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A REVIEW OF GENETIC IMPROVEMENT OF MAIN FRUIT TREES THROUGH MODERN BIOTECHNOLOGICAL TOOLS AND CONSIDERATIONS OF THE CULTIVATION AND RESEARCH OF THE ENGINEERED PLANT RESTRICTIONS

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This review describes the regeneration and genetic transformation strategies for the main fruit trees aimed to produce cis- or trans-genic editing tools; the risks and benefits derived from the proper use of these technologies are likewise discussed. plants and aimed to describe the most important goals achieved up to now, included those got with genome
The plants designed and realized with genetic transformation technology for a sustainable and more profitable agriculture, are also projected to produce specific proteins for pharmaceutical field and suitable for the climate changing to, and they are fundamental to better understand the gene function. For this reason, this technology will still be useful or essential and, with appropriate corrections, despite the progress the new recent technologies will survive for a long time. This technique also allows us greatly accelerate the development of improved plants by access to the readily available and huge national local germplasm, already gathered and preserved, avoiding the loss of important gene pool. Gene transformation technologies are carried out over two decades and in the majority of the cases, are used to improve specifically the plants' weak traits, providing an answer to farmers' demands, while leaving untouched all others traits of the most value varieties.
At moment all these improved plants are subjected to the same restrictive laws, but many optimistic people hope in some deregulation for plants obtained by using the new tools of genome editing, beside trans-grafting and cis-genic techniques. Meanwhile techniques to produce improved non-transgenic plants from genetically engineered mother plants are explored as well as the techniques used to avoid the transmission of the transgenes to other compatible plants nearby.
The consequences of the total veto imposed by mostly EU governments to cultivate these plants, and in one case, as in Italy, even the field trials, making the farmers and minor breeder companies dependent on few big companies and unable to defend national local germplasm with an obvious negative impact on economy and on science progresses.
Keywords: Genetic transformation, fruit plants, genome editing, marker genes, plant regeneration promoters.

INTRODUCTION

Genetically modified organisms (GMOs), particularly genetically modified plants (GMPs), while being very lucrative topic and the center of much debate, are still not well understood by the public. Primarily both acronyms are inappropriately used to indicate the plants obtained by recombinant DNA technology, while more accurately they should be named as Genetically Engineered Plants (GEPs). The GEPs are the result of changes and selection process according to the needs of the day, made by human over the centuries with any breeding technique. However, the GEPs are obtained with changes of the genome through introduction of genes isolated from any living organism and introduced with biological (bacteria and viruses), physical or chemical vectors. At present these plants are generically called "trans-genics", which contain the expression of a foreign gene or the suppression of an endogenous protein to modify a function.
Genes/DNA can be altered in species but they still do not distinguish the origin of the genes used for the modification. This technology has given rise to strong discussions in communities that opposed the transfer of genes between genotypes that may not hybridize naturally. Following these complaints, the cis-genic and intragenic concepts were introduced about ten years ago to distinguish the plants engineered on the basis of the origin of introgressed genes or the changes made by the individual genes of the plant subjected to modifications. Therefore, these plants were named "cis-genic" when crop plants have been genetically modified with one or more genes (containing introns and flanking regions such as native promoter and terminator regions in a sense orientation) isolated from a crossable donor plant. Whereas, the technique "intragenic" confers to the

modifications made in individual genes with *in vitro* rearrangement of genetic elements, i.e. they can have genetic elements from different genes and loci, thus expression of genes can be modified by using different promoter or terminator regions (Schouten *et al*., 2006). To date, from the legislative point of view, both types of plants (Cis or Trans) are considered to be same. These two types still have a common phenomenon for insertion of the new sequence in a random position in the genome and / or the selection gene and any non-coding sequences of bacterial or viral origin vector (e.g. the T-DNA borders).

Although irrelevant, but some people believe that it is important to replace the "left and right border" with sequences of plant origin, that could be possible with the use of new carriers of the P-type DNA (Plant-derived transfer DNA) designed specifically for the cis-genic production plants (Rommens *et al*., 2007). Although recently the cis-genesis and intra-genesis technology had a further positive evolution, by eliminating the selectable markers in modified apple through heat-induced recombinase, and carrying the cis-gene FB_MR5 and its native regulatory sequences, conferring a resistance to fire blight (Kost *et al*., 2015). Although everybody is conscious about the needs of new plants suitable for the rapid increase of world population and climate changes, the acceptability is still very low by the public, particularly in Europe.

It is well known that the estimated average crop production of cultivated plants in the world is about 40% of the total potential crop production and the losses are endorsed by diseases (10-20%), drought stress (25%), weeds (5-10%), cold stress (16%), anoxia (16%), salinity stress (5%), heavy metals (3%) and poor soils (20%). The current demand for food is inferred as an approach to justify the profits of multinationals, beyond wastage and poor distribution that could not be fulfilled with only the cultivation of virgin fertile areas that are not sufficient. But with the contribution of those territories that are currently inhospitable due to unavailability of suitable plants and the traditional techniques of genetic improvement does not appear to be particularly effective for the purpose. Biotechnology aims to satisfy this need by providing plant varieties with higher production, quality, health and sustainability, with the ultimate goal of limiting pre and post-harvest losses in relatively short time.

In addition, these technologies, allow to change even the plants to make them suitable for "phyto assisted bioremediation" or "bio assisted phytoremediation", i.e. create plants with extensive and dense root systems to provide a huge surface area adsorption and an extended rhizosphere, which together will form the root exudates a microhabitat conducive to microbial community responsible to carry out the biodegradation of organic contaminants in soils. This modification can be done in plants already known for the ability to accumulate toxic metals to enhance their potential for accumulation.

The gene transfer has happened, not only for the governed characters by single genes but also by multiple genes and the GEPs may also be useful in pharmaceutical and nutraceutical to produce new products, enhancing the attitude to the synthesis and accumulation of metabolites, that will be extracts from the plant through in vitro cultured tissue, using then the plants as "bio-factories".

However, some methodological obstacles still persist for many plant species, like difficulties for gene transfer in the selection methods and inefficient protocols of *in vitro* regeneration of plants from cell or tissues, particularly from those of somatic origin. However, the progress is still undergoing (Shahzad *et al*., 2013; Mehmood *et al*., 2013, 2014, 2016; Nafees *et al*., 2015; Kareem *et al*., 2018).

Despite the difficulties of several kinds it has been a significant progress, especially in herbaceous plants, favored by short life cycle and their small size makes them suitable to be assayed *in vivo* in small spaces. This technology however is particularly suitable for those plant species characterized by a long juvenile phase, by a high level of heterozygosity or by a low genetic variability. As compared to any other known technology, it allows swift functioning and accuracy in alleviating the defects of the commercial cultivars/rootstocks without modifying or altering their main features. Contrary to believes, these techniques could save the old varieties from extinction, which still contribute to the characteristics of our products. Subject to minor procedures of "gene therapy", to correct the most serious defects, allows us to maintain a wide biodiversity including the cultivated varieties (Ercan *et al*., 2018).

Now days the aims of using the GEPs have widen compared to the past when it was only aimed at increasing productivity through improved defenses against pathogens and environmental adversity and reducing production costs by facilitating the agronomic practices. Subsequently the purpose has been moved to improve the quality of the final products and ultimate aim to create products with new properties such as vaccines, antibodies, vitamins, hormones, therapeutic enzymes of human or animal origin, cosmetics and bioplastics. It is direly needed to accelerate the production of varieties capable of producing foods with protein content similar to that of meat, with the aim of reducing the consumption of meat.

Although over forty years have been passed since the discovery of this technology and over twenty years of food from engineered plants is available in Europe. The European legislation does not allow this food to be used for human consumption, because of the suspicion part of the population that is heavily influenced by the information of the GEPs products and their authenticity. The issues of authenticity and expertise of the products is leading the companies to adjust their communication and marketing strategies. Public information on the subject has contributed strongly to hinder the acceptability of these foods, due to frequently incorrect,

confusing, biased and manipulative information. The EU has taken the most important decisions related to the GEPs on the United States by shifting the conflict from the political and legislative level to the administrative and judicial level that has prohibited field trials of GEPs in Italy, even for research purpose. The consequence of these politics the research in this area has drastically reduced in some European countries such as Italy, that was very active at the end of last century, even in research in the sector of in vitro plant regeneration from tissues which I fundamental in the success of gene modification with the recent biotechnological technique of genome editing.

The present review is an update of previous works done by Baldoni and Rugini (2002); Petri and Burgos (2005); Bhatti and Jha (2010); Vidal *et al.* (2010); Gambino and Gribaudo (2012); Limera *et al.* (2017). It describes the techniques and the steps necessary for field trials and commercialization of GEPs and recent interesting results achieved in fruit crops. These will be accompanied by a brief description of the strategies adopted to obtain transgenic genotypes, to improve the performance of the GEPs, obtained through the new technology of genome editing. The review describes the mechanisms and applications of these biotechnological tools for the improvement of fruit trees and enlightens their relationship with the European Union biosafety regulations for GMO plants and their products obtained through these techniques.

