24 Future Trends

24.1 Genetic Improvement of Wheat

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24.1.1 Introduction

About 7,000 species of plants are grown by farmers somewhere in the world. Of these, only 30 species provide 90 percent of our calorific intake. The three main crops are wheat, rice and maize. They occupy 35% of all global arable land, wheat being cultivated on 230 million hectares (mio ha) followed by rice and maize with 151 and 140 mio ha respectively (FAOSTAT, 2005). The importance of bread wheat (*Triticum aestivum* L.) as a food crop originates mainly from the viscoelastic properties of the gluten proteins in the seed's endosperm that allows the dough to be expanded during fermentation and enables bread production.

For thousands of years, improvement of wheat was achieved by careful selection of the best grain from well-adapted plants. During the last century, new wheat varieties were produced by selected crossing and breeding. Desirable traits such as ease of harvesting, shorter growing seasons, better milling and baking qualities, improved disease resistance and higher yields are combined by crossing selected wheat varieties. It takes about ten to twelve years of testing at a cost of around 0.5 mio USD for a new variety to be released (NAMA, 2005).

For breeders and the wheat industry, the time and high costs are an incentive to accelerate the development of new elite varieties. This is achieved in modern breeding programmes by marker-assisted selection (MAS). For diagnostic purposes, DNA is isolated from plants from which selection is to be conducted, e.g. different varieties or accessions of wheat, and is screened for sequence variations. The knowledge that a certain DNA sequence variation is linked to a desirable trait in the plant allows the breeder to select the most promising plants within large populations without waiting for their performance in the field. Selection takes place in the laboratory. To make marker-assisted selection efficient, the whole genome of a plant has to be covered by markers. For wheat with its large hexaploid genome (about 40 times the size of rice or 5 times the size of the human genome) this is a major task taken on by the wheat science community worldwide. The application of marker-assisted selection and the introgression of chromosome regions from wild relatives were particularly important for the success of wheat breeding. Besides MAS, biotechnology offers another tool for improving wheat, namely genetic engineering (Langridge *et al.*, 2001).

In future, wheat breeding will have to keep responding to a growing world population that has chosen wheat as one of the most favoured staple foods at a time when the increase in yield is slowing down worldwide (FAOSTAT, 2005). In addition, wheat varieties will have to serve a wider range of end uses with differing but specific quality requirements. As outlined by wheat breeder K. Brunckhorst (see chapter 2), current farming practices tend towards wheat growing without crop rotation and with reduced tillage, mainly for economic reasons. Both factors promote the development of soil-borne diseases and require varieties equipped with corresponding resistances. All of these demands can only be addressed if a high level of genetic diversity exists from which the breeder can choose.

24.1.2 Genetically Engineered Plants

Mutations occur randomly in any genome, they are either without effect or are most likely to be disadvantageous to the organism. As a consequence they will be discarded by natural selection or, in the case of wheat, by the wheat breeder. To increase the mutation rate in plant genomes with the aim of increasing the frequency of advantageous mutations, the induction of mutations with chemicals or ionizing radiation and subsequent selection was quite popular in breeding in the 1980s and 1990s. Mutagenesis breeding is still carried out, but to a much lesser degree.

In contrast to mutagenesis breeding, genetic engineering is highly specific since defined DNA fragments that encode a well characterized protein are transferred. Only single genes or a few genes are integrated into the genome of the transgenic plant, the genetically modified organism (GMO), whereas in breeding whole genomes are "shuffled" to combine desirable traits. In the case of mutagenesis breeding random, mostly detrimental, mutations occur somewhere in the genome.

In the following the term "gene" is used to describe a DNA fragment that is transcribed into mRNA and subsequently translated into a protein. Either alone or through interaction with other proteins (or DNA) the protein finally confers a certain phenotype. The term "expression" of a gene of interest in this context can equally mean transcription or translation. Fig. 219 illustrates the expression of a gene into a protein from DNA via mRNA as it occurs in all living cells with a few exceptions (i.e. some viruses).

Results of genetic engineering first became widely noticed in 1994 when the FlavrSavr® tomato from Calgene Inc. became the first transgenic food that was granted a licence for human consumption by the U.S. Food and Drug Administration (FDA). A decade of testing at a cost of 525 mio USD had been invested to prove that the tomatoes were as safe as traditionally bred varieties. Although the FlavrSavr® tomatoes were more resistant to rotting while maintaining their taste, consumer reluctance and their relatively high price meant that production ceased only a few years later.

By 2002 transgenic crops had gained greater acceptance, at least in the USA, Argentina, Canada and China. Together these countries made up 99% of the worldwide area under transgenic crop cultivation. In total, 16 countries were growing genetically modified (GM) crops in 2002 (James, 2002). The traits that have been introduced into GM plants are mainly various tolerances to herbicides and resistances to insects, alone or in combination. The current global status of all approved GM plants is summarized in Tab. 136, in alphabetical order (agbios, 2005). To obtain the certification "approved genetically modified plant" in the USA and Canada, a risk assessment process lasting 7 to 10 years from the stage of discovery via confined field trials up to a food, feed and environmental assessment has to be conducted. The transgenic plants are examined with respect to the same types of risk as traditionally generated plants, e.g. toxins, potential allergens, weediness and pest potential.

