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Review

Recent progress on environmental biosafety assessment of genetically modified trees and floricultural plants in Japan

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Abstract A broad spectrum of living modified organisms (LMOs) have been developed and commercial production of them have already been started. It is the global requirement to conduct environmental biosafety after the implementation of Cartagena Protocol on Biosafety. Environmental biosafety assessment is of cardinal importance even in confined field trials and at the deliberate release to an environment for practical uses. It is necessary to collect various categories of data prior to fields; not only about the morphological and physiological traits of the LMOs in some confined experimental facilities, but also about characteristics of host organisms in the natural habitat and field for intended releases. Environmental release of LMOs is possible only after collected information is examined systematically and comprehensively in a scientifically-sound manner, and target end-points of such uses are well-identified. Environmental biosafety assessments principally require case-by-case evaluation. Gene-flow, allelopathy and invasiveness are three key aspects in GM plants. In Japan, many crop species do not have their wild relatives due to their historical introduction from overseas. Furthermore, crops grow properly only under agricultural practices, and invasive weediness of these plants is very likely low. In contrast, floricultural plants and trees have related native species located around the fields that can cross with the transgenic counterparts in many cases. In this review, we examine the environmental biosafety assessment of LMO floricultural plants and trees enumerating the example of field trials in Japan, and describe concepts that should be noted for the commercial cultivation and environmental release of these species.

Key words: Environmental biosafety assessment, GMOs, Field trial, Trees, Floricultural plants.

Over the past two decades, products of genetic engineering such as genetically engineered microorganisms have seen wide use in contained commercial applications. In the past 10 years, commercial production systems have been implemented with field dissemination of biotech crops such as herbicide-tolerant soybean and canola and insect-resistant corn. Such transgenic organisms are often referred to as genetically modified organisms (GMOs); however, in this text, the term living modified organism (LMO) is used as the legal term in the Cartagena Protocol on Biosafety (http://www.cbd.int/biosafety/articles.shtml?a=cpb-03).

Transgenic organisms are altered with a few rational genes to improve their original characteristics. In plant breeding, the technology helps to introduce useful genetic variation into conventional genotypes and alleviates the pitfalls of using distantly related species for breeding. LMOs are used in medicine, industry, and agriculture. Because LMOs are generated without ordinary crossing and with gene transfer beyond species, their potential impact on people and the environment must be considered.

The risk assessment concept has been developed over decades through participatory discussion and reexamination by various shareholders, such as regulatory agencies, decision makers, and scientists of diverse professional disciplines, at domestic and international forums in scientific communities, governmental consultation bodies like the National Research Council (NRC) in the United States, or the Japan Bioindustry Association and the Organization for Economic Cooperation and Development (OECD). The feasibility of the risk assessment process and its components has also been discussed with potential LMO developers (Watanabe et al. 2005).

The initial international framework for discussion was established as an OECD guideline in 1994 and included the long-term examination of the content by various parties from OECD member countries. The content was reflected well in Article 15 and Annex II of the Cartagena Protocol on Biosafety. The elements of the risk assessments have been well documented in these international templates; however, the actual modality of the measurements, for example, specific assessment methods or experimental procedures, was kept flexible, as risk assessment requirements vary with the given transgene and host species combinations and specific environments. Thus, a broad range of risk assessment methods exist, in contrast to the international standardization of specific experimental procedures such as PCR of a specific transgene, the primary evaluation method for soil microorganism diversity by community-level physiological profiles (CLPP), or the various specific lab methodologies for evaluating allelopathy. For this reason, LMOs are always evaluated carefully with a step-by-step precautionary approach in physically confined facilities, biologically confined condition, and fields (National Research Council 2002; Pythoud 2004).

Environmental biosafety assessment is of cardinal importance in the field trial and release to an environment of a LMO. Before field release, we must collect data about host organisms, crossing systems, pollinators, pollination distance, the natural habitat including related wild species, hybridization with related wild species, and competitiveness, including allelopathy and weediness in the natural habitat and test field (Teutonico 2006). Before releasing LMOs into the environment, we must comprehensively evaluate the collected information about host species, results of field trials, and the physiological traits of the LMOs; only after considering all these factors is the environmental release of LMOs permitted. In the case of food crops, risk assessments evaluating the LMO as a food item are also performed before the LMO is made available on the market (Watanabe et al. 2005).