***Techniques and strategies to get transgenic plants*:** Transformation of plant cells comprises direct and indirect methods. For indirect method the agrobacteria (*Agrobacterium tumefaciens* or *A. rhizogenes*) or viruses are used as vectors. Whereas, the direct methods also called biolistic include cell bombardment with gold micro projectiles coated with DNA, or direct insertion of DNA into protoplasts for means of liposomes or micro syringes or by electroporation. The most efficient vector for the transfer of exogenous DNA in fruit plants up till now is represented by the Ti plasmid (Tumor-inducing plasmid) of the *A. tumefaciens*, carrying the gene marker for selection of gene of interest. The bacterium is engineered with one or more genes of interest and a marker gene needed for selection of cells that host the genes. Alternatively, the biolistic transformation represents a direct method that allows shooting a spherical gold or tungsten bullets of 0.4 to 1.2 pm, covered with constructs of gene of interest at speeds of 300-600 m/sec. These constructs can reach the nucleus as well as chloroplasts and/or mitochondria, in which DNA will be integrated more decisively. For genome editing technologies to escape from GMO regulations, the *Agrobacterium*-mediated transformation method is not reliable unless the first regenerated plants is submitted to self- or back-crossing with the wild type to eliminate the transgenic complex of modified plant. This practice however is not advisable to use for the woody plants because they are heterozygous resulting the offspring would be very different from the original plants submitted to modification.

***Markers and selection*:** The selected marker genes, depending on their types, are able to confer the cell resistance against toxic products (for example an antibiotic) or their ability to metabolize a particular specific product from which it derives energy for growth. In the presence of culture medium of these products only the cells that have introgressed marker gene together with the gene of interest can metabolize it and thus survive. However, under appropriate culture conditions, these cells may give rise to somatic embryos or shoots and then conversion to plants. Finally, molecular analysis and the selection of plants with the desired gene expression will complete the selection. However, it is also possible to abandon inserting the marker gene and in this case the selection of modified plants will be made *in vivo* through a careful phenotypic evaluation. In this case it is essential to produce a large number of plants with high employment of energy to grow *in vitro*, and it will be vital to have a very efficient method of regeneration from adult material, which is not commonly available in majority of the fruit species, unless zygotic material is used.

Public opinion does not accept the use of marker genes for resistance to antibiotics (i.e. *nptII gene*) or herbicides; however, the European Food Safety Authority (EFSA) does not consider this restriction. Although these genes are taken from microorganisms ubiquitous in nature so there is a fear that it can be transferred to human intestine bacteria, making them resistant to the antibiotic or other microbes used in the production of cheese or yoghurt. For this reason, since 31 December 2004, in Europe, it is not possible to use resistance markers antibiotics (Directive 2001/18/EC), contrasting to the USA or other countries. However, for the transformation of species with short reproductive cycle that are propagated through seed can be used for co-transformation (gene of interest on a plasmid and marker gene on another). In this way, following the intersection and segregation, the selection can be done only among offspring that do not contain the marker gene. In woody plants multi-auto-transformation vectors (MAT) could not produce satisfactory results, as in the case of citrus fruits (Ballester *et al.,* 2008) and apricot (Lopez-Noguera *et al.,* 2009). These vectors contain a morphological marker (*rol* or *ipt* genes) associated with a site-specific recombination system which allows the removal of undesired sequences, leaving only the useful genes integrated.

The new generations of gene constructs contain marker genes that allow the metabolism of compounds that are normally not metabolized by plants. A characteristic example is the transformation with *manA* gene, which codes for the Mannose-6-phosphate isomerase enzyme (MPIs); that enables the cells transformed with this gene to survive in the culture medium due to the presence of mannose as a carbon source. With the exception of the grapevine which is able to metabolize it, some fruit, such as apples, papaya, and almond

have been transformed by this method (Petri and Burgos, 2005. Bhatti and Jha, 2010; Breyer *et al.,* 2014). Finally, an 'inducible site-specific recombinase' has been developed, that implies the elimination of the marker gene by activation of recombinase enzymes, as a result of chemical or thermal shock treatments (Chong-Pérez *et al.,* 2012). Progress is also being made in developing reporter genes derived from the larger class of myeloblastosis (MYB) transcription factors that are involved in anthocyanin pigment activation in plants (Elomaa *et al*., 2003). In woody plants, this approach has been applied in grapevines by Kandel *et al.* (2016), where the MybA1 (MYB-related transcription factor VvMYBA1) reporter gene was suitable for identification of gene expression events at the cell culture level (Kandel *et al.,* 2016).

***Application of new promoters*:** So far, most of the transgenic plants have been produced with constitutive promoters, in particular with the *35S* from the cauliflower mosaic virus. These promoters are able to express the gene in all tissues in a continuous manner; therefore, it can cause little alterations in the growth and development or even cause the silencing of the transgene. Several plant promoters such as the site-specific promoters and inducible promoters are available. However, they express the transgene separately at particular times (as a result of various stimuli, such as wounds and abiotic stresses of various kinds) and in specific organs (leaf, root, vascular system, flower, etc.). The promoter of calmodulin (*uidA* apple) has been inserted in a hybrid rootstock of *Prunus* (Maghuly *et al.,* 2008) and the promoter of *cinnamoyl CoA reductase* inserted in grape (Gago *et al*., 2011) to increase the expression in vascular tissues. While, the promoter of *espansine* was inserted in tomato to improve the shelf life of berries (Karaaslan and Hrazdina, 2010). *Rubisco* is a very powerful promoter, similarly to *35S*, but of vegetable origin belonging to the plants, is being studied intensively. *DefH9* is also promising promoter, characterized by high specificity of expression in the placenta and in the ovule. This gene combined with the *iaaM* gene, which codes for the tryptophane-monossigenase enzyme that converts tryptophan to indol-acetammide, and produces indole acetic acid by making up the chimeric gene (*DefH9-iaaM*), which supports the development of the fruit without fertilization (partenocarpy) (Spena and Rotino, 2001). One of the most studied promoters induced by abiotic and biotic stress is the promoter for the *osmotin* gene, which produce the homonymous protein, known to be active in the defense against these types of stress (Raghothama *et al.,* 1993).

***Plant material and techniques used for plant regeneration*:** Essential prerequisite for successful application of these techniques in fruit trees is the regeneration from cells through both somatic embryogenesis or shoots organogenesis from somatic tissues of high agronomic and commercial value of cultivars with restriction to one or few specific defective characters to be altered (Silvestri *et al.,* 2016). However, sometimes, due to the difficulty of regeneration encountered in those cultivars it is necessary to resort the varieties of lesser value with the results of critical spells by GMO detractors. Normally immature or mature zygotic embryos are less desirable although they possess high morphogenetic capacity in some fruit trees, but they differ from each other as well as their mother plant due to their heterozygosis nature.

In attempt to reduce chimeric tissues in regenerated plants, when possible, organogenesis is preferred as compared to somatic embryogenesis. Organogenesis normally produces non-chimeric plants because single initial cell is involved in the regeneration process, particularly if selectable genes are not used. Direct regeneration is also preferred rather than mediated by callus in order to avoid the somaclonal variability that could make further changes to the final transgenic plants. It is advisable to use the technique of "Double Regeneration" for tissues that have very poor regenerative capacity. This novel technique is still not well known, although it was tested for the first time in olive (Rugini and Caricato, 1995; Rugini and Silvestri, 2016) and later in apple (Rugini and Muganu, 1998), pear (Abdollai *et al.,* 2006) and cherry that increased the regeneration frequency and consequently the transformation events. This technique involves the use of leaflets, from an adventitious bud, originated from any tissue *in vitro* of 1 to 2 mm long. These leaflets placed in regeneration medium possess higher ability to regenerate directly from their tissues and callus that can acquires higher organogenesis capacity (somatic embryos or shoots) for numerous subcultures (Abdollahi *et al.,* 2006).

***Procedures for field testing and marketing of transgenic plants in Europe*:** The field trials must be authorized by the authorities of the State (i.e. in EU each member country issue the permission, in Italy the Ministry of Environment is the responsible body), regulated by the Directive (2001/18 / EU) of the European Parliament '. It is compulsory for the European countries to fill in a series of documents for each transgenic genotype (Notification) in a very detailed manner and submit to the competent authority of the country where the trial is requested; a) General information related to people and their training; b) Information related to GEPs; c) Information concerning the terms of issue and the potential host environment; d) information on the interactions between GEPs and the environment; e) A direct monitoring plan to evaluate the effects on human and animal health and the environment; f) information on control plans, waste management and remediation in case of emergency. It must also contain the risk assessment for environment, agro-biodiversity, agricultural systems and the food industry. Finally, the notification must be transferred to the EU competent office with the main characteristics of the GEPs. The single EU member States, according to the European directorates (European Directive 2001/1), must chose he field trial sites and approve the experimental protocols for each species and plans of concurrence with traditional crops. If the

countries do not follow the rules, they cannot release the permission for field trials; Italy as a member state, has not complied with these directives yet. During the year 2012 they did not grant an extension to the University of Tuscia for regular trials to complete the experiments despite of 9 notifications and intimated the destruction of field trials of 360 ten-year-old fruit trees, including those modified with *A. rhizogenes* wild type (Meldolesi, 2012) which is not an engineered vector. By using these tactics, contrary to European directives, the field trials of GMO plants are forbidden in Italy since 2002.

The procedures for the cultivation of these plants are very long and costly that also includes the assessment of risk to human health and the environment. It generally requires more than 10 years, that is a period equal to that for evaluating a chemical product for the defense of the plants. It requires only 1-2 years to approve a cosmetic product while 7-8 years for a product for biological defense of plants. It is well known that production of one transgenic plant needs 50,000 euro on average while it takes more than 50,000,000 euro for their marketing. It depicts that only multinationals can afford this technology, thus creating a monopoly and inhibiting the public research for patent and commercialization of any transgenic plant. It should be noted that the plants produced from any other genetic improvement technique does not require any control prior to marketing. Although it is well known the risks can also arise with the conventional breeding methods when wild species are used to improve the cultivated ones. It is commonly known that the intersection with the rearranging of genes that occurs in the progeny can create new combinations of genes that are members for production of protein and other compounds, such as allergens, which could cause damages to the consumers. We cannot forget that in the recent past in the US some varieties of potatoes were withdrawn from the market by the trade authorities when a lot of deaths occur due to their toxicity. This will not happen in case of GEPs because the controls are very complex and accurate, and safe for both animals and humans before the products are marketed.