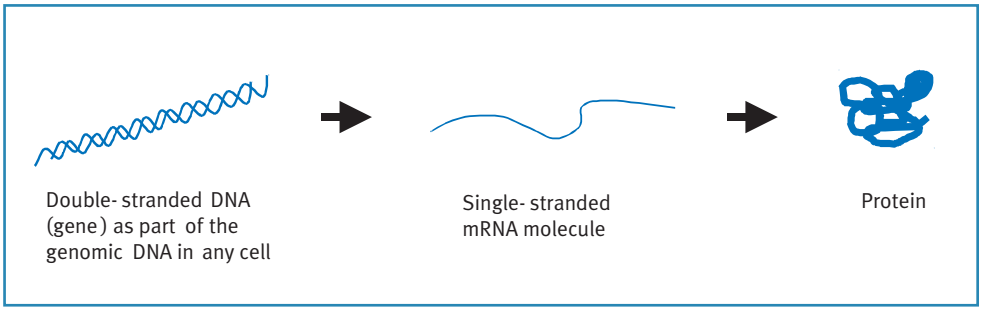


Fig. 219: From the DNA or gene via mRNA to the protein

Tab. 136: Global status of all approved genetically modified plants to date (June 2005)

Crop name	Phenotypic trait	Year and country of first approval
Argentine canola (*Brassica napus*)	Herbicide tolerance	1995 Canada
Argentine canola	Modified seed fatty acid content	1994 USA
Argentine canola	Herbicide tolerance and restored fertility	1995 Canada
Carnation	Increased shelf-life and herbicide tolerance	1995 Australia
Carnation	Modified flower colour and herbicide tolerance	1995 Australia
Chicory	Herbicide tolerance and restored fertility	1996 European Union
Cotton	Insect resistance	1995 USA
Cotton	Herbicide tolerance	1994 USA
Creeping bentgrass	Herbicide tolerance	2003 USA
Flax, linseed	Herbicide tolerance	1996 Canada
Maize	Herbicide tolerance	1995 USA
Maize	Insect resistance	1995 USA
Maize	Insect resistance and herbicide tolerance	1995 USA
Maize	Herbicide tolerance and male sterility	1995 USA
Papaya	Virus resistance	1996 USA
Polish canola (*Brassica rapa*)	Herbicide tolerance	1997 Canada
Potato	Insect resistance	1995 USA, Canada
Potato	Insect and virus resistance	1998 USA
Rice	Herbicide tolerance	1999 USA
Soybean	Herbicide tolerance	1994 USA
Soybean	Modified seed fatty acid content	1997 USA
Squash	Resistance to viral infection	1994 USA
Sugar beet	Herbicide tolerance	1998 USA
Tobacco	Herbicide tolerance	1994 European Union
Tobacco	Reduced nicotine	2002 USA
Tomato	Increased shelf-life through delayed ripening	1994 USA
Tomato	Insect resistance	1998 USA
Tomato	FlavrSavr¤ tomato with delayed softening at maturity	1992 USA
Wheat	Herbicide tolerance	2004 USA

In the case of wheat, a herbicide-tolerant variety is the only GM variety that has been approved for release to date. Considering the many acknowledged field trials as part of the risk assessment process for GM plants the number of transgenic wheat varieties finally released is expected to increase drastically in the coming years. In the USA alone, as at April 2005, about 350 field trials have been acknowledged just for wheat. Nearly half of these field experiments investigate herbicide tolerance. The second-largest group of field

Little Glossary of Wheat Genomics

anthesis = the period of time when the flower is open and the pollen is released
auxin = a plant growth hormone
BYDV = barley yellow dwarf virus
callus = Latin callum means "thick skin", a mass of unorganized plant cells, developed as the result of wounding or culture on nutrient media; the plural is calluses or calli
CIMMYT = International Maize and Wheat Improvement Center (Centro Internacional de Mejoramiento de Ma z y Trigo).
DNA = deoxyribonucleic acid; a linear, double-stranded large molecule carrying the genetic code of life
RNA = ribonucleic acid; sections of the DNA are copied into single-stranded messenger RNA molecules
mRNA = messenger RNA
explant = living organ, piece of tissue or cell which is taken from the natural site of growth and placed in a medium for tissue culture
genome = the complete set of genes in an organism
in vitro = Latin for "in glass"; refers to work done in a test tube or culture medium in the laboratory
phenotype = the visible properties of an organism as a consequence of its genes
somatic = adjective of Greek soma means "the body"; somatic wheat embryos are derived from "normal" tissue cells that behave like a zygotic embryo without fertilization
transcription = process of copying the information from DNA into mRNA; carried out by enzymes
transgene = "transferred gene", the DNA fragment that has been introduced into the genome of an organism by genetic engineering
translation = process by which the mRNA is converted to a sequence of amino acids forming a protein; also carried out by enzymes
WSMV = wheat streak mosaic virus
zygotic = originating from a fertilized egg; e.g. the zygotic wheat embryo is derived from a pollinated and thus fertilized egg cell and there is only one per seed

trials (24%) assesses transgenic wheat with potentially improved resistance to fungal diseases caused by *Fusarium* ssp., powdery mildew and *Septoria* leaf blotch or viral infections (BYDV, WSMV). Further trials have verified the performance of genetically modified wheat in the field with respect to higher yields, altered starch or seed protein composition and drought tolerance. For more details of field trials classified according to individual countries we recommend the web link http://www.nbiap.vt.edu/cfdocs/globalfieldtests.cfm.

24.1.3 Gene Transfer Techniques to Transform Wheat

The two prerequisites for the generation of transgenic cereals are the availability of *in vitro* culture techniques and techniques to transfer the gene of interest into the genome of the cell. The gene of interest has to be integrated into cells that are totipotent. Totipotency describes the ability of a cell to regenerate a complete individual. The gene transfer into the totipotent cell guarantees that each cell of the regenerated plant contains the genetic information encoded by the transgene.

Unlike many other crops, cereals, especially wheat, were thought to be recalcitrant to *in vitro* culture. When the regeneration of whole wheat plants from embryos of immature seeds first succeeded in 1978 (Shimada, 1978), they became the explant of choice, and ongoing optimization of culture conditions has since enabled plants to be regenerated efficiently from many different wheat cultivars. The formation of numerous somatic embryos and their regeneration is controlled by concentrations of phytohormones in the synthetic growth medium. The medium also supplies the cells with carbohydrates, minerals and vitamins.

The major steps of an *in vitro* culture system for wheat are shown in Fig. 220.

A. Immature seeds are harvested from wheat spikes 10 - 15 days after anthesis, **zygotic embryos** are isolated and transferred to a synthetic growth medium.
B. Cells within the isolated immature embryo are induced to form callus. Callus induction is achieved by the phytohormone auxin in the medium. Each callus bears numerous **somatic embryos** with the potential to regenerate into individual plants. A change in auxin concentrations allows **regeneration** into plants.

