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review

Gene Transfer and Modulation for the Production of Food with Enhanced Quali-Quantitative Values: Potentials, Promises and Achievements

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Summary

We present an overview of the research and achievements of applications of molecular tools based on gene transfer and gene modulation (gene knock-down and knock-out), aimed at enhancing food production, improving food properties and producing various valuable compounds for human nutrition. Selected cases of genetically manipulated plants (biofortification and allergene silencing) and animals (fish and livestock) are examined. Promises and accomplishments are considered when giving topic examples of the potentials offered by some applications of molecular biology for obtaining goods, among them milk, with enhanced value, and of their impact on society at large.

Key words: biofortification, functional foods, gene silencing, gene suppression, health hazards, milk production, human nutrition, transgenic plants and animals, transpharmers

Introduction

This review focuses on the research, promises and achievements of applications of molecular tools based on gene transfer and modulation of genes (knock-down and knock-out) carried out in plants and animals, aiming at improving food properties and producing various valuable compounds for human nutrition. The field is huge since a great amount of research has already been developed, and a number of promising results have been achieved. Various documents concerning the state of the art, in particular the regulatory aspects of experimental and market releases of transgenic products, are available on the websites of the institutional organizations involved in agro-food policies, among them the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (*1*), the Food and Agriculture Organization of the United Nations (FAO) (*2*), the International Centre for Genetic Engineering and Biotechnology (ICGEB) (*3*), the Joint Research Centre of the European Commission (JRC) (*4*) and the European Food Safety Authority (EFSA) (*5*).

This topic is a remarkable example of the controversial interactions of science, society and politics which characterize various innovations in biology research. Such innovations, also known as 'bio-objects', leave the laboratories and break into our every-day lives bearing their load of promises and concerns. The products of gene transfer and modulation, in fact, are constructed and manipulated biologies potentially useful for enhancing human life quality, balancing on the fine line between the natural and the non-natural/artificial, and challenging conventional natural, cultural, scientific and institutional orderings (*6*). Details of their impact on society

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can be found in specific literature (*7–9*) and in our previous works concerning the analysis of consumer acceptability and perceived risk related to genetically modified organisms (GMOs) (*10*), and some bio-social implications of biology innovation and food (*11,12*). Moreover, additional viewpoints can be found on the websites of various associations and non-profit organizations involved in bio-social issues and environmental protection.

Here we report a few selected cases of genetic manipulation of plants and animals which we consider most representative and relevant in scientific achievement for their impact on society at large and from a historical point of view. The aim is to build up a portrait of the potentials offered by molecular biology for obtaining food with enhanced value. This portrait is not meant to be a complete *magnum opus;* conversely, it is intended to stimulate readers' interest to further explore this fascinating field. Besides the interest in the scientific aspects, in fact, the production of fortified food, where the properties of food and medicine are strictly linked, points our attention to the deep connection between food consumption, culture and lifestyle, a relationship known since ancient times.

Transgenic Crops for Improved Food Production

The first transgenic plant achievement dates back 30 years, when generations of antibiotic-resistant tobacco (*13–14*) and petunia (*15*) were announced at the Miami Winter Symposia of 1983 by three independent groups, followed a few months later by a publication on transgenic sunflower expressing the bean phaseolin gene (*16*). Since then, technical improvements and continuous successful gene transfer have been reported in innumerable plants and, according to the 2011 report of the ISAAA on the Global Status of Commercialized Biotech/GM Crops (*1*), nowadays 160 million hectares are cultivated with biotech crops, with a globally upward trend. Soya bean, maize, cotton and canola continued to be the principal biotech crops in 2011, and herbicide tolerance has consistently been the dominant trait.

Notwithstanding this commercial background, focusing mainly on the industrial and feed crops which have been genetically modified for traits useful to farmers as well as biotech companies, there is a constant interest in the scientific world in exploiting gene transfer techniques for improving food properties and producing various valuable compounds for human nutrition. This is reflected by the shift from the first generation of transgenic plants to the second generation (plants with improved quality and reduction of allergenic components) and further (plant molecular farming, production of renewable resources). Accordingly, many genetically modified plants have been generated with the aim of producing pharmaceutical and nutraceutical compounds for use by humans, a technology known as biopharming. Vaccine production in transgenic plants is one of the best known examples of these new generation biotech crops. In particular edible vaccines, produced in edible parts of the plants, in their dual function of food and medicine, have been designed to be easily delivered, and to overcome the drawbacks of classical vaccine manufacturing based on microorganisms and mammalian cells (*17*).

In some applications of gene transfer for relevant compound production, both plants and animals have been used to reach the same goal, and further experimentation would help in identifying the best system, considering efficiency, cost and acceptability of the products. This is the case of lactoferrin, a multifunctional protein involved in several biological functions, such as regulation of iron transport, antimicrobial defence and antitumour mechanisms. Of the transgenic systems utilized to produce human lactoferrin, besides yeast, transgenic cows and rice have also been successfully used. The recombinant human lactoferrin (rhLF) is produced on an industrial scale from *Aspergillus awamori* by Agennix (Houston, TX, USA), from rice by Ventria Bioscience (Sacramento, CA, USA) and from transgenic cows by Pharming (Leiden, The Netherlands), and other companies in China and Argentina, as described below. In plants, expression of rhLF has been also achieved in tobacco, potatoes, tomato, maize, barley and rice (*18*).

A number of reviews of the scientific literature produced over the years concerning genetically modified plants, including the above-mentioned new generation ones, have been produced; as the total amount of available papers and data have highly increased, they have focused on various different aspects (among the latest papers see *19–24*).

In the present review, we concentrate on some selected examples concerning gene transfer for improved food production, and in particular for increasing nutritional value, an approach also known as biofortification. Biofortification of plant-derived foods appears to be a promising strategy for the alleviation of nutritional deficiencies. Various examples of genetically engineered crops in order to increase their nutritional value through improvement of the level of vitamins/antioxidant compounds are already available.

Modification of fatty acid content in food is a relevant objective pursued both in animals (as described in the section below) and plants, since scientific evidence has implicated the quantity and/or quality of dietary fats in the development of several illnesses, including cardiovascular diseases, some cancers and arthritis. Accordingly, in canola (*Brassica napus*) seeds, high levels of ω-3 fatty acids have been accumulated by transferring the genes of the enzymes involved in the fatty acid biosynthetic pathway (D6 and D12 fatty acid desaturases from the commercially grown fungus *Mortierella alpina* and the D15 fatty acid desaturase from canola) (*25*).

Several attempts have been made to engineer higher lycopene levels in tomato fruits. With this aim, the bacterial *crtI* gene was introduced in tomato (*26*) resulting in an unexpected threefold increase in β-carotene but not in lycopene. Overexpression of lycopene β-cyclase (*β-Lcy*) was obtained in tomato by introducing *via Agrobacterium* a construct containing the *Arabidopsis β-Lcy* cDNA fused with the tomato *Pds* promoter, which is up-regulated in ripening fruits. Three transformants showed a significant increase in fruit β-carotene content and an orange/orange-red colour of fruits (*27*).

