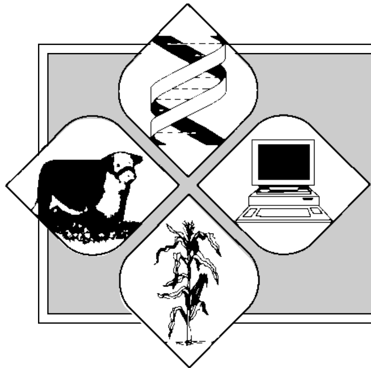

ISB NEWS REPORT

COVERING AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY DEVELOPMENTS

DECEMBER 2002

In This Issue:

USDA Biotechnology Risk Assessment Research Grants Program FY03 Update 1

Report on Talk to the United Nations 2

Engineering Fruit Quality via Novel Genetic Intervention 3

The Fingers of Gene Regulation in Plants 5

Gene Modification of an Endogenous Gene in Rice Plants 7

Enhanced Resistance to Water Deficit Stress in Transgenic Tomatoes 8

Transgenic Pigs Expressing Human Decay Accelerating Factor 9

Don't Panic - It's Organic 10

NEWS AND NOTES

USDA BIOTECHNOLOGY RISK ASSESSMENT RESEARCH GRANTS PROGRAM FY03 UPDATE

The Fiscal Year 2003 Request for Applications (RFA) for the USDA Biotechnology Risk Assessment Research Grants Program (BRARGP) is being developed. The 2002 Farm Bill amends the legislative authority for the BRARGP as follows:

I. Funding for the program is essentially doubled. Approximately $3 million is expected to be available to support research grants from the BRARGP in fiscal year 2003.

II. Research Priorities for the BRARGP have been expanded to include:

(1) Research designed to identify and develop appropriate management practices to minimize physical and biological risks associated with genetically engineered animals, plants, and microorganisms.

(2) Research designed to develop methods to monitor the dispersal of genetically engineered animals, plants, and microorganisms.

(3) Research designed to further existing knowledge with respect to the characteristics, rates, and methods of gene transfer that may occur between genetically engineered animals, plants, and microorganisms and related wild and agricultural organisms.

(4) Environmental assessment research designed to provide analysis, which compares the relative impacts of animals, plants, and microorganisms modified through genetic engineering to other types of production systems.

(5) Other areas of research designed to further the purposes of this program.

The FY2003 RFA will be posted to the BRARGP website as soon as it is available. Please visit: http://www.reeusda.gov/crgam/biotechrisk/biotech.htm for additional details.

THE ISB NEWS REPORT

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REPORT ON TALK TO THE UNITED NATIONS

Jennifer Thomson

On November 6, 2002, I participated in the second lecture in a series arranged at the request of Kofi Annan, Secretary-General of the United Nations. The title of my lecture was Genetically Modified Crops for Developing Countries. There were two speakers, Dr Daphne Preuss of the University of Chicago and myself. The audience consisted of UN delegates, UN staff members, and various NGOs.

Before the talks, the Secretary-General hosted a lunch, which was also attended by the Deputy Secretary-General, Madame Louise Frechette, the Assistant Secretary-General, Michael Doyle, and the President of the US National Academy of Science, Dr Bruce Alberts. This luncheon provided an opportunity for us to discuss some of the issues that would be raised during the lectures with a few of the UN delegates who are particularly interested in this topic.

Dr. Preuss, who works on whole chromosomes in plants, gave an overview of classical plant breeding, explaining how genetic modification differed and describing the relative advantages of each breeding method. She also discussed intellectual property and market forces. I focused on how GM crops can make a difference in developing countries. I gave examples of how GM crops are improving the lives of small-scale farmers in countries such as Indonesia, China, India, and various parts of Africa. Planting insect-resistant cotton gives farmers an increased yield of superior cotton and dramatically decreases the use of pesticides with concomitant improvement of the environment and the health of farmers and others who are exposed to such sprays. I discussed the type of developments that would specifically improve the lives of people in developing countries, such as ß-carotene enriched golden rice and cassava resistant to African Cassava Mosaic Virus. I then dealt with perceptions of lack of food safety associated with GM food and described the complexity of tests that GM crops and foods derived from them have to undergo in order to be declared safe. Finally, I discussed potential environmental impacts such as pollen transfer, horizontal gene transfer, and weediness.

Question time was lively, and I had an interesting interchange with the Ambassador from Zambia, a country that has refused maize food aid from the USA, as it might contain GM maize—this food aid refusal is in the face of major starvation among poor Zambians. The Ambassador accused scientists of not informing them of

the true facts concerning GM food. Having myself visited Zambia recently and spoken with a number of scientists, and having talked to fellow scientists who have either also visited Zambia or have hosted Zambian politicians and scientists in the USA, I don't believe this to be the case. On the contrary, I believe the politicians' opinions of the scientists' testimonies may be biased by political aims.