The time required to regenerate a wheat plant by *in vitro* culture from immature embryos is about three months. The efficiency of an *in vitro* culture system depends on the number of regenerated plants per isolated embryo. For a culture system to be efficient enough for gene transfer, numerous somatic embryos per callus have to be formed, as only a small percentage of callus cells stably integrate the transferred gene.

When the *in vitro* culture system is used to generate transgenic plants, the gene transfer occurs immediately or 2 - 3 days after isolation of the immature embryos. Together with the gene of interest, a selectable marker gene is co-transformed. The co-transformed selectable marker gene provides resistance to the selection agent in the medium component, usually an antibiotic or herbicide. So only successfully transformed cells can survive, while cells that have not integrated the selectable marker gene cannot detoxify the antibiotic or the herbicide and die off. In this case the resistance to an antibiotic (e.g. kanamycin) or herbicide (e.g. BASTA®) serves the sole purpose of allowing the identification of the transformed cells and is actually an undesirable trait once the transgenic plant is ready for field testing. Consumers are concerned that the use of antibiotics as selectable markers might increase the antibiotic resistance of human pathogens. Although no conclusive evidence exists that

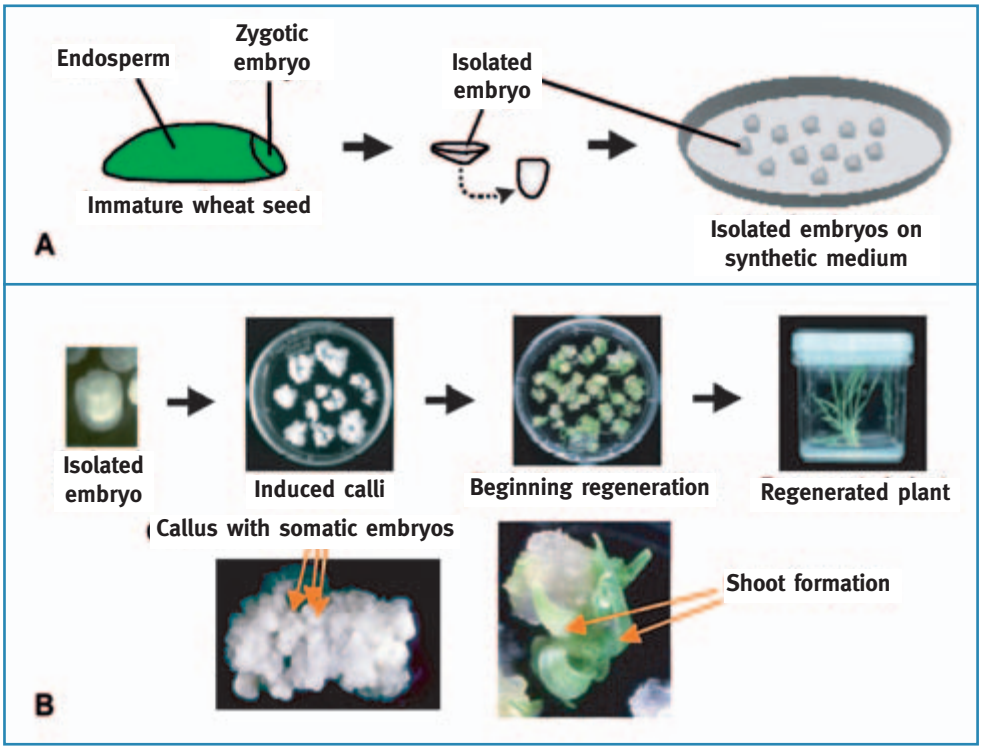


Fig. 220: In vitro *culture steps to regenerate wheat plants from immature embryos*

these antibiotic resistance genes cause harm to humans, development of marker-free transgenic plants is a major field of research in genetic engineering in order to increase public acceptance of the technology.

The two most established techniques for wheat transformation are the biolistic method using the particle gun, also called microprojectile-mediated gene transfer, and transformation using *Agrobacterium tumefaciens*.

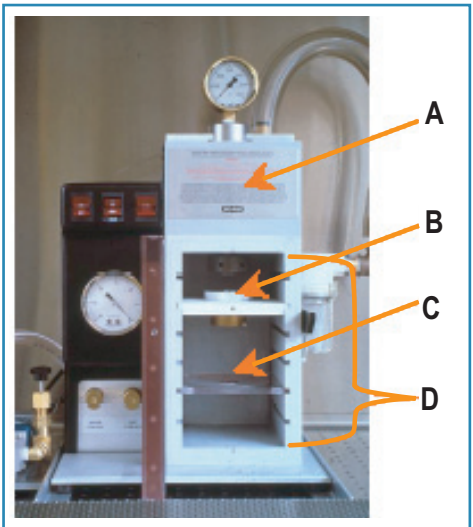
The Biolistic Transformation Method

The biolistic transformation method was first described in 1987 by John Sanford and colleagues at Cornell University (USA) who used DNA-coated tungsten particles to literally shoot them into onion cells (Sanford *et al.*, 1987). The principle of direct gene transfer using the particle gun (Fig. 221) is still the same today, although gunpowder has been replaced by gas pressure and the chemically inert metal gold is used instead of tungsten.

