

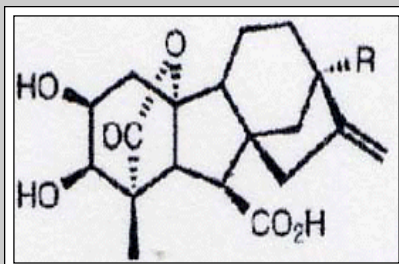
ISB NEWS REPORT

COVERING AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY DEVELOPMENTS

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INSECT RESEARCH

INSECT RESISTANCE TO Bt CROPS: LESSONS FROM THE FIRST SEVEN YEARS

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Problems with insecticides have spurred the search for alternative means of insect control. Not only do conventional insecticides cause environmental and safety hazards, more than 500 species of pests have evolved resistance to them. Insecticidal proteins from the common bacterium *Bacillus thuringiensis* (Bt) are an environmentally friendly alternative to conventional insecticides.

Bt toxins kill insects by binding to and disrupting midgut membranes. Unlike broad spectrum insecticides, Bt toxins kill certain pests but cause little or no harm to most nontarget organisms including wildlife, insect natural enemies, and people. For decades, sprays containing Bt toxins have been useful in organic and main-stream pest control.

Transgenic crops that produce Bt toxins control some key pests, thus decreasing reliance on insecticide applications¹. Surprisingly, after seven years of large scale planting of Bt crops, pest resistance to Bt crops in the field has not been documented².

Large scale planting of Bt crops began in 1996 and grew quickly to more than 10 million ha per year. The cumulative area of Bt crops grown globally from 1996 to 2002 exceeded 62 million ha, enough to cover the states of California and Iowa. More than 99% of this area was planted with either Bt corn or Bt cotton producing Bt toxins Cry1Ab or Cry1Ac to kill larvae of lepidopteran pests. These Bt crops expose pests to Bt toxin throughout the growing season.

The widespread and prolonged exposure to Bt toxins represents one of the largest selections for resistance in insects the world has ever seen. Before Bt crops were grown commercially, many scientists predicted that pests would evolve resistance quickly. This view was supported by pervasive resistance to conventional insecticides, lab-selected resistance to Bt toxins in many pests, and field-evolved resistance to sprays of Bt toxins in diamondback moth (*Plutella xylostella*)³.

To counter the threat of resistance, the refuge strategy⁴ has been adopted widely. Growers provide refuges of non-transgenic host plants along with Bt crops to

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enhance survival of susceptible pests. Ideally, rare resistant adults emerging from Bt crops mate with more abundant susceptible adults from refuges. Modeling results suggest that resistance can be substantially delayed if the heterozygous offspring from such matings are killed by the Bt crop. The refuge strategy is based primarily on theoretical calculations and limited experimental evidence from small-scale experiments with diamondback moth. No rigorous large scale tests of the refuge strategy have been reported.

Although the refuge strategy works beautifully in theory, some scientists thought that real world deviations from its assumptions could doom Bt crops to early failure. Contrary to this expectation, researchers from the University of Arizona, the University of California, and Cornell University recently reported that field-evolved resistance to Bt crops has not been documented yet². This conclusion is based on a review of published results of resistance monitoring efforts in the U.S. and China, which account for the vast majority of Bt crops grown worldwide. To enhance understanding of this surprising outcome, we review below the status of pest resistance to Bt crops, including responses of resistant strains in laboratory and greenhouse tests, and frequencies of resistance in field populations targeted by Bt crops.

Survival of Resistant Strains on Bt Plants in Laboratory and Greenhouse Tests

A key finding is that resistance to Bt toxins in artificial diet or leaf dip bioassays does not necessarily confer the ability to survive on Bt plants. Problems surviving on Bt plants despite resistance to Bt toxins in bioassays could be caused by longer exposure to toxins in tests with plants, higher toxin concentrations in Bt plants, differences between sets of toxins in Bt plants and those tested in bioassays, and interactions between plant chemistry and Bt toxins.

Many pests have been selected for resistance to Bt toxins in the laboratory, but only the diamondback moth has evolved resistance to Bt toxins in the field². Some field populations of diamondback moth in the U.S., Asia and elsewhere that were treated repeatedly with sprays of Bt toxins have evolved resistance to them. This pest attacks cruciferous crops such as cabbage and broccoli. It is not targeted by commercial Bt crops, but has been tested in lab and greenhouse experiments against Bt crucifers created primarily for resistance research. Successful development on Bt crucifers is well documented for at least three independent strains of diamondback moth that evolved resistance via field and lab selection.

While no pests have evolved resistance to Bt crops in the field yet, survival on Bt cotton is reported for at least two lab-selected resistant strains of each of two major cotton pests, pink bollworm (*Pectinophora gossypiella*) and cotton bollworm (*Helicoverpa armigera*). In contrast, a lab-selected strain of another major



cotton pest, tobacco budworm (*Heliothis virescens*) had 10,000-fold resistance to Cry1Ac in artificial diet but did not survive to pupation on Bt cotton or on non-Bt cotton. This developmental failure on cotton plants could reflect fitness costs associated with resistance, inbreeding depression, or both.