***Scientific and administrative bodies involved in the gep controls and their products*:** Due to a lack of information, very few people know the extent of work and the diligence that various national and international agencies undergo to control the products derived from GEPs before reaching to the consumers. Miscommunication is a major factor that lowers the trust and increase fear of public towards accepting the GEPs. The control starts from the isolation of the gene until the plant is evaluated in the greenhouse /field and processed to the market. In the US, the NIH (National Institute of Health) issues the guidelines, that USDA (U.S. Department of Agriculture) carries out the controls from the agronomic point of view. The EPA (U.S. Environmental Protection Agency) monitors the safety to the environment and ultimately the FDA (U.S. Food and Drug Administration) exerts controls on food safety. In EU the GEPs are under the control of the EEA (European Environmental Agency) and EFSA (European Food Safety Authority) that are responsible for the control of GEPs and products derived from GEPs in the European Union. They also decide that which type of genetically modified seeds can be imported and which can be grown in Europe. The authorization includes a chemical, biological and genetic series of analysis done in more than 40 laboratories in Europe. In Europe, the precautionary principle has become standard control for entering into the GEPs. This principle is a precautionary policy with regard to political and economic decisions on the management of scientifically controversial issues.

In United States the labeling of food containing GEPs, as compared to conventional products, there is no need for labeling, while in Europe the foods with higher content of 0.9% with ingredient GMOs is authorized and above 0.5 % for GMOs will be labeled with only the positive opinion of the EFSA (49/2000) in order to facilitate the free choice of the consumers.

From published studies concerning the safety of GEPs (about 3500) and the data obtained from a series of studies funded by the European Union during last 15 years and costing 70 million euro and involving 400 public research centers concluded that GEPs do not exhibit a different behavior from that of traditional crops. Approximately 1,783 experiments have been carried out (original research articles, reviews, relevant opinions and reports) and published during the period from 2002 to 2012. Many of them resulted from European projects in order to analyze the risks of GEPs for humans, animals and environment. There is not even single reference who witnessed a real risk associated with the use of genetically engineered plants (Nicolia *et al.,* 2014). The studies done on the harmful effects of GEPs by independent scientific academies and numerous other agencies concluded that commercialized GEPs and their products are safe for human consumption as well as for the environment. The recent meta-analysis of Klümper and Qaim (2014) has consolidated these claims and confirmed the significant high agronomic and economic benefits of GEP crops with equally significant reductions in the use of pesticides. Moreover, the various work carried out in gene expression between lines derived from crossing and transgenic lines, particular in wheat, have suggested that the presence of the transgenes do not significantly alter the gene expression. Therefore, the transgenic plants can be considered substantially equivalent to the non-transformed parental lines (Baudo *et al.,* 2006).

Table 1. Main results in genetic transformation in fruit trees by using gene transfer technology, including genome editing tools (*)

Objectives	Species or cultivar	Gene	Results	References
Insect protection	Apple (M9)	*AtCys*,	Resistant to *M. melolontha*	Basso *et al.*,2006
	Persimon	*CrylA(c)*.	Resistant to *Virachola*	Tao *et al.*,1997
	Pomegranate	*CrylA(b*)	*isocrates*	Verma et al., 2014
Fungal protection	Kiwifruit (Hayward)	*osmotin*	Tolerance to botrytis,	Rugini *et al.*, 2011
	Olive (Canino)	*osmotin*	Cadofora	Rugini *et al.*, 1999; 2000
	Citrus	*Chitinase (chit42)*	Tolerant to *Spilocea*	Gentile *et al.*, 2007
	Pomegranate	*stilbene syntax* and *PGIP*	*oleagina*)	Szankowski, 2003
	Pomegranate (Gala, Elstar).	*Vf* of *Malus floribunda*	botrytis	Paris *et al.*2009; Szankowski
	Strawberry	gene *rolC*	To be verified	*et al.*,2009
	Apple	*Rvi6*	*Venturia inequalis*	Landi *et al.*, 2009
	Grapes	*Chitinase* and *β-1,3-*	Tolerant to *Phytophthora* and	Krens *et al.*, 2015
	(*) Apple (*Malus Domestica*	*glucanase*	more productive	Nookaraju and Agrawal,
	Borkh)	*Rvi6*	Scab resistance	2012
	(*) Grapevine (*Vitis vinifera*	*VVTL-1*	Downy mildew resistance	Wurdig et al., 2015
	L.)	*MdMLO19*	Resistance to Apple scab	
	(*) Apple (*Malus domestica*)	*MLO-7*	(*Venturia inaequalis*) strain	
	(*) Grapevine (*Vitis vinifera*		104	Dhekney et al., 2011
	L.)		Resistance to Powdery	
			mildew	Pessina et al.,2016
			(*Erysiphe necator*)	
			Resistance to powdery	Malnoy et al., 2016
			mildew (*Podosphaera*	
			leucotricha)	
			Resistance to powdery	
			mildew (*Podosphaera*	
			leucotricha)	
Protection against virus	Apricot and Plum	(*CP*) of the virus PPV	virus sharka (PPV)	Machado *et al.*, 1994; Scorza *et al.*, 1994; Malinowski *et*
	Papaya	(*CP*) of the virus	Tolerant to virus (PRVQ	*al.*, 2006
	Citrus spp	(*CP*)	tristeza (CTV)	Kohli e Criostou, 2008
	Apricot (Japanese apricot)	*ParPDS*	Apple latent spherical virus	Rai, 2006
			(virus induced gene	Kawai *et al.*, 2014
	(*) Sweet cherry (*Prunus*	*PNRSV*	silencing)	
	avium)			Zhao and Song, 2014
			Non-transgenic scion grafted onto the transgenic rootstock showed resistance to PNRSV (Prunus necrotic ringspot virus)	
Protection against diseases	(*) Plum (*Prunus domestica* L.)	*PPV-CP*	Transgenic plum clone Honeysweet resistant to sharka disease	Scorza et al., 2013
	(*) Citrus sinensis Osbeck	*EBEPthA4* of the of the *CsLOB1* promoter	High rate of resistance to citrus canker	Peng et al., 2017
Protection against bacteria and Nematodes	Pear	*Lactoferrin* bovina	Tollerant to *Erwinia*	Malnoy *et al.*, 2003
	Apple	*Lc* (Leaf Color) of maiz	Tolerant to *Erwinia*	Flachowsky *et al.*, 2010
	Sweet Orange	Sovraesp. *Spermidina*	Cancer Tolerant	Fu *et al.*, 2011
	Pear (Conference)	*synthetase*	Erwinia	Rugini, not published
	Orange	gene Glucose oxidase (*Gox*)	Nematodes?	Rodriguez *et al.*, 2011
	Banana	Silencing of limonene synthase	Resistance to banana xanthomonas wilt	Namukwaya *et al.*, 2012
	Sweet orange	Ferredoxin like protein (*Pflp*)	Citrus canker	Kobayashi *et al.*, 2017
	Apple	gene	Fire blight	Broggini *et al.*, 2014
	Sweet Orange	Sarcotoin *IA*	Citrus variegated chlorosis	Caserta *et al.*, 2017
	Plantain	*FB_MR5*	Nematodes resistance	Roderick *et al.*, 2012

