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An Insight into the Strategies and Applications

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GENETIC ENGINEERING - AN INSIGHT INTO THE STRATEGIES AND APPLICATIONS

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Notice

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Meet the editor

Dr. Farrukh Jamal received his PhD in biochemistry from Dr. Ram Manohar Lohia Avadh University, Faizabad, India. His experience in the area of biochemistry includes reproductive biochemistry, plant proteins, and enzymes in wastewater treatment and insect pest management of agriculturally important food crops. Dr. Jamal took up professional teaching in 2001 as an assistant professor of biochemistry at Dr. Ram Manohar Lohia Avadh University, Faizabad, India. At present, his focus is on addressing the growing public concern over the toxicity and carcinogenicity of synthetic and recalcitrant dyes. He has contributed on novel defense proteins/proteinaceous protease inhibitors present in plants and their effectiveness on insect pests for applications in integrated pest management. Dr. Jamal has several independent projects, participated in several seminars and conferences, and published his work in internationally acclaimed books and journals.

Contents

Preface

None of the biological molecules have fascinated the investigators of life sciences as much as the molecule that serves to store the information about the cellular microcosm. The discovery of novel enzymes and improvisation of molecular techniques paved the way to look at the genes differently. The activity of manipulating the gene at will opens the door of transferring desirable characteristics and translating them into products that had numerous utility. The recent debate on the controversies and myths surrounding the genetically modified organisms and products served as a blessing in disguise to prove and disapprove such research. The book unfolds with an article by Brankov et al.on controversies floating around the gene revolution in the past decade. With the progressive debate on gene revolution and adoption of genetically modified food crops in sustaining the global population and meeting out the hunger challenges, the author argues the failure of transgenic technology to eradicate hunger.

The genetic engineering of plants has always gathered the attention of *Agrobacterium tumefaciens*, a plant pathogen, to serve as a biological tool for delivering novel genes into plant cell. Tumor-inducing plasmids are blessed with natural tendency of transforming plant cells and have been widely used in engineering of crop plants. Yıldız et al. dwell on new transformation protocols which are interesting and can be used to increase the transformation efficiency via *Agrobacterium tumefaciens* in most plant species. Yoshikawa et al. provide an insight into a new plant breeding technique using Apple latent spherical virus vectors to shorten the breeding periods of fruit trees like apple and pears. Such approaches are certainly a boon and are expected to see future application.

Gürel et al.'s presentation on genome editing is worth reading as the technique represents a major breakthrough in the field of genetic engineering which will certainly benefit research community engaged in the area of developing transgenic plants in a more convenient and safer way.

Addressing the implications of genetic engineering, the book doesn't end up in limiting the technology to only plants. Sen et al. provide an interesting article on genetic engineering for therapeutic purpose, especially in cases of diabetes and obesity. Viral vectors could serve as a wonderful tool for potent genetic modification to arm the stem cells in resisting apoptosis upon exposure to hyperglycemic environment.

Science has always been fascinating, and flight of imagination has always motivated to get an insight into the working of microcosmic biological system. This compilation is simply the tip of the iceberg, and what will follow in years to come would change the outlook of the past perception. The contribution from all the authors is deeply acknowledged, and their efforts will serve as the foundation in architecting the nascent as well as established scien-

tists, students, and logical minds. Above all, genetic engineering has always existed in nature, and the book presents a reflection of the understanding to the curious investigators.

Ms. Andrea Korić, as Publishing Process Manager, has always been prompt to reply to the queries and extend her kind support as and when required. Her attitude and humbleness are gratefully acknowledged.

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Chapter 1

Gene Revolution in Agriculture: 20 Years of Controversy

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Bozidar Popovic and Vladimir Bozovic

Additional information is available at the end of the chapter

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Abstract

In the spirit of the general debate over genetically modified (GM) food which is not slowing down, we wanted to answer some questions, such as: Is Gene Revolution an answer to world hunger? Do GM crops with more complex transformation contribute to the enrichment of multinationals? Why U.S. increases food aids? To this end, we firstly describe the diffusion of GM crops around the world during the previous 20 years. Starting from 1996, we present global progress with adoption of biotech crops, its distribution in developed and developing countries, global area by trait, adoption rate and global value of biotech crops. The findings reveal 10 countries, four crops, and two traits domination. The findings of this study clarify the failure of transgenic technology to eradicate hunger. In addition, the results have shown statistically significant correlation between stacked trait and global market value of biotech crops as well as between raising production of biotech crops in U.S. and an increase in U.S. food aid through World Food Program (WFP).

Keywords: gene revolution, famine, stacked traits, global market value, U.S. food aid

1. Introduction

Although a significant amount of food, which is obtained from genetically modified (GM) plants, is involved in a food chain, public debate on the issue is not slowing down. There is a large expert disagreement about environmental and health effect as well as about socioeconomic implications [1–4].

The consumer willingness to use and pay GM products varies among the nations [5–8]. From the very beginning, the U.S. supported biotechnology industry. The same policy that considers

GM and conventional foods substantially equivalent carried out all administrations, from R. Reagan to B. Obama administration. At present, the total U.S. revenues from GM systems reached at least $350 billion, the equivalent of approximately 2.5% of GDP [9]. Directly or indirectly numerous international organizations have contributed to the spread of GM products [10]. Among them, the most important are World Bank (WB), World Trade Organization (WTO), Consultative Group on International Agricultural Research (CGIAR), Food and Agriculture Organization (FAO), various international foundations, research centers, and universities. The WTO's Agreement on trade-related intellectual property rights (TRIPS) negotiated at the end of the Uruguay Round obliges member states to patenting biotech inventions (products and processes) and plant varieties and for the first time provides a legal means for the protection of intellectual property rights. Patent protection in this field gives the corporations unprecedented control over research and development as well as over whole food chain [11]. This strongly accelerated already started transformation process of the commercial seed industry from a sector composed primarily of family-owned small firms to a small number corporate domination [12]. After many acquisitions and joint ventures formation, the top three seed firms currently control 85% of transgenic corn patent in the U.S. [13]. Global recession has contributed to the further strengthening of the monopoly power of the biggest biotech companies [14]. Because overall industry data are not shared publicly, *Farm Journal* magazine has reported market shares by interviewing consultants, industry executives, and other sources to assemble the data [15]. According to this source in 2014, DuPont, Pioneer, and Monsanto dominate, accounting for 70% of the corn seed business and 60% of the soybean seed business.

While majority of farmers worldwide still rely on saving their own seeds, the prevalence of this practice, especially in developed countries, is declining rapidly [16]. In the leading GMOs country, the U.S., the rate of saving soybean seed fell to 10% in 2001 from 63% in 1960 [17], while the rate of saving corn seed fell to less than 5% [18]. Ignoring the interests of the poor countries can be observed from the fact that multinational corporations from North America and Europe take advantage of natural wealth located mainly in the Global South [10, 19]. The best example is maybe the neem tree, *Azadirachta indica*, which has religious and cultural significance throughout India. U.S. Patent Office issued Patent No. 5,124,349 to W.R. Grace & Co. on the particular derivative of the neem tree. Long before any official discovery, farmers in India had been using a neem, among many other purposes, as a powerful insecticide that is not harmful to humans [20].

2. Research methodology

In the spirit of the general debate, which is not slowing down in this chapter, we wanted to answer some questions, such as Is Gene Revolution an answer to world hunger? Do GM crops with more complex transformation contribute to the enrichment of multinationals? Why U.S. increases food aids? To this end, we have set up the following research hypotheses:

1. GM food has failed to feed the hungry.

2. There is a statistically significant correlation between stacked trait and global market value of biotech crops.

3. There is a correlation between raising production of biotech crops in the U.S. and an increase in the U.S. food aid through World Food Program (WFP).

For the purpose of general understanding of the issue at the beginning, we present global progress with adoption of biotech crops, its distribution in developed and developing countries, global area by trait, adoption rate, and global value of biotech crops. The first hypothesis "GM food has failed to feed the hungry" we set because agribusiness segment has often promoted transgenic technology as the next hunger-quenching Green revolution. "There is a statistically significant correlation between stacked trait and global market value of biotech crops" hypothesis has been settled because the multinationals claim that this complex transformation can allow farmers to address multiple old problems with two or more traits in the same seed and are more effective at meeting the needs of farmers and consumers than the traditional, mono-trait seed varieties [21]. Finally, our last hypothesis "There is a correlation between raising production of biotech crops in the U.S. and an increase in the U.S. food aid through WFP" was tested due to the controversy over the food aid. Excluding the many benefits of emergency humanitarian aid, recent finding shows that an increase in U.S. food aid increases the incidence of armed civil conflict in recipient countries by prolonging existing conflicts [22]. In addition to this aspect, a disagreement over the food aid in terms of GM food arose in 2000s when several African countries and countries in transition (such as Serbia and Montenegro) refused to accept U.S. aid due to GM ingredients presence. "A fiery" debate continues via WTO Doha Round in 2003 and 2004, when the other countries have put pressure on the U.S., as the principal donor country, to move away from in-kind food aid. The great power of lobby groups in America could be visible due to the U.S. Congress rejection of Bush administration proposal that one-quarter of U.S. food aid should be cash-based [11]. The principal reason why the U.S. continues to insist on giving its food aid in-kind may be the inability to find export market for its GM maize [23].

Data required for the analysis were collected from relevant sources: ISAAA, FAO, USDA, Earth Policy Institute. The arithmetic means of the confidence interval as a measure of variability in the descriptive statistics have been used. The method of parametric statistics (*t* test, ANOVA) was applied in processing the results, while Pearson's correlation coefficient and simple linear regression were used as a measure of association. To detect trends in the variables considered, the collected data were compared by linear regression analysis using the factor time as independent variable. Data were analyzed using Microsoft Excel and R Studio software.

3. Global diffusion

3.1. International diffusion of biotech crops

In the period 1996–2015, up to approximately 18 million farmers grew biotech crops annually. Global biotech hectares increased more than 100-fold, from 1.7 million hectares in 1996 to

179.7 million hectares in 2015. In other words, GM crops are grown on approximately 3.7% of the world's total agricultural land and 13% of arable land and by <1% of the world's farming population [24]. In the 19 years period 1996–2014, the growth rate of area under GM was continually growing. Total crop plantings were decreased for the first time in 2015 comparing with 2014 (-1%). A clear linear trend of the total biotech hectarage growth can be expressed by equation:

$$y = 9.919x - 5.781\left(R2 = 0.993\right) \tag{1}$$

As represented in **Figure 1**, linear trend of GM crops diffusion is obvious in both developing and developed countries. Linear trend equation for developing and developed countries, respectively, is as follows:

$$y = 5.749x - 16.79\left(R2 = 0.969\right) \tag{2}$$

$$y = 4.161x + 11.07\left(R2 = 0.946\right) \tag{3}$$

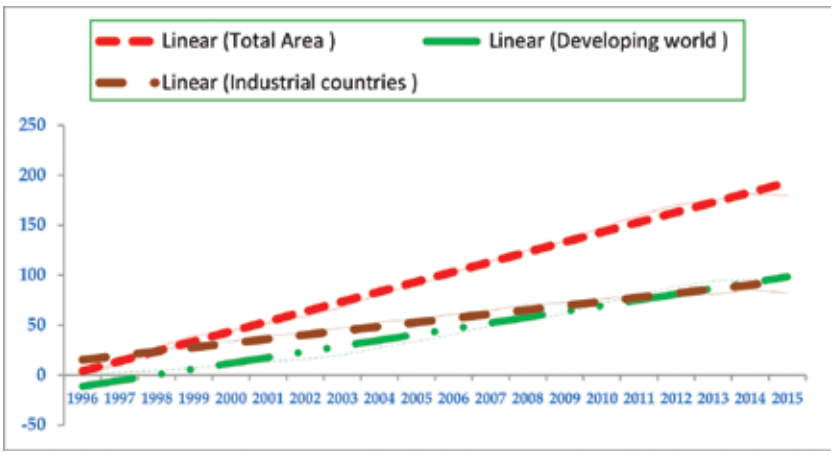

Figure 1. Total area under GM, distribution in developing and developed countries (million hectares). Source: Author's calculations based on data from James (1996–2015).

For the first time in 2012, developing countries planted more biotech crops than industrial countries, 51.2 and 48.8%, respectively. Higher participation of developing countries was also observed in subsequent years. Latin American, Asian, and African farmers collectively grew 53.6% of the global biotech hectares in 2013, 52.9% in 2014, and 54% in 2015.

The number of producer countries also grew by time, starting from 6 in 1996 to 28 in 2015 (**Figure 2**). The first countries that engaged themselves in the transgenic production were U.S.,

China, Argentina, Canada, Australia, and Mexico. In 1998, South Africa, Spain, and France grew transgenic crops for the first time. Portugal, Rumania, and Ukraine joined them in 1999 and Bulgaria, Uruguay, Indonesia, and Germany in 2000. India planted GM crops for the first time in 2002. In the same year, Colombia and Honduras started their production. Two countries, Brazil and the Philippines, approved planting of GM crops in 2003. In 2004, for the first time, Paraguay reported its cultivation. New producing countries become Iran and Czech Republic in 2005; Slovakia in 2006; Chile and Poland in 2007; Burkina Faso, Egypt, and Bolivia in 2008; Costa Rica joined them in 2009; Pakistan, Myanmar, and Sweden in 2010; Sudan and Cuba in 2012; Bangladesh in 2014; Vietnam in 2015. From Graph 2, it can be seen a significant increase in the producing countries in numbers. For example, the number of developing countries increases from 2 in 1996 to 20 in 2015 [21, 25–43]. It is very important to stress that total number of producing countries has not increased since 2010. This is an indication of problems in the geographical diffusion of GMOs.

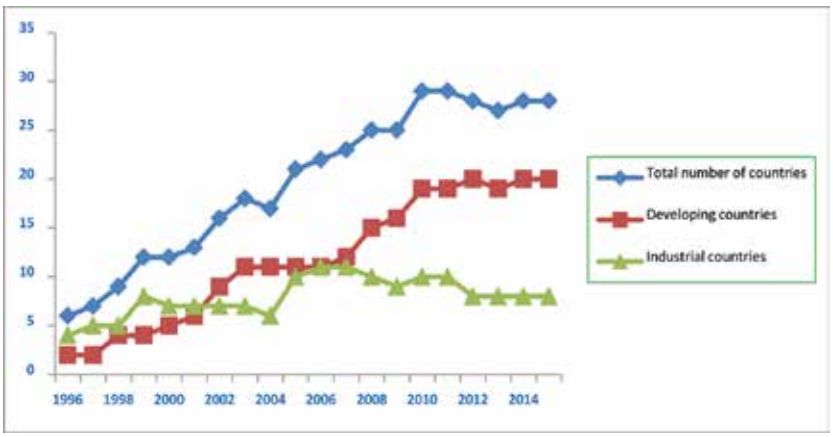

Figure 2. Producing countries developing and developed. Author's calculations based on data [21, 25–43].

From the very beginning, U.S. is absolute leader in the production, but U.S. involvement has decreased with the inclusion of the other countries. For example, U.S. accounted for 64% in the total area under GM in 1997, while their share in 2015 was 39.4%. Brazil, the second largest producer, started relatively late with the production but at present accounts for 24.6% in the total areas. Argentina, the third biggest producer, participates in the total acreage of 13.6%. India and Canada areas reached 6.4 and 6.1% of global hectarage, respectively. China share in the total area is reduced significantly, from 16% in 1997 to 2% in 2015. Australia produces GM on just 0.7 million hectares (0.4% of total area under GM crops), which is a just slight increase from 1997 (0.1 million hectares). In 2015, there were 28 countries that grew GM crops, but in reality, a large majority of the area planted to GM crops still remains in just a few countries. Top 10 countries accounted for 98% of the total global GM hectarage. The list of countries that grew GM crops in 2015 includes Bangladesh, which grew as little as 25 hectares of GM crops in 2015, and Costa Rica, which grew 38 hectares in 2014 [21, 25–43]. After the initial years of high growth rates, the global growth of GM crops is, in fact, slowing down, as the amount of

land under GM crops in the few countries that are large-scale adopters has become saturated [24]. Annual global growth rates were between 3 and 10% in the past seven years, with the global GM area in 2013 and 2014 approximately 3% higher than the years before [43].

At first glance, this diffusion is impressive but certainly is not without problems. Despite the great support to genetically modified organisms (GMOs) in the several countries of Latin America, there is a resistance on this continent. Venezuela with over 30 million inhabitants approved a new law on December 23, 2015, that imposes one of the world's toughest regulations on GMOs. The Seed Law seeks to consolidate national food sovereignty, regulates the production of hybrid seed, rejects the production, distribution, and import of GMO seeds, and also bans transgenic seed research [44]. Similarly, in the Middle East, Iran, country which initiated its rice biotech activities in 2005 with several hundred farmers growing 4000 hectares of Bt rice on their farms and cloned the first GM animal, sheep in 2006 [45] very soon, has decided to drop the commercialization of GMOs. Currently, government is preparing new law to facilitate GMO production. At the same, time the resistance of certain groups strengthens [46].

Apart from Russia where situation is more than clear and where the food safety regulator Rosselkhoznadzor bans GMO production and import [47] and Prime Minister has described as amounting to little more than a form of biological warfare weapon [48], the biggest challenge in terms of adoption and acceptance of GMOs remains Africa and Europe. For a long time, South Africa was the only country on the continent of Africa to commercialize biotech crops. GMO proponents have expressed their pleasure because of broad geographical coverage in Africa in 2008 when Egypt as represent of North Africa and Burkina Faso from West Africa become GM producer countries [37]. Historically, important event was also the involvement of Sudan in 2012. But, all commercial production in Egypt is currently stalled due to Ministerial Decree 378 (MD378), 2012, rescinding the registration of Ajeeb-YG corn variety for its commercial use [49]. Bt cotton remains the only crop commercialized in Burkina Faso [50]. Sofitex, Burkina Faso's major cotton production association, on April 5, 2015, decided to switch its production to 100 percent conventional methods and has announced a rejection of GM cotton in the next three years due to the disappointing yields and poor quality fiber [51]. South Africa, the largest producer country in Africa, has experienced the greatest reduction—23% less for its GM maize crop hectarage in 2015 comparing to 2014. Sudan, GM cotton producer country, seized a shipment of GM soybeans reportedly imported from the U.S. [52]. Nigeria, one country being targeted for expansion, has recently submitted a written petition (more than 100 groups representing at least 5 million Nigerians) to the country's authorities in hopes of impeding GMO entrance [53].

Cultivation in the EU fell 10.4% in 2015 to only 128.103 hectares, almost all of that insect-resistant corn grown in Spain (93.7%). Share of GM corn in total corn area is just 1.3%. Commercial cultivation of GE crops is minimal in other EU producing countries: Portugal, Czech Republic, Romania, Slovakia, France, Germany, and Poland. According to USDA Foreign Agriculture Services, EU member states can be divided into three categories depending on GMOs view. The adopters include the GM producer countries as well as countries where the government and industries mostly favor biotechnology (Denmark, Estonia, Finland,

Flanders in northern Belgium, the Netherlands, and the United Kingdom). Conflicted members are those states where scientific community, farmers, and the feed industry are willing to adopt the technology, but consumers and governments, influenced by activist Green parties and NGOs, reject it (France, Germany, Poland, Southern Belgium (Wallonia), Bulgaria, Ireland, Lithuania Sweden, Germany). In the opposed member states, most stakeholders reject the technology and government generally supports organic agriculture and geographical indications (Austria, Croatia, Cyprus, Greece, Hungary, Italy, Malta, Slovenia, and Latvia) [54]. One of the leading opposed countries is Hungary which initiated a joint alliance of EU member states rejecting the use of GE crops with the objective to make the entire EU free from GM crops.