Diretto *et al.* (*28*) aimed at improving potato with a mini-pathway of bacterial origin, driving the synthesis of β-carotene (provitamin A) through three exogenous

genes: phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*) and lycopene β-cyclase (*CrtY*). Expression of all three genes under tuber-specific promoter control resulted in tubers with a deep yellow phenotype. In these tubers carotenoids increased approx. 20-fold, to 114 μg per g of dry mass and β-carotene 3600-fold, to 47 μg per g of dry mass, sufficient to provide 50 % of the recommended daily allowance of vitamin A with 250 g (fresh mass) of the transgenic potatoes.

A research project named BioCassava Plus (*29*) was developed by a public network of researchers, aiming to reduce micronutrient malnutrition by increasing the nutritional value of cassava, a staple crop in Africa. The objective of the project was to produce and field test a transgenic variety of TME7 cassava enhanced with β-carotene in Nigeria, such that it contains 40 μg of carotene per g of dry mass and 40 μg of iron per g of dry mass.

Overcoming vitamin deficiency: the case of Golden Rice

Among the crops already produced in the various biofortification projects, Golden Rice has a ringside seat since it is still the paradigmatic example of the potentialities that a transgenic crop can have, but also of the problems it can cause. This project represents a scientific breakthrough, being the first case of biochemical pathway engineering. Moreover, Golden Rice was intended as a product aiming at solving a major humanitarian world problem rather than to benefit producers (*30,31*). Nonetheless, it encountered fierce opposition, and thereafter few new transgenic Golden Rice lines have been produced throughout the years.

The first paper concerning Golden Rice was published in Science in 2000 (*32*). The authors stated that at that moment as many as 124 million children worldwide were deficient in vitamin A. This deficiency causes symptoms ranging from night blindness to xerophthalmia and keratomalacia, leading to total blindness. Even subclinical vitamin A deficiency can have broader consequences in developing countries in terms of child morbidity and mortality. Oral delivery of vitamin A was problematic because of the lack of infrastructure, therefore the supplementation of a major staple food, rice, with provitamin A seemed an effective strategy.

In an attempt to develop vitamin-A-producing rice, a project started in 1992, led by Ingo Potrykus of the Institute of Plant Sciences at the Swiss Federal Institute of Technology (ETH, Zürich, Switzerland), together with Peter Beyer of the University of Freiburg, Germany, with the support of the Rockefeller Foundation (New York, NY, USA) and others. *Agrobacterium*-mediated gene transfer was used to introduce the entire β-carotene biosynthetic pathway into rice endosperm (the edible part of rice) with different vectors. The vector pB19hpc combined the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*Narcissus pseudonarcissus*) with the sequence coding for a bacterial phytoene desaturase (*crtI*) originating from *Erwinia uredovora,* placed under the control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV 35S promoter, respectively. The *psy* cDNA contained a 59-bp sequence coding for a functional transit peptide, and the *crtI* gene contained the transit peptide sequence of the pea Rubisco small subunit: this plasmid directed the formation of lycopene in the endosperm plastids. Provitamin A was produced in the absence of heterologous β-cyclase (which converts lycopene to β-carotene): the absence of lycopene in Golden Rice showed that the carotenoid pathway proceeded beyond the transgenic end point and thus that the endogenous pathway must also be acting (*33,34*).

The scientists involved in the project intended to make the technology freely available to developing countries but had to face a number of legal constraints coming from pending licences, since part of their financial support derived from the European Community Carotene Plus project, whose industrial partners had rights to protect. An agreement was reached with Zeneca (Fernhurst, UK), corporate sponsors of the Carotene Plus program, and subsequently with most of the Intellectual and Technical Property Rights owners (some 32 companies and institutions hold 70 patents for various technologies used to create the enriched rice), so that it was possible to grant freedom to operate to public research institutions in developing countries to proceed in introducing the trait into local varieties. The cut-off line between humanitarian and commercial use, *i.e.* the maximum annual income for a small-scale farmer to be exempted from royalty payment, was set at US$ 10 000 (*35*).

A substantial improvement in the Golden Rice was achieved by Paine *et al.* (*36*), from Syngenta biotechnology company, who hypothesized that the daffodil *psy* gene was the limiting step in β-carotene accumulation. They identified a *psy* gene from maize and introduced it in combination with the *Erwinia uredovora crtI* gene, used to generate the original Golden Rice. This Golden Rice 2 (GR2) showed an increase in total carotenoids of up to 23-fold (maximum 37 μg/g) compared to the original Golden Rice, now called GR1 (whose highest provitamin-A-producing line contained 1.6 μg per g of carotenoids in the endosperm), and a preferential accumulation of β-carotene. β-Carotene derived from GR2 was effectively converted to vitamin A in humans (*37*).

An in-depth analysis of the potential economic effects of Golden Rice and the prospective impacts of countries adopting this technology was given by Nielsen and Anderson in 2003 (*38*). To have the greatest impact at a low cost, Golden Rice varieties should be adapted for widespread cultivation in Asia and should deliver as much β-carotene as possible (*39*); the findings of Stein *et al.* (*40*) suggested that related investments were worthwhile. The characteristics of Golden Rice may be transferred to locally adapted varieties and ecotypes in rice-growing countries to limit the reduction of biodiversity. Both the original GR1 and GR2 originated from Japonica rice cultivars; researchers were then backcrossing GR1 and GR2 lines with the Indica varieties popular among Asian farmers. The initial perspective of making Golden Rice cultivars available to farmers in a few years failed, however, and the first field trial of Golden Rice in Asia was only started in 2008 (*41*).

The most productive rice variety in Bangladesh, BRRI Dhan-29, engineered at the International Rice Research Institute (IRRI) in Los Baños, Philippines with the β-carotene genes from corn, was successfully field-tested

at the IRRI in February 2011. Besides BRRI Dhan-29, the IRRI variety IR-64 and the Filipino variety RC-28 were genetically engineered to have greater expressions of the corn gene responsible for producing β-carotene. According to IRRI, if Golden Rice is shown to be safe and to improve the vitamin A status, a delivery program will be designed to ensure that Golden Rice is acceptable and accessible in vitamin-A-deficient communities (*42*).

A considerable amount of literature and documents concerning the opposition to the Golden Rice project has been published, mostly signed by various institutions and international non-governmental organizations (NGOs), and can be found on the Internet. Besides risks related to the environmental and biodiversity protections, bio--social concerns and disapproval of the policies adopted for the empowerment of developing countries are the most relevant issues pointed out against the adoption of this crop. One of the most embraced criticisms of Golden Rice is that the amount of transgenic rice to be consumed during an everyday human diet to meet the daily requirement of vitamin A would be largely unfeasible. Figures reported by scientific papers, however, support results quite divergent from this assumption (*36*,*37*), proving the fact that the Golden Rice project has raised a wealth of controversial opinions since its beginning and the debate on the issue is far from being closed.