Lab selection of a major corn pest, European corn borer (*Ostrinia nubilalis*), has produced strains that resist Bt toxins in artificial diet, but cannot survive on Bt corn. Unlike the aforementioned resistant strain of tobacco budworm, a resistant strain of European corn borer survived on non-transgenic corn, but not on Bt corn. In this case, inability to survive on the Bt plants is caused by the high concentration of Bt toxin in the plants or the interaction between Bt toxin and other plant traits.

In summary, at least seven resistant strains of three species of pests have survived on Bt crops in lab and greenhouse tests. Three resistant strains of diamondback moth survived on Bt crucifers created for resistance research. Two resistant strains of pink bollworm survived on commercially grown varieties of Bt cotton that produce Cry1Ac. Two resistant strains of *H. armigera*, which has inherently lower susceptibility to Cry1Ac, also survived on Bt cotton.

Frequency of Resistance to Bt Toxins in Field Populations

As far as we know, field-evolved resistance to Bt crops has not yet occurred. Indeed, no increase in resistance to Bt toxins was detected in careful monitoring for two to six years of field populations of four major pests targeted by Bt crops (Table 1).

The frequency of pink bollworm resistance to Cry1Ac in

Table 1. Monitoring resistance for two to six years in field populations of pests targeted by Bt crops. So far, no studies show increases in resistance.

Estimated frequency of resistance ^a				
Crop	Insect	Region	Initial (year)	Final (year)
Bt corn	European corn borer	U.S.	0 (1996)	0 (2001)
		France	0 (2000)	0 (2001)
Bt cotton	<i>H. armigera</i>	N. China	0.0095 (1998)	0.0022 (2000)
	<i>H. zea</i>	N. Carolina	0.00043 (2000)	0 (2001)
	Pink bollworm	Arizona	0.16 (1997)	0.075 (2001)

^aValues are estimates of resistance allele frequency except for *H. armigera*, which are survival to third instar with 1 microgram Cry1Ac per ml diet.

Arizona was surprisingly high in 1997, but did not increase from 1997 to 2001. The frequency of resistance in this pest has been estimated by collecting bolls from cotton fields statewide, rearing progeny of individuals from these bolls, and testing the progeny using bioassays with Cry1Ac in artificial diet. Experiments show that 10 micrograms Cry1Ac per ml diet kills essentially all homozygous susceptible and heterozygous larvae, but few or no resistant homozygotes. Thus, the resistance (r) allele frequency can be estimated as the square root of the frequency of survivors, assuming Hardy-Weinberg equilibrium.

The first estimate of pink bollworm r allele frequency was 0.16 (with 95% confidence limits of 0.05 – 0.26) in 1997, which is surprisingly high. This estimate is more than 100 times higher than the highest previous estimate of Bt r allele frequency in a pest targeted by a Bt crop (0.0015 for *H. virescens*) and well above the values typically assumed for modeling the refuge strategy. Despite this deviation from ideal conditions and use of Bt cotton on more than half of Arizona's cotton acreage, the frequency of resistance did not increase from 1997 to 2001.

Several factors probably contributed to the observed lack of increase of pink bollworm resistance to Bt cotton. In resistant strains of pink bollworm studied so far, inheritance of resistance to Bt cotton is completely recessive, which fits the ideal conditions for the refuge strategy. Although resistant strains can complete development on Bt cotton, their performance on Bt cotton is diminished compared with their performance on non-Bt cotton. This disadvantage is called "incomplete resistance." Further, on non-Bt cotton, resistant strains show fitness costs that reduce their performance relative to susceptible strains.

Using methods similar to those applied to pink bollworm, researchers found no increase in the frequency of *H. armigera* resistance to Cry1Ac in northern China from 1998 to 2001. In this region, use of Bt cotton increased from 10,000 ha in 1997 to 1 million ha in 2000, representing about 90% of the cotton planted. In lab bioassays, survival of larvae exposed to diet with 1 microgram Cry1Ac per ml diet was 0.95% in 1998 but only 0.22% in 2001. Likewise, resistance to Cry1Ac in the cotton pest *Helicoverpa zea* did not increase in North Carolina from 2000 to 2001. Extensive screening of European corn borer populations from the U.S. and France has not detected any individuals with genes that confer resistance to Bt corn.

Conclusions

Although at least seven strains of three pests have sur-

vived on Bt plants in lab and greenhouse tests, field-evolved resistance to Bt crops has not been documented yet. Refuges of non-Bt crops grown along with Bt crops are probably important for delaying pest resistance, even though conditions for success of the refuge strategy are not ideal in some cases.

The first generation of Bt crops, which produce one of two closely related toxins (Cry1Ab or Cry1Ac) targeting lepidopteran pests, has remained effective longer than some scientists predicted. Second generation Bt crops, such as Bt cotton producing both Cry1Ac and Cry2Ab, have great promise. The success of Bt crops so far has exceeded expectations of many, but does not preclude resistance problems in the future. In light of current uncertainty, it is best to remain humble about predictions and vigilant in efforts to delay and monitor resistance.