3.2. Four crops domination

Between 1986 and 1995, 56 GM crops were field tested around the world the majority of which were on eight crops [25]. Besides expiration that there are more than 85 potential new products in the pipeline now being field tested [43], 20 years later, only 10 crops are grown commercially (soybean, corn, cotton, canola, alfalfa, sugar beet, papaya, squash, potato, and eggplant/brinjal). There are no commercialized GM varieties of a number of key global staple crops, including wheat, rice, barley, millet, sorghum, cassava, and yam. The first generations of GM potato that resist blight were planted on 162 hectares in 2015 after FDA approval [55]. In significant quantities (about 99%), only four crops soybean, corn, cotton, and canola have been grown over the past 20 years. Principal four crops are generally used in food system as ingredients in processed food and in animal feed. Exceptions used as whole foods are some fruit and vegetables GM sweet corn, squash, papaya, eggplant/brinjal, potato which together with sugar beet and alfalfa collectively account for only 1% of global GM crop hectares. Area under all four crops, soybean, maize, cotton, and canola, depicts linear trend of growth, respectively (**Figure 3**):

$$y = 4.740x + 1.742\left(R2 = 0.986\right) \tag{4}$$

$$y = 3.265x - 5.836\left(R2 = 0.959\right) \tag{5}$$

$$y = 1.43x - 2.2\left(R2 = 0.964\right) \tag{6}$$

$$y = 0.445x + 0.367\left(R2 = 0.954\right) \tag{7}$$

As could be seen in **Figure 4**, maximum share of soybeans in total areas was in 2001 (63.3%), and overtime, it decreases to 51.2% in 2015. Share of corn stabilized at the level of about 30% in 2015 such as it was in 1998. Share of cotton increases from 9% in 1996 to 13.3% in 2015. The biggest drop is recorded in the share of canola in the total GM area, from 8.6% in 1998 to 4.7%

in 2015. GM sugar beet production started in 2008, but until now areas under this crop are about 0.5 million hectares, which means there is no enlargement as it was the case with previous crops. Regarding global adoption rate, it can be noticed from **Figure 5** that only soybean rate continuously was growing but with a sharp slowdown after 2009. Cotton adoption rate reached its maximum value in 2011 (82%) and then fell to 75% in 2015. Similarly, maximum corn value was 35% in 2012, after slipped to 29% in 2015. Canola global adoption rate slipped from max 30 to 24% in 2012 and 2015, respectively.

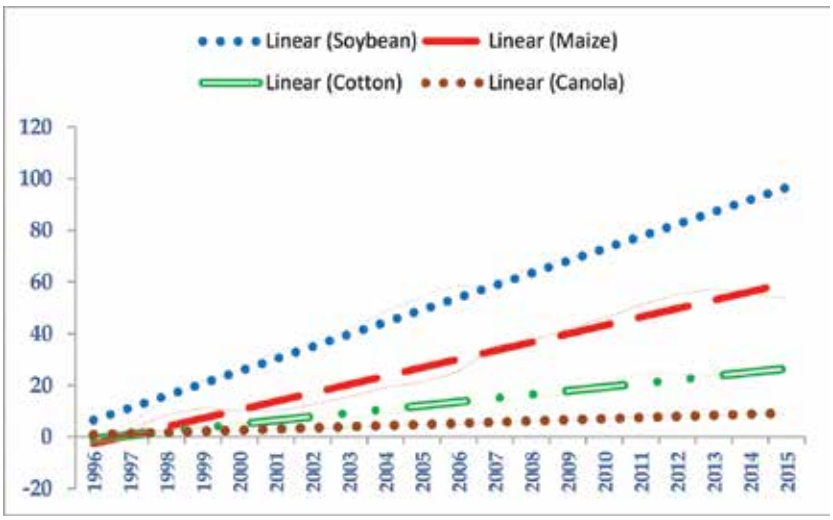

Figure 3. Linear growth trend for four principal GM crops (million hectares/year). Author's calculation based on Refs. [21, 25–43].

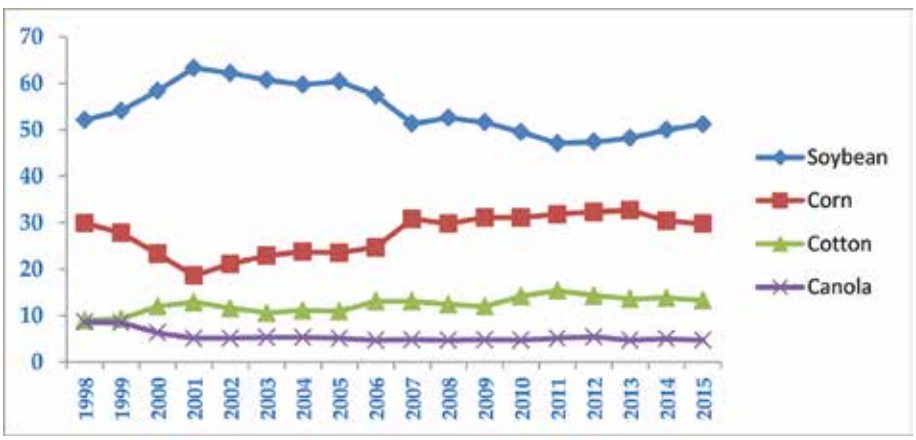

Figure 4. Percentage share of the most important crops in the total area under GMOs (1996–2015). Author's calculation based on Refs. [21, 25–43].

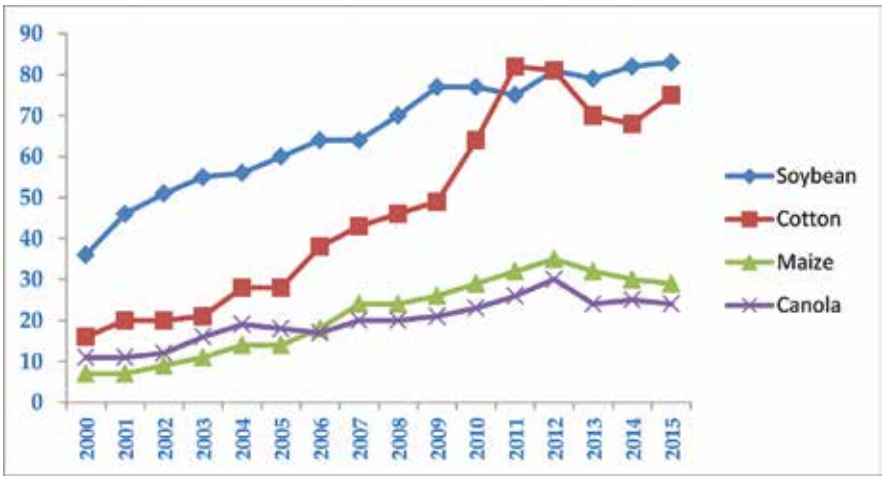

Figure 5. Global adoption rate of the principal GM crops (2000–2015). Author's calculation based on Refs. [21, 25–43].

One look at the slower growth of plantation area is due to the saturation of adoption rate in countries with mature GM models. For example, adoption rate of GM cotton in Australia is 99%, in Canada 95% of canola is GM, 95% cotton in India is GM, 99% cotton and 100% soybean in Argentina are GM, 95% of soybean in Paraguay is GM and over 90% of all four principal GM crops in U.S. is already GM [56]. Another view indicates that the resistance becoming stronger by opposition from consumer and environmental groups, regulatory hurdles, and, in some cases, scientific obstacles. There are opinions that the problems with diffusion of GM canola lay in the fact that canola is worst candidate crop species for practical segregation of GE and non-GE because it is inherently promiscuous [57]. Also, there are reports that state "Hundreds of thousands of farmers have died in India, after having been allegedly forced to grow GM cotton instead of traditional crops. The seeds are so expensive and demand so much more maintenance that farmers often go bankrupt and kill themselves" [58]. One might ask whether the refusal of consumers around the world to accept GM sugar is the reason for the stagnation of GM sugar beets production. Anyway, the fact is that the U.S. corn and soybeans more and more are channeled into biofuels production. For example, in the marketing year MY Aug/Sept 2015/2016, 38.8% of the total corn disappearance was ethanol share, 10.1% of total use was directed to total food, seed, and industrial use (not including ethanol), feed and residual use accounted for 38.8%, while export accounted for 12.2% of the total corn disappearance. The situation was very different in MY Aug/Sept 1995/1996: 4.6% of the total use was ethanol use, 14.4% was total food, seed, and industrial use (not including ethanol), 54.9% was dedicated to feed and residual use, and 26.1% for export [59]. In the period 1995–2015, U.S. almost doubled its corn production, from 7.4×10^9 to 13.6×10^9 bushels [60]. Taking into consideration problems with exports market, it is logical routing of surplus into ethanol production. Share of soybean oil used for biodiesel increased from 0.3% in 2000/2001 to 23% in 2014/2015. In the same period of time, soybean oil production has increased for just 11.7% (from 18.4×10^9 to 20.6×10^9 pounds). It can be observed that share of the U.S. soybean oil export in total use significantly declined from 17.6% in 2009/2010 to 10% in 2014/2015 [59]. The reasons

for these trends may lay in the efforts of the European retailers and major manufacturers (including REWE Group, Lidl, Edeke in Germany, Carrefour in France, and many of the retailers in Austria, along with Waitrose in the United Kingdom) to support the transition from GMO to non-GMO for animal production. The most prominent initiative Danube Soy was launched in 2012 as a "mainstream" vehicle for providing access to non-GMO soy for EU markets. It is undergoing rapid growth as a source of sustainable and non-GMO soy for Europe, and soya production in the Danube area (excluding Ukraine) has increased from 560,000 hectares in 2011 to 960,000 hectares in 2015 [61]. Signing this declaration means that EU wants to increase self-sufficiently in soy production. As of 2011, the EU imported 72% of its protein feed needs (majority coming from the U.S., Argentina, and Brazil—GM producing countries), so the plan is to provide at least 50% soybean needs until 2025 from non-GMO Danube region.

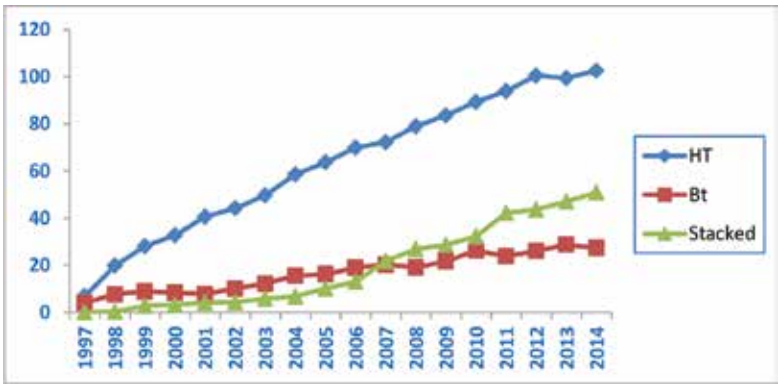

Figure 6. GM plants, by traits 1997–2014 (million hectares) [21, 26–42].

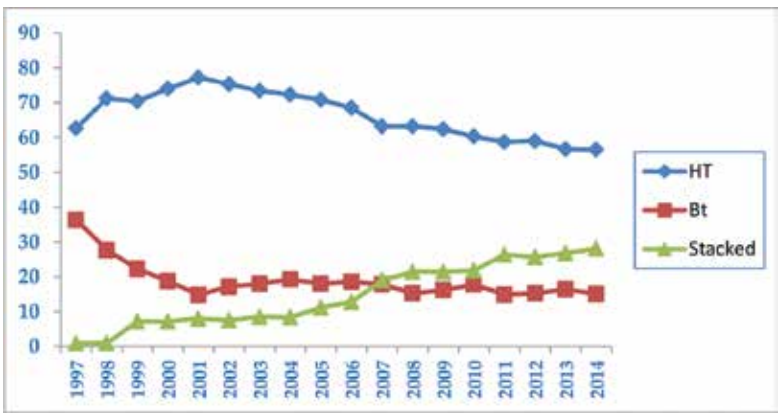

Figure 7. Percentage share of the most important traits in the total area under GMOs (1997–2014). Author's calculation based on Refs. [21, 26–42].

3.3. Herbicide tolerance and insect resistance domination

GMOs diffusion is uneven if we are speaking about traits also. The most widely used commercial GM traits are herbicide tolerance (HT) and insect resistance (IR). HT crops are developed to survive application of specific herbicides, which would otherwise kill the crop plants. "Roundup Ready" is the most common HT crops today. These crops have been created by Monsanto in order to tolerate applications of the company's glyphosate-based herbicide "Roundup." IR crops are engineered with a gene from the bacteria *Bacillus thuringiensis* (Bt), which is toxic to some insects. When certain insects ingest the protein produced by Bt, the function of their digestive systems is disrupted, producing slow growth and, ultimately, death. In recent years, various combinations of these two properties are used in creation of stacked plants. Gene stacking refers to the process of combining two or more genes of interest into a single plant; the combined traits resulting from this process are called stacked traits. As represented in **Figure 6**, HT plants occupied from the very beginning the most of area under GMOs. From 2007, areas under stacked crops are higher than the areas under Bt crops. In 2014, 56.4% of the GM crops worldwide were engineered to be HT, 15.1% were engineered to be Bt, and 28.1% were stacked with both HT and Bt traits (**Figure 7**). These numbers are significantly different in 2001 when 77.2% of crops were HT, 14.8% were Bt, and 8% were stacked. It is important to stress that referred data do not mean that the area under HT crops was reduced. Contrarily, it was increased. When we consider stacked and single trait crops together in 2014, 84.5% of all GM crops in the world are engineered to be tolerant to herbicides. Companies that have patent protection over GM seeds in the same time are herbicides producers. This means that multinationals in the same time sell both seeds and pesticides. HT and Bt crops account for almost all the GM crops grown commercially over the past 20 years. Other traits, virus resistance and drought tolerance, are very important for poor small-scale farmers in developing countries, collectively account for <1% of global GM crop hectares [43].

3.4. Global market value of biotech crops

Global market value of transgenic crops has increased overtime 165 times in terms of current prices and 115 times in terms of constant prices (2002 = 100) (**Figure 8**). Linear trend of growth in current prices can be expressed through equation:

$$y = 899.0x - 2028\left(R2 = 0.969\right) \tag{8}$$

The trend growth in constant prices is expressed as:

$$y = 691.3x - 940.7\left(R2 = 0.978\right) \tag{9}$$

We have also obtained the simple linear trend for transgenic crops global market value per hectare in current prices and polynomial trend of transgenic crops global market value per hectare in constant prices (**Figure 9**):

$$y = 2.029x + 47.20\left(R2 = 0.919\right) \tag{10}$$

$$y = 0.050x2 - 0.477\mathrm{x} + 59.36\left(\mathrm{R}2 = 0.561\right) \tag{11}$$

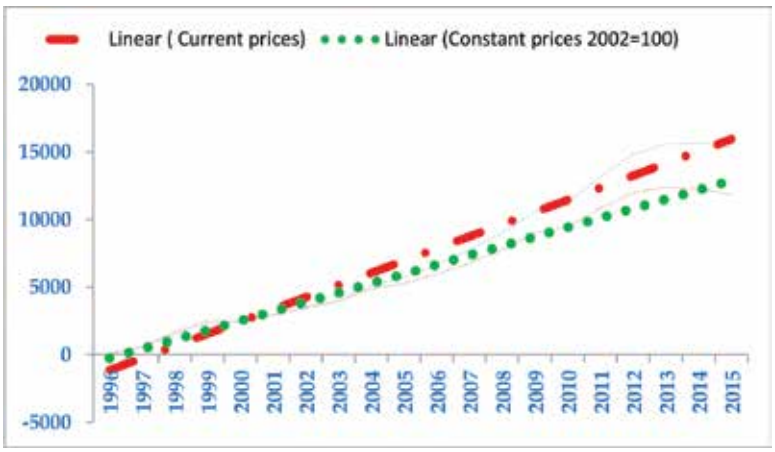

Figure 8. Global market value of biotech crops (million US$). Authors calculation based on Refs. [21, 25–43].

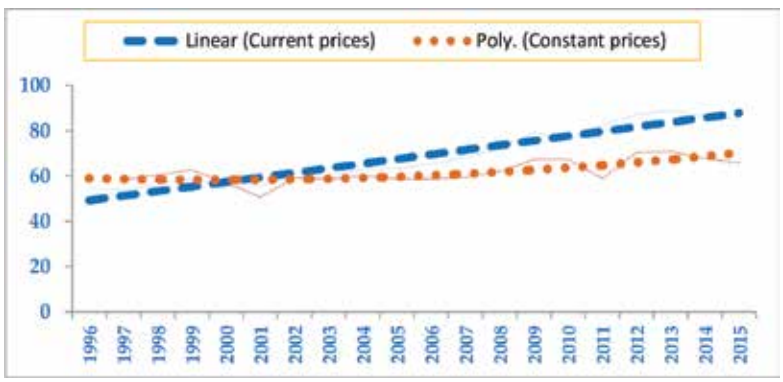

Figure 9. Transgenic crops global market value per hectare in current and constant prices. Source: Author's calculation based on Ref. [21, 25–43].

4. Results of hypotheses testing

In order to test our first hypothesis "GM food has failed to feed the hungry," we calculate the significance of the difference between *per capita* grain production and surplus/deficit cereal productions in the two periods, period before and after GM commercialization, 1958–1995 and

1996–2012. We found that there is no statistically significant *per capita* grain production differences between the two observed periods ($t = -1.481$; df = 53; $p = 0.145$) (**Figure 10**). Average cereals production level in the period 1958–1995 was 305.13 kg/person/year (CI 297.57–312.69) and 313.94 in the period 1996–2012 (CI 307.54–320.34). Transgenic technology has not contributed to the improvement in food balance, since there is no statistically significant difference in surplus/deficit cereal production between the two observed periods $t = 0.941$; df = 51; $p = 0.51$) (**Figure 11**). Average value of this parameter was 7.91×10^6 tons (CI −5.02 to 20.86) in the period before transgenic plant cultivation and -4.0×10^6 tons (CI −30.48 to 22.48) in the period after GMOs cultivation. These results clearly demonstrated that transgenic technology has failed to

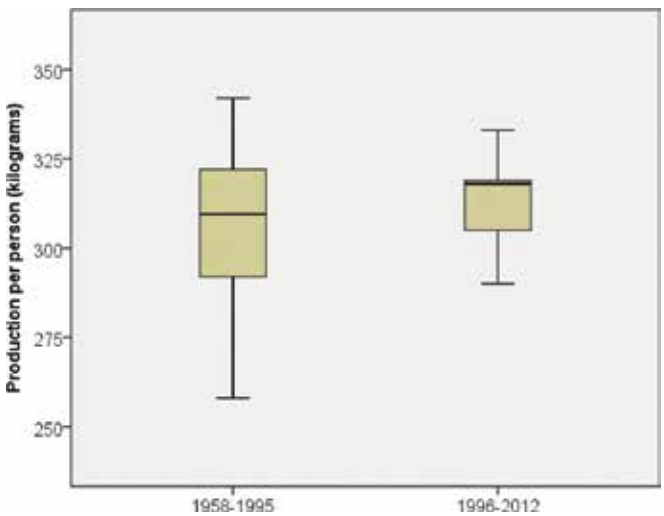

Figure 10. Cereals production (1958–1995, 1996–2012). Authors' calculation based on Ref. [62].

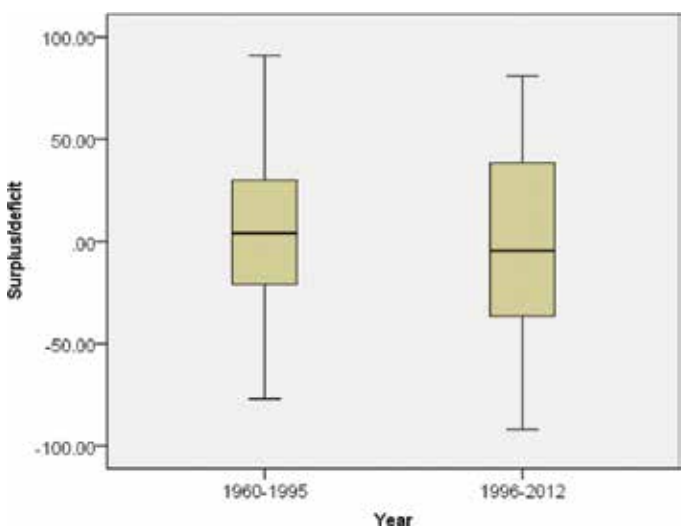

Figure 11. Cereal surplus/deficit before and after GM cultivation. Author's calculations based on Ref. [62].

contribute to hunger and famine reduction. Unfortunately, food insecurity and world hunger are still a recurring problem in a significant number of countries.