Still, several years after its first production, whilst the scientific community values Golden Rice as a flag of the humanitarian engagement of public research, opponents, on the contrary, present this bio-object as an example of technology aimed at further enriching the markets of the already rich countries rather than elevating the economies of the developing countries (*43*,*44*). This assertion is well exemplified in the words of philosopher Vandana Shiva, the environmental activist and ecofeminist, one of the leaders and board members of the International Forum on Globalization and a figure of the global solidarity movement known as the alter-globalization movement: 'While the complicated technology transfer package of Golden Rice will not solve vitamin A problems in India, it is a very effective strategy for corporate take-over of rice production, using the public sector as a Trojan horse' (*45*).

The promise of resveratrol for healthier transgenic food production

Stilbenes are natural, biologically active phenolic compounds occurring in a number of plant families, including Vitaceae and (within this family) *Vitis vinifera* L., which is one of the most economically important horticultural crops. In particular, resveratrol (3,5,4'-trihydroxy-*trans*--stilbene) and viniferins are present in grapevine as constitutive compounds of the woody organs (roots, canes, stems) and as induced substances (in leaves and fruit) acting as phytoalexins in the mechanisms of grape response to pathogens and abiotic stresses (*46*,*47*). Several other fruits and vegetables naturally produce stilbenes, as resveratrol has been found in cranberries, blueberries, mulberries, peanuts and jackfruit (*48*).

Stilbenes also exhibit a broad spectrum of antibiotic and pharmacological activities, and have generated considerable interest as nutraceuticals, owing to their diverse health-promoting properties and their supposed role against cardiac ailments and cancer. The first evidence of stilbene synthesis in Vitaceae species was reported by Langcake and Pryce in 1976 (*49*). Since then, intense research aimed at elucidating the role of stilbenes has been carried out, although the topic still needs further studies. Resveratrol, one of the best known plant secondary metabolites, has also been detected in red wine (it is synthesized in the berry skin and the maceration period during winemaking allows for its extraction into the resulting wine) (*46*).

At the beginning of this millennium, much emphasis was given to the so-called French paradox by the media, *i.e.* the fact that French people suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats: this conflicting data was hypothetically explained by the consumption of red wine, which should decrease the incidence of cardiac diseases (*50*,*51*). Thereafter, a great number of studies in model animals and epidemiological observations in humans concentrated on proving the effects of polyphenols and resveratrol in wine, as well as in determining the most suitable dose assumed to produce these beneficial attributes.

Accordingly, moderate wine consumption has been proposed in the prevention of various chronic pathologies, in particular those related to cellular oxidative stress, including diabetes, high blood pressure, atherosclerosis, cholesterol reduction and certain cancers (*52*–*55*). Other studies (*56*,*57*), however, raised doubts about the healthy effects of dietary resveratrol consumption. Recently, works conducted on the beneficial effects of resveratrol at the University of Connecticut Health Center (UCHC, Farmington, CT, USA) have been subjected to an extensive research misconduct investigation by UCHC itself and the US Office of Research Integrity (ORI, Rockville, MD, USA) (*58*,*59*). The significance of this case of fabrication and/or falsification of data, however, seems to be an example of scientific research fraud rather than an invalidation of the many studies on the health benefits of resveratrol. Possibly, the potentialities of this compound have been overestimated and at the present time, the long-term effects of supplementation of resveratrol and other stilbenes in human diet still need to be ascertained.

Nonetheless, the interesting biological properties and the potential positive effects of these compounds have driven many scientists to study them and to attempt the production of functional food with an enhanced content of resveratrol and related compounds *via* gene transfer. The role of resveratrol as a phytoalexin has been widely studied, in particular in the defense against fungal pathogens (*60*).

Transfer of stilbene-encoding genes to plants unable to produce (or having a limited production of) phytoalexins has been attempted, starting from the early 1990s. The first gene transfer experiment was performed by transferring a stilbene synthase gene from peanut (*Arachis hypogea*) to tobacco cells, resulting in *de novo* resveratrol synthesis after induction with UV-light and an elicitor (*61*). Genes involved in the biochemical pathway of stilbenes in grapevine were also used in gene transfer experiments, such as two grapevine genes coding for stilbene synthase to tobacco. The obtained transgenic tobacco

plants were more resistant to *Botrytis cinerea* infection than the control (*62*). Since those pioneering works, stilbene synthase genes from grapevine or peanut have been transferred to a number of plants to increase their tolerance/resistance to plant pathogens or to exploit their positive effects on human health; in some cases both aims were pursued.

Among the gene transfer experiments with the final goal of improving the health protection properties of different plant species, genetically modified alfalfa (*Medicago sativa*) was used as a model in applying biotechnological approaches to cancer prevention. This transgenic alfalfa was generated with a peanut cDNA encoding resveratrol synthase, which produced *trans*-resveratrol-3-*O*-β-D-glucopyranoside (RG), whose constitutive accumulation increased resistance to *Phoma medicaginis* (*63*). Transgenic alfalfa or synthetic RG included in mice diets were unable to inhibit the formation of preneoplastic lesions (that directly correlate with the risk of colon cancer and tumour size in humans) in the colon of mice, probably due to the metabolism and biological uptake of the compound in the upper gastrointestinal tract, but when these diets were supplemented with the exogenous enzymes β-glucosidase (*64*) or α-galactosidase (*65*), the development of the preneoplastic lesions was significantly reduced.

Resveratrol was extracted and purified from transgenic tobacco plants. Having observed an increased accumulation of human breast adenocarcinoma cells in G_0 and G_1 phases of cell cycle in the cells treated with this resveratrol as compared to the untreated cells, it has been concluded that resveratrol from transgenic plants merits further investigation as a potential cancer chemopreventive agent in humans (*66*).

Stilbene synthase genes from *Vitis vinifera* were transferred to *Lactuca sativa*, resulting in a transgenic red lettuce capable of producing resveratrol in amounts higher than red wine (*67*). Fruit tree species were also genetically modified with genes coding for resveratrol synthesis. cDNA encoding stilbene synthase from *V. vinifera*, transcriptionally regulated by an enhanced cauliflower mosaic virus (CaMV) 35S promoter, was transferred to Spadona pear (*Pyrus communis*) resulting in piceid and resveratroloside accumulation in transgenic plants (*68*). A similar approach was adopted in Elstar and Holsteiner Cox apples (*Malus domestica*), where stilbene synthase gene (*Vst1*) transfer caused the accumulation of a resveratrol derivative, *trans*-resveratrol-3-*O*-β-D-glycopyranoside (piceid), in fruit tissues (*69*). The possible influence of the novel biosynthetic pathway on the accumulation of other phenolic compounds naturally present in apple fruit was investigated, and in none of the transgenic apple lines that accumulated piceid was a negative correlation between levels of piceid and the amounts of flavanols, flavonols, phloretin derivatives and hydroxycinnamic acids observed, except for the flavonol content, which slightly decreased (*70*).

Stilbene synthase production induced by transferred exogenous genes has been obtained in a wide range of plant species, including tomato (*71*), barley and wheat (*72–74*), rice (*75*), poplar (*76*), pea (*77*), hop (*78*), and purple sweet potato (*79*). These experiments were usually aimed at the production of transgenic plants with increased resistance to fungal pathogens, but the positive effects of resveratrol on human health were also considered. However, not all the resulting transgenic lines showed an increased tolerance to pathogens when specific bioassays were performed.