Because the suitability of releasing LMOs into the environment is a factor of the combination of host plant, transgene, and cultivation environment, environmental biosafety assessments principally require case-by-case evaluation (Watanabe et al. 2005). Therefore, it is difficult to distinguish the evaluation method of crop and other LMOs plants clearly. However, crop plants generally lack related natural species, with the exception of the soybean (*Glycine max*) in Japan, which is related to the natural species *Glycine soja* (Kaga et al. 2006). Furthermore, most established crop plants are subjected to regular agricultural maintenance, and the weediness of these plants is very low. In contrast, floricultural plants and trees may have related natural species located around the fields that in many cases can cross with the genetically modified (GM) species (Charest 1995; Valenzuela et al. 2006).

In this review, we specifically examine the environmental biosafety assessment of GM floricultural plants and trees, and describe concepts that should be noted for the commercial cultivation and environmental release of these types of plants.

Floricultural plants

Flower color is one of the most important characters of floricultural plants. Consumers always appreciate novel flower color. Traditional breeding has been successful to widen the range of flower colors available. However, it is rare for a single species to have all varieties of flower color because a single species often has limited genes that determine its flower color. Genetic engineering, with which any genes from any organisms can be utilized, has liberated plant breeding from these previous genetic constrains. Flower color modification with genetic engineering has been reviewed (Chandler and Lu 2005; Tanaka 2006; Tanaka et al. 2005; Tanaka and Brugliera 2006) and regulatory and commercial issues of genetically modified floricultural plants have been reviewed (Chandler 2003; Chandler and Tanaka 2007).

Rose, chrysanthemum, carnation, lily and gerbera that account for about 60% of world cut flower market do not have violet and blue varieties. Absence of these flower colors can be attributed to the absence of the flavonoid 3′, 5′-hydroxylase (F3′5′H) gene in these ornamental plants. The gene is essential for plants to synthesize the delphinidin-based anthocyanins that most violet and blue species produce.

Significant efforts have been made to express F3′5′H gene in the plant species mentioned above to yield novel flower colors with blue hues. The first transgnenic violet carnation expressing F3′5′H gene was marketed first in Australia in 1996 followed by Japan, USA and Europe. By changing the host variety and combination of promoter and coding sequences, further varieties of violet carnations were developed and a total of six varieties called Moon series (www.florigene.com) have now been successfully marketed (Chandler 2003; Chandler and Tanaka 2007).

It is necessary to obtain government permission to grow, sell and import transgenic flowers. Before the Cartagena Protocol, these transgenic carnations were regulated by the guidelines and/or laws of each country in which they were grown or sold. After Japan and Europe adopted the protocol, local laws were amended, emphasizing protection of domestic biodiversity. The legislative procedures for development and commercialization of genetically modified plants are often expensive, time-consuming and even painful in the amount of data required and the regulatory procedure varies from country to country to large extent. There is a trend to increase scrutiny (Chandler 2003).

So far five carnation varieties have been granted general release permission in Japan, based on the Cartagena protocol. The summary of the application documents can be seen at http://www.bch.biodic.go.jp/english/law.html. The application documents include detailed description of the particular plant, especially its reproduction. Carnation (*Dianthus caryophyllus*) originated from the Mediterranean area but current cultivated carnations are hybrid cultivars that have been made by extensive breeding. Carnations are usually vegetatively propagated. Their sexual fertility, especially pollen viability, is low and carnations only set seed in controlled breeding conditions Since the anthers of carnation cultivars exist deep under the multiple petals, there is little possibility of pollen dispersion. In case of the color modified carnations, they rarely produce viable pollen. If the carnation produced fertile pollen, hybridization experiment with Japanese wild *Dianthus* species (*D. superbus* L., *D. kiusianus* Makino, *D. japonicus* Thunb., and *D. shinanensis* (Yatabe) Makino, *D. superbus* var. *longicalicinus* (Maxim.) F. N. Williams, and *D. superbus* var. *speciosus* Reichb) might be required.