The second hypotheses "There is a statistically significant correlation between stacked trait and global market value of biotech crops" have been confirmed as presented in **Figure 12**. A statistically significant correlation exists between global market value (G) (const price, 2002 = 100) of biotech crops and areas under stacked crops (S) ($r = 0.984$; $p < 0.001$). The regression analysis suggests that an increase in 1 million hectares under stacked crops causes an increase in global market value by 198.3 million in terms of 2002 US$ (**Table 1**). This can be represented by the following equation:

$$G = 198.3xS + 243.834 \tag{12}$$

The results can lead us to conclusion that stacked traits obtained through more complex transformations potentially leading to a further enrichment of multinational companies.

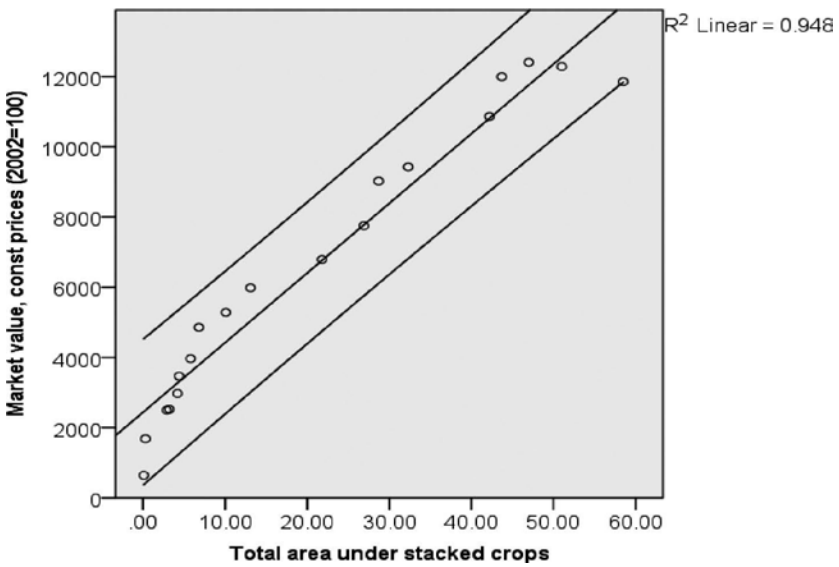

Figure 12. Correlation between global market value of transgenic crops and stacked crops. Authors' calculations based on Refs. [21, 25–43].

		Coefficients		*t*	*p*
		B	**Std. Error**		
1	Constant	2438.834	320.191	7.517	0.000
	S	198.335	11.247	17.734	0.000

Table 1. The regression analyses (stacked crops/global market value).

Finally, we pay attention to U.S. food aid which is in all forms procured by the U.S. Department of Agriculture (USDA) and administered by either the USDA or the U.S. Agency for International Development (USAID). A substantial portion of the U.S. food aid is channeled through the WFP where the U.S. as the major donor provided over the last 18 years around 43% of total WFP contributions [63]. With some fluctuation, U.S. food aid increased 3.2 times in const prices and 4.5 times in current prices from 1996 to 2015 (**Figure 13**). Maximum value reached in 2008 in terms of 2002 US$ prices. Polynomial trend line equations in current and constant prices are as follows:

$$y = 0.009x6 - 0.556x5 + 11.99x4 - 122.8x3 + 604.4x2 - 1169x + 1183 \left(R2 = 0.826\right) \quad (13)$$

$$y = 0.008x6 - 0.482x5 + 10.5x4 - 108.8x3 + 538.9x2 - 103.4x + 1147 \left(R2 = 0.721\right) \quad (14)$$

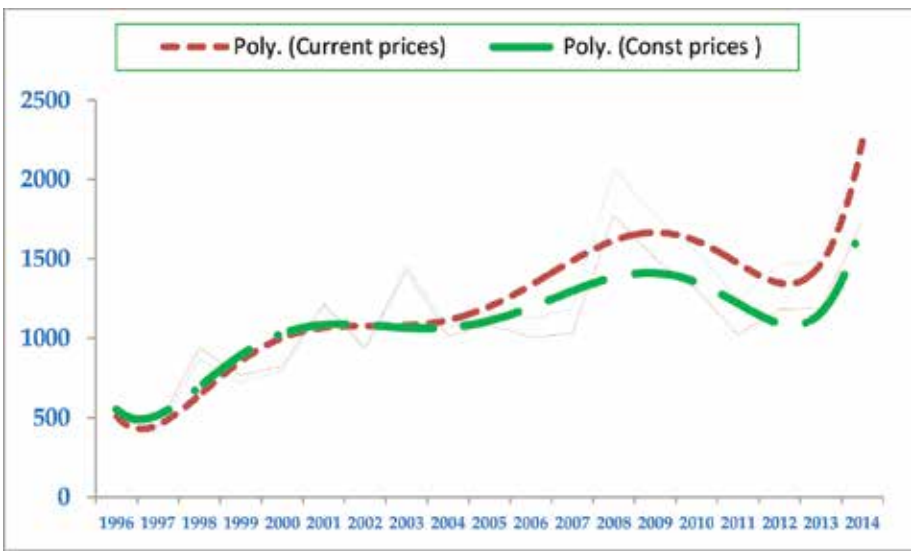

Figure 13. The U.S. food aid (1996–2014). Author's calculations based on Refs. [63, 64].

Taking into consideration that the U.S. increases in food emergencies in the last two decades, we have settled our third, last research hypothesis: "There is a correlation between raising production of biotech crops in the U.S. and an increase of the U.S. food aid through WFP." The results have shown significant correlation between U.S. GMOs areas (G) and the U.S. aid (A) ($r = 0.72$; $p = 0.001$) (**Figure 14**). The regression analysis (**Table 2**) suggests that an increase in 1 million hectares under biotech crops causes an increase in U.S. aid for 11.1 million US$ (const price, 2002 = 100) as expressed by the following equation:

$$A = 11093.17xG + 574475.15 \quad (15)$$

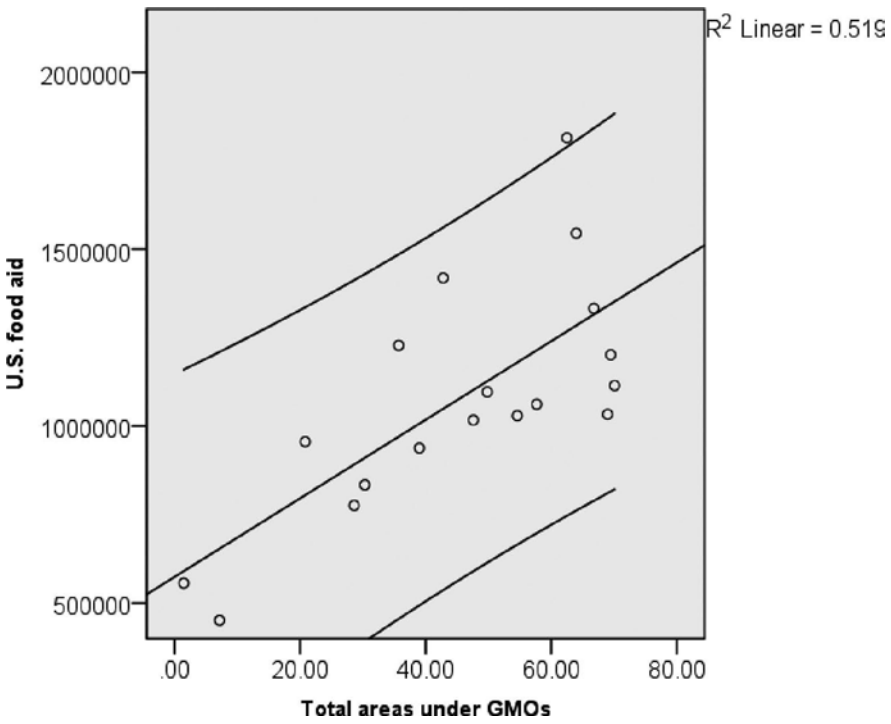

Figure 14. Correlation between U.S. GMOs areas and U.S. food aid. Authors' calculation based on Refs. [21, 25–43, 63, 64].

		Coefficients		*t*	*p*
		B	Std. Error		
1	Constant	574475.15	133289	4.310	0.009
	G	11093.17	2669.74	4.156	0.000

Table 2. The regression analyses (GMOs/U.S. aid).

5. Conclusion

Unlike the Green revolution, in which public research institutes developed technologies and freely disseminated them around the world, Gene revolution is led by multinational corporations, which place on the market GM crops protected by patents. Despite the claims of GMOs impressive diffusion, unequal distribution is present in all segments. Herbicide tolerance and insect resistance are the main GM traits that are currently under commercial cultivation, and the main crops are soybean, maize, canola, and cotton. Soybean and maize share in the total acreage was 81% in 2015. The reason for such a large share is their widespread use as food, feed, for industrial purposes and for biofuels production. These two plant species are broadly

present as ingredients of processed foods. For example, soya components are in margarine, cooking fat, mayonnaise, biscuits, coffee creams, fried products, ice creams, baked goods, chocolate, smoothies, tofu, sauces, as well as in meat, meat products, milk, and eggs (as 75% of soy worldwide is used for animal feed, especially for poultry and pigs) [65].

GM crops are grown by <1% of the world's farming population on 13% arable land mostly located in Americas and in some part of Asia. Transgenic technology is facing problems on all continents. Russia has learned a lesson from the past when even a strong nuclear power failed to provide food self-sufficiency. That's why the modern tendency of the Russian Federation is to ensure food security. In case, it comes to fruition that Russia will be a key player in reshaping tomorrow's global food system. Because the U.S. is powerless to oppose the Russian ban on GMOs and because of strong possibility of further strengthening of the European opposition, the U.S. intensifies the pressure on week, poor countries or countries in transition to accept transgenic technology. Apart from several African countries (Cameroon, Egypt, Ghana, Kenya, Malawi, Nigeria, and Uganda) where multinationals conducted in recent years field trials on GM crops as the penultimate step prior to its approval, in the Balkan region Serbia faces the greatest pressure to accept this technology. The reason lays in the facts that Serbia is leading agricultural country in the region, among the 10 largest world exporter of corn (non-GM), one of the biggest European producing countries of non-GM soya exporting soybean products into five continents and because the U.S. has lost export market for soybean meal. So far, 127 municipalities in Serbia have signed "declaration against GMOs." On May 29, 2009, National Parliament adopted new law on GMOs that fully prohibit the possibility of commercial growing of GMOs, or trade and products derived from GMOs. On the other hand, corrupt politicians and livestock agribusiness sector are pressuring to amend the Law on GMOs. Although multinationals conduct court disputes with farmers who sown GM seed without paying from the countries that allow the GM cultivation, in Serbia as a part of its expansion, strategy does not require protection of patent rights for illegally planted seeds.

Transgenic technology has failed to contribute to hunger and famine reduction since there is no statistically significant *per capita* grain production differences between the two periods, period before GM cultivation and period after it. In order to ensure food security for a growing population, it is necessary to launch a new revolution in food production or revoke the patent protection of transgenic technology and develop crops and traits vitally important for poor farmers and developing countries. At present, generally speaking, the main winners are multinationals who as time passes more and more strengthening its financial position. It can be seen, *inter alia*, on the basis of a correlation between the stacked trait and global market value of biotech crops. U.S. food aid will still remain a controversial issue as there is a significant correlation between raising production of biotech crops in the U.S. and an increase in the U.S. food aid through WFP.

Finally, it is important that governments around the world provide a transparent source of information for consumers on GM foods, which clearly listed of what GM foods are on the market. That way, consumer rights to informed choice will be satisfied.

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Chapter 2

New Approaches to *Agrobacterium tumefaciens*-Mediated Gene Transfer to Plants

Mustafa Yildiz, Murat Aycan and Sunjung Park

Additional information is available at the end of the chapter

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Abstract

Agrobacterium tumefaciens, a plant pathogen, is commonly used as a vector for the introduction of foreign genes into plants and consequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plants and induces diseases known as crown gall. The bacterium has a large plasmid that induces tumor induction, and for this reason, it was named tumor-inducing (Ti) plasmid. The expression of T-DNA genes of Ti-plasmid in plant cells causes the formation of tumors at the infection site. The molecular basis of *Agrobacterium*-mediated transformation is the stable integration of a DNA sequence (T-DNA) from Ti (tumor-inducing) plasmid of *A. tumefaciens* into the plant genome. *A. tumefaciens*-mediated transformation has some advantages compared with direct gene transfer methods such as integration of low copy number of T-DNA into plant genome, stable gene expression, and transformation of large size DNA segments. That is why manipulations of the plant, bacteria and physical conditions have been applied to increase the virulence of bacteria and to increase the transformation efficiency. Preculturing explants before inoculation, modification of temperature and medium pH, addition chemicals to inoculation medium such as acetosyringone, changing bacterial density, and cocultivation period, and vacuum infiltration have been reported to increase transformation. In this chapter, four new transformation protocols that can be used to increase the transformation efficiency via *A. tumefaciens* in most plant species are described.

Keywords: *Agrobacterium tumefaciens*, gene transfer, gamma radiation, magnetic field, squirting cucumber's fruit juice, osmotic pressure

1. Introduction

Genetic engineering could use the genetic resources that exist in nature without any limitation. With the use of these techniques, a gene cloned to an organism from any living being (human,

animal, plant, and microorganisms) could be easily transferred. For instance, a gene cloned from bacteria could be placed in the plant cell to give resistance to the pests, and this transfer gives an opportunity for the production of the organisms expressed as "genetically modified" or transgenic. These transferred genes replicate with the natural plant genes after they are placed in the organism and produce protein. The process of the studies on modern technology includes (i) recognizing, (ii) characterizing, (iii) isolating, and (iv) transferring desired genes to new hosts.

The basis of the techniques used for gene transfer to plants is the integration of a DNA segment including the gene of interest into chromosomes of the plant cells and thereof the recovery of transgenic plants from transformed cells by using tissue culture methods. In general, the rate of transformed cells in tissue is quite low. That is why the prerequisite of success in gene transfer is high-frequency shoot regeneration. Most commonly used technique in gene transfer to plants is the bacterium *Agrobacterium tumefaciens*. *A. tumefaciens* is known as a "natural genetic engineer of plants" due to this trait [1]. *Agrobacterium*-mediated transformation method has been a widely used gene transfer method. The advantages of the method are wide host range of plants: agronomically and horticulturally important crops including soybean, cotton, rice, wheat, flowers, and various trees [2] and transferring a small copy number of the transfer-DNA (T-DNA) into the cytoplasm and resulting in stable integration into plant chromosome. Although it has merits as compared to other transformation methods, such as particle bombardment, electroporation, and silicon carbide fibers, it is still hard to achieve high transformation efficiency and gene expression using this method.

2. Molecular mechanism of *A. tumefaciens*-mediated DNA transfer

Agrobacterium, of the family *Rhizobiaceae*, is a genus of Gram-negative bacterium that genetically transforms host plants and causes crown gall tumors at wound sites [3] (**Figure 1**). *Agrobacterium* can transfer DNA to a broad group of organisms: plants, fungi such as yeasts, ascomycetes, and basidiomycetes, and protist such as algae [4, 5]. *Agrobacterium* is usually classified by the disease symptomology (type of opine) and host range. The genetic mechanism of

Figure 1. Crown gall in sugar beet caused by wild (oncogenic) *Agrobacterium* strain.

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host range determination is still obscure, but it was reported that several virulence (*vir*) genes on the tumor-inducing (Ti) plasmid, *virC* [6], *virF* [7], and *virH* [8] were involved in determination for the range of plant species.

Bacterial recognition of monosaccharide and phenolic compounds secreted by the plant wound site initiates the tumor induction. "Activated" *Agrobacterium* transfers a particular gene segment called transfer DNA (T-DNA) from the Ti plasmid. After T-DNA is stably integrated into the chromosomal DNA in the nucleus of the host plant, genes for opine synthesis and tumor-inducing factors on the T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumors, providing increased synthesis and secretion of opine for bacterial consumption [9]. Opine is the condensation of an amino acid with a keto acid or sugar and is a major carbon and nitrogen source for *Agrobacterium* growth. Different *A. tumefaciens* strains produce different opine phenotypes of crown gall tumors because a particular opine expressed in the tumor is used for particular bacterial growth. Most common *Agrobacterium* strains produce an octopine or a nopaline form of opines [10]. Octopine and nopaline are derivatives of arginine. Agropine is discovered in octopine-type tumors, and it is derived from glutamate [11].

The studies on (virulent) *Agrobacterium* strains that create tumor have demonstrated that a small and round DNA molecule that exists in bacterium with a size of 150–250 kb creates tumor and opine synthesis [1]. This DNA molecule that exists in *A. tumefaciens* bacterium is called Ti (tumor inducing) plasmid [12]. Several components of *Agrobacterium* are necessary for transferring the piece of bacterial DNA into the plant cell [13]. These are the following:

1. The region on the Ti plasmid is called the transfer DNA (T-DNA) [14]. The T-DNA region is a small segment that integrates into the plant genome by being transferred from bacterium into the plant cell [15]. Previous studies have demonstrated that some genes in the T-DNA region (TMS1, TMS2 and TMS3) induce tumor genesis and opine synthesis in infected cells [16].

   The T-DNA region of octopine and nopaline type plasmids is marked by right and left borders of 25 bp long random nucleotide sequences, and they have the genes that form tumor [17]. Previous studies have shown that any DNA segment inserted between these borders is easily transferred into the plant cell. Besides, it has been found out that removing the genes that forms tumor from the T-DNA region via restriction enzyme does not affect gene transfer into the plant cell [1]. The plasmids with no genes that form tumor are named as nononcogenic Ti-plasmids.

2. The second condition that plays an important role in gene transfer from *Agrobacterium* to the plant cell is the virulence (*vir*) region that is outside of the T-DNA and close to the left border with a nearly 25 kb length. Previous studies have shown that *vir* region contains six main genes (*virA*, v*irB*, *virC*, *virD*, *virE*, and *virG*) [18]. *VirA* codes for a receptor that detects and correlates with phenolic compounds leaking out of damaged plant cells, and as a consequence, *virG* is stimulated. Stimulated *VirG* takes charge of the transcription operator task for itself and the other *vir* genes. *virC* enables to separate from the borders, while *virD* gene provides the regeneration of the T-DNA strand; *virB* and *virE* genes facilitate the move of the T-DNA from the bacterium to the plant cell [19].

3. The third condition that is influential in gene transfer into the plant cells is the compounds coded by three loci (*chvA*, *chvB* and *pscA*) in the chromosomes of *Agrobacterium*, being of great importance for the bacterium to attach to the plant cell and to respond to the specific chemical (chemotaxis) [20].