Judging from the questions we received, it was clear that many in the audience had very little knowledge of farming practice, for instance, that maize is a hybrid crop and unless farmers buy seed every year, whether GM or not, their yield will be severely diminished due to lack of hybrid vigor. I discussed open-pollinated varieties from which farmers could plant their own seed, but explained that these varieties would not be suitable for large scale cultivation and certainly not for export.

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Plant Research

Engineering Fruit Quality via Novel Genetic Intervention

Autar K. Mattoo

In almost every sphere of life, quality is always better than just the quantity. That's perhaps one reason the present day scenario is more focused on quality time. As we prosper more, we want better things in our lives, of which improved food quality has highest priority. This is because we are becoming increasingly aware of the fact that we become what we eat and that our body does better with balanced food. Foods rich in vitamins, minerals, and fiber are naturally of significance to maintaining a healthy body. Thus, consumers hear more and more about recommendations for several courses per day of vegetables and fruits as part of our meals—several courses rather than just one because the levels of nutritive compounds in vegetables and fruits are much below the recommended daily allowance (RDA). Both the increasing awareness and the demand for high quality produce requires that scientists/agriculturists find the ways and means to enrich vegetables and fruits with value-added and health-beneficial compounds. The consumption of fresh produce in the US comprises at least 18% of the American diet (on fresh weight basis).

Quality is a combination of various desirable and economically significant attributes that make vegetables and fruits attractive and acceptable, such as size and shape, color, taste, flavor, gloss, presence of external and internal defects, texture, and softening. These attributes comprise complex parameters and are determined by a number of characteristics. For example, textural quality is determined, among other things, by fruit firmness or softness, fibrousness or toughness, succulence, and sensory qualities. A large number of cellular chemicals contribute to tomato aroma, while fruit taste is determined by acidity caused by organic acids and sugars. Thus, it is conceivable that each of these quality features is a result of highly regulated, multiple processes inherent in the commodity. Modern research has shown that most fruit quality attributes are controlled by sets of genes.

Many factors can negatively affect nutritive composition of a commodity, two of which are short shelf life and microbial infestation. Over-ripening, senescence, and physiological disorders, including chilling injury, are the major causes of post-harvest losses of fruit and vegetables. The genetic and biochemical bases of most of these processes remain to be explored. Losses during post-harvest storage are an economic drain and represent one of the greatest threats to a grower. For instance, tomato fruit decay during post-harvest results in a loss of 20% of marketed fruit, comprising the single greatest economic impact on tomato fruit commerce. Pathogen stress results in decay and development of off-flavor in fresh produce, rendering it unsuitable for consumption. To reduce the threatening impact of fruit rot, chemical fungicides are extensively applied, often on a weekly basis. Most chemicals used to control microbial infestation are toxic and contribute to an unfriendly environment.

Our focus is on using basic biology underlying fruit/vegetable development and ripening/senescence to develop novel, high nutritive-quality and stress-tolerant vegetable and fruit germplasm for the fresh market and processing industries. To reach these goals, we are applying an integrative approach comprised of biochemistry, molecular genetics, and biotechnology, using tomato as a fruit/vegetable model. The development of novel and improved vegetable crops using genetic engineering technology holds great promise. This molecular approach has, in principle, opened the entire living kingdom as a source of genes for introgression into established cultivars, and at the same time made possible rapid transfer of individual genes from the resource population to a particular cultivar, or for the reinsertion of native loci in increased copy number after

laboratory manipulation. Biotechnology can be effectively applied in enhancing the nutritional quality of foods—for example, it is desirable to decrease the levels of toxic metabolites like allergens and to increase the levels of selected nutrients such as lycopene, polyamines, vitamin E, and glutathione, which work as anti-oxidants and decrease the risk of nutritional deficiencies.

Ripening of tomato fruit involves differentiation of chloroplast into chromoplasts, and most of the nutrients beneficial to human health—ß-carotene, vitamin E, and lycopene—accumulate in the chromoplasts. Careful genetic manipulation of processes or metabolites that can stabilize and maintain these chromoplasts in an anabolic state for a longer duration should allow development of transgenic plants with enhanced phytonutrient content. A major obstacle, however, in achieving these objectives is the limited basic information on genes of horticultural and economic importance, particularly since regulatory genes are expressed in low quantities, are highly regulated, and are expressed only in specific tissues at a specific time.

