonstrated (Fuchs *et al.*, 1997). The use of multiple transgene constructs appears to be a much more efficient means of introducing multiple resistance genes into a well-adapted genotype than combination by conventional breeding and multiple cycles of selection.

It will be important for scientists developing transgenic plants for disease resistance to conduct thorough trials to evaluate resistance, and to include field trials in which growers can see the effectiveness of transgenic resistance in comparison to existing

genetic resistance (or lack of resistance). Unless growers are convinced of the economic and ecological benefits of utilizing transgenic resistance approaches, it is unlikely that disease-resistant transgenic ornamentals will be widely grown or reach consumer acceptance, despite the promising results reported from research to date. Cooperation between scientists and growers will be necessary in order to realize the potential for combining effective resistance to multiple pathogens into horticulturally desirable cultivars of a wide range of ornamentals.

Compared to conventional breeding for virus resistance, genetic engineering provides a quicker and more precise technology to obtain plants that are resistant to viruses; however, most transgenic virus-resistant plants are still under laboratory development. The few commercially grown virus-resistant crops include papaya expressing *Papaya ringspot virus* coat protein (Ferreira *et al.*, 2002) and multiple virus resistant cucurbits (Fuchs *et al.*, 1997). Ten years after genetically enhanced varieties of major crops have become commercially available; adaptation of these varieties by U.S. farmers has become widespread (USDA-ERS, 2006). As we move forward in modern agriculture, the difficulty lies not so much in the world of science but rather in commercialization and adaptation of genetic engineering by the public. The ultimate success of agricultural biotechnology will depend on our ability to identify and measure its potential benefits and risks as well as their contribution.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
61~72

Establishment of a Rice Mutant Library for Functional Genomics

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ABSTRACT

Rice has been a model monocot for functional genomics research due to its small genome size (430 Mb) relative to other cereals, its ease of transformation, and its economic importance. With the completion of genomic sequencing analysis, the next challenge will be to assign functions of 37,000 predicted rice genes. During years 2002 to 2006, we have generated nearly 60,000 gene knockout or activation transgenic rice mutant lines using a T-DNA insertional mutagenesis approach. These mutant lines provide important resources for functional analysis of cereal genes using both forward and reverse genetics approaches. The forward genetics approach would screen for mutants with abnormal phenotypes grown under various conditions or treatments, and the reverse genetics approach would identify mutants based on information of flanking sequence tag (FST). By applying these approaches, currently, we have identified many mutants with altered phenotypes of important agronomic traits. Functions of genes responsible for these phenotypes are being analyzed. Novel genes with essential functions will be employed for cereal crop improvement through genetic engineering approaches.

INTRODUCTION

Rice (*Oryza sativa*) is one of the most important crops in the world. Rice, wheat, and maize together account for 60% of the world's food production, and rice itself is the principal food of nearly 50% of the world's population. These cereal crops share a large degree of synteny, making rice an excellent model cereal crop for genomics research (Gale and Devos, 1998). Rice was the obvious choice for the first genome sequencing of a crop plant for the following reasons: 1) Rice has the smallest genome

size (400 Mb) among the major cereal crops. 2) Rice can be transformed on a large scale on a routine basis. 3) Isolation of genes from rice could facilitate isolation of homologous genes from other cereal crops. 4) Much molecular and genetic information (ESTs, markers, genetic and physical maps, etc.) about rice is available.

With the completion of rice genomic sequencing, the challenge of the post-genome era is to understand the functions of the huge number of genes predicted by sequence information. To facilitate such an effort, several approaches have been developed. An important and direct approach of defining the function of a novel gene is to alter or eliminate its function by insertional mutagenesis. Insertional mutagenesis, using T-DNA or a transposable element, has the advantage that the inserted element acts as a tag for gene identification. Gene knockout by insertion of *Agrobacterium tumefaciens* derived transfer DNA (T-DNA) has been developed for tagging genes in various plant species. The advantages of using T-DNA tags are that T-DNA insertion is a random event, and that the inserted genes are stable through multiple generations (Azpiroz-Leehan and Feldmann, 1997). Additionally, T-DNA is usually inserted at low copy number in the plant genome, e.g., 1.5 copies per diploid genome in Arabidopsis and rice (Feldmann, 1991; Jeon *et al.*, 2000), which facilitates establishment of linkage between T-DNA-inserted gene and mutant phenotype.

T-DNA insertional mutagenesis has been successfully used to obtain loss-of-function mutants and identify genes from Arabidopsis (Azpiroz-Leehan and Feldmann, 1997; Krysan *et al.*, 1999) and rice (Jeon *et al.*, 2000; Jung *et al.*, 2003). However, this gene knockout tagging approach has some limitations, i.e., difficulty in identifying the function of redundant genes, or of genes required in early development. An alternative approach, gene activation tagging, has been designed to obtain dominant

mutants by activating genes adjacent to enhancers integrated into the genomic DNA through transformation. Advantageous features of the gene knockout/activation tagging approach for functional genomics include: 1) disruption of gene function leading to loss-of-function mutations, 2) activation of gene expression causing gain-of-function mutations, 3) the technique being a direct way to determine the function of a gene product *in situ*, and 4) the inserted marker being available for subsequent identification of disrupted genes.

Development of a large population of insertional mutant rice lines will be extremely valuable for functional analysis of rice genes. While these lines are important resources for forward genetic studies of gene function, their importance for reverse genetic studies also increases with the availability of rice genome sequence. An important approach for reverse genetic studies is FST analysis. Sequences flanking T-DNA are determined for each transgenic line thereby leading to the development of a database of T-DNA tagged genes that can be searched electronically.

Although a large number of rice mutants tagged by T-DNA have been produced worldwide, the number (88,000) of gene activation lines remains small relative to the number (288,000) of gene knockout lines (Guiderdoni *et al.*, 2006). As described above, gene activation lines have advantages not offered by gene knockout lines, we set out and significantly expanding the collection of gene activation lines currently available to researchers. The T-DNA was also specially designed for trapping of genes or promoters that are active in special tissues, growth stages or under certain growth conditions. Currently, we have generated 60,000 fertile independent transgenic rice lines tagged with T-DNA and analyzed their FSTs. A total of 15,000 FSTs have been obtained from 30,000 transgenic lines, mapped to the genome of rice

cultivar Tainung 67, and analyzed (Hsing *et al.*, 2006). This resource provides a valuable tool for the determination of rice gene functions on a genomic scale, and a searchable FST database is available at the Taiwan Rice Insertional Mutant (TRIM) website (http://trim.sinica.edu.tw/).

MATERIALS AND METHODS

Generation of gene knockout or activation tagged rice mutant lines

Rice cultivar (*Oryza sativa* cv. Tainung 67) have been transformed with the multi-functional plasmid pTag8 (Fig. 1) *via Agrobacterium*-mediated transformation. T0 transgenic rice plants regenerated from tissue culture have been shipped to Taiwan Agriculture Research Institute (TARI), with an average rate of approximately 1000-1500 lines per month. Insertion of T-DNA into different genic regions lead to either gene knockout or gene knockout (Fig. 2). The pipeline for generation of T-DNA tagged rice mutant library and its application in basic research and crop improvement is shown in Fig. 3. The consortium that integrates the multidiscipline expertise and resources for team efforts is shown in Fig. 4. The examples of some mutants are shown in Fig. 5. The standard procedures for study a mutant tagged by T-DNA is shown in Fig. 6.

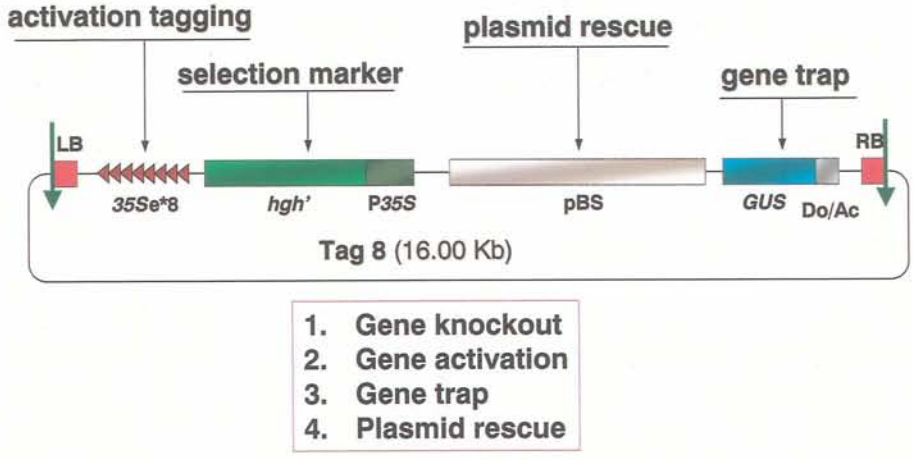


Fig. 1. Multiple-functional T-DNA for Gene Knockout/Activation Tagging

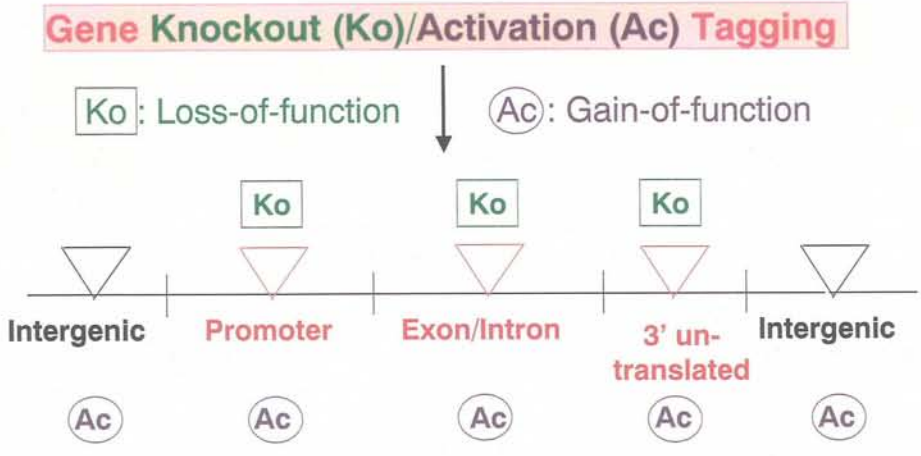


Fig. 2 T-DNA insertional mutagenesis leads to gene knockout or activation

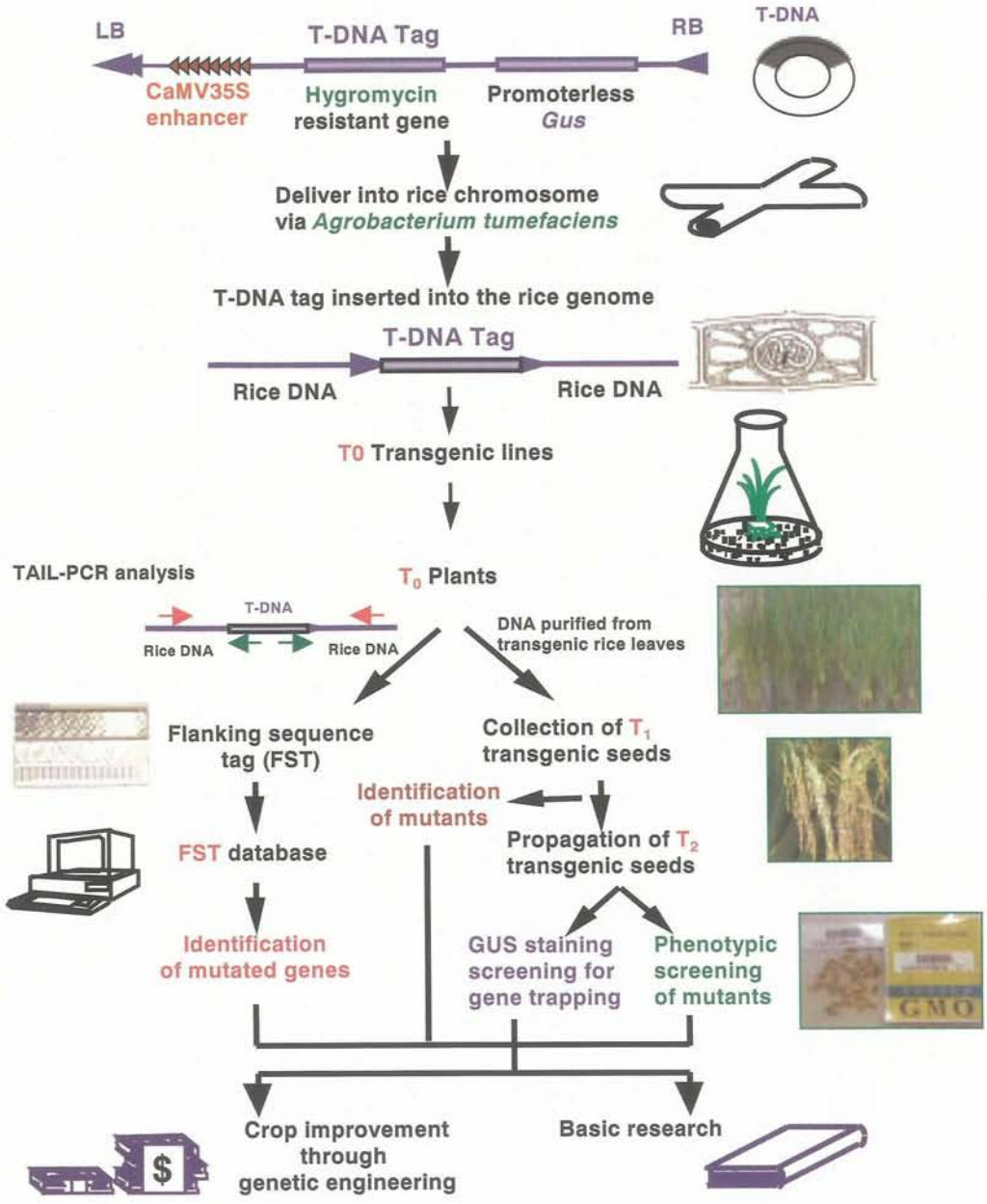


Fig.3 Generation and Utility of Rice Gene Knockout/Activation Library

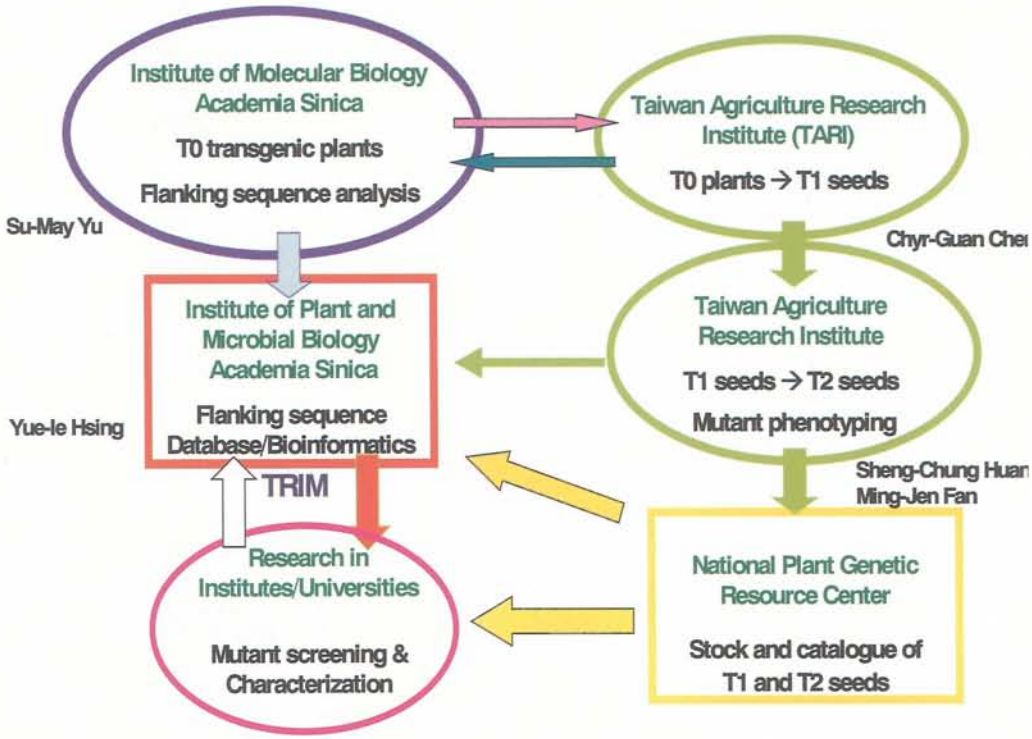


Fig. 4. Consortium for generation of T-DNA tagged rice mutant library.

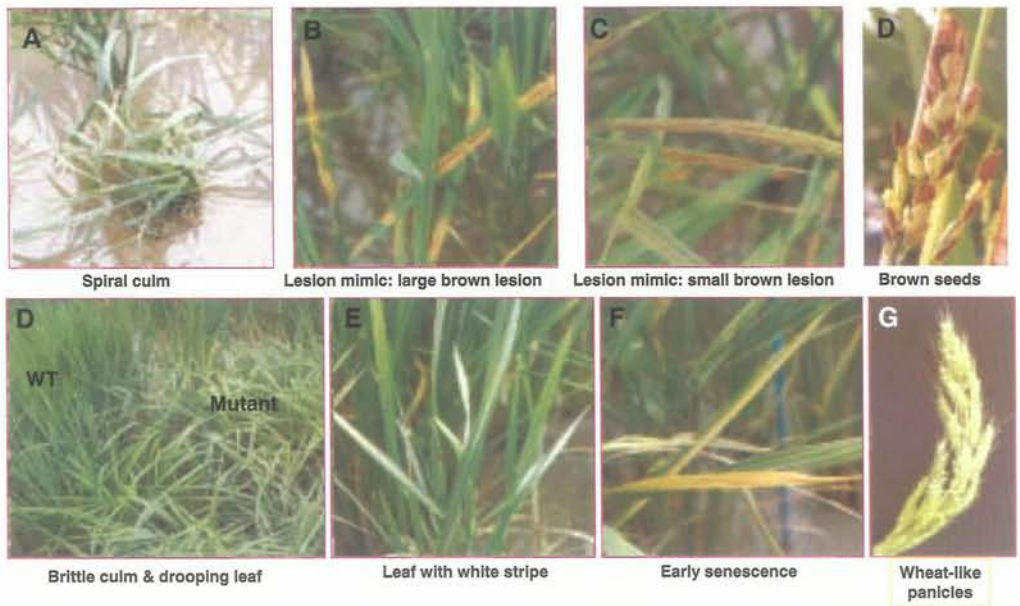


Fig. 5. Examples of rice mutants selected for study.

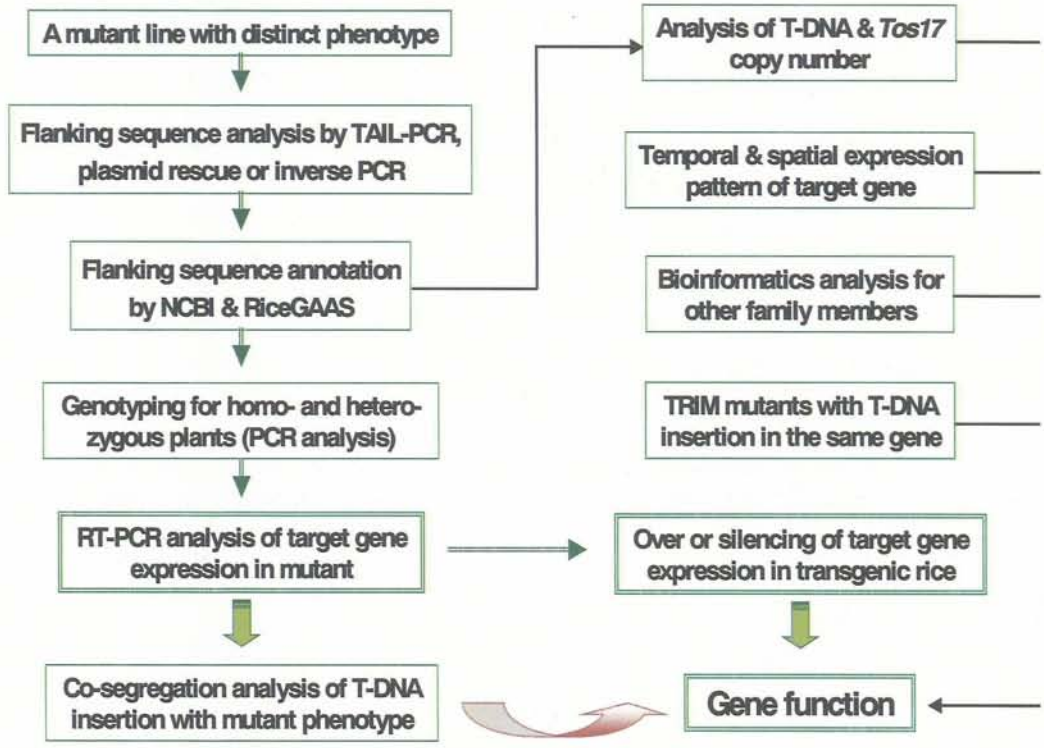


Fig. 6. Standard procedure for studying T-DNA tagged rice mutants.

RESULTS

Several labs joined the research team effort for screening and characterization of rice mutants

Currently, about two dozens of labs have obtained T2 seeds of the TRIM mutant library from the National Plant Genetic Resource Center and are screening and studying mutants with altered responses to biotic or abiotic stresses, or with altered growth and development phenotypes. Among these labs, 12 of them have applied and/or are currently carried out National Agricultural Biotechnology Program

projects. Several putative mutants identified through forward genetics approaches, including (a) insensitive or hypersensitive to salt and water stresses; (b) insensitive or hypersensitive to sugar or hormones; (c) altered growth rate, seed size and color, height, nutrient uptake, flowering time and yield; and (d) resistant to disease and insect, have been identified. Many mutants with essential genes, mainly signaling and transcription factors, being tagged by T-DNA, through reverse genetics approaches, have also being selected. These mutants and mutated genes are being characterized. The examples of some mutants are shown in Fig. 5.

The TRIM mutant library is a precious research resource for science and agriculture biotechnology

The TRIM mutant resources offer excellent tools for both basic and applied research of rice and other cereals and are available to the science community in Taiwan. The TRIM database facilitates identification of genes that are functional in biochemical pathways, physiological processes, or growth and development of cereal crops. Detailed characterization of the gene knockout/activation mutants would validate functions of these genes. Many of these genes could be valuable for crop improvement, e.g., to enhance biotic and abiotic stress tolerance, productivity, and quality of cereal crops, through genetic engineering approaches. Accomplishment of this project has a major impact on the development of agriculture biotechnology in Taiwan as well as promotion of the Taiwanese rice functional genomics projects in the worldwide arena.

CONCLUSIONS

The TRIM mutant library provides an opportunity for international collaborations

The TRIM project is launched in 2002, several years behind other labs at the beginning. However, the project has been progressed at a good pace. Currently, TRIM mutant resources ranked No. 3 among the four largest T-DNA insertion libraries (China, Korea, Taiwan and France) in the world. However, in terms of the user-friendly utility, information provided, and number of available T2 seeds, the TRIM mutant resource is ranking close to the top. Promotion has been conducted to encourage the plant science community in Taiwan to make the best utilization of this library for rice functional genomics research. Many labs are expected to be productive in next several years. Due to the distinguished achievement in establishing the TRIM mutant resources, Dr. Yu was recently invited to contribute two articles, together with many internationally well-known scientists, for the book *Rice Functional Genomics* to be published by Springer. Establishment of the TRIM resources has set up a milestone for rice functional genomics research for the Taiwanese science community as well as opportunity for promoting worldwide collaborations for cereal crop improvement.

ACKNOWLEDGEMENTS

The TRIM mutant library projects are supported by grants from the National Science and Technology Program for Agriculture Biotechnology, Academia Sinica, National Science Council, and Council of Agriculture of R.O.C.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
73~92

Dealing with Environmental Issues of GM Crops in an Isolated Place We Call Home

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ABSTRACT

Island populations have a much higher risk of extinction than their mainland counterparts for a number of reasons. Particular concern has been voiced that gene flow and hybridization between agricultural crops and native plant species may exacerbate their precarious position, especially if the gene flow occurs from crops developed through recombinant DNA technologies. Horizontal gene transfer (HGT) and vertical gene transfer (VGT) are the two possible ways for gene flow and introgression to occur. VGT is more likely to facilitate gene transfer between agricultural crops and native plant species, although this too is dependent on a variety of factors. In this critical review phylogenetic tribal boundaries were used as a limit to hybridization potential. Overlap was found between agricultural crops and native species in four tribes: *Heliantheae*, *Gossypieae*, *Solaneae*, and *Phaseoleae*. In each tribe the factors which increase and decrease the likelihood of hybridization were evaluated and distribution analyses performed. In general it is concluded that hybridization potentials are low for most species (except *Gossypium tomentosum* which is known to hybridize with its cultivated relatives), however, small scale pollination studies should be performed for each tribe to quantify the risk and to better manage populations of native species.

Key words: Gene flow; Genetically engineered; Hybridization; Introgression.

INTRODUCTION

The Hawaiian archipelago is the most isolated island group in the world, separated by almost 1,600 km to other islands and nearly 4,000 km to the next major land mass. Due to this isolation, and 1-5 million years of evolution on the different islands (McDonald *et al.*, 1986), Hawai'i has become home to more than 1,000 native flowering plant species. There are 88 families and 211 genera represented by these species, of which over 90% are endemic (Sohmer and Gustafson, 1987). Rare species have a high priority in conservation programs because of their vulnerability to extinction by natural processes and human disturbances (Levin *et al.*, 1996).

Island populations have a much higher risk of extinction than mainland populations (Frankham, 1998; Smith *et al.*, 1993) due to human activities, over-exploitation, habitat destruction, and the introduction of competitive species (Reid and Miller, 1989; Levin *et al.*, 1996; Rieseberg, 1991).They are susceptible to hybridization because of their small numbers and their frequent proximity to more abundant congeners (Crawford *et al.*, 1987; Carlquist, 1974). Insular species are more prone to interbreeding because they tend to be less genetically divergent and to have weaker crossing barriers than their continental counterparts (Crawford *et al.*, 1987). Another factor that increases hybridization rates in insular populations is that they often have poorly differentiated floral architecture, which facilitates interspecific cross-pollination (Carlquist, 1974) or restricts self pollination (Fryxell, 1979). Rare insular species are also particularly vulnerable to hybridization because gametic wastage, reduced seed-set, and the production of ill-fit progeny on the one hand, and the swamping effect of gene flow on the other, are not likely to be counterbalanced by immigration from nonspecific populations (Levin *et al.*, 1996).

A key point that needs to be made regarding the central focus of this paper, potential for gene flow from agricultural crops to wild species, is that gene flow occurs widely in nature. This gene flow is independent of whether transgenes are involved (Haygood *et al.*, 2003). Pollen from agricultural crops often reaches wild plants growing nearby, and when the wild species are closely related to the crops, hybridization often ensues (Ellstrand *et al.*, 1999). In order for crop genes to be transferred to wild populations via hybridization, crop plants and their wild relatives need to occur sympatrically, overlap in flowering time, and be cross-compatible (Keeler and Turner, 1991). In short, if a crop and wild relative co-exist, the frequency of hybridization under natural conditions will strongly depend both on their relatedness and on the phenology and pattern of pollen dispersal (Lavigne *et al.*, 2002).

Due to the sensitivity of island ecosystems, and the fact that a large percentage of the endemic flora of Hawaii are federally listed as endangered species, concern has been voiced that gene flow and hybridization between agricultural crops and these native species may worsen their precarious position. Of particular concern is that gene flow occurs from agricultural products that have been modified through recombinant-DNA or biotechnological techniques to display traits such as insect or herbicide resistance, and the potential creation of "super plants" which could disperse freely into native ranges.

In this review two potential methods of gene transfer are considered, namely horizontal gene transfer (HGT) and vertical gene transfer (VGT). Which native genera overlap with agricultural crops was also determined by considering phylogenetic relationships. Following determining which native genera are

potentially at risk, the factors increase or decrease the likelihood of gene flow in an attempt to justify the potential for hybridization and gene flow are considered.