3. Structure and function of Ti plasmid

Ti plasmid contains 35-kb virulence (*vir*) region, which is composed of seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*). Vir proteins are involved in signal recognition, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import, and probably T-DNA integration into the plant chromosome. Expression of *vir* genes is triggered by phenolic compounds, which are secreted from the wound site of the host plant. After bacterial and plant cell binding, *virA* gene within the bacterium is activated by signal molecules, such as sap with acidic pH (5.0–5.8), phenolic compounds, acetosyringone, and lignin or flavonoid precursors secreted from plant wound [21]. Monocyclic phenolics, such as acetosyringone, are the most effective *vir* gene inducers [22]. Uninjured plants do not produce these phenolic compounds or produce them at low levels. Phenolic compound is dramatically increased in the wounded plant and enhances transferring T-DNA during bacterial infection. Sugars also assist activation of the major phenolic-mediated wound-signaling pathway. The major role of the VirA coupled with VirG protein is activating other *vir* genes. VirA activates VirG, which is a cytoplasmic DNA-binding protein and works as a transcriptional factor to induce the expression of other *vir* genes. Heterologous system, VirD1 and VirD2, proteins act like endonucleases that cut between the third and fourth base pairs of 24 bp right and left border repeats of the T-DNA bottom strand [23]. A linear single-stranded copy of the T-DNA region is oriented from 5′ to 3′ direction, initiating at the right T-DNA border and terminating at the left border [24]. Howard and Citovsky [25] reported the structural model of the T-strand when it is transferred out of the bacterium into the plant cells. They suggested a protein-nucleic acid complex called a T-complex. This T-DNA transport intermediate has at least three components: a T-strand molecule, VirD2, and VirE2 single-strand DNA-binding protein. Citovsky et al. [26] showed that VirE coats the single-stranded DNA and forms strong, stable unfolded VirE2-ssDNA complex that is protected from external nucleolyic activity. VirC1 also helps generate a T-strand when VirD1 and VirD2 are limiting. VirD2 and VirE2 have specific nuclear localization signal (NLS) to drive T-complex into the nucleus. The direct relevance of the NLS for T-strand transfer to the host plant nucleus was confirmed by NLS deletion mutants of VirD2 and VirE that resulted in reduced T-DNA expression and tumor formation [27]. The T-DNA transfer system is similar with interbacterial conjugative transfer system of broad host range plasmids. Eleven *virB* genes in the Ti-plasmid make proteins that seem to be involved in T-DNA transfer [28]. VirB proteins are primarily linked with the cytoplasmic and periplasmic membranes to be a part of putative trans-membrane pore or channel for transferring bacterial DNA to another bacterium or host plant [29]. The main proteins for pilus formation were identified, and they are studied to find the mechanism for pili formation [30]. Three VirB proteins (VirB1, VirB2, and VirB5) are the structural subunits of the promiscuous conjugative pilus structure. VirB1, VirB2 and VirB5 needed each other for the stability and cellular localization [31].

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Another main component of Ti plasmid is T-DNA, which is actually integrated into the plant cell chromosome. The T-DNA is on average 25 kb, ranging from 10 to 30 kb in size. T-DNA region is flanked and delineated by two 25-bp direct repeats, known as the right border and left border [21]. These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). Excised single strand of T-DNA from the Ti plasmid is exported from the bacterial cell to the plant cell by the activity of the other *Agrobacterium* Vir proteins through pili. The studies have shown that a deletion of segment in the left border does not affect genetic material transfer from the bacterium into the plant cell, while the right border nucleotide sequence of the T-DNA is of vital importance for *Agrobacterium* pathogenicity, and the transfer of the T-DNA is directed from the right to left border by creating polarity [32]. Wild-type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. They are *tml*, *tms*, and *tmr* regions for leafy tumor, shooty tumor, and rooty tumor, respectively, in a plant wound site [33]. After T-DNA is integrated into the host plant, opine is synthesized, and then secreted out and imported into *Agrobacterium*. The absorbed opine molecule is catabolized by a specific enzyme in *Agrobacerium* and degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth. The Ti plasmid also has other components: opine catabolism region, conjugal transfer region, and vegetative origin of replication of the Ti plasmid (*ori*V).

The successful expression of the transgene depends on where the T-DNA integrates within the chromosome. T-DNA can be inserted near or far from transcriptional activating elements or enhancers, resulting in the activation of T-DNA-carried transgenes. The failure of transgene expression (gene silencing) can be caused by methylation or posttranscriptional gene silencing of multiple copies of transgenes. RNAs from these transgene copies may interfere with each other and then be degraded. This is one of the important merits of *Agrobacterium*-mediated transformation method because fewer gene copies are integrated compared to the direct gene transfer method (e.g., polyethylene glycol liposome-mediated transformation, electroporation, or particle bombardment) [34].

4. Adoption of plant molecular biology

A. tumefaciens has been used for plant genetic engineering extensively. Plants were genetically engineered for the purpose of developing resistance to herbicides, insect, or virus, tolerance to drought, salt, or cold, and increasing the yield [35]. The *Agrobacterium*-mediated transformation method has not only been used for commercial purpose but also for basic biology research to test study gene regulation or protein function in transgenic plants [36]. The *Agrobacterium*-mediated transformation method was improved by the strategy of developing modern binary Ti plasmid. Ti plasmids have been engineered to separate T-DNA and *vir* regions into two distinct plasmids, resulting in a binary vector and a *vir* helper plasmid, respectively [37]. Many *Agrobacterium* strains containing nononcogenic *vir* helper plasmids are called disarmed plasmid. LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105 bacterial strains have been commercially developed to have disarmed plasmid [4]. The wild-type Ti plasmid was around 200 kb, and the sizes of the processed binary

vector from wild-type Ti plasmid were reduced to less than 10 kb, which resulted in increasing transformation efficiency. Binary vector has a replication origin for both *Escherichia coli* and *Agrobacterium*, an antibiotic selectable marker for bacteria and plants, a reporter gene, such as β-glucuronidase (GUS), luciferase, or green fluorescent protein (GFP), and a T-DNA region containing a multicloning site in which genes of interest can be inserted into. In the binary vector system, T-DNA region on the binary vector from the bacterium is transformed into the host plant with the help of another plasmid containing *vir* genes [38].

5. *Agrobacterium*-mediated plant transformation protocol development

Transformation efficiency can be increased through the manipulation of either the plant or the bacterium by enhancing competency of plant tissue and *vir* gene expression, respectively [2]. To increase the virulence of bacterium by inducing the *vir* gene expression, temperature, media pH, chemical inducers such as acetosyringone [2] has been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria and host plants. Manipulation of other factors such as bacterial density, co-cultivation duration, surfactant, and vacuum infiltration has also increased transformation efficiency in many experiments [39].

Temperature is an important environmental factor that mainly affects transfer pilus (T-pilus; pili) biogenesis in *Agrobacterium*. *Agrobacterium* produced higher amounts of exocellular assembly of the major T-pilus components, VirB2 and VirB5, at 20°C and expression of VirB2 and VirB5 was inhibited at 26°C to 28°C [40]. Schmidit-Eisenlohr et al. [31] reported that optimized virulence gene induction was detected from the *Agrobacterium* grown on an agar plate at 20°C. Co-cultivation temperature effects on plant transformation have been studied. Dillen et al. [41] tested optimal co-cultivation temperature for *Nicotiana tabacum* and *Phaseolus acutifolius* transformation and highest GUS expression was detected at 19°C or at 22°C co-cultivation. A temperature of 22°C resulted in higher GUS expression than at 28°C in tobacco *Agrobacterium* mediated-transformation study [42]. It implies that low temperature during co-cultivation induces pili formation and results in high T-DNA transformation efficiency into host plants.

Other factors affecting transformation efficiency were studied. Whalen et al. [43] first reported that Silwet L-77 could be used to increase the susceptibility of *Arabidopsis* leaves to *Agrobacterium* infection by reducing surface tension with low phytotoxicity to allow aqueous droplets to spread evenly over leaf surface and to penetrate the stomatal opening. Due to these characteristics, Silwet L-77 enhances entry of bacteria into relatively inaccessible plant tissues. Silwet L-77 (0.001%) resulted in the development of disease phenotypes and toxicity at concentrations above 0.1% in *Arabidopsis* transformation study. Surfactant was reported as a most critical factor for increasing GUS activity. GUS activity was enhanced with 0.01% Silwet L-77 treatment, and highest activity was detected at 0.05%. When the concentration was greater than 0.05%, most of the immature wheat embryos could not survive [44]. Curtis and Nam [45] compared other surfactants, Pluronic F-68 and Tween-20, to Silwet L-77. It was confirmed that 0.05% (v/v) Silwet L-77 treatment was the most beneficial.

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Vacuum infiltration has also been used mostly as an aid for efficient *Agrobacterium* inoculation for flowering stage of *Arabidopsis* [46], *Brassica napus* [47], or Chinese cabbage [48] transformation. Kapila et al. [49] reported that vacuum infiltration, ranging from 1 to 0.1 mbar for 20 min application to *Proteus vulgaris* leaf transformation via *A. tumefaciens*, resulted in high transient expression, and all infiltrated leaves showed high GUS expression sectors. Dillen et al. [41] used the vacuum infiltration method on tobacco leaves and *P. vulgaris* leaf transformation to increase transformation efficiency. Vacuum and Silwet L-77 combination effect has been studied in *Arabidopsis* flower dipping method and tobacco leaf disk transformation study, and vacuum with the low concentrations of the surfactant together resulted in the highest transformation efficiency in both studies [50].

Effect of bacterial growth phase and cell density on transformation efficiency had been considered as an important factor also. In standard protocol, cells are grown to the stationary phase ($OD_{600\ nm} \approx 2–2.4$), pelleted and resuspended in inoculation medium to stationary or log or mid-log phase ($OD_{600\ nm} \approx 0.1–1.15$). High concentrations of bacteria at the stationary phase have normally been used for rice, legume, and tobacco transformation [49], and low concentrations of bacteria at the log or mid-log phase have been used for broccoli [51], cabbage [52], wheat [44], cottonwood [53], and tobacco [54]. Clough et al. [46] reported that different bacterial concentration ranging from 0.15 to 1.75 of $OD_{600\ nm}$ resulted in different transformation efficiency in *Arabidopsis* transformation.

Co-cultivation duration also affects transformation efficiency. Co-cultivation for 2–5 days has been normally used in *Agrobacterium*-mediated transformation under various co-cultivation temperature [55]. Coculture for 3 days resulted in high transformation efficiency and reached to a maximum at day 5 in citrange (*Citru sinensis* L. Osbeck × *Poncirus trifoliate* L. Raf.) [56]. Co-cultivation period of more than 5 days caused bacterial overgrowth and decreased the transformation efficiency. Many transformation experiments in different plant species, such as tea (*C. sinensis* L.), cauliflower, white spruce (*Picea glauca*), and citrange, showed that 2–3 days of co-cultivation gave rise to higher transformation efficiency under room temperature [2]. Therefore, 2–3 days co-cultivation has been routinely used in most transformation protocols, since longer co-cultivation causes bacterial overgrowth, which covers the leaf tissue and brings toxicity under room temperature co-cultivation condition.

Bacteria preculture on minimal media for 3 days has been routinely practiced to induce pili formation before co-cultivation. Clough et al. [46] compared *vir* genes induction in liquid medium culture to the plate culture for 20 h at 19°C. However, they reported that two different preculture methods showed similar transformation in *Arabidopsis*.

Acetosyringone (AS) is a phenolic compound produced from wounded plant cells. AS concentration has been known as a very important factor affecting transformation efficiency. Fullner and Nester [57] reported that *Agrobacterium* did not produce pili without 200 μM AS at both 19°C and 25°C. Results from a wheat inflorescence transformation experiment showed that T-DNA cannot be transformed to the plant tissue without AS [39]. These results indicated that AS is the main factor in the low temperature co-cultivation condition that induces VirB protein, which is a subunit of pili. Various optimal concentrations of AS depended on the plant species. But in most experiments, optimal AS concentrations for different plant species are in the range of 50–400 μM AS.

6. New methods for high-transformation frequency via *A. tumefaciens*

6.1. Utilizing explant's negative atmospheric pressure for increased gene transformation

In the study conducted by Beyaz et al. [58], the aim was to increase transformation efficiency in flax (*Linum usitatissimum* L.) by increasing osmotic pressure of the tissue as plant material flax cultivars "Madaras," "Clarck," and "1886 Sel." were used in the study. Sterilized seeds got cultured on Murashige and Skoog (MS) [59] medium for germination and seedling establishment. *A. tumefaciens* strain, GV2260 harboring plasmid p35S GUS-INT that contains neomycin phosphotransferase II (*npt-II*) gene, was used for inoculation. GV2260 strain carrying p35S GUS-INT plasmid was grown overnight in a liquid Nutrient Broth (NB) medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 28°C in a rotary shaker (180 rpm) (0.6 of $OD_{600\ nm}$) and used for transformation. In the study, conventional transformation method in which hypocotyls were directly cultured on co-cultivation medium after inoculation with 500 μl bacterial solution for 20 min was compared to the method in which 7-old-day flax seedlings having cotyledon leaves without root system dried for 35 min in laminar flow were inoculated with 500 μl bacterial solution for 20 min (**Figure 2**). In both the transformation methods, after inoculation, hypocotyl segments—0.5 cm in length—were cultured on co-cultivation medium for 2 days. Then, the explants were transferred to a regeneration medium supplemented with 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, 100 mg l^{-1} kanamycin, and 500 mg l^{-1} augmentin and cultured for 4 weeks in a culture room at a temperature of 24 ± 1°C. Shoots were transferred to a rooting medium containing 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1}

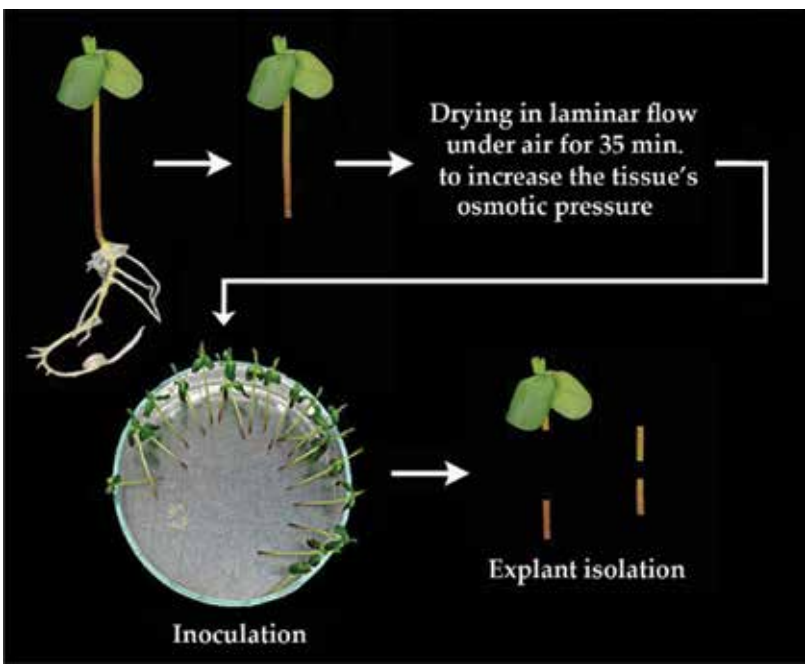

Figure 2. Explant isolation from inoculated 7-old-day flax seedlings having cotyledon leaves without root system.

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kanamycin in Magenta vessels to culture for 3 weeks at 24 ± 1°C. After root formation, plantlets were transferred to pots in a growth room for 3 weeks to recover putative transgenic plants. The presence of neomycin phosphotransferase II (*npt-II*) gene in transformants was confirmed by PCR.

The lowest results were noted in the first inoculation method in which hypocotyls were directly cultured on co-cultivation medium after inoculation (**Table 1** and **Figure 3a**). Keeping 7-day-old seedlings having cotyledon leaves without root system under air in laminar flow was aimed to enable seedlings to intake bacterial solution rapidly toward inner cells by increased osmotic pressure and consequently to increase the transformation efficiency. The highest results of all characters examined in all cultivars were obtained from a newly described inoculation method in which 7-day-old sterile flax seedlings having cotyledon leaves and no root system were inoculated with 500 μl bacterial solution for 20 min after drying in sterile cabin for 35 min (**Table 1** and **Figure 3b**). Shoot regeneration percentage, mean shoot number per explant, mean shoot number per petri dish, mean shoot number rooted per Magenta vessels, total plant number growing in soil, total PCR+ plant number, and transformation efficiency were recorded in a newly described transformation method as 70.83, 1.58, 29.50, 27.00, 97.66, 82.00, and 84.19%, while they were 34.50, 1.09, 9.25, 6.91, 18.00, 5.66, and 30.45% in routinely used transformation method (**Table 1**).

Transformation efficiency was recorded as 84.19% from hypocotyl explants excised from inoculated 7-day-old sterile seedlings having cotyledon leaves without a root system, while it was

Cultivars	Inocu. method	Regeneration (%)	Mean shoot number per explant	Mean shoot number per petri dish	Mean shoot number rooted per Magenta vessel	Total plant number growing in soil[1]	Total PCR+ plant number[2]	Transfor. efficiency (%) (2/1 × 100)
"Madaras"	1	41.00[b]	1.09[b]	11.25[b]	8.50[b]	25.00[b]	8.00[b]	30.76[b]
	2	71.25[a]	1.65[a]	30.00[a]	28.75[a]	104.00[a]	88.00[a]	84.61[a]
"Clarck"	1	42.50[b]	1.03[b]	10.50[b]	8.25[b]	18.00[b]	6.00[b]	33.33[b]
	2	73.75[a]	1.65[a]	33.25[a]	28.75[a]	103.00[a]	82.00[a]	79.61[a]
"1886 Sel."	1	20.00[b]	1.15[b]	6.00[b]	4.00[b]	11.00[b]	3.00[b]	27.27[b]
	2	67.50[a]	1.46[a]	25.25[a]	23.50[a]	86.00[a]	76.00[a]	88.37[a]
Mean of cultivar[s]	**1**	**34.50**	**1.09**	**9.25**	**6.91**	**18.00**	**5.66**	**30.45**
	2	**70.83**	**1.58**	**29.50**	**27.00**	**97.66**	**82.00**	**84.19**

Values followed by the different letters in a column are significantly different at the 0.01 level. Each value is the mean of 4 replications containing 25 explants per replication. All experiments were repeated 2 times.

1. Hypocotyls were directly transferred to co-cultivation medium after inoculation with 500 μl bacterial solution for 20 min.

2. Seven-day-old sterile seedlings having cotyledon leaves without root system were dried in sterile cabin for 35 min. Then, they were inoculated with 500 μl bacterial solution for 20 min. Finally, hypocotyls excised from inoculated seedlings were placed on co-cultivation medium.

Table 1. The development of shoots from hypocotyls inoculated with *A. tumefaciens* with two different methods on selection medium 4 weeks after culture initiation and transgenic plant development.

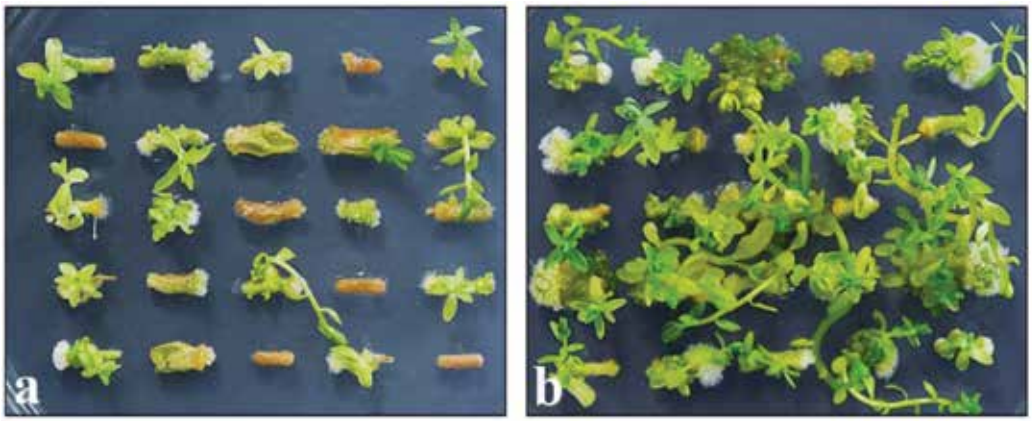

Figure 3. Shoot regeneration from hypocotyl explants of cv. "Madaras" inoculated by two different methods (a) first inoculation method, (b) second inoculation method on selection medium 4 weeks after culture initiation.

30.45% in routinely used transformation method where hypocotyls were directly cultured on selection medium after inoculation (**Table 1**). In the study, a new inoculation method was based on enabling seedlings to intake bacterial solution rapidly toward inner cells by increased osmotic pressure of explants to increase the number of cells inoculated and consequently to higher transgenic shoots was developed.