While stilbenes can be recovered as an extract from a selected number of plants, these products are not suitable for many applications in the food/pharmaceutical sectors due to high levels of impurities as well as the overall low concentration of resveratrol and its derivatives in the extract (*80*). Therefore, it is important to develop an effective method of producing this compound commercially. To deliver a highly defined and enriched resveratrol product, hairy root cultures of peanut were studied as a bioproduction system for resveratrol and associated derivatives. In this system, 99 % of the total resveratrol produced was secreted into the culture medium at levels of 98 μg per mg of the medium dried extract (*80*).

Given the increased interest in these compounds and the increasing number of experiments aimed at the generation of transgenic plants overexpressing them, some comprehensive reviews have recently been published, focusing on the role and activity of resveratrol (*47*), its molecular engineering in plants (*81,82*), and methods for obtaining it, from plant extraction to chemical synthesis and biotechnological production (*83,84*). In these papers gene and promoter options are discussed, as well as factors modifying transgene expression and epigenetic modifications, the incidence of these compounds in plant metabolism and development, and the use of biotechnology through recombinant microorganisms and plant cell suspensions.

Transgenic Animals for Improved Food Production

Gene transfer in animals was pioneered at the end of the 1970s, starting with a mouse expressing foreign DNA sequences of tumour virus SV40 (*85*), and nowadays this technique has been successfully applied in a considerable number of farm animals such as rabbit, pig, sheep (*86*), cattle (*87*) and goat (*88*).

Direct microinjection of genetic material into the pronuclei of fertilized one-cell eggs to be implanted into the oviducts of pseudopregnant surrogate female mice has been the first effective strategy developed (*89*) and, even though laborious, costly and hampered by relatively low efficiency and species-dependent embryo survival, is still reported as the most accepted method of production of transgenic animals (*90*). Alternatively, starting from the pioneering studies developed in the 1970s based on retrovirus vectors (*91*), lentiviral vectors have been efficiently used for microinjecting fertilized eggs, unfertilized oocytes and zygotes, and nowadays lentivirus-mediated transgenesis is the most strongly emergent technology for delivering genes as well as for transfecting synthetic small interfering RNA (siRNA) molecules aimed at targeting gene knockdown (*90,92*). The combination of somatic cell nuclear transfer, an established tool to yield copies of selected individuals in different animal species, with genetic engineering is currently reported as the most suit-

able method for generating transgenic large animals for both agricultural and biomedical applications (*93*).

It is worth remembering that male germinal cells (spermatozoa and spermatogonial stem cells) have been used to direct the transfer of exogenous DNA into female cells for generating transgenic animals (*94*). Embryonic stem cell-mediated gene transfer has also been developed (*95*) by inserting a desired DNA sequence *via* homologous recombination into an *in vitro* culture of these totipotent cells, which are then incorporated into an embryo at the blastocyst stage of development. DNA constructs can also be transfected by electroporation into fetal cells, such as fibroblasts, which can thereafter be used to clone transgenic animals by nuclear transfer (*96*). Combination of classical breeding with gene transfer may also be used, as in transgenic cows which, besides insertion of a desired gene into embryos, can be produced by mating wild type cows with genetically modified bulls for the generation of transgenic progeny (*97*).

To date, according to the results obtained in animals relevant to food production, gene transfer seems to be a costly strategy, and not as efficient as initially promised, compared with conventional selection. Moreover, transgenic farm animal production for biomedical applications encountered more acceptability and provoked less public concern, thus influencing the industrial application of GM animals. Consequently, gene transfer has primarily been used for biomedical rather than agricultural applications. In medicine, genetically modified pigs have been adopted as organ sources for xenotransplantation (*98*). The generation of transgenic mice capable of mimicking human genetic diseases provides a suitable whole animal model for studying human genetic diseases in order to develop pathological and pharmacological studies (*99*). The production of drugs for pharmaceutical use is another relevant application of transgenic animals, in particular to overcome the limitation of recombinant bacterial systems, which are less suitable for producing complex proteins such as monoclonal antibodies or coagulation blood factors. Moreover, compared to plants, the high yield and low-cost production as well as the high quality of the proteins obtained are the features which make animal systems appealing tools (*100*). The most common system is milk from transgenic farm mammals; nevertheless, chicken egg white and blood, seminal plasma, urine, silk glands and insect larval haemolymph have also been exploited (*100*).

As for food improvement, animals have been genetically modified to obtain improved livestock and quali-/quantitatively enhanced food sources. Productivity traits, such as feed conversion, rate of gain, reduction of fat, and improved quality of meat are the most pursued objectives (*101*). The productivity traits are however quantitative, thus controlled by numerous genes, of which only a few are presently known. For this reason, besides the current low level of public acceptance, the progress of agricultural applications of livestock transgenic technology is less developed compared to biomedical applications. Among the hundreds of quantitative trait loci (QTL) already mapped in livestock, two mutations underpinning the QTL have been identified in dairy cattle: the first concerns the 1DGAT1 (acyl-CoA:diacylglycerol acyltransferase) locus on chromosome 14 as a gene contributing to fat composition in milk (*102*) and the second concerns the ABCG2 (ATP-binding cassette, subfamily G (WHITE), member 2) locus on chromosome 6 as a gene contributing to fat and protein concentration in milk (*103*).

More advanced is the knowledge of monogenic traits of economic and biological interest, and a number of causative mutations have already been identified, as reported in the valuable database Online Mendelian Inheritance in Animals (OMIA) (*104*), where numerous single-locus traits are described in 192 animal species other than human and mouse, including food sources as interesting as cattle, pig, sheep, goat, chicken and horse, according to the different food ethics of various countries, which either assign the status of food source to animals or designate their consumption as a taboo (*105*).

In the near future, the achievements of genome projects will offer useful information for better understanding of the most economically important genes in livestock species in order to accomplish the selection for desirable traits, or conversely, against undesirable traits (*106*), and in the main species, like bovine ones, genome sequencing has already been accomplished (*107*).

Growth enhancement in transgenic animals

Growth enhancement is one of the most pursued applications of transgenic techniques. Pigs, fish and cattle have been engineered in an attempt to provide more food per animal, generally by transferring growth hormone genes (*108*). Growth hormone (GH) is a peptide hormone naturally synthesized, stored, and secreted by the pituitary gland, which stimulates growth, cell multiplication and regeneration (known as anabolic effects) in humans and other animals. Over- and under-production of GH are related to relevant pathologies.

Production of superfish has received the most attention because of its success in generating marketable products in shorter periods of time and with lower production costs, and has been applied to a wide range of fish species including the coho (*Oncorhynchus kisutch*), Atlantic (*Salmo salar*) and Japanese (*Oncorhynchus masou*) salmons. As detailed in Maclean and Laight's review (*109*), the growth enhancement of fish species can be dramatically improved by transgenesis based on the GH gene: in the Atlantic salmon and coho salmon, more than tenfold larger G_0 fish than control counterparts were obtained, and in *Tilapia* sp. up to more than threefold mass increase was recorded. Results in carp, northern pike and channel catfish were not so dramatic, but still relevant, possibly because of the considerable strain selection over many hundreds of years of human exploitation rather than because of the failure of transgenesis.