Native Plant Species vs. Cultivated Crops

As this proposal considers the potential for gene flow from agricultural crops grown in Hawaii to the native plant species, crops currently being produced in Hawaii are considered primarily. However, crops that are currently the focus of research with transgenic characteristics, and which may be considered for Hawaii in future, were also included. Crops for which permits have been acknowledged or are pending in Hawaii are: rice, wheat, corn, pineapple, soybeans, coffee, lime and tobacco. Furthermore, deregulated transgenic corn, soybeans and papaya are being, or have been, grown commercially in the State of Hawaii.

To determine which native plants are related to agricultural products that possibly fall into this category as well as to determine which native species are potentially at risk, a phylogram of plants depicting evolutionary relatedness based on recently published mostly molecular phylogenies was obtained (Angiosperm Phylogeny Group (APG), 1998). In this way the interrelationship between the flowering containing native genera of the Hawaiian Islands or agriculturally significant crops could be visualized to indicate where a potential overlap occurs. This shows the relatedness between these organisms, since cross compatibility is an important factor in determining if hybridization is likely to occur between two species.

Subsequently, the phylogenetic information was related to the agriculturally relevant crops produced in Hawai'i (http://www.nass.usda.gov), focusing particularly on crops with known wild relatives. It is unlikely for species belonging to different

tribes to exhibit cross-compatibility (G. Carr, personal communication). In this way it was possible for us to further hone the list of native species potentially at risk. The family and tribe of each relevant agricultural product, as well as the native genera found in each tribe are cross listed (Table 1). Subsequently cross compatibility was determined between the native genera and the agricultural product (i.e. physiological crossing barriers) to determine if potential for hybridization exists between the two species. An evaluation was then made of the locations of native species relative to regions where agricultural crops are being grown (i.e. the role of spatial distance on gene flow) (Ellstrand, 2003). The relationship and potential for hybridization is discussed individually for each crop.

Table 1. Phylogenetic relationship between agriculturally significant crops and native plant genera. Tribes showing overlapping agricultural crops and native plant genera are shown in bold.

Family	Tribe	Agricultural Product	Genera containing native species
Araceae	*Colocasieae*	Taro- *Colocasia*	
Poaceae	*Oryzeae*	Rice- *Oryza*	
	Triticeae	Wheat- *Triticum*	
	Andropogoneae	Sugarcane- *Saccharum*	
	Maydeae	Maize- *Zea*	
	Agrostideae		*Agrostis, Calamagrostis, Deschampsia*
	-----		*Cenchrus*
	Andropogoneae		*Chrysopogon, Ischaemum*
	Paniceae		*Dichanthelium*
	-----		*Dissochondrus*
	Eragrosteae		*Eragrostis*
	Poeae		*Festuca, Poa, Trisetum*
	-----		*Isachne*
	Leptureae		*Lepturus*
	Paniceae		*Panicum*
Bromeliaceae	-----	Pineapple- *Ananas*	
Musaceae	-----	Banana- *Musa*	
Fabaceae	***Phaseoleae***	**Soybeans- *Glycine***	***Canavalia, Erythrina, Mucuna, Strongylodon, Vigna***
	Acacieae		*Acacia*
	Caesalpinieae		*Caesalpinia*

	Mimoseae		*Kanaloa*
	Robinieae		*Sesbania*
	Sophoreae		*Sophora*
	Vicieae		*Vicia*
Caricaceae	-----	Papaya- *Carica*	
Brassicaceae	*Brassiceae*	Rapeseed- *Brassica*	
	Lepidieae		*Lepidium*
Malvaceae	***Gossypieae***	**Cotton- *Gossypium***	***Gossypium, Kokia***
	Abutileae		*Abutilon*
	Hibisceae		*Hibiscus*
Rubiaceae	*Coffeae*	Coffee- *Coffea*	
	Bobeinae		*Bobea*
	Canthieae		*Canthium*
	-----		*Coprosma*
	Gardenieae		*Gardenia*
	Hedyotideae		*Hedyotis*
	Morindeae		*Morinda*
	Spermacoceae		*Nertera*
	Psychotrieae		*Psychotria*
Solanaceae	-----	Tobacco- *Nicotiana*	
	-----	**Potato- *Solanum***	***Solanum***
	Lycieae		*Lycium*
	-----		*Nothocestrum*
Asteraceae	***Heliantheae***	**Sunflower- *Helianthus***	***Argyroxiphium**, Bidens, Dubautia*, Lipochaeta*, Wilkesia****
	Artemisieae		*Artemisia*
	Vernonieae		*Hesperomannia*
	-----		*Lagenifera**
	-----		*Remya**
	-----		*Tetramolopium**

* Genera currently in the family: Compositae. Some will be considered as *Heliantheae* for reasons based on morphology, historical classification, and their potential for intergeneric hybridization.
----- No tribal classification.

Sunflower (*Helianthus annuus* L.)

Native Genera: Argyroxiphium (DC)- {Hawaiian: *ʻĀhinahina*}, Silversword, ʻEke silversword, Kaʻū silversword; Bidens (L.)- {Hawaiian: *Koʻokoʻolau, koʻolau*}, Spanish needle, beggartick; Dubautia (Gaud.)- {Hawaiian: *Naʻenaʻe, kūpaoa*}; Lipochaeta (DC)- {Hawaiian: *Nehe*}; Wilkesia (A. Gray)- {Hawaiian: *Iliau*}.

Factors affecting gene flow from sunflower to native genera

Cultivated sunflower has been shown to be cross-compatible with wild *H. annuus* and other species in the genus, which are largely endemic to western North America (Rogers *et al.*, 1982).Hand pollinations between wild sunflowers and cultivated sunflowers have resulted in fertile hybrids (Burke *et al.*, 2002). Spontaneous hybridization at substantial rates and over distances of up to 1,000 m from the crop has been detected. 10% (299 of 3,000) of seeds tested for hybridization with wild *H. annuus* planted at various distances were hybrid individuals (Arias and Rieseberg, 1994). In some crosses, the fertility of the hybrid plants did not vary significantly from the purely wild plants (Snow *et al.*, 1998). Introgression of crop alleles into wild *H. annuus* is likely to be impeded rather than prevented by the lower fitness of the hybrids (Ellstrand *et al.*, 1999).

Both cultivated and wild sunflowers are pollinated primarily by honey bees. Bumble bees, wild bees, and other insects may also be pollinators (http://www.iia.msu.edu). Similarly, *A. sandwicense* is pollinated by a bee belonging to the genus *Nesoprosopis* spp., whereas *Bidens* spp. is visited by bees, moths, and butterflies for pollination. Sunflower pollen is spiny, and thus well adapted to being transported by insects and therefore may be transported over great distances. Since the pollen is relatively heavy, wind pollination is negligible (Fick, 1978). A shared pollinator may effect intergeneric hybridization, even at great distances.

Regulations implemented for transgenic plants by the United States Department of Agriculture - Animal and Plant Health Inspection Services (USDA-APHIS), requires a separation distance of ~800 m between hybrid (or non-hybrid) sunflowers and a source of pollen which has the potential to hybridize with these, as well as a distance

of ~1,600 m between oil and non-oil sunflower types and between either type and other volunteers or wild types (http://www.aphis.usda.gov).

In order to evaluate the native species that fall into areas where *Helianthus* pollen may be found, a distribution analysis was performed for the islands of Kaua'i, O'ahu, Maui, and Hawaii. It was found that *Argyroxiphium* and *Wilkesia* do not naturally occur in any regions where viable sunflower pollen is likely to be found at any significant levels, however, many populations of *Bidens, Dubautia*, and *Lipochaeta* spp. fall directly within the agricultural areas, or close enough to the borders, to potentially be at risk.

Although cultivated sunflower generally matures more rapidly than wild sunflowers, they often exhibit some degree of phenological overlap (Burke *et al.*, 2002). Due to the climatic conditions in the Hawaiian Islands, sowing of crops is not generally restricted by seasonal factors. Consequently, *Helianthus* may potentially be in flower at any time of the year, and the native plant genera have variable flowering times which may coincide with *Helianthus* flowering

Cotton (*Gossypium barbadense* L.; *G. hirsutum* L.)

Native Genera: Gossypium (Nutt. ex Seem.)- {Hawaiian: *Ma'o, huluhulu*}; Kokia (Lewton)- {Hawaiian: *Koki'o, hau hele 'ula*}.

Factors affecting gene flow from cotton to native genera

Fryxell (1979) suggested, on the basis of morphology, that *G. tomentosum* is at risk of extinction as a result of hybridization with *G. hirsutum.* The ability of these species to hybridize has been demonstrated in breeding programs

(http://www.aphis.usda.gov). The two species interbreed freely as they are closely related, and swamping of the genotype increases the risk of extinction.

G. tomentosum is reported to be cytogenetically similar to and equidistant from *G. barbadense* and *G. hirsutum* (DeJoode and Wendel, 1992). Ironically, although all the information suggests a threat to Hawaiian cotton from *G. hirsutum*, the only reported natural hybridization was between *G. tomentosum* and *G. barbadense* in western O'ahu (Stephens, 1964). Intermediacy was found in the hybrid with respect to pubescence, leaf nectarines, corolla colors, and petal spots. A population of hybrids (*G. barbadense* x *G. tomentosum*) is known from the Nanakuli area (western O'ahu) (C. Morden, personal communication).

Cultivated cotton is a facultative self-pollinator and an opportunistic out-crosser when insect pollinators are present (Oosterhuis and Jernstedt, 1999; Free, 1993). Cotton pollen is relatively large and heavy, and not easily dispersed by wind (Fryxell, 1979; Jenkins, 1992). Insects, especially honey bees (*Apis mellifera* L.), are considered the major vectors of cotton pollen (Mamood *et al.*, 1990). The pollen remains viable for approximately 12 hours after release (Govila and Rao, 1969). Gene flow from transgenic to non-transgenic cotton has been observed in several cases through pollen dispersal studies. The transgene was found in the progeny seed at a significant value in the first border row, but this value dropped below 1% within 7 m. A low level of outcrossing (<1%) could be measured sporadically to about 25 m (Umbeck *et al.*, 1991). In a similar Australian study, the outcrossing distance was even shorter (Llewellyn and Fitt, 1996).

The USDA-APHIS regulation previously required a minimum isolation of ~30 m, if the contaminating cotton source differs by easily observable morphological characteristics from the field to be inspected. The isolation distance was ~200 m for Foundation, Registered, and Certified seed classes. A recent amendment to the regulation stipulates that, "a 12 m-wide perimeter of non-transgenic cotton could surround the transgenic plants to act as pollen sink for insect pollinators." The perimeter cotton would be disposed of by harvesting, disking and monitoring." (http://www.aphis.usda.gov).

Though *Gossypium* spp. are normally fully self-compatible, *G. tomentosum* has developed a very long style through evolution. This style elevates the stigmatic surface above the androecium making spontaneous self-pollination an extremely rare event. The result is that without a pollinator, *G. tomentosum* fails to set seed. Other evolutionary adaptations of *G. tomentosum* include: the absence of a dark petal spot, the presence of a distinctive glossy yellow petal color, the ability of the flowers to remain open during the night, and a weakly developed floral odor. These adaptations suggest that *G. tomentosum* is pollinated by moths (Fryxell, 1979).

K. kauaiensis is the only species in the *Kokia* genus that survives in its natural habitat, although this too is severely threatened. The other species in the genus survive as cultivated park or street specimens and are therefore unlikely to come into contact with a hybridizing pollen source.

In this distribution analysis, it was that found many populations of both *G. tomentosum* and *Kokia* spp. which fall into agricultural areas on the main islands of Hawai'i. Though their distribution is not extensive, the knowledge of the

hybridization potential (*G. tomentosum* x *G. barbadense*) and their low population numbers place *G. tomentosum* and *Kokia* spp. in a very precarious position.

Potato (*Solanum tuberosum* L.)

Native Genera: *Solanum* (L.)

Factors affecting gene flow from potato to native genera

The genus *Solanum* exhibits allopolyploidy amongst many of its species. *S. tuberosum* ssp. *tuberosum* L., an allohexaploid (2n = 72) is grown worldwide. *S. tuberosum* ssp. *andigena* Juz. *et* Bu., an allotetraploid (2n = 48), is cultivated mainly in Mexico and Central and South America (Hanneman, 1994). The Hawaiian *Solanum* species are allodiploids (2n = 24) (Wagner *et al.*, 1999).

Potato can self pollinate or cross pollinate (McPartlan and Dale, 1994). Early spring potatoes may be harvested before flowering, however, for maximum yields harvesting does usually not occur before the vines have begun to die (Marr, 1992). Potato flowers are unattractive to honeybees as they produce no nectar, and bumblebees (*Bombus* spp.) have been observed as the main pollinators of potatoes (McPartlan and Dale, 1994). Another important pollinator of *S. tuberosum* is the potato beetle (*Meligethes aeneus*) (Skogsmyr, 1994). Many potato cultivars are fertile while other cultivars are fully or partially sexually sterile. This sterility may limit opportunities for hybridization (Ellstrand *et al.*, 1996).

Most tuber-bearing *Solanum* species are cross compatible; although *S. tuberosum* is strongly incompatible with tuber-bearing *Solanum* species in the United States and Canada (Love, 1994). No evidence has been reported of naturally occurring intergeneric hybrids with *Solanum* as one parent.

Isolation barriers are strong between *S. tuberosum* and the thousands of non-tuber-bearing *Solanum* species (Ellstrand, 2003). McPartlan and Dale (1994), performed a pollination study in England to determine whether *S. tuberosum*_will spontaneously mate with two non-tuber-bearing species, *S. nigrum* and *S. dulcamara*. No hybridization was detected between the cultivated and these non-tuber-bearing *Solanum* species. No studies regarding the hybridization between *S. tuberosum* and the native Hawaiian *Solanum* species have been published.

Pollen dispersal in potatoes is dependant on the potato cultivar fertility, the type of pollinator, and the prevailing weather conditions (Treu and Emberlin, 2000). McPartlan and Dale (1994), found gene flow between transgenic and non-transgenic "Desiree" plants to be 2% at 3 m distance from the pollen source, and 0.017% at 10 m. The USDA-APHIS, recommends an isolating distance of ~9 m between male-fertile transgenic potato plants and non-transgenic varieties to prevent gene flow from occurring (http://www.aphis.usda.gov).

S. tuberosum does not represent a major agricultural crop in Hawaii (http://www.nass.usda.gov). The concern is based on subsistence cultivation of potatoes by homeowners. This cultivation could potentially aid pollen dispersal into areas where wild *Solanum* species may be encountered. A distribution analysis was performed between the major agricultural areas and the reported *Solanum* populations in order to provide an overview of populations that could potentially be at risk. Areas where both native and cultivated *Solanum* species may overlap and where potential exists for hybridization on all of the major islands were identified.

Soybeans (*Glycine max* L.)

Native Genera: *Canavalia* (Adans.) *Erythrina* (L.)- *Mucuna* (Adans.)- *Vigna* (Savi)-

Factors affecting gene flow from soyabeans to native genera

Cultivated soybeans (*G. max*) represent one of the few known members of the tribe: *Phaseoleae* that show stabilized polyploidy. Other members in this tribe generally have diploid genomes with 2n = 20 or 22 (e.g. *Canavalia* spp., *Mucuna* spp., *Vigna* spp.), with the exception of *Erythrina* spp. (2n = 42) which is apparently an ancient allotetraploid (Lackey, 1980). Interspecific hybridization resulting in wild plants morphologically intermediate to *G. max* and *G. soja* has been shown to spontaneously occur near Asian fields when *G. soja* is present (Abe *et al.*, 1999). The resulting stabile hybrid (*G. gracilis*) is intermediate to and interfertile with both *G. max* and *G. soja* (Ellstrand, 2003; Nakayama and Yamaguchi, 2002). No report was found of a natural intergeneric hybridization with *G. max* as the pollen parent.

Since the cultivated soybean is a predominantly self pollinated species (Nakayama and Yamaguchi, 2002), pollen drift is unlikely to play a major role in hybridization with wild relatives. Cross-pollination rates ranging from 0.03% to 0.44% at 1 m from the pollen source were observed in a three-year study in Arkansas (Caviness, 1966). At distances of more than ~4.5 m from the pollen source, natural cross-pollination was rare and did not vary greatly. However, during this study it was also shown that it is not possible to completely eliminate cross-pollination at distances greater than 4.5 m (Caviness, 1966). Furthermore, pollination normally occurs prior to the flower opening (Boerma and Moradshahi, 1975), thereby further reducing the potential for cross-pollination. Nevertheless studies have shown that soybeans may be pollinated by insect vectors, the most likely being the honey bee (*A. mellifera*). Bumble bees and pollen feeding thrips may also act as pollinators (Nakayama and Yamaguchi,

2002). Pollination studies performed investigated the pollen movement within and between rows to male-sterile soybeans suggest that a distance of approximately 18 m would be sufficient to restrict pollen contamination below a significant level (Boerma and Moradshahi, 1975). The highest rate of cross-pollination recently reported was when white flowered and purple flowered *G. max* cultivars were alternately grown within a row (15.2 cm apart). The dominance of purple flower color over the white color was used to identify cross-pollination and the rates ranged from 0.65 to 6.32%, with an average of 1.8%. The rates of cross-pollination was significantly less (0.03 to 0.05%), when the white flower color cultivar was grown at a distance greater than 5 m from the purple color cultivar in natural crosses between rows. These results are comparable to values previously reported (Ray *et al.*, 2003).

Regulations concerning isolation distances between transgenic soybean varieties and non-transgenic varieties, implemented by the USDA-APHIS, require a distance adequate to prevent mechanical mixture (http://www.aphis.usda.gov).

Since no information was found concerning the possibility of intergeneric hybridization, it was assumed that this represents a potential risk to the native Hawaiian plant species, until shown otherwise. For this reason a distribution analysis of the five native Fabaceae species in relation to the major agricultural areas was performed. In general overlapping areas where both native and cultivated plants may be present are on all the major islands, and though cross pollination does not occur over vast distances, some species may still be at risk.

FINAL CONCLUSIONS AND RECOMMENDATIONS

In this study, four agricultural crops, namely sunflowers (*H. annuus*); cotton (*G. barbadense*, *G. hirsutum*); potatoes (*S. tuberosum*); and soybeans (*G. max*) were considered for their potential to hybridize with native relatives in the Hawaiian archipelago. Tribal boundaries were considered to be a primary limit to hybridization potential since, at greater intergeneric distances, hybridization has not been documented for any taxa. Genotypic relationship is not the only factor that determines whether gene flow is likely to occur. Biological barriers, physical barriers and environmental factors all play a role in limiting or enhancing gene flow by vertical gene transfer (Grant, 1975; Ellstrand *et al.*, 1999; Wolf *et al.*, 2001).

Insular species are at greater risk of extinction than mainland populations (Levin *et al.*, 1996; Wolf *et al.*, 2001), and hence hybridization is a potential threat. Various factors contribute to a higher extinction rate of insular species, including inbreeding depression, a loss of genetic variation through genetic assimilation, or genetic adaptation to island conditions. Insular species are coming into contact with non-native species at higher frequencies due to human processes that increases the potential for gene flow between these groups (Levin *et al.*, 1996).

The USDA-APHIS suggests isolation distances between transgenic and non-transgenic plant species, to minimize the potential for hybridization to occur. It is recommended that the USDA-APHIS isolation distances be strictly employed to separate any agricultural crop from native Hawaiian plant relatives. Further, laboratory pollination studies should be performed for the crops and native genera that were considered in this report to confirm whether cross-pollination is possible among these species.

It is crucial to determine whether hybridization may occur under natural conditions, while hand pollination should provide sufficient evidence to determine if hybrid progeny are produced. Any hybrid progeny should be evaluated for fertility. Although hybridization seems unlikely, quantifying this risk with experimental data is important.

In addition to the agricultural products discussed in this study, many native plant species have relatives that have become naturalized in the Hawaiian Islands. One such example is the genus *Solanum.* It seems unlikely for *S. tuberosum* to hybridize with any of the four native members of the *Solanum* genus, however, of the eleven naturalized non-tuber-bearing *Solanum* species that exist in the Hawaiian archipelago all may have a higher likelihood to hybridize to the native species. Often the naturalized relatives share habitats that bring them into close proximity of indigenous species. It is recommended that hybridization and introgression between these groups be further investigated.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
93~100

Regulatory Framework of Genetically Modified Plants in Japan

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ABSTRACT

Japan regulates genetically modified plants within a framework of safety evaluations related to their consumption as food or animal feed and their impact on biological diversity. The evaluations are conducted by the Food Safety Commission under the Cabinet Office, the Agricultural Materials Council under the Ministry of Agriculture, Forestry, and Fisheries (MAFF), and the Biodiversity Impact Assessment Group under the MAFF and the Ministry of the Environment. Japan adopts the point of view that genetic modification technology shall be "used with close attention to its safety". As of 2006, genetically modified plants are not cultivated in Japan. However large amounts of genetically modified crops, such as soybeans, corn, rapeseed, and cotton, are imported into Japan as commodities. It is ironic that the special attention given to genetically modified components in the labeling requirements for processed foods may be impeding public acceptance of genetic modification.

Keywords: GMO; Regulation; Food; Feed; Environment; Public acceptance (PA).

INTRODUCTION

Genetic modification technology holds hopes for providing technological solutions to social issues on the one hand and uncertainties on the other because it is a new technology with which we have little historical experience. Society should recognize that although this technology may be beneficial to humans, its impact on the health of humans and animals alike, and particularly on the environment, also needs to be scientifically evaluated so that the technology can be used in conjunction with appropriate control systems. In order for the government to respond to such demands in a responsible manner, it must establish a framework and provide it with laws and other regulatory means as well as adopting approaches and specific methods to perform impact assessments. This report describes the regulatory framework for genetically modified plants in Japan.

Japan's Basic Point of View on the Regulation of Genetically Modified Plants

Japan has adopted the position that genetic modification technology shall be "used with close attention to its safety". This means that it will use genetic modification technology with an understanding of its potential and importance and with valid confirmation of its safety to the environment and health based on the latest scientific findings. Furthermore, it is the government's basic policy to disclose accurate information to the public in an appropriate manner and ask the public to participate in discussions on safety issues. This policy conforms to global trends. Based on such a point of view, the MAFF promotes studies of genetic modification for the purpose of putting genetically modified organisms to practical use. The ministry also conducts safety evaluations during development as well as use. In response to criticisms that information on genetic modification technology has been scarce and difficult to understand, the government plans to conduct surveys so that it can provide accurate

and easy-to-understand information. The results of the surveys will be used for public communications and will be reflected in government policies to promote public understanding of the importance and safety of genetic modification technologies.

Japan's Regulatory Programs for Genetically Modified Plants

In Japan, genetically modified plants are examined for their safety both as food and as feed, as well as their impact on biodiversity.

(1) Safety as food

The safety of genetically modified plants for food use is examined according to the ***Basic Law for Food Safety*** by the Food Safety Commission under the Cabinet Office. Information, however, is disseminated by the Ministry of Health, Labor, and Welfare. Food derived from genetically modified plants may be imported for market distribution only if the Food Safety Commission has confirmed its safety. Stacks, combinations of different events, are also subject to examination. Major items examined include:

- ∞ The safety of host plants, genes used for modification, and vectors;
- ∞ Genes used for modification;
- ∞ The safety of proteins produced as a result of genetic modification, particularly their allergenicity (allergenicity is assessed by the ease of breakdown of proteins and comparisons with amino acid sequences of known allergens);
- ∞ The potential for unexpected transmutations as the result of genetic modification; and
- ∞ The potential for significant changes in the nutrient content of food.

As of August 15, 2006, the Commission had confirmed 75 food items to be safe.

(2) Safety as animal feed

The safety of genetically modified plants for use in animal feed is examined according to the ***Law Concerning the Safety and Quality Improvement of Feed*** (known as the ***Feed Safety Law***) by the Agricultural Materials Council under the MAFF. Feed derived from genetically modified plants may be imported and sold only if the Agricultural Materials Council has confirmed its safety. Stacks are excluded from the examination. Major items examined include the following:

- ∞ Any significant changes in components of feed in comparison with existing crops; and
- ∞ The potential for the production of toxic substances (ranging from the potential production of toxic substances in genetically modified plants to the potential production of new toxic substances in animals by the action of a genetically modified component on the metabolic systems of the animals).

As of March 31, 2006, the Council had confirmed the safety of 47 items.

(3) Impact on biodiversity

As a result of the November 21, 2003 ratification of the Cartagena Protocol on Biosafety of the Convention on Biological Diversity, which came into effect internationally on September 11 of that year, Japan has developed a legal basis framework with respect to the potential impacts of genetic modification on biodiversity. Although a protocol of an international convention is not binding in itself, signatories to the convention are required to develop a regulatory framework using domestic laws to ensure that the protocol rules are observed and implemented in their countries. Japan enacted the ***Law Concerning Securing of Biological***

Diversity by the Regulation of the Use of Genetically Modified Organisms (known as the ***Cartagena Law***) on February 19, 2004 to implement assessment requirements to deal with the impacts of genetically modified organisms on biodiversity.

The impacts of genetically modified plants on biodiversity are examined according to the above-mentioned ***Cartagena Law*** by the Biodiversity Impact Assessment Group under the joint jurisdiction of the MAFF and the Ministry of the Environment. Genetically modified plants are divided into two classes according to use: class 1 plants are used outdoors, while class 2 plants are used in an indoor environment equipped with measures to prevent their spread. So far there have been no examples of class 2 plants; all plants classified to date have been class 1. Class 1 plants are further divided into segregated cultivation for limited growing periods and general cultivation.

Japan allows only plants which have been confirmed to be safe by the Biodiversity Impact Assessment Groupfor general cultivation outdoors and for market distribution. Ornamental foliage plants, which are not used for food or feed, are also subject to examination, as well as stacks. Major items examined include the following:

- ∞ Competitive superiority;
- ∞ Potential production of toxic substances; and
- ∞ Cross-fertility.

As of September 5, 2006, 31 items had been approved for general cultivation in Japan.

Table 1 summarizes the framework for the evaluation of genetically modified plants for their safety as food and feed as well as their impacts on biological diversity.

Current general acceptance of genetically modified plants in Japan

As of 2006, there is no cultivation of genetically modified plants in Japan. A certain type of genetically modified carnation is sold, which used to be cultivated in Japan but is now grown in Colombia and imported into Japan. One of the reasons that genetically modified plants are not cultivated in Japan is the lack of public acceptance of them for food and cultivation.

Nevertheless, Japan, the world's largest importer of agricultural products, imports large amounts of genetically modified soybeans, corn, rapeseed, and cotton as commodities, and uses them in animal feed, food oils, and processed food. The public is generally unaware of this fact.

Labeling problem

Labeling regulations require the disclosure of any genetically modified ingredients, such as soybeans, used in processed food. Labeling of non-use of such ingredients is optional. In reality, a large majority of labels emphasize the "non-use" of genetically modified ingredients. Expressions such as "no genetically modified ingredients" appear to be used as a commercial catchword. This practice may be persuading consumers that genetic modification is harmful.

Issues

Economidis (2006) of the European Commission summed up the results of 20 years of studies on the safety of GMOs by saying "GMOs as well as non-GMOs, are neither inherently risky nor inherently safe; whether or not the application with an organism poses a risk to human health or the environment depends on the

characteristics of the organism and the circumstances of the application", and acknowledged that risks associated with GMOs were no greater than risks inherent in the host organisms. Public acceptance, however, is a prerequisite for the use of genetically modified plants. Regulations are relatively loose in the United States where the acceptance of GMO crops is well advanced. In Japan, however, the lack of public tolerance has resulted in a relatively stricter regulatory framework. This strictness, in turn, has created the undeniable impression that the GMO plants are dangerous. A dilemma of increasingly tighter controls and the reluctance to accept GMO organisms on the part of the public remain an issue.