6.2. The effect of squirting cucumber (*Ecballium elaterium* (L.) A. Rich) fruit juice on *A. tumefaciens*-mediated transformation

Squirting cucumber (*E. elaterium* (L.) A. Rich.), from the cucumber family, contains cucurbitacins such as α-elaterin (cucurbitacin E), β-elaterin (cucurbitacin B), elatericine A (cucurbitacin D), and elatericine B (cucurbitacin I) [60] that are poisonous and showed antibacterial activities [61]. However, it was found out that mature fruit juice of the plant stimulated growth of *A. tumefaciens* and increased gene transfer frequency in tobacco [62]. That was why, this study supported by a project numbered 113O280 from the Scientific and Technological Research Council of Turkey (TUBİTAK) was conducted to determine the effect of squirting cucumber (*E. elaterium* (L.) A. Rich.) fruit juice on *A. tumefaciens*-mediated gene transfer in flax (*L. usitatissimum* L.).

Flax cultivar "Madaras" obtained from "Northern Crop Science Laboratories," North Dakota, USA, was used in the study. Seed sterilization was achieved according to the protocol described by Yildiz and Er [63] and then seeds were sown on Murashige and Skoog (MS) [59] medium for germination and further seedling development. The mature fruits of squirting cucumber were squeezed manually and fruit juice collected in the glass jar was filter-sterilized by using 0.45 μm filters and stored at −20°C. Different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800, and 1600 μl l^{-1}) were added to regeneration medium after autoclaving in order to determine the most effective concentration on shoot regeneration from hypocotyl explants. The highest shoot regeneration was recorded in 400 μl l^{-1} squirt-

ing cucumber fruit juice concentration. *A. tumefaciens* strain GV2260 harboring plasmid p35S GUS-INT that contains neomycin phosphotransferase II (*npt-II*) gene was used for inoculation. GV2260 strain carrying p35S GUS-INT plasmid was grown overnight in a liquid NB (Nutrient Broth) medium containing 50 mg l^{-1} kanamycin, 50 mg l^{-1} rifampicin, and different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800 and 1600 μl l^{-1}) at 28°C in a rotary shaker (180 rpm) (0.6 of $OD_{600\ nm}$) and used for transformation. Hypocotyl explants of 7-day-old flax seedlings were inoculated with bacteria in a liquid medium having different squirting cucumber fruit juice concentrations (0-control, 200, 400, 800 and 1600 μl l^{-1}) for 20 min. After inoculation, hypocotyl explants were transferred to a solid medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, and 400 μl l^{-1} squirting cucumber fruit juice for co-cultivation for 2 days in a culture room at a temperature of 24 ± 1°C. Explants were then transferred to the medium that had the same content as co-cultivation, supplemented with 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid for selection for 4 weeks. Regenerated shoots were transferred to a rooting medium supplemented with 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1} kanamycin in Magenta vessels for 3 weeks at 24 ± 1°C. After root formation, plantlets were transferred to pots in a growth room for 3 weeks to recover putative transgenic plants. The presence of neomycin phosphotransferase II (*npt-II*) gene in transformants was confirmed by PCR.

At the end of the study, it was determined that 400 μl l^{-1} squirting cucumber fruit juice added to bacterial growth and inoculation medium was found the most effective fruit juice concentration on gene transformation frequency. The highest shoot regeneration percentage on selection medium, having antibiotics was recorded 54.00% as the highest from the medium containing 400 μl l^{-1} fruit juice 4 weeks after culture initiation. It was 41.25% in control treatment having no fruit juice. The highest shoot number per explant was recorded in the treatment where 1600 μl l^{-1} fruit juice was used as 2.06. From the medium having 400 μl l^{-1} fruit juice, 1.16 shoots per explant were recovered. The highest shoot length was noted as 1.73 cm from growth medium containing 1600 μl l^{-1} fruit juice. Total shoot number per petri dish was noted as 12.53 as the highest from medium containing 400 μl l^{-1} fruit juice (**Table 2**). In 1600 μl l^{-1} fruit juice concentration in which the highest results were recorded in shoot number per explant and the highest shoot length per explant, total shoot number per petri dish was achieved as 7.75—the lowest.

After a 4-week-cultivation on selection medium, rooted explants were directly transferred to soil by skipping *in vitro* rooting stage (**Figure 4a**). From the medium having 400 μl l^{-1} fruit juice, 11.00 rooted explants were transferred to soil, and finally, 3.00 putative plantlets were grown in soil, reached maturity, and all were morphologically normal (**Figure 4b**). On the other hand, 5.57 putative transgenic plantlets were grown from a medium containing 800 μl l^{-1} fruit juice. Out of 8.70 rooted explants were transferred to soil, only 1.86 putative transgenic plants were grown in soil in control application where no fruit juice was used (**Table 2**). After PCR analysis, all the plants grown in soil from selection medium containing 400 μl l^{-1} fruit juice were confirmed to be transgenic, while two plants were found transgenic from medium having 800 μl l^{-1} fruit juice. The highest transformation efficiency was noted as 100.00% in 400 μl l^{-1} fruit juice treatment. In control where squirting cucumber fruit juice was not used, transformation efficiency was 0.00% meaning that no transgenic plants were recovered (**Table 2**).

Fruit j. concent. (μl l^{-1})	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil[1]	The number of PCR+ transgenic plants[2]	Transfor. efficiency (%) (2/1 × 100)
0	41.25[b]	1.17[b]	1.24[b]	9.53[b]	8.70[ab]	1.86[c]	0.00[b]	0.00
200	49.00[a]	1.04[b]	1.05[b]	10.19[ab]	9.00[ab]	2.65[b]	1.00[b]	37.73
400	54.00[a]	1.16[b]	1.50[a]	12.53[a]	11.00[a]	3.00[b]	3.00[a]	100.00
800	42.00[b]	1.07[b]	0.97[b]	8.98[b]	7.65[b]	5.57[a]	2.00[ab]	35.90
1600	38.75[c]	2.06[a]	1.73[a]	7.75[c]	6.25[c]	2.00[c]	0.00[b]	0.00

Values followed by the different letters in a column are significantly different at the 0.01 level.
Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 2. The effect of squirting cucumber fruit juice on shoot regeneration from hypocotyl explants inoculated with *A. tumefaciens* on selection medium containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid 4 weeks after culture initiation and recovery of transgenic plants.

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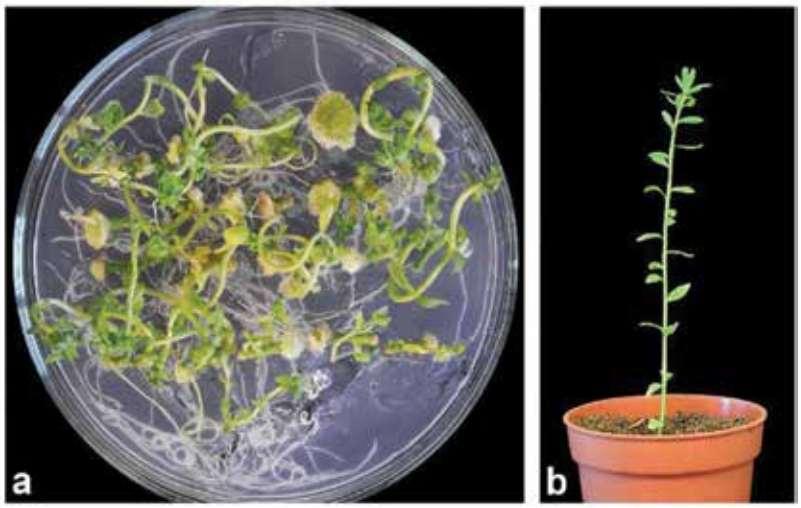

Figure 4. Shoot regeneration from hypocotyl explants of cv. "Madaras" inoculated with *A. tumefaciens* on selection medium containing 100 mg l^{-1} kanamycin, 500 mg l^{-1} duocid, and 400 μl l^{-1} fruit juice 4 weeks after culture initiation (a) and PCR-confirmed transgenic plant growing in soil (b).

Results showed positive effects of squirting cucumber's fruit juice on regeneration and transformation at 400 μl l^{-1} concentration as compared to control. At fruit juice concentrations over and below 400 μl l^{-1} in the culture medium, shoot regeneration and transformation were hindered significantly. Kanamycin-resistant shoots were formed in a medium containing high concentration (100 mg l^{-1}) of kanamycin that prevents the escaped shoots in the selection medium efficiently. PCR analysis confirmed that all raised plants were transgenic in the medium having squirting cucumber's fruit juice at a concentration of 400 μl l^{-1}.

The data presented here clearly indicates that the addition of squirting cucumber's fruit juice to bacterial growth, inoculation, and co-cultivation media improved the transformation frequency of flax significantly. We conclude that squirting cucumber's fruit juice induces *vir* genes leading to increased transformation efficiency.

6.3. Use of magnetic field strength for high-transformation frequency via *A. tumefaciens*

Exposure of seeds to magnetic field for a short time was found to help in accelerated sprouting and growth of the seedlings. It was reported that magnetic conditions stimulated plant growth [64–66]. The current study was aimed to examine the effects of magnetic field strength on *A. tumefaciens*-mediated gene transfer in flax (*L. usitatissimum* L.). Seeds of flax cv. "Madaras" obtained from "Northern Crop Science Laboratories," North Dakota, USA, were used. First, seeds were exposed to different magnetic field strengths (0-control, 75, 150, and 300 mT) for 24 h. Then, they were surface sterilized with 40% commercial bleach containing 5% sodium hypochlorite at 10°C for 12 min with continuous stirring, and they were washed three times with sterile distilled water at the same temperature. Sterilized seeds were germinated on MS medium in Magenta vessels. Hypocotyl explants excised from 7-day-old seedlings were used

for regeneration. GV2260 line of *A. tumefaciens* having p35S GUS-INT plasmid containing *npt-II* gene that determines kanamycin resistance was used in transformation studies. Hypocotyls were kept in petri dishes containing 50 ml sterile water with 500 µl bacterial solution during 20 min for inoculation. Inoculated hypocotyls were then cultured on MS medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, for co-cultivation for 2 days in culture room at a temperature of 24 ± 1°C. Then, explants were transferred to selection medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, 50 mg l^{-1} kanamycin, and 500 mg l^{-1} duocid and cultured for 4 weeks. Shoots were kept in a rooting medium containing 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1} kanamycin in Magenta vessels for 3 weeks at 24 ± 1°C. Then, plantlets were transferred to pots in a growth room for 3 weeks to obtain putative transgenic plants. The presence of the *npt-II* gene was verified by PCR analysis in candidate plants.

The highest results with respect to regeneration percentage, shoot number per explant, the highest shoot length per explant, total shoot number per petri dish, the number of rooted explants transferred to soil, the number of putative transgenic plants growing in soil, and the number of PCR+ transgenic plants were recorded in the treatment, where seeds were exposed to 75 mT magnetic field strength as 82.00%, 2.40, 3.40, 27.60, 16.40, 12.60, and 8.00, respectively. In control application where no magnetic field strength was used, the lowest results were obtained in all characters examined. Out of 12.60 putative transgenic plants growing in soil, 8.00 was found PCR positive (**Figure 5**).

Results clearly showed that 75 mT magnetic field strength increased *A. tumefaciens*-mediated transformation frequency in flax. At 75 mT magnetic field strength, out of 12.60 putative transgenic plants, 8 were confirmed transgenic after PCR analysis that meant 63.49% transforma-

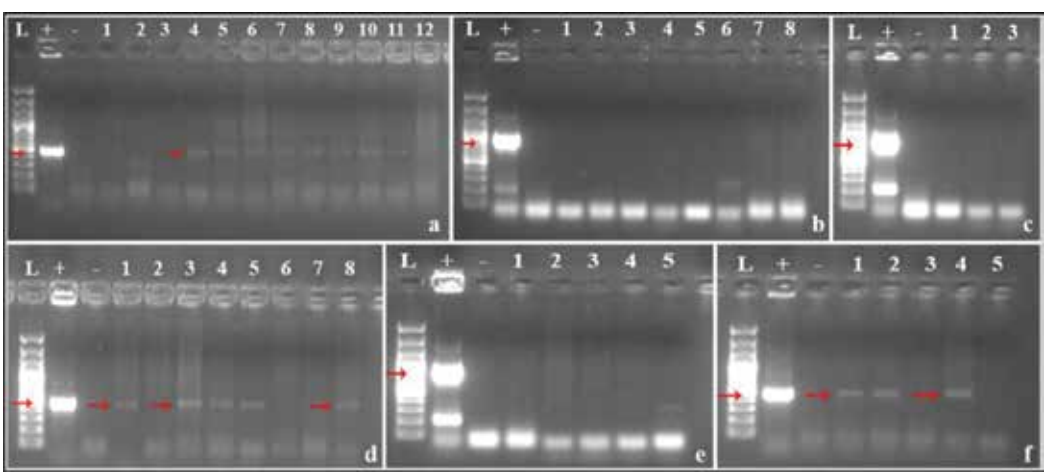

Figure 5. PCR analysis of genomic DNA from putative transgenic plants cv. "Madaras" for amplification of 458 bp *npt-II*. *L* DNA ladder 100 bp, + Plasmid as a positive control. Water as a negative control. (a) Detection of the *npt-II* gene in 75 mT magnetic field strength, (b) detection of *chv* gene in 75 mT magnetic field strength, (c) detection of *chv* gene in 300 mT magnetic field strength, (d) detection of the *npt-II* gene in 150 mT magnetic field strength, (e) detection of the *chv* gene in 150 mT magnetic field strength, (f) detection of the *npt-II* gene in 300 mT magnetic field strength.

Mag. field stren. (mT)	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil[1]	The number of PCR+ transgenic plants[2]	Transfor. efficiency (%) (2/1 × 100)
0	41.25[b]	1.17[c]	1.24[c]	9.53[d]	8.70[d]	1.86[d]	0.00[d]	0.00
75	82.00[a]	2.40[a]	3.40[a]	27.60[a]	16.40[a]	12.60[a]	8.00[a]	63.49
150	78.00[a]	2.36[a]	2.76[b]	24.80[b]	15.60[b]	8.40[b]	5.00[d]	59.52
300	46.00[b]	1.70[b]	2.54[b]	12.80[c]	9.20[c]	5.50[c]	3.00[c]	54.54

Values followed by the different letters in a column are significantly different at the 0.01 level.
Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 3. The effect of magnetic field strengths on transgenic shoot regeneration and recovery of transgenic plants from hypocotyls inoculated with *A. tumefaciens* on selection medium containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid 4 weeks after culture initiation and recovery of transgenic plants.

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Gamma dose (Gy)	Regeneration (%)	Shoot number per explant	The highest shoot length per explant (cm)	Total shoot number per petri dish	The number of rooted explants transferred to soil	The number of putative transgenic plants growing in soil[1]	The number of PCR+ transgenic plants[2]	Transfor. efficiency (%) (2/1 × 100)
0	41.25[c]	1.17[c]	1.24[c]	9.53[d]	8.70[c]	1.86[a]	0.00[d]	0.00
40	70.00a	2.20[b]	1.79[a]	30.80[a]	13.20[a]	25.00[a]	25.00[a]	100.00
80	66.00[b]	2.11[b]	1.58[b]	27.85[a]	14.00[a]	25.00[a]	22.00[b]	88.00
120	61.00[b]	2.06[a]	1.57[b]	25.13[a]	12.20[b]	25.00[a]	20.00[c]	80.00

Values followed by the different letters in a column are significantly different at the 0.01 level.
Each value is the mean of 5 replications containing 10 explants per replication. All experiments were repeated 2 times.

Table 4. The effect of gamma radiation on transgenic shoot regeneration from hypocotyls inoculated with *A. tumefaciens* on selection medium containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} duocid 4 weeks after culture initiation.

tion efficiency (**Table 3**). To our knowledge, this was the first study indicating that magnetic field strength could increase *A. tumefaciens*-mediated transformation.

This study was supported by a project number 113O280 from the Scientific and Technological Research Council of Turkey (TUBİTAK).

6.4. The effect of gamma radiation on *A. tumefaciens*-mediated transformation

Gamma rays give rise to cytological, biochemical, physiological, and morphological changes in cells and tissues via producing free radicals in cells [67]. Although higher doses of gamma radiation were inhibitory [68], lower doses are stimulatory. Low doses of gamma rays have been reported to increase cell proliferation, germination, cell growth, enzyme activity, stress resistance, and crop yields [69–72].

In the study supported by the project number 113O280 from the Scientific and Technological Research Council of Turkey (TUBİTAK), the effects of gamma radiation of radioactive cobalt (^{60}Co) γ rays on *A. tumefaciens*-mediated gene transfer to flax were examined. Flax seeds of cv. "Madaras" were irradiated with different gamma doses (0-control, 40, 80, and 120 Gy), and then surface sterilized by using the protocol described in Section 6.3. Sterilized seeds were then sown in Magenta vessels having MS medium for germination. Hypocotyls of 7-day-old seedlings were used for regeneration. GV2260 line of *A. tumefaciens* having p35S GUS-INT plasmid containing *npt-II* gene that determines kanamycin resistance was used in transformation studies. Hypocotyls were kept in petri dishes containing 50 ml sterile water with 500 μl bacterial solution during 20 min for inoculation. Inoculated hypocotyls were then cultured on MS medium containing 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, for co-cultivation for 2 days in a culture room at a temperature of 24 ± 1°C. Then, explants were transferred to selection medium conta gamma dose with respect ining 1 mg l^{-1} BAP, 0.02 mg l^{-1} NAA, 50 mg l^{-1} kanamycin, and 500 mg l^{-1} duocid, and cultured for 4 weeks. Shoots were then transferred to a rooting medium having 3 mg l^{-1} indole-butyric acid (IBA) and 100 mg l^{-1} kanamycin in Magenta vessels for 3 weeks at 24 ± 1°C. Plantlets were transferred to pots to develop for 3 weeks. The presence of the *npt-II* gene in putative transgenic plants was verified by PCR analysis.

The highest results were recorded in 40 Gy gamma dose with respect to the number of putative transgenic plants growing in soil, the number of PCR+ transgenic plants, and transformation efficiency as 25.00, 25.00, and 100.00% (**Table 4**). It could be concluded that low gamma radiation increased transformation efficiency significantly compared with control application where no gamma radiation was used.

7. Conclusion

A. tumefaciens as a plant pathogen naturally infects the wound sites in dicotyledonous plants and induces disease known as crown gall, and this bacterium has been widely used for the introduction of foreign genes into plants and consequent regeneration of transgenic plants. However, *A. tumefaciens*-medited gene transfer is quite difficult in most of the plant species.

The success of genetic transformation via *A. tumefaciens* is limited due to the fact that plant's defence mechanism will be active when pathogen attacks. That is why manipulations of the plant and bacterium and physical conditions have been applied to increase the virulence of bacterium and to increase the transformation efficiency. To our knowledge, the four new transformation protocols described in this chapter are new and not reported elsewhere before. And newly reported protocols can be easily used to increase the transformation efficiency in most plant species. We hope that transformation protocols described in this chapter may help researchers to increase the success of transformation studies via *A. tumefaciens*.

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Chapter 3

A New Plant Breeding Technique Using ALSV Vectors to Shorten the Breeding Periods of Fruit Trees

Noriko Yamagishi and Nobuyuki Yoshikawa

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66059

Abstract

Fruit trees have a long juvenile phase. For example, the juvenile phase of apple lasts for 6–12 years and is a serious constraint for creating new varieties by breeding based on crossing and selection. In this chapter, we report a novel technology using the *apple latent spherical virus* (ALSV) vector to accelerate flowering time and life cycle in apple and pear seedlings. Inoculation of apple and pear cotyledons immediately after germination with ALSV-AtFT/MdTFL1 concurrently expressing *Arabidopsis FLOWERING LOCUS T* (*AtFT*) gene and suppressing apple *TERMINAL FLOWER 1-1 (MdTFL1-1)* gene can shorten the period from seeding to flowering to 1.5–3 months after germination and generation times in order to obtain next-generation seeds in 1 year or less. Most next-generation seedlings obtained from ALSV vector–infected plants were free of the virus. We also developed a method for eliminating ALSV vectors from infected apple and pear plants by only high-temperature treatment. A method combining the promotion of flowering in apple and pear by ALSV vector with an ALSV elimination technique is expected to see future application as a new plant breeding technique that can significantly shorten the breeding periods of apple and pear.