Plant growth, development, and senescence are regulated by a set of plant growth substances that function individually or in unison at specific stages in a plant's life. A set of these, such as auxins, gibberellins, cytokinins, and brassinosteroids, are generally considered as promoters of growth and development, while methyl jasmonate, abscisic acid, and ethylene promote senescence and cell death. In addition to these plant hormones, polyamines have been implicated in cell division, embryogenesis, root formation, floral initiation and development, fruit development and ripening, pollen tube growth, and senescence. However, direct evidence for any physiological role(s) polyamines may have in plant growth, development, and senescence is just emerging. Polyamines are considered anti-senescence in nature and have been implicated in regulating or limiting the action of the pro-senescence hormone ethylene.

Ethylene is synthesized from S-adenosylmethionine (SAM) by a sequential action of two key ethylene-biosynthesis enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. Ethylene is a simple, gaseous hydrocarbon that acts as a dominant hormone in post-harvest physiology and senescence of plant organs. The last century witnessed the cloning and characterization of several ripening-specific gene transcripts, genetic analysis of the various single-gene ripening mutants of tomato, the identification and cloning of genes encoding for each step in ethylene biosynthesis, and identification of ethylene receptors.[1] Genetic manipulation of one or more of these genes has contributed to the practical application of this knowledge to extend the shelf life of horticulturally-important crops.

Biosynthetic pathways for ethylene and polyamines in plants share SAM as a key intermediate. In this regard, it would appear that a plant cell has the potential to commit the flux of SAM either into polyamine biosynthesis, ethylene biosynthesis, or both.[2] Polyamines are ubiquitous in flowering plants and mainly comprise putrescine, cadaverine, 1,3-diaminopropane, spermidine, and spermine; however, other modified forms are also known in plants. Putrescine is formed from either arginine via an intermediate agmatine, a reaction catalyzed by arginine decarboxylase (ADC), or from ornithine by ornithine decarboxylase (ODC). Spermidine is synthesized from putrescine and the aminopropyl group donated by decarboxylated SAM, which is a product of SAM decarboxylation. In turn, spermidine incorporates another aminopropyl group (from decarboxylated SAM) to form spermine.[3]

To unambiguously demonstrate the role(s) of polyamines in the ripening process, whether through an effect on ethylene biosynthesis or ethylene action or other signals, my laboratory in collaboration with Prof. Avtar Handa of Purdue University (West Lafayette, Indiana) used a transgenic approach to accumulate polyamines at higher levels during ripening in tomato fruit. We reconstructed the yeast SAM decarboxylase (ySAMdc) gene[4] by fusing it with a ripening-inducible promoter[5] and introduced it into tomato plants using *Agrobacterium*-mediated gene transfer. Segregation analysis indicated Mendelian inheritance of the gene. Among four independent lines found homozygous with the introduced ySAMdc gene, two were fully characterized. The two transgenic lines produced fruit with several-fold increases in the levels of spermidine and spermine, while the wild type and azygous lines showed a consistent decrease during ripening in these polyamines. The accumulation of polyamines in the transgenic lines paralleled the accumulation of the ySAMdc transcripts. The effects of unusual accumulation of spermidine and spermine during ripening of the fruit resulted in delayed ripening of the fruit on the vine by two weeks, as compared to the fruit from the wild-type plants, and the juice quality was vastly improved.[6] There was no marked change in the fruit yield between the lines.

Further, we found that the transgenic fruit we developed accumulated several-fold more of the carotenoid lycopene compared to the non-transformed fruit.[6] Red tomato fruit color is primarily determined by lycopene content. Transformation of chloroplasts into chromoplasts during tomato fruit ripening leads to chlorophyll loss and increased content

of carotenoids such as lycopene. Carotenoids are lipid soluble pigments found in chloroplast and chromoplast membranes.[7] In addition to their role in photosynthetic reaction centers, they are essential for photoprotection. Moreover, they are also precursors of abscisic acid (ABA), a plant hormone that modulates developmental and stress processes. Polyamines are, among other processes, likely involved in stabilization of chromoplast function in plants.

Further investigation into this area should reveal novel regulation of plant metabolic processes, which may yet present us with new approaches to engineer metabolic pathways. These high polyamine-accumulating transgenic tomato lines are providing an excellent model system to investigate the role of polyamines in regulating gene expression, metabolism, growth, and development in plants and fruit ripening/senescence processes. For instance, we are investigating if polyamine accumulation results in changing the cellular redox systems due to their anti-oxidative nature, what processes are involved in poly-amines-mediated stabilization of cellular membranes, which gene clusters are regulated by polyamines-mediated signaling, and how polyamines tilt the developmental balance towards anabolic from catabolic metabolism.

I thank my colleagues listed in the references for collaboration and stimulating research.