Some North American multinational companies have already developed transgenic salmon, trout, and tilapia designed to grow faster than their conventional siblings. This is the case of the AquAdvantage® Salmon produced by AquaBounty Technologies (Waltham, MA, USA), which presents the product with the following words: 'AquAdvantage® Salmon (AAS) includes a gene from the Chinook salmon, which provides the fish with the potential to grow to market size in half the time of conventional salmon and, in all other respects, AAS is identical to other Atlantic salmon' (*110*). This salmon, which repre-

sents a profitable solution to increasing fish demand in the coming years, will be recorded as the first transgenic animal for human consumption approved by Food and Drug Administration (FDA) (*111*). A lengthy authorization process started in 1995, when AquaBounty Technologies applied for official US Government approval to develop this salmon commercially. In 2010, the FDA announced that there was enough information available to review the GM salmon, and in May 2012 it completed its environmental assessment. In December 2012, a draft was published declaring that the production of the GM fish is unlikely to have any detrimental impact on the wider environment, opening the doors for commercialization and yet again raising a strong anti-GMO reaction (*112*).

Initially, GH genes from mammals, principally human, bovine and rat, were the first exogenes used for developing the superfish under the control of the metallothionein B promoter. Recently, sequences derived from fish itself ('all fish' genetic material) carrying both promoters and genes from fish origin have been employed on the basis of a presumed higher likelihood of acceptability by consumers, as in the case of cisgenic plants (*113*). Oppositions to genetic manipulation of animals, as for plants, however, seem to ignore this assumption. In 2001, the European Patent Office (EPO) granted its first patent (Patent No. 0578653) on genetically engineered fish concerning a gene construct for production of transgenic fish produced by Canadian Seabright Corporation Ltd., Toronto, Canada (*114*). The construct carried a promoter sequence of the antifreeze protein derived from ocean pout. This promoter proved to provide better gene expression and regulation in the fish (*115*). For instance, transgenic Atlantic salmon, generated with all fish GH chimeric gene constructs carrying this promoter linked to a chinook salmon GH, presented an amazing growth rate since, when one year old, the average increase was 2- to 6-fold and the largest transgenic fish was 13 times bigger than the non-transgenic controls (*116*).

Transgenic superfish have been studied in depth and specific tests have been used to measure their performance, including manoeuvrability, speed of escape response, sprint speed and propulsive efficiency. Some evidence to support the view that growth-enhanced transgenic fish may be inferior to their wild-type counterparts has been published, since in transgenic rainbow trout a behaviour related to an increased predation mortality has been seen, and in transgenic coho salmon a lowered swimming performance (*117*,*118*) has been observed. Application of gene transfer to fish is relatively simple, but the 'mobility' of transgenic fish and the consequent need for containment are related problems and, besides strict containment measures, sterilization has been proposed to overcome this disadvantage (*109*,*119*).

GH gene transfer has also been exploited in pigs and resulted in transgenic animals having faster mass gain, higher (17 %) efficiency in converting feed into meat and reduced (down to one-fifth) fat in the carcass compared to the wild-type counterparts. However, various deleterious effects have also been observed (*120*). This is the case of a pig named Beltsville pig after the Agricultural Research Station in Beltsville, MD, USA, where it was generated, and nicknamed super-pig for its large size. This animal was produced by zygote microinjection with a construct carrying an ovine metallothionein 1a-ovine growth hormone fusion gene. High-level expression of the transgene resulted in a dramatic reduction in carcass fat with a concomitant increase in carcass protein and moisture content as compared to the control littermates. However, the high expression of this transgene due to its ineffective regulation hindered these results, and various health problems (lethargy, lameness, uncoordination, exopthalmus, gastric ulcers, severe synovitis, degenerative joint disease, pericarditis and endocarditis, cardiomegaly, paraketosis, nephritis, and pneumonia) related to its enormous size were the side effects in this experimental pig, which at the end could not support its own mass (*120, 121*). Such severe problems were thought to be the result of the constant high level of circulating GH, but attempts to control expression by the use of inducible promoters have not yet been successful. Consequently, the super-pig could never be used for ethical reasons, was therefore put down to end its suffering and served as an example for critics of transgenic animal research (*122*).

In sheep, transgenic GH lambs did not present faster growth nor more efficient feed conversion compared to controls; however, they were much leaner and presented a lack of body fat, this latter trait being attributed to the result of hyperglycemia and glycosuria (*123*,*124*). Transgenic lambs with elevated GH also had a number of other pathologies, including joint problems and degenerative kidney disease (*120*).

Overexpression of GH genes in transgenic mice has been applied in order to understand the effects of long-term GH excess. In these models, bovine, ovine or rat GHs (*i.e.* hormones with actions closely resembling, if not identical to, those of endogenous mouse GH) exhibited growth enhancement, increased adult body size, reduced lifespan and various endocrine and reproductive abnormalities, *i.e.* a variety of direct and indirect actions at the hypothalamic, pituitary, gonadal, and reproductive tract levels (*125*).

Quality improvement of food

Production of food with improved quality, as a result of enhanced/enriched/reduced contents of specific components, is another main goal of livestock genetic improvement. Modification of polyunsaturated fatty acids (PUFAs) is one of the most relevant goals, as clinical studies have shown that long-chain ω-3 PUFAs have a beneficial effect on human health, whilst high levels of n-6 PUFAs in human bodies are closely related to cancer, cardiovascular diseases and mental disorders (*126*). Since mammals lack n-3 desaturases, the n-3 and n-6 PUFAs are not interconvertible. Accordingly, products from sea fish are the main dietary source of n-3 fatty acids for humans. For this reason farmers and the livestock production industry feed animals with flaxseed, fish meal or other marine products for enriching foods, contributing on the other hand to the depletion of marine fish stock as well as running the risk of potential contamination of fish products with mercury and other chemicals (*96*).

Following a promising experiment where exogenous expression of a humanized *fat-1* gene (*hfat-1*) encoding an n-3 fatty acid desaturase from *Caenorhabditis elegans* in mice resulted in a significant increase of n-3 fatty ac-

ids in various tissues and in milk, as well as in a sharply decreased ratio of n-6/n-3 fatty acids (*127*), this exogene was transferred in pig and cow to produce healthier livestock. In both experiments, fetal fibroblast cells were used for gene transfer and transgenic animals were cloned from these cells. In piglets, in the major tissues (muscle, liver, kidney, heart, spleen, tongue, brain and skin) of the various founder animals recovered, and in their offspring, n-3 PUFAs were significantly increased, while n-6 PUFAs were decreased, resulting in a greatly reduced n-6/n-3 ratio (*96*).

In cows, only a transgenic calf was generated; however, this animal exhibited promising features. Analyses of fatty acids from ear tissues, in fact, showed that all major n-3 fatty acid peaks were elevated, whilst all major n-6 peaks were at a lower level, thus proving suitable expression of n-3 desaturases (*128*). Moreover, this animal grew healthy and, after artificial insemination, naturally delivered a healthy calf. The content of n-3 PUFAs in milk was significantly increased and the ratio between n-6 and n-3 was reduced fourfold compared to the non-transgenic counterparts. These results showed that the transgenic cow was capable of normal reproduction, the transgene was properly expressed in the mammary gland and the n-3 fatty acids were enriched in the milk (*128*).