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PLANT RESEARCH

GENERATING SEMI-DWARF RICE BY GENETIC MANIPULATION OF GIBBERELLIN METABOLISM

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Dwarf character is one of the most valuable traits in crop breeding because semi-dwarf cultivars are not only more

resistant to damage by wind and rain (lodging-resistant), but also enable an increase in grain yield rather than straw biomass in response to fertilizer application. Indeed, the introduction of the semi-dwarf trait into cereal crop cultivars, a breakthrough referred to as the 'Green Revolution', enabled dramatic increases in wheat and rice production.

Dwarf mutants have been isolated and extensively characterized in many plant species. The endogenous phytohormone gibberellin (GA) is one of the several compounds associated with dwarf phenotype, and many GA-related dwarf mutants (deficient or insensitive) have been identified in various plant species, including rice. The precise control of GA biosynthesis and degradation is important for normal shoot growth; therefore, genetic manipulation of bioactive GA level is a practical strategy to generate dwarf plants through modification of endogenous GA content¹.

There are two approaches to reduce the endogenous GA content—suppression of GA biosynthesis and enhancement of GA catabolism. To suppress bioactive GA synthesis, we have generated antisense transformants for rice GA biosynthetic gene, *OsGA3ox2*, which encodes a GA biosynthetic enzyme, GA 3-oxidase (GA3ox; Figure 1). However, only a few transformants showed a semi-dwarf phenotype with reduced bioactive GA content, and the phenotype was not stably inherited in the next generation². These phenotypic instabilities may be due to compensation for changes in bioactive GA levels by the homeostatic system. In fact, a reduced bioactive GA content stimulated

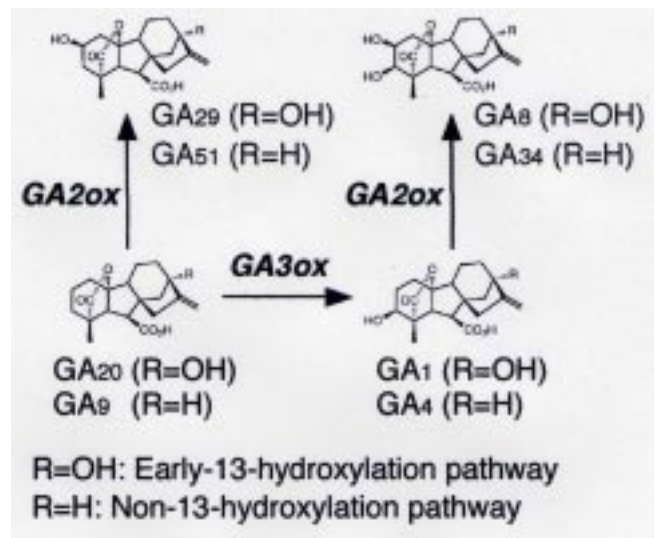


Figure 1. Bioactive GA metabolism in rice. Bioactive GAs, GA₁ and GA₄, are formed by GA 3-oxidase (GA3ox) from GA₂₀ and GA₉, respectively. These 4 GAs are turned over to inactive catabolites by GA 2-oxidase (GA2ox). In rice, vegetative organs mainly synthesize 13-hydroxylated GAs (such as GA₁), while reproductive organs specifically accumulate non-13-hydroxylated GAs (such as GA₄).



the feedback up-regulation of other GA biosynthetic genes. Increased expression of these genes should lead to higher levels of substrates of target enzymes in order to compensate for the reduced activity of the target enzyme by antisense expression.

Another approach is overexpression of GA catabolic gene, which encodes GA 2-oxidase (GA2ox; Figure 1). Most transformants overexpressing a rice GA2ox gene, *OsGA2ox1*, showed extremely dwarfed phenotype³. These results suggest the possibility that plant height could be controlled by modifying the expression levels of GA2ox gene. A correlation between the severity of dwarf phenotype and the expression levels of introduced *OsGA2ox1* gene supports this idea. However, we also observed severe defects in flower development and seed formation in these transformants. It has been suggested that GAs are also involved in reproductive development in various plant species. Overexpression of *OsGA2ox1* in the shoot apex and floral organs by a constitutive promoter should impede phase transition and floral organ development elicited by bioactive GAs. Therefore, it is necessary to restrict the *OsGA2ox1* expression in transgenic rice by using an organ-specific promoter when we want to produce dwarf plants without any defects in flower development and seed formation.

Because of their agronomic importance, a large number of dwarf mutants of rice have been isolated and several of these were characterized as GA-deficient. Biochemical studies revealed that rice has an organ-specific GA biosynthesis pathway. Indeed, vegetative organs of rice mainly synthesized 13-hydroxylated GAs, such as GA₁, while in reproductive organs, especially the anther, non-13-hydroxylated GAs, such as GA₄, were specifically accumulated at a high level (Figure 1).