Technically, the gun works as follows: helium gas flows into the pressure chamber, where pressure builds up behind a plastic disc of defined strength, the rupture disc. When the gas pressure in the top chamber reaches a threshold value, the rupture disc bursts and releases the pressure downwards into the vacuum chamber. The pressure accelerates the DNA-coated gold particles that reside close to the burst rupture disc until they hit the cells of the explants, the immature embryos on a Petri dish containing medium. The microprojectiles have to penetrate the walls of the plant cells so that the DNA can get into solution in the aqueous cell content. Only a small proportion of the cells that have been hit will integrate the DNA into their genome. In most cells the DNA is degraded by enzymes or lost during subsequent cell divisions. In these latter cases the transformation is called "transient" in contrast to the desired "stable" transformation. The first stable transformations of cereal plants using immature embryos were achieved at the beginning of the 1990s. Shortly after the success with rice in 1991 (Christou *et al.*, 1991) wheat followed in 1993 (Vasil *et al.*, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994). The initially low success rate for generating transgenic wheat plants of about 1% (one transgenic plant regenerated out of 100 isolated and bombarded immature embryo explants) has since increased dramatically and may be as much as 60% with certain cultivars, depending on the laboratory reporting.

The biolistic transformation method makes use of physical processes to transfer the gene into the plant cells. This makes it a versatile and effective transformation method that is not limited to a cell type, species or genotype. Discussed disadvantages of the biolistic method are a tendency to integrate multiple copies of the transgene into the genome together with observed fragmentation of transgene copies, especially when large DNA fragments are transferred. Multiple integrations of the same sequence can cause inhibition of gene expression by the plant so that the trait encoded by the transgene is not displayed.

An alternative to the biolistic transformation method, *Agrobacterium tumefaciens*-mediated



A: Helium-gas pressure chamber
B: DNA-coated gold particles
C: Tray for Petri dish with explants
D: Vacuum chamber

Fig. 221: Particle gun for biolistic gene transfer (Photograph courtesy of A. Milligan, ACPFG, Australia)

transformation, is now being used for genetic transformation of a wide range of species.

Agrobacterium-Mediated Gene Transfer

Agrobacterium tumefaciens is a soil bacterium causing crown gall tumours on several dicotyledonous plant species, e.g. tobacco or *Arabidopsis*. Tumours are formed at sites where wounding has occurred. The bacterium induces tumour growth by genetic transformation of the plant cell (Fig. 222). At wounding sites plant cells release small compounds such as phenolic acetosyringone. This is perceived by the bacterium, which then activates genes localized on the so-called tumour-inducing (Ti) plasmid (circular DNA molecule). The activation results in the release of a DNA fragment from the Ti plasmid, called Transfer-DNA or T-DNA. The T-DNA stably integrates into the plant genome. It contains genetic information to modify the hormone levels in the plant, causing tumour development.

For genetic engineering of plants the Ti plasmid had to be disarmed so that it no longer induced tumours. The tumour-causing genes of *Agrobacterium* wild-type strains that are located on the T-DNA are now replaced by the gene of interest and a selectable marker gene. As with biolistic gene transfer, *Agrobacterium*-mediated gene transfer is carried out using immature wheat embryos.

Agrobacterium-mediated transformation of cereals using immature embryos as explants first succeeded in 1994 with rice (Hiei *et al.*, 1994); it was followed by maize two years later (Ishida *et al.*, 1996) and finally by barley and wheat in 1997 (Tingay *et al.*, 1997; Cheng *et al.*, 1997). A major advantage of using *Agrobacterium* for transformation is the higher rate of single copy insertions compared to the biolistic method.

The selectable marker genes co-transformed with the gene of interest can be the same as the ones used in the biolistic transformation method, mediating antibiotic or herbicide resistance of transformed cells. A new generation of selectable marker genes enables transformed cells to metabolize nutrients that plants cannot normally utilize. For example, marker genes encoding the enzymes xylose isomerase or mannose phosphate isomerase allow selection with sugars like xylose or mannose respectively. Non-transformed cells starve to death, as no carbon source is available to them.

Both DNA transfer methods are used for genetic transformation of wheat, but a review of the

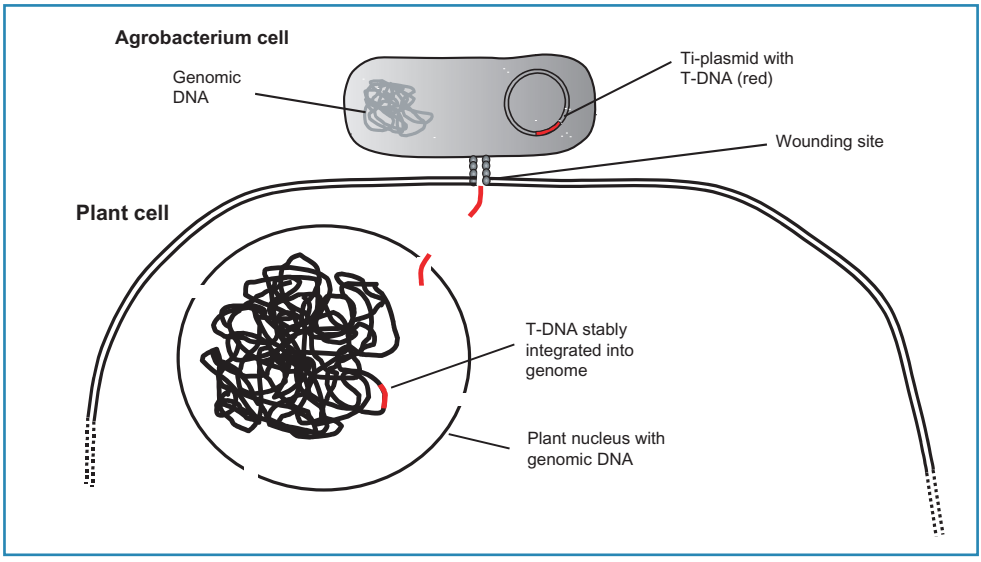


Fig. 222: T-DNA transfer from Agrobacterium *to the plant nucleus and integration into the genomic plant DNA*

scientific literature up to 2003 reveals that most foreign genes are transferred into wheat by microprojectile bombardment (Sahrawat *et al*., 2003). The reasons for this are two-fold. First the transformation of wheat by the bombardment method preceded *Agrobacterium*-mediated transformation by five years, so the technique was well established in many laboratories. Secondly, it was difficult to establish the *Agrobacterium*-mediated transformation system for wheat in different laboratories where different wheat genotypes are used. Different *Agrobacterium* strains show different degrees of effectiveness on different wheat varieties. Conditions for the co-cultivation of the bacterium with the wheat tissue require optimization, whereas the purely physical method using the particle gun does not call for the adjustment of two organisms.