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The Fingers of Gene Regulation in Plants

Roger N. Beachy and M. Isabel Ordiz

The machinery that controls gene expression in all organisms consists of a series of intracellular processes in which specific proteins, broadly referred to as transcription factors, play a significant role. Transcription factors have a modular structure: a DNA binding domain which targets the protein to specific DNA sequence(s); and a regulatory domain that acts to increase or decrease transcription of the gene(s) that contains the DNA binding domain.

Of all transcription factors, zinc finger-containing proteins are the most abundant in nature. It is estimated that 700 genes encode such factors in humans, while the model plant *Arabidopsis thaliana* contains about 85 genes of this class. Natural zinc finger proteins contain three zinc finger domains. Each single domain contains 30 amino acids that create a structure of two β-sheets and one α-helix (ββα), stabilized by hydrophobic interactions and the presence of a single zinc ion. The α-helix structure facilitates the interaction of the protein with three nucleotides (triplet). Polydactil six-zinc finger proteins that recognize eighteen consecutive nucleotides have been artificially engineered, thus creating a protein that is highly specific for 18 nucleotides.

By combining the binding characteristics of a synthetic six-zinc finger with a regulator domain, e.g., the herpes simplex virus VP16 activation domain, we explored the use of zinc finger technology in plants to control the expression of novel and endogenous genes.

In our experiments, we used a well-characterized six-zinc finger protein, 2C7-protein, and its DNA recognition sequence. To measure the interaction of the two elements, β-glucuronidase reporter genes that contained the 2C7 DNA binding site were constructed and introduced to cells along with genes encoding the 2C7 proteins. In tobacco protoplasts, there was significant induction of reporter gene

expression, subject to a variety of conditions.[1] For example, the expression of the reporter gene was dependent on the distance between the 2C7 sites and TATA box of the reporter gene construct. The addition of the herpes simplex virus VP16 activation domain or a four times repeat of the minimal activation domain from VP16 (referred to as VP64) to the 2C7 protein increased expression of the reporter gene from 5 to 30 fold in tobacco protoplasts. In transgenic plants, expression of the reporter gene increased by as much as 450 fold.[1]

In related experiments, the zinc finger activator protein was expressed from a phloem-specific promoter in transgenic plants that also contained a reporter gene construct. In these plants, the reporter gene was expressed only in the vascular tissues. Expression of the transgenes was stably inherited.[1]

Stege and colleagues used a viral vector (based on tobacco mosaic virus) to test a variety of gene constructs in transient assays in BY-2 tobacco and maize protoplasts. The results of these studies indicate that the system can be useful in monocot as well as dicot cells. The use of C7 proteins that contain three-zinc fingers resulted in a higher level of gene activation (by about 4x) when compared with 2C7 six-zinc finger proteins. The authors suggested that the smaller DNA binding site might increase the occupancy of the protein on the C7 binding site.[2]

This paper also analyzed the effect of several known repressor domains (e.g., KRAB-A and SID) in this assay system. The KRAB-A domain of the Kruppel-associated box-A domain is known to act at the level of chromatin modification and remodeling; the mode of action of mSin3 (SID repressor domain) of the human MAD1 protein is not known. When attached to the six-zinc finger protein, the KRAB domain caused no specific repression of the reporter gene in the tobacco protoplasts; in contrast, the SID domain exhibited a 5-fold level of repression.

These data confirmed that it is possible to increase as well as decrease the expression of exogenous genes in plants using synthetic six-zinc finger proteins, thereby opening the possibility of controlling endogenous genes in plants.

To explore that possibility of regulating a native gene, Guan and colleagues developed a synthetic six-zinc finger protein that binds to a specific sequence upstream of the promoter of the *Arabidopsis thaliana AP3* gene; *AP3* controls floral organ identity.[3] The effect of the zinc finger protein was tested with a reporter gene comprising the *AP3* promoter and the β-glucuronidase open reading frame. The positive or negative effect of the factor was mediated by the fusion of the zinc finger proteins with an activator (VP64) or repressor (SID) domain, under control of the *AP1* promoter. The *AP1* promoter is expressed throughout young floral primordia and in sepal and petal primordia in mature flowers. Plants co-transfected with the recognition sequence (AP3::GUS) and the activator protein (AP1::ZFP^{AP3}-VP64) showed expression of β-glucuronidase in all primordia and in petals and sepals in later stages in a manner similar to the expression of *AP1*. Some plants had five or more petals and a reduced number of sepals; the stamens were normal and fertile.

In plants that contain the repressor protein AP1::Sid-ZFP^{AP3} the β-glucuronidase was expressed in stamens only. Petals in some flowers were absent and some were converted to sepals; these plants were fertile. When a constitutive promoter (UBQ3) was fused to the repressor protein, most of the plants were sterile, the petals were narrower and shorter than normal, and stamens were reduced in size with respect to the normal plant. The phenotype of these plants was similar to the phenotype previously described for *ap3* and *sap* mutants. These characteristics were transmitted to the progeny in at least two consecutive generations,[3] confirming that the zinc finger regulatory proteins can continue to exert their regulatory effect, as do endogenous regulatory genes.