The rice *d18* mutant has been characterized as a GA-deficient dwarf and recently demonstrated that it is defective in the GA3ox gene, *OsGA3ox2*⁴. In rice, GA3ox is encoded redundantly by 2 genes, *OsGA3ox1* and *OsGA3ox2*. It is noteworthy that reproductive development was not affected in the null alleles of *d18* mutant, although their vegetative development was severely defected (Figure 2). The phenotype suggests that bioactive GA production is severely defected in shoot but not in reproductive development. This means that only one gene (*OsGA3ox2*) functions in shoot elongation, whereas another gene (*OsGA3ox1*) functions redundantly in reproductive organ development. Indeed, *OsGA3ox2* was broadly expressed in both vegetative and reproductive organs, while *OsGA3ox1* was specifically expressed in



Figure 2. Phenotype of rice *d18* mutant. The null alleles of the *d18* mutant were severely dwarfed. It is noteworthy that reproductive development was not abolished in these plants, although their vegetative development was severely defected. The phenotype suggests that bioactive GA production for shoot elongation is severely defected but that for reproductive development is less affected. This indicates that one gene (*OsGA3ox2/D18*) contributes to the GA biosynthesis in shoot elongation, whereas another gene (*OsGA3ox1*) functions redundantly in the reproductive organ development.

reproductive organs of wild-type rice (Sakamoto *et al.*, manuscript submitted), and both *OsGA3ox1* and *OsGA3ox2* proteins produced in *E. coli* are functional in vitro⁴.

The finding that *OsGA3ox2* (*D18*) is an important gene for shoot elongation but not for reproductive development gave us a good promoter for overexpression of *OsGA2ox1* to generate dwarf plants without defects in reproductive development. As we expected, most transformants carrying the *D18* promoter-driven *OsGA2ox1* (*D18:OsGA2ox1*) showed a suitable semi-dwarf phenotype with a height of 70% to 90% that of wild-type³ (Figure 3). These transformants did not show any abnormalities in flowering time, panicle and grain structure, or fertility.

The use of *D18* promoter may have two merits over other vegetative organ-specific promoters in terms of the site of expression and response to GA. Overproduced *OsGA2ox1* consumes both GA₂₀, the substrate for GA₁ production, and bioactive GA₁ itself. Therefore, both biosynthesis and accumulation of GA₁ were severely inhibited in transformants constitutively expressing *OsGA2ox1*. However, the *D18* promoter restricts *OsGA2ox1* expression by a similar amount and at the same site as it restricts endogenous GA3ox gene (*OsGA3ox2*) expression, when we selected suitable transformants to ensure that the *D18* promoter of the transgene functioned in the same manner



Figure 3. Phenotype of *D18:OsGA2ox1* transgenic rice. Typical phenotype of transgenic rice plants carrying the *D18* promoter driven *OsGA2ox1* (*D18:OsGA2ox1*). Most transformants showed a suitable semi-dwarf phenotype (right plant) with a height of 70% to 90% that of wild-type (left plant). These transformants did not show any abnormalities in panicle and grain development.

as the endogenous promoter. Under such conditions, GA_{20} will be shared between catabolic (*OsGA2ox1*) and biosynthetic (*OsGA3ox2*) enzymes, and consequently GA_1 synthesis should be partially maintained.

Another merit of the *D18* promoter for stabilizing the semi-dwarf phenotype is its GA-responsiveness. As mentioned above, the homeostatic system controlling the bioactive GA levels was a serious obstacle when we modified the GA levels in transgenic plants to generate dwarf plants. However, feedback regulation caused by decreased GA_1 content equally up-regulated the expression of both endogenous *D18* (*OsGA3ox2*) and transformed *OsGA2ox1* genes⁵. As the ratio of these two (biosynthetic and catabolic) enzymes would not be changed in *D18:OsGA2ox1* transformants, the promoter consequently contributes to the stability of the semi-dwarf phenotype in transgenic rice.

Our results demonstrate the feasibility of controlling plant height by modifying the expression of the GA catabolic gene in transgenic plants under the control of the promoter for a GA biosynthesis gene. Although we used rice as a model plant, this strategy can, in principle, be adopted for other plant species. Because most of the known dwarfing genes are recessive, this strategy makes it possible to introduce a single dominant dwarfing gene that enables us to increase the grain yield into rice and other transformable crops without the need for a conventional, long-term breeding program.

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INFLUENCE OF ORGANIC ACID EXUDATION IN ALFAFA ON ALUMINUM TOLERANCE, NUTRIENT ACQUISITION, AND BACTERIAL DIVERSITY

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Acid soil is a major constraint to crop production in many parts of the world. Although soil acidification occurs in most soils over time, superimposing agricultural practices on soil systems accelerates acidification. Mineral acid soils develop from leaching of basic cations due to rainfall and irrigation, because of nitrification of ammonium fertilizer, and from cation uptake by nitrogen-fixing legumes. Furthermore, acid rain is increasing soil acidification in many locations worldwide. Many factors contribute to the phytotoxicity of acid soils, depending on soil composition. In acid soils with a high mineral content, aluminum (Al) and manganese (Mn) ions released from soil minerals are taken up by root cells and interfere with root growth and a wide range of cellular processes. In addition, plants in acid mineral soils suffer from nutrient deficiencies in calcium, magnesium, and molybdenum.