Keywords: apple latent spherical virus (ALSV) vector, apple, pear, promotion of flowering, elimination of ALSV

1. Introduction

Woody fruit trees have a long juvenile phase—the period between germination and flowering of plants. In apple and pear, the vegetative growth (juvenile phase) generally lasts for 6–12 years with no flowering and fruiting. After transition from the juvenile phase to the adult phase, the trees flower/fruit every year [1–3].

Several apple varieties have been bred to in order to impart resistance to diseases and insect pests, as well as for quality improvement of the fruit. Breeding of fruit trees is conventionally conducted by crossing and selection [4, 5]. So, it is necessary to cultivate many hybrid seedlings to examine their characteristics for breeding new varieties. For example, "Fuji," an apple variety that currently has the world's highest production, was selected from 787 hybrid seedlings obtained by crossing "Kokkou (Ralls Janet)" with "Delicious" apples. This variety flowered and fruited approximately 12 years after seeding [6]. Since apple fruits are produced continuously on the same tree over dozens of years, breeding of a high-quality variety is very important for apple production.

A long juvenile period of apple seedlings is a major barrier to the breeding of new varieties. Moreover, breeding of fruit trees requires large fields for cultivation of seedlings and considerable labor for their management. Technologies for shortening the juvenile phase, including grafting onto dwarfing rootstocks, have been developed; however, despite the use of these technologies, several years are required for flowering/fruition [1, 2].

In recent years, global warming is advancing owing to an increase of greenhouse gas concentrations in the atmosphere. Fruit trees are susceptible to global warming because their important physiological phenomena, such as flowering and dormancy, are dependent on environmental climates. In Japan, the influences of global warming on the production of apples have already begun to appear, with poor coloration of fruit, increased frost injury due to early flowering phase, and damage by harmful insects reported [7]. It is presumed that a further shift of land suitable for cultivation of fruit trees and changes in the distribution of diseases and pests caused by global warming will be accelerated in the future; therefore, it is necessary to implement rapid fruit breeding technologies. The improvement in efficiency of fruit tree breeding, particularly shortening of the juvenile phase, is gaining a great deal of attention.

Many genes involved in plant flowering have been identified in the past 20 years in model plants such as *Arabidopsis thaliana* and rice. The most important gene among them is the *FT* gene, which encodes the flowering hormone, "florigen." Although it has been known since its discovery that florigen is a mobile signaling substance which is synthesized in leaves and transferred to the shoot apical meristem, it is sensitive to photoperiod. Interestingly, it took approximately 70 years to identify this molecule [8]. The *FT* gene encodes an approximately 20-kDa water-soluble protein belonging to the phosphatidylethanolamine-binding protein (PEBP) family, and was reported for the first time in *A. thaliana* (*FLOWERING LOCUS T of A. thaliana* (*AtFT*) gene) and rice (*Heading gate3a* (*Hd3a*) gene) [9–12]. The *FT* protein is conserved widely in angiosperms and has a common function of promoting flowering [8]. In fruit trees, it has been reported that flowering was promoted in genetically modified orange and pear expressing the citrus florigen-like gene, *CiFT* [13, 14]. Early flowering also occurs in genetically modified apples where the florigen-like gene, *MdFT*, is expressed [15, 16].

Proteins belonging to the PEBP family contain the *A. thaliana TFL1* gene [17–19]. The *TFL1* gene is highly homologous with the *AtFT* gene but has an adverse function that it suppresses

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flowering. Fruit trees have a gene homologous to the *TFL1* gene; the suppression of *MdTFL1-1*, an apple *TFL1*-like gene, was reported as leading to early flowering [20, 21]. Similarly, in pear, suppression of *PcTFL1-1* and *PcTFL1-2* led to the induction of early flowering [22]. In research using other genes, Flachowsky et al. reported that an early flowering apple line T1190 expressing *BsMADS4*, a transcription factor involved in the initiation of flower bud formation, could be used for breeding a disease-resistant variety via a rapid cycle breeding system where a generation was completed in 1 year [23].

Viral vector technology is a tool to express or suppress the target gene in the virus-infected plant [24, 25]. Infection of a plant by a plant virus vector integrated with the target gene for expression results in the occurrence of expression of the gene in the infected plant. Conversely, when attempting to suppress the expression of a gene, infection of a plant by a viral vector with a part of the target gene leads to induction of suppression of the target gene in the infected plant by virus-induced gene silencing (VIGS). Viral vectors have the advantage of allowing us to evaluate phenotypes rapidly. Recently, we constructed apple latent spherical virus (ALSV) vectors by adding cloning sites to the ALSV genome. The ALSV vector system can be used for the expression of a foreign gene and VIGS in various plant species [26–38].

In this chapter, we introduce an ALSV vector-based technology for early flowering and shortening of a generation time in the apple and pear. Use of this technology allows apples and pear to complete a generation within 1 year, which reduces a breeding term of fruit trees substantially. Moreover, because the viral vectors can easily be removed from both next-generation seedlings and infected plants, this technology is considered not applicable to regulations of the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Protocol).

2. Apple latent spherical virus (ALSV) vectors

ALSV is a spherical virus with a diameter of approximately 25 nm originally isolated from an apple tree and is composed of two RNA genome segments (RNA1 and RNA2) and three types of coat proteins (Vp25, Vp20, and Vp24) [39–41]. The apple is the only natural host of ALSV; however, ALSV has a relatively wide range of hosts, and it can experimentally infect not only herbaceous plants such as *Solanaceae, Cucurbitaceae, Fabaceae,* and *Gentianaceae* but also fruit trees belonging to *Rosaceae* [26–38]. ALSV is characterized by latent infection causing no symptoms in almost all host plants and invasion of the shoot apical meristem of infected plants.

Previously, we used ALSV vectors constructed using pUC18 plasmid. These vectors had to be inoculated to *Chenopodium quinoa* plants for virus propagation [42]. Currently, we constructed RNA1 and RNA2 vectors using Ti plasmid where cloning sites for foreign gene were introduced in both RNA vectors [43, 44]. As shown in **Figure 1a**, RNA1 vector has a cloning site in the 3′-noncoding region (SM), and RNA2 vector has two cloning sites in the ORF (XSB) and

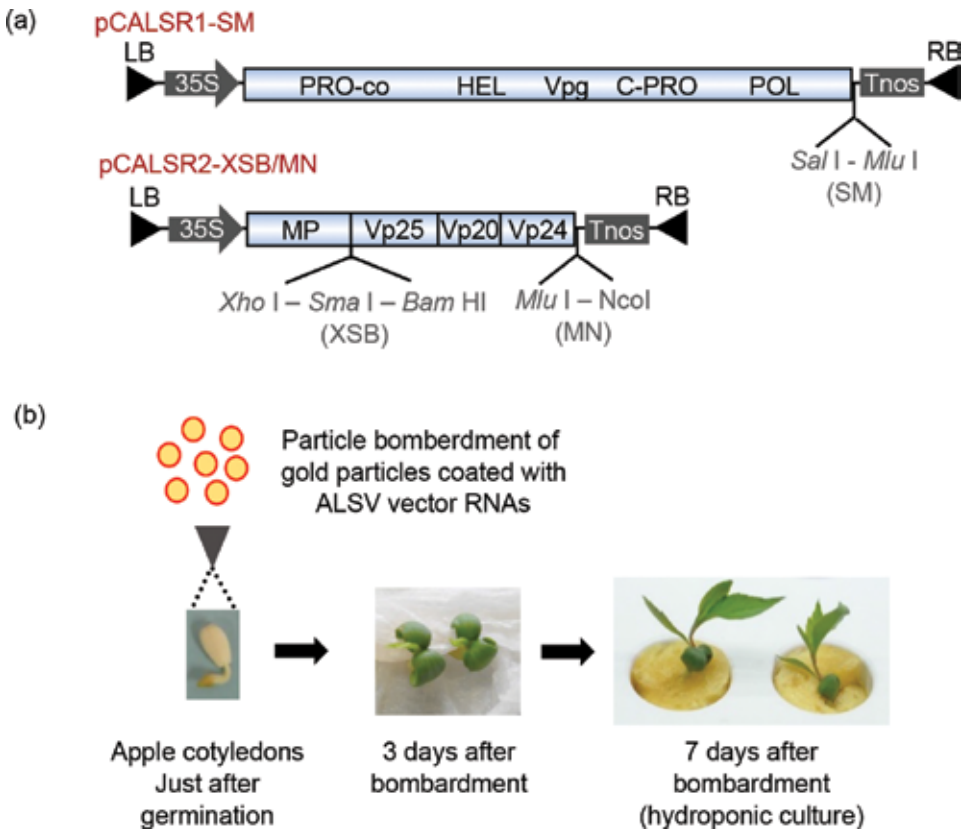

Figure 1. (a) Genetic map of ALSV binary vector (pCALSR1-SM and pCALSR2-XSB/M). LB and RB, left and right borders, respectively. 35S, CaMV 35S RNA promoter. Tnos, nopaline synthase terminator. SM, MN, and XSB, cloning sites. (b) Outline of biolistic inoculation of ALSV vectors to apple cotyledon just after germination.

the 3′-noncoding region (MN). These vectors can be inoculated to *Nicotiana benthamiana* by agro-infiltration [43, 44].

3. Efficient inoculation method of ALSV vector

Generally, it is difficult to directly inoculate cDNA clones of ALSV vector to apple and pear seedlings because cDNA clone results in a very low infection rate. Therefore, we first inoculated the clones to an experimental plant, *N. benthamiana* by agro-infiltration, which have been established to introduce many plant viruses to plants, for viral multiplication. Then, we reinoculated the virus preparation (crude sap of infected *N. benthamiana* leaves) to a propagation host *C. quinoa* for preparing the high-titer inocula for fruit trees. We established an efficient inoculation method using RNA sample extracted from the infected *C. quinoa* leaves by particle bombardment [42, 45]. Inoculation of apple seedlings immediately after germination

by this method (**Figure 1b**) allowed us to achieve 90–100% infection rate. Cotyledons immediately after germination of fruit trees appear to be highly susceptible to viral infection.

4. Induction of early flowering of apple and pear by ALSV vector infection

We first constructed an ALSV vector expressing *AtFT* (ALSV-AtFT) and inoculated this vector to cotyledons of apple seedlings as shown in **Figure 1a**. The results indicated that approximately 30% of the infected seedlings formed flower buds and flowered 1.5–2 months after the inoculation at the stage 7–8 true leaves [30]. This was likely the result of expression of *AtFT* from ALSV-AtFT in the shoot apical meristem of the infected seedlings. The flowers induced by ALSV-AtFT infection showed an apparently normal morphology. Pollens collected from these flowers were used for pollination of apple flowers that flowered naturally, which led to the formation of fruits with normal seeds. Thus, inoculation of ALSV-AtFT allowed us to achieve early flowering in apple seedlings. However, the flowering rate was no greater than approximately 30% of the infected seedlings, and the flowered seedlings shifted to vegetative growth and flowered again only rarely. In the following experiments, we constructed ALSV-MdTFL1 which a part of *MdTFL1-1* gene was inserted in a cloning site in the XSB site of RNA2 vector. This vector suppressed the expression of an *MdTFL1-1* gene in infected apple [29], and the infection of this vector resulted in the formation of flower buds 1.5–3 months after inoculation (stage 8–19 true leaves). The infected apple seedlings showed continuous flowering where they formed flower buds on the extending auxiliary buds for several months. Unfortunately, the rate of flowering by ALSV-MdTFL1 infection was as low as approximately 10% of the infected seedlings.

Finally, we constructed an ALSV vector (ALSV-AtFT/MdTFL1) that expresses *AtFT* and suppresses *MdTFL1-1* gene concurrently [46]. Surprisingly, greater than approximately 90% of ALSV-AtFT1/MdTFL1-infected apple seedlings formed flower buds and flowered 1.5–3 months after inoculation (stage 7–22 true leaves) (**Figure 2a**), and the majority of these early flowering seedlings showed continuous flowering in which they flowered continuously over several months (**Figure 2b**). It was confirmed that their pollens were fertile and pollination between early flowering individuals led to fruit formation (**Figure 2c**). Their fruit skin color displayed green, yellow, and red coloring in the process of maturation depending on the individual (**Figure 2d**). Their fruit size was approximately 2.5–4.5 cm, and seeds formed inside, with seeds germinating and growing normally after breaking of dormancy (**Figure 2d**) [46]. In addition, the total soluble solids (TSS) determined by a refractometer was showed 7.8–13.5% in their juice. From the above, fruits formed in seedlings infected with ALSV-AtFT/MdTFL1 were likely to be used for evaluating quality regarding their skin color and sugar content.

It was revealed that infection of apple seedlings by ALSV-AtFT1/MdTFL1 allowed us to shorten one generation (from seeds of the current generation to formation of the

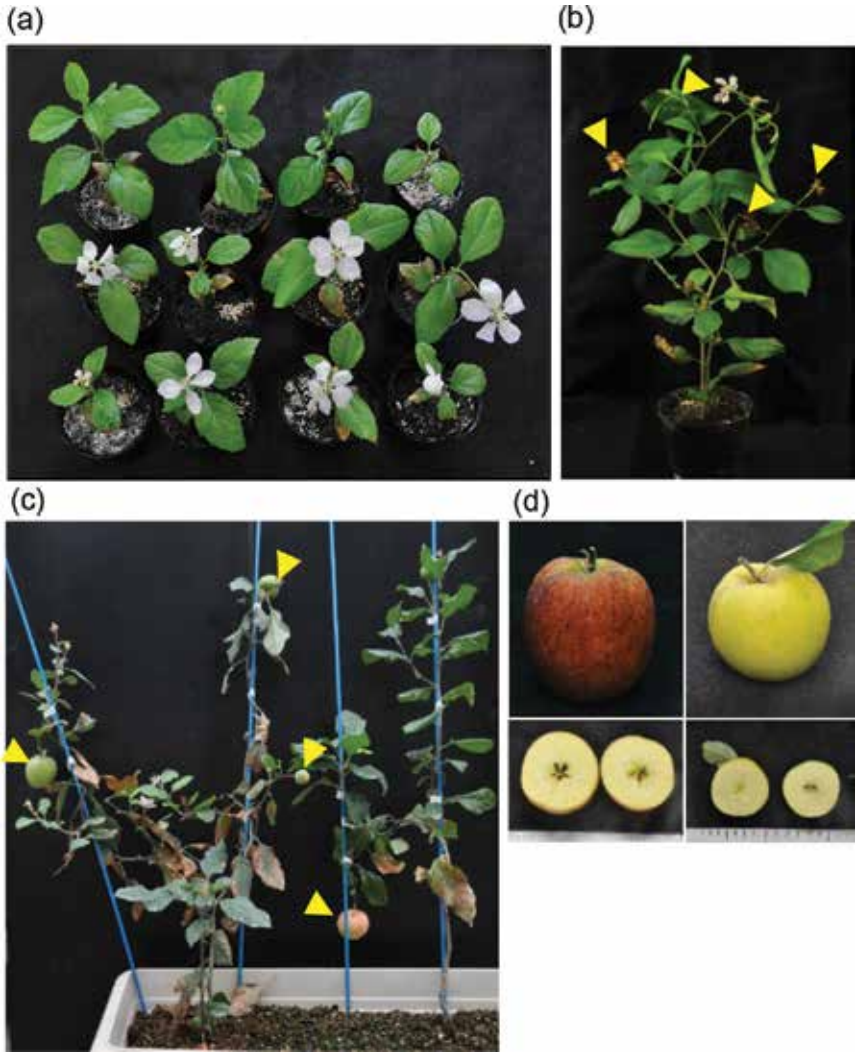

Figure 2. (a) Precocious flowering of apple seedlings infected with ALSV-AtFT/MdTFL1 (45 days postinoculation (dpi)). Eleven of 12 apple seedlings infected with ALSV-AtFT/MdTFL1 produced flower buds within 2 months. (b) A seedling (3 months postinoculation (mpi)) showing continuous flowering. Arrows indicate the flowers. (c) Apple seedlings with fruits (arrows) 8.5 mpi. (d) Ripe fruits on ALSV-AtFT/MdTFL1 infected apple seedlings (10 mpi) with viable seeds.

next-generation seeds) of apples that usually takes 5–12 years, to a year or less. This new technology is expected to be able to shorten substantially the period for breeding a new variety of apple via crossbreeding. In addition, as conventional breeding of fruit trees requires large fields, the use of this new technology enables completion of one generation in a growth chamber (**Figure 3**).

We also verified whether the technology of inducing early flowering using ALSV vector was applicable to pear [46]. We inoculated cotyledons of pear seedlings immediately

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Figure 3. (a) Precocious flowering and fruit production of apple seedlings infected with ALSV-AtFT/MdTFL1 (7 mpi) in a growth chamber with a hydroponic cultural apparatus. Arrows indicate fruits. (b) Ripe fruits in seedlings shown in (a) with viable seeds. Scale bars indicate 1 cm.

after germination with ALSV-AtFT/MdTFL1 and confirmed that approximately 33% of the infected individuals flowered and showed continuous flowering where they flowered continuously over several months as the apple seedlings did (**Figure 4a, b**). We also constructed an ALSV vector (ALSV-AtFT/PcTFL1) that simultaneously performs *AtFT* expression and suppression of *PcTFL1-1* expression and inoculated to cotyledons of pear seedlings. The results indicated that approximately 86% of the infected individuals showed continuous flowering. This effect is likely due to replacement of the apple gene (*MdTFL1-1*) by pear counterpart (*PcTFL1-1*). This confirmed that the sequence identity is important for efficient gene silencing as reported elsewhere [28, 47, 48]. In addition, the pollens of early-flowered pear seedlings were fertile and triggered fruition of the infected individuals via pollination with their pistils (**Figure 4c**). We presume that this technology is likely to be feasible for all fruit trees that are susceptible to ALSV infection, and substantially contributes to optimization of breeding by crossing of new fruit tree varieties.

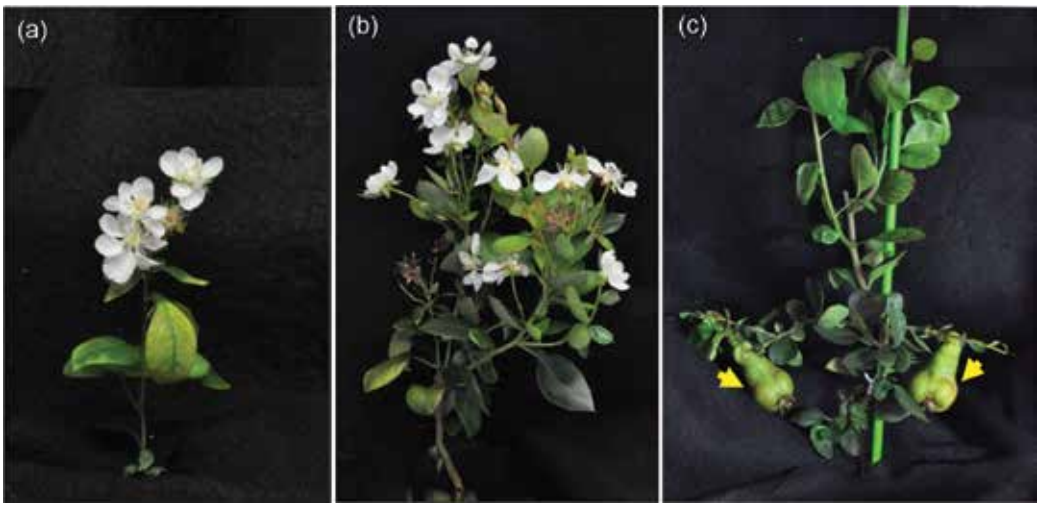

Figure 4. (a) Precocious flowering of a pear seedling infected with ALSV-AtFT/MdTFL1 (45 dpi). (b) Continuous flowering of a pear seedling infected with ALSV vector (3 mpi). (c) Fruition on a pear seedling infected with ALSV vector (7 mpi). Arrows indicate fruits on a pear seedling.