The limitation for generating transgenic wheat plants is not so much the gene transfer technique as the *in vitro* culture performance of many wheat varieties. The rate of callus induction and subsequent regeneration is an intrinsic factor of a variety, and so far it cannot usually be overcome by modifying the culture conditions. The problem is circumvented by screening for wheat varieties with a high regeneration efficiency in order to study the function of a gene of interest. Wheat and other cereals are more than just model systems for functional studies, unlike practical model plants like *Arabidopsis*. With the increasing use of agronomically important transgenes, commercial wheat varieties will become the focus of genetic improvement by means of gene transfer, and it can be predicted that efforts will increase to improve their regeneration efficiency.

24.1.4 Genetic Engineering of Agronomically Important Traits

In the following, four examples of genetic engineering of wheat are discussed that have reached or are about to reach the field-testing stage. Agronomically important traits that have been modified in wheat, besides herbicide tolerance, are resistance or tolerance to biotic and abiotic stresses and the modification of starch and seed protein composition. The examples have been chosen because they show the spectrum of gene transfer: addition, modification, enhancement or removal of certain characteristics in the transgenic plants.

Utilizing puroindoline genes to modify grain hardness and disease resistance

In the USA, several field trials to test the performance of transgenic wheat with a modified puroindoline content have been acknowledged in recent years. A number of related patents claim their exclusive right to use cereals with a genetically modified puroindoline content and point out the potential of puroindoline proteins.

Discovery of puroindolines

For commercial purposes, wheat grain is divided into three distinct quality classes: "soft" and "hard" hexaploid wheats, and "very hard" tetraploid durum wheat. The hardness or texture determines the milling and end-use qualities of wheat. Wheat grain with a soft texture requires less energy to mill, generates smaller flour particles and less starch damage, and absorbs less water than hard wheat.
In the 1980*s* and 1990*s*, biochemical studies identified a correlation between grain hardness and the presence of a protein which was given the names friabilin, grain softness protein (GSP) or "non-stick" protein, depending on the laboratory working on grain hardness (Hogg *et al*., 2005; Morris, 2002). When several labs partially sequenced their protein they found it was very similar or even identical to another group of proteins called puroindolines (Blochet *et al*., 1993), indicating that these are one and the same. Puroindoline proteins were also isolated from the endosperm of wheat kernels but hypothesized to be involved in plant defence mechanisms against pathogens as they resembled other proteins with a proven antimicrobial effect.

So it was that the findings from two apparently unrelated research topics, grain hardness and disease resistance, converged with the discovery of the puroindoline proteins PINA and PINB.

It is now accepted that the two PIN encoding genes *puroindoline-a* and b determine grain hardness (or rather softness) and that even a single amino acid change in one of them can be sufficient to generate hard wheat grain. Further support for the hypothesis that the puroindolines control grain texture in wheat was obtained by transferring *puroindoline*[45] genes into rice and hard grain wheat. The kernels of transgenic rice and wheat plants indeed had reduced grain hardness as measured by reduced force required to crush kernels, reduced starch damage and an increased proportion of small endosperm particles after milling. The grain softness in these plants correlated with the expression level of the transgenes: the more the transferred puroindoline gene was transcribed, producing active PIN protein, the softer was the transgenic grain (Krishnamurthy *et al.*, 2001; Beecher *et al.*, 2002; Hogg *et al.* 2004).

Agronomic importance of puroindolines and the use of transgenic wheat

Besides the modification of grain texture with transgenic wheat, the genes can be used to produce PIN protein in easy-to-culture organisms like bacteria or yeast. The protein then can be purified and applied as an additive to modify flour characteristics.

In the case of puroindolines the range of applications goes further because of their antimicrobial potential. In 2004 it was confirmed that the wheat *puroindoline-b* gene improves the resistance of transgenic apple to fungus-induced scab disease; this fungus has started to overcome the resistance gene commonly used in apple breeding in Europe (Faize *et al.*, 2004).

The economic potential of puroindoline proteins is reflected in the number of patents that have been granted over the last few years (2001 to 2005).

A search of the US patent database (http://www.uspto.gov/patft/index.html) shows that the obvious and more specific aspects of the use of puroindoline shave already been detected. The patents protect, for example, the modification of cereal grain hardness through expression of recombinant *puroindoline* protein in transgenic plants, transgenic plants that express puroindoline and show a reduction in damage caused by plant pathogens such as fungi and bacteria, the use of antimicrobial peptides like puroindolines to treat infections and the use of puroindoline proteins as additives for preparing biscuits.

Transgenic wheat with modified expression of *puroindoline* genes can kill two birds with one stone: modified grain softness and resistance to pathogens. Though PINA and PINB protein are seed-specific proteins, it is possible to express a gene of interest in a tissue-specific manner. In the case of resistance improvement, foliar pathogens could be addressed by directing PIN protein synthesis to the leaves. Severe yield losses in wheat are caused by *Fusarium* species, a pathogenic fungus that infects the spikes, causing head blight and the production of toxins detrimental to human and animal health. PIN proteins have been shown to inhibit the growth of *Fusarium* species when tested *in vitro*, and field trials are in progress which might prove PIN proteins to be a practical tool for generating increased resistance to damage caused by *Fusarium* (Gerhardt *et al.*, 2002).

Whereas puroindolines are responsible for grain hardness, the group of gluten proteins gives wheat flour its uniqueness in forming dough that exhibits the rheological properties required for the production of leavened bread and the diversity of foods that result from this feature. Gluten proteins are glutenins and gliadins. Glutenin proteins are the focus of the work of several research groups worldwide that are studying the impact of variable compositions on the functional properties of dough using transgenic wheat. Gliadins are the most abundant wheat seed proteins. Bearing in mind that wheat is the most popular staple food and also rich in protein (9 - 15%), gliadins are clearly one of the proteins most consumed by humans.