The possibilities of using synthetic proteins to regulate at will the expression of endogenous genes will have many applications, including the study of gene function, in regulating the expression of novel genes and developing novel pathways for controlling expression of genes in plants, as well as other organisms. Clearly, the targeted application of this technology opens a wide range of possibilities in plant science and agricultural biotechnology.

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Gene Modification of an Endogenous Gene in Rice Plants

Shigeru Iida and Rie Terada

Rice (*Oryza sativa* L.) is an important staple food for more than half of the world's population. It is a model plant for other cereal species because of several characteristics: its small genome (~430 Mb); the similarity of its sequences and constellation of the genes with other cereals; the availability of large expressed sequenced tag (EST) and cDNA databases; and its efficient use of *Agrobacterium*-mediated transformation.[1] Moreover, the draft sequences of both *japonica* and *indica* subspecies were published in April 2002,[2,3] and an international effort by the International Rice Genome Sequencing Project is supposed to complete the entire genome sequence of the *japonica* subspecies cv. Nipponbare by the end of the same year (see http://rgp.dna.affrc.go.jp/index.html).

A comparison of the *Arabidopsis* genome sequence with the available rice sequences indicates that a large proportion of the approximately 40,000 predicted rice genes have no recognizable homologues in *Arabidopsis.* Under these circumstances, the development of reverse genetics procedures to study rice gene function becomes extremely important. Among various procedures that modify genomic sequences, including generation of insertion mutants with transposons and T-DNA sequences, gene targeting by homologous recombination is one of the most powerful tools of such reverse genetics because it can alter an endogenous gene into a designed sequence. In higher plants, however, only limited success has been achieved in targeting endogenous natural genes in *Arabidopsis*—a single plant has been engineered with the targeted *AGL5* MADS-box regulatory gene, and three targeted plants have been rendered herbicide-resistant by homologous recombination of the endogenous gene for protoporphyrinogen oxidase (PPO).[4,5] No attempt to modify an endogenous gene in monocotyledonous plants, including rice, has been reported.

Shigeru Iida and coworkers have reported an efficient and reproducible procedure for gene targeting by homologous recombination in rice that is based upon three main components: 1) optimization of *Agrobacterium*-mediated transformation; 2) utilization of strong positive/negative selection; and 3) stringent PCR screening for the targeted allele.[6] The authors chose the rice *Waxy* gene as a model gene to be targeted because its mutants affect the quality and quantity of rice grain and because its phenotype in pollen and in endosperm can easily be assessed by simple iodine staining (see Figure 1). The single locus *Waxy* gene encodes granule-bound starch synthase, a key enzyme in amylose synthesis.

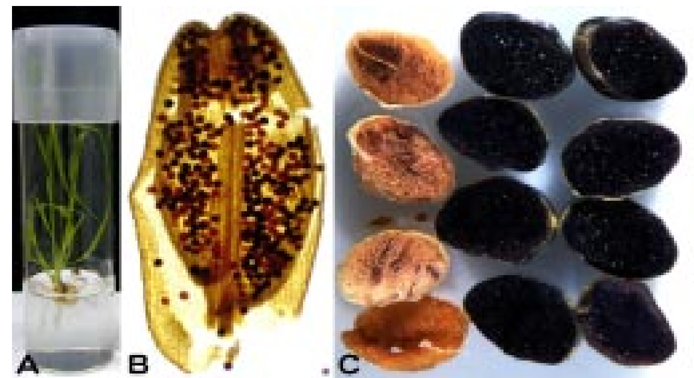

Figure 1. *Targeted rice plant (A) and its Waxy phenotype detected by the iodine staining in pollen (B) and endosperm (C). The Waxy and waxy phenotypes are indicated by the dark and light brown staining with iodine, respectively.*

The group has been engaged in developing *Waxy* gene targeting for more than five years. In six separate experiments using the *japonica* rice variety cv. Nipponbare, they obtained, using positive/negative selection, six independent targeted rice plants from the PCR-screened calli from among the approximately 1% of survivors. All of these six fertile transgenic plants were true recombinants and were not comprised of undesirable events produced by ectopic recombination (integration of the sequence produced by homologous recombination into a genome other than the correctly targeted site) and/or simultaneous ectopic integration of the introduced transgene (drug-resistant, positive selection marker). The occurrence of such ectopic recombination or ectopic integration events was often observed in gene targeting of the *PPO* gene in *Arabidopsis*.[5]