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Table 1. Japanese framework for an examination of the safety of genetically modified plants for food and feed use and the assessment of their impacts on biological diversity

	Examining body	Jurisdiction	Governing law	Major item examined
Safety as food	Food Safety Commission	Cabinet Office	Basic Law on Food Safety	∞ Safety of host plants, genes used for modification, and vectors ∞ Genes used for modification ∞ Safety of proteins produced as a result of genetic modification, particularly their allergenicity. (allergenicity is assessed by the ease of breakdown of proteins and comparisons with amino acid sequences of known allergens) ∞ Potential for unexpected transmutations as the result of genetic modification ∞ Potential for significant changes in the nutrient content of food
Safety as animal feed	Agricultural Materials Council	Ministry of Agriculture, Forestry, and Fisheries	Law Concerning the Safety and Quality Improvement of Feed (the Feed Safety Law)	∞ Any significant changes in components of feed in comparison with existing crops ∞ Potential for the production of toxic substances (ranging from the potential production of toxic substances in genetically modified plants to the potential production of new toxic substances in animals by the action of a genetically modified component on metabolic system of the animal)
Impact on biodiversity	Biodiversity Impact Assessment Group	Ministry of Agriculture, Forestry, and Fisheries Ministry of the Environment	Law Concerning Securing of Biological Diversity by the Regulation of the Use of Genetically Modified Organisms	∞ Competitive superiority ∞ Potential production of toxic substances ∞ Cross-fertility

International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
101~110

Biosafety Assessment of Plants with Novel Traits in Canada

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ABSTRACT

Canada has a unique system for regulating products of agriculture biotechnology, based upon familiarity, substantial equivalence, and trait novelty. Plants with novel traits (PNTs) are regulated; such plants can be transgenic or non-transgenic. Safety assessments are required before a PNT can be used as a food, livestock feed, or released unconfined into the environment. This paper outlines the regulation of PNTs, environmental safety assessments of PNTs, and a case assessment study of herbicide-resistant *Brassica napus* canola in Canada. Since 2005, 62 PNTs have been authorized for environmental release, possessing such traits as herbicide resistance, insect pest and virus resistance, and oil compositional changes. Future challenges in regulation include the interpretation of novelty, new classes of PNTs (e.g., stress tolerance, molecular farming, biofuels production), and evaluating long-term and non-target effects.

Key words: *Brassica napus*; Biosafety risk assessment; Transgenic crop.

INTRODUCTION

Canada is the fourth largest producer of transgenic crops, after the United States, Argentina, and Brazil (Beckie *et al.* 2006). Canada regulates plants with novel traits (PNTs). A PNT is defined as "a plant containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species in Canada. PNTs may be produced by conventional breeding, mutagenesis, or more commonly, by recombinant DNA (rDNA) techniques" (CFIA 2004). The primary assumption underlying this approach is that there are similar environmental risk issues regardless of the process of developing a PNT. Therefore, in contrast to other countries, Canada regulates a broader range of plants, including those with traits selected following mutagenesis or introgressed following outcrossing. There are two main concerns by industry or breeders of this regulatory approach. A plant not produced by rDNA technology is subject to regulation, whereas the same plant in other countries is not. Secondly, products requiring regulation are not as easily identified as with a process designation (i.e., products produced using rDNA techniques), causing uncertainties among cultivar developers.

Canada's regulatory approach is based on the principles of familiarity (familiar counterpart as a comparator), substantial equivalence (defines the threshold of risk), and trait novelty. 'Novel' does not necessarily equate with 'risk'. A product may be considered novel if it: (1) possesses a new trait(s) or characteristic(s) – e.g., herbicide resistance, insect resistance; (2) possesses a modified trait(s) or characteristic(s) – e.g., disease resistance outside the range of normal variation within that species; or (3) is considered for a new use (e.g., as a food or livestock feed). Most products of

breeding programs would not be considered PNTs. Similarly, many products of mutagenesis may not be considered PNTs (e.g., trait changes within normal ranges). Risk assessment focuses on unintended, not unexpected effects. This paper outlines the regulation of PNTs, environmental safety assessments of PNTs, and a case assessment study of herbicide-resistant (HR) Brassica napus canola in Canada.

Regulation of Plants with Novel Traits in Canada

Mandatory, pre-market safety assessments are required by government regulators before a PNT can be used as a food, livestock feed, or released unconfined into the environment. Therefore, PNTS are assessed based on potential impact on the environment, potential impact on livestock health, and potential impact on human health. The Plant Biosafety Office (PBO) of the Canadian Food Inspection Agency (CFIA) is responsible for the regulation of the environmental release of PNTs and feed assessments (Fig. 1). CFIA responsibilities include approval and inspection of confined field trials of PNTs, approval of unconfined release of PNTs, and assessment of import applications for PNTs. Health Canada conducts food safety assessments. Food, feed, and environmental release authorizations are linked; all three are required before a PNT is approved.

The key element of the Canadian federal regulatory framework for PNTs is to use existing legislation and regulatory institutions; there is no "Gene Act" or "Biotechnology Agency" (Fig. 1). Fig. 2 outlines the regulatory approval process for PNTs. The main concepts for the regulation of PNTs are: (1) science-based and product-based; (2) case-by-case; (3) regulatory transparency (decision documents and consultations available online); (4) harmonized approvals between Canadian regulators and bilateral initiatives with the United States (i.e., data requirements); and

(5) research in support of the regulatory system.

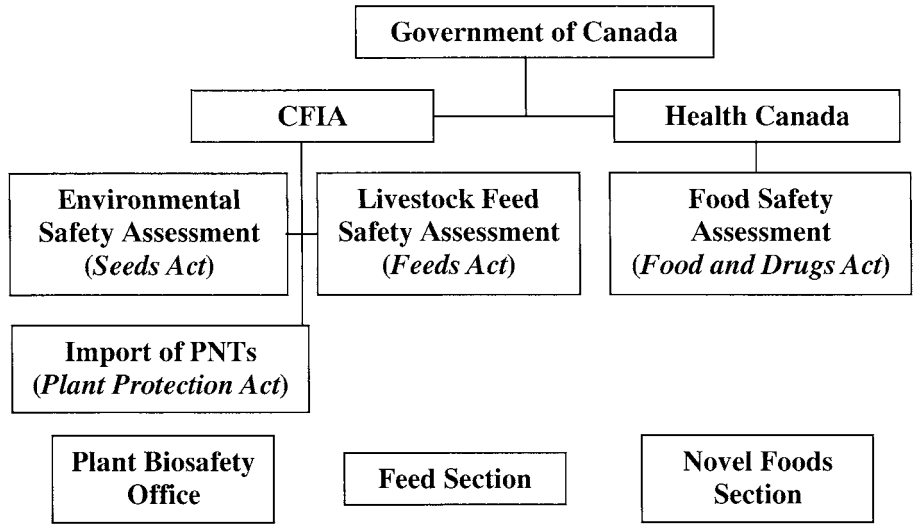


Fig. 1. Regulation of plants with novel traits in Canada using existing legislation.

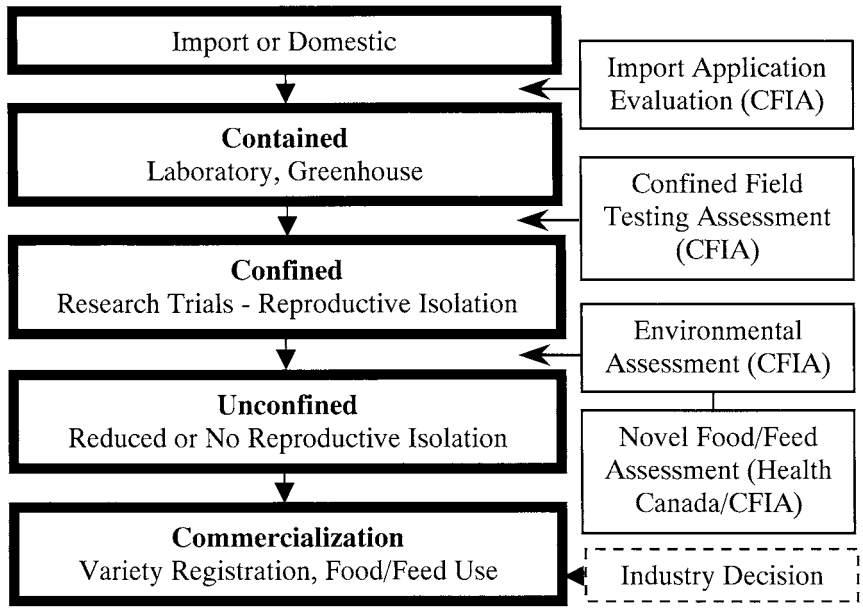


Fig. 2. Regulatory approval process for plants with novel traits in Canada.

Approvals for unconfined environmental release of PNTs have been granted since 1995. Since 2005, 62 PNTs (52 of which are transgenic) have been authorized for environmental release. These PNTs include canola (oilseed rape, *Brassica napus*; turnip rape, *B. rapa*), corn, soybean, wheat, cotton, flax, lentil, potato, soybean, squash, sugar beet, and tomato. Traits include herbicide resistance; insect pest and virus resistance; hybrid systems; and oil compositional changes. The actual release is a commercial decision. The time to commercialize a PNT in Canada has been 8 to 10 years (Beckie *et al.* 2006).

The first approved PNTs possessed enhanced input traits, mainly herbicide resistance, that enabled them to tolerate the application of selective (imidazolinones, IMIs; bromoxynil, BX) or non-selective (glyphosate, GLY; glufosinate, GLU) herbicides. The GLY-, GLU-, and BX-HR crops in Canada were produced by rDNA techniques through the insertion of bacterial genes (i.e., transgenic), whereas IMI resistance was attained by chemical mutagenesis (i.e., non-transgenic) (CFIA 1995a, 1995b, 1995c, 2004). Five field crops are currently grown commercially in Canada (Table 1). Corn hybrids may have stacked traits (i.e., HR and *Bt*). Canola and spring wheat are grown predominantly in western Canada, whereas soybean and corn are grown mostly in eastern Canada. Canola occupies over 80% of the area cultivated to novel-trait crops, followed by soybean, corn, and wheat in order of decreasing area (Beckie *et al.* 2006).

Table 1. Field crops with novel traits grown commercially in Canada since 1995 (NA, not required; adapted from Beckie et al. 2006).

Species	Herbicide resistance	Variety registration	Regulatory approval	Breeding system
Canola, *Brassica napus*	Glyphosate	Yes	Yes	ca. 30% outcrossing
	Glufosinate	Yes	Yes	
	Imidazolinone	Yes	Yes	
	Bromoxynil	Yes	Yes	
Soybean, *Glycine max*	Glyphosate	Yes	Yes	Highly selfing
	Glufosinate	No	Yes	
Corn, *Zea mays*	Glyphosate	NA	Yes	Highly outcrossing
	Glufosinate	NA	Yes	
	Imidazolinone	NA	Yes	
	Sethoxydim	NA	Yes	
Wheat, *Triticum aestivum*	Imidazolinone	Yes	Yes	Highly selfing
Lentil, *Lens culinaris*	Imidazolinone	Yes	Yes	Highly selfing

Environmental Safety Assessments

PNTs may be granted approval for confined environmental release (i.e., field research trials), which may be followed by unconfined environmental (commercial) release. Confined environmental release comprises risk mitigation measures, plus inspection and monitoring. All confined field trials are restricted to a maximum of 1 ha per site, maximum of 10 sites per province, with a cumulative size of no more than 5 ha per province. Reproductive isolation distances are the following: *Brassica rapa* canola, 400 m; *Brassica napus* canola, 200 m; corn, 200 m; soybean, 10 m; wheat, 30 m; and potato, 1 m. Other options to reduce pollen flow include cages, nets, or bags, guard/border rows, or flower removal. The applicant is responsible for monitoring the trial site on a regular basis and for keeping the trial in compliance. The applicant must keep monitoring records. CFIA inspects all sites, unannounced, for compliance to the terms and conditions. All plant materials must not enter the food or feed chain without federal government approval must be stored securely if not disposed of, and may be disposed of in a manner which destroys viability. Post-harvest restrictions and monitoring include trial plots not planted to the same crop for a specified period of time (e.g., 3 years for *Brassica napus* canola, 1 year for corn), and each site must be monitored during the post-harvest growing seasons for volunteers and related/prohibited species. Applications for confined field trials peaked in 1997 (Figure 3). Figure 4 shows trait trends in such trials.

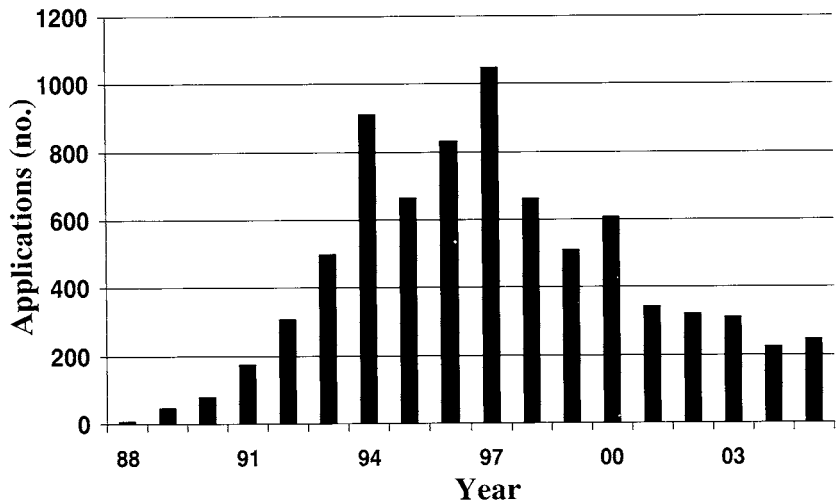


Fig. 3. Number of applications for confined field trials of PNTs in Canada.

Criteria for unconfined environmental release of a PNT includes: (1) potential to become a weed of agriculture or be invasive of natural habitats; (2) potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive; (3) potential to become a plant pest; (4) potential impact (including its gene products) on non-target species, including humans; and (5) potential impact on biodiversity. The applicant must address the identity and origin of the PNT; the properties of the novel gene and gene products; the relative phenotypic expression of the PNT compared to a similar counterpart, where differences are anticipated; and anticipated or known relative effects on the environment resulting from the release. Information regarding the PNT must include a description of the PNT; description of the modification of the PNT; description of the inheritance and stability of introduced traits functional in the plant; description of parental genome; number of generations removed from the original modification; and description of novel traits and how they were introduced. Information required for unconfined release assessment also includes the biology, ecology, and interactions of a PNT. Such information includes description of the biology of the plant species prior to modification; selection of a suitable counterpart; phenotype of the PNT; cultivation of the PNT; interactions with sexually-compatible species; residual effects and toxicity on non-target organisms; and other environmental interactions.

Information is needed to answer key questions, such as have you created a weed?; what are the consequences of gene flow?; does it cause disease in plants?; does it have unintended effects on non-target organisms? For intra-specific crosses, once a PNT is authorized, all its progeny and sister lines which have been derived from the original transformation and their respective progenies are also authorized for unconfined release provided the applicant has determined that no inter-specific crosses were performed, the intended uses are similar, and these plants do not display any additional novel traits. For inter-specific crosses, once a PNT is authorized, plants from the first inter-specific cross may require an environmental safety assessment unless the applicant has determined there is no new transformation event; the intended uses are similar; these plants do not display any additional novel traits based on their characterization; and the novel genes are expressed at a level similar to that of the authorized line. For intentional trait

stacking, the applicant must contact the PBO 60 days prior to the anticipated environmental release. The PBO may request and review data to support the safe use of the modified plant in the environment. Stacking of approved traits with potential incompatible management requirements, possible negative synergistic effects, or where production of the plant may be extended to a new area of the country may elicit an environmental safety assessment.

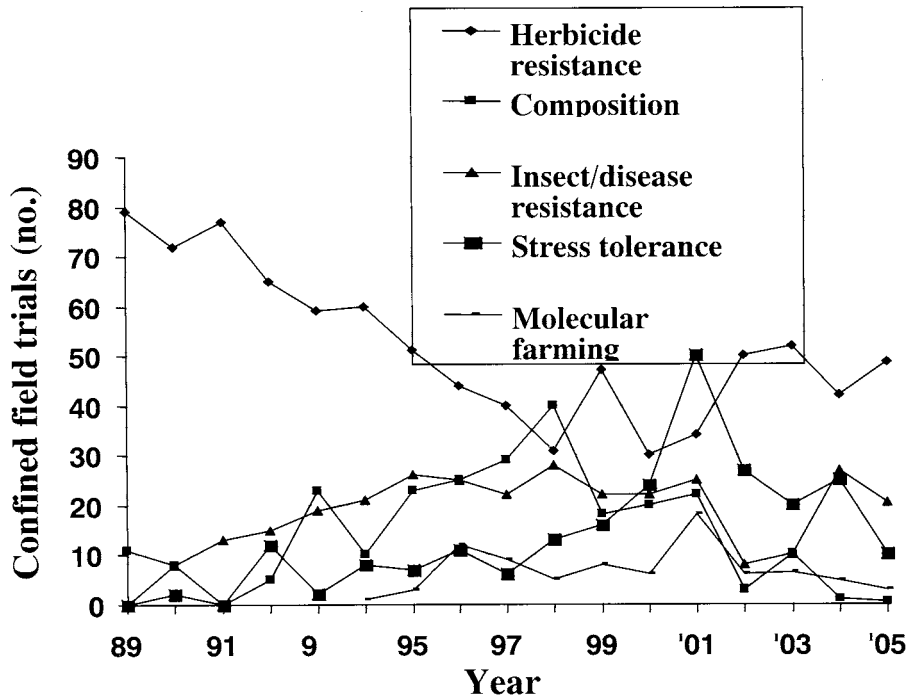


Fig. 4. Types of traits in confined field trials in Canada.

The applicant is required to outline stewardship plans (e.g., insect or herbicide resistance management, post-release monitoring). For herbicide resistance management, the applicant should address control of volunteers, changes in usual agronomic practices that may arise and result in reduced sustainability or have significant impacts on soil conservation; selection of herbicide resistance in weeds resulting from the potential continued application of the same herbicide mode of action in subsequent rotations; introgression of novel trait into related species; management of the HR crop during the growing season, particularly where multiple herbicide resistance due to cross-pollination could arise in volunteers in subsequent growing seasons;

communication to growers as well as an efficient mechanism allowing growers to report problems to the developer; and monitoring the effectiveness of the stewardship plan. Companies are required to report the results of their own monitoring activities to CFIA.

CASE STUDY

Biosafety Assessment of Herbicide-resistant *Brassica napus* Canola

Canola is grown primarily as an oilseed crop; after the oil is extracted, the meal is used as a livestock feed. Because significant populations of weedy bird's rape (*Brassica rapa*) is found in eastern Canada, glyphosate is used to control the weed, and outcrossing with *B. napus* canola occurs, approval of glyphosate-resistant *B. rapa* canola was restricted to western Canada. The PNT is no longer commercialized in Canada.

Sustainability underlines the decision-making process; the introduction of a new HR crop should have at least a neutral impact. In these environmental assessments (CFIA 1995a, 1995b, 1995c), the key environmental question was what advantage would be conferred outside of managed environments in the absence of selection by the specific herbicide? HR crops are not more fit; transfer of herbicide resistance does not confer fitness outside of managed environments (Beckie *et al.* 2006). What are the consequences of gene flow - e.g., the loss of the use of the herbicide for control of crop volunteers and possibly for related weeds? Other questions to address: are there suitable management options for volunteers and how does the new trait affect current crop management practices by both adopters and non-adopters?; are growers able to use the same crop management practices? will herbicide selection pressure for evolution of HR weeds increase significantly? Agronomic problems can become environmental problems. For HR canola, growers are using similar crop management techniques and similar rotations (Beckie *et al.* 2006). Gene stacking occurs in canola volunteers; this was acknowledged in the decision documents. However, such volunteers are not more difficult to control than non- or single-HR plants and can be managed without increasing herbicide use or tillage (Beckie *et al.* 2006); integrated weed management (e.g., crop and herbicide rotation) is essential in HR canola production systems to

mitigate herbicide resistance in weeds (Beckie 2006). To this end, growers must be made aware of proper herbicide resistance management practices and tactics.

FUTURE CHALLENGES

Future challenges in the regulation of PNTs are (1) to develop clear guidance on the interpretation of novelty that can be applied to all types of plants, e.g., crops, ornamental plants, and trees; (2) develop guidelines for molecular farming; (3) regulatory streamlining (import vs. domestic, PNTs vs. novel plants); (4) how to evaluate long-term and non-target effects (e.g., suitable indicator species, acceptable protocols); and (5) how to regulate new classes of PNTs, e.g., canola grown for biodiesel.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
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Biosafety Assessment of Transgenic Crops in the Soil Environment

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ABSTRACT

Transgenic papaya expressing the PRSV gene is the first transgenic crop developed in Taiwan. This paper describes the horizontal gene transformation (HGT) of the kanamycin-resistant gene, *nptII,* from transgenic papaya, and the persistence of transgenes in the soil. Two strains of *Acinetobacter* spp., BD413 and BD413 (pFG4Δ*nptII*), were used as recipient cells. Our experiments suggested that HGT of genomic DNA transfer might be less likely to occur in soil. The persistence of transgenes in the soil was evaluated by seven soil DNA extraction methods, and the method of using CTAB/SDS/gel (cetyltrimethylammonium bromide /sodium dodecyl sulfate/gel purification) was the best with 45.8% DNA recovery. The detection limit of transgenic DNA in soil for real-time polymerase chain reaction (PCR) was 0.3 ng/g soil (dry). Specific transgenic sequences inserted in the genome of transgenic papaya were detected in soil for 5 months, and the persistence of the 398-bp fragment (pBI121/NOS-t) in soil samples was less than 0.16 μg/g soil, whereas the residues of the 769- (35S-P/PRSV-CP) and 200-bp fragments (NOS-P/nptII) were less than 2.0×10^{-4} μg/g soil. Concentrations of the *nptII* gene in soil were very low; therefore, the risk for horizontal gene transfer of the *nptII* gene in soil is very low.

Key words: Papaya; Horizontal gene transformation; Persistence.

INTRODUCTION

Most genetically modified crops use the neomycin phosphotransferase gene (*nptII*) as a selection gene (Flavell *et al.*, 1992); this is the gene that encodes the enzyme which inactivates the drug kanamycin. The World Health Organization and Food and Agriculture Organization published several reports on the issue of the safety of genetically modified foods of plant origin in 2000, and suggested that the possibility of transfer of antibiotic-resistant genes to pathogenic microorganisms and possible clinical implications must be considered.

Selective marker genes of kanamycin resistance from genetically modified (GM) maize was retrieved by transformation of an *Acinetobacter* BD413 strain (de Vries and Wackernagel, 1998). Without the introduced homologous DNA in the recipient cell, no horizontal gene transformation (HGT) was detected.
Horizontal gene transfers from transgenic sugar beets were studied in the bacterium *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*), and the data showed that the bacterium took up and integrated transgenic sugar beet DNA under optimized laboratory conditions (Gebhard and Smalla, 1998).

Transgenic DNA may persist in soil for a long time. The released chromosomal and plasmid DNA in soil remains active during transformation from several hours (Recorbet *et al.*, 1993) to more than 2 months (Romanovski *et al.*, 1993). Gebhard and Smalla (1999) reported that long-term persistence of transgenic DNA from transgenic sugar beets could be found under field conditions for up to 2 years, and HGT from plants to bacteria was possible in soil microcosm conditions, but was not detected under field conditions.

Therefore, it is important to investigate the persistence of the transgenic *nptII* gene from genetically modified crops in soil, because transgenic genes which bind to soil particles can resist degradation, and retain biological activities for genetic transformation when incorporated in pathogenic microorganisms.

Papaya ringspot virus (PRSV) is the most destructive disease of papaya (*Carica papaya* L.) in Taiwan, and transgenic papaya resistant to PRSV was thus developed here (Cheng *et al.*, 1996). The coat protein gene of the PRSV (PRSV CP) and the neomycin phosphotransferase gene, *nptII*, of pBI121 were included in a Ti-binary vector, and then the CP gene was transcribed by the cauliflower mosaic virus (CaMV) 35S promoter (35S-P) and NOS terminator (NOS-T) (Fig. 1).

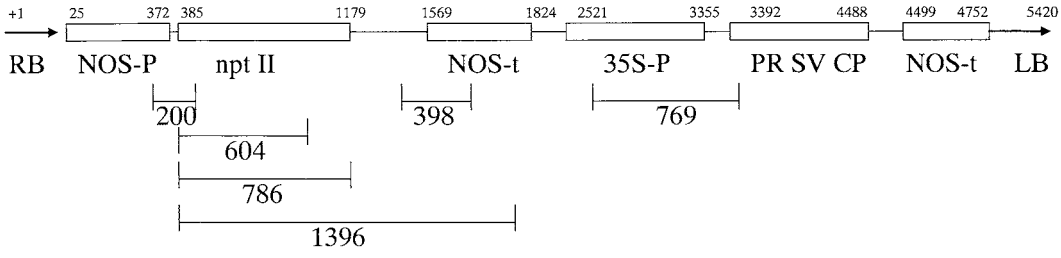


Fig. 1. Genetic construct in the transgenic papaya. PCR amplification of regions of 200, 398, 604, 769, 786 and 1396 bp are shown.

The objectives of this study were to evaluate the potential for HGT of the *nptII* gene from transgenic papaya (genomic DNA and PCR products of 604, 786, and 1396 bp), and to monitor the persistence of transgenic papaya DNA in soil where transgenic papaya has been grown. Transgenic DNA sequences were quantified by real-time polymerase chain reaction (PCR), and fragments of transgenic genes of 769-bp of the 35S-P/CP region, 398-bp (pBI121/NOS-T), and 200-bp of the NOS-P/nptII region were analyzed.

MATERIALS AND METHODS

Horizontal Gene Transformation

Genomic papaya DNA and PCR products of 604, 786, and 1396 bp amplified from transgenic genomic papaya DNA were used as plant DNA for transformation.

Bacteria

Acinetobacter sp. BD413 was selected, because this bacterium contains no nptII sequence that can be used to promote homologous recombination with transgenic papaya DNA. *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) with a deletion of 313 bp in the plasmid-harbored *nptII* gene was use to test for homologous recombination from transgenic papaya DNA (Gebhard and Smalla, 1998). Transformation was conducted on a filter and in a soil microcosm (Nielsen *et al.*, 2000).

Plant DNA Extraction

Genomic DNA of transgenic papaya (line: 18-2-4) and nontransgenic papaya (TA-2) were extracted by the cetyltrimethylammonium bromide (CTAB) method (Lipp *et al.*, 1999). Specific PCR products, containing the transgenic DNA in the regions of NOS-P/nptII, pBI121/NOS-T, and 35S-P/PRSV CP, were amplified by PCR (Fig. 1, Table 1). Dilutions of amplified PCR products of different lengths were used as DNA for transformation, and standards for gel electrophoresis and real-time PCR.

Table 1. Primers used for the standard PCR and real-time PCR amplifications

Location	Primer sequences (5'~3')	Amplified product (bp)	$T_{annealing}$ (°C)	
			Standard	Real-time
nptII-1-5'	ATGATTGAACAAGATGGATTGC	604	54	-[a]
nptII-1-3'	GGCCATTTTCCACCATGATA			
nptII-2-5'	GAACAAGATGGATTGCACGC	786	57	-
nptII-2-3'	GAAGAACTCGTCAAGAAGGC			
nptII-2-5'	GAACAAGATGGATTGCACGC	1396	56	-
NOS-3-3'	TTATCCTAGTTTGCGCGCTA			
35S-P 5217-F	AACCAAGGCAAGTAATAGAG	769	60	60
CP-right 9389-R	TAGTTGACACATCGTTTCC			
pBI 3677-F	CTGCCATCACGAGATTTCGAT	398	70	60
pBI 4074-R	CCGAAGCCCAACCTTTCA			
pBI 2695-F	GTCGCCTAAGGTCACTATCAG	200	60	60
pBI 2894-R	TAGCCTCTCCACCCAAGC			
P1	TGCTAAAGGAAGCGGAAC	1384[b]	60	-
P2	AGGTCAACAGGCGGTAAC			
P1	TGCTAAAGGAAGCGGAAC	1697[c]	60	-
P2	AGGTCAACAGGCGGTAAC			

[a] Not run.
[b] Confirmation of recipient cell
[c] Confirmation of transformed cell

Soil DNA Extraction

The upper 15 cm of soil was collected from December 2003 to April 2004 from a confined experimental field at Wufong, where transgenic papaya and non-transgenic papaya are grown. The soil texture (sandy loam) was composed of 30% silt, 12% clay, 58% sand, 0.44% organic matter, and 6.17 cmol/kg cation exchange capacity (CEC).