5. Elimination of ALSV from infected apple and pear trees

We tested 487 seeds obtained using pollens of ALSV-infected apple trees as the pollen parent, as well as 450 seeds from fruits on ALSV-infected apple trees, by ELISA and RT-PCR to test seed transmission. The rates of seed transmission from pollens and ovules were 0.38 and 4.5%, respectively [49]. We also investigated the rate of seed transmission from ovules using qRT-PCR, indicating that approximately 1% seedlings (two individuals out of 192 individuals) were infected with the virus [50]. Examination of 47 next-generation apple seedlings obtained from early-flowered seedlings using ALSV technology (ALSV-AtFT1/MdTFL1) showed that none of them were infected with ALSV vector, indicating that virus-free individuals can be obtained successfully [46].

Elimination of ALSV vectors from infected plants may allow the use of early flowering plants as breeding materials without genetic modification. We sometimes observed a phenomenon in which ALSV multiplied in inoculated leaves but not move to upper un-inoculated leaves [38]. We incubated ALSV-infected apple and pear seedlings for four weeks in a 37°C chamber, then returned them to a 25°C, and investigated the distribution of ALSV in infected plants. It was revealed that ALSV stopped movement to new tissues after the 37°C treatment, and no ALSV multiplication was observed in new tissues developed at 25°C [38]. We attempted detection of ALSV from the shoot apical meristem tissue of ALSV-infected apple and pear seedlings after incubation at 37°C by *in situ* hybridization; however, no ALSV was detected from the shoot apical meristem tissue after incubation at 37°C. It is likely that exclusion of ALSV from the shoot apical meristem tissue by high-temperature treatment (37°C) leads to cessation of subsequent ALSV movement to newly developed tissues [38] (**Figure 5**).

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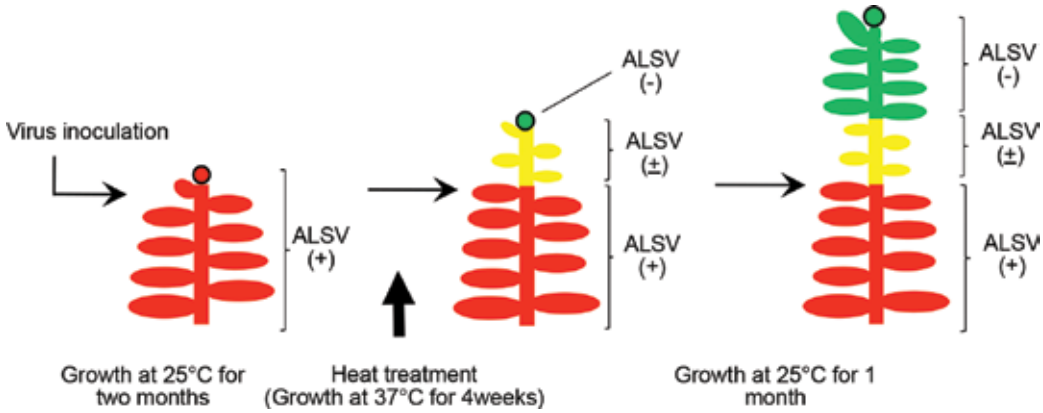

Figure 5. ALSV distribution in apple and pear seedling before and after heat treatment. *Red color* indicates the tissues which developed before heat treatment and systemically infected with ALSV. *Yellow color* indicates the tissues which developed during heat treatment and in which ALSV was weakly detected and/or not detected. *Green color* indicates the tissues which developed after heat treatment and in which ALSV was not detected. *Black circles* indicate the shoot apical meristems.

The results indicate that ALSV free tissues could be easily obtained from infected plants that flowered early by ALSV-AtFT1/MdTFL1 infection. Use of virus-free tissues as scions is likely to allow us to grow virus-free plants.

6. Discussion and perspective

The long juvenile phase of fruit trees is a significant barrier for efficient fruit tree breeding [3, 51]. The technology developed in the present study substantially shortens one generation of fruit trees via infection of the trees with an ALSV vector for promotion of flowering in fruit tree breeding. Conveniently, the majority of individuals of the obtained next-generation seedlings were free of ALSV because of low seed transmission rate of ALSV. Our ALSV vector technology, which is different from recombinant DNA technology, induces no mutation on the genome of the infected fruit tree. It is difficult to distinguish between normal plants and the plants after removal of ALSV vector. It is also possible to remove the virus easily by heat treatment from the infected materials, with these fruit trees not distinguishable from normal fruit trees. Therefore, these trees are likely to be used for breeding materials.

Velázquez et al. reported that they constructed a *clbvlNpr* vector from the *Citrus leaf blotch virus* and induced citrus in the juvenile phase to flower early via *AtFT* expression by the vector [52]. ALSV infects not only *Rosaceae* fruit trees but also citrus and grape; we can expect that ALSV will be used for the promotion of flowering in a greater variety of fruit trees in the future.

In recent years, determination of the full genome sequence of fruit trees has advanced, leading to publication of these sequences [53, 54]. This information is expected to accelerate bioinformatics, identification of molecular markers, marker selection, and omics research in fruit trees

even more [3, 51, 55–59]. The combination of virus-induced flowering technology described here with information obtained from these research is expected to lead to further optimization of fruit tree breeding in the future.

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Chapter 4

Plant Genome Editing and its Applications in Cereals

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Additional information is available at the end of the chapter

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Abstract

Recently developed methods for genome editing, representing a major breakthrough in the field of genetic engineering, will enable researchers to produce transgenic plants in a more convenient and safer way. Double-strand breaks (DSBs) are triggered by synthetic nucleases that later induce DNA repair mechanisms known as nonhomologous-end joining (NHEJ) or homology-directed repair (HDR) in the presence of a donor DNA. Gene targeting (GT) was earlier demonstrated in rice and maize genomes by exploiting several genes (*Acetohydroxyacid synthase, waxy, ALS, OS11N3* etc.), while zinc finger nucleases (ZFNs) were used to modify *IPK1* gene in maize. Clustered regularly interspaced short palindromic repeats (CRISPR-CAS) system has been shown to be efficient for targeted mutagenesis in wheat that has a hexaploid complex genome, rice, maize, and recently in barley. The CRISPR system is considered as advantageous over previous approaches due to its easy use and efficiency, however, needs to be improved for high off-target effects.

Keywords: genome editing, TALENs, ZFNs, CRISPR-CAS, rice, maize, wheat, barley

1. Introduction

Genome editing refers to the ability to perform controlled changes in the genome using specific nucleases. The ability of a recombination initiation by inducing double-strand breaks (DSBs) is a breakthrough in efficient genome editing and engineering of plants. Site-directed mutagenesis and gene replacement have been possible by these mechanisms that will lead to crop improvement and progress in functional genomics studies. Cereals, on the other hand, represent an important group in agriculture as those directly supply main carbohydrate sources for human food and animal feeding, e.g., rice for Asia, wheat for the whole world, and maize for the Americas. Grass family (known as *Poaceae*) consists of agronomically important plants such as wheat, rice, maize, sorghum, oat, and barley whose grains have high nutritional value having a rich source of fibers, vitamins, and minerals. Substantial amount

Plant	Explant	Transformation method	Genome-editing approach	Gene/locus	Reference
Maize	Embryogenic cell cultures	Whisker-mediated	ZFNs	*Inositol pentakisphosphate 2-kinase (IPK1)*	Shukla *et al*. [8]
Rice	Embryogenic cells	*Agrobacterium tumefaciens*	TALENs	*Os11N3*	Li *et al*. [9]
Wheat	Cell suspensions	*Agrobacterium tumefaciens*	CRISPR	*Inositol oxygenase* (*INOX*) and *phytoene desaturase* (*PDS*)	Upadhyay *et al*. [10]
Rice	Callus	Particle bombardment	CRISPR	*Chlorophyll A oxygenase 1* (*CAO1*) and *Lazy 1*	Miao *et al*. [11]
Maize	Callus	*Agrobacterium tumefaciens*	GT by HR	*Bar*, *gfp*, *npt II*	Ayar *et al*. [12]
Barley	Immature embryos	*Agrobacterium tumefaciens*	CRISPR	*HvPM19*	Lawrenson *et al*. [13]
Wheat	Protoplasts, immature embryos	Polyethylene glycol, particle bombardment	CRISPR	*TaGASR7*	Zhang *et al*. [14]
Rice	Callus	*Agrobacterium tumefaciens*	CRISPR	*OsERF922*	Wang *et al*. [15]
Maize	Protoplast callus	Polyethylene glycol, *Agrobacterium tumefaciens*	CRISPR	*Zmzb7*	Feng *et al*. [16]

ZFNs: zinc finger nucleases; TALENs: transcription activator-like effector nucleases; CRISPR: clustered regularly interspaced short palindromic repeats (CRISPR/CAS system); GT: gene targeting; HR: homologous recombination.

Table 1. Genome-editing applications in cereals.

of research has been conducted during the past two decades for the improvement of cereal varieties through conventional or molecular breeding, or a combination of both. Conventional methods such as hybridization, selection, and hybrid breeding have been applied and a large number of genes and quantitative trait loci (QTLs) for various traits have been tagged with molecular markers to apply marker-assisted selection (MAS) for trait improvement [1]. It is now possible to measure gene expression, and with the recent methods, obtaining knockout plants for different genes, which lead to an understanding of the roles and functions of genes and their effects under changing environments.

Genome modification studies have been launched in plants two decades ago with low-targeted integration frequencies [2]. By the discovery of nucleases inducing DSBs in specific loci, GT frequencies dramatically enhanced. In maize, acetohydroxyacid synthase genes (*AHAS108* and *AHAS109*) were modified using chimeric RNA/DNA oligonucleotides (ONDs) with a frequency of 10^{-4}, which was higher than spontaneous mutations and GT

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by homologous recombination (HR) [3]. GT experiments were conducted and improved by various studies in which different genes were targeted in maize, rice, wheat, and barley. A negative/positive selection approach was demonstrated for targeting the waxy gene of rice [4]. Gao *et al*. [5] used a sequence-specific meganuclease *I-CreI* for NHEJ-mediated targeted mutagenesis in liguleless1 locus of maize. HR-mediated targeting studies in agronomical traits such as herbicide tolerance have been the main object in model cereals [3, 6]. Besides conferring herbicide tolerance, genes that are difficult to mutate by conventional mutagenesis have been successfully targeted and analyzed for their putative functions, e.g., *ROS1* of rice, which is associated with cytosine DNA demethylation and thus epigenetic modifications in plants [7].

By the advancement of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR-CAS) system, these technologies were performed in *Arabidopsis* and tobacco, as models, and also cereals, as summarized in **Table 1**, to modify specific gene/locus. In this chapter, the major technologies for genome editing are described with an emphasis on the applications of cereal model species.

2. Principles of genome editing

The basis of genome editing relies on the formation of DSBs at specific loci and triggering DNA repair mechanisms. DSBs can be formed in eukaryotic cells by chemical and physical factors (reactive oxygen species, ionized-radiation, etc.) or by natural events like meiotic recombination. During the last decades, it was demonstrated that DSBs can be induced by synthetic nucleases and lately by the bacterial defence system CRISPR as well. A common feature of these synthetic nucleases is the combination of the bacterial type IIS restriction endonuclease *Fok*I nuclease subunit with a synthetic DNA-binding domain. This combination results in a specific DNA-binding domain for target and a nonspecific DNA cleavage domain. Zinc finger nucleases (ZFNs), TALENs, and dCas9-Fok (hybrid of *Fok*I nuclease subunit with deactivated Cas9) are all based on this principle. It should be noted that mutations generated by *Fok*I-based nucleases show small deletions or small deletions with insertions (so-called "indels").

The strategies for genome editing are based on the endogenous cellular processes related to DNA repair and recombination. It is well known that recombination occurs naturally during meiosis and in many cases, involves chromatin exchange between homologous sequences. Such a recombination is designated as homologous recombination (HR) and is the governing recombination type and DNA repair mechanism in lower organisms such as bacteria, yeasts, and moss [17]. Homologous recombination frequency in lower organisms such as yeast and the moss *Physcomitrella patens* can reach to over 10% or even 90% of transformants, respectively [17, 18].

DNA repair through homologous recombination is designated as homology directed repair (HDR). This pathway is initiated by a DSB in DNA, which is a result of DNA damage or an

endonuclease activity. In the presence of a donor DNA template and specific endonucleases, this pathway enables the replacement of a specific sequence. Therefore, one can use oligonucleotides such as triplex forming oligonucleotides (TFOs), short ssDNA or dsDNA donors such as T-DNA to induce HR. It should be noted that while T-DNA of *Agrobacterium* is infiltrated in the plant cell as a ssDNA coated by VirE2, it turns into dsDNA shortly after and probably integrates into the plant genome as dsDNA [19, 20]. While unassisted HR levels are very low, and it is highly induced by specific genomic DSBs [21]. Therefore, to induce gene replacement, one needs a nuclease/nickase to induce single specific DSB/nick and a donor DNA with homology arms to the genomic target sequence. The designed donor can then be used from a single known mutation of one base to a complete gene replacement and the integration of a new sequence into the genome.

When looking at DNA repair mechanisms, a nonlegitimate recombination or nonhomologous-end joining (NHEJ) is the dominant repair pathway in higher organisms such as flowering plants and humans. In NHEJ pathway, two broken ends of DNA were ligated together without the need of a homologous template (**Figure 1**). This pathway can be looked on as an "SOS" pathway, where the cell is "panicked" and quickly repair the damaged DNA with putative errors in the process. The NHEJ pathway is usually recognized with many errors and, therefore, is an excellent choice for gene disruption. NHEJ can achieve all editing objectives, i.e., mutations including small deletions or insertions [22], as well as gene insertion and gene replacement [19, 23]. However, while getting a mutation is certain, the mutations are completely random, and unlike the homologous directed recombination (HDR), there is no way to predict which mutation will occur and what will be the final result.

Gene insertion is a combination of single DSB with a supplied donor DNA. Here, we mimic the T-DNA integration by *Agrobacterium*, which is known to integrate randomly into an existing genomic DSBs [24]. Integration of supplied donor DNA either as T-DNA or simple

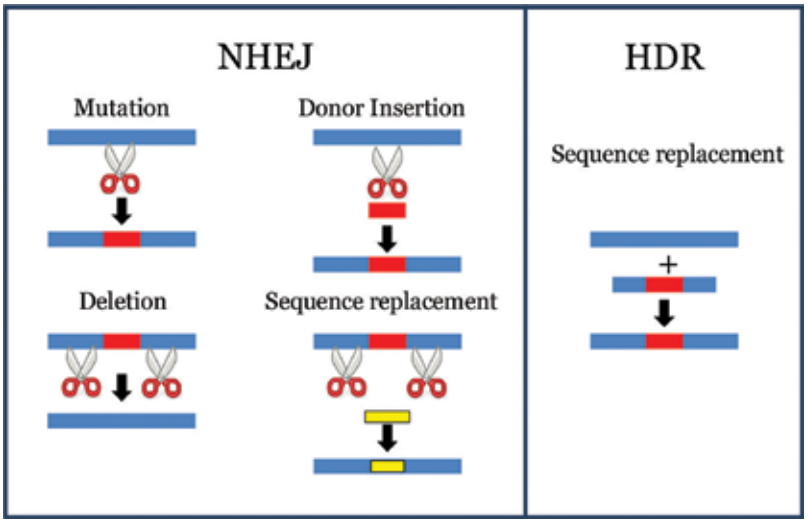

Figure 1. Two DNA repair mechanisms underlying genome-editing studies.

dsDNA (such as PCR product) into the desired location are increased by causing a specific DSB [24, 25]. However, the donor will incorporate also into many other genomic locations, and massive screening should be taken to exclude undesired integrations.

Gene replacement can be achieved by generating DSBs flanking the gene of interest and supplying a donor DNA. This may result in deletion and targeted insertion leads to a gene replacement [19].

In both NHEJ and HDR, the main challenge is screening and recognizing the relevant HDR event. When designing genome editing, these two DNA repair pathways (NHEJ and HDR) would be the main guidelines that should be considered. In general, sequence replacement can be achieved by HDR while mutations, deletions, and insertions can be achieved utilizing NHEJ (**Figure 1**).

3. Zinc finger nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are synthetic endonucleases combining zinc finger DNA-binding domain with a nuclease subunit (typically *Fok*I endonuclease). ZFNs have evolved from transcription factors harboring zinc-finger domain in their DNA-binding domain. ZFNs are built from C_2H_2 zinc-finger domains where each finger recognizes three nucleotides. Therefore, a 3-finger nuclease will bind to a DNA sequence of nine nucleotides. The first ZFN known as Zif268 is a combination between transcription factor Zif268 DNA-binding domain and *Fok*I nuclease subunit [26]. *Fok*I forms a dimer to cleave DNA thus two ZFNs' different monomers should be designed and used to cleave a single target sequence.

Major problems in using ZFNs are low specificity resulting in genotoxicity [27–30] and high complexity leading to low success rates of the designed enzymes [31].

DSBs by ZFNs have been applied mainly as a proof of concept and for research [32–34] in model plants and thus, all genome-editing strategies were explored by this pioneering system. In maize, inositol pentakisphosphate 2-kinase (*IPK1*) gene was targeted by generating a panel of 66 ZFNs against five intragenic positions [8] (**Table 1**). *IPK1* gene was chosen for its importance in phytate reduction as an agronomic and ecological trait. Sequencing of genomic PCR products confirmed that addition of PAT gene conferring the herbicide tolerance into *IPK1* had occurred precisely in a homology-directed manner. A recent study was conducted to explore noncoding genomic regions suitable for site-specific integrations to ensure stability and high gene expression in rice, using ZFNs. As a result, 28 genomic regions including only one noncoding have been discovered for safe integration of ZFN constructs carrying a *β-glucuronidase* gene [35].

4. TAL effector proteins (TALENs)

Transcription activator-like effector nucleases (TALEN) are synthetic nucleases combining *Fok*I nuclease subunit with DNA-binding domain composed of repeats. Repeat number may

vary and is typically between 16 and 30 forming a protein encoded by about 3.7 Kb open reading frame (ORF). Each repeat binds to a single nucleotide and is composed of 33–34 amino acids. Amino acids 12 and 13 are variable and known as repeat variable diresidue (RVDs). These variations enable the binding of different nucleotides, whereas NI for adenosine, HD for cytosin, NN for guanine, and NG for thymidine [36].

TALENs where evolved from the *Xanthomonas* AvrBs3 superfamily of type III effectors acting as transcription factors *in planta* [36, 37]. The different proteins in this family contain different number of DNA-binding repeats that govern the pathogen host range. Analysis of repeats/targets resulted with the discovery of a new set of DNA-binding domains that are simpler and more specific than the zinc finger sets used for ZFNs. The new repeat combination enables TALENs to have high target specificity and high DNA affinity, which results in both low genotoxicity and high genome-editing rates. TALENs are probably the most accurate systems for genome editing with high success levels but the system suffers from several drawbacks.

Similar to ZFNs, TALENs use *Fok*I nuclease subunit working as a dimer and, therefore, two monomers should be designed for each genomic target. The resulted ORF size is huge and, therefore, cannot be used in viral vectors. Furthermore, size and the need to synthesize a new pair of enzyme for each genomic target may hinder the ability to edit several genomic targets.

TALEN-directed mutations were generated in *Os11N3* gene in rice, which is normally activated by TAL-effectors (named AvrXa7 or PthXo3) of a rice pathogen causing bacterial blight disease [9]. That study showed that TALENs can be successfully employed for the modification of a S gene promoter to prevent its induction by bacterial effectors. The authors discussed the possibility of editing multiple susceptibility genes to confer resistance to other forms of bacterial blight.

5. CRISPR-CAS system

CRISPR or clustered regularly interspaced short palindromic repeats is a bacterial defence mechanism against bacteriophages. Although usually this system is composed of a cascade of many different proteins, in *Streptococcus pyogenes*, most cascade proteins are provided as a single self-operating protein designated as Cas9.