All the targeted rice plants were heterozygotes at the *Waxy* locus—a wild-type *Waxy* allele and a targeted recombinant *waxy* allele having a selective drug-resistant marker were integrated within the *Waxy* gene. Indeed, both the *waxy* and drug-resistant traits cosegregate into their selfed progeny in Mendelian fashion (see Figure 1 for the *Waxy* characters detected by the iodine staining). Southern hybridization analysis revealed that the targeted *waxy* region of about 35 kb comprised the anticipated structure, and that the drug-resistant marker used for selecting transformants was integrated only within the targeted *waxy* gene in the genome. No sequence aberrations could be detected in the sequenced junction regions where somatic homologous recombination should have taken place. Based on these results, it is concluded that the rice *Waxy* gene

can be specifically disrupted by substitution of the drug-resistant marker sequence for the targeted segment within the *Waxy* gene through homologous recombination.

The next obvious question is whether the procedure is applicable for other rice genes. Since the adapted strategy is independent of the gene-specific selection demonstrated in *Arabidopsis*,[5] it can, in principle, be applied to any gene, including essential genes, because all of the six targeted plants obtained were heterozygous. It would be also interesting to see whether only "clean and true" targeted events can also occur in other rice genes and how often either ectopic recombination or simultaneous ectopic integration is accompanied by the precise homologous recombination. Certainly, "clean and true" gene targeting without any ectopic events would become the most powerful tool for characterizing the functions of a number of rice genes, the function of which remains to be identified. Another obvious question would be whether the procedure is applicable to other plants. Since both the conditions for transformation and the introduced vectors used are designed to be optimized for gene targeting in rice calli, appropriate modifications and refinements would be necessary for other plants. However, a strategy based upon the three main components mentioned above must be applicable to other plants. Therefore, the method can, in principle, be used to obtain various gene-targeted or knockout lines of rice and presumably other plants including other cereals.

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Enhanced Resistance to Water Deficit Stress in Transgenic Tomatoes

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Unlike migratory birds and other animals, most angiosperm plants cannot escape environmental stresses and hence have developed sophisticated mechanisms for defense. Research has revealed that many of the stresses, e.g., oxidative, salinity, drought, high light influence, water deficit etc., likely have interconnected defense responses. Therefore, examining individual stress responses independently may not provide complete information or afford optimal solutions. Fundamental research is beginning to unravel the molecular basis of abiotic stress resistance and has shown that transgenic plants exhibiting an elevation of enzymes that scavenge toxic oxygen species have increased abiotic stress tolerance. Stress responsive genes like *Lea* (late embryogenesis), *rd* (responsive to dehydration), and *erd* (early responsive to dehydration) have been isolated.

Many environmental stresses ultimately lead to dehydration in plants. A research team from the Institute of BioAgricultural Sciences (Taiwan) and the National Graduate Institute of Life Sciences (Taiwan) has developed transgenic tomato plants with an enhanced resistance to water deficit stress by over-expressing the *CBF1* (C repeat/dehydration-responsive element binding factor 1) gene. *CBF1* genes are regarded as 'master switches' that in turn activate expression of *COR* genes, increasing freezing tolerance in transgenic plants in the absence of cold stimulation. A plasmid, containing a DNA cassette consisting of an *Arabidopsis CBF1* cDNA driven by a 35S promoter, and *nos* terminator, ß-Glucuronidase (GUS), and *NPTII* reporter genes, was inserted into the tomato genome via *Agrobacterium tumefaciens* transformation.

Twenty-one unique transgenic tomato lines were obtained. Some progenies of transgenic plants were evaluated for resistance to water deficit stress. For the water deficit treatment, wild type and transgenic T1 plants were grown in the same pots at 24°C, without water, for various periods, 0, 7, 14, 21 and 28 d, up to four weeks. The transgenic plants were more resistant to water deprivation than wild type—the water content of transgenic plants remained high during water deficit treatments, thereby reducing the tissue damage, while a marked reduction in water content was observed in wild type plants. The leaves of wild type plants became wilted and curled whereas transgenic plants did not. Less than 6% of the wild type survived after four weeks of water deficit. Gibberellic acid (GA3) pretreatment of both wild type

and transgenic tomato plants had little or no impact on water deficit resistance.

Proline content was greater in transgenic plants than wild type under both normal and water deficit conditions. Absence of further elevation of Pro content during water deficit treatment indicates that over-expression of *CBF1* under non-stress conditions protects plants from subsequent stresses. GA3 treatments did not affect Pro content in wild type and transgenic tomato plants. These results suggest that transgenic tomato plants possess an inherent resistance to water deficit conditions due to the expression of *CBF1*. However, phenotypically the transgenic tomato plants were shorter than wild type plants, with decreased fruit and seed numbers and fresh weights compared to those of wild type plants under normal growth conditions. Exogenous GA3 application improved fruit size, seed number, and fresh weight, though they did not reach the same levels as those of wild type plants. Results from additional chilling treatments conducted on transgenic tomato and wild type lines were similar to water deficit treatments—the transgenic plants were more resistant to chilling than wild type.