[45] *italic* type indicates a gene, normal type a protein

Transgenic Approaches to Obtaining Flour Without Celiac Disease Potential

While mediating the superior baking quality of wheat, a subgroup of the gliadin proteins, α-gliadins, seem to be responsible for causing celiac disease in susceptible humans. In Europe and North America about one person in 300 is affected by the severe dietary syndrome. Celiac disease is a disease of the small intestine characterized by an allergic reaction to the gluten protein. Inflammation destroys the lining of the small intestine and reduces the absorption of nutrients. Severe nutritional, vitamin and mineral deficiencies and their symptoms are the consequence. A reduction of the allergen is not sufficient to prevent an allergic reaction, and complete avoidance is the only treatment currently recommended. This is not easy to achieve, as wheat gluten protein can be found not only in obvious wheat flour products such as bread but also in a very long list of processed foods where it has been used as an additive.

Research projects, supported by the German Federal Ministry of Education and Research, industrial partners and associations, were launched in 2000 to tackle the celiac-causing problem of the gliadins by means of molecular genetic approaches. Two subprojects use transgenic plants to avoid the allergenic potential of gliadin proteins: genetically engineered maize containing wheat storage protein genes (Gahrtz *et al.*, 2004) and genetically modified wheat with inactivated gliadin genes (Folck *et al.*, 2004).

Transgenic maize with wheat glutenin proteins

Maize flour contains starch and protein but does not cause celiac disease symptoms. Unfortunately, maize flour does not have baking quality comparable to that of wheat. As the α-gliadins are probably the allergenic protein in wheat flour, *glutenin* genes transferred into maize have the potential to improve the baking characteristics of maize flour without causing a problem for people allergic to gliadins.

In a first step, different glutenin genes are transferred separately into maize by microprojectile bombardment. As in wheat, their synthesis in transgenic maize is restricted to the endosperm. Flour from plants containing an individual glutenin gene makes it possible to test the corresponding glutenin protein for allergenic potential. Once the allergenic potential of each glutenin protein introduced into transgenic maize is excluded, glutenins can be combined by crossing the different transgenic maize lines. The next step is to analyze the transgenic maize flour with respect to its ability to form gluten-like structures in maize dough, finally improving the baking properties of maize flour (Gahrtz *et al.*, 2004).

Transgenic wheat with a silenced gliadin gene function

The above examples of transgenic approaches in plants employed genetic engineering to add a feature in the form of a gene to a plant. As well as adding a gene and the trait it encodes, one can also remove a gene function. Genetic transformation with inverted genes or gene fragments can inactivate genes in the plant. The transferred DNA must have the same sequence as the gene it is supposed to inhibit. This genetic approach is derived from a natural phenomenon called "gene silencing". It does not remove the gene from the plant but blocks its activity in the sense that the gene will no longer be translated into a protein.

To apply a "silencing technique" to inhibit the synthesis of allergenic α-gliadins is challenging, as the number of genes that possibly encode for α-gliadin proteins in wheat is estimated to be up to 150 (Anderson *et al.*, 1997). Initial attempts to use the silencing technique to remove the expression of α-gliadin genes in wheat have proved successful. Analyses of extracted gliadin proteins indicated that only the group of α-gliadins was massively reduced in these plants, whereas other, non-allergenic γ- and ω-gliadins remained unchanged. As a next step the flour from the transgenic wheat plants will be analyzed to investigate its baking characteristics and allergenic potential (Folck *et al.*, 2004).

On the basis of the current results it is estimated that the introduction of non-toxic wheat characteristics into elite breeding varieties

and their approval for commercial use could take another 10 years before new products are available for persons with celiac disease.

In the case of *puroindoline, glutenin* or *gliadin* genes, the gene encodes the trait directly in wheat, as the trait is the protein itself. Modifying the expression of these genes directly modifies the trait. Plants' responses to environmental stresses can be more complex and involve the function of multiple known and unknown genes. The activity of stress-response genes is controlled by the environmental stimulus. The stress as a stimulus has first to be perceived by the plant; the perceived signal then has to be transmitted to activate the responsive genes that finally cope with the stress. The response genes can be activated by regulatory proteins that bind to them and proteins. These regulatory proteins are therefore called transcription factors. One transcription factor can activate numerous responsive genes, acting as an amplifier, and induce complex protein activity changes that occur, for example, when a plant is stressed.

Genetic Engineering for Improved Stress Tolerance

One outcome of basic research is the knowledge of regulatory genes that control the activity of other genes. Among these are the above-mentioned transcription factors like DREB1A (dehydration response element binding factor 1A). In the model plant *Arabidopsis* DREB1A regulates the expression of drought-tolerance genes. The DREB1A gene has recently been transferred stably into bread wheat using the biolistic transformation method. The expression of the gene was modified in such a way that DREB1A protein is produced only under conditions of environmental stress, and then activates other genes that mediate stress tolerance (Pellegrineschi *et al*., 2004). Stress-inducible expression of DREB1A is desirable, as permanent activity of such a regulatory protein could interfere with other regulatory pathways. It would impose an unnecessary cost on the plant in terms of energy to produce proteins in non-stress situations and might result in lower yield.

The transgenic wheat plants expressing the DREB1A gene demonstrated significant resistance to water stress in comparison to non-transgenic control plants of the same genotype under greenhouse conditions. The transgenic plants showed a 10-day delay in wilting when water was withheld as compared to controls. The research was carried out at CIMMYT, Mexico, a non-profit research organization for the sustainable development of wheat and maize farming for developing countries. Five years after a moratorium on planting transgenic maize under field conditions in 1998, the above greenhouse trial for transgenic wheat was approved in Mexico in 2003, and following the positive results a second, larger-scale trial is awaiting approval (Anon., 2005).
Drought is probably the world's most important limitation to agricultural production, affecting not only many developing countries but also important wheat producing countries like Australia. It is considered a complicated stress to study, as under natural conditions drought is usually accompanied by other stresses like heat and specific soil characteristics such as mineral deficiencies or toxicities. Increasing drought tolerance in wheat and other species is a challenge for breeding as well as for genetic engineering and might require both approaches in order to succeed.
More complex solutions are required when the addition of a trait cannot be achieved by one gene alone. A genetic engineering effort that involved the transfer of several genes is illustrated by transgenic rice that was enabled to synthesize provitamin A in its endosperm.