The soil DNA extraction method was evaluated with three commercial kits, three chemical extractants (SDS, CTAB, and guanidine isothiocyanate (GTC)), and two physical disruption methods (Bead/SDS and freeze-thawing). The objective of this investigation was to select a reliable soil DNA extraction method which is accurate and reproducible.

Standard PCR and real-time PCR

Standard PCR products of the expected sizes were used to confirm specific genes. Standard curves of real-time PCR were obtained using the threshold cycle (C_T) against serial dilutions of transgenic PCR products of 769, 713, 398, 200, or 102 bp. All determinations were performed in triplicate on an Mx3000P real-time PCR system (Stratagene).

RESULTS AND DISCUSSION

Transformation on the Filter

A high transformation frequency was found when a 1396 bp PCR product was used as the donor DNA, and 10- and 100-fold drops in the transformation frequencies were observed when the length of the PCR products was reduced from 1396 ($(6.8 \pm 0.9) \times 10^{-6}$, 0.4 μg) to 786 bp ($(4.3 \pm 3.2) \times 10^{-7}$, 0.4 μg) and to 604 bp ($(1.2 \pm 0.3) \times 10^{-8}$, 0.4 μg) (Table 2), respectively.

No transformants were detected in either recipient bacterial cell when genomic DNA of transgenic papaya was used as the donor DNA (Table 2). The negative detection was possibly due to dilution of the transgenic DNA by the entire plant genome. No transformation of the concentrated PCR DNA was detected in *Acinetobacter* sp.

BD413, as expected by a recipient with no homologous DNA.

Table 2. Effect of DNA length and dose on the transformation of *Acinetobacter* sp. strain BD413 (pFG4Δ*nptII*) and *Acinetobacter* sp. BD413 on filters (at 30 °C). DNA used was genomic DNA and PCR products of transgenic papaya.

Bacterium	DNA	Dose	Transformation frequency [a]
Acinetobacter sp. BD413 (pFG4Δ*nptII*)	604 bp	0.4 μg	$(1.2 \pm 0.3) \times 10^{-8}$
		3.6 μg	$(1.3 \pm 0.03) \times 10^{-8}$
		14.3 μg	$(2.8 \pm 0.5) \times 10^{-8}$
		28.6 μg	$(3.2 \pm 2.0) \times 10^{-8}$
		57.1 μg	$(2.5 \pm 0.7) \times 10^{-8}$
	786 bp	0.4 μg	$(4.3 \pm 3.2) \times 10^{-7}$
		3.6 μg	$(2.9 \pm 0.6) \times 10^{-7}$
		14.3 μg	$(3.2 \pm 1.0) \times 10^{-7}$
		28.6 μg	$(2.9 \pm 0.1) \times 10^{-7}$
		57.1 μg	$(5.8 \pm 3.6) \times 10^{-7}$
	1396 bp	0.4 μg	$(6.8 \pm 0.9) \times 10^{-6}$
		3.6 μg	$(1.6 \pm 0.2) \times 10^{-6}$
		14.3 μg	$(2.0 \pm 0.5) \times 10^{-6}$
		28.6 μg	$(6.8 \pm 3.0) \times 10^{-6}$
		57.1 μg	$(7.7 \pm 2.7) \times 10^{-6}$
	Genomic DNA	0.4~57.1 μg	$< 10^{-10}$
		5.7 mg	$< 10^{-10}$
Acinetobacter sp. BD413			
	604~1396 bp	0.4~57.1 μg	$< 10^{-10}$
	Genomic DNA	0.4~57.1 μg	$< 10^{-10}$

[a] Mean of transformation frequency ± standard deviation.

Transformation in Soil Microcosms

Transformation in soil microcosms was less than the detection limit (10^{-9}~10^{-10}, number of transformed cell /number of recipient cell) when genomic DNA was added (Table 3). The result of the assessment of DNA availability to bacteria in the soil showed that a higher transformation frequency ($(4.8 \pm 0.9) \times 10^{-8}$, 28.6 μg) was found when the 1396 bp PCR product was added simultaneously with the bacteria. A lower transformation frequency ($(3.3 \pm 0.9) \times 10^{-9}$, 28.6 μg) was observed when cells were added first, and the DNA was added 24 h later (Table 3).

Table 3. Effect of DNA availability on the transformation of *Acinetobacter* sp. BD413 (pFG4Δ*nptII*) in the sterile soil microcosm. DNA was added simultaneously with the bacteria, or 24 h later. The DNA dose was 12.7 μg for genomic DNA, and 28.6 μg for the 1396 bp PCR product.

DNA	Treatment	Transformation frequency [a]
Genomic DNA	Cell + DNA (simultaneously) [b]	$< 10^{-9}$
	Cell + DNA (24 h later) [c]	$< 10^{-10}$
1396 bp	Cell + DNA (simultaneously) [b]	$(4.8 \pm 0.9) \times 10^{-8}$
	Cell + DNA (24 h later) [c]	$(3.3 \pm 0.9) \times 10^{-9}$

[a] Frequency ± standard deviation.
[b] DNA was simultaneously added to cells.
[c] Cells were added first, and then DNA was added 24 h later.

Comparison of Soil DNA Extraction Methods

The sizes of the soil DNA extracts obtained by the SDS/GTC and CTAB/SDS methods (23 kb) were generally greater than those of the other methods (< 20 kb). DNA recovery was the highest with the CTAB/SDS/gel method (45.8%), followed by the SDS/GTC/gel method (40.8%), and the bead/SDS/gel method (19.5%) (Table 4). Soil DNA extracts purified by the gel method produced higher DNA recoveries than those by PEG treatment (Table 4).

One of the most important factors for residual soil DNA analysis is its DNA purity. The purity of the soil DNA extracts for the residual analysis should be evaluated by PCR not by the absorption ratios of A_{260}/A_{280} and A_{260}/A_{230}, because soil DNA extracts usually produce low ratios of A_{260}/A_{280} and A_{260}/A_{230}, but a successful PCR reaction can be achieved even when the ratios of A_{260}/A_{230} and A_{260}/A_{280} are lower than 0.8 and 1.5, respectively.

Table 4. Recovery (%) of DNA by different soil extraction methods [a]

Method	769 bp (%)	398 bp (%)	200 bp (%)	Mean
UC-Kit	0.03	0.15	0.09	0.09
SDS/GTC				
PEG purification	3.4	11.5	2.0	5.7
Gel purification	42.8	30.2	49.3	40.8
CTAB/SDS				
PEG purification	3.7	12.3	2.4	6.1
Gel purification	57.8	32.8	46.9	45.8
Bead/SDS				
PEG purification	1.3	7.1	5.9	4.8
Gel purification	10.4	18.4	29.8	19.5

[a] Recovery was conducted by adding 0.8 μg of the PCR product to 0.1 g of a soil blank, and calculated by real-time PCR.

Detection Limit of the Real-Time PCR

The detection limit of DNA in the real-time PCR was 0.5 fg based on the threshold cycles (C_Ts), which were 27.0 for the 769 bp, 23.9 for the 398 bp, and 23.2 for the 200 bp (PCR products) (Fig. 2). Thus, the detection limit of transgenic DNA in the soil was set at 30 pg/0.1 g or 0.3 ng/g soils. The smaller the C_T is, the higher the DNA concentration would have been in the original solution.

Persistence of Transgenic DNA in the Soil

Persistences of the 398-bp (fragment amplified by PCR) in soil samples were all < 0.16 μg/g soil, and were high in December 2003 (0.16 μg/g soil), then decreased sharply from January to March 2004 (0.06 μg/g soil) due to rainfall and agricultural activities which occurred in that season (Fig. 3). The 35S-P/PRSV-CP and NOS-P/*nptII* genes in the soil could be detected for 5 months, but concentrations were < 2.0×10^{-4} μg/g soils (Fig. 3).

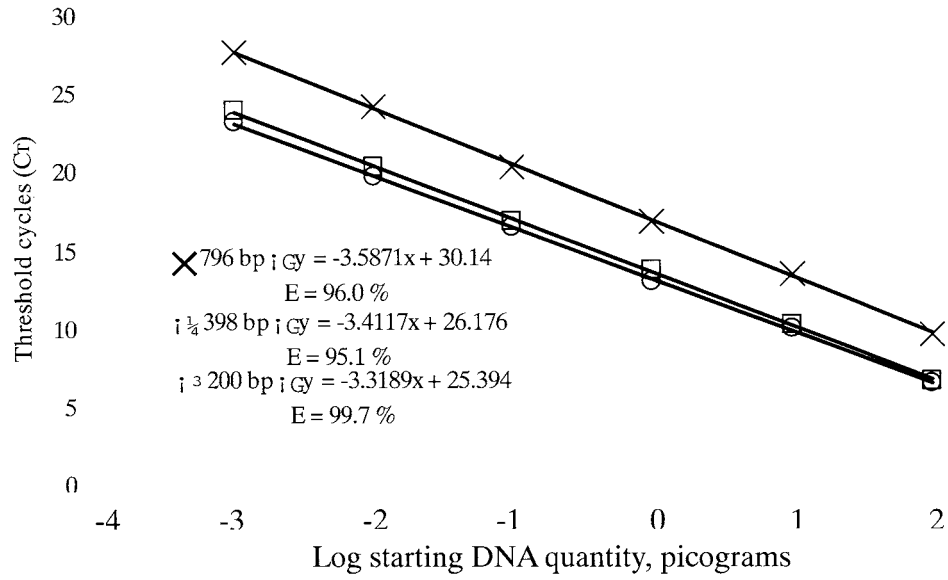


Fig. 2. Standard curves of the 769 (×), 398 (□), and 200 bp (□) (PCR products) obtained from the real-time PCR. The PCR efficiencies were in the ranges of 95.1~99.7%.

A previous study showed that homologous gene transformation in *Acinetobacter* BD413 (pFG4Δ*nptII*) could be detected in sterile soil, when transgenic PCR products were added at 28.6 μg/1.2 g sterile soil (or 23.8 μg/g dry soil) (Lo *et al.*, 2005). Our studies indicated that residues of 200-bp in soil were lower than 2.0×10^{-4} μg/g soil, which was 8.4×10^{-6} times less than the dose required for gene transformation in soil (residue DNA: DNA for gene transformation = 2.0×10^{-4}: 23.8 μg/g soil).

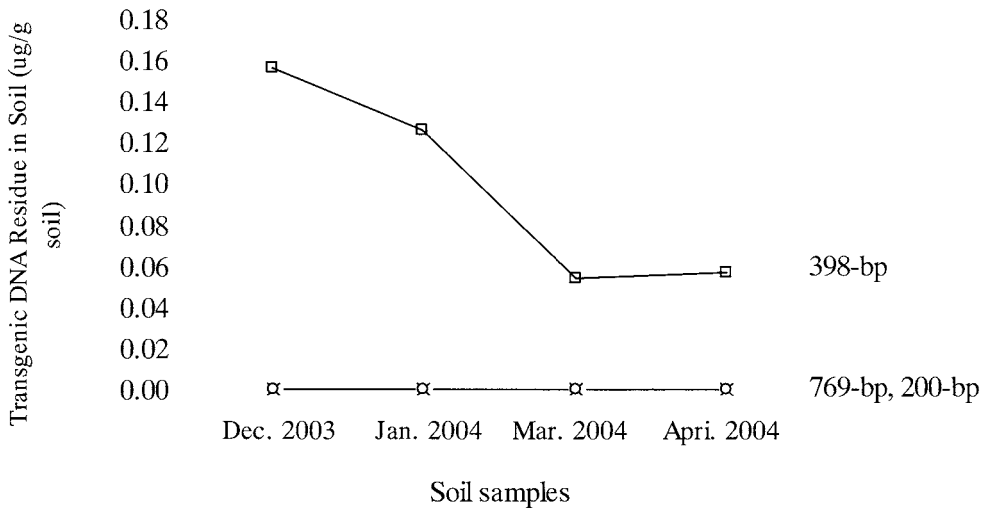


Fig. 3. Persistence of transgenic papaya DNA in soil where transgenic papaya had been grown. Soil DNA was extracted by the CTAB/SDS/gel method. The residues of 398-bp were less than 0.16 μg/g soil, whereas the residues of 769- and 200-bp were less than 2.0×10^{-4} μg/g soil.

CONCLUSIONS

In summary, the transformation of transgenic papaya DNA is less likely to occur in soil than on filters, and may even be less likely to occur in natural conditions. The residual data also revealed that the chance of antibiotic *nptII* gene transfer from transgenic papaya to soil bacteria was very low.

ACKNOWLEDGMENTS

This study was supported by the Council of Agriculture and National Science Council, Taiwan. The authors are grateful to Dr. K. Smalla (Germany) for providing *Acinetobacter* sp. BD413 (pFG4Δ*nptII*), and Dr. K. M. Nielsen (Norway) for introducing the transformation technique.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
125~137

Biosafety Evaluation of Transgenic Potatoes: Gene Flow from Transgenic Potatoes

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ABSTRACT

A potential concern associated with the release of transgenic crops is the risk of gene flow to neighboring crops of the same species or to related species. In potato there is little accurate information or experience on effective pollination distances for gene flow between plants. The incidence of intraspecific or interspecific pollination as a basis for gene flow has only received minimal attention since true seed production is unimportant for plant propagation. Gene flow of transgenes from potato crops can arise from several different paths. The most obvious is from true seeds arising on neighboring non-genetically modified (GM) plants, either potatoes or other *Solanum* species, following pollen dispersal from transgenic potato crops. Alternatively, it may arise from true seeds developing on the transgenic potato crops which accumulate in the seed bank for germination in future years. This may arise from either self pollination within transgenic potato crops or pollen dispersal from neighboring non-transgenic potato crops or other *Solanum* species. Germination of such seed may occur many years later, and the resulting plants may contribute to transgene flow *via* either seed or pollen dispersal. In a similar manner, gene flow

may arise from volunteer plants arising in the season following the transgenic potato crop from tubers remaining after harvest. This paper summarizes information relevant to these pathways for gene flow from transgenic potato crops and discusses approaches for minimizing such gene flows.

Key words: Transgenic potato; GM potato; Gene flow; Hybridization; Isolation distances.

INTRODUCTION

The release of genetically modified (GM) crops into agricultural production has raised considerable debate, especially among the general public, politicians, and bureaucrats (Nap *et al.*, 2003). A main concern associated with the release of transgenic crops is the risk of gene flow from transgenic crops to neighboring crops of the same species or related species (Conner *et al.*, 2003). This may undermine the containment of transgenes and compromise the segregation of transgenic and non-transgenic crops. A common concern raised about gene flow involving transgenes is the potential for the resulting plants to become agricultural weeds or invade natural habitats and compromise natural biodiversity (Conner *et al.*, 2003).

Many decades of experience in seed production of specific cultivars in many crops have defined well-established isolation distances that are rigidly enforced to maintain seed purity to defined legal specifications (Frankel and Galum, 1977). However, true seed production is unimportant in asexually propagated crops such as potatoes. Consequently there is very little accurate information or experience on effective pollination distances in potato and isolation required to mitigate intraspecific or interspecific gene flows (Conner, 1994). Such information is essential for regulatory

authorities to set realistic isolation distances and management strategies to contain gene flow from transgenic potato crops.

This paper summarizes the potential pathways by which gene flow can occur from transgenic potato crops to neighboring non-transgenic potato crops or other *Solanum* species. The incidence of such gene flow is discussed along with various approaches that can be used to minimize or prevent such events.

POLLEN-MEDIATED GENE FLOW FROM FIELD-GROWN TRANSGENIC POTATOES

The total number of field tests on transgenic potatoes is estimated to have exceeded 2000 over the last 20 years (Conner 2006). However, the incidence of pollen-mediated transgene dispersal has only been reported from a small number of these field tests. This represents a wasted opportunity, and more effort in recording and reporting this data would have greatly increased the confidence in the conclusions made to date and facilitate future applications for field testing transgenic potatoes (Conner 2006).

Pollen-mediated transgene dispersal has been investigated by estimating the frequency of transgenic progeny produced on non-transgenic pollen-trap cultivars planted at varying distances surrounding the transgenic plots. In such studies, synchronized flowering times for the transgenic and pollen-trap plants was ensured by removing flower buds from the pollen-trap when necessary. The majority of these independently performed studies have reported very similar results, with very limited dispersal of pollen over short distances from the edge of transgenic potatoes. The frequency of transgenic progeny from non-transgenic potatoes growing in rows

adjacent to transgenic potatoes ranged from 1% to 24% and rapidly fell to negligible levels within 3~5 m (Tynan *et al.*, 1990; Dale *et al.*, 1992; Conner, 1993, 1995; McPartlan and Dale, 1994). One study concluded that pollen-mediated transgene dispersal can occur at high frequencies up to 1000 m from field trials of transgenic potato (Skogsmyr, 1994). However, this latter study has been discredited and considered to be based on a high frequency of false positives rather than on actual gene flow (Conner and Dale, 1996). Based on the collective data it was recommended that a distance of 20 m is generally adequate for containing novel gene constructs during initial field tests of transgenic potato (Conner and Dale, 1996).

More recently, we completed another comprehensive study from seven field test sites over six seasons that screened a total of over 1.3 million progeny from non-transgenic pollen-trap buffer rows (Erasmuson *et al.*, 2005). The accuracy of this phenotypic screening was verified by PCR in a sample of the progeny. In the buffer row immediately adjacent to the field trial, the frequency of transgenic progeny ranged from 0.7 to 5.9 per 10,000 and declined to between 0 and 0.5 per 10,000 at the third buffer row from the field trial (a distance of 2.25 m). This also confirms that isolation distances of 20 m are sufficient for the initial field evaluation of transgenic potatoes containing novel genes. If such data had been repeatedly verified in many environments and for additional potato cultivars, some regulatory bodies might no longer require the removal of flower buds during field tests of transgenic potato or may have set more-realistic isolation distances (Conner 2006).

All of the gene flow studies from transgenic potato field tests have only involved small-scale field tests. Such field tests may often involve transgenic lines, some of which exhibit poor agronomic performance, slow growth, and a failure to flower

(Conner, 2006). When commercial fields of transgenic potatoes are grown on a larger scale with lines of equal agronomic performance to the parent cultivar, the load of transgenic pollen may be considerably higher. This suggests the need to demand greater isolation distances for commercial transgenic potato fields to mitigate pollen-mediated gene flow. However, anecdotal information from potato breeders and farmers suggests that pollen dispersal in commercial fields also only occurs over short distances. Berry formation on male-sterile potato cultivars is only occasionally observed on plants growing immediately alongside male-fertile cultivars, suggesting that effective pollen dispersal in potato only occurs over a few meters (Conner and Dale, 1996).

VOLUNTEER POTATOES AS SOURCE OF GENE FLOW

In an agricultural context potatoes often appear as "weeds" in the same field or neighboring areas in seasons following a potato crop. These may arise from true seeds or from tubers remaining in the ground after harvest. Both sexual and asexual propagation can therefore produce transgenic potato plants in subsequent seasons from which gene flow pathways can arise in later years.

The number of true seeds produced in commercial fields of fertile potato cultivars is highly dependent upon the cultivar grown, environmental conditions, and pest/pathogen activity, but can be as high as 150~250 million per hectare (Accatino 1980; Lawson 1983). Seeds may remain dormant in the soil for up to 2 years and have been reported to retain viability over a seven-year rotation. Consequently, true potato seeds have the potential to become a weed problem in subsequent crops (Lawson 1983). Furthermore, the potato berries can be consumed by various vertebrates which can provide a vector for seed dispersal beyond the field where the

potato crop was grown. Germination of such transgenic seed and potential establishment of plants can provide a pathway for gene flow. Management practices to minimize such gene flow pathways may be difficult to implement because germination from the seed bank is unpredictable and may occur many years later and/or at sites other than that at which the seed was dispersed.

Potato plants as weeds can also arise from the inability to remove all tubers from a field during harvest, resulting in the appearance of volunteer (ground keeper or self-set) plants the following year. Up to 367,000 tubers per hectare have been estimated to remain in the field after mechanical harvest (Lutman, 1977). Depending of the severity of the winter, many of these tubers will remain viable until the next spring. Potato tubers require 50 frost-hour equivalents at -2 ºC or below to kill tubers, e.g., 25 h at -2 ºC, or 5 h at -10 ºC, etc. (Lumkes and Sijtsma, 1972). In the temperate British climate, up to 80% of tubers left after harvest died in even mild winters (Lutman, 1977). The remaining viable volunteer tubers do not remain dormant and will always sprout the next season.

The incidence of volunteer plants following the growing of transgenic potatoes has only been reported from a small number of field tests from New Zealand (Conner, 1993, 1995; Reader *et al.*, 2005). Even when transgenic potatoes are carefully hand-harvested, the frequency of volunteers ranged from 0.4/m2 to 2.9/m2. Transgenic volunteer potatoes appeared less frequently than volunteers of the non-transgenic parental cultivars. This was consistent when calculated on a relative basis to both the number of plants in the field trial and the number of harvested tubers. Therefore, in small-scale field trials, transgenic potato lines are, on average, likely to be less invasive than the non-transgenic parental cultivars. This is not unexpected since

many transgenic lines in such small-scale field tests exhibit poor agronomic performance and lower tuber yields (Conner, 2006). However, when commercial fields of transgenic potatoes are grown with transgenic lines of equal agronomic performance to the parent cultivar, the frequency of volunteers is likely to be similar for transgenic and non-transgenic cultivars.

Despite the potential for volunteer potatoes to become a weed problem, they do not usually persist for more than one or two seasons and are rarely seen outside cultivated fields because they do not have the invasive potential of most weeds (Conner *et al.*, 1997). The expression of transgenes is not anticipated to increase the invasiveness of potato cultivars any more than the traditional breeding for traits such as resistance to pests, diseases, drought, and frost (Conner *et al.*, 1997). However, the presence of such volunteers provides a potential pathway for gene flow in seasons following the transgenic potato crop.

INTERSPECIFIC TRANSGENE FLOW TO OTHER SOLANUM SPECIES

The potential of gene flow from transgenic crops to other related species is commonly raised as a concern. The introgression of transgenes into other species may increase the survival and spread of weeds that may invade agricultural land or natural habitats and may impact natural biodiversity (Conner *et al.*, 2003). Natural gene flow between cultivated potato crops and related *Solanum* species *via* hybrid swarms is well recognized in the Peruvian/Bolivian center of origin for the potato (Ross, 1986), where 130 wild potato species occur (Spooner and Hijmans, 2001). As part of a biosafety assessment for transgenic potato releases in this region, interspecific hybridization was confirmed between potato and six species found close

to potato plots (Celis *et al.*, 2004). The recovery of hybrids between these species (*S. albicans, S. acaule, S. chomathophilum, S. raphanifolium, S. bukasovii, and S. sparsipilum*) were all confirmed through AFLP markers. Spontaneous field hybridization was also confirmed over short distances from a potato pollen source and between potato and *S. acaule, S. bukasovii,* and *S. sparsipilum.*

In most other regions of the world, outside the center of origin for potato, the potential of spontaneous hybridization between potato and other *Solanum* species is substantially less. However it is important to consider the species that grow in the region where transgenic potatoes are to be cultivated. In temperate regions investigations have focused on the potential for gene transfer to *S. nigrum* and related nightshade species since these are commonly found in and around potato fields. Sterile F1 hybrids have been reported between potato and black nightshade, but only after resorting to very early embryo rescue and *in vitro* culture of the embryos (Eijlander and Stiekema, 1994). The resulting interspecific hybrids were very weak and difficult to keep alive. Similar attempts to make this *S. tuberosum* x *S. nigrum* cross elsewhere have failed (Dale, 1993). Other attempts to hybridize *S. dulcamara* and *S. tuberosum* have also failed (Eijlander and Stiekema, 1994; Dale 1993). Gene transfer from transgenic potatoes to black nightshade was monitored during one of our earlier contained field trials in the 1989/1990 season. No evidence for gene transfer was obtained, despite screening over 53,000 black nightshade seedlings (Conner, 1993, 1995). Similar results using 7,600 *S. nigrum* seedlings were observed in Australia (Conner, 1994).

As part of the biosafety assessment for field trials on transgenic potatoes in New Zealand, we have investigated the potential for hybridization *via* hand pollination

between three potato cultivars and a range of *Solanum* species found in New Zealand. These included the three indigenous species (*S. americanum, S. aviculare,* and *S. laciniatum*) and ten exotic or garden species (*S. brevidens, S. muricatum, S. nigrum, S. furcatum, S. chenopodioides, S. dulcamara, S. jasminoides, S. physalifolium, S. pseudocapsicum*, and *S. melongena*). All attempted interspecific crosses failed to produce seed, whereas control intragenic crosses were successful for all species except *S. jasminoides* (A. J. Conner *et al.*, unpublished data).

When spontaneous interspecific hybridization between potato and related species is possible, gene flow can occur in either of two directions. The most obvious is from hybrid seed formation on neighboring *Solanum* species, following pollen dispersal from transgenic potato crops. Alternatively, interspecific hybrid seeds may develop on the transgenic potato crops following pollen dispersal from neighboring *Solanum* species. Such hybrid seed may accumulate in the seed bank for germination in future years. Germination of such seed may occur many years later, and the resulting hybrid plants may contribute to transgene introgression into other *Solanum* species by further backcrossing.

MITIGATING GENE FLOW FROM TRANSGENIC POTATOES

An approach often suggested to mitigate gene flow from transgenic potatoes involves the use of male sterility. Some existing potatoes cultivars are already male sterile. For example, the Andean cultivar "Revolucion" has been used as a parental cultivar in the transgenic development of nematode resistance for proposed release in the Peruvian/Bolivian center of diversity of potato (Celis *et al.*, 2004). Alternatively, male sterility in potato can be derived through transgenic development by targeting pollen expression of a suicide gene such as barnase (citations to be inserted).

However, this approach only targets mitigation of pollen-mediated gene flow from transgenic potatoes to neighboring non-transgenic potatoes or related *Solanum* species. The use of male-sterile potatoes does not eliminate hybridization with pollen from neighboring non-transgenic potatoes or related *Solanum* species. The resulting true seed can accumulate in the seed bank and contribute to gene flow to non-transgenic potatoes or introgression into related species following germination in later years.

To completely eliminate the possibility of gene flow would require the development of non-flowering genotypes. In most crops, this would not be feasible since flowering is an essential prerequisite for either grain or fruit production, as well as seed propagation. However, for potatoes, flowering is not required for either plant propagation or the harvested produce. In an attempt to recover non-flowering lines of important New Zealand potatoes cultivars we field-screened large numbers of plant lines regenerated from cell culture. While several non-flowering somaclonal variants were identified, these lines unfortunately also possessed a range of poor attributes (A.J. Conner *et al.*, unpublished data). Further screening might be successful. Another approach might be to design a transgenic strategy to knock-out flowering.

An approach has recently been described for eliminating transgenes during pollen development. This involves the recombinase-mediated auto-excision of transgenes controlled by a tightly regulated microspore-specific promoter to remove either selectable marker genes or all of the transgenes during pollen development (Mlynárová *et al.*, 2006). This provides a system to mitigate gene flow *via* pollen dispersal and could provide a valuable containment system for the coexistence of GM and non-GM agriculture. This is a convenient approach in potatoes since they

are clonally propagated and transgenic pollen formation is unnecessary for cultivar propagation. A similar approach could also be taken to effect transgene excision from ovules and mitigate sexual transmission and gene flow of transgenes.

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Designing Biotechnology for Biosafety

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ABSTRACT

Public perception of transgenic plants has often been based upon information provided by vocal, anti-transgenic groups. Companies marketing transgenics have not been viewed as an unbiased source of information, and presentations have not been effective. Government regulatory agencies provide the public with reassurance that consumption and environmental risks are being addressed, but these agencies provide only limited public presentations on risk: benefit studies of transgenic plants. Scientists developing transgenic plants are well positioned to explain how they develop the plant materials and why they are safe, yet they fail to interact with the public and provide this much need information.