Several strategies have been pursued to manage acid soils including liming and application of phosphorus, organic soil amendments, and development of tolerant plant varieties. Liming is often not practical or economical, and incorporation of organic matter has only a temporary ameliorative effect on Al toxicity. Natural variation for tolerance to acid soils is found in some crop species, and characterization of



tolerant plants has led to a better understanding of the mechanisms of Al tolerance and identified candidate genes for ectopic expression to enhance Al tolerance in plants. In a number of tolerant genotypes, excretion of organic acids has a central role in Al tolerance. Upon exposure to Al under acid conditions, organic acids released from roots of tolerant plants form strong complexes with Al ions. This external chelation appears to exclude entry of toxic Al ions into sensitive root cells.

Alfalfa (*Medicago sativa*) is highly sensitive to Al toxicity in acid soil conditions and little tolerance has been found in germplasm collections. Developing acid soil tolerance for this crop is important due to its many important roles in agriculture in the U.S. and around the world. Alfalfa provides nutritious animal forage, increases soil fertility, and is an important conservation buffer preventing nutrient runoff and soil erosion. In an effort to increase tolerance of alfalfa to Al, we generated transgenic alfalfa plants with gene constructs designed to increase organic acid synthesis¹.

In the first report of this tactic, a citrate synthase (CS) gene from *Pseudomonas aeruginosa* was over-expressed in tobacco and papaya plants². Of the organic acids, citrate has the highest binding activity for Al followed by oxalate, malate, and succinate. We hypothesized that over-expression of plant genes for two other enzymes involved in organic acid synthesis, phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), would enhance organic acid synthesis and Al tolerance in alfalfa. MDH is a ubiquitous plant enzyme that occurs in a number of forms due to its diverse roles in plant metabolism³. The nodule-enhanced form (neMDH) from alfalfa, which is primarily expressed in nitrogen-fixing root nodules, has an exceptionally high turnover rate (k_{cat}) for the production of malate compared to other isoforms⁴, making it an attractive candidate for increasing synthesis of malate in roots.

Transgenic alfalfa plants containing an alfalfa neMDH cDNA or nePEPC cDNA under the control of the constitutive CaMV 35S promoter were generated and evaluated for enzymatic activity, organic acid production, and Al tolerance¹. Increases in enzyme protein in root tips generally corresponded with increases in enzyme activity. Selected transgenic plants with a 1.6-fold increase in MDH specific activity showed a 4.2-fold increase in citrate, oxalate, malate, succinate, and acetate in root tissues compared to the control untransformed line. A plant line containing the PEPC transgene with a 2-fold increase in PEPC activity had increased amounts of malate compared to the control. When cultured in quartz sand, trans-

genic lines containing the neMDH transgene exuded up to 7.1-fold more citrate, oxalate, malate, succinate, and acetate than the untransformed line. The line containing the PEPC transgene did not show a significant increase in organic acids in root exudates. In acidic solution culture assays, plants expressing the neMDH or nePEPC transgene showed enhanced root elongation compared with the control untransformed line¹. In assays with 20 μM AlCl_3 , root growth of the transgenic lines was 2- to 3-fold greater than the growth rate of the untransformed control. When subjected to culture with 100 μM AlCl_3 , the roots of untransformed control plants showed no growth, while transgenic lines continued to grow, albeit at a reduced rate. After growth in acid soil (pH=4.0, $\text{Al}_{\text{KCl}}=71 \mu\text{g/ml}$), plants containing the neMDH transgene had twice the root biomass of the untransformed control. This suggests that expression of neMDH can confer broad acid soil tolerance. We are currently investigating the effect of a stronger constitutive promoter and a root tip-specific promoter on gene expression and tolerance to Al in acid culture.

The mechanism of Al tolerance in transgenic alfalfa constitutively expressing the neMDH cDNA has not been investigated. However, it is likely that exclusion of Al from the root is occurring due to exudation of organic acids as well as chelation of Al with organic acids inside root cells. Aluminum content in roots of plants over-expressing neMDH grown in acidic soil was significantly higher than in control plants¹, suggesting the possibility of internal detoxification of Al by complexing with organic acids. Interestingly, these plants also had higher root phosphorus (P) content. Acid soils often have low levels of available P for plant uptake and much of the soil P is complexed with Al and iron hydrous oxides. Organic acids secreted from plant roots have been shown to increase P availability in acid soils. Enhanced P accumulation in transgenic alfalfa tissues suggests that excretion of organic acids is occurring in soil, which results in increased P solubility.