Objectives	Species or cultivar	Gene	Results	References
	Citrus	*rpfF*	Citrus greening	Dutt *et al.*, 2015
	(*) Apple (*Malus domestica*) protoplasts	Maize cystatin *NPR1* *DIPM-1, 2, and 4*	Resistance to fire blight disease	Malnoy et al., 2016
Protection against abiotic stress	Kiwifruit	antiporter gene *AtNHX1*	Tolerant to salinity	Tian *et al.*, 2011
	Citrus	Gene proline synthesis	Drought resistant	Molinari *et al.*, 2004
	Apple	*Osmyb4*di Rice	Water/Cold Stress	Pasquali *et al.*, 2008
	Olive (Canino)	*osmotine*	Drought resistant	Rugini *et al*,2000;
	Peach, Blackcurrant, Dwarf Apple	*ppdhn; dhns*; *MbDREB1*	Tolerant to cold, salinity and drought.	Lanham *et al.*, 2001; Yang et al, 2011).
	Olive (Canino)	*osmotine*	Cold and salinity	Rugini *et al*, 2000; D'Angeli and Altamura, 2007
	Rough lemon	Yeast *(HAL2)*	Salt tolerance	Ali *et al.*, 2012
	Apple	*MdbHLH104*	Tolerance to iron deficiency	Zhao *et al.*, 2016
	Wild grapevine	*VaCPK20*	Cold and drought tolerance	Dubrovina *et al.*, 2015
	Apple	*MdSIMYB1*	Multiple abiotic stresses	Wang *et al.*, 2013
	Banana	*CBF1*	Cold hardiness	Hu *et al.*, 2016
	Plum	*cytapx, cytsod*	Salt tolerance	Vivancos *et el.*, 2013
Multiple Characters and bioactive molecules	Apple, Banana and Papaya	antigen; various transcription factors	human disease vaccines; Biotic and abiotic stress	Kumar *et al.*, 2005; Hernandez *et al.*, 2007; Lau e Korban, 2010
	(*) Grapevine (*Vitis vinifera* L.)	L-idonate dehydrogenase gene (*IdnDH*)	100% mutation frequency in the transgenic cell mass (CM) as well as corresponding regenerated plants expressing sgRNA1/Cas9	Ren et al., 2016
Modifications of vegetative reproduction habitus, and reduction in juvenility,	Citrus culture *Citrange troyer*	Over expression *Of Phy a of rice*	Reduction in apical dominance.	Distefano *et al.*, 2013 Distefano *et al.*, 2013
	Papaya	*Phy b* of *Arabidopsis thaliana*	Alternate bearing	Rugini *et al.*, 1994
	Grapes		Reduction in size	Nakano *et al.*, 1994
	Rootstock culture	*riT-DNA*	Reduction in size	Rugini and Gutierrez-Pesce, 1999;
		riT-DNA	Reduction in size of rootstock and scion	Rugini *et al.*, com. pers.
	Kiwifruit (Hayward).	*riT-DNA*	Large fruits, tolerant to drought	Rugini *et al.*, 1991; Rugini and Mariotti, 1991; Rugini *et al.*, 1999
	Kiwifruit (Hayward and GTH), Citrus	*rol B*	Reduced size of flowers, tolerant to drought	Gentile *et al.*, 2004; La Malfa *et al.*2011
	Olive cv. Canino	*rol ABC*	Dwarfed scion of plants	Rugini *et al.*, 1999, 2000 Zhu 2001; Smolka *et al.*, 2010; Welander *et al.*, 1998; Holefors *et al.*, 1998
	Apple	*rol ABC* *rol B and rol ABC*	Dwarfed scion of plants	Zhu *et al.*, 2008
	Apple rootstock M9	*ga* of Arabidopsis thaliana that	Reduction in inter nodal length	Fagoaga *et al.*, 2007
	Citrus	reduces sensitivity to *GAn antisense gene CcGA20ox1*	Dwarfed phenotype	Rugini, 1984, 1986; Caboni *et al.*, 1996; Rugini e Gutierrez-Pesce, 1999
	Olive, Almond, Walnut, F12/I, MRS Culture	*The A. rhizogenes wt NCPPB 1855 And strain 232)*	Easy rhizogenesis, but it can modify vegetative habitus	Pena *et al.*, 2001
	(*)Citrus		Reduction of juvenility	Flachowski *et al.*,2007; Matsuda *et al.*, 2009
	Pear	*LEAFY, APETALA1, AP1* *BpMADS4 of* betulla and	Reduction of juvenility	Endo *et al.*, 2005
	Trifoliate Orange (*Poncirus trifoliata* L. Raf.)	*CiFT*	Blooming after one year	Tanaka *et al.*, 2014
	Apple Kiwifruit	gene *CiFT*	Perpetual flowering	Wu *et al.*, 2017
	Avocado	*FT*	Prevent premature bud break	Ziv *et al.*, 2014
	Apple	*SVP2*	Induce early flowering	Freimen *et al.*, 2015
		PaFT	Delays dormancy and leaf senescence	Artlip *et al.*, 2014
	Apple	*PCFT2*		
		CBF		

Objectives	Species or cultivar	Gene	Results	References
	(*) Apple (*Malus domestica*)		Enhance growth, delayed bud break and early entry to	
		MdGA20-ox	dormancy	Zhao et al., 2016
	(*) Apple (*Malus domestica*)		Transgenic apple lines with reduced height, shorter internode length, and higher	
		MdAG-like genes:	number of nodes	Klocko et al., 2016
		MdMADS15 and *MdMADS22*	Trees with polypetalous flowers. Reduced male and female fertility of flowers	
Quality of	Apple	*A6PR*	Change in sugar contents	Cheng *et al.*, 2005
fruits,	Grapes	Overexpression (*Adh*)	Change in sugar contents, pigments	Tesniere *et al.*, 2006.
auto-compatibility	Apple (Artic Apple)	Silencing of *PPO*	Reduction in browning	Xu, 2013
and parth-	Apple	Silencing of gene *SI*	self-fertility	Broothaerts *et al.*, 2004
enocarpy,	Kiwifruit	*defh9-IAAM*	seedlessness	Rugini and Spena unpublished
maturation,	Strawberry, Grapes	*DefH9-IAAM*	Quantity and quality of fruits	Mezzetti *et al.*, 2004;
flavor, pulp consistency	Strawberry	foliage anthocyanin *MYB10* (transcription factor)	high anthocyanin levels	Costantini *et al.*, 2009 Lin-Wang *et al.*, 2010
	Apple	*MdMYB10*	high anthocyanin levels	
	Kiwifruit	genes x carotenoids	ß-carotenes in fruits	Espley *et al.*, 2007
	Apple	biosynthesis	allergens reduction more fruit	Kim *et al.*, 2010
	Apple, Kiwifruit, Pear,	antisense *Mal d 1*	setting, reduction of the	Gilissen *et al.*, 2005
	Papaya, Strawberry	Antisense ethylene	aroma	Dandekar *et al*., 2004; Nieuwenhuizen *et al.*, 2012;
	Strawberry	Gene x regulation. cell wall	Increase percentage of pulp	Gao *et al.*, 2007; Lopez-
	Litchi	*SAMDC* gene	Increasing shelf life	Gomez *et al.*, 2009; Lee e
	Strawberry	*Menollin* gene	Increasing sweetness	Kim, 2011
	Litchi	*PISTILLATA* cDNA	Parthenocarpy	Lee and Kim, 2011
	Persimmon	*DkLAC1*	Increase proanthocynidin	Das *et al.*, 2016
	Loquat	*IAAM*	(Pas)	Min *et al.*, 2015
	Banana	2a (*MtPsy2a)*	Parthenocarpy	Padilla *et al.*, 2013
		ZmPsy,	Increasing concentration of	Mo *et al.*, 2015
		PaCrtI	pro-vitamin, A	Tao *et al.*, 2015 Paul *et al.*, 2016

***State of the art research on geps in the world on fruit species in view of the new tools of "genome editing*"**: Now days, the genetic transformation and the recent technology of genome editing, associated with traditional breeding, are technologies to be preferred as compared to other techniques, as the most advanced technology in agriculture. Comparing the genetic transformation techniques with those of classical hybridization is like comparing a latest generation smart-phone with a telegraph wire. This biotechnological technique is the standard and logical evolution of classical hybridization techniques in biology as well as in pharmacological and agriculture sector in particular. In fact, it allows a more targeted genetic improvement released from sexual compatibility by increasing the genetic variability (biodiversity) and achieving the objectives in relatively short time. These objectives are hardly achievable with classic cross breeding techniques, which transfer whole chromosomes or large segments of DNA. If GMOs were not so criticized and hindered, they could provide unbelievable progress in a very short time, both in agriculture and in the pharmaceutical industries by creating countless high-income jobs and attracting investment from multinationals. Recently some anti-Ebola drugs have been developed from *Nicotiana benthamiana* plants produced in the USA. However, the first steps in the development of these plants as bioreactors for drugs and for the production of monoclonal antibodies was started in Italy, where, unfortunately, the field trials are forbidden along with many other obstacles are hindering the research activities.

Combination of this technique with the traditional cross breeding in fruit trees is a way to establish mother plants with very short juvenility. It also enables to the transmission of the transgene, which shortens the juvenility period of the offspring up to 50% during crossing with any other compatible variety. It is possible to select the desired genotypes of transgene-free offspringthat are not subjected to the existing GMO regulations. In this way one can select non-GM woody plants also in species with long juvenile period in

a very short time (Cressey, 2013). Some experiments have been successfully carried out under field trials (Tab. 1).

The research is aimed at modifying defects, enhancing or reducing the expression of existing genes of commercially important cultivars by challenging the difficulties that often compete with the *in vitro* regeneration. However, the sequencing of plant genomes is also in progress in different fruit trees (Dondini and Tartarini, 2014). This technique allows identifying the genes in the same plant to be edited and modify them according to our requirements without importing them from other species. It allows using more and more techniques of Gene 'stacking' or 'pyramiding' and antisense RNA technique. The technique of Gene 'stacking' or 'pyramiding', is already being used in traditional breeding, i.e. the combination of desired traits in a single line. This strategy is rapidly gaining popularity in biotech crops through the co-expression of multiple genes that are necessary for some biochemical pathways (multimeric enzymes, proteins that control different 'choke points', improving the tolerance to abiotic stress, such as: high and low temperatures, water scarcity, limestone and acidity of the soil, deficits and excesses of light, pollutants, etc.). Transgenic strawberries were produced with this method at the University of Tuscia with three genes (Osmotin, PR1, chitinase) that were destroyed by "No Global" authorities in 2002 during field trials. Until that time, they had not shown any symptoms of fungal diseases in the greenhouses nor in the field as compared to controls. Osmotin is one of these genes has proved to be a homologous human hormone adiponectin, involved in glucose metabolism. It is promising to be the basic of new therapies for the treatment of different diseases including diabetes, cancer and certain diseases of the central nervous system (Naseer *et al.,* 2014).

There is a trend to use genes belonging to the species inserted in sense and antisense to better understand their function. The "antisense RNA" technique is normally used in plants to block the expression of endogenous genes, using fragments of cloned genes in reverse orientation near a promoter that reduces in a more or less obvious production of endogenous protein. To block the activity of some genes for slowing down the ripening of the fruits is considered as harmful (eg. silencing the genes responsible for Polygalacturonase (PG) and ethylene biosynthesis). The RNAi (RNA interference) is the most advanced technique based on a process of post-transcriptional gene inactivation, particularly present in plants and animals that is triggered by double stranded RNA (DsRNA) homologous to the sequence the suppression of harmful genes (Voinnet, 2008; Parent and Vaucheret, 2012). All these techniques are originated from the results obtained using first and most rudimentary techniques of genetic transfection that cause the insertion of the random transgene: duplications, doubles, triples insertions, antisense insertions and especially the unwanted antisense gene silencing caused by the interference of exogenous and endogenous RNA.