It is essential to compare growth characters between a transgenic line and its host in a field trial (type 1 experiment) in Japan. This is in order to measure that no significant difference that could affect biodiversity is present in the transgenic carnations. It is also necessary to assess if a transgenic plant produces toxic or allelopathic substances. This is done by lettuce seed germination bioassay tests in soil containing carnation debris and counting the number of microorganisms in the soil. Various molecular analyses such as Southern, Northern and sequence of whole T-DNA are also necessary. It was concluded that gene dispersal to Japanese wild *Dianthus* is most unlikely and general release in Japan was granted. Moon carnations are the only transgenic plants on sale in Japan. They are well accepted among consumers.

Transgenic roses producing delphinidin which have flowers with a blue hue have been also made by expressing F3′5′H gene (Tanaka 2006; Tanaka and Brugliera 2006). Two transgenic lines are being assessed following the Cartagena law in Japan. Cultivated roses have been produced through extensive breeding efforts by using about eight wild rose species and are categorized into an artificial species, *Rosa hybrida*. Unlike the case of carnation, the transgenic roses produce viable pollen which increases the complexity of field trial and general release application. There are about 10 wildrose species in Japan and three of them (*R. multiflora*, *R. wichuraiana*, *R. rugosa*) have been utilized to generate *R. hybrida*. However, *R. hydrida* are tetraploid and most wild species including the three species were diploid. It is therefore assumed that they are very unlikely to hybridize with each other in natural condition (Fukui, H. personal communication). This is supported by the fact that wild roses have not hybridized to *R. hybrida* even though rose is cultivated in large numbers all over the Japan. No cross hybridization between *R. hybrida* and the wild species could be demonstrated in the field trial (the details will be published elsewhere at a later date).

More recently, transgenic chrysanthemum with modified *cry1Ab* gene showing strong insect tolerance had been developed by Shinoyama et al. in Fukui Prefecture (2003). The plants are expected to reduce insecticide use, improve yield and cut-flower quality, and reduce production cost. The transgenic chrysamthemeum plants produce fertile pollen grains and so have a potential to cross-pollinate to wild relatives native to Japan. This group is now accumulating experimental data relating to environmental biosafety assessment using the plants cultivated in a closed greenhouse and a special-netted house.

Trees

Over 200 field cultivations on genetically modified trees have been performed in recent years (COP-8 2006). At a commercial level, only two cases of environmental release of GM trees have occurred. The first was the GM papaya in Hawaii in 1997 and the second was the GM poplar in China in 2003. In 1996, the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture reviewed the environmental assessment of GM papaya and found no significant impact to the environment. The genetically engineered papaya was comparable to conventional papaya varieties, did not exhibit characteristics of weediness, and had no effect on non-target organisms or the general environment (Valenzuela et al. 2006). Those commercial cultivations were subjected to almost the same evaluation items as used for GM crops, since no country has a specific environmental biosafety assessment for GM trees (Valenzuela et al. 2006). Therefore, for both commercially released GM tree species, the environmental biosafety assessment was performed in a manner similar to that used for other GM crops. As mentioned above, LMOs should undergo case-by-case evaluation. Tree species have characteristics distinct from other crop plants, and GM trees must be evaluated based on relevant parameters. Because trees are perennial plants with a long rotation time and remain in the same place for a considerable period, long-term evaluation of the impact of GM trees on the surrounding environment is required (Owusu 1999). Furthermore, for many trees, little is known of their physiology and genetics (Owusu 1999; Valenzuela et al. 2006), and in many cases, tree species generate interspecific hybrids (e.g., *Quercus*, *Populus* and *Eucalyptus*) (Barbour et al. 2003; Ubukata 2003; Brooker 2000). Their pollen and seed dispersal distances are not well-known, and unforeseen acceptors of GM pollen undoubtedly exist around the plantation area.