Genetic Engineering of the Provitamin A Pathway in "Golden Rice"

Rice is milled to remove its oil-rich outer layers that become rancid upon storage in a warm climate. The remaining endosperm lacks essential nutrients like vitamin A. In countries where mainly rice is consumed, vitamin A deficiency is a serious health problem leading to blindness and death in the poor populations of Asia, Africa and Latin America. Breeding for increased vitamin A is not possible by conventional methods as no rice cultivars are available

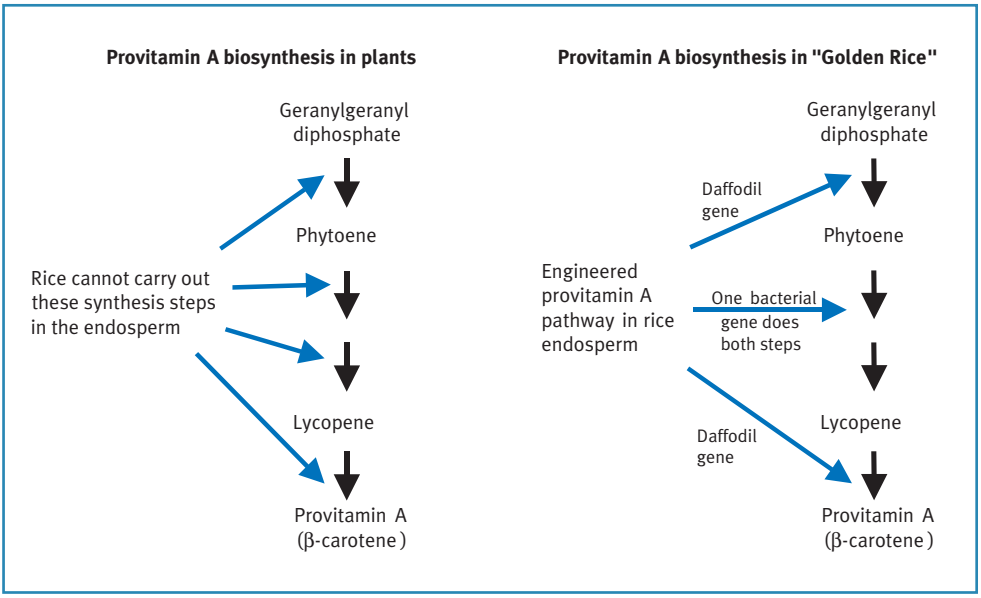


Fig. 223: Provitamin A synthesis in plants and in "Golden Rice"

which contain provitamin A (β-carotene), a precursor of vitamin A, in their endosperm (Ye *et al.*, 2000). Genetic engineering of provitamin A was possible, since its precursor geranylgeranyl diphosphate is present in rice endosperm (Fig. 223).

In plants, it takes four enzymes to synthesize provitamin A starting from geranylgeranyl diphosphate, but using a bacterial gene a short cut to generate provitamin A via three enzymatic steps is possible (Fig. 223).
Transgene rice plants containing all three transferred genes were selected; they showed normal vegetative growth and were fertile. The endosperm of mature seeds from transgenic rice plants was yellow in most cases, indicating carotenoid formation, in contrast to the white endosperm of non-transgenic rice plants (Fig. 224). The presence of carotenoids in transgenic lines was further confirmed by other methods, and provitamin A concentration was estimated to be at least 2 µg provitamin A per 1 g endosperm (Ye *et al.*, 2000; Beyer *et al.*, 2002; Potrykus, 2001). So the daily intake of 300 g "Golden Rice" would provide the equivalent of 100 µg of pure vitamin A. Even if the 100 µg vitamin A equivalent does not correspond to the 100% daily allowance for vitamin A according to the RDA (recommended dietary allowances), nutritionists estimate that the provitamin A provided by "Golden rice" will nevertheless have a definite beneficial effect on vitamin A-deficient people (Potrykus, 2001).

It is planned to apply this approach to wheat, cassava, sweet potato, banana and further basic food security crops also, in order to guarantee a supply of provitamin A worldwide. Besides being a precursor of vitamin A, provitamin A also acts as a free radical scavenger and can help prevent diseases of the cardiovascular system and various cancers, an activity that pure vitamin A does not have to the same degree. Issues involving intellectual and technical property rights have meanwhile been solved, and field experiments are envisaged (Potrykus, 2001).

Genetically engineered synthesis of provitamin A is representative of an emerging trend in breeding and also genetic engineering called "biofortification". Biofortification is the

process of generating food crops that are rich in bioavailable micronutrients. Plant parts intended for consumption are enriched with minerals and vitamins. Another important target is iron, for example, since iron is the most common micronutrient deficiency worldwide. In this case the mere enrichment of iron in the seeds of cereals would not be sufficient, as absorption is inhibited by phytic acid in plants. Phytic acid is especially abundant in cereals and legumes. A possible solution might be a protein that degrades phytic acid and keeps iron available for uptake. This solution has been proposed and is already realized in rice with the transfer of a phytase gene. This has, in fact, drastically reduced the inhibiting effect of phytic acid on iron availability (Lucca *et al.*, 2001).

Fig. 224: Golden rice seeds (top) compared with non-transgenic rice seeds (bottom) (photograph courtesy of Peter Beyer, University of Freiburg i. Br., Germany)

In wheat, provitamin A, iron and zinc are the most evident deficiencies and affect countries with high population densities and widespread micronutrient malnutrition such as Pakistan and India. International alliances of scientific institutions have been formed to address these problems of less privileged countries. For more information, use weblink http://www.harvestplus.org/about.html. Biofortification can also be beneficial for developed countries where major health risks like cardiovascular disease or cancer could be approached using biofortified basic staple foods. Wheat as the most popular cereal is likely to have the biggest impact.