The restrictive rules and social barriers associated with transgenic plants can be overcome in the near future through the new tools called "genome editing", ZSFNs, TALENs and CRISPER/Cas9 (Bogdanove *et al.,* 2010) and Oligonucleotide-Directed Mutagenesis (ODM). These innovative methods allow insertion of a new gene at precise position of DNA chains, to avoid any contiguous genetic disorder. These techniques can also provide the ability to change base of plant DNA, improving the same genes without tracing, as they use the same segment of DNA for insertion site that needs to be modified or even transferred as RNA sequences without insertion into the genome to generate a simple knock-out (gene-specific silencing). Biomolecular techniques are already available that allow us to see immediately before the plant becomes adult if genetic modification has been satisfactory (Kanchiswamy *et al.,* 2015; Lu-Xiao*et al.,* 2015). Application and description of these new technologies of Gene editing in fruit plants are reported in a review by Limera *et al.* (2017). Recently, the ZFNs protocol was developed in apple and fig trees by Peer et al. (2015) where whole plants were regenerated by repairing *uidA* gene; as an alternative to ZFNs for genome editing. Whereas, transcription activator-like effector nucleases (TALENs) have rapidly emerged that can alter transcription of genes in host plant cells, but in our knowledge, it seems not to be applied yet in fruit plants.

CRISPR/Cas9 system has been used in several woody fruit species in order to induce precise gene mutations. The most recent application of CRISPR/Cas9 system in inducing disease resistance in woody fruit species has been done in citrus to increase resistance against citrus canker by Peng *et al*. (2017). The Oligonucleotide-directed mutagenesis **(**ODM**)** is a gene-editing technique which aims to introduce a new mutation in a plant genome by replacing one or few base pairs (Lusser *et al.,* 2011). This site-specific mutation occurs by the introduction of chemically synthesized DNA oligonucleotides or chimeric DNA-RNA of 20–100 nucleotide fragments, delivered into the plant cells by biolistic methods or electroporation of protoplasts (Breyer *et al.,* 2009; Sauer *et al.,* 2016), while it is not advisable to use *Agrobacterium*-mediated transformation method for the reason mentioned above.

The strategies of genetic improvement are described briefly as below and the most promising genes that could be used for fruit plants, as well as the most interesting results are prescribed in table 1. Particular emphasis is given to biotic and abiotic stresses that represent the main causes of production losses, despite the massive use of agrochemicals.

Transformation for protecting plant from biotic stresses**:** ***insects, fungi, viruses, bacteria and nematodes*****:** Insects and fungi are more destructive than viruses and bacteria. In some cases, it was possible to introduce resistance genes from wild species into commercially cultivated species by the traditional breeding that is time consuming and pose well-known

problems related to woody plant breeding. Resistance genes through conventional breeding have not been found yet effective and durable for other species.

***Plant protection from insects*:** Now days insect resistance is widely perceived by the private laboratories to produce transgenic crops in the market, after the herbicide resistance, the peculiarities of a toxin (*Bt* protein) produced by the *Bacillus thuringiensis* bacterium is used as a biological and natural organic insecticide. When sprayed on the foliage of plants to defend the insect pests that swallow along with the plant tissue and their death occur as a result of rupture of the intestinal wall due to the toxin of bacterium. The genes (Cry genes) responsible for the production of toxin that are integrated into the genome of the plants induce their tissues to produce the same deadly toxins for insects. To limit the occurrence of resistance to *Bt* toxins by insects, which can occur both with the intensive use of the distributed bacterium on the plant for biological control purposes. For spreading transgenic plants, it is recommended to cultivate 20% non-transgenic plants alongside transgenic ones. In order to avoid the occurrence of resistance after cultivation of 20% of non-transgenic plants, it is necessary to produce plants with high levels of *Bt* toxin in tissues using multiple methods or pyramiding genes (Gassmann *et al.,* 2011).

Currently this strategy works well to defend plants from lepidopteran pests (European corn borer) and some beetles (corn rootworm), while some difficulties are encountered by other pests. Transformation experiences with this gene are also reported in the literature on apple and persimmon (Table 1). Although the *Bt* toxin is degraded in the acidic range, particularly in the stomach of mammals, however, to assure the public opinion it is desirable to produce the toxin only in tissues of plant organs that are not used for human consumption, with the use of site-specific or inducible promoters (e.g. wound promoters). New strategies are now being carried out in herbaceous plants to make transgenic plants able to imitate the chemical warning 'alert pheromone' produced by insects like aphids when they are under attack by their predators. In this way the insects are warned by the danger coming from the transgenic plants and they do not approach genetically modified plants (Cressey, 2013).

***Plant protection from fungi*:** With the development of new strains of virulent pathogens, resistant cultivars tend to become sensitive over time and pathogens spread rapidly, although control is carried out with various techniques including quarantine, hygiene, breeding and clonal selection of varieties and application of fungicides. The uncontrolled use of pesticides not only leads to an increase of production costs and environmental degradation, but also induces new forms of resistance in pathogens, forcing the manufacturing of other new pesticides. These problems have encouraged to find biotechnological solutions to cope with fungal diseases. Particularly the identification of genes involved in the resistance, both coding for enzymes involved in the biosynthesis of toxic fungal compounds or coding for toxic proteins which directly inhibit fungal growth (Cornelissen and Melchers, 1993; Terras *et al.,* 1998), with the aim to introduce them in susceptible plants or to replace the promoters of antifungal genes with other more efficient genes. Several proteins have been reported with antifungal activity, and classified in eleven classes, known as pathogenesis-related proteins (PR). Some of them have also shown antiviral and antibacterial activity. The defense-related genes have been extensively described by Baldoni and Rugini (2002), some of them are described here.

The *osmotin* gene (*Osm*) encoding a PR protein is expressed in all the plants under biotic and abiotic stress conditions (Liu *et al.,* 1994; Zhu *et al.,* 1996; Yun *et al.,* 1998). The proteins of this family are now being studied in plants to induce tolerance to cold and drought stress.

The *osmotin* gene of tobacco was introduced in kiwifruit CV Hayward and in olive CV Canino under the control of the constitutive 35S promoter. In the first case, by artificial post-harvest infection, the fruits showed a strong tolerance to *Botrytis cinerea* (gray mold) and *Cadophora luteum-olivacea* (cadophora) (Fig 1) (Rugini *et al.,* 2011; Rugini, 2012); but the study on health properties could not be possible due to the intimate destruction of plants by the Ministry of the Environment. In the second case of olive, one of the three clones came from different event of transformation were examined both in pots and in the field conditions for ten years that showed a better tolerance to the peacock eye (*Spilocea oleagina*) and the less susceptibility was correlated with an higher content of intracellular protein in the leaves (Rugini *et al.,* 2000) (Tab. 1).

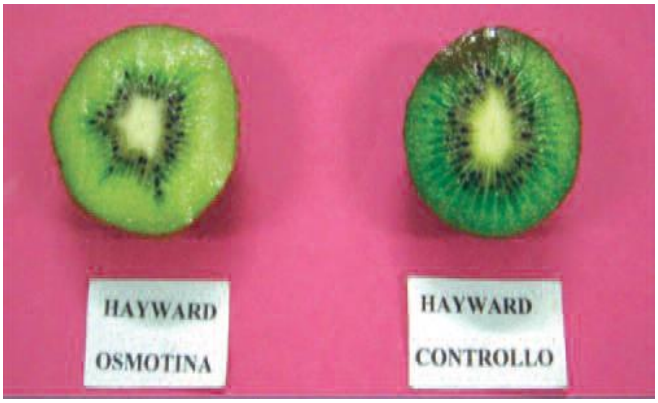

Figure 1. Kiwi, CV Hayward, transformed with osmotin gene: the fruits resulted more tolerance to botrytis and cadophora following artificial inoculation (Rugini *et al.*, 2011).

The use of endochitinase, polygalacturonase (PGIP) inhibiting proteins and stilbene synthase for the defense is similar to the transformation of lemon with *chit42*, chitinase of the fungus *Trichoderma harsianum* showing a protective

effect against *Botrytis*. Transformation of apple with the stilbene synthase grape genes and kiwifruit with PGIP gene are described in Table 1.

To overcome the problems encountered by classical breeding to transfer *Vf* gene resistant to scab (*Venturia inequalis*) from *Malus floribunda* to the cultivated apple plants having very small fruits. With the aim to obtain offspring with marketable fruits size, the *Vf* gene was isolated from *Malus floribunda* (Belfanti *et al.,* 2004) and subsequently transferred via *Agrobacterium,* to cvs Gala and Elstar. It resulted more resistant to the fungal disease maintaining the native morpho-physiological characteristics of original cultivar. Transgenic strawberry with *rolC* gene resulted in more tolerance to *Phytophthora cactorum*, more productive and with better fruit quality and same results were found with the transfer of pyramidated *rol* genes (*rolABC*) (Tab. 1) (Fig. 2).

Figure 2. Strawberries transformed with rolC (top right) (Costantini *et al.*, 2009). The other photos show a field trial of transgenic strawberries with *rolABC* and *osmotin* genes at the University of Tuscia (Rugini, unpubl. data). All plants were more tolerant to pathogenic fungi.