In Japan, commercial cultivation or environmental release of GM trees has not occurred, and written policies and regulations for such releases do not exist. Only two field trials of GM trees have taken place in Japan, eucalyptus in 2005 (Japan Biosafety Clearing-House 2005; Kikuchi et al. 2006) and poplar in 2007 (Japan Biosafety Clearing-House 2007). The trials were performed in a small confined field and the GM trees were managed meticulously. The necessary regulations for these plantations were the same as for GM crops. The first trial of GM trees was performed at Tsukuba University in Ibaraki on a *Eucalyptus camaldulensis* conferred with salinity tolerance (Japan Biosafety Clearing-House 2005). Generally, in Japan, a field trial is judged based on biological properties of host plants, characteristics of the transgene, and the differences between the GM plants and host non-GM plants. In the case of eucalyptus, this evaluation was carried out using GM plants and host plants grown in a special-netted house (Figure 1). The main items evaluated were growth properties (Figure 2), influence on soil microbes (Table 1), and production of allelopathic substances. In the case of GM crops, several case studies have examined the biological properties of host plants in test areas. However, little information exists on the cultivation and biological properties of *E. camaldulensis* because

Figure 1. The special-netted house in which the GM and host eucalyptus plants were grown.

Table 1. Soil microbe assessment for cultivated soil of transgenic and non-transgenic eucalyptus in a special-netted house. Evaluation was carried out by the plate culture method (Shiomi et al. 1992). No significant difference was detected among those samples.

Lines	Fungus	Ray fungus	Bacteria
Non-transformant	1.95×10^{5}	4.92×10^{5}	9.64×10^{6}
Line 12-5B	1.92×10^{5}	4.77×10^{5}	9.66×10^{6}
Line 12-5C	2.21×10^{5}	4.31×10^{5}	1.11×10^{7}
Line 20-C	1.81×10^{5}	6.47×10^{5}	1.02×10^{7}

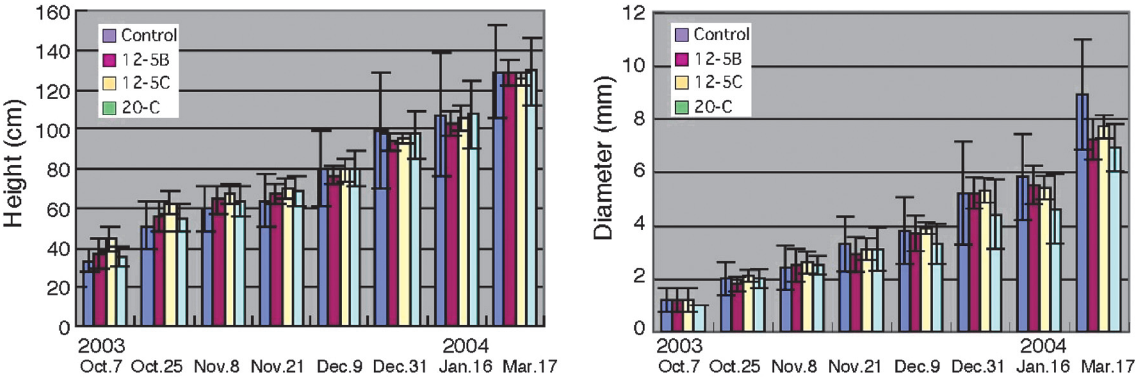

Figure 2. Comparison of growth profiles between transgenic lines and non-transformed *Eucalyptus camaldulensis* in the special-netted house. Transgenic lines and non-transformants had similar growth profiles.

Figure 3. Evaluation of the competitiveness against natural vegetation, wintering habit, and weediness of non-transformed *Eucalyptus camaldulensis* in Tsukuba. White arrows indicate the planted *Eucalyptus camaldulensis*. *Eucalyptus camaldulensis* was not a dominant competitor against native species and did not exhibit weediness.