Milk production by transgenic animals

Milk-producing transgenic animals are the objects of extensive research aimed at manufacturing compounds to be available in, or extracted at low cost from, their milk. Transgenic animals containing human proteins in their milk are also known as transpharmers and various animal species have already been adopted as transpharmers (*129*).

The expression vector carrying the gene encoding for the protein of interest fused to milk-specific regulatory elements is generally introduced into the germline of the chosen species *via* pronuclear microinjection of one-cell embryos or transfection into a primary cell population suitable for somatic cell nuclear transfer. Mice are mainly used for experimental assays and for testing the constructs prior to generating larger transgenic founder animals. Transgenic rabbits are not suitable for large-scale production because of their small lactation yield and labour-intensive milking, whilst sows have been successfully used for the considerable amount of milk (100–200 L) they can produce. Transgenic ruminants are obvious candidates since thousands of years of genetic selection have yielded breeds of sheep, goats, and cattle that can produce prodigious quantities of milk (*129*).

More than 60 therapeutic proteins, including plasma proteins, hormones, monoclonal antibodies, vaccines, cytokines, enzymes, fibrinogen and insulin have already been produced experimentally from the milk of transgenic cows, sheep and goats (*97*). Amongst them is the anticoagulant antithrombin (ATryn), the first biological drug from genetically engineered animals which was manufactured by the US company GTC Biotherapeutics (Framingham, MA, USA) from the milk of a transgenic goat and approved in February 2009 by the US Food and Drug Administration (see the FDA archive, *130*). Transgenic milk has also been exploited for industrial applications, like the high-value polymers produced in goat milk as a result of exogenous expression of the silk gene from spider (*131*).

Gene transfer has been used for improving livestock growth, and for health and survivability of milk-fed newborns. In pigs, for instance, where milk production is a limiting factor for piglet growth, overexpression of bovine α-lactalbumin, which plays a key role in the lactose synthesis and regulation of milk volume, resulted in increased milk lactose (thus an enhanced carbohydrate source) associated with a 20–50 % increase in milk yield. As a result, the growth and survival of suckling piglets improved significantly (*132*).

The introduction of new antimicrobial properties into milk is a desirable aim to provide passive immunity for the suckling young, but also for humans consuming the milk. This was the goal of a work aimed at overexpressing the human form of the antimicrobial protein lysozyme in the goat's milk, which was used for feeding piglets as human models and resulted in a beneficial effect on their intestinal microflora (*133*).

The mammary gland of dairy cattle is considered ideal for large-scale production of heterologous proteins due to its large capacity for protein synthesis, efficient secretion, and low feed and housing costs of dairy animals compared to *in vitro* fermentation or tissue culture systems (*18*). Accordingly, a number of research projects have concentrated on transgenic milk production both for medical and nutritional properties. As functional food, engineered milk links together the attributes of medicinal and nutritional, becoming a medicinal food. Bovine milk, in fact, is an important food for humans, with a consumption of milk and milk products *per capita* variable among world regions.

Cow's milk is a source of lipids, proteins, amino acids, vitamins and minerals and contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides (*134*). In western societies, however, a decreasing trend in milk consumption has been noted during the last decades, probably due to claimed negative health effects that have been attributed to milk and milk products because of its high saturated fatty acid content (*134*). Thus improved milk, particularly as far as qualitative attributes are concerned, is an attractive goal. Here we consider an improvement based on additional gene transfer, whilst some interesting perspectives of gene modulation and silencing are reported in the next section.

Reducing the susceptibility of livestock to pests and diseases *via* genetic engineering has long been an ambitious goal, which would also result in improved production and reproductive performance and could ultimately provide safer food products of superior quality (*135*). This concerns, for example, mastitis, a bacterial infection of the mammary gland, which is one of the most costly diseases in cattle breeding, and can also result in death. In dairy cattle, mastitis is mostly caused by *Staphylococcus aureus* infection, a pathogen particularly hard to control with antibiotic treatments. A transgenic strategy has proved to be promising for the prevention of mastitis in cattle by expressing exogenous lysostaphin, an endopeptidase with lytic properties on a *Staphylococcus* cell wall.

In fact, transgenic cows producing lysostaphin in their milk showed a high degree of protection when challenged with *S. aureus.* Moreover, increased antimicrobial properties in the milk of mice associated with the overexpression of the endopeptidase lysozyme or lactoferrin provided evidence for the potentials offered by this approach to prevention of mastitis in dairy cattle (*135*).

As for the enhancement of the functional properties of dairy milk *via* a transgenic approach, some promising results regard the production of milk with less lactose or cholesterol (*136*). New Zealand has been a leader in research concerning transgenic milk production and, since 2000, scientists at AgResearch (*137*) have been successfully producing transgenic cows that yield modified milk containing therapeutic proteins to treat human diseases. Various experimental field trials, in proper containments, have been approved by the Environmental Risk Management Authority of New Zealand (ERMA). Among the various results, qualitatively improved dairy milk composition with higher casein content (up to 20 % more) was achieved by transferring additional copies of the genes encoding bovine β- and κ-casein (*138*). This result was quite interesting, considering the relevance of casein in milk composition, representing 80 % of the overall proteins, having important nutritional and processing properties and therefore being one of the prime targets for milk composition improvement. Moreover, this was the first proof that transgenic technology could be used to modify milk composition in cows following previous research focused on transgenic cows to produce therapeutic recombinant proteins.

The production of cheap surrogates for human breast milk for the newborn has been a relevant goal in the food industry and as an alternative to infant formula, transgenic technology for producing cow's milk with increased similarity to the properties of human milk has been pursued by a few companies in different parts of the world. Besides the absence of the species-specific quali-quantitative composition of minerals, carbohydrates, proteins, fat, prebiotics and antibodies which make human milk unique to a baby's nutrition, hard digestibility and allergenicity are the most relevant drawbacks of cow's milk (*139*).

Human lactoferrin, the iron-binding protein also present in human breast milk and in particular in colostrum, has been regarded as a suitable candidate to enrich cow's milk on the basis of its recognized physiological properties, which are anti-inflammatory and have antipathogenic functions and roles in iron absorption and/or excretion in newborns as well as in the promotion of intestinal cell growth (*18,135,140*). In milk, recombinant human lactoferrin (rhLF) has been overexpressed in concentrations high enough to meet the needs of human children, with the goal of obtaining functional dairy milk with increased protection against infections and improved gastrointestinal health properties. The obtained recombinant protein was highly similar to the human natural counterpart for its most prominent biological activities, *i.e.* iron binding and release and antibacterial activity, thus paving the way for safe usage of rhLF in humans (*141*). Moreover, that work proved the potential of transgenic cattle as bioreactors in terms of capacity (*140*). The milk was obtained from the female offspring of one of the first notable transpharmer animals, Herman the Bull, which was engineered to carry a gene for lactoferrin by Gen Pharm International (Mountain View, CA, USA) (*142*). Herman fathered eight calves in 1994, following a breeding program established at Gen Pharm's European laboratory (Leiden, The Netherlands); in 2004 was euthanized for stopping osteoarthritis sufferance and, after taxidermy mounting, exposed as bio-object icon in the Naturalis Biodiversity Center, Leiden, the Netherlands (*143*).