The researchers looked for *CBF1* responsive genes using subtractive hybridization and isolated an upregulated *CATALASE1* (*CAT1*) gene in the transgenic tomato plants. They further found that *CAT1* expression and catalase activity were increased, and H_20_2 concentration was reduced, in the transgenic tomato plants. The authors suggest that the upregulation of *CAT1* might be due to the over-expression of *CBF1*. This phenomenon supports the hypothesis that activation of antioxidant genes confers the water deficit resistance seen in these transgenic tomato plants. The results further suggest that enhancement of the stress tolerance phenomenon in transgenic tomato plants might be conferred by multiple defense systems due to presence of *CBF1*, which acts as a master switch controlling various pathways responsive to stress.

The researchers are planning to isolate other upregulated genes like *P5CS* and others responsive to heterologous *CBF1* that are important in transgenic tomato plants with water deficit resistance. Characterization of these stress responsive genes will contribute to an understanding of stress resistance and stress signal reduction pathways. In conclusion, the study suggests that heterologous *CBF1* could improve environmental stress resistance in agriculturally important crop plants.

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Animal Research

Transgenic Pigs Expressing Human Decay Accelerating Factor

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Thousands of people die in the US each year awaiting an organ transplant due to the shortage of available organs. One approach to address this chronic shortage of human organs is xenotransplantation, which is the transfer of tissues or organs from one species to another. Pigs' tissues have been proposed as the most likely alternative to human organs because of the similarity in anatomy and physiology between pig and human organs. The direct transplantation of pig organs, however, into humans would trigger a rapid immune response, resulting in destruction of the transplanted tissue.

Scientists have sought to develop a pig that has been genetically altered to allow pig-to-human organ transplants. Two approaches have been utilized recently. In the approach pursued by PPL Therapeutics (Blacksburg, VA) and the University of Missouri, the alpha galactosyltransferase gene, which synthesizes an antigenic alpha galactose sugar on the cell surface, was deleted using a combination of gene targeting and nuclear transfer technology. In theory, pig cells unable to synthesize the alpha galactose sugar should be less antigenic to the human immune system. The other approach is to create transgenic pigs that express a human gene that suppresses the complement-mediated lysis of foreign cells.

In the October 29, 2002, issue of the *Proceedings of the National Academy of Sciences, USA*, a team of Italian researchers reports the production of transgenic pigs expressing human decay accelerating factor, a complement-inhibiting factor. This group used sperm-mediated gene transfer (SMGT) as a method of producing the transgenic pigs. Sperm-mediated gene transfer in mice was highly controversial when first published in 1989 because many groups subsequently had difficulty replicating the

technique. However, since that time a number of reports have appeared showing that SMGT has been successfully used to generate transgenic fish, honeybee, sea urchin, chicken, and sheep.

Standard approaches to generating transgenic livestock include microinjection of DNA into fertilized eggs or transfer of foreign DNA into cultured cells followed by nuclear transfer into enucleated oocytes. In both cases, successes have been realized. However, with the microinjection method, the efficiency is low and with the nuclear transfer method the health of the cloned animals appears to be an issue.

To generate transgenic pigs, the Italian group first treated sperm from pre-selected boars with a DNA construct containing the human decay accelerating factor (*hDAF*) gene and then used this treated sperm for artificial insemination of eggs. Eight fertilizations in 15 gilts total were performed, resulting in the birth of 93 piglets, of which 53 (57%) carried the *hDAF* gene. Two fertilizations produced no transgenic piglets, while the other six resulted in 46-88% transgenic piglets. The transgene was stably integrated into the pig genome and was transmitted to progeny in a normal Mendelian fashion.

Human *DAF* mRNA and protein were detected in the transgenic pigs. In 34/53 (64%) of the transgenic piglets, the human *DAF* gene was expressed in all tissues examined. Expression of human *DAF* mRNA varied considerably between animals and varied in different tissues of the same animal. *DAF* mRNA expression was stable from birth to 6 – 12 months of age. Furthermore, human DAF protein was detected in the appropriate membrane fraction of transgenic pig heart and red blood cells.

The ability of human DAF to protect cells from lysis by antibodies and complement in fresh human serum was examined. Macrophages from transgenic pigs showed increased resistance to human serum compared with non-transgenic control pigs. With cells from control pigs, less than 10% of the cells survived treatment with human serum, whereas with cells from transgenic pigs, greater than 75% and often closer to 100% of the cells survived. Similar results were found when aortic endothelial cells from transgenic and control pigs were tested.