Mobilization of other soil nutrients has been related to complexation with organic acids from root exudates. An analysis of macro- and micronutrient availability in rhizosphere soil of transgenic alfalfa plants with the neMDH construct and control untransformed plants from field plots showed significant differences between the soil samples⁵. Significant increases in soil P, K, Mg, Mn, Cu, and Zn content were found in the transgenic alfalfa rhizosphere. The ability to liberate soil nutrients may improve plant performance in nutrient poor field soils.

In addition to their effect on nutrient availability, root exudates also have a major influence on soil microbial

activity and microbial diversity. Plant roots release as much as 20% of assimilated carbon as organic acids, amino compounds, sugars, and phosphate esters. We investigated bacterial density and diversity in the rhizosphere of transgenic alfalfa over-expressing neMDH and control untransformed alfalfa grown in the field for 53 weeks⁵. Relative abundance of bacterial groups was determined by extracting total soil DNA from each rhizosphere and then amplifying and sequencing 16S rDNA clones. Qualitative differences in bacterial groups occurred between the two rhizospheres. The major group of sequences obtained from both rhizospheres belonged to the proteobacteria. The transgenic alfalfa rhizosphere supported more a- and d-proteobacteria and fewer g-proteobacteria than the untransformed alfalfa rhizosphere. The rhizosphere soil from transgenic alfalfa also supported more bacteria from the high G+C gram-positive, fibrobacter, and nitrospira groups than the rhizosphere soil from untransformed plants. We assessed soil microbial functional diversity by determining carbon source utilization patterns with Biolog GN microtiter plates. Bacteria from the transgenic alfalfa rhizosphere utilized significantly more substrates and showed significantly greater functional diversity than bacteria in the untransformed alfalfa rhizosphere. These results suggest that the transgenic alfalfa plants are producing root exudates that lead to differences in bacterial community structure and composition between the two rhizospheres.

Because of the numerous roles of malate and other organic acids in plant processes, there may be many physiological consequences for MDH over-expression. We have found that transgenic alfalfa plants over-expressing the neMDH cDNA show enhanced Al tolerance, increased P accumulation, and the ability to modify the rhizosphere for improved nutrient acquisition. In nitrogen-fixing root nodules, malate is the primary energy source for bacteroid respiration. Initial experiments to assess the effect of neMDH over-expression on nitrogen fixation showed significantly improved fixation³. Thus, modification of MDH expression in alfalfa shows promise for improving plant adaptation and plant performance in marginal soils.

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HEAVY METAL TOLERANT TRANSGENIC PLANTS

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Heavy metals such as cadmium, chromium, copper, lead, arsenic, nickel, and zinc contaminate urban soils at many sites throughout the world. Nations that continue to use leaded gasoline find toxic levels of lead in agricultural areas, making it difficult to raise animals and crops. Other heavy metals accumulate in soil and water from mining operations, industrial manufacturing facilities, recycling plants, and solid waste disposal sites. Military munitions are also major worldwide sources of groundwater and soil heavy metal contaminants, which wind or rain can sometimes disperse great distances from their point of use or disposal.

Traditional methods of removing heavy metals from soil and water are expensive and laborious, and often disrupt the environment. Contaminated soils can be excavated from the site and placed in a sanitary landfill, at a cost of approximately \$2,000,000 per acre. Not only is this method of heavy metal removal expensive, it may risk the spread of contaminated soil during removal. Alternatively, heavy metals can be stabilized and somewhat detoxified *in situ* using chelators. An example would be the addition of phosphate to soils contaminated with lead, forming an insoluble pyromorphite compound that remains inert in the soil. The cost of this fixation method is about half that of excavation. Another alternative is the use of plants to remediate heavy metals from soils. Phytoremediation can cost less than a quarter of the price of removal or fixation; however, the process can take much longer to be effective. Excavation and fixation of contaminated soils both require six to nine months on average for completion; by comparison, phytoextraction can take between 18 and 60 months.

Studies by the U.S. Environmental Protection Agency and the U.S. Military investigated the feasibility of phytoremediation for heavy metals cleanup¹. Their re-



search looked at four mechanisms of heavy metal uptake by plants: phytoextraction, phytovolatilization, phytostabilization, and rhizofiltration.

The process of phytoextraction uses plants to absorb, concentrate, and precipitate heavy metals from soil. The metals accumulate in plant tissues where they are permanently stored. Plants called hyperaccumulators are preferred because they take up 100 times the concentration of metals over other plants. The plants are then discarded or processed to reclaim the metals.

Phytovolatilization is used to extract volatile metals such as mercury and selenium from sludge and soils and release them through transpiration to the atmosphere as a detoxified vapor.

Phytostabilization is used in sludge, soils, and spoils matrices. Plants are used to stabilize the metals by reducing water and wind erosion. In addition, the mobility of the contaminants is reduced by either being concentrated in root tissue, adsorbed onto roots, or precipitated in the root zone. Secretions into the rhizosphere precipitate the metals and bind them to solid particles in the matrix. The plants also dehydrate the matrix reducing the bulk needed for disposal. This procedure works well for keeping arsenic, cadmium, and lead from leaving the contaminated matrix.