24.1.5 References

- *agbios, accessed June 2005, http://www.agbios.com/main.php.*
- *Anderson OD, Litts JC and Greene FC. 1997. The α-gliadin gene family. I. Characterization of ten new wheat α-gliadin genomic clones, evidence for limited sequence conservation of flanking DNA, and Southern analysis of gene family. Theor. Appl. Genet. 95:50-58.*
- *Anon., 2005. http://www.monsanto.co.uk/news/ukshowlib.phtml?uid=8203, accessed June 2005.*
- *Becker D, Brettschneider R and Lörz H, 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J. 5(2):299-307.*
- *Beecher B, Bettge A, Smidansky E and Giroux MJ, 2002. Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet. 105:870-877.*
- *Beyer P, Al-Babili S, Ye X, Lucca P, Schaub P, Welsch R and Potrykus I, 2002. Golden Rice: Introducing the β-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. J. Nutrition 132(3):506-510.*
- *Blochet J-E, Chevalier C, Forest E, Pebay-Peyroula E, Gautier M-F, Joudrier P, Pezolet M and Marion D, 1993. Complete amino acid sequence of puroindoline, a new basic and cystine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Lett. 329:336-340.*
- *Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW and Wan Y, 1997. Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol. 115:971-980.*

• *Christou P, Ford TL and Kofron M, 1991. Genotype-independent stable transformation of rice (Oryza sativa) plants. Bio/Technol. 9:957-962.*

• *Faize M, Sourice S, Dupuis F, Parisi L, Gautier MF and Chevreau E, 2004. Expression of wheat puroindoline-b reduces scab susceptibility in transgenic apple (Malus domestica Borkh.). Plant Sci. 167:347-354.*

• *FAOSTAT, accessed June 2005, http://apps.fao.org*

• *Folck A, Wieser H, Knies P, Lörz H and Becker D. 2004. Silencing the α-Gliadins in Wheat. Poster ABIC 2004,http://vvgvg.org/pdf/Anne%20WEizen%20Hamburg%202.pdf.*

• *Gahrtz M, Wieser H, Schröder M, Lörz H and Becker D, 2004. Expression of wheat glutenin genes in maize endosperm. Poster ABIC 2004, http://vvgvg.org/pdf/Poster%20ABIC%202004c.pdf.*

• *Gerhardt SA, Balconi C and Sherwood JE, 2002. Control of scab with puroindoline-containing transgenic wheat. National Fusarium Head Blight Forum Proceedings, Dec. 7-9, Erlanger, KY, USA, p 28.*

• *Hiei Y, Ohta S, Komari T and Kumashiro T, 1994. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271-282.*

• *Hogg AC, Beecher B, Martin JM, Meyer F, Talbert L, Lanning S and Giroux MJ, 2005. Hard wheat milling and bread baking traits affected by the seed-specific overexpression of puroindolines. Crop Sci. 45:871-878.*

• *Hogg AC, Sripo T, Beecher B, Martin JM and Giroux MJ, 2004. Wheat puroindolines interact to form friabilin and control wheat grain hardness. Theor. Appl. Genet. 108:1089-1097.*

• *Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T, 1996. High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nature Biotechnol. 14:745-750.*

• *James C, 2002. Preview No. 27: Global Status of Commercialized Transgenic Crops 2002. ISAAA Briefs, 27, pp http://www.botanischergarten.ch/UNIDO/ISAAA_Briefs_No._27.pdf and http://www.isaaa.org.*

• *Krishnamurthy K and Giroux MJ, 2001. Expression of wheat puroindoline genes in transgenic rice enhances grain softness. Nature Biotechnol. 19:162-166.*

• *Langridge P, Lagudah ES, Holton TA, Appels R, Sharp PJ and Chalmers KJ, 2001. Trends in genetic and genome analyses in wheat: a review. Australian J. Agric. Res. 52(11):1043-1078.*

• *Lucca P, Hurrell R and Potrykus I, 2001. Genetic engineering approaches to improve the bio-availability and the level of iron in rice grains. Theor. Appl. Genet. 102:392-397.*

• *Morris CF, 2002. Puroindolines: The molecular genetic basis of wheat grain hardness. Plant Mol. Biol. 48:633-647.*

• *NAMA (North American Millers' Association), accessed June 2005, http://www.namamillers.org.*

• *Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K and Hoisington D, 2004. Stress-induced expression in wheat of the Arabidopsis thaliana DREB1A gene delays water stress symptoms under greenhouse conditions. Genome 47(3):493-500.*

• *Potrykus I, 2001. Response to Greenpeace comments on "Golden Rice", http://www.biotech-info.net/IP_response.html, accessed June 2005.*

• *Sahrawat AK, Becker D, Lütticke S and Lörz H, 2003. Genetic improvement of wheat via alien gene transfer, an assessment. Plant Sci. 165(5):1147-1168.*

• *Sanford JC, Klein TM, Wolf ED and Allen N, 1987. Delivery of substances into cells and tissues using a particle bombardment process. J. Part. Sci. Technol. 5:27-37.*

• *Shimada T, 1978. Plant regeneration from the callus induced from wheat embryos, Jap. J. Genet. 53:371-375.*

• *Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettel R. 1997. Agrobacterium tumefaciens-mediated barley transformation. Plant J. 11(6):1369-1376.*

• *Vasil V, Castillo AM, Fromm ME and Vasil IK, 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technol. 10: 67-674.*

• *Vasil V, Srivastava V, Castillo AM, Fromm ME and Vasil IK, 1993. Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technol. 11:1553-1558.*

• *Weeks JT, Anderson OD and Blechl AE, 1993. Rapid production of multiple independent lines of fertile transgenic wheat (Triticum aestivum). Plant Phys. 102:1077-1084.*

• *Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P and Potrykus I, 2000. Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287: 303-305.*