The Cas9 has several functional characteristics including enabling binding of sgRNA, searching for complementary sequence, and cleaving the target sequence (HNH domain that cleaves the complementary DNA strand and RuvC domain cleaves the noncomplementary DNA strand). The most important is the PAM recognition domain that distinguishes the bacterial encoding RNA from the bacteriophage target sequence. In Cas9, this sequence requires NGG downstream to the targeted sequence. The Cas9 first binds the PAM sequence and then opens the DNA, allowing RNA/DNA hybridization or R-loop formation and then cleaves both DNA/RNA and ssDNA strands [38–40].

In 2013, several articles have been published reporting the plant genome engineering, using CRISPR system that five of them resulted in the generation of mutant plants with specific

targeted loci [41]. Besides the applicability of CRISPR, the use of protoplast cultures for transient expression assays and agroinfiltration of leaf tissues have been preferred due to their advantages. Upadhyay *et al*. [10] targeted inositol oxygenase (*INOX*) and phytoene desaturase (*PDS*) genes in the suspension cultures of wheat, which has a complex hexaploid genome (17 Gb). The authors reported that CRISPR system was simpler than ZFNs and TALENs with high efficiency even in large genomes at each of the multiple targeted locations. In the same year, targeted mutagenesis by CRISPR-CAS in *Chlorophyll A oxygenase 1* (*CAO1*) and *Lazy 1* genes were demonstrated in rice [11]. These genes were selected for their easily detectable phenotypes for screening, e.g., pale green leaves for *CAO1* and tiller-spreading appearance for *lazy 1* gene, respectively. Finally, induction of heritable mutations (transmitted stably to T_2 plants) by CRISPR has been shown in barley which is an important model with its diploid nature [13]. In another study, high activities of sgRNA were optimized using protoplast cultures of wheat and both protoplasts and immature embryos were tested as a broadly applicable system for genome editing [14]. Recently, insertion and deletion mutations were successfully introduced to a rice gene *OsERF922*, coding an ERF family transcription factor and resistance to rice blast was enhanced in the resulted plants [15].

6. Conclusions and future perspectives

There have been substantial efforts to develop efficient technologies for GT in plants including cereals. For this aim, synthetic nucleases, including the mitochondrial *I-SceI* from yeast and chloroplast *I-CreI* from *Chlamydomonas reinhardti*, have been used for higher GT frequencies [5, 12, 42]. However, it was recently shown that CRISPR-CAS system can greatly facilitate the modification of targeted locus in rice, maize, wheat, and barley tissue cultures [13–16]. The primary application of genome-editing tools is obtaining knockout plants, and in time, the other applications that extend to crop improvement are expected, e.g., abiotic stress tolerance will be important for near future to resolve stress response and adaptation pathways [43]. For example, barley has been used for a long time in genomics studies as a highly adaptive and tolerant model for environmental stresses [44, 45].

Frequency of HDR in plants (*Arabidopsis* and tobacco) is typically 10^{-4}–10^{-5} [46], whereas gene replacement by HR in plants may be increased to 10^{-2} through transient expression of meganucleases, which induces double-strand breaks (DSBs) [2]. NHEJ levels as shown in the form of T-DNA integration are 3–15 times higher when compared to HDR events in plants and transgenes integrated into the correct site in about 1% of the transformants [23].

There are several considerations and limitations for the major genome-editing technologies. TALENs are considered as the most precise genome-editing system for today. This suggests not only hitting the correct genomic location but more importantly less cytotoxicity from off-targeting effect. The high precision enables targeting multiple targets with confidence. High efficiency in genome editing is translated to the amount of screened plants in order to reach the desired modified plant. ZFNs are considered to be less efficient than TALENs and Cas9 shows higher efficiency. Both ZFNs and TALENs have to be redesigned for each target, while CRISPR-based methods require redesign of RNA molecules. Therefore, CRISPR methods are

easier to employ and are more suitable for large genomic screenings or multiplex gene targeting. However, more evidence has raised Cas9 low specificity effect [47] and low homologous recombination ratio [48], whereas several approaches were taken to overcome these limitations of which dCas9-Fok might be the most promising [49–53], none addresses the size constrains presented by Cas9.

The huge size constrains of the current genome-editing tools prevent applying plant viral vector as genome-editing tool, thus the researcher should use meganucleases for these applications. In general, Cas9 and its derivative technologies would be sufficient for research of *Agrobacterium* transformable and regenerative plants. Nevertheless, the need for a more precise and smaller system exists, and we can expect that future technologies will answer these restrictions.

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Chapter 5

Genetic Modification of Stem Cells in Diabetes and Obesity

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Additional information is available at the end of the chapter

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Abstract

Genetic modification, or gene transfer, represents a method of treatment for several diseases. It has been used extensively in the context of cardiovascular diseases; however, its role in the context of metabolic diseases, such as diabetes and obesity, has remained largely unexplored. In this chapter, we will review the use of adult stem cells, focusing on endothelial progenitor cells (EPCs) and mesenchymal stromal cells (MSCs), in the context of diabetes. We have highlighted the use of viral vectors, particularly DNA viruses, as a tool for genetic modification to help stem cells survive and resist apoptosis in a hyperglycemic environment. We then discuss genetic modification of EPCs and MSCs to treat complications of diabetes and obesity. Although there are several unanswered questions in the field of metabolic diseases, the future application of gene transfer technology along with genetic modification of stem cells prior to the therapy holds significant therapeutic promise.

Keywords: gene transfer, endothelial dysfunction, apoptosis, vascular complications, superoxide dismutase, stem cells, diabetes, viral vectors, mesenchymal stromal cells, endothelial progenitor cells

1. Introduction

In the past few decades, there have been many important advances in the treatment of diabetes and its associated complications that have dramatically improved the lives of patients. Despite this progress, there are still many unresolved issues with pharmaceutical therapies and, therefore, a cure is still elusive. The estimated number of adults worldwide with diabetes is

over 400 million and it has been predicted that between 2010 and 2030, there will be an increase in the number of people with diabetes in both developing and developed countries (69 and 20%, respectively) [1, 2]. With this alarming rise, there is also a dramatic increase in patients who have cardiovascular comorbidities [3].

Currently, injections of exogenous insulin are still the mainstay therapy for type 1 diabetes. Although whole pancreas or islet transplantations have become clinically available, this approach is limited by the short number of donors, the adverse effects of immunosuppressive therapy and possibility of acute and chronic transplant rejection is high. For type 2 diabetes, there are a multitude of medications on the market, yet a tight glycemic control remains a challenge for patients.

Here, we will discuss the use of two different adult human stem cells: endothelial progenitor cells (EPCs) and mesenchymal stromal cells (MSCs) (**Table 1**) and the role they can play to treat diabetes and its complications.

Adult stem cells:	**EPCs**	**MSCs**
Origin	Bone marrow and hematopoietic cells	Any mesenchymal tissue
Harvested from	Peripheral blood or bone marrow	Fat, bone, cartilage, muscle, liver, bone marrow
Surface markers*	CD34/133/ KDR (+) or combination, CD45(−)	CD73/90/ 105 (+) and negative for CD34/45/ 11b/14/19 and HLA class II
Immunogenicity	High incidence of rejection	Low, cells can be transplanted across species
Cell shape/size	Size of a white blood cell, circular	Similar in size to a muscle cell, spindle shaped
Responds to	Acute injury, necessary for vasculo-genesis	Chronic need of the body to maintain mesenchymal tissues
Helps to form	Endothelium *Progenitor cell*	Any adult mesenchymal tissue: fat, bone, cartilage, muscle. *Multipotent*
Gluco-toxicity	High incidence of apoptosis	Chronic injury, leads to intra-cellular ROS accumulation

*As per, International Society for Cellular Therapy (ISCT).

Table 1. Characteristics of EPCs and MSCs.

EPCs, which can be harvested from peripheral blood and from bone marrow, are broadly classified as hematopoietic stem cells. These cells come in the circulation in increased numbers from bone marrow (BM), in response to an acute ischemia or injury. MSCs, on the other hand, are resident in all mesenchymal tissue and bone marrow and unlike EPCs, these cells are multipotent [4]. In adults, EPCs are acute response stem cells which increase in number in order to increase tissue perfusion, whereas MSCs are cells that are necessary for chronic regeneration in a mesenchymal tissue. Both EPCs and MSCs have been used for the therapy of multiple disease states. For the purpose of this review, we will discuss therapies involving MSCs and EPCs in the context of diabetes, obesity and myocardial ischemia including the use of genetically modified stem cells where genetic modification has been carried out using viral vectors.

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2. Endothelial progenitor cells

As previously mentioned, EPCs are precursors of the endothelium. The specific makers that have been used to define EPCs are CD34+, CD34/KDR+, and CD133+ [5–7].

The endothelium plays an important role in the regulation of vascular tone and homeostasis through its paracrine properties [8, 9]. The roles of the endothelium include the following: forming a blood vessel wall monolayer, maintain vasodilation and promote angiogenesis. Endothelial dysfunction leads to vasoconstriction and inflammation [10]. When the endothelial function is impaired, there is a reduced production of nitric oxide (NO) and an increased production of reactive oxygen species (ROS) [11]. This leads to cellular apoptosis and aggravated inflammation which promotes atherosclerosis through platelet aggregation, adhesion and plaque formation [10, 12]. Therefore, endothelial dysfunction is a major cause of cardiovascular diseases (CVD) such as stroke, myocardial infarction (MI) and peripheral vascular disease (PVD) [13, 14].

A prime cause of endothelial dysfunction is diabetes mellitus, which is caused by hyperglycemia and glucose intolerance due to insulin deficiency/resistance [15]. Chronic hyperglycemia, associated with both type I and type II diabetes mellitus, leads to endothelial dysfunction and cardiovascular disease (CVD) [16]. A hyperglycemic state damages the endothelium and impairs EPC number and function. This interferes with vasculogenesis, poor healing and impaired overall endothelial function [17]. Therefore, preventing hyperglycemic environment induced damage to endothelium and EPCs is very important in order to prevent endothelial dysfunction and associated cardiovascular disease.

One approach to treat endothelial dysfunction in diabetes is non-pharmacological therapy. Lifestyle changes, such as diet and exercise can help to reduce body weight and improve endothelial function. We have previously reported a significant improvement in endothelial function, measured by flow mediated dilatation and endothelial progenitor cells (EPCs), specifically CD34+ cell number, function and gene expression after 6 weeks of aerobic exercise [18].

Pharmacological therapies that improve endothelial function include calcium antagonist, beta blocker, angiotensin-converting-enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), statins, insulin resistance reducing drugs and even erythropoietin [19].

Another manner in which endothelium dysfunction can be treated is with EPCs themselves. Several studies were performed to treat endothelial dysfunction by transplantation of EPCs and it was determined that these cells could be a powerful tool for cell-based therapy primarily through their paracrine properties. EPCs from bone marrow (BM) can circulate in peripheral blood and repair damaged endothelium either by transforming into mature endothelium or through their paracrine properties [20, 21]. These cells can also be used as a biomarker for endothelial dysfunction [18].

Unfortunately, the number of EPCs found in diabetic patients is reportedly lower than their healthy patient counterpart [22]. In addition, the EPCs also lose their ability to migrate to

damaged areas [16]. In order to prevent that scenario, researchers have looked into modifying the EPC genes themselves. Di Stefano and Cols. reported that a gene deletion can reverse the high-glucose caused defects of BM-derived EPCs and increase angiogenesis by reducing oxidative stress-related apoptosis [22]. Manipulation of the gene expression of EPCs may facilitate an increased efficacy of diabetic patient's endothelial progenitor cells [22]. These cells could then be used to treat many of the side effects associated with diabetes.

One of the major side effects of high glucose exposure, or diabetes, is ischemia. Ischemia could be responsible for poor cardiac function as well as peripheral vascular disease (PVD). We have previously demonstrated the use of genetically modified rat EPCs to treat myocardial infarction (MI)-related complications in Sprague-Dawley rats. Adeno-associated virus (AAV) mediated IGF-1 expression reportedly increased cardiac function by increasing cardiomyocyte proliferation and capillary density in the myocardium and by decreasing cardiomyocyte apoptosis in a myocardial infarction rat model [7]. Both adenovirus and recombinant AAV are DNA virus which, remains as an episomal character without integrating with host genome. This reduces chances of mutagenesis and avoids unnecessary prolonged over-expression [4, 23]. Another study showed that genetically modified human EPCs (hEPCs) with a tissue kallikrein (TK) gene upregulation helps hEPCs to minimize oxidative stress-related apoptosis, as well as, enhances vascularization and offers protection against ischemia-induced MI [24]. Similarly, VEGF-165 gene upregulated EPCs also significantly improved cardiac function by promoting angiogenesis in rats with an ischemic myocardium [25]. In order to address peripheral vascular disease, Goto and cols. examined the overexpression of beta integrin in EPCs and found that it helped to promote angiogenesis and improve blood flow in mouse hind limbs [26]. In order to combat the increase in apoptosis caused by high glucose environments, the silencing of apoptotic genes p53 has been explored. Silencing p53 showed better survivability of the EPCs in high glucose. When p53 silenced EPCs were transplanted into a diabetic mouse model, an increased blood flow and vascularization was found [27, 28]. Another study also demonstrated increased blood flow in ischemic mouse hind limb post-genetic modification of EPCs. This study showed that the inhibition of glycogen synthase kinase-3beta (GSK3beta) boosts survival of EPCs and increases migration of vascular endothelial cells to the ischemic hind limb–thereby improving angiogenesis [29].

Systemic inflammation is directly related to diabetes. It has therefore been hypothesized that a reduction in inflammation could positively affect endothelial dysfunction. An interesting study showed that the genetic modification of EPCs, to overexpress A20 (an anti-inflammatory protein), decreases endothelial inflammation. This could be a cell-based therapy to reduce inflammation caused by diabetes [30].

Erectile dysfunction is another complication of diabetes that can manifest due to vasculopathy and neuropathy that could also be treated with genetic modification. Cell-based treatment for diabetes mellitus-induced erectile dysfunction (DMED) is possible by over expressing human telomerase reverse transcriptase (hTERT) EPCs. Endothelial nitric oxide synthase (eNOS) expression increased significantly after EPCs-hTERT treatment as well as the reduction in apoptosis and the resistance to oxidative stress. Therefore, EPC-hTERT treatments helped to improve erectile function in DMED rats [31].

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3. Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) have attracted scientific and clinical interest for the role they could play to establish an effective therapy in regenerative medicine. Considering the regenerative and immunomodulatory properties of MSCs, these cells have been considered as a promising cell-based therapy to treat diabetes [32]. MSCs can be obtained from different sources such as umbilical cord blood, bone marrow, adipose tissue, pancreatic islet, fetal liver, lung and other tissues [33–35]. These cells are easily expanded in culture. The specific markers that are used to define the MSCs are CD44, CD73, CD90, CD105 but not CD31, CD34, CD45 [36]. MSCs can be differentiated into osteoblasts, adipocytes, myocytes and chondrocytes [37].

When transplanted into a streptozotocin (STZ)-induced diabetic mouse model, bone marrow-derived MSCs were able to improve β-cell mass and increased insulin production, which resulted in a reversal of hyperglycemia [38–41]. Additionally, similar results were found when bone marrow-derived MSCs were obtained from donors with newly (6 weeks) diagnosed type I diabetes [41].

Regarding the use of MSCs to treat type 2 diabetes and obesity the most promising results come from experiments performed in obese diabetic mouse models. Diet-induced obese (DIO) mice fed with high fat diet (60% of calories from fat) for several weeks do become obese with a decreased glucose tolerance and insulin sensitivity. However, after mouse adipose tissue-derived MSC transplantation, DIO mice demonstrated a reduction in blood glucose levels and improved glucose disposal [42]. Interestingly, the DIO mice that had received MSCs showed reduced body weights, a decrease in serum triacylglycerol coupled concomitantly with an increase in HDL levels [42]. The mechanisms by which MSCs can reverse the complications caused by diabetes and obesity are still unclear. The literature indicates that MSCs can migrate to an injured pancreas, suppress pro-inflammatory cytokines and prevent β-cell apoptosis. However, one cannot exclude the fact that MSCs could also differentiate into pancreatic β-cells [42, 43].

Recently, our group has demonstrated that high glucose level, as typically observed in diabetic patients, promotes adipogenesis, increases accumulation of intracellular reactive oxygen species (ROS), upregulates inflammatory genes and decreases cellular oxygen consumption rate (OCR) in human adipose-derived MSCs [44]. Nevertheless, these effects promoted by hyperglycemia on MSCs can be minimized or reversed by upregulation of mitochondrial (rather than cytosolic, or extracellular) antioxidants such as superoxide dismutase-2 (SOD-2) also known as manganese-dependent superoxide dismutase (Mn-SOD).

In our study, we induced SOD-2 overexpression in human MSCs with the use of an adenoviral vector serotype 5 (AdSOD-2), a DNA virus, by gene transduction. As mentioned before the adenovirus remains as an episomal character, not integrating with the host genome and is therefore considered a safe approach to induce the over-expression of antioxidants in human cells for translational research [4].

Remarkable results were observed when SOD-2 upregulated MSCs were transplanted in db/db leptin receptors deficient obese diabetic mice. First, we noted that when human MSCs

are delivered intra-peritoneally (IP), they reach distal intra-peritoneal fat pockets. Therefore, when injecting SOD2 upregulated MSCs intraperitoneally (one single infusion), we expected the cells to reach these distal fat pockets and cause a reduction in ROS and thus help to reduce inflammation. Following a reduction in ROS in the local adipocyte pockets, the next step was to verify whether this approach could increase insulin sensitivity and reduce fat mass as well as blood glucose levels. In fact, at four weeks post-delivery of SOD-2 upregulated MSCs, a significant improvement in glucose tolerance and total body weight was found in db/db mice. The control for this experiment was db/db mice that received green fluorescent protein (GFP) transduced MSCs (AdGFP upregulated human adipo-derived MSCs) [44]. It was not elucidated whether the benefits promoted by genetically modified MSCs are restricted to local adipocyte pockets or if there is systemic improvement. However, the overall effect of improvement in glucose tolerance is clear. Different studies suggest that the "homing-in" and benefits of MSCs (genetically modified or not) depends on the route of cell delivery. Thus, the target of this cell-based therapy might change considering intra-peritoneal versus cells delivered into the tail vein [42, 44].

Injection of MSCs into the tail vein may help cells to reach the hepato-biliary system rather than fat in the peritoneal space. Additionally, injecting via intrasplenic route was found to be more effective at reversing hyperglycemia than the intrapancreatic route in a STZ-induced diabetic mouse model [45]. The effects of upregulation of other antioxidants on MSCs to treat diabetes in different high fat diet induced obese diabetic mouse models are under investigation in our laboratory [46].

4. Conclusion

In this review, we have briefly described promising studies that have used genetically modified EPCs and MSCs to reduce endothelial dysfunction and rejuvenate mesenchymal tissue (i.e. adipose tissue), respectively, in disease states such as diabetes and obesity. We have described the use of these stem cells post-genetic modification that can lead to a novel, yet safe therapy to improve the lives of the diabetic and obese population. Finally, we have outlined the use of these cells as a disease and therapy bio-marker in order to predict the disease progression of both prediabetes and diabetes.

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This collection presents various interesting aspects of genetic engineering. Many thought-provoking queries like "Is gene revolution an answer to the world hunger? Do GM crops with more complex transformation contribute to the enrichment of multinationals? Why the US increases food aids?" have been analyzed. Transformation protocols and retrieval of recombinants are essential to the success of genetic engineering. The book throws light on new transformation strategies which can be used to increase the transformation efficiency in most plant species. Genetic engineering offers potentially viable solution to look for alternatives beyond Bt toxins with similar pattern of toxicity. An interesting chapter is dedicated to in vitro fig regeneration and transformation systems. To address the long juvenile phase of fruit trees, the book includes a chapter on plant breeding technique that can significantly shorten the breeding periods. The book dwells on aspects of genome editing which will enable researchers to produce transgenic plants in a more convenient and safer way to genetic modification of stem cells holding significant therapeutic promise to treat complications of diabetes and obesity. I hope this book will serve as a seed for further investigations and novel innovations in the area of genetic engineering.

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