Water is cleared of heavy metal contaminants using rhizofiltration. Plants growing in an *ex situ* or *in situ* hydroponics system are used to absorb, concentrate, and precipitate the metals, which remain in the roots. This technique works best with water tolerant plants having fibrous root systems. Cadmium and lead have been removed from contaminated water using this technique.

A recent summary of heavy metal phytoremediation concluded that the process could significantly decrease contamination over traditional methods, producing a 95% reduction in contaminated material disposed in landfills. However, the method has limitations. Natural plants have limited feasibility for remediation because of the toxicity of the metals to the plants and other inadequate growing conditions. In addition, because phytoremediation is confined to the area covered by the depths of the roots, the method is restricted to shallow contamination sites and does not fully prevent the leeching of contaminants into groundwater. Also, complete remediation is a prolonged procedure because of the slow uptake of metals and small biomass of the plants. Finally, an increased threat of bioaccumulation occurs if the plants enter the food chain in the ecosystem.

Much of the earlier research on improving phytoremediation focused on finding hyperaccumulating plants. The research of Charles Rhyne and Sumita Ghosh at Jackson State University in Mississippi illustrates the criteria for plants regarded as cadmium and lead hyperaccumulators (refer to http://www-esd.lbl.gov/CEB/BEST/ann_rpt99/inter_9story.html). However, hyperaccumulators specific for particular compounds are difficult to find, and many of the plants take up the compounds under prescribed conditions that restrict their use in the field.

Youngsook Lee at the National Research Laboratory for Phytoremediation in Pohang, Korea, devised a strategy for reducing some of the limitations of heavy metal phytoremediation². Her team developed transgenic plants capable of tolerating high levels of accumulated cadmium and lead. These plants take up heavy metals more rapidly than traditional bioremediation plants, making them potential hyperaccumulators with application for phytoextraction and rhizofiltration in the field.

Lee observed that certain *Saccharomyces cerevisiae*, which possess the YCF1, or yeast cadmium factor 1 protein, is known to pump cadmium (Cd(II)) into vacuoles, and tested whether YCF1 would also confer resistance to lead (Pb(II)). Also known as vacuolar glutathione S-conjugate transporter, YCF1 belongs to the ATP-binding cassette superfamily^{2,3}. Lee's team confirmed that *YCF1* gene expression permitted *S. cerevisiae* to withstand the toxic effects of 3 mM lead (Pb II) and 0.1 mM cadmium (Cd II) concentrations in growth media. This protection against lead and cadmium toxicity was due to the uptake and storage of the heavy metals in yeast vacuoles. Next, Lee's group attempted to determine if *YCF1* expression in plants produced the same results.

Arabidopsis thaliana was investigated as a model for *YCF1* expression. First, the *YCF1* gene was created using RT-PCR from YCF1 expressing yeast. For expression in *A. thaliana*, Lee and colleagues subcloned the *YCF1* gene into two vectors—PBI121 and pCambia1302. To enhance expression in plants, the pCambia1302 vector was cloned with four copies of the CaMV 35S promoter. *Agrobacterium tumefaciens* was used for transformation of *A. thaliana*. Green fluorescent protein tagged to *YCF1*, used as an expression reporter, indicated the presence of YCF1 protein in the vacuolar as well as in the plasma membrane of the transformed *Arabidopsis* cells.

Lee and coworkers investigated the uptake and sequestering of lead and cadmium in the plants. Transformed *A.*

thaliana was grown on gravel supplemented with half-concentration Murashige-Skoog agar medium containing 0.75 mM lead or 70 uM cadmium. After three weeks, the plant tissues were analyzed for metal uptake using atomic absorption spectroscopy. Lee's findings showed that the transgenic plants were as effective as naturally occurring hyperaccumulators. Although the transgenic plants accumulated less than two fold higher concentrations of Cd and Pb compared to wild type, this is likely much less than the hyperaccumulator plants (mentioned above).

Plants used for metal phytoremediation have few purposes after they have done their work, as the levels of metal taken up by the tissues may make them unsuitable for agricultural use, although they may have value if methods for inexpensively reclaiming the metals from the plant tissues are refined. Alternatively, heavy metal accumulating plants can be incinerated and the ashes disposed, which is much easier than excavating and disposing the contaminated soil. Although it is highly unlikely heavy metal accumulating plants will ever be used for food, production of non-toxic crops in heavy metal-contaminated areas may be developed from plants that exclude heavy metals, as recently described in *Plant Physiology* by Lee and colleagues⁵.

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ANIMAL RESEARCH

THREE BRITTLE PIGS

Eric Wong

The production of cloned pigs by nuclear transfer has been proposed as a step toward the development of transgenic pigs as a source of transplantable organs for humans. Ironically, the journal article describing an improved method for generating cloned pigs was published in print at about the same time as a news release announcing that these cloned pigs suddenly died at six months of age due to heart failure. This setback reemphasizes the fact that much of the biological details about animal cloning remains a mystery.