This report presents two significant findings: 1) the extremely high rate of transgenesis using sperm-mediated gene transfer; and 2) the generation of transgenic pigs expressing human decay accelerating factor. If the SMGT results can be verified, then this would represent a very simple, efficient, and cost effective method of producing transgenic animals. However, the one caveat is that this method allows for only the addition of genes, not the deletion of genes as has been accomplished with the alpha galactosidase gene. Nevertheless, the protection from complement-mediated lysis conferred upon the transgenic pig cells represents a promising step forward in the development of animal organs for human transplantation.

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REGULATORY NEWS

DON'T PANIC - IT'S ORGANIC

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On October 12, and after more than a decade in development, the US Department of Agriculture implemented its National Organic Program. The program creates a set of national standards that food labeled "organic" must meet, replacing an assortment of state and private certification standards for organic status.

According to the USDA, the term "organic" can only be used on labels of goods that have been produced and handled in accordance to the new regulations. For example, produce must be grown and handled without the use of various substances (e.g., pesticides), ionizing radiation, or excluded methods. These excluded methods include processes used to genetically modify organisms by means not possible under natural conditions, such as cell fusion and recombinant DNA technology. The USDA does not consider traditional breeding techniques and *in vitro* fertilization to be excluded methods. Detailed information about the program is available from the USDA's National Organic Program website (http://www.ams.usda.gov/nop).

Greg Conko, director of food safety policy at the Competitive Enterprise Institute, (a non-profit public policy organization located in Washington, D.C.), has suggested that, because the federal organic standards prohibit the use of

bioengineered ingredients, they negate the need to create separate labeling for genetically-modified (GM) food. Not everyone shares this view.

A measure on the November ballot in Oregon would have required the labeling of all GM food and products containing ingredients made from GM food. Measure 27, the first of its kind to go before US voters, would have called for the labeling of such items sold in stores and restaurants, and any produced in the state. Opponents of Measure 27 argued that it would necessitate meticulous tracking of GM food and ingredients to ensure proper labeling of food sold in the state and products made or housed in the state and distributed to other states. This would create a logistical albatross for farmers, food makers, and supermarkets.

The federal government did not care for the bill either. In early October, the Food and Drug Administration's deputy commissioner, Lester Crawford, sent a letter to Oregon's governor warning that, if Measure 27 passed, the state of Oregon might impermissibly interfere with manufacturers' ability to market their products on a nationwide basis. Such interference could be viewed as a violation of the US Constitution's commerce clause, which prohibits states from impeding the flow of interstate commerce. Despite public opinion polls indicating that consumers are anxious for a GM food label, on November 5 Oregon voters rejected Measure 27 by a ratio of about three to one.

An interesting aspect about Lester Crawford's letter is that it reinforced the FDA's position that, in its scientific judgment, the agency has found no significant difference between bioengineered foods and conventional counterparts. The FDA's emphasis on the products that enter the marketplace contrasts with USDA's process-based national organic system.

In Europe, there is a clear focus upon the methods used to produce food. The European Union has not allowed any new GM food or crop to enter its market since October 1998, a policy that has caused major trade friction with the US and has created a setback for European agbiotech companies. In an effort to put an end to the four-year moratorium, the EU's Directive 2001/18 became law on October 17. Some believe that this measure will pave the way for authorization of new GM crops by providing improved safety testing and consumer choice.

The new directive puts into place a step-by-step approval process based on the assessment of risks to human health and the environment before any GM product or product containing a GM organism can be released into the environment or placed on the market. The directive includes mandatory monitoring requirements and rules on mandatory labeling and traceability. The traceability requirements impose new obligations on business operators to transmit and retain information at each stage of market placement, so that it will be possible to trace GM organisms through the production and distribution chain.

Yet internal divisions among EU member states may render the new rules meaningless. In October, Europe's council of agricultural ministers met and failed to agree about the rules. The Commission had proposed that all food containing more than 1% of GM ingredients should be labeled, but Austria and Italy call for tighter limits, while Sweden insists on zero tolerance. On the other hand, Britain opposes the labeling of food with minute traces of GM ingredients on the grounds that it is neither practical nor achievable. A meeting of Europe's environment ministers also failed to achieve an agreement about the rules.

Frustrated with the EU's slow progress, agricultural groups recently urged the Bush administration to file a complaint with the World Trade Organization against the European moratorium. Even if EU member states agreed to end the moratorium, it is unlikely that implementation of the new Directive will smooth trade friction with the US, considering that the US State Department's Alan P. Larson (Under Secretary for Economic, Business, and Agricultural Affairs) characterizes the EU's tracing and labeling regulations as "onerous" and "discriminatory."

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