Keywords: Public; Self-regulation.

INTRODUCTION

Consumption of Early Development Transgenic Plants.

Many of the initial transgenics submitted for field release and potential commercial use were straightforward in their scientific design. They relied on well characterized

genetic systems found in nature. The development of Bt toxin crop plants were based on the long term, safe usage of the bacterium *Bacillus thurengiensis* for control of lepidopteran larvae. As a control treatment favored by organic growers, the bacteria may be applied anytime up to harvest, ensuring eventual ingestion by consumers. From a scientific perspective the risks are negligible. Virus resistant transgenics, expressing a portion of the target virus, results in consumption of a small fraction of the viral transcript and protein found in non-transgenic, virus infected food crops. Again, scientists would feel there is no consumption risk. A third major area for transgenics was herbicide resistance. In cases where the herbicide resistance was derived from herbicide-insensitive plants, resistance was attributed to a single amino acid change in the target enzyme. Changes in a single amino acid were perceived as unlikely to induce consumption risks.

In all of the transgenic plants, antibiotic resistance is incorporated as a by-product of the initial selection process. Selection on kanamycin would not introduce resistance to a medically widespread antibiotic, and DNA transfer has proven unlikely (Gay and Gillespie, 2005). None of the early transgenic plant material has been shown to pose a consumption risk, representing well designed scientific choices for transgenic plants. Interestingly, traditional breeding has generated greater consumption risk, as evidenced by recalls of a high glycoalkaloid potato and a high cucurbitacin summer squash (Rymal et al, 1984, Zitnak and Johnston, 1970).

Environmental Impact of Early Development Transgenic Plants.

Controlling crop seed viability was an issue addressed by scientists at an early stage of transgenic development. This was seen in the proposed, although never demonstrated, technique referred to as the "terminator". Unfortunately, commercial

acquisition of the technology cast a negative impression on use of the technology. Thus early attempts to avoid genetic dispersion were put on hold (Lee and Natesan, 2006). Efforts to limit genetic dispersion, and other potential problems with transgenic plants in the environment, were relegated to agricultural producers, who were relied upon to carry out proper crop management practices. These practices include crop rotation, suitable field spacing relative to non-transgenic fields, herbicide rotations and planting of non-transgenic refuge crops.

Scientific input on environmental issues is generally focused on refuting data presented by groups opposed to transgenic technologies. Scientific scrutiny of environmental risks has yet to find evidence of impact any greater than the general impact found with commercial agriculture. Unfortunately, scientists that develop transgenic crops do not have the opportunity to explain the rationale behind their designs, and the potential environmental benefits they hold. Benefits such as reduced exposure to chemical pesticides, reduced soil erosion due to less tillage, and lower energy inputs are not being defined by scientists. The public perception of transgenics comes largely from the corporations selling the plant material, while independent public scientific presentation and discussion is desperately needed.

Future Transgenic Plants.

As is the case with most technologies, complexity grows with our increased understanding of the science driving the technology. Transgenic technology is advancing on two major fronts, promoter regulation and incorporation of multi-genic traits (Halpin, 2005). Promoter regulation is a scientist's answer to environmental containment, and to consumption risks that may be posed by certain genes introduced to control insects and plant pathogens.

Promoter regulation provides the opportunity to produce seedless fruits, providing containment and value-added crop traits simultaneously (Ficcadenti *et al.*, 1999). In cases where there is no need for seeds (eg. potatoes, tobacco, and alfalfa), promoter regulation can provide plants unable to produce pollen. Promoter regulation may also be useful when expressing genes encoding antimicrobial peptides. A wide array of peptides is known that provide inhibitory activity to a range of plant pathogens. Certain peptides are also known to regulate biochemical aspects of insects. Current expression technologies, involving fusion proteins, allow peptides of 6 to 12 amino acids in length to be produced in plants (Jones *et al.*, 2004). Deployment of antimicrobial peptides requires special attention due to our limited knowledge of how they may interact after ingestion. Single amino acid changes in small peptides can represent a ten percent change in amino acid composition, producing unforeseen consequences (Groot *et al.*, 2006). Scientific rationale would suggest that antimicrobial peptides be limited, through promoter regulation, to non-edible portions of plants, although expression in edible portions of plants may be possible after consumption risks have been evaluated.

Multi-genic traits are evaluated scientifically through understanding what changes occur to the overall metabolic profile of the transgenic plant. The same could be said for introduction of individual genes that are part of a biochemical pathway. Understanding the biochemical pathways allow the scientist to predict what other effects may arise in the transgenic plant, and this knowledge is incorporated into decision making and engineering. Scientists have developed methods of metabolic profiling (metabolomics) to try and determine if non-target characteristics are being affected by the transgene expression. Scientists are well aware of the limits to

metabolomic studies. First there is insufficient baseline data available to know what a "normal" profile should look like. Variations between cultivars of a given plant species, and variations due to environmental conditions, can easily exceed differences between transgenic and non-transgenic (Catchpole *et al.*, 2005, Matthews *et al.*, 2005, Rischer and Oksman-Caldentey, 2006). Overall risks in pathway modifications may be even less than early stage transgenics, as attributes such as enhanced nutritional quality would not be expected to carry a consumption risk or an environmental risk.

Scientific Self Regulation.

While countries such as the United States have an elaborate framework of regulations governing transgenic plants, it cannot be assumed that constraints needed to be placed on scientists developing transgenic plants. Scientists are in general a very rational group of professionals, and rank among the top five most respected professions in the US and UK. They are quite unlike the mad scientists portrayed by anti-transgenic groups as creating "Frankenfoods". Transgenic material being sent for regulatory approval has already met the safety criteria of peer-reviewed science and should be fairly easy to approve. Any regulatory judgment on safety requires guidance from the scientific community.

Since the advent of molecular biology, scientists have shown their concern for issues of safety and environmental impact. A landmark meeting in Asilomar, California in 1975 called for a temporary self-imposed moratorium on molecular cloning. This was followed by agreement among scientists on the proper procedures for carrying out cloning experiments, beginning with use of bacteria unable to survive out of the laboratory (Berg *et al.*, 1975). Subsequently, government regulations were put in

place for recombinant DNA studies. Advances in molecular biology have brought scientists to a new era of self regulation. Development of "synthetic biology", wherein a basic form of microbe is created by introduction of wholly synthetic DNA, has called for new agreements among scientists on the procedures that need to be adhered to for safety and public approval (Service, 2006). Some scientists have called for formal training of new scientists in the art of self governance (Davies and Wolf-Phillips, 2006). This has already occurred, without training, however the idea of training scientists to be more interactive with the public would promote acceptance of advances in biotechnology.

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Impact of Roundup Ready® Canola on Plant Pathogen and Its Suitability as Animal Feed: A Case Study in Canada

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ABSTRACT

The steady increase in world production of genetically modified (GM) or transgenic crops since their introduction in the 1990s has caused some concerns, regarding the bio-safety on persistence and stability of recombinant DNA from GM crops. A field experiment was established in 2000 at the Agriculture and Agri-Food Canada Lethbridge Research Centre to investigate the long-term environmental impacts of some GM crops approved for production in Canada, including GM canola, corn and potatoes. One of the objectives of this study was to investigate horizontal gene transfer (HGT) from Roundup Ready® (RR) canola (*Brassica napus* event RT73) to *Sclerotinia sclerotiorum*, causal agent of stem blight of canola. The investigations focused on the *cp4 5-enolpyrulylshikimate-3-phosphate synthase* (*cp4 EPSPS*) gene, which endows RR canola with herbicide tolerance towards glyphosate. PCR and Southern hybridization were used to test sclerotia of *S. sclerotiorum* produced in diseased stems of RR canola and conventional canola, which were collected from the field in 2004 and 2005, for the presence of the 1363 bp *cp4 EPSPS* transgene and four of its fragments; three of which were construct-specific and one that was gene-specific. Results of PCR showed that *cp4 EPSPS* was present in diseased RR canola

stems but was absent in sclerotia of *S. sclerotiorum* formed inside the same stems. These results were further confirmed by Southern blotting and hybridization. The study concludes that there is no evidence for recombinant DNA transfer of *cp4 EPSPS* or any of its constituent fragments from RR canola to the pathogen *S. sclerotiorum*. From our studies with animals we found that feed-ingested DNA fragments do survive the terminal Gastrointestinal (GI) tract, but recombinant DNA in the gut is processed similar to any other endogenous feed-ingested genetic material. Plant DNA from GM feed degrades very rapidly upon its release into the rumen fluid. We did not detect any *cp4 EPSPS* fragments in the microbial DNA fraction of *in vitro* ruminal cultures, suggesting that bacterial transformation did not occur. Based on our investigations it was clear that uptake of transgenic DNA fragments by ruminal bacteria is precluded or time-limited by rapid degradation of plant DNA upon plant cell lysis. Thus use of GM canola as animal feed is safe and should be encouraged based on the scientific findings.

Key Words: Bio-safety; cp4 EPSPS gene; Sclerotia; *Sclerotinia sclerotiorum*; Canola; *Brassica napus*; Horizontal gene transfer; Roundup Ready® canola; Glyphosate.

INTRODUCTION

Canada was the fourth world largest country in the world for commercial production of genetically modified (GM) crops in 2005 with canola, corn and soybean as the major GM crops (James, 2005). The GM canola crops, Roundup Ready® (glyphosate tolerant) (RR canola) and Liberty Link® (glufosinolate tolerant) (LL canola), were first registered in Canada in 1995 (Stringham *et al.*, 2003). They constitute over 75% of all canola production in Canada (Canola Council of Canada, 2001; Stringham *et al.*, 2003). In 2003, the area for RR canola was 3.2 million ha in

Canada (Traxler, 2006). The estimated economic benefits of RR canola in Canada in 2000 were about $47 million (29%) accruing to producers, $93 million (57%) accruing to industry, and $21 million (14%) accruing to consumers (Philips, 2003). Consumer acceptance of GM canola has generally been very good in Canada and its trading partners, except in Europe where there is a ban on the use of GM canola and its products (Stringham *et al.*, 2003).

Despite the overall benefits of growing transgenic crops, there are several concerns regarding their ecological and environmental biosafety. These include higher reported levels of *S. sclerotiorum* infection in glyphosate tolerant crops (Lee *et al.*, 2003); evolution of herbicide resistant weeds (Llewellyn *et al.*, 2002; Owen and Zelaya, 2005); recombinant DNA movement within and across species; and persistence, stability and potential uptake of the transgenic DNA by viruses, bacteria, plant, animal and fungal cells (Ho *et al.*, 2000a; 2000b; Fincham *et al.*, 1989).

Horizontal gene transfer (HGT) has been implicated in the evolution of fungi (Rosewich and Kistler, 2000) but the exact mechanism(s) and reason behind such an event has not been clearly understood. *Sclerotinia sclerotiorum* is a fungal pathogen which often produces black sclerotia on diseased canola plants. Such intimate host-pathogen relationships make this pathogen an ideal candidate for studying horizontal gene transfer from GM canola to the pathogen. Since canola meal is an important constituent of animal diets, both monogastrics and ruminants, it is pertinent to assess the fate of the recombinant DNA arising from GM canola when used as animal feed.

A long-term (12 years or longer) field study was initiated in 2000 at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta, to assess the

environmental and economic impact of GM crop production in Canada. Specific objectives of this study were to determine: 1) population dynamics of weeds, diseases and insects (of target and non-target species), 2) soil microbial diversity, 3) herbicide resistance development, 4) gene flow, 5) crop yield and quality, and 6) economic opportunity and risk to the farmer. The objectives of this report will focus the discussion only in two of these areas: the impact of RR canola on the pathogen *Sclerotinia sclerotiorum* and the suitability of RR canola as animal feed.

RESULT AND DISCUSSION

Design of the Field Experiment of GM Crops

The field experiment was established in 2000 in a 10-ha area at the Lethbridge Research Centre, Agriculture and Agri-Food Canada, to investigate the long-term environmental impacts of some GM crops approved for production in Canada, including RR canola, LL canola, RR maize, LL maize, *Bt* maize and *Bt* potato. The experiment consisted of 10 treatments with four replicates arranged in a randomized complete block design. The individual plot size was 15 m x 35 m (W x L). There were 3 m pathways between plots and 20 m pathways between replicates to maintain treatment integrity over many years.

Diseases of GM Canola and Conventional Canola in the Field (2000-2005)

When canola plants in plots of RR canola, LL canola and conventional canola (CC) reached maturity stage (mid-August) in each year, they were examined for natural occurrence of diseases. Plants were rated for each disease by inspecting five sites in each plot, with 100 plants (50 per row) in two rows per site. Sites were located in an

X pattern, with 5 m separation between sites. At each site, the number of plants with symptoms for each disease was recorded.

Results of disease survey showed that no diseases were observed in all canola (RR canola, LL canola and CC canola) in the first year (2000). During 2001-2005, three diseases were detected, including sclerotinia stem rot *(Sclerotinia sclerotiorum)* (Table 1), staghead (*Albugo candida*) (Table 2), and alternaria pod spot (*Alternaria brassicae* and *A. raphani*) (Table 3). However, incidence of these diseases was low (<7%) and, for each disease, there was no significant differences among the treatments of RR canola, LL canola and CC canola (Tables 1-3). Incidence of alternaria pod spot was 16.8% in CC canola in 2002 and 16.2% in RR canola in 2004 (Table 3). This high incidence of alternaria pod spot was associated with damage from a flea beetle infestation.

Table 1. Incidence of Sclerotinia rot (*Scleroitnia sclerotiorum*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Sclerotinia Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.7	0.0	0.1	1.6	2.9a
Liberty Link®	0.0	0.3	0.0	0.4	0.4	3.2a
Roundup Ready®	0.0	0.3	0.0	0.1	1.3	2.8a

Table 2. Incidence of staghead (*Albugo candida*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Staghead Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.6	0.0	0.0	0.3	0.3
Liberty Link®	0.0	0.3	0.0	0.0	0.2	0.2
Roundup Ready®	0.0	0.2	0.0	0.0	0.2	0.4

Table 3. Incidence of alternaria pod spot (*Alternaria brassicae* and *A. raphani*) in Roundup Ready® canola, Liberty Link® canola and Conventional canola.

	Alternaria Disease Incidence (%)					
Treatment	2000	2001	2002	2003	2004	2005
Conventional	0.0	0.2	16.8*	6.1	4.0	2.0
Liberty Link®	0.0	0.2	2.3	3.1	2.1	2.2
Roundup Ready®	0.0	0.1	3.8	6.5	16.2*	2.6

* Alternaria infection was associated with flea beetle damage

Assessment of Horizontal Gene Transfer from RR Canola to *Sclerotinia sclerotiorum*

Samples of canola stems and sclerotia of *S. sclerotiorum* collected in 2004 and 2005 were used in this study. In each year, canola stems infected by *S. sclerotiorum* and sclerotia produced inside the stems were collected from plots of RR and CC canola in early September. Healthy stems of canola from these plots as well as sclerotia of *S. sclerotiorum* produced on diseased bean plants in another field were also collected for use as controls. Sclerotia were washed in running water for 10 minutes in a sieve,

and rubbed between two layers of paper towel to remove all the remaining plant material from the sclerotial surface. The stems and sclerotia were then used for testing presence of the RR-gene by PCR analyses and Southern hybridization (Sharma *et al.*, submitted) (Table 4).

Table 4. Detection of *cp4 EPSPS* and its fragments in *Sclerotinia sclerotiorum,* ruminal cultures and animal digesta/tissues.

Detection of *cp4 EPSPS*	Duration of the Study	Findings	Reference
Sclerotinia sclerotiorum	2 crop seasons	Negative	Sharma *et al.* (submitted)
Ruminal *in vitro* cultures	48 h	Transgene fragments detected up to 48 h; no transformation of ruminal bacteria	Sharma *et al.*, 2004
Ruminal fluid	4 h	Detected up to 10 min	Alexander *et al.*, 2004
Animal GI* tract contents	Feeding trial (3-4 months)	Detected in digesta samples of lambs and swine.	Sharma *et al.*, 2006
Animal GI* tract tissues	Feeding trial (3-4 months)	Detected at a low frequency from lambs and swine	Sharma *et al.*, 2006

*GI, gastrointestinal tract

PCR analysis of conventional and Roundup Ready® canola stems for the 1363 bp *cp4 EPSPS* transgene confirmed the GM trait of the canola plants from which the sclerotia samples were derived; all the CC stems showed absence of 1.3 kb transgene whereas all the RR stems tested positive (Fig.1). PCR assays for the 1363 bp *cp4 EPSPS* did not yield positive results for any of the samples of sclerotia of *S. sclerotiorum* collected from plots of RR canola in 2004 and 2005 (Fig. 2A). Representative gel for the non-detection of three different construct- or gene-specific fragments (F1, F3, F4) ranging in size from 278 to 527-bp are also shown in Fig. 2

(B, C, D). The non-detection of 1363 bp *cp4 EPSPS* is not surprising since previous study using *in vitro* culture indicates that the complete transgene degrades rapidly depending on the extent of processing of the sample (Alexander *et al.*, 2002). Thus, the possibility of gene transfer of *S. sclerotiorum* is low despite the intimate association between GM canola and the pathogen due to the well-known active mechanisms of degradation and excision of foreign DNA.

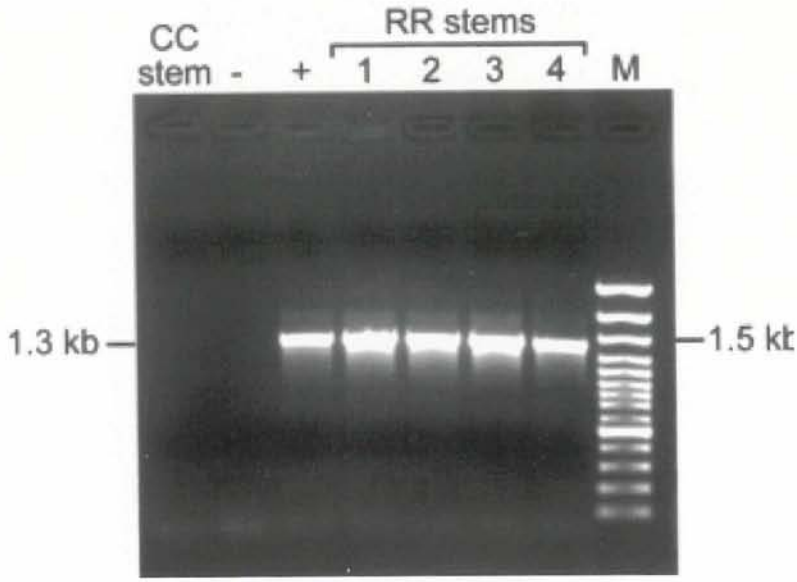


Fig.1. PCR analysis of 1363 bp *cp4 EPSPS* from diseased Roundup Ready® canola stems. Lane CC: conventional canola lacking the transgene (negative extraction control); Lane -: negative PCR control (no DNA template); Lane +: positive control (Roundup Ready® leaf DNA as template); Lanes 1-4: PCR product from four diseased stems.

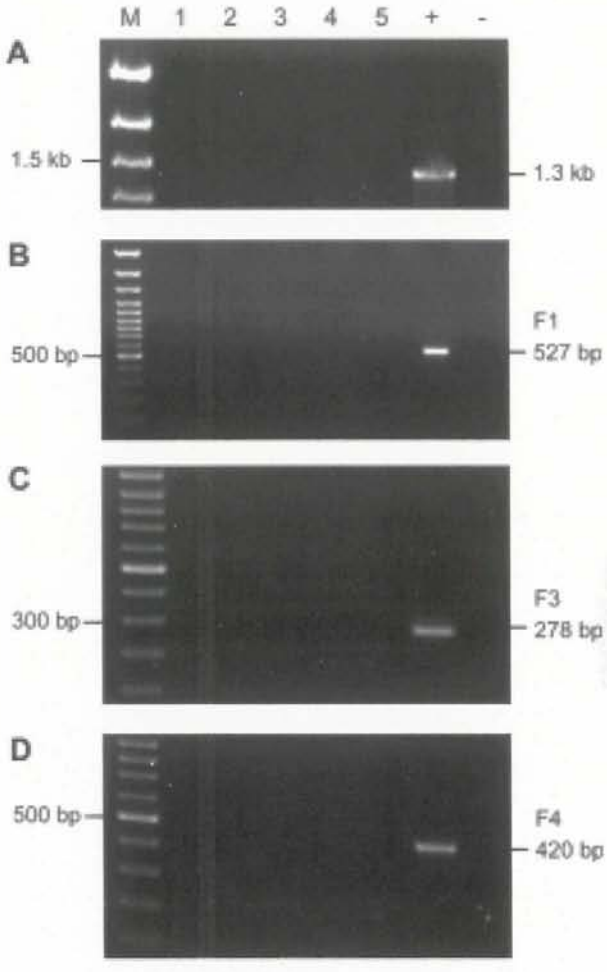


Fig. 2. Representative gel for PCR amplification of *cp4 EPSPS* and fragments F1, F3, F4 from sclerotia collected from diseased Roundup Ready® stems. A: absence of the 1363 bp *cp4 EPSPS*. Lanes 1, 2, 4, and 5: PCR products from four different sclerotia. Lane 3: blank (no PCR product loaded). B: Absence of fragment F1 from sclerotia. Lanes 1-5: PCR products from five different sclerotia. C: Absence of fragment F3 from sclerotia. Lanes 1-4: PCR products from four different sclerotia. Lane 5: blank. D: Absence of fragment F4 from sclerotia samples. Lanes 1-5: PCR products from five different sclerotia. For all the panels Lane M: 100 bp DNA ladder; Lane +: positive control (Roundup Ready® leaf DNA as template); Lane - : negative control (no DNA template).

Effects of RR Canola on Ruminant Animals and Ruminal Bacteria

We evaluated Roundup Ready® canola meal in barley based diets for lambs and found that digestibility, feed efficiency, growth performance, carcass characteristics or meat quality of lambs fed the transgenic diet was no different than the animals fed conventional canola diets (Stanford *et al.*, 2003). While assessing the fate of recombinant DNA in mixed ruminal cultures we observed that upon processing of canola, DNA large enough to contain intact plant gene remains unlike the report by Chiter *et al.* (2000) where treatment of canola meal resulted in complete degradation of DNA and heating maize grains to 95° C for 5 min resulted in an inability to PCR amplify a 577-bp sequence. In mixed ruminal cultures, plant DNA was found to be rapidly degraded upon its release into the rumen fluid. However we could detect *cp4 EPSPS* (1363 bp) up to 8 h from meals and 4 h from mixed diets (Alexander *et al.*, 2002). Research from our laboratory provided a comparison for the first time on the stability of complete transgene versus the smaller transgene fragments. We found that detection of fragments was representative of persistence of the whole transgene (Sharma *et al.*, 2004) and that no transformation was observed in the ruminal bacteria up to at least 48 h. Transformation of gut micro-organisms with the recombinant DNA does not seem possible given the high nuclease activity (i.e. enzymes that degrade DNA or RNA) in the rumen and the gastrointestinal tract. It is likely that the plant DNA released in the digestive tract is degraded into multitude of smaller fragments of different sizes which may exhibit differential stability. However, if these fragments are associated with the undigested feed particles, these are excreted via feces.

Using extremely low limit of detection of the PCR assay (12.5 pg), we elucidated that no *EPSPS* fragments are amplifiable in microbial fraction of the ruminal cultures suggesting that transformation had not occurred during 48 h incubation. Further our

results indicate that uptake of transgenic DNA fragments by ruminal bacteria is probably precluded or time-limited by rapid degradation of plant DNA-transgenic or otherwise upon cell lysis (Sharma *et al.*, 2004). Using quantitative real-time PCR we found that free DNA is rapidly degraded at neutral pH of duodenal fluid reducing the likelihood of transgenic DNA uptake in the Gastrointestinal (GI) tract. The *cp4 EPSPS* gene and four of its fragments were detected from ruminal fluid up to only 10 min, however these could be detected from the feces up to 2 - 4 h (Alexander *et al.*, 2004).

In a feeding trial conducted using sheep (n=11) and pigs (n=36) containing 6.5% and 15% Roundup Ready® canola, respectively, the transgene was tracked in digesta and GI tract tissues. We found low-copy *ribulose-1,5-bisphosphate carboxylase /oxygenase* (*rubisco;*186- and 540-bp) fragments were present at slightly lower, variable frequencies in digesta (18-82%) and intestinal tissues (9-27% of ovine and 17-25% of porcine samples), and infrequently in visceral organs (1 of 88 ovine samples; 3 of 216 porcine samples). Each of the five *cp4 EPSPS* transgene fragments (179 to 527 bp) surveyed were present in at least 27% of ovine large intestinal content samples (max. 64%) and at least 33% of porcine cecal contents samples (max. 75%). We found that transgenic DNA has the same fate as endogenous plant DNA when used as animal feed.

Effect of RR Canola on Weed Community Dynamics

Weed density by species was determined 1) before seeding, 2) before in-crop herbicide application, and 3) before crop harvest. Weed counts were made in fifteen quadrats (0.25 m^2) per plot utilizing a 'W' pattern. The weed seed bank was determined at the beginning of the experiment and it will be determined again at the

conclusion of the study. Visual assessments of potential herbicide resistant plants were made throughout the growing season in each year.

Preliminary results showed that weed densities were lower in both RR canola and LL canola compared to conventional canola (CC canola). This usually resulted in higher canola yield (R. E. Blackshaw *et al.* unpublished data). No weeds resistant to glyphosate (Roundup herbicide) or glufosinate (Liberty herbicide) were found in this experiment or anywhere in Canada.

CONCLUSIONS

The field study at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta, reveals that the transgene *cp4 EPSPS* for tolerance to glyphosate in RR canola is incapable of spreading onto *S. sclerotiorum*, despite the intimate host-parasite relationship. There is no significant difference in occurrence of diseases between GM canola and CC canola as the incidence for each of the three diseases remains low during the period of 2000-2005.

Regarding the use of Roundup Ready® canola as animal feed we found that transgenic DNA behaves no differently than plant endogenous DNA. We did not find any uptake of transgene or its fragments into the ruminal microbial population under the period of time investigated. The transgenic DNA could be detected from the digesta in the GI tract at variable frequencies in lambs and swine fed the transgenic diets.

The trend toward an increase in production of transgenic crops will likely continue around the world. The negative findings on horizontal gene transfer from GM canola to *S. sclerotiorum*, ruminal bacteria, and fecal material of sheep and pig at the Lethbridge Research Centre in Canada support the view of Traxler (2006) that the environmental effects of transgenic crops have been strongly positive to date. Nevertheless, the long-term environmental concerns of GM crops or GM organisms deserve continued monitoring, because of the novel nature of the GM technology. This include long-term studies to determine whether gene flow from RR canola to related plant species will have a negative effect on biodiversity or on control of weeds.

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
163~190

High-level Tryptophan Accumulation in Seeds of Transgenic Rice and Its Limited Effects on Agronomic Traits and Seed Metabolite Profile*

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*This paper was published in Journal of Experimental Botany, page 1-10, August 14, 2006.
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ABSTRACT

Metabolic manipulation of plants to improve their nutritional quality is an important goal of plant biotechnology. Expression in rice (*Oryza sativa* L.) of a transgene (*OASA1D*) encoding a feedback-insensitive *α* subunit of rice anthranilate synthase results in the accumulation of tryptophan (Trp) in calli and leaves. It is shown here that the amount of free Trp in the seeds of such plants is increased by about two orders of magnitude compared with that in the seeds of wild-type plants. The total Trp content in the seeds of the transgenic plants was also increased. Two homozygous lines, HW1 and HW5, of *OASA1D* transgenic rice were generated for

characterization of agronomic traits and aromatic metabolite profiling of seeds. The marked overproduction of Trp was stable in these lines under field conditions, although spikelet fertility and yield, as well as seed germination ability, were reduced compared with the wild type. These differences in agronomic traits were small, however, in HW5. In spite of the high Trp content in the seeds of the HW lines, metabolic profiling revealed no substantial changes in the amounts of other phenolic compounds. The amount of indole acetic acid was increased about 2-fold in the seeds of the transgenic lines. The establishment and characterization of these *OASA1D* transgenic lines have thus demonstrated the feasibility of increasing the Trp content in the seeds of rice (or of other crops) as a means of improving its nutritional value for human consumption or animal feed.