***Plant protection from Virus*:** From the observation about the properties of the known "cross-protection", (scilicet is a virus that produces only mild effects and make plants capable to protect against most damaging viruses), there was a perception that presence of capsid protein (CP-Coat protein) would be sufficient for the virus even if produced by the plant itself. It has been observed that transgenic plants are capable of producing the CP manifest a resistance to virus infection, as demonstrated in various horticultural crops, including varieties in danger of extinction, such as San Marzano tomatoes as well as fruit trees such as apricot, plum, citrus fruits (Table 1). This technology has helped to safeguard the cultivation and industrial sectors of papaya in Hawaii, making the transgenic Papaya resistant to "Papaya Ringspot Virus" the first fruit plant authorized to cultivation for fruit marketing. Over 10 years of cultivation it has demonstrated the integrity and stability of the gene also in the offspring (Kohli and Criostou, 2008) and these fruits also did not cause any problem in animals feed. Some effectiveness in protecting the plants from pathogenic viruses has also confirmed the use of recombinant scFv antibodies (Cervera *et al.,* 2010).

***Plant protection from bacteria and nematodes*:** Fire blight in *Rosaceae* (apple, pear, quince and other ornamental species), caused by *Erwinia amylovora* and bacterial canker (*Pseudomonas syringae*) in stone fruit, black spots (*Xanthomonas campestris* pv *juglandis*) of walnut and citrus canker (from *Xanthomonas citri*) are among the diseases that recently caused problems for fruit production in the world. The research has been focused on the genes that produce anti-microbial proteins, as lytic peptides (cecropin, manganin, attacin, harpin and synthetic analogs) (Petri and Burgos, 2005; Bhatti and Jha, 2010; Mendes *et al.,* 2009). The results of other strategies are shown in Tab. 1 that have been effective to control the bacteria in fruit trees such as: the expression of *bovine lactoferrin* in a plant that reduces the availability of iron, the overexpression of colored pigments with *Lc* (Leaf Color) gene and spermidine synthase. The later approach seems interesting to defend the plants from both abiotic and biotic stresses; and the transformation with the gene glucose oxidase (*Gox*) of *Aspergillus niger*, which induces the production of H_2O_2 that triggers cell death. It also seems effective to increase the synthesis of some terpenes in controlling nematodes, considering the high quantities of these compounds found in the roots of resistant herbaceous plants.

Transformation to protect plants from abiotic stresses

***Tolerance to drought / salinity and high pH*:** About one-fourth of the cultivated land in the world is susceptible to the damage from salts, particularly sodium chloride (NaCl), but also sulfates of Ca, Na and Mg, in addition to very common potassium chloride (KCl) and sodium carbonate (Na_2CO_3). The salt stress causes various types of damages, including the reduction of growth, inhibition of photosynthesis, reduced absorption of nutrients, membrane disorders and the production of toxic metabolites. The pathway followed to make the plants tolerant or resistant to abiotic stress is linked with increasing the capacity of the genes involved in detoxification of free radicals (reactive oxygen species -ROS-). Osmo-protectants produced by plants in response to osmotic stress such as drought and salinity (heat-shock proteins -HSPs-) are accumulated by the plants as a result of heat stress. Furthermore, many genes are involved to control salt stress, but the transport of ions and organic osmolytes appear to be particularly promising targets, particularly when combined in a pyramidal approach (Jain and Selvaraj, 1997). Kiwifruits have been produced with the antiporter gene AtNHX1 to improve salt resistance (Tian *et al.,* 2011).

Trehalose, a disaccharide that allows the survival of some micro-organisms in the absence of water and high temperatures, serves as an osmo-protectant against environmental stresses, in particular to salinity, but also to the cold or high temperatures and water stress. This strategy produced good results in transgenic potato (Kwon *et al.,* 2004) and it could also be applied in fruit trees. Proline is one of the most promising osmo-regulators, which is accumulated in the tissues as a result of osmotic stress, giving a more tolerance to water stress. By using this strategy, transgenic plants of Carrizo, a gene encoding the enzyme is expressed that limit the speed of proline pathway and accumulated a large quantity of proline in the leaves to withstand long periods of drought (Molinari *et al.,* 2004). Similarly, in the transgenic plants of apple with rice Osmyb4 gene improved the adaptive response to water and cold stress (Pasquali *et al.,* 2008). Transgenic plants of olive cv. Canino in field and greenhouse over-expressing osmotin gene from tobacco (D'Angeli *et al.,* 2001), grew in the field only in absence of water supply, while those plants with irrigation were dead (Rugini *et al.,* 2000). These results were confirmed by experiments conducted in the pots with two-years-old olive transgenic plants (Fig 3) with minimum water supply. The transgenic plants resulted growing very healthy after 4 weeks, while the control plants of un-transformed Canino and Canino grafted on *rolABC* rootstock died. In vitro experiment of these plants showed a normal growth rate in the presence of 2 and 4% PEG (polyethylene glycol) along with increased accumulation of proline in the tissues. While controls at same osmotic concentrations showed evident symptoms of leaf damage with reduced growth. In addition, the activity of enzymes related to water stress was also increased in transgenic shoots as compared to control ones. Moreover, the transgenic shoots also showed higher proline accumulation supporting the hypothesis that the osmotin gene conferred increased tolerance to drought stress in transgenic shoots as compared with the wild type (Silvestri *et al.,* 2017). Another forward step was made with the discovery of the NCED-3 gene (Ruggiero *et al.,* 2004) responsible for the production of a key enzyme in the biosynthesis of abscisic acid (ABA), a hormone liable to the response of plants under environmental stresses. This gene act as a promoter to inhibit cyclin kinase 2alfa (ICK1) enzymes responsible for cell growth and actives only under stress and inhibits the growth of the plant exposed to salt stress just before the accumulation of toxic ions in the cells. This strategy could be promising in fruit tree plants, since the experiments have already been carried out in rice by changing in the levels of the NCED-3 expression and downstream elements of this new pathway (Yang *et al.* 2011a).

Figure 3. Olive plants (cv Canino) transformed with the *osmotin* gene (right). The transgenic plants resulted more resistant to water stress and cold than the control (left).

Tolerance to cold stress: The different tissues of plants of temperate climates respond differently when exposed to freezing temperatures (Wisniewski and Arora, 1993). The defense strategies and the biotechnology approaches to select plants tolerant to cold stress have been described in detail by Baldoni and Rugini (2002). In short, the acclimatization changes involve the carbohydrates metabolism, the composition of plasma membranes and the accumulation of some classes of proteins with cryo-protective function (Chen *et al.,* 1995). The plants adopt two main strategies to defend against cold stress: tolerance or avoidance. In the first case the plants form extracellular ice to lower the freezing point of the cells. While on the other hand in case of avoidance, the internal fluids in cells get super-cooled to prevent the occurrence of cold stress (Wisniewski and Arora, 2000; Pirzadah *et al.,* 2014). Normally in fruit trees the super-cooling is about -2 to -4 ° C (Ashworth and Kieft, 1995). The genes responsible for the phenotype of ice, "ina+", were cloned from different species (Warren, 1995) and the specific proteins (antifreeze), such as deidrine (a subset of LEA proteins), were directly involved in acclimatization to cold. Other proteins such as those of cortex reserves (BSP), some enzymatic systems (plasma membrane ATPase; glutathione reductase) and phytochromes are also associated with adaptation to cold stress. The antifreeze proteins isolated from different plants have a different structure and probably different mode of action; some of them are similar to the PR (Hon *et al.,* 1995). Dehydrin-like genes (*dhns*) were isolated from cold-acclimated leaves of blackcurrant plants using RT-PCR with a guessmer primer based on known dehydrin DNA sequences (Lanham *et al.,* 2001). Several types of proteins over-expressed in response to cold stress were also over-expressed in response to other types of stress, like biotic stresses. O*smotin* is one of these genes that have been identified in response to salt stress and subsequently to cold stress and others (Zhu *et al.,* 1996). In field trials, the

transgenic *osmotin* olive trees, cv Canino, showed that protein is positively involved in the induction of the cell death program, that is produced during acclimatization period due to cold and overexpression of *osmotin* enhances its role as a cryoprotectant (D'Anageli and Altamura, 2007) Table 1). Proteins 1, 3, like endoglucanases, chitinase and thaumatin, have anti-freeze and *in vivo* antimicrobial properties as well (Pearce, 1999). Corresponding genes have been isolated and subsequently inserted into different woody plants including fruit. (Tab. 1)

***Transformation to produce bioactive molecules and transcription factors for multiple character*: *control*:** Studies are underway to verify the possibility of transforming cells in order to induce them to differentiate. The *VvWOX* genes in grapevine appeared to have a role in somatic embryogenesis regulation (Gambino *et al.,* 2011). The fruit plants can also be used to produce bioactive molecules; the kiwifruit and the citrange have been transformed with the human HEGF (human epidermal growth factor). Transgenic apple trees are able to produce the antigen against the viral infection of respiratory tract (Lau and Korban, 2010); Finally, banana and papaya were transformed for the production of vaccines against hepatitis B, (Kumar *et al.,* 2005) and *Taenia solium* (Hernandez *et al.,* 2007) respectively.

Some classes of transcription factors seem to be promising for the defense of plants against biotic and abiotic stresses. Two genes coding for *VpWRKY* 1 and 2, isolated from Chinese wild grape expressed in *Arabidopsis* have improved resistance to fungi and osmotic stress (Li *et al.,* 2010); the *VvWRKY11* gene has helped to increase the tolerance to osmotic stress (Liu *et al.,* 2011), while the *VvWRKY2* gene influenced the lignin pathway in tobacco (Guillaumie *et al.,* 2010). The gene MbDREB1 of dwarf apple (*Malus baccata*) increased tolerance to low temperatures, salt and drought stress (Yang *et al.,* 2011b) (Table 1). These studies open the way to produce *cis-genic* fruit trees, possibly destined to colonize inhospitable areas of the Earth and to produce fruits that can control certain human diseases in underdeveloped countries.