In 2011, the media gave resonance to two experiments occurring in laboratories in different parts of the world (Argentina and China), both claiming the first achievement, aiming at producing transgenic cow's milk using human genes that allow the animal to produce the equivalent of a (human) mother's milk. On April 2, 2011, The Telegraph announced that the Chinese Academy of Engineering had led a research which generated cows producing human lactoferrin (*144*). What was new in this research, according to official statements, was that, after seven years of research, by using a property technology, the produced milk contained the highest level of lactoferrin in the world. The Chinese Ministry of Agriculture approved the testing on genetically modified dairy cows for human breast milk, and official media reported that this milk would become the first transgenic animal product in commercial use with prospects for use in infant formula milk powder, health foods and medicines.

A few days later, the Argentinean National Institute of Agricultural Technology (INTA; Buenos Aires, Argentina) announced that a cloned cow, named Rosita ISA was the first cattle born in the world with two human genes coding for proteins (lysozyme and lactoferrin) present in breast milk (*145*). The lysozyme is an enzyme present in (human) breast milk at high concentrations during the first week of lactation. Lactoferrin exists in all mammals but is specific to each species, and therefore bovine lactoferrin does not affect humans. 'As an adult, the cow will produce milk that is similar to humans', INTA's statement said. The goal was to increase the nutritional value of cow's milk with lactoferrin, which provides infants with antibacterial and antiviral protection and improves iron absorption, and lysozyme, which is also an antibacterial agent.

Transgenic milk production, and in particular the above-reported case of AgResearch in New Zealand, has been rightly considered a remarkable case of the impact of scientific innovation on society, and has been a case study in analyzing the contested representation of the research in its promotion, its governance, and the opposition sparked amongst environmental/antigenetic modification groups, including the Maori community (*146*). Quite interesting, and worth devoting significance to, was the opposition against this research, and the government environmental risk management authority (ERMA) approval of the experimental field trials, conducted by the New Zealand women's organization Mothers Against Genetic Engineering in Food and the Environment (MADGE), a 'network of politically non-aligned women who are actively resisting the use of genetically engineered material in our food and on our land', led by artist Alannah Currie (*147*). In particular, two campaigns, a masquerade in the parliament building and billboards displayed in Wellington and Auckland, New Zealand, based on a

strong, creative and provocative symbolic communication, were renowned for their astonishing approach and related media reactions. They were meant, really, to condense metaphors and visual and emotional excesses in order to provoke a public debate about the social and cultural ethics of genetic engineering in New Zealand. The topic, on the other hand, was quite inspiring, considering emotional and cultural implications concerning animal milk and mother's breast milk.

Gene Modulation and Silencing for Improved Food

Molecular techniques for food improvement have mostly been based on gene transfer into target genomes. Nonetheless, gene silencing of undesired traits would be a promising tool, and it is expected that besides (or maybe instead of) improvement based on traditional gene transfer, further research will be focused on adopting this strategy in plants and livestock.

Exogenous RNA-based gene regulation

Besides the various kinds of ribonucleic acids (RNA) present in organisms, such as messenger (mRNA), ribosomal (rRNA) and transfer (tRNA), many eukaryotes have an additional, highly abundant class of non-coding RNAs (20 to 30 nucleotides) generally referred to as small RNAs (sRNAs) (*148*). In the cell, sRNAs have various functions, such as regulation of gene expression, development and chromatin structure, and represent an important plant defense mechanism against viral infections and transposons. sRNAs are distinguished in two main classes: micro RNAs (miRNAs) and small interfering RNAs (siRNAs). The first derive from single-stranded endogenous RNA transcripts folding themselves up to produce imperfect double-stranded stem loop precursors, whilst siRNAs are processed from long, perfectly double-stranded RNA (dsRNA) precursors from endogenous (*trans*-acting siRNAs, natural antisense transcript-derived siRNAs, heterochromatic siRNAs) or exogenous sequences, for example from viruses, which are cleaved in the cell to short RNA fragments (*149*).

Numerous studies have demonstrated that siRNAs in cultured cells of plants and animals can trigger highly efficient gene silencing through degradation of the endogenous mRNA, whose sequence is complementary to the siRNAs. The most commonly applied methods for gene silencing based on siRNAs are virus-induced gene silencing (VIGS), RNA interference (RNAi), miRNA as in the case of artificial miRNA (amiRNA) and miRNA-induced gene silencing (MIGS) (*150*).

The process of introducing RNA molecules into cells to suppress the expression of a gene of interest, for obtaining RNA interference (RNAi), is nowadays a crucial tool in functional genomic studies, and various technologies have been developed. In plants, several constructs adapted to *Agrobacterium*-mediated gene transfer or virus vectors are available (*151*), whilst in animals transfection *via* liposomes, electroporation and viral gene transfer *via* recombinant viral vectors based on retrovirus, adeno-associated virus, adenovirus, and lentivirus are the most applied strategies (*152*). Further development in the knowledge of plant and livestock genomes is expected to enlarge the list of candidate genes to be modulated for useful application in enhanced food production.

RNAi in plant-derived food

In plants, RNAi can be used to improve plant nutritional value, as in the case of improved content of essential aminoacids. Lysine, for instance, is an essential amino acid necessary for human health, which has to be introduced through the diet since humans are not able to synthesize it. In the selected maize lines, Illinois High Protein (IHP), whose extremely high protein value is however affected by no lysine content against a too high level of zein, RNAi anti α-zeins have been obtained by *Agrobacterium*-mediated transfer of a construct carrying inverted repeated gene sequences encoding RNAs for silencing the 22- and 19-kDa α-zeins (*153*). Analysis of protein content showed a change in protein composition, in particular concerning the ratios of zein and non-zein fractions of total proteins. Moreover, whilst an increase of lysine resulted in seeds with enhanced nutritional value, an incomplete α-zein reduction maintained a desired property of the kernel, *i.e.* a vitreous, hard endosperm. This result proved that RNAi strategy is effective for obtaining kernels where three relevant traits (high-protein and high-lysine contents and vitreous, hard endosperm) are combined, *i.e.* a result that has never been achieved by conventional breeding.

In food improvement, RNAi can also be applied to silence the genes coding for antinutritional or harmful components. In plants, a very attractive application of gene silencing is the reduction of the allergenic properties of plant-derived food, and some interesting research has already been developed in this framework. In peanut, RNAi technology was used to eliminate the immunodominant Ara h 2 protein, the major allergen in peanut by transferring into peanut hypocotyls, *via Agrobacterium*, the inverted repeats of the coding region of Ara h 2 genomic DNA cloned in the a RNAi-inducing plant transformation vector pHANNIBAL. Crude extracts of several transgenic seeds of the T_0 plants showed the absence of this protein and their allergenicity was checked as IgE-binding capacity in the sera of patients allergic to peanuts. Showing, by ELISA test, a significant decrease in the IgE-binding capacity of transgenic seeds compared to the wild-type, the feasibility of alleviating peanut allergy by RNAi technology was proved (*154*).