Key words: Amino acids; Anthranilate synthase; AS; Noncontainment greenhouse; IAA; Isolated field; 5MT; *OASA1D*; *Oryza sativa.*

INTRODUCTION

Metabolic manipulation of plants to improve their nutritional value is a primary goal of plant biotechnology. Essential amino acids such as Lys, Met, Thr, and Trp contribute substantially to the nutritional quality of plantbased foods for humans and domestic animals, but the amounts of such amino acids are limited in many crops. Dietary supplementation with Trp increases the growth rate of pigs and poultry (Subcommittee on Poultry Nutrition, 1994; Subcommittee on Swine Nutrition, 1998). An inadequate supply of Trp leads to a marked reduction in food intake in pigs as a result of the reduced synthesis of serotonin in the brain (Henry *et al.,* 1992; Se`ve, 1999; Eder *et al.,* 2001). Indeed, Trp has been used as a pharmaceutical agent in the treatment of depression (Massey *et al.,* 1998). The ability to increase the level of Trp in food crops by metabolic engineering is thus desirable from both nutritional and

clinical viewpoints. The achievement of an increase in the amino acid content of seeds by genetic engineering, however, has been limited to Lys (Falco *et al.,* 1995; Mazur *et al.,* 1999; Zhu and Galili, 2003) and Met (Movig *et al.,* 1997; Lai and Messing, 2002).

The accumulation of free Trp in plants has been achieved by the introduction of genes encoding feedback-insensitive α subunits of anthranilate synthase (AS), which catalyses the conversion of chorismate to anthranilate. This approach has thus yielded increased levels of Trp in the roots of the forage legume *Astragalus sinicus* (Cho *et al.,* 2000) and in the leaves of tobacco (Zhang *et al.,* 2001). A mutant *OASA1* gene, *OASA1D* [*formerly referred to as OASA1 (D323N)*], that encodes a feedback-insensitive α subunit of rice AS (Tozawa *et al.,* 2001) has previously been generated. Rice calli and leaves as well as potato plants and tubers that express *OASA1D* accumulate large amounts of free Trp (Tozawa *et al.,* 2001; Yamada *et al.,* 2004).

Manipulation of metabolic pathways in plants by genetic engineering thus has the potential to improve the nutritional value of crops and to allow the production of desired natural products in higher plants (DellaPenna, 2001; Morandini and Salamini, 2003). The proteins encoded by the introduced genes might affect not only the abundance of the target metabolite, however, but also, through changes in metabolic networks, that of related compounds. Moreover, such changes may influence the physiology or morphology of plants. Little is known, however, of the consequences of such manipulation for metabolite profiles and agronomic traits in plants.

Metabolism of Trp in plants is associated with the generation of a range of secondary compounds such as indole alkaloids and indole acetic acid (IAA). Manipulation of the Trp biosynthetic pathway might thus be expected to influence the synthesis of such metabolites and thereby elicit a pronounced change in metabolite profile. Indeed, overexpression of Trp decarboxylase, which catalyses the conversion of Trp to tryptamine, resulted in a marked reduction in the amount of indole glucosinolate in canola plants (Chavadej *et al.,* 1994) and a decreased abundance of Trp, Phe, and chlorogenic acid in potato (Yao *et al.,* 1995).

Evaluation of transgenic plants of improved nutritional value for effects of the transgene on agronomic traits and metabolite profiles is thus essential. However, the possible effects of the accumulation of Trp or of any other essential amino acid in seeds on agronomic traits and metabolite profiles have not been determined to date. Demonstration that Trp is the only major metabolite that accumulates in transgenic rice seeds, for example, would be likely to increase their acceptability for consumption by humans and farm animals.

The effects of *OASA1D* expression in rice both on the amounts of Trp and other amino acids in seeds and on agronomic traits of plants cultivated in isolated field trials have now been studied. The profile of phenolic compounds as well as the content of IAA in the transgenic seeds was also determined. Rice is one of the major crops in the world and its use for animal feed has recently been developed (Sakai et *al.,* 2003*a*). The results of the increase of Trp in transgenic rice prove the usefulness of the gene for improving the nutrition of other crops for animal feed.

MATERIALS AND METHODS

Plant materials and growth conditions

Seed calli of rice (*Oryza sativa* L. cv. Nipponbare) were transformed with the use of *Agrobacterium* as described by Hiei *et al.* (1994). The generation of rice plants transgenic for *OASA1D* or *OASA2*, with both genes controlled by the promoter of the maize ubiquitin gene, has also been described (Tozawa *et al.*, 2001; Yamada *et al.*, 2004). Rice plants transgenic for the β-glucuronidase gene (*GUS*) were also generated as described by Urushibara *et al.* (2001). All transgenic plants contained the hygromycin phosphotransferase gene (*hpt*) under the control of the 35S promoter of the cauliflower mosaic virus. Transgenic rice plants (R0 to R4) and non-transformed control plants (Nipponbare) were grown at 28℃ and 60% humidity under natural light conditions in pots (1/10 000a) containing podosol soil (Sumitomo Chemical, Osaka, Japan) in a containment greenhouse.

Two *OASA1D* transgenic lines, HW1 and HW5, were also grown in a non-containment greenhouse (R5) and an isolated field (R6). In 2002, seeds of the HW lines and Nipponbare were sown on 15 April and the seedlings were transferred individually to pots (1/10 000a) containing podosol soil on 15 May. The plants were maintained in the non-containment greenhouse under natural light and temperature conditions at the National Institute of Crop Science (NICS). No additional fertilizer was applied. In 2003, seeds of the HW lines and Nipponbare were sown on 14 May and the seedlings were transferred to an isolated paddy field (10 m x 1.2 m) at the National Institute for Agro-Environmental Sciences in Tsukuba on 4 June. For each line, 40 seedlings were planted in duplicate in an area of 15 cm x 30 cm per plant. The total amount of fertilizer applied m^{-2} included 6 g of N, 6 g of P_2O_5, and 6 g of K_2O and was added at planting. Other practices followed the cultivation standards of

Ibaraki Prefecture (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990).

Evaluation of agronomic traits

Agronomic traits, including heading date, culm length, and morphological characteristics, were evaluated by standard protocols (Ibaraki Prefecture Standard Lowland Rice Cultivation, 1990). Plants in the isolated field were harvested individually, air-dried, and analysed for yield and seed germination. All panicles were harvested from each plant, dried at 38 ℃ for 4 weeks, and then maintained in a freezer at -30 ℃ until analysis of amino acids and of IAA and metabolite profiling. For germination analysis, 15 or 20 seeds of individual lines were transferred to a filter paper that had been moistened with distilled water and placed in a Petri dish (6 cm in diameter); the analysis was performed in duplicate. The Petri dish was maintained at 35℃ for 15 d in the dark, and seed germination was assessed each day according to the modified standard method of NICS based on viviparity (Sakai *et al.*, 2003*b*).

Amino acid analysis and determination of total nitrogen content

Dehulled seeds were autoclaved individually with 50 μl of water in a 1.5 ml Eppendorf tube for 15 min, and extracts were prepared from each seed as previously described (Wakasa and Widholm, 1987). The amounts of free amino acids were then quantified with the use of the PICO.TAG analysis system (Waters, Milford, MA). For quantitation of total Trp, five dehulled grains were pulverized with a mortar and pestle and heated in 5 M NaOH for 28 h at 110 ℃; the hydrolysate was then acidified with 6 M HCl and subjected to analysis with an L-8800 High Speed Amino Acid Analyser (Hitachi High-Technologies, Tokyo, Japan).

Dehulled rice seeds were weighed and decomposed in concentrated sulphuric acid in the presence of salicylic acid and sodium thiosulphate. The ammonia formed was distilled, and was determined colorimetrically at 640 nm using the indophenol method.

Metabolite profiling

Four dehulled seeds were pulverized with a mortar and pestle and subjected to extraction for 1 h with 10 vols (v/w) of a mixture of water: methanol: acetic acid (249: 250: 1, by vol.). The extract was centrifuged at 16 000 g for 20 min, the resulting supernatant was passed through a SepPak C_{18} cartridge (Waters, Milford, MA), and the eluate was subjected to reversed-phase HPLC (LC-10Avp system; Shimadzu, Kyoto, Japan) with a Cadenza Column CD-C18 [250 mm x 4.6 mm (inner diameter); Imtakt, Kyoto, Japan). Elution was performed with a mixture of acetonitrile and 0.02% aqueous trifluoroacetic acid (3: 97 v/v, at 0 min; 30: 70 v/v at 40 min; 98: 2 v/v at 75 min) at a flow rate of 0.85 ml min^{-1} and a temperature of 40 ℃; it was monitored with a photodiode array detector (Shimadzu SPDM10Avp) over a wavelength range of 190-400 nm.

IAA analysis

Six dehulled seeds were pulverized with a mortar and pestle and then soaked for 3 h at 4℃ in 10 vol (v/w) of 80% acetone in water containing 2.5 mM diethyl dithiocarbamate. This extraction procedure was repeated three times. The combined extract was divided into three portions that were respectively subjected to quantification of free IAA, free plus ester forms of IAA and total IAA. The free IAA in the extract was partially purified by solid-phase extraction and quantified by liquid chomatography and tandem MS (LC-MS/MS) as previously described by Matsuda *et*

al. (2005). For determination of the amount of free plus ester forms of IAA, the original extract was acidified to pH 2 with aqueous HCl and then analysed as for free IAA. For determination of the amount of total IAA, the extract was subjected to hydrolysis with 7 M NaOH for 3 h at 100℃ under N_2 before analysis. [Phenyl-$^{13}C_6$]IAA was used as the internal standard in these analyses.

RESULTS AND DISCUSSION

Generation of rice plants expressing OASA1D

Transgenic rice plants that express *OASA1D* were generated and subjected to the analyses of Southern blot and northern blot as described previously (Tozawa *et al.,* 2001; Yamada *et al.,* 2004). Given that expression of *OASA1D* confers resistance to 0.3 mM 5-methyltryptophan (5MT), some transgenic plants were generated from calli selected with 5MT instead of with hygromycin (Yamada *et al.,* 2004). Transgenic lines selected by growth in the presence of 5MT or hygromycin are denoted by M or H, respectively.

Spikelet fertility of greenhouse-grown plants

Almost all regenerated plants of >120 transgenic lines grown in pots exhibited normal growth, with exceptional instances of dwarfism or slow growth presumably being attributable to somaclonal mutations (Phillips *et al.,* 1994). However, for plants grown in a greenhouse, the spikelet fertility of transgenic plants tended to be lower than that of the wild type (Table 1). The mean spikelet fertility of the transgenic plants was thus only 31%, compared with a value of 76% for seed-grown Nipponbare. Given that a decrease in spikelet fertility has previously been observed in transgenic rice plants generated by tissue culture (Hiei *et al.,* 1994; Urushibara *et al.,* 2001), part of the reduction in spikelet fertility apparent in *OASA1D*-expressing plants might be due to the regeneration process. However, transgenic rice plants that overexpress the

wild-type gene (*OASA2*) for another α subunit of rice AS but do not accumulate Trp in calli or leaves (Tozawa *et al.*, 2001) exhibited a spikelet fertility (61%) higher than that of plants that express *OASA1D*, but lower than that of Nipponbare, suggesting that accumulation of Trp might also contribute to the reduced spikelet fertility of the *OASA1D* transgenic plants. Analysis by genomic Southern hybridization of R0 (regenerated) plants of *OASA1D* transgenic lines with a spikelet fertility of >50% revealed that they had a relatively high copy number (three to seven) of the transgene (data not shown), suggesting that the copy number was not a principal cause of low spikelet fertility.

Table 1. Spikelet fertility of OASAID transgenic plants (R0) grown in a greenhouse

Transgene	Trp content	Spikelet fertility (%)						Total no. of plants
		≦20	21-40	41-60	61-80	81-100	Mean±SD	
OASAID	Increased	25	19	12	7	0	30.7±21.2	63
OASA2	Normal	5	4	6	14	11	61.4±27.2	40
NB[a]	Normal	0	1	2	3	6	76.2±19.7	12

[a] Seed grown Nipponbare plants.

Trp content of seeds produced by greenhouse-grown plants

Seeds of 12 *OASA1D* transgenic lines with a spikelet fertility of >50% were analysed for free and total Trp contents in the second or third generation (R2 or R3 seeds) of plants grown in a greenhouse. All lines showed a marked increase in the amount of free Trp, with the mean free Trp content ranging from 3037 to 23 705 nmol g^{-1} of dry seed weight (Table 2); these values correspond to increases of 55- to 431-fold compared with the free Trp content of non-transgenic Nipponbare seeds (55 nmol g^{-1}). The amount of free Trp as a percentage of total Trp in the transgenic seeds (34–87%) was also greatly increased compared with that in wild-type seeds (1.3%).

Given that seeds of a transgenic line expressing *GUS*, which encodes β -glucuronidase, exhibited a Trp content similar to that of Nipponbare seeds, it was unlikely that the Trp accumulation apparent in seeds of *OASA1D* transgenic plants resulted from an abnormality caused by the transformation process.

The transgenic seeds analysed for Trp content were a mixture of those homozygous or heterozygous for *OASA1D*. To examine the possible influence of genotype on Trp content, seeds from a single transgenic plant of the H17 line in the R1 generation were divided into two groups. One group of 26 seeds showed segregation of *OASA1D* by PCR analysis (14 positive, 12 negative). The other group of 32 seeds, which also should have been a mixture of transgene genotypes, all contained an increased level of free Trp (9134±3740 nmol g^{-1}). These results thus indicated that Trp accumulation in seeds is determined primarily by the genotype of the mother plant. However, given that the free Trp content of seeds of the H17 or M34 lines showed some variability (Table 2), the level of free Trp in seeds might also be influenced by seed genotype. Although the physiological conditions of plants and seeds also affect amino acid content, the increase in the amount of Trp in the seeds of transgenic rice expressing *OASA1D* was sufficiently high to be attributed to the activity of the transgene.

It is the total Trp content, including both free Trp and Trp in proteins, that is important for the nutritional value of seeds. The increase in the amount of free Trp in seeds of *OASA1D* transgenic plants was accompanied by an increase in the total Trp content (Table 2). The total Trp content of seeds of the various transgenic ines thus ranged from 8100 to 48 519 nmol g^{-1}, values that correspond to increases of 1.9- to 11.6-fold compared with that for Nipponbare (4188 nmol g^{-1}).

The weight of individual dehulled seeds tended to be s aller for *OASA1D* transgenic lines than for Nipponbare (Table 2). However, a reduced seed weight was also apparent for plants harbouring a *GUS* transgene, suggesting that this effect might be attributable to the transformation process. *OASA1D* transgenic line M34, whose seeds showed the highest Trp content, also manifested the lowest seed weight. However, the seeds of line M21, which exhibited a medium level of Trp accumulation, were also of low weight. No clear correlation between Trp content and seed weight was thus apparent.

Generation of homozygous OASA1D *transgenic rice lines for evaluation of agronomic traits*

Two *OASA1D* transgenic lines, M121 and H41, were advanced to obtain homozygotes for evaluation of agronomic traits. The resulting homozygous lines were designated HW1 for M121 and HW5 for H41. The early generations of these homozygous lines exhibited a spikelet fertility of >50% and normal morphological features. Genomic Southern blot analysis of selfed progenies and of F_2 plants of F_1 hybrids between either HW1 or HW5 and Nipponbare revealed that HW1 contained three copies of the transgene and HW5 harboured four copies. No segregation of hybridized bands was observed in F_2 plants, indicating that the transgenes were integrated at one locus (data not shown).

Table 2. Total and free Trp contents of seeds from *OASA1D* transgenic plants grown in a greenhouse.

Transgenis Line	Seed generation	Seed Weight (mg)	Total Trp (nmol g^{-1})	Free Trp (nmol g^{-1}) Means±SD
Nipponbare		18.6	4188	55.0±21.7
GUS 16[a]	R2	14.2	5173	41.0±8.0
M25	R2	11.7	8100	
M121	R3	14.9	8810	3036.6±233.4
M31	R2	17.1	9237	
M21	R2	9.9	9264	
H29	R2	14.1	11 320	
H36	R2	17.0	14 425	7642.3±830.3
H17	R3	16.2	14 178	12 309.0±5764.9
H37	R2	14.0	15 619	
H42	R2	11.9	16 967	
H40	R2	12.7	17 322	
M45	R2	12.7	20 830	14 495.3±3528.0
M34	R2	7.7	48 519	23 705.3±11 681.2

[a] Transgenic line harbouring *GUS* and hph (*OASA1D* lines contain *OASA1D* and *hph*).

Five seeds of one panicle from each transgenic line were used for measurement of total Trp content and of average seed weight. Total Trp content and seed weight of Nipponbare were determined as average values for two plants. Free Trp was measured in three to five single seeds, and values presented are means ±SD. Trp content is shown as nmol Trp g^{-1} dry seed weight.

Trp content of seeds produced by field-grown OASA1D *transgenic plants*

The free and total Trp contents as well as the nitrogen content of R5 and R6 seeds of the HW lines grown in a greenhouse were higher than those of Nipponbare seeds (Table 3), and all of these values were slightly higher for HW1 than for HW5. The levels of free and total Trp in seeds were stable during growth of the HW lines in a greenhouse for 2 years (data not shown). In the field condition, the free Trp content of seeds of the HW lines was increased about 2-fold compared with the corresponding values for seeds of greenhouse-grown plants (Table 3). This increased

accumulation of free Trp in the seeds of fieldgrown plants was not accompanied by a similar increase in the total Trp content. The level of total Trp in seeds of the HW lines was thus stable under different growth conditions. The nitrogen content of seeds of field-grown HW lines and Nipponbare was lower than the corresponding values for seeds of greenhouse-grown plants. The amounts of free and total Trp in Nipponbare seeds were also lower in the field condition than in the greenhouse, with the result that the relative values to Nipponbare for seeds of the fieldgrown HW lines were increased accordingly (Table 3). The levels of Trp in the seeds of Nipponbare grown in another field were similarly low (data not shown).

Table 3. Nitrogen and Trp contents of seeds produced by HW lines under field or greenhouse conditions

Growth condition	Line	N content (%)	Free Trp		Total Trp	
			(nmol g^{-1})	R.V.[a]	(nmol g^{-1})	R.V.[a]
Greenhouse	HW1	1.39±0.12 (2)	3403±283.4 a (6)	55.9	7883±151.2 a (3)	1.9
	HW5	1.30±0.10 (2)	1762±333.3 b (6)	41.0	6949±387.6 b (3)	1.7
	NB	1.24±0.04 (2)	43±15.7 c (6)	1	4118±237.0 c (3)	1
Field	HW1	1.13±0.03 (2)	5294±2672.6 a (6)	311.0	8477±1139.0 a (4)	14.1
	HW5	1.04±0.01 (2)	3288±1366.1 a (6)	193.0	5684±1818.0 b (4)	9.4
	NB	0.96±0.13 (2)	17±9.4 b (9)	1	602±413.0 c (3)	1

[a] Relative value compared with Nipponbare.

Five seeds of Nipponbare (NB) or HW lines were analysed for nitrogen content (percentage of dry seed weight) and total Trp content (nmol g^{-1} dry seed weight) in each of the indicated (in parentheses) number of experiments, and data presented are means ±SD. Values for free Trp content (nmol g^{-1} dry seed weight) are presented as means ±SD for the numbers of seeds indicated in parentheses. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

The opposite effects of field growth on the free Trp content of seeds of the HW lines and of Nipponbare seeds might be attributable, in part, to a response of the transgene promoter to the cooler temperatures or to the difference in temperature between night and day in the field. The *OASA1D* gene was driven by the ubiquitin gene promoter, which is responsive to stress (Takimoto *et al.,* 1994) and might therefore be activated by low temperatures, resulting in increased expression of *OASA1D* and a greater accumulation of free Trp.

Amino acid composition of seeds produced by field-grown OASA1D *transgenic plants*

The increased Trp content of rice calli expressing *OASA1D* does not result in substantial changes in the amounts of other amino acids (Tozawa *et al.,* 2001). The marked accumulation of free Trp in the seeds of the HW1 and HW5 lines grown under field conditions was accompanied by an increase in the amounts of other amino acids to some extent (Table 4). Their increases were relatively small compared with that of Trp. The total amount of free amino acids was increased 4.2- and 2.9-fold in HW1 and HW5, respectively, compared with the value for Nipponbare, with the maximal change in the content of any one amino acid (other than Trp) being limited to a 5.2-fold increase. However, with the exception of Ala (and Trp), the ratio of the amount of each amino acid to the total amount of free amino acids was reduced or remained virtually the same in the transgenic lines compared with Nipponbare.

Table 4. Free amino acid composition of seeds produced by field-grown HW lines.

Amino acid	Free amino acid content(nmol g^{-1})							
	NB		HW1			HW5		
	Mean±SD	(%)[a]	Mean±SD	(%)[a]	Relative value[b]	Mean±SD	(%)[a]	Relative value[b]
Gly	51±29 a	1.9	153±95 b	1.4	3.0	142±26 b	1.8	2.8
Ala	154±95 a	5.8	794±581 b	7.3	5.2	608±315 b	7.9	3.9
Val	32±19 a	1.2	84±37 b	0.8	2.6	68±16 b	0.9	2.1
Leu	13±7 a	0.5	36±20 b	0.3	2.7	32±8 b	0.4	2.5
Ile	8±5 a	0.3	28±15 b	0.3	3.5	26±9 b	0.3	3.3
Ser	172±95 a	6.5	288±107 a	2.6	1.7	277±68 a	3.6	1.6
Thr	37±30 a	1.4	70±37 a	0.6	1.9	57±31 a	0.7	1.5
Met	5±4 a	0.2	8±2 a	0.1	1.6	6±2 a	0.1	1.2
Asp	575±333 a	21.9	1285±727 a,b	11.7	2.2	1136±139 b	14.7	2.0
Asn	595±760 a	22.6	1269±616 a	11.6	2.1	746±296 a	9.7	1.3
Glu	660±359 a	25.1	810±303 a	7.4	1.2	739±165 a	9.6	1.1
Gln	51±31 a	1.9	47±13 a	0.4	0.9	54±8 a	0.7	1.1
Arg	67±71 a	2.5	239±123 b	2.2	3.6	102±48 a	1.3	1.5
Lys	16±7 a	0.6	65±34 b	0.6	4.1	48±14 b	0.6	3.0
His	28±17 a	1.1	115±48 b	1.1	4.1	116±75 b	1.5	4.1
Phe	7±4 a	0.3	30±15 b	0.3	4.3	25±7 b	0.3	3.6
Tyr	91±55 a	3.5	208±74 b	1.9	2.3	171±28 b	2.2	1.9
Trp	20±9 a	0.8	5294±2673 b	48.4	264.7	3288±1366 b	42.5	164.4
Pro	49±31 a	1.9	125±60 b	1.2	2.6	96±17 b	1.2	2.0
Total	2629±2001 a	100	10 947±4484 b	100	4.2	7734±1810 b	100	2.9

[a] Amount of each amino acid as a percentage of total amino acids.
[b] Amount of each amino acid relative to that in Nipponbare.

Data are expressed as nmol of amino acid g^{-1} dry seed weight and are means ±SD of values from six seeds of Nipponbare (NB) or HW lines. Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

The levels of Lys and Phe were low in Nipponbare seeds but their absolute amounts were increased in the HW seeds, with the result that their percentage contributions to the total amount of free amino acids were the same in the HW lines and in the wild type. Given that Gln is the amino donor for the synthesis of anthranilate, its abundance might have been expected to be changed in the seeds of the transgenic lines. Its absolute amount in seeds of the HW lines was similar to that in Nipponbare seeds, however. Serine is also a precursor for Trp synthesis, but its amount in HW seeds was not significantly increased compared with that in Nipponbare seeds.

The increases in the absolute amounts of free amino acids in the seeds of the transgenic lines suggest the existence of regulatory mechanisms that increase amino acid synthesis in response to Trp accumulation. The *opaque-2* mutation in maize (Oh545*o2*) is associated with an increased level of free amino acids in mature endosperm (Wang and Larkins, 2001). Genetic analysis suggests that the gene for aspartate kinase 2 is the gene responsible for the effect of this mutation on free amino acid content (Wang *et al.,* 2001). A mutation in a transcriptional regulator of AS genes also renders Trp biosynthesis insensitive to Trp concentration (Bender and Fink, 1998). Our results therefore suggest that Trp accumulation in rice seeds might increase the transcription of genes that encode enzymes responsible for amino acid synthesis.

Agronomic traits of field-grown* OASA1D *transgenic plants

HW lines grown in a greenhouse appeared similar to Nipponbare with regard to most agronomic traits analysed (Table 5). For plants grown under field conditions, however, differences in traits related to seed productivity were apparent between the transgenic lines (especially HW1) and the wild type. Both HW lines grown in the field thus exhibited a spikelet fertility lower than that of Nipponbare, although pollen

fertility (Table 5) and anther size (reflecting the number of pollen grains) (data not shown) for the transgenic lines were similar to those for Nipponbare. Moreover, the average spikelet number per panicle was significantly smaller for the HW lines than for Nipponbare. The reduction in the number of spikelets per panicle and the low spikelet fertility likely contributed to an observed decrease in harvested seed weight for HW1 and HW5 to 52.5% and 69.6% of the value for Nipponbare, respectively. The harvested plant weight was similar for the HW lines and Nipponbare (data not shown). Whereas the individual brown seed weight varied among transgenic lines in early generations (Table 2), it did not differ markedly among HW lines and Nipponbare under field conditions (Table 5).

The high concentration of Trp in the HW lines is likely to be the primary cause of the differences in agronomic traits between these lines and Nipponbare grown under field conditions. Spikelet number per panicle is determined at an early stage of development of the inflorescence apex and is influenced by several conditions, such as nitrogen and carbon availability as well as temperature (Takeoka *et al.,* 1993). Accumulation of Trp might increase the sensitivity of plants of the HW lines to environmental stress and thereby reduce spikelet number and fertility in the field condition.

The culm length of HW lines grown in the field was smaller than that of Nipponbare, although this difference was statistically significant only for HW1 (Table 5). No difference in plant height was observed between HW lines and Nipponbare (data not shown). A short culm length has often been observed in plants regenerated from tissue culture (Phillips *et al.,* 1994), suggesting that this characteristic of the HW lines might be attributable to somaclonal mutation.

Table 5. Agronomic traits of HW1 and HW5 lines

Growth condition (year)	Line	*n* [a]	Day to Heading ±SD	Culm length (cm)	Panicle length (cm)	No.of tillers plant^{-1}	Spikelet fertility (%) ±SD[b]	No.of spilelets panicle^{-1}	Mean dry matter of harvested seeds (30 plants g^{-1}) ±SD[c]	Pollen fertility (%)[d]	Brown seed weight (1000 grains g^{-1})[e]
Green house (2002)	HW1	10	129±0.64	78.1 a	16.1 a	13.7 a	79.5±6.84			98.3	20.1 a
	HW5	10	132±2.04	74.9 a	15.7 a	13.1 a	88.3±5.19			97.5	20.1 a
	NB	23	130±1.13	80.1 a	17.0 a	11.6 a	88.2±6.65			98.9	20.1 a
Field (2003)	HW1	2	100±0.00	73.3 b	18.1 a	15.5 a	65.0±18.21	71.6 b	443.6±35.64	97.6	20.2 ab
	HW5	2	101±0.50	75.7 ab	17.8 a	13.2 a	81.3±11.00	85.6 b	588.4±50.77	98.5	21.0 a
	NB	2	100±0.00	81.3 a	20.1 a	12.2 a	91.6±2.91	122.3 a	844.8±27.86	99.4	20.0 b

[a] n means repetition. The number of plants or plots for the greenhouse and field conditions, respectively. Heading date, culm length, panicle length, and the number of tillers were recorded for each of 10 or 23 plants in the greenhouse condition and for each of 15 plants per plot in the field condition.

[b] Spikelet fertility was recorded for all panicles of 10 plants in 2002 and for three panicles of main culms of 60 plants in 2003.