***Transformation to change the architecture of the canopy and reducing the period of juvenility*:** The growth and development of plants can be modified by varying the expression of phytochromes (*Phy*) that regulate the perception of light, thus affecting the apical dominance (Vince-Prue and Canham, 1983). The modification of light perception can change the distribution of the products of photosynthesis in vegetative organs and thus the shape of the crown for the advantage of high-density plantation systems. The modification of the phenotype can also be achieved with the overexpression of genes that alter hormonal perception, that can cause poor accumulation of carbohydrates in the branches due to a weak growth (Zerche and Druege, 2009) or a hormonal imbalance in the tissues (auxin/cytokinin ratio).

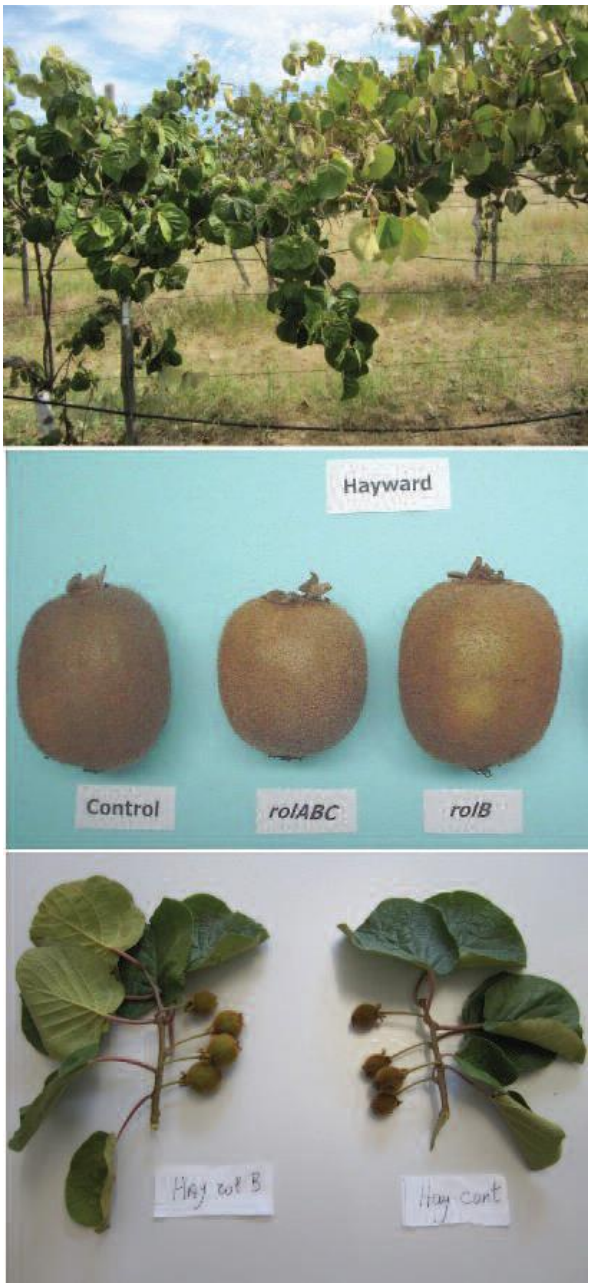

Figure 4. Kiwi plant CV Hayward transgenic for *rolB* retains the same morphology of the control plant, but it is more resistant to drought and produces larger fruits than control. Note the shriveled fruits of the control in comparison to those of the *rolB* line which are turgid and larger under not irrigated condition. The *rolABC* Hayward plant instead shows a smaller size, high resistance to water stress but produces smaller fruits with round shape, similarly to derived F1 (Hyward x GTH *rolABC*) progeny than the respective controls (Rugini, 2012).

Figure 5. Field trials at University of Tuscia in the first decade of this century: cherry and plum (right) rootstocks, induced to root with *A. rhizogenes* WT infection. These plants resulted smaller and with crinkled leaves than the respective ones which were induced to root with auxin treatment. It should be stressed that the roots, differentiated following bacterium infection, not always result transformed (in this case the morphology and phenology of the plant do not change).

Figure 6. *In vitro* (left) and *in vivo* (right) regeneration of Colt rootstock. In both cases the roots originated *in vitro* from the explants infected with *Agrobacterium rhizogenes* WT. *In vivo* regeneration may be used when it is difficult to regenerate *in vitro*, but in some cases the riT-DNA suckers, paradoxically may be recalcitrant *in vivo* to produce roots.

The single (*rolB*, *rolC*) or pyramidal (*rolABC*) genes or all the *riT-DNA* of *A. rhizogenes* (De Paolis *et al.*, 2019) are known to determine the reduction of plant size when transferred in it (Tab. 1) (Fig 4), whereas reduce the plant size of the grafted cultivars used as rootstocks, although the vegetative / reproductive habits of scion could also be changed (Rugini *et al.*, 2015).

Figure 7. Field trials of "trans grafting" plants at experimental farm of the University of Tuscia. Top with a view of the cherry grove with CV Lapins: the small plants are grafted on ri-TDNA rootstock Colt derived from different infection events, in comparison with those grafted on Colt WT. The plants were sprayed with a chemical before extirpation, ordered by the Italian Ministry of Environment. Below: a view of incineration of cherry trees and several other transgenic fruit tree species (360 plants in total) on 29 October 2012.

This bacterium can simply be used to induce rooting in recalcitrant genotypes, (Figure 5, 6, 7); thus the neoformed roots may or may not contain the transgene. If the roots contain the riT-DNA, the scion can also be modified. According to present legislation concerning GMO plant modified with this bacterium, WT should not be considered as transgenics because no manipulation has been done in plasmid.

The unproductive period can be shortened or nearly eliminated by over-expression of flowering genes in plants. (Pena *et al.*, 2001; Endo *et al.*, 2005) Tab. 1 and Fig. 8.

Figure 8. Citrus spp overexpressing LEAFY (LFY) genes and APETALA1 (AP1) (Pena *et al.*, 2001) and the gene *CiFT* are able to differentiate flowers after one-year hardening in greenhouse (Endo *et al.*, 2005).

***Transformation for quality, ripeness, flavor and fruit size, self-compatibility and parthenocarpy*:** The targets for improving the quality of the fruits are generally carbohydrates, secondary metabolites, shelf life, softening of the pulp, ripening and suppression of allergens. Currently strawberry is selected as a model for its short development cycle, to study the ways to improve the quality of the fruits of the *Rosaceae* spp. The strategies followed by herbaceous trees can be applied to those trees. Sweetest fruits but with few calories, produced by expressing the super-sweet thaumatin, protein has already been tried successfully in potatoes and cucumbers (Witty and Harvey, 1990; Szwacka *et al.,* 1996). Similar proteins have also been found in cherry (Fils-Lycaon *et al.*, 1996) and grapefruit (Tattersall *et al.*, 1997) that give a sweet taste during ripening, as well as performing an antifungal role. Another target is the production of bananas, almost only food for some populations, having high β-carotene content and micronutrients, including iron (Cressey, 2013).

Transgenic apple trees were produced with the aldose 6-phosphate reductase (*A6PR*) (Cheng *et al.,* 2005) to increase sucrose and reduce sorbitol level in the leaves. Transformation was done in grapes with constructs of the 35S constitutive promoters to modify the activity of alcohol dehydrogenase (*Adh*); when the gene was over-expressed, a lower content of sucrose and a better polymerization of the proanthocyanidins were observed (Tesniere *et al.,* 2006).

Apples have been modified to encourage the consumption of fresh-cut apple products. The fruits (Arctic Apple) do not quickly turn brown after cutting or biting through the silencing of poly-phenol oxidase (PPO), a key enzyme involved in the chain of biochemical events that cause browning. If this modification attracts the consumer, then it could also be applied to other fruit bearing plants like avocado, pear and even some vegetables. On February 13, 2015 U.S. Department of Agriculture's Animal and Plant Health Inspection Service decided to deregulate the first two varieties of apple that does not turn brown, as Arctic Golden and Arctic Granny.

In *Fragaria vesca*, *Fragaria x ananassa* and *Rubus idaeus*, DefH9-IAAM plants have shown a greater number of flowers per inflorescence and an increase in the number of inflorescences per plant, resulting in an increase in the number of fruit, in addition to the weight and size of transgenic fruits (Mezzetti *et al.*, 2004). In transgenic clones of grape cvs 'Silcora' and 'Thompson Seedless' transgenic for *DefH9-IAAM*, grown in open field have shown a higher grape production due to an increase in the number of inflorescences and a content of IAA with a substantial equivalence of nutritional characteristics of the berries (Costantini *et al.*, 2007) (Fig. 9). Research is being carried out to produce plums for industrial use without a woody endocarp, to prevent wood residues, due to the crumbling of the core during the transformation process and may end up in processed foods (Cressey, 2013).

Figure 9. Grape, CV Silcora, and raspberry, transformed with DefH9-IAAM gene. The plants produced great number of flowers and fruits resulted larger than the respective controls (Mezzetti *et al.*, 2004).

Control of fruit ripening is possible by regulating the activity of enzymes such as polygalacturonase and ethylene (Table 1). However, the possibility of delaying the softening and ripening are often associated to a reduction of typical aromas of ripening.