Allergenicity cross-reaction between pollen and edible crops is known to afflict the population who suffer from allergies. This is the case of people who are allergic to birch pollen and also become allergic to apple fruit because of the immunological similarities between the major allergen in birch, Bet v 1, and the major allergen in apple, Mal d 1. To overcome this suffering, inhibition of Mal d 1 expression in apple plants by RNA interference has been successfully attempted (*155*). A T-DNA construct coding for an intron-spliced hairpin RNA carrying a *Mal d 1*-specific inverted repeat sequence, separated by a *Mal d 1*-specific intron sequence, was introduced into apple leaf explants *via Agrobacterium*. This construct efficiently silenced the target gene, as confirmed by quanti-

tative real-time PCR. Moreover, silencing was unaffected by grafting and remained stable for more than 3 years, throughout all the developmental stages (*156*). Among other interesting examples of successful application of RNAi for producing crops with reduced/absent allergenic properties, it is worth mentioning soya bean, where transgenic suppression was successful in down-regulation of the major allergene P34 accumulation in seeds, with no apparent negative consequences on the normal seed development (*157*), and tomato, where the genes for allergens Lyc e 1.01 and Lyc e 1.02 were successfully silenced (*158*).

It has been observed that some substances that are toxic for humans, are, however, very important for plants. This is the case of gossypol, a natural phenol derived from the cotton plant (genus *Gossypium*) with known anti-insect properties. Whilst it has been estimated that global cottonseed production would potentially provide the protein requirement for half a billion people per year, this plant is under-utilized as a food crop because of the presence of toxic gossypol in the whole cotton plant, including seeds. Accordingly, the elimination of gossypol from cottonseed has been a long-standing goal of breeders.

RNAi was successfully used to break down gossypol biosynthesis in cottonseed by targeting a gene coding for the key enzyme δ-cadinene synthase (*159*). Transgenic cotton was generated by transferring *via Agrobacterium* a hairpin RNA construct carrying a trigger sequence of the *Gossypium hirsutum* δ-cadinene synthase gene, under the control of a highly seed-specific cotton α-globulin B gene promoter. The level and stability of gossypol in cottonseed and other tissues were determined in T_1 and T_2 plants and seeds. In seeds, gossypol quantity was significantly reduced in a stable and heritable manner. Moreover, since the levels of gossypol and related terpenoids in the foliage and floral parts were not decreased, and thus their function in plant defense against insects and diseases remained unaffected, this strategy proved to be interesting for producing plants with both good agronomical characteristics and enhanced food properties.

RNAi in animal-derived food

Applications of gene down-regulation or silencing may also open up interesting perspectives in animal-derived food. Myostatin null mice, for instance, generated by gene targeting, showed a dramatic and widespread increase in skeletal muscle mass compared to wild-type counterparts, due to an increased number of muscle fibres without a corresponding increase in the amount of fat (*160*). Accordingly, reduction of myostatin expression could be an attractive application of gene silencing for enhancing muscle development in the livestock industry, in particular for beef animals, and would be a promising alternative to the use of GH genes which, as pointed out above, gave disappointing results in mammals.

Myostatin, in fact, also known as growth differentiation factor 8 (GDF8), proved to have an inhibitory effect on muscle differentiation and growth, as animals lacking myostatin or treated with substances that block the binding of myostatin to its receptor showed significant muscle hypertrophy (*161*). This protein has been the object of various studies and myostatin genes have been characterized and found to be highly conserved among vertebrate species. Two breeds of cattle, Belgian Blue and Piedmontese, which were characterized by increased muscle mass because of their increased ability to convert feed into lean muscle (double-muscling trait) proved to carry mutations (nonsense and frame shift mutations) in the myostatin coding sequence.

It should be pointed out, however, that the selection of double-muscled animals has resulted in a huge increase in the incidence of dystocia, *i.e.* difficult calf delivery, as in double-muscled Belgian Blue cows, Caesarean section is routinely required (over 90 %) for reducing calf losses (*162*). The increased muscle mass and feed efficiency, however, have been evoked as appealing features, balancing such a drawback (*160*).

Nonetheless, in mouse, another interesting selective gene targeting strategy to interfere with the myostatin pathway has been achieved, and it could open the door to further application in cattle production where different expression of traits between the sexes would be desirable. This strategy is based on the generation of transgenic animals for the Y-chromosome-linked muscle-specific expression of a dominant negative myostatin pro-domain, which resulted in the production of males showing a 5–20 % increase in skeletal muscle mass, whilst females were non-transgenic and thus not affected in their growth phenotype (*135*).

Molecular strategies would also be promising tools for preventing some fatal diseases, which also have deleterious effects in humans through ill animal consumption, like the neurodegenerative prion diseases or transmissible spongiform encephalopathies, including scrapie and the mad cow disease (BSE). In these syndromes, a misfolded isoform of the cellular prion protein (PrP) proved to accumulate and act as a novel infectious agent; thus, the introduction of mutated prion protein genes, gene knockout or RNAi-mediated knockdown of PrP expression would be expected to produce livestock animals resistant to prion diseases. Some research has been done in mouse, and initial studies in sheep, goats and cattle seem promising; *in vitro* assays with brain tissue homogenates derived from PrP-deficient cattle produced *via* PrP knockout demonstrated resistance to prion propagation and the cattle were apparently normal in all analysed aspects (*135*).

As for the improved nutritional value of milk, with its relevant implications for health of the many human adults who lack intestinal lactose-hydrolyzing enzyme activity, an interesting application of RNAi would be the reduced presence of lactose based on α-lactalbumin expression knock-down. Compared to transgenic knock-out, which has already been obtained in experimental mouse and resulted in the production of lactose-free milk, down-regulation would produce milk with the adequate lactose content to allow osmotic regulation and concentration of milk (*163*). Similarly, RNAi knock-down expression of the β-lactoglobulin gene would reduce the allergenicity of cow's milk (*135*).

Concluding Remarks

As described in this review, the opportunities offered by molecular tools both in plants and animals for production of food with enhanced quali-quantitative values

are quite numerous, and notable. Moreover, the amount of new information, in particular the data continually accumulated with the genome projects, predicts even greater potential. However, according to the state of the art, the significant amount of research efforts fulfilled in the last decades seems to have produced more promises than concrete achievements to offer to the market.

A series of issues can be mentioned as explanations for this drawback: first of all, the considerable amount of resources and time required in order to acquire reliable knowledge on gene involvement in biological pathways related to food properties and to develop sound techniques for applying the knowledge in the production of goods for human nutrition. Besides, there is a series of central issues, including risk evaluation process, bureaucratic approval, regulatory procedures and public acceptability, which complement, whilst often delaying, scientific achievements, as exemplified by the above-described cases of Golden Rice, AquAdvantage® Salmon and the surrogates for human breast milk for the newborn.

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