Tsukuba is not a natural habitat or plantation area of eucalyptus (Pryor 1976; Nishimura 1987). Therefore, non-transformed *E. camaldulensis* was cultivated for a few years in a test field (Figure 3), and competition against natural vegetation, wintering habit, and weediness were evaluated. *Eucalyptus camaldulensis* grown in Tsukuba experienced some damage during winter and lower growth in spring than nearby herbaceous plants, suggesting that it would not have a competitive advantage over natural vegetation and that weediness would not be an issue (Kikuchi et al. 2006). In general, cross-hybridization between GM trees and natural related species must be investigated, but no domestic species related to the eucalyptus grows in Japan (Pryor 1976; Nishimura 1987). Although in principle, considering exotic species is not necessary under the Cartagena Protocol, little information on gene flow and cross-hybridization exists excluding Australia, the natural habitat of the eucalyptus (Boland et al. 1980; Hingston and Potts 2005), and some kinds of eucalyptus are used as ornamental trees in Tsukuba. Therefore, the excision of the floral bud was included in the use regulations as a preventive measure to deter the spread of the transgene to planted ornamental trees around the test field.

Thus, no differences have existed in the regulation of GM trees and GM crops in field trials because only two field trials of GM trees have been performed in small confined fields in Japan. A basic principle of the evaluation of LMOs is step-by-step analysis. In fact, the commercial cultivation and environmental release of GM trees is never permitted without small-scale field trials. Furthermore, another principle of the evaluation of LMOs is a case-by-case analysis. While the basic properties of LMOs are provided by the characteristics of the host species and the transgene, the traits of LMOs are affected considerably by the environment (e.g., biological, climatic, and geographic conditions). Therefore, imposing uniform regulations is difficult and we must apply a case-by-case evaluation for the commercial cultivation and environmental release of GM trees.

What kind of evaluation will be necessary for commercial cultivation and environmental release of GM trees? Many scientists have discussed the components of such evaluations (Charest 1995), and the three primary issues are summarized as follows. First, long-term biological assessment should be conducted at the small field-scale under controlled conditions without gene flow because of the relatively long life spans and persistence of trees (Charest 1995; Owusu 1999; Valenzuela et al. 2006). Second, evaluating gene flow and identifying species that may cross-hybridize with host trees is critical (Charest 1995; Valenzuela et al. 2006). In commercial cultivation and environmental release of GM trees, GM trees are planted in environments suitable for host plants. Thus, the possibility is great that host and related species are found in the same environment. Third, ecological assessment of forests formed by the host species should also be done (OECD 1999a, b; 2000). Some tree species are apt to form simple monocultures, and forests are important habitats for many kinds of organisms, e.g., animals, insects, plants, fungi, and bacteria.

The first issue of above is a basic principle that should be applied to all cases of GM tree plantations. The second one might be necessary as evaluation item. If transgenes have a neutral impact on the host tree and related species, this evaluation item might not be needed. The third item might be important in extreme cases in which the transgene is expected to have a large impact on the ecosystem. Resistance genes for insects or diseases may correspond to this case when the host tree forms a large forest. In the case of insect resistance, a decrease in the number of target insects may lead to a reduction in the number of natural predators that are not the targets of the GM tree. In the case of disease resistance, a threat exists of resistant strains appearing. The necessary evaluation items should depend on the transgene and the properties of the host tree, with long-term evaluation of the biological impact of the GM tree.