[c] Mean dry matter of harvested seeds which were air-dried (assumed moisture content of seeds was approximately 10–15%).

[d] Pollen fertility was recorded for three spikelets of each of three randomly selected plants. It was estimated from microscopic analysis of the shape and color of the pollen stained with I-KI solution (1%).

[e] The weight of 1000 seeds was determined from that of 20 or 100 grains in the greenhouse and field conditions, respectively.

Means followed by the same letters are not significantly different at the 5% level (Student's *t* test).

Germination of seeds from field-grown OASA1D *transgenic plants*

The growth condition markedly influenced seed germination in HW lines. Whereas the germination percentage for seeds of greenhouse-grown plants was similar for HW lines and Nipponbare, it was greatly reduced for seeds of fieldgrown HW1 plants compared with that for field-grown HW5 or Nipponbare plants (Table 6). The time to germination was also increased for seeds from both HW lines grown under field conditions, as well as for seeds from HW1 plants grown in the greenhouse.

Table 6. Germination percentage and time to seedling establishment for HW lines.

Line	Greenhouse		Field	
	Germination (%)	Time to establish seedlings (d)	Germination (%)	Time to establish seedlings (d)
HW1	95	6.3	53.3	10.1
HW5	100	3.1	93.3	5.3
NB	100	3	100	2.8

The germination test was performed with 20 seeds from greenhouse grown plants and 15 seeds from field-grown plants. Data are means of values from two experiments.

Poor seed germination has been observed in other plants with increased levels of an essential amino acid. Transgenic soybean with a large increase in free Lys content thus manifested reduced seed viability (Falco *et al.,* 1995). Seeds of transgenic *Arabidopsis thaliana* with a high Lys content also exhibited retarded germination and seed establishment (Zhu and Galili, 2003). The seeds of HW1 plants grown in the field showed the lowest frequency of and greatest delay in germination as well as the highest content of free Trp compared with seeds of HW1 or HW5 plants grown in the greenhouse and HW5 plants grown in the field. These observations suggest that an increase in Trp content over a certain threshold level might substantially influence germination. The importance of amino acid metabolism such as Gln, Lys, and Met in maize germination efficiency has been shown (Limami *et al.,* 2002; Anzala e*t al.,* 2006). Trp has been shown to be an endogenous inhibitor of embryo germination in white wheat (Morris *et al.,* 1988).

It might prove possible to ameliorate the unfavourable traits observed in the HW lines grown under field conditions by controlling the extent and tissue distribution of Trp accumulation with the use of a different promoter to drive *OASA1D* expression.

Potential promoters for this purpose include those of embryo-specific genes or of genes that are not responsive to stress.

Metabolite profile of seeds from field-grown* OASA1D *transgenic plants

Changes in the composition of aromatic components in the seeds of field-grown HW1 and HW5 plants were analyzed by reversed-phase HPLC. Elution was monitored with a photodiode array detector over a wavelength range of 190–400 nm. Typical chromatograms obtained at 280 nm, the most effective wavelength for detection of changes in the composition of anthranilate-related metabolites, are shown in Fig. 1. The aromatic metabolite profiles of dehulled seeds revealed no apparent marked accumulation of components other than Trp in the transgenic seeds (Fig. 1, inset). Magnification of the chromatograms revealed small differences between HW lines and Nipponbare (Fig. 1). A peak with a retention time of 10 min, for example, was specifically detected in both transgenic lines. Essentially, identical results were obtained by monitoring elution at wavelengths other than 280 nm (data not shown). These results are surprising given the high levels of Trp in the transgenic seeds and that the Trp biosynthetic pathway gives rise to various secondary metabolites, such as the indole alkaloids and indole glucosinolates, in many plants. To date, no remarkable secondary metabolites of Trp origin have been reported in rice plants and the present study suggested that the absence of such Trp-derived secondary metabolites was probably not due to a shortage of Trp supply, but to a very low capability of relevant Trp utilization in rice. Furthermore, Trp decarboxylase probably plays more important role in the divergence of Trprelated carbon flow into the secondary metabolism, as has been observed in transgenic plants overexpressing Trp decarboxylase (Chavadej *et al.,* 1994; Yao *et al.,* 1995).

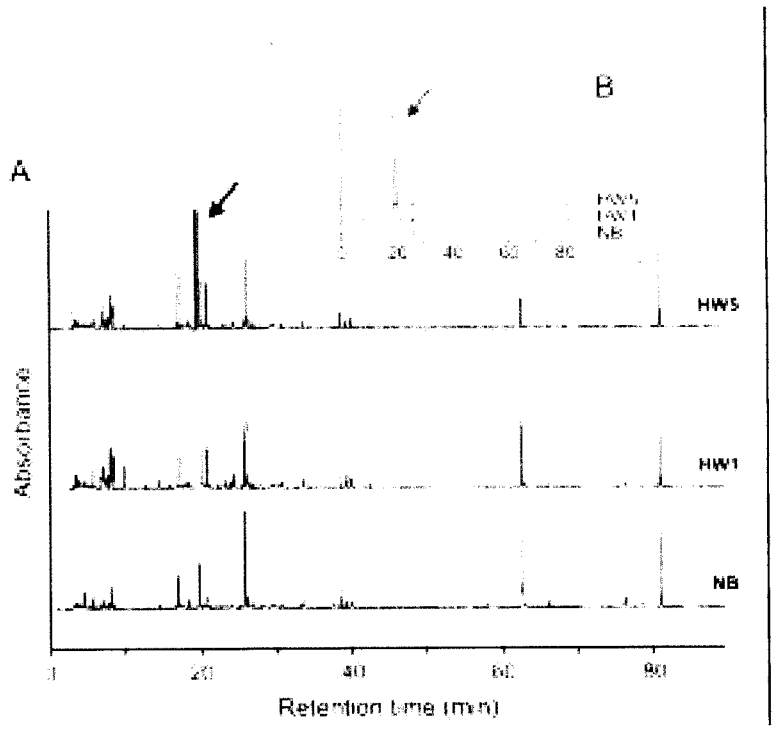


Fig. 1. Aromatic metabolite profiles of seeds derived from field-grown HW lines. Seed extracts of HW1, HW5, and Nipponbare were analysed by reversed-phase HPLC with detection at 280 nm. The traces in the inset are shown at higher magnification in the main panel. The arrow indicates the peak corresponding to Trp.

It was specifically investigated whether the accumulation of Trp in the seeds of the field-grown HW lines affected the amount of IAA, given the close relations between the Trp biosynthetic pathway and IAA production. Therefore the levels of free IAA, of free IAA plus its ester conjugates, and of total IAA (including amide conjugates) were measured in the seeds of the HW lines and Nipponbare (Fig. 2A). The amounts of free and conjugated forms of IAA were each increased about 2-fold in the seeds of both HW lines compared with those in Nipponbare seeds. The increase in the level of

free IAA in seeds was consistent with our previous demonstration of IAA accumulation in rice calli expressing *OASA1D* (Morino *et al.,* 2005). Increased auxin content has been associated with Trp accumulation in cultured carrot and potato cells resistant to 5MT (Widholm, 1977; Sung, 1979). The level of IAA conjugates was also found to be increased in the *Arabidopsis* mutant *Amt1*, which expresses a feedback-insensitive AS and accumulates Trp (Kreps and Town, 1992; Ludwig-Muller *et al.,* 1993). In addition, 5MT-resistant mutants of *Lemna gibba* showed an approximately 3-fold increase in the amount of free IAA (Tam *et al.,* 1995). Seeds of rice and maize normally contain higher concentrations of IAA than do other organs of these plants (Bandurski and Schulze, 1977). The increase in the amounts of free and total IAA apparent in the seeds of the HW lines thus suggests that rice seeds are able to accumulate IAA to especially high levels.

Given that IAA affects multiple aspects of plant growth, the low spikelet fertility and density as well as the impaired seed germination of the HW lines might reflect the increased abundance of IAA rather than that of Trp. IAA markedly inhibited the germination of wheat embryos excised from caryopses that were highly dormant (Ramaih *et al.,* 2003); Trp, the precursor of IAA, was shown to be equally inhibitory in this instance, however. In the case of these HW lines, the impairment in seed agronomic traits was greater for HW1 than for HW5, whereas the amount of total IAA was slightly higher in the seeds of HW5 than in those of HW1. The impairment thus appeared to be more correlated with Trp content than with total IAA (Fig. 2). Regardless, the growth of HW1 and HW5 seedlings after germination overtook that of Nipponbare and no differences in plant growth at the harvesting stage were detected between Nipponbare and the HW lines (data not shown).

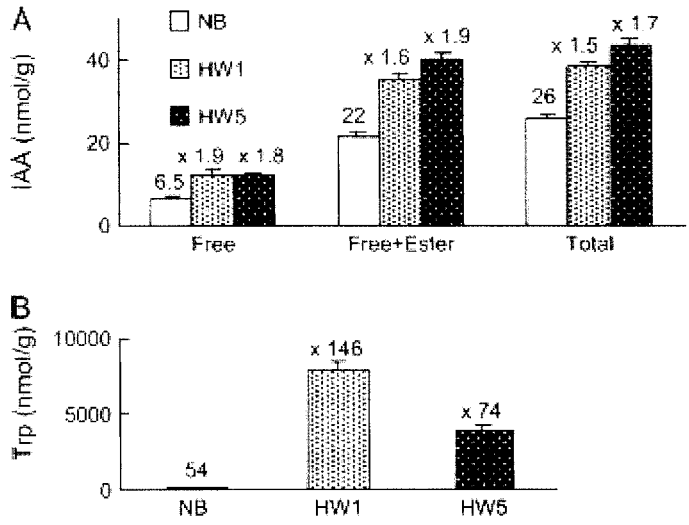


Fig. 2. IAA and Trp contents of seeds derived from field-grown HW lines. The amounts of IAA (A) and free Trp (B) were quantitated in the same seeds of HW1, HW5, or Nipponbare (NB) plants. Data are expressed as nanomoles of analyte per gram of dry seed weight and are means ±SD of values determined from three groups of six seeds.

CONCLUSION

These results have revealed that the seeds of rice plants expressing the *OASA1D* transgene accumulate free Trp to high levels and in a stable manner. This accumulation of Trp was not accompanied by substantial changes in the amounts of other phenolic compounds. The growth of *OASA1D* plants under the field conditions used here revealed a reduction in spikelet fertility and in the number of spikelets per panicle, as well as in the efficiency of seed germination compared with Nipponbare. However, these differences with Nipponbare were more prominent in the HW1 line than in the HW5 line, the latter being largely similar to the wild type with regard to many agronomic traits.

For use as human food, it is preferable that transgenic crops do not contain antibiotic resistance genes. An advantage of *OASA1D* transgenic rice is that the transgene confers sufficient resistance to 5MT to allow the selection of transformed cells with this agent, thereby obviating the need for another gene as a selectable marker (Yamada *et al.,* 2004). Although rice is primarily grown for human consumption, its use for animal feed has been recently developed (Sakai *et al.,* 2003*a*). An increased Trp content of rice seed would thus also prove beneficial for animal nutrition.

ACKNOWLEDGEMENTS

We thank T Kohama, M Kobayashi-Kawagishi, M Ohtake, M Sakurai, and T Kobayashi for help with the maintenance of materials. This work was supported by CREST of the Japan Science and Technology Agency (JST) and by the Multidisciplinary Research of the National Agriculture and Bio-oriented Research Organization (NARO).

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International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
191~208

The Road to Commercialization of Transgenic Products in Taiwan –A Bridge Too Far

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ABSTRACT

The government of Taiwan commenced a national effort of investing in biotechnology very early in the 1980s and steadily increased public investment in various national initiatives and programs. Today, after 10 years of the National Science and Technology Program for Agricultural Biotechnology (NSTP/AB), it has established a solid research foundation, yet has achieved no notable successes in its goals of commercialization. While no one has any doubts over the technological capacity that Taiwan possesses, there are clearly gaps yet to be identified that are preventing the access of bridging R&D and markets. This paper discusses strategies in product development cycles and possible deficiencies that Taiwan must overcome to realize this great potential.

Key words: Agricultural biotechnology; Industrialization platform; Genetically modified organisms (GMOs); Regulatory science; Capacity building.

INTRODUCTION

Since 1982, the government of Taiwan has consistently promoted biotechnology and invested heavily in research and development (R&D), human capital, and infrastructure necessary to build the industry in Taiwan. By 2004, investment reached US$1.6 billion of public funds, with $35 million (2.27%) for agricultural biotechnology. These efforts over the past two decades, however, have produced few successes. Even less investment and output were observed within the private sector

To study the problem, a special project was commissioned in 2005 by the Science & Technology Policy Research and Information Center (STPI), National Applied Research Laboratories. The STPI reported its findings in 2006 in the publication, *Strategic Planning on the Development of the Taiwan Agricultural Biotechnology Industry*, in which a new strategy (hereafter referred to as the STPI model) modeled on product development cycles used in the biotech industry was identified and applied to bridge the gap in the path from R&D to commercialization.

The STPI model proposes a strategy whereby the government focuses on scarce public resources by creating an "industrialization platform" to aid in product development. The proposal was subsequently adopted and implemented by the National Science and Technology Program for Agricultural Biotechnology (NSTP/AB) as a new national strategy for Taiwan (STPI, 2006).

The STPI model was developed from the successful experiences of the biotech industry in the US (Fig. 1A, and see Appendix, Table 3, for more details). The industrialization platform identifies both early product development and advanced development phases as missing links which Taiwan needs for future R&D to succeed.

This strategy seems reasonable when one considers that all of the transgenic products in the pipeline that have been developed in Taiwan remain at the 'proof of concept' phase or earlier.

There are problems with such a view. Although it is extremely helpful for identifying the product development cycle by learning from successes of the biotech industry, currently there is no industry-wide consensus, or even nomenclature, for describing, comparing, or evaluating product development cycles in agricultural biotechnology. McElroy (2004) suggested that a standard nomenclature for describing the steps in agrobiotech product development cycles would enable better valuation of products during the commercialization process (Fig. 1B).

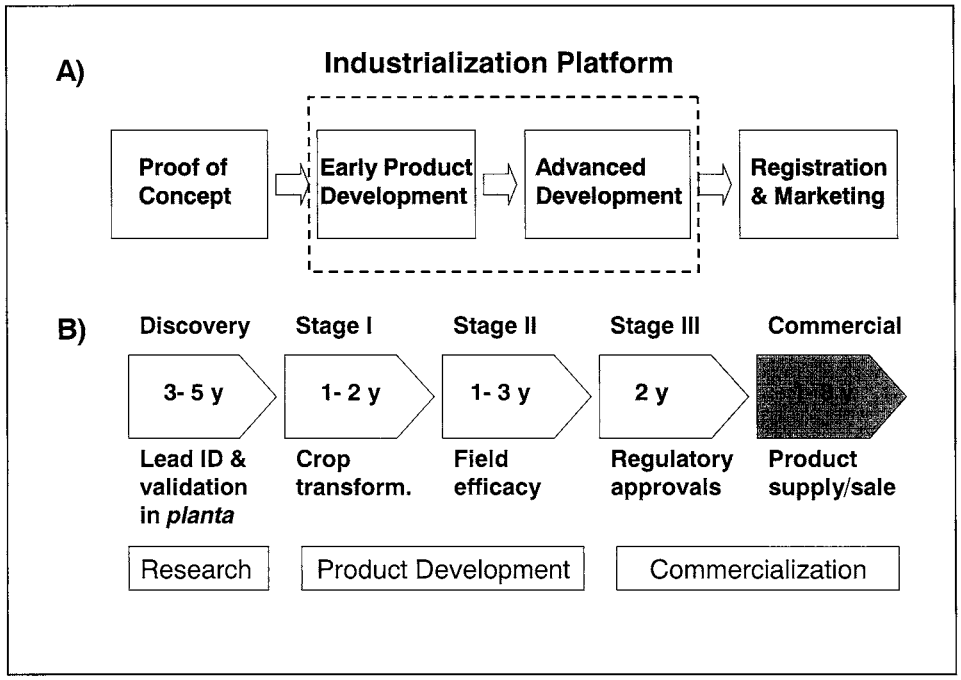


Figure 1. Product developm3ent cycle in agricultural biotechnology. A) STPI Model; B) McElroy Model.

While the new industrialization platform may be useful for building the necessary infrastructure for traditional products, the STPI model falls short for transgenic products (i.e., genetically modified organisms; GMOs) that need to pass through the heavily regulated phase of registration (Fig. 1A) to obtain regulatory approval at stage III in the McElroy model (Fig. 1B).

The industrialization platform -- equivalent to stages I and II in the McElroy model – is an area that is much less clearly defined by industry in the product development cycle among companies with diverse experiences. Most companies, however, would agree that the bottleneck in the product development cycle to market and the most costly stage really lie beyond the industrialization platform, in particular in stage III, where regulatory approval decides whether a product may legally be placed on the market.

The task at stage III is to register the product with the authorities to obtain regulatory approval to enter the market. The requirements for registration are often for biosafety evaluations – to protect plant, animal, and human health when products are placed on the market and released into the environment. The compliance costs for biosafety at stage III are very high and vary according to the market location, in that each country has different standards for biosafety dictated by their laws and regulations. In recent years, biosafety compliance has become the critical path to gaining market access. This is especially true of GMO products where biosafety is of great concern to the public and thus heavily regulated worldwide.

The Costly Registration Stage

Biosafety requires that all biotech products be submitted to a battery of tests and regulatory scrutiny prior to commercialization. Regulatory reviews and approvals for the cultivation and food/feed consumption are country-specific. Therefore, developers must decide very early in which country's markets they will seek regulatory approval for their products. The compliance costs for producing safety data for Japan may differ greatly from that for China, or for the European Community. It has been estimated that it may currently cost multinational companies, with a global market in mind, from $5~10 million to $20~30 million per transgenic crop product (McElroy, 2003). A more-realistic study measuring the costs of biosafety regulation arrived at an estimate of $7.06~15.44 million for an insect-resistant corn (Table 1) and $6.18~14.610 million for an herbicide-resistant corn (Kalaitzandonakes *et al.*, 2006).

High regulatory costs limit the commercialization of transgenic crops to a few multinational companies. Compliance costs in stage III effectively create a barrier that blocks academic and government research institutions and small businesses from commercializing transgenic crops (PEW, 2004). It further discourages the establishment of new biotechnology firms and the flow of venture capital that finances them (McElroy, 2003).

In Taiwan, new biotech firms are growing very slowly. Government investment and outputs for research in agricultural biotechnology look disproportionate (Table 2). In the years 2002~2004, the Taiwanese government spent US$102 million to fund 1365 research projects in agrobiotech. Although the investment did produce a significant number of academic papers (932 in 3 years), it contributed little to commercialization

in terms of patents generated, the number of successful technology transfers, and new biotech companies derived from the results. After a closer look at nearly a thousand papers, we were also disappointed to find no studies were related to the science and technology of biosafety that would be needed for the stage III regulatory requirements.

Table 1. Compliance Costs for Insect-Resistant Corn, in US$1,000

Cost categories	Range of costs incurred
Preparation for hand-off of events into regulatory	20~50
Molecular characterization	300~1200
Compositional assessment	750~1500
Animal performance and safety studies	300~845
Protein production and characterization	162~1725
Toxicology (90 days in rats) – when performed	250~300
Protein safety assessment	195~ 853
Production of tissues	680~2200
Agronomic and phenotypic assessments	130~460
Non-target organism studies	100~600
ELISA development, validation, and expression analysis	415~610
EPA expenses for PIPs	150~715
EU import (detection methods and fees)	230~405
Environmental fate studies	32~800
Canada costs	40~195
Stewardship	250~1000
Facility and management overhead costs	600~4500
Total	**7060~15,440**

Table 2. Government Investment and Research Output in Agricultural Biotechnology

Year	Number of projects	US$ (x 10^6)	Papers published	Number of patents	Technology transfers	New companies
2004	423	35.1 (NT$1166)	413	0	6	0
2003	519	30.1 (NT$1000)	246	5	1	3
2002	423	36.8 (NT$1221)	273	0	4	2
Total	1365	102.0 (NT$3387)	932	5	11	5

Source: STPI, 2006.

During the period of 2001~2003, there were actual studies on the food safety of GM papaya developed by National Chung Hsing University, Taichung. The research was funded by the Council of Agriculture, and four student Master's theses were produced. Unfortunately, no results were published in peer-reviewed journals. The data were seriously flawed and grossly deficient in meeting the data requirements of the Guidance of Safety Assessment for Genetically Modified Foods published by the Department of Health (DOH). GM papaya never had a chance to complete a dossier for food safety for submission to the DOH for registration and commercial approval.

The lessons learned from the GM papaya experience were multifold. Not only are the scientists who become product developers largely unaware of the regulatory costs, but additionally, the gap between the regulatory science, practiced by industry scientists, and academic science, practiced in universities, has fundamentally been underestimated.

Industry of multinational company spends huge amounts of money on regulatory science to establish high-quality laboratories producing large amounts of data to fulfill the safety requirements demanded by regulators in order to complete product

registration. What is often not obvious to academic scientists in this field of endeavor is that much of the experimental data generated for regulatory requirements are not publishable, either for academic reasons or for business reasons of confidentiality.

The study by Kalaitzandonakes *et al.* (2006) did not take into account the large overhead costs for biotech companies. They surmised that it may cost 20% more, and prove difficult to scale up, information that has remained "business confidential" for many companies.

In 2002~2004, with the goal of commercialization, Taiwanese government investment in research reached an average of US$34 million a year. That total amount has sufficient capacity, on the scale of multinational companies, to enable several successful products worth $6~15 million each to reach the global market. So far, none has been produced. Instead of utilizing public funds in focused product development, $102 million was divided by 1365 projects where each research project got US$75,000 on average for very diverse research goals. Focus is always an issue, and while this has been criticized, there has been no resolution reached in government programs.

Since the registration process usually takes years to complete, it is necessary to maintain technical teams to respond to all newly-generated data requirements during the review process. The longer the approval process takes, the more costly it is for developers. An uncertainty with the approval process adds to the costs. Non-transparent processes in government increase the uncertainties. The speed of regulatory decision-making is an important constraint on the ability of industry to bring new products to market.

The Slow Regulatory Process

In spite of some successes over the last decade, many observers have been disappointed at the rate of development and commercialization of new biotech products in recent years. Indeed, accumulating evidence suggests that innovation and product development have slowed down, and high compliance costs for regulatory approval have been cited as a key culprit (McElroy, 2003; Bradford *et al.*, 2005; Jaffe, 2006; Kalaitzandonakes *et al.*, 2006). Over time, stage III continues to evolve, and has developed into a system with increasing sophistication in scientific methodologies and regulatory complexity.

The regulatory process of many agencies often may take years and may vary case by case. In recent years, evidence has shown that the regulatory process is getting longer and decision making is becoming slower. Some factors contributing to this slowdown in some countries may come from data sophistication that has evolved over time. Others may come from the increasing complexity of a regulatory system that is continually growing. Anti-GMO movements by NGOs and low public acceptance undoubtedly have also forced regulators all over the world to take more-precautionary measures and enact more-stringent legislative safeguards.

In the US, federal agencies engaging in product review have slowed down to more than double the length of time (Jaffe, 2006). The regulatory process for USDA in 1994~2000 took 6.1 months on average, increasing to 15.4 months in 2001~2005. The process in the FDA took 6.5 months in 1994~2000, which grew to 15.2 months in 2001~2005.

In Taiwan, the review process appears to have also slowed down in recent years. In

2001, the DOH implemented legislation to require mandatory registration of GM soybeans and corn. The first approval, Roundup Ready soybean, was completed in July 2002. By July 2006, only 12 cases had been completed for approval. In 2005, only one case completed the process, and the same so far in 2006, (Fig. 2A). The average review time took 347 days at the DOH, in contrast to 199 days for the GM soybean and corn cases processed by the USFDA (Fig. 2B).

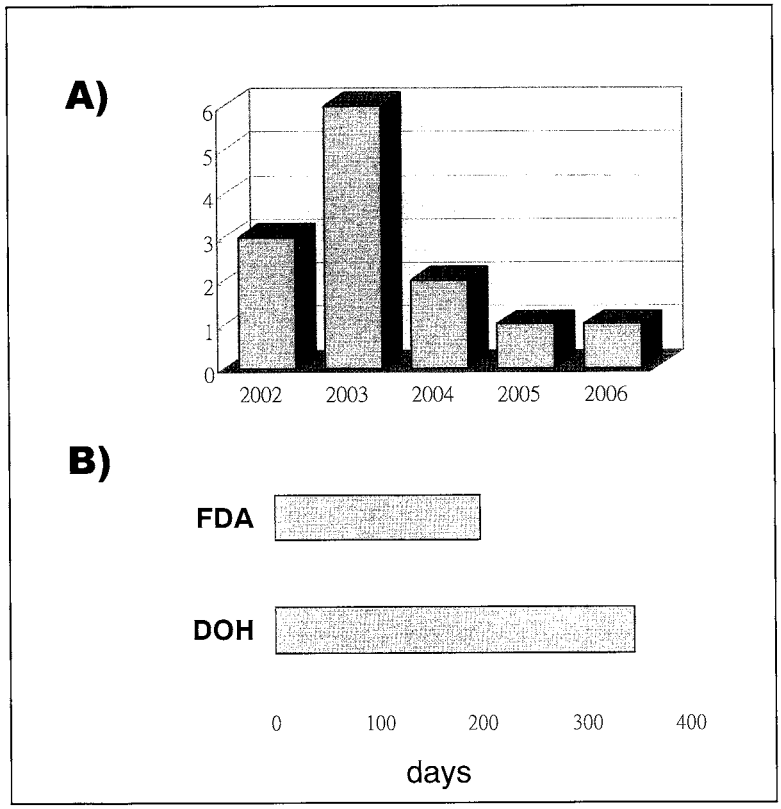


Figure 2. The regulatory process at DOH. A) Number of GM food approvals, 2002-2006. B) Comparison of approval process, in days, taken for GM soybean and GM corn at DOH and USFDA.

Despite a small portfolio of GM products covered by the current legislation (soybeans and corn), DOH has yet to develop the capacity to catch up with the number of other GM products that need to be reviewed and approved for market. In comparison to neighboring countries, for example Japan and Korea, each of which imports the same commodities from essentially the same sources as Taiwan, the DOH seriously lags behind in terms of regulatory approval of many more GM food cases that are already commercialized and are in the pipeline waiting to be processed (Fig. 3).

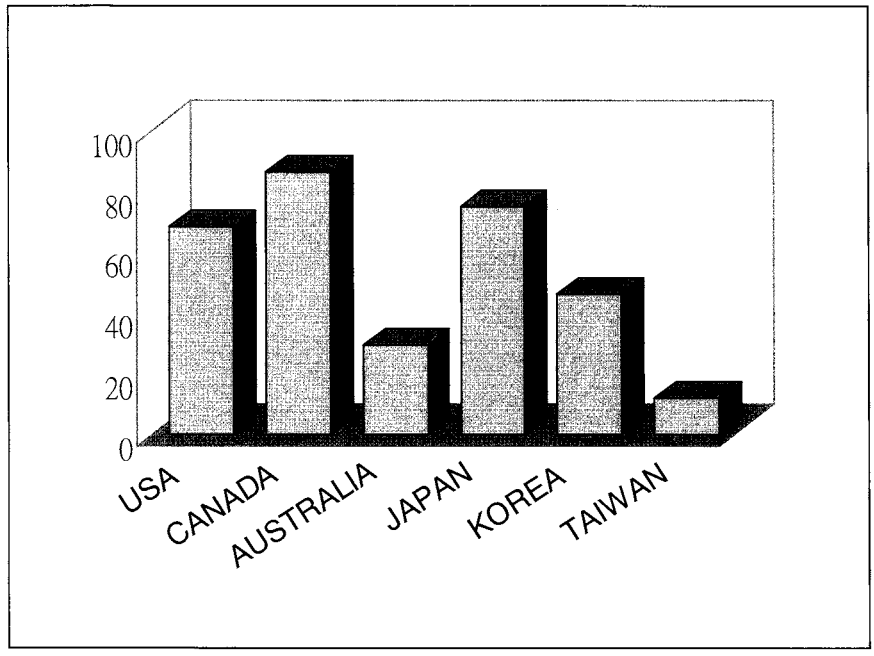


Figure 3. Number of GM Food approvals in exporting NAFTA countries and importing Asia Pacific countries (as of July 2006).