The introduction of parthenocarpy character in self-sterile species may allow the control of development of the fruit even in prohibitive environmental conditions for pollination and could increase fruit size. Broothaerts *et al.* (2004) demonstrated the silencing of the gene (*SI*), that prevents self-fertilization in many tree species, it was possible to produce fruits in apple tree lines capable of fertilization (Fig 2). The parthenocarpy character is often polygenic and therefore more difficult to deal with traditional techniques, therefore genetic mutation and alteration of ploidy level have been recently observed in olive (Rugini *et al.*, 2016). Transgenic parthenocarpic fruits of tobacco, eggplant and tomato were obtained successfully by Rotino *et al.* (1997 and 1999). These plants contain the coding region of the *IAAM* gene (gene for tryptophan monooxygenase) in their genome, the enzyme that converts tryptophan to indolacetamide, a precursor of IAA, under the control of the sequence of a gene regulator *defh9* placental-specific. The expression of *defh9-IAAM* begins during the development of the flower mimicking the hormonal effects of pollination and embryo development by increasing the content and / or the activity of auxin in the ovule. Kiwifruit CV 'Hayward', expressing these genes was produced 15 years ago and currently maintained *in vitro*, are waiting to be tested in the field (Rugini and Spena, unpublished.)

***Transformation for production of secondary metabolites*:** Much attention is now being directed towards the effects of secondary metabolites, in particular flavonoids (i.e. quercetin and kaempferol) and anthocyanins, which possess antioxidant properties and vasodilating action, with consequent protection from cardiovascular diseases. The gene of Petunia chalcone isomerase, an enzyme involved in the biosynthesis of flavonoids could be used to transform a lot of fruit trees, such as plum, grapes, strawberries, orange, grapefruit to strengthen the natural color of fruits, (Baldoni and Rugini, 2002; Petri *et al.*, 2008; 2011). Numerous works have been carried out on fruit plants in recent years (strawberry, citrus, grapes, apple, plum, and kiwi) to study the function of various genes involved in the production of these pigments, by using the way of either the overexpression or silencing of certain genes. Since the expression of anthocyanins (belonging to the flavonoid family) is regulated by a transcription factor *MYB*, the transformation with *FaMYB10* (Lin-Wang *et al.*, 2010) and *MdMYB10* (Espley *et al.*, 2007) respectively in strawberry and apple tree, has produced high levels of anthocyanins in the plant as well as is in the fruits. Decreasing the level of flavonoids with antisense chalcone synthase (*chs*) in strawberry has increased sensitivity to *Botrytis cinerea* (Hanhineva *et al.*, 2009). The kiwifruit transformed with genes for the biosynthesis of carotenoids (Kim *et al.*, 2010) has increased β-carotene in fruits. Gilissen *et al.* (2005) obtained a drastic reduction of the allergen expression in apple *Mal d 1* (Tab. 1 and Fig. 2).

***Trans Grafting Method*:** It is well known that grafting has been extensively used in horticultural crops to improve their quality and productivity and mainly to overcome the difficulties of rooting ability of the varieties under adverse conditions of soil. The rootstock and scion influence each other, while maintaining their genetic integrity; particularly rootstock can alter the phenotype in term of its vigor, fruit set and the phenological phases of scion, nutrient, and water uptake (Haroldsen *et al.*, 2012b). When the rootstock is genetically modified by modern technologies is called "Trans grafting". Several experiments have been done in fruit crops like apple and cherry (Rugini *et al*, 2015) (table 1), with the advantage that the scion acquires benefits and traits conferred by transgenes in the rootstock. The end products, such as

fruits, do not contain the transgene and hence are not genetically modified (Schaart and Visser, 2009; Haroldsen *et al.,* 2012a; Lemgo *et al.,* 2013). Several studies, as reported in the review paper by Limera *et al*. (2017) demonstrated that the benefits are due to some specific RNA molecules which can induce direct epigenetic modifications at the DNA level (Molnar *et al.,* 2010). Whereas, the microRNAs and transacting siRNAs have also been associated with the transmission of silencing signals systemically via phloem and from cell to cell through the plasmodesmata (Nazim and Kim, 2013; Zhao and Song, 2014). Considering these properties, the use of genetically modified rootstocks is encouraged, since avoiding the presence of transgene in the fruits by taking the same advantage of transgenic scion without requiring the level of biosafety of the traditional genetically modified plants (GEPs).

***Genetic stability*:** When a gene is transferred or modified by physical or chemical agents in a genome of a plant, it is essential that its expression remains stable over time and should be inherited by the progeny. This requirement is essential for the propagation of gamic plants, but it is equally important for vegetatively propagated crops for further subsequent improvements of the species by using the sexual method. Many studies on the genetic stability and inheritance have been carried out in both woody and herbaceous plants. The loss of functionality over time is observed rarely. The observations in woody plants were made both with the marker genes, such as the gene for resistance to kanamycin and with the target genes (Rugini *et al.,* 1997; 1999; 2008). Firstly, the stability and inheritance were observed in the apple tree (James *et al*., 1996), and secondly in the kiwifruit transgenic for *rolABC*, both for the male (cv. GTH) and female line (cv. Hayward). The transgenic kiwifruit plants have preserved the typical phenotype "hairy root" for the entire observation period of about 25 years, including the period in the greenhouse and in the field. In addition, the progeny derived from "normal transgenic male x female" maintained their phenotype during the expression for more than 16 years of observation. Same applies to the transgenic olive for *rolABC* and *osmotin* (Rugini *et al.,* 2008) and the rootstock of Colt cherry, containing *riTDNA A. rhizogenses* retaining the ability to reduce the vigor of the cv Lapins grafted (Rugini and Gutierrez-Pesce, 1999; Rugini *et al.,* 2015), for the transgenic strawberry for *rolABC* and *osmotin* and apricot transgenic for the virus coat protein (Laimer, Personal communication.). A lot of work has been done in the US by Scorza and collaborators of transgenic plants of *Prunus domestica* transgenic for the "Plum pox virus coat protein" (PPV- CP), through *Gus* and *nptII* genes. The expression remained stable for over 5 years both in the original plants and the progeny derived from them after crossing (Ravelonandro *et al.,* 1997).

Conclusions: The fruit tress normally need frequent varietal renewal; the frequency at present time is emphasized by the climate changing. In several countries, the rare old local varieties still kept in cultivation need replacement due to natural aging and not capable to give the due incomes. Meanwhile the hundreds of local varieties have been saved from extinction by the researchers through public initiatives, expected to be genetically improved, as they cannot be cultivated in present situations. Farmers continue to employ varieties often selected in different environments, whose fruits often exhibit different characteristics from other fruits that have been obtained by the hybridization of local genotypes or from varieties improved by "gene therapy", through recombinant DNA technology. One cannot deny the extraordinary success of cultivation of transgenic papaya in the Hawaii and the plum tree in USA, both preserved by genetic engineering through the dangerous virus that devastated the orchards. Similar benefits could be achieved from other fruit species improved with these technologies for some agronomic characteristics, if they had not been hampered by fierce resistance, which brought in some countries, like Italy, even the strange ban on field testing, undermining the potential of developing technology.

It is necessary to make people understand that agriculture occupies relatively little space on planet earth and if this space is exploited more efficiently with proper rotations, such as good agricultural practices and with the use of efficient varieties, will make more "green" surface and more extensions will be available. While in some European countries research on this branch of biotechnology has been blocked or severely curtailed but private companies and public institutions in many other countries are rapidly pursuing their goals and extraordinary results. China is currently the sixth largest user of GM crops and claims to be a great "experimental" greenhouse for these technologies. Park *et al.* (2010) describes a thorough analysis to affirm that it is not worth to ignore the transgenic crops as one of the tools that can help to come up with sustainable developments while the world's population is continually increasing.

To reduce the impact and for further reassurance of the public opinion, the use of new markers for the selection is a good alternate. Such as the *ipt* gene from *A. tumefaciens*, which induces the regeneration of transformed cells, allowing the selection visually, in order to replace those practiced so far (resistant to antibiotics or herbicides). For genotypes with high ability to regenerate can eliminate any marker gene, operating through *in vitro* selection, in a greenhouse or in the field by using physiological (response to toxins, filtered culture of pathogens, salt resistance / drought, etc.) or morphological parameters, as already reported in apple (Malnoy *et al.,* 2010).

In near future the research will substantially have main objectives like 1) the identification and evaluation of genes for useful traits with their specific promoters, 2) the identification of markers linked to important agronomic characteristics and quality to accelerate intersection

programs, as an alternative approach to gene transfer or reinforcement of the same, and 3) the regulation of expression gene of the transgene in the plant, and 4) the development of increasingly targeted and less invasive technologies at present, such as new genetic editing, compared to classical genetic transformation of gene transfer including Talens and CRISPRs.

It is therefore, necessary to find right balance if it is nothing more than to exercise reciprocal control. Preventing the experimentation in the field, as in Italy, creates confusion in the evaluation of transgenic plants; often what occurs in protected conditions is not a reliable indicator for that happens outside too, as scientifically described by Mittler (2006).

Unfortunately, some countries are outlined from this extraordinary technology that could contribute to produce innovations for the benefit of everyone, including many young scientists who are forced to immigrate to do private research for commercial lobbies and some producers' organizations, as well as short-sighted politicians.

The information about science should be reinforced and overall should be treated by field specialists. The attitude of GMOs is an example of anti-scientific nature, often the defects are attributed only to GMO with known methods of genetic improvement (e.g. resistance to diseases), with risk to demonize all genetic improvement and the progress done up till now.

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