Currently, GM trees are roughly classified by transgene types into five groups: marker, drug resistance, physiological control including woody quality, abiotic stress tolerance, and biotic stress tolerance (Smalla et al. 2000). Rough standards for the penetration of transgenes into the environment must be established that depend on the transgene type. Regarding gene flow of transgenes through the environmental release of GM trees, marker and drug-resistance transgenes have weak impacts on the environment (National Research Council 2002; Stewart et al. 2003); these types of transgenes may not benefit or harm trees that are given the transgene. The physiological control and abiotic stress-tolerance transgenes have a certain impact when the transgene confers increased fitness to the species (National Research Council 2002); the GM trees become better competitors and may become dominant or acquire a new habitat. In these cases, we must evaluate the effect of the transgene and the behavior of GM trees under controlled conditions in cultivated fields before commercial cultivation or environmental release. When a transgene has a negative impact on a species, some researchers have concerns about weakening the species (COP-8 2006). For example, a GM tree with reduced lignin content is less resistant to wind, insects, and disease because lignin confers physical strength and improves a tree’s resistance to insects and disease (Pilate et al. 2002). However, the species might only be weakened transiently, as these negative transgenes would be eliminated from the genome of the species. For this reason, we do not need to be concerned about this type of transgene. Finally, the biotic stress-tolerance genes may have a greater impact on the environment, as mentioned above. This type of GM tree has an obvious biological target. Spreading the transgene to the surrounding environment could create a biological crisis for the target species. Because no species is completely independent of others, the ecosystem surrounding the GM trees would be disrupted, or a disease-resistant strain may appear in the future. In general, plantations of GM trees belonging to this group require detailed environmental biosafety evaluations from the total ecosystem point of view.

As stated above, case-by-case analysis is needed in the evaluation of LMOs. A rough classification of items to consider includes the properties of the host (trees), the place of use (planting), the dimensions of use (planting), and the transgene. Based on the combination of these parameters, some cases are worthwhile and others should be carefully considered before GM trees are planted. If gene flow, including seed dispersal, were completely controlled, most cases of GM plantations would be permitted. A few studies have reported successful reductions in pollen (Takada et al. 2005, 2007) and the prevention of flowering in some species (Lemmetyinen et al. 2004). However, gene flow cannot currently be completely controlled, and if spread, we may not be able to remove a transgene from the environment and to return to the conditions that existed before the GM tree planting. For commercial plantations and environmental release, the benefits and environmental risks of GM tree planting should be thoroughly evaluated beforehand.

GM trees are expected to provide future benefits in the form of environmental remediation and the sustainable use of resources (Kopriva and Rennenberg 2000). However, the appropriate applications of this technology must still be identified. For example, isolated locations where gene flow from GM trees does not succeed may be suitable for commercial plantations and environmental release. Such places may include remote solitary islands or regions where the host species and related species do not exist. In the former case, the transgene would be isolated on an island, removed from the gene flow of the host species. In the latter case, GM trees are already isolated biologically because the host species is an exotic. In both situations, it would be possible to completely eliminate all GM trees including their progeny, if necessary, because of the long life cycle and considerable juvenile period of most trees. Such a scenario may only need adjusting in extreme cases. We would need to perform a large-scale simulation using a method that can be reset, if circumstances demand, before commercial cultivation or environmental release of GM trees. Accumulating the scientific results of environmental biosafety assessments and clarifying the range of risk involved in commercial plantations and the environmental release of GM trees are of key importance. The benefits of GM trees and the risks of plantations should be clarified, and the utilization (plantation) of GM trees should be discussed in detail. Effective utilization of LMOs may be an important component in future sustainable development.

Conclusion

Although considerable public resistance to the planting of GM crops exists at the moment, public opinion is currently comparatively generous toward nonfood GMOs. Societal acceptance is indispensable for the development of a new technology. Society must become familiar with GM plants and attractive GM plants must be developed. A concept of GM plant development in which the benefit to consumers and society in general is greater than that to the producer is required. In addition, environmental risk assessments are essential. The environmental biosafety of GM planting should be evaluated scientifically and the latent risk of GM planting should be clarified. Environmental biosafety assessments of LMOs are currently not prioritized in scientific research. Although utilization of LMOs is an extremely effective choice for sustainable development, the scientific value of the environmental biosafety assessment tends to be disregarded. In fact, it is difficult to obtain previous environmental biosafety assessment data. Environmental biosafety assessments should be treated as scientific research and the data published. The evaluation of LMOs is a step-by-step and case-by-case analysis. In addition, more studies need to be performed and the results made available for subsequent trials (COP-8 2006). Scientific technology has two opposite faces, which are useful and harmful for human. When the technology is under our control, we can enjoy the profit. But if the technology would be out of our control, we would be sometime backfired from the technology or nature. To control LMOs, including the use of GM plants, proper evaluation and environmental biosafety assessments are essential to scientific research.

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