Not only is the DOH slow to catch up with this much-needed work, but the legislation itself is not ready. In November 2003, the DOH published advanced notice that it would broaden the scope of registration for all GM foods and crops. The legislation would enable domestically developed biotech products, e.g., GM papaya, GM rice, etc., to begin the approval process. Until 2006, the legislation still has not been implemented for GM products other than soybeans and corn.

Bridging the Gap – The Regulatory System

The slowdown has drawn concerns not only from industry but from foreign governments as well. Taiwan is the sixth largest agricultural market for the US and imported almost US$1 billion of US biotech products in 2005. While trade has not been interrupted, the slow approval process for new products worries the US government (USDA, 2006). In the 2006 National Trade Estimate Report on Foreign Trade Barriers, the US Trade Representative sounded the alarm "...with a number of products entering the regulatory approval pipeline and a lack of investment in a strong domestic regulatory infrastructure, delays in approvals have become more frequent." (USTR, 2006). In a comparative study of the regulatory systems in Japan and Taiwan, Chen and Watanabe (2006) observed 'pitfalls in implementing biosafety regulatory framework systems' in Taiwan and recommended that this is an urgent task for the Taiwanese government.

A 2005 survey by the Taiwan Institute of Economic Research that studied the limiting factors preventing the willingness of corporate investment in Taiwan also lists the "incompleteness of laws and regulations" as the number one factor (STPI, 2006).

Bridging the Gap – Biosafety Research

Taiwan has demonstrated great potential in developing and utilizing these technologies. Between 2002 and 2004, the Taiwanese government funded 1365 research projects in agricultural biotechnology which produced a respectable volume of academic publications, 932 papers in total (Table 2). So far, almost all the research papers produced in Taiwan are 'discovery' or stage I studies. The innovative initiative of building the "industrialization platform" will undoubtedly bring the fruits of research closer to commercialization. Those efforts do not appear to be sufficient to get past stage III of the regulatory process, and to the commercial stage where post-market compliance to various regulations plays a part.

It is important to consider the post-market commercial stage. The well-known case of StarLink, a GM corn once approved in the US, has taught everyone a good lesson. Dubious biosafety data resulted in the EPA decision to allow it for feed use but not for food use. This allowed StarLink to reach the market where it quickly made a disastrous failure, losing close to a billion dollars and resulting in many lawsuits which finally resulted in it being pulled off the market.

Since the 2001 StarLink fiasco, much progress has been made in upgrading both the technology of biosafety research and the regulations. With such a knowledge-intensive field and heavy investment, naturally, people might generally expect that the research would maximize the output of biotechnology. A study of two major science literature databases that track broad trends in plant transgenic science knowledge from 1973 to 2003 reports a similar observation (Vain, 2006). The study identified 30,624 papers, of which 14.8% were related to technology development, 71.3% were related to application of technology, and 13.8% were related to

development of genetically modified (GM) crops or feed. The expanding gap between technology development and applications may limit future transgenic science and the ability to address issues related to GM crops. Although we have not been able to analyze the nature of papers published in Taiwan in such detail, most scientists would agree that those in the category of original technology development are scarce.

The paucity of studies on technology development and on food safety/environmental risk assessment has not only hampered national efforts to commercialize GMO products to the market but has also dampened the development of science-based regulations (NRC, 2000; IOM, 2004; Bradford *et al.*, 2005; Pelletier, 2006). Above all, in order to bridge the gap from product development to commercialization, Taiwan needs studies in biosafety to fulfill the registration requirements, both locally and elsewhere in the world.

The Developing Country Model

The STPI and McElroy models are drawn from experiences in industrialized countries. In 2005, there were 21 countries in the world cultivating transgenic plants. Many of these are developing countries (James, 2005). There is much to be learned from the experiences of those developing countries.

In commercial cultivation of GM crops, India and China have far more experience at the local farmers' level, with thousands of hectares of GM crops and much accumulating knowledge and experience for future development of crop technology and associated risk management. Even Japan lags behind these Asian countries (Watanabe, 2003).

Like Japan, Taiwan has so far had no success in commercializing its own GM crops. China, however, had already succeeded in commercializing 31 products by 2000 (Rozelle *et al.*, 2001). This is not entirely surprising because developing countries, such as China and India, also invest heavily in R&D and have become the third- and fifth-ranked, respectively, behind the US, the UK and Germany, in the number of GM crop publications produced every year (Vain, 2006). The success in developing countries clearly demonstrates that R&D in these countries can and does support commercialization without the astronomical costs to achieve the same goal of reaching the market seen in industrialized nations.

While one might question that biosafety was compromised due to 'sub-standard' measures adopted in these countries, and admittedly, while much research is still needed, 10 years of global commercialization has sufficiently demonstrated that transgenic technology per se is definitely not intrinsically hazardous. Proper regulatory management within the paradigm of risk analysis could provide adequate protection to plant, animal, and human health. In that context, it is only rational to challenge the current trend which continues to move in the direction of overregulation. At the same time, there are also certain advantages to study and learn from the experiences generated by developing countries where minimal costs from public funds were used to produce many commercial products with minimal regulatory requirements, but while not necessarily compromising safety.

Like Japan, Taiwan needs regulatory agencies that have clear responsibilities, and clear, workable risk-management schemes in R&D institutions. That is especially true considering Taiwan's biotechnology R&D resources rely almost exclusively on

the public sector through such programs as the NSTP/AB. In the foreseeable future, there is little opportunity for Taiwan to build up its own private biotech companies to the scale required to compete with the biotech giants in industrial countries. Instead of building a commercial model in the pattern of the biotech giants, Taiwan has no option but to create a unique path to future agrobiotech development, perhaps taking the lead from those developing countries where small farm practices are the rule rather than the exception.

CONCLUSIONS

Ultimately, the biotechnology products grown in Taiwan are not just for domestic consumption. To survive in the global trade market, Taiwan urgently needs to build its own capacity in government by improving the domestic legislation system and in science and technology of biosafety research. The capacity for biosafety research in turn can promote policy and regulations based on sound science that would allow Taiwan to cope with the ever-evolving complex international regulatory environment.

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Appendix

Table 3. Monsanto Model of Product Development Pipeline Phases

Discovery	Phase I	Phase II	Phase III	Phase IV
Gene/Trait Identification	Proof of concept	Early development	Advanced development	Pre-launch
Average duration, 2~4 years	1~2 years	1~2 years	1~2 years	1~3 years
Average probability of success, 5%	25%	50%	75%	90%
1.High throughput screening, 2.Model crop testing	1.Gene optimization 2.Crop transformation	1.Trait development 2.Large-scale transformation 3.Pre-regulatory data	1.Trait-integration 2.Field testing 3.Regulatory data generation	1.Regulatory submission 2.Seed bulk up 3.Premarketing

Source: Fraley (2005). Available at http://www.monsanto.com/.

International Sympoium (2006)
Ecological and Environmental Biosafety of Transgenic Plants
209~220

A Novel Strategy for Plant Virus Resistance Using Artificial miRNA

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ABSTRACT

Plant micro-RNAs (miRNAs) are 21-nt-long small RNAs that regulate the abundance of target mRNAs by guiding cleavage at the sequence complementary region. We found that DNA sequences encoding Arabidopsis pre-miRNAs can be expressed from a 35S promoter in transgenic plants to produce mature miRNAs. Moreover, sequences in pre-miRNAs can be modified to produce artificial miRNAs (amiRNAs) of a pre-determined sequence. Here, we describe a novel strategy to express amiRNA designed to target and degrade offending viral RNAs thereby conferring specific resistance. The amiRNA-mediated approach should have broad applicability for engineering multiple virus resistance in crop plants.

INTRODUCTION

Viruses are among the most important groups of plant pathogens in agriculture world-wide. Plant viral diseases inflict serious economic losses in major crops by

reducing yield and compromising quality (Kang et al., 2005). Crops can be protected from virus infection by resistance encoded by genes present in existing germplasms. Quite a number of dominant resistance genes (R gene) e.g. *Ry* for resistance to *Potato virus Y* (PVY) in potato (Kang et al., 2005); *Rx1* and *Rx2* for *Potato virus X* (PVX) in potato (Bendahmane et al., 1999); S*w5* for resistance to *Tomato spotted wilt virus* (TSWV) in tomato (Brommonschenkel et al., 2000), etc, have been identified. Some of these dominant genes have been used to confer virus resistance by transgenic approach. Other than dominant R genes, natural recessive resistance genes have also been reported. Several host genes, e.g. the eukaryotic initiation factor (iso) 4E (eIF4E iso), are known to be required for plant viruses to complete its infection cycle in host cells. Mutant plants with deficiency in one of these genes display resistance to potyvirus (Duprat et al., 2002).

The successful incorporation of foreign DNAs into plant cells and the generation of transformed cells into transgenic plants in 1983 opened up new opportunities to use transgenic technologies for the development of virus resistance in plants. During the ensuing two decades, pathogen-derived resistance (Sanford and Johnston, 1985) has been broadly used to genetically modify crop plants for virus tolerance/resistance world-wide. The availability of such virus-resistant crop plants promises to generate enormous economic benefit for agriculture. Nevertheless, concerns have been raised concerning possible ecological impacts of large scale and long term use of such transgenic plants in the field. In this article, we describe a novel strategy to genetically engineer virus resistance using artificial miRNA (amiRNA) technology which may mitigate several of these concerns.

Pathogen-derived Resistance

The concept of pathogen-derived resistance was first propounded by Sanford and Johnston (1985) who suggested using a portion of a pathogen's own genetic material for host defense against the pathogen itself. The underlying rationale was that certain pathogen-derived molecules (RNAs or proteins) may be critical for viral pathogenesis. Non-functional forms of such molecules could act in a dominant negative manner and interfere with virus replication, assembly or movement. Therefore, expression of such dominant negative forms of the pathogen-derived molecule in host cells may confer virus resistance in the expressing host (Sanford and Johnston, 1985).

The feasibility of pathogen-derived resistance was first examined by the expression of the *Tobacco mosaic virus* (TMV) coat protein (CP) gene in tobacco plants (Abel et al., 1986). When challenged with TMV, transgenic plants expressing the TMV CP either did not display symptoms of TMV infection or showed a delay in symptom development. The use of CP expression to confer resistance was quickly verified for several viruses. This type of pathogen-derived resistance is generally referred to as protein-mediated resistance or CP-mediated resistance. In earlier experiments, the prevailing notion was that the virus resistance level is directly related to the CP expression level of transgenic lines.

Further investigations by several laboratories, however, led to the surprising finding that that some transgenic lines with high virus resistance levels in fact did not express any viral CP. Moreover, the CP RNA level was very low or even not detectable in these resistant plants. Subsequent work clarified this apparent discrepancy of non-expressing transgenic plants with virus resistance. We now know

that these transgenic lines were resistant to virus because the expressed CP mRNA triggered post-transcriptional gene silencing (PTGS) and provided RNA-mediated resistance to virus by the siRNA pathway.

In addition to CP mRNA, RNA-mediated virus resistance can be brought about by expression of satellite RNA, defective interfering (DI) RNA or even noncoding region of viral genome RNAs which compete and interfere with virus replication (Baulcombe, 1996). This type of resistance can also be accomplished by expression of viral sequences in the sense or antisense orientation (Smith et al., 1994; Waterhouse et al., 1998) or in double-stranded forms (Helliwell and Waterhouse, 2003). In all these cases, expression triggers degradation of both the transgene RNA and the corresponding viral RNA via the siRNA pathway.

The siRNA Pathway

The siRNA pathway targets double-stranded (ds) RNA for degradation by DICER-like proteins (DCLs) in a sequence-specific manner through the production of siRNA. Whereas DCL2 cleaves dsRNAs from replicating viruses (Xie et al., 2004), DCL3 cleaves dsRNAs derived from endogenous transcripts through the activity of RDR2 and RDR6 (Dalmay et al., 2001; Mourrain et al., 2000). The siRNAs produced are incorporated into RNA-induced silencing complexes (RISC), which guide cleavage of target RNAs. In RISC, siRNAs mediate sequence-specific binding and cleavage of target RNAs (Baulcombe, 2004). Once cleaved, the RNA is further degraded by exonucleases in the cytoplasm. Alternatively, siRNAs are used as primers for RDR polymerase, using target RNA as a template to generate more dsRNA and produce additional siRNAs. This RDR activity expands the pool of siRNA and amplifies PTGS resulting in more potent silencing activity and effective defense against plant viruses.

Plant MicroRNA (miRNA) Genes and the miRNA Pathway

Recently, novel small RNAs, known as micro-RNAs (miRNAs), have been identified as important regulators of gene expression in both plants and animals. miRNAs are single-stranded RNAs 21 nucleotides (nt) in length, generated from processing of longer pre-miRNA precursors (Bartel, 2004) by DCL1 in Arabidopsis (Xie et al., 2004). These small RNAs are recruited to the RISC complex. Using RNA:RNA base-pairing, miRNAs direct RISC in a sequence-specific manner to down-regulate target mRNAs in one of two ways. Limited miRNA:mRNA base-pairing results in translational repression, which is the case with majority of the animal miRNAs studied so far. By contrast, most plant miRNAs show extensive base-pairing to, and guide cleavage of, their target mRNAs (Jones-Rhoades et al., 2006). So far, 117 miRNA genes have been identified in *A. thaliana* and Arabidopsis miRNAs have been shown to be important regulators of plant developmental processes (Jones-Rhoades et al., 2006).

The Use of Artificial miRNAs (amiRNAs) to Confer Virus Resistance

Previous reports have shown that alterations of several nucleotides within a miRNA 21-nt sequence do not affect its biogenesis and maturation (Guo et al., 2005; Vaucheret et al., 2004). This finding raises the possibility to modify miRNA sequence to target specific transcripts, originally not under miRNA control. In human cells, miR30 precursor has been modified to generate an amiRNA to down-regulate gene expression probably by translational inhibition (Boden et al., 2004; Dickins et al., 2005; Stegmeier et al., 2005; Zeng et al., 2002). In plants, Schwab et al. (2006) (Schwab et al., 2006) and Alvarez et al (2006) (Alvarez et al., 2006) have recently

reported the successful down-regulation of Arabidopsis gene expression by amiRNAs targeting either individual transcripts or groups of endogenous transcripts. These amiRNAs were constructed using precursors of miR164b, miR172a and miR319a as backbones.

In an independent study, we have also successfully produced several different amiRNAs using pre-miR159a and pre-miR169c as backbones (Niu et al, 2006). High levels of amiRNAs can be detected in transient expression assays using leaves of *Nicotiana benthamiana* and also in transgenic *Arabidopsis thaliana* carrying 35S-amiRNA transgenes. (Fig. 1). The essential features of the application of this technology for virus resistance are outlined in Fig. 2.

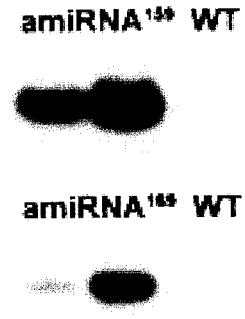


Fig.1. Transient expression of amiRNAs in *N. benthamiana* by agro-infiltration.

To explore possible biotechnological applications of the amiRNA technology, we used pre-miR159a to generate artificial pre-miRNAs^{159} (pre-amiRNAs^{159}) containing sequences complementary to genomes of two plant viruses, *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV). Transgenic lines carrying both 35S-pre-amiRNA^{159} transgenes can express the appropriate amiRNA at high levels.

Moreover, amiRNA transgenic plants showed specific resistance to either TYMV or TuMV, depending on the expression of the cognate amiRNA (Niu et al., 2006). Finally, transgenic plants that expressed both amiRNAs were resistant to both viruses and the virus resistance trait is heritable through at least 3 generations.

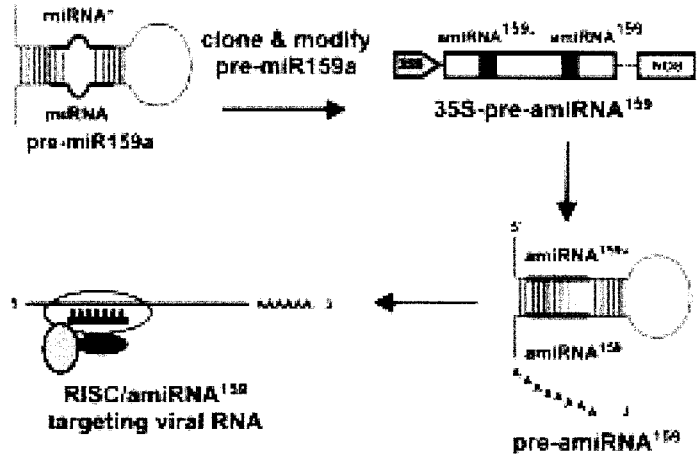


Fig.2. Engineering of amiRNA to target and degrade viral RNA.

It has been reported that virus and transgene-mediated RNA-silencing becomes attenuated at low temperatures which inhibit siRNA accumulation in insect, plant and mammalian cells (Fortier and Belote, 2000; Kameda et al., 2004; Szittya et al., 2003) This temperature sensitivity explains why siRNA-mediated virus resistance breaks down at 15 degrees C (Szittya et al., 2003). We found that miRNA accumulation was hardly affected at low temperatures. Consistent with this finding, transgenic lines expressing amiRNAs maintained their specific virus resistance even at low temperatures (Niu et al., 2006).

Comparison between Pathogen-derived Resistance and amiRNA-Mediated Resistance

Concerns have been raised regarding the large scale and long-term use in the field of transgenic plants (Tepfer, 2002). Potential risks that might occur include the following (Aaziz and Tepfer, 1999; Falk and Bruening, 1994; Hammond et al., 1999; Rubio et al., 1999; Tepfer, 2002): (1) Novel pathogens might be generated via recombination between the virus-derived transgene and non-target viruses; (2) Through possible trans-encapsidation unrelated viruses might be transmitted to host plants; (3) Transcripts expressed from the transgene with pathogen-related sequences might synergize with unrelated viruses to exacerbate symptoms; (4) There might be gene flow from transgenic pollen to weedy relatives, and (5) Transgenic plants might produce new allergens or toxic proteins. With the exception of item #4, these concerns are minimized in the case of transgenic plants engineered to express amiRNAs. We believe that the use of such amiRNA plants will improve bio-safety and reduce any possible environmental impact.

CONCLUSIONS

In this work, we describe a novel strategy to engineer specific virus resistance by the expression of appropriate amiRNA. Because of the functional conservation of miRNA action in plants this strategy should have broad applicability to other viruses as well. It should also be possible to prevent any breakdown of resistance by expressing in the same plant 2-3 amiRNAs targeting different essential regions of a virus. In addition, broad spectrum resistance to several viruses can also be obtained by co-expression of appropriately-designed multiple amiRNAs (Niu et al., 2006).

ACKNOWELEDGMENTS

We thank Drs. Enno Krebbers, Richard Broglie, Karen Broglie and Barbara Mazur for helpful suggestions and stimulating discussions. This work was supported by a grant from DUPONT to N.H.C.

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Programme Schedule

Date/Time	Topic/Activity
Wed., 6 Dec. 2006	Arrival of the foreign participants
18:00-	Hotel Accommodation: **The Splendor- Taichung**(台中金典會館) http://www.splendor-taichung.com.tw/tce/index.htm 1049 Chien Hsing Rd., Taichung Taiwan Tel. 886-4-23288000 Fax: 886-4-23229000
Thu., 7 Dec.	
09:00-10:00	Registration at ARI
10:00-10:20	Inaugural Session Welcome Address Dr. Chien-Yih Lin, Director General, TARI Opening Remarks Dr. Jen-Chyuan Lee, Deputy Minister, COA Special Remarks Dr. Qian Dai, Deputy Minister, NSC Special Remarks Dr. Gwo-Chen Li, the Executive Secretariat, NSTP·AB
10:20-10:30	Group Photo Session
10:30-10:50	Tea/Coffee Break
Session A: Moderator: Dr. Jei-Fu Shaw, President, National Chung Hsing University	
10:50-11:40	Assessment of Emerging Virus Threats for Application of Transgenic Papaya Resistant to Papaya Ringspot Virus. By Prof. Shyi-Dong Yeh (National Chung Hsing Univ.)
11:40-12:30	Environmental Risk Assessments and the Regulation of Genetically Engineered Plants in the United States. By Dr. David Heron (Policy Coordination Division of Biotechnology Regulatory Services, USDA, APHIS)
12:30-14:00	Lunch
Session B: Moderator: Prof. Anthony John Conner, Senior Scientist, Crop & Food Research	
14:00-14:50	Engineering Resistance and Disease Management in Ornamental Crops. By Dr. Hei-Ti Hsu (Microbiologist, USDA, ARS)

14:50-15:40 Establishment of a Rice Mutant Population for Functional Genomics. By Dr. Su-May Yu (Institute of Molecular Biology, Academia Sinica)

15:40-16:00 Coffee Break

Session C: Moderator: Dr. Hung-Chang Huang, Principal Research Scientist, Agriculture & Agri-Food Canada, Research Centre

16:00-16:50 Dealing with Environmental and Societal Issues of GM Crops in an Isolated Place We Call Home. By Dr. Ania M Wieczorek (Dept. of Tropical Plant & Soil Sciences, University of Hawaii, USA)

16:50-17:40 Regulatory Framework of Genetically Modified Plants in Japan. By Dr. Junichi Tanaka (Biotechnology Safety Division, Agriculture, Forestry and Fisheries Research Council, Ministry of Agriculture, Forestry and Fisheries, Japan)

17:40-18:30 Poster Section: Poster Exhibition & Field Trips of Biosafety Assessment at ARI

18:30- Welcome Party

20:00- Hotel Accommodation: **The Splendor- Taichung**(台中金典會館)
http://www.splendor-taichung.com.tw/tce/index.htm
1049 Chien Hsing Rd., Taichung Taiwan
Tel. 886-4-23288000 Fax: 886-4-23229000

Fri., 8 Dec.

Session D: Moderator: Dr. Hei-Ti Hsu, Microbiologist, USDA-ARS

08:40-09:30 Biosafety Assessment of Plants with Novel Traits in Canada. By Dr. Hugh Beckie (Saskatoon Research Centre, Canada)

09:30-10:20 Biosafety Assessment of Transgenic Crops in Soil Environment. By Dr. Chi-Chu Lo (Research Fellow, ACTRI, Taiwan)

10:20-10:40 Coffee Break

Session E: Moderator: Dr. Yu-Tsai Huang, Director General, Agriculture & Food Agency, COA

10:40-11:30 Biosafety Evaluation of Transgenic Potatoes. By Prof. Anthony John Conner (Crop & Food Research, NZ)

11:30-12:20 Designing Biotechnology for Biosafety. By Dr. Richard W. Jones (Vegetable Laboratory, USDA, ARS)

12:20-14:00 Lunch

Session F: Moderator: Dr. David Heron, Assistant Director, Biotechnology Regulatory Services, USDA-APHIS

14:00-14:50 Impact of Roundup Ready® Canola on Plant Pathogen and Its Suitability as Animal Feed: A Case Study in Canada. By Dr. Hung-Chang Huang (Agriculture & Agri-Food Canada, Research Centre)

14:50-15:40 Characterization of Transgenic Rice with High Level of Trypotophan in the Field. By Prof. K. Wakasa (Tokyo University of Agriculture, Japan)

15:40-16:00 Coffee Break

Session G: Moderator: Dr. Liang-Jwu, Chen, Professor, Molecular Biology Institute, National Chung Hsing University

16:00-16:50 The Road to Commercialization of Transgenic Products in Taiwan - A Bridge Too Far. By Dr. Wen-Yen Kao (Biotech Regulatory Affairs Manager, Syngenta, Taiwan)

16:50-17:40 Novel Strategy to Confer Virus Resistance Using miRNA Technology. By Dr. Shih-Shun Lin (The Rockefeller University, NY, USA)

Session H: Moderator: Dr. Chien-Yih Lin, Director General, TARI, Dr. Tuan-Hua David Ho, Director, Institute of Plant & Microbial Biology, Academia Sinica, Dr. Yu-Tsai Huang, Director General, Agriculture & Food Agency, COA, Dr. Tzu-Bin Huang, Director General, Dept. of Sciece & Technology, COA

17:40-18:30 Round Table Discussions (invited participants)

18:30- Dinner (Banquet Hosted by Dr. Chien-Yih Lin, Director General, TARI)

20:00- Hotel Accommodation: **The Splendor- Taichung**（台中金典會館）
http://www.splendor-taichung.com.tw/tce/index.htm
1049 Chien Hsing Rd., Taichung Taiwan
Tel. 886-4-23288000 Fax: 886-4-23229000

Study tours & discussion

Sat., 9 Dec.

07:30-09:10 From The Splendor Taichung Hotel to Taiwan Orchid Biotechnology Park (TOBP)

09:10-10:10 Visiting TOBP

10:10-11:00 From TOBP to Taiwan Livestock Research Institute (TLRI, COA)

11:00-12:00 Visiting TLRI, Transgenic animals/Animal Genebank

12:00-12:30	From TLRI to Tainan District Agricultural Research Station
12:30-13:30	Lunch at Tainan
13:30-	Sightseeing at Tainan
19:30-	Hotel Accommodation: Evergreen Plasza Hotel - Tainan No.1, Lane 336, Sec.3, Chung-Hua E. Road, Tainan, 701, Taiwan. Tel：06-2899988, Fax：06-2896699

Sun., 10 Dec.

08:00-09:30	From Tainan to Floricultural Research Center (FRC), TARI
09:30-10:20	Visiting FRC
10:20-11:20	From FRC to Endemic Species Research Institute (ESRI)
11:20-12:00	Visiting ESRI
12:00-13:30	Lunch at Ji-Ji
13:30-14:30	From Ji-Ji to Sun-Moon Lake
14:30-18:00	Sightseeing at Sun-Moon Lake
18:30-	Dinner at Sun-Moon Lake
19:30-	Hotel Accommodation: Suian Hotel - Tantou Tel: 0492850566 Fax: 0492850090

Mon., 11 Dec.

08:00-11:00	From Sun-Moon Lake to Taipei
11:00-12:00	Visiting Taipei 101 Building
12:00-13:30	Lunch at Hai-Pa Wang
13:30-14:00	To National Palace Museum
14:00-16:00	Visiting National Palace Museum
16:00-17:00	From National Palace Museum to the Council of Agriculture (COA)
17:00-18:00	Visiting Dr. Tzu-Bin Huang, Director General, Department of Science & Technology, COA.
18:30-	Dinner hosted by COA
20:00-	Hotel Accommodation: City Suites -Taipei 169 Nanjing West Rd., Taipei, Taiwan Tel. 886-2-25561700 Fax: 886-2-25561770

Tue., 12 Dec.

Return of participants to respective countries.

Special Publication of ARI No.126
ISBN-13:978-986-00-7734-6
ISBN-10:986-00-7734-7

國家圖書館預行編目資料

Ecological and Environmental Biosafety of Transgenic Plants / Edited by Sheng-Chung Huang, Shu Chen, Chien-Yih Lin/Agricultural Research Institute, Council of Agriculture/Taichung

2006(民 95)

Cover:19*26cm

ISBN-13 : 978-986-00-7734-6

ISBN-10 : 986-00-7734-7

GPN : 1009503692

Ecological and Environmental Biosafety of Transgenic Plants

發行人：林俊義
編　者：黃勝忠、陳述、林俊義
出版者：行政院農業委員會農業試驗所
地　址：台中縣霧峰鄉(41301)萬豐村中正路 189 號
電　話：04-23302301，傳真：04-23338162
網　址：http://www.tari.gov.tw
出版年月：2006 年 12 月
展售門市：國家書坊台視總店　台北市八德路三段 10 號
(02)2578-1515ext643
五南文化廣場　台中市中山路 2 號
(04)2226-0330
中華民國 95 年 12 月初版
定　價：新台幣/ 300 元 (平裝)



Published by
Taiwan Agricultural Research Institute, Council of Agriculture
December, 2006