In the September 2003 issue of *Biology of Reproduction*, a team of researchers from the University of Connecticut and Taiwan reported the production of cloned pigs by a new method known as whole-cell intracytoplasmic microinjection. To date, three basic methods have been used to produce cloned animals. The original method involved the placement of the donor cell into the perivitelline space surrounding the enucleated oocyte and fusing the donor and recipient cells with electrical pulses. The second method, which was first utilized for cloning mice, involves the microinjection of isolated donor nuclei into enucleated recipient oocytes. The third and most recent method, whole cell intracytoplasmic microinjection, involves the direct injection of whole cells into the cytoplasm of an enucleated oocyte. This latter method eliminates the need for electrofusion or nuclei isolation and is similar to the intracytoplasmic sperm injection method that has been used in human infertility clinics for a number of years. The key difference is that in intracytoplasmic sperm injection the recipient oocyte has not been enucleated and retains its haploid DNA content and the injected cell is a haploid sperm.

One of the initial concerns with the whole-cell intracytoplasmic microinjection method was whether the cellular membrane would break down in the oocyte and form a pronucleus. These studies show that six hours after whole-cell intracytoplasmic microinjection, the plasma membrane was no longer detectable and that by 12 hours after oocyte activation nuclear swelling was clearly evident. In vitro, these reconstructed oocytes were able to support embryo development to the hatched blastocyst stage in 37% of the injected oocytes, which represents an improvement in the efficiency of development to the blastocyst stage. In



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contrast, in previous pig nuclear transfer studies, a 10–23% success rate to the blastocyst stage was reported for the electrofusion method and a 31% success rate for nuclear injection.

Following experiments to optimize the conditions for whole-cell intracytoplasmic microinjection, the researchers attempted to clone pigs starting with skin fibroblasts from the ear of a sow transgenic for the porcine lactoferrin and human factor IX genes. Six hundred eighty-five fibroblast cells were injected into enucleated, *in vitro*-matured oocytes. These reconstructed oocytes were transferred into nine recipients. At day 21, 6/9 (67%) of the recipients were confirmed to be pregnant by ultrasound. Six piglets aborted from three recipients between days 23 and 28 of gestation. Four piglets were born and one piglet died three days after birth due to infection and abnormal spine development. All live born and aborted piglets tested positive for both transgenes, verifying that they were derived from the transgenic donor cells.

The three surviving piglets unexpectedly and suddenly died at less than six months of age due to heart failure, a condition dubbed “adult clone sudden death syndrome.” These deaths and the untimely deaths and health problems of other cloned animals have underscored the dangers and risks associated with animal cloning. In the case of cloned pigs, these animals have been proposed as organ donors for humans. Although some cloned animals appear perfectly healthy, others suffer serious health problems. This variability is likely due to the degree to which the donor cell nucleus is reprogrammed in the recipient oocyte.

This setback, however, should not spell an end to animal cloning, because the progeny of a cloned animal should not be affected by problems related to incomplete nuclear reprogramming. Because the genome is naturally reprogrammed during the development of sperm and egg in the cloned animal, the second generation animal should be normal. As with any new technology, there will always be setbacks along with successes. These results should merely remind researchers to proceed with caution during the development of any new technology.

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ISB ANNOUNCES NEW AND ENHANCED DATABASES

Information Systems for Biotechnology has created and enhanced several of the databases available through the ISB web site (<http://www.isb.vt.edu>).

Group Reports from the Workshop on Future Directions and Research Priorities for the USDA Biotechnology Risk Assessment Research Grants Program

Group Reports from the USDA BRARG workshop held in June, 2003 are now available through the ISB web site.

The reports were compiled by each of the breakout groups: Plants–unintended effects, Plants–resistance management, Plants–gene flow, Microorganisms, Fish, Shellfish and Insects, and Animals. The six group reports identify research needs and priorities for future funding through the BRARG program. The reports are available at http://www.isb.vt.edu/brarg/brarg_wshop/brarg_meeting.htm.

Annotated Bibliographies for Environmental/Ecological Impacts of Transgenic Organisms

To increase awareness and accessibility of peer-reviewed journal articles with data addressing environmental and ecological impacts of transgenic organisms, Dr. LaReesa Wolfenbarger has compiled an annotated bibliography with abstracts. Abstracts are sorted into three categories: data papers (empirical or theoretical), issue papers (no data), and papers on other topics. Abstracts that indicated the paper contained original data (and those where it was ambiguous) are sorted into five topics corresponding to the breakout group topics from the BRARG Workshop above: Plants–unintended effects, Plants–resistance management, Plants–gene flow, Microorganisms, and Animals.

The bibliographies are available in five formats: Acrobat™, MS-Word™, Rich Text, HTML, and Endnote™ .enl files for direct importation into bibliography management programs. The bibliographies will be updated on a regular basis and are accessible at http://www.isb.vt.edu/eeito_bibs/eeito_bibs.cfm or through the Risk Assessment menu option.

Field Tests Currently in Effect

In addition to the other search criteria available, users may now view only those field test permits that are currently in effect. The field test database is available at <http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>.

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