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Recombinant DNA and gene cloning technology in agricultural plant protection (A review)

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Bitki korumada değiştirilmiş DNA ve gen klonlama teknolojileri (Derleme)

ÖZET

Rekombinant DNA teknolojisi araştırmacıların laboratuar şartlarında DNA sekanslarını izole edip istekleri doğrultusunda düzenlemelerini olanaklı kılar. Bu teknolojiyi kullanarak herhangi bir kaynaktan kodlanmış herhangi bir genin ekspresyonunu gerçekleştirmek mümkündür. Bitkilerden, funguslardan, bakterilerden veya laboratuarda sentezlenmiş bir sekanstan bile herhangi bir canlıya bilgi aktarılabilir ve orada çalışması sağlanabilir. Bu araçlar bitkilerin geliştirilmesine, bitki korumada yarayışlı genlerin aktarımı ile bitki patojenlerine ve zararlılara karşı dayanıklılık kazandırılması gibi avantajlar sağlamaktadır. Bu derlemede bitki koruma ile ilgili yeni biyoteknolojik yöntemler tartışılmaktadır.

ANAHTAR KELİMELER: Değiştirilmiş DNA, dayanıklılık, gen aktarımı

SUMMARY

Recombinant DNA technology allows researchers to isolate and manipulate DNA sequences *in vitro*. Using this technology, it is possible to express virtually any kind of coding sequence from any possible source. Selected genes or sequences from animals, plants, fungi, bacteria, or even sequences synthesized *in vitro* can be introduced into and expressed in almost any other organism. These tools allow the development of novel crop plants and in plant protection with the improved characteristics gained by the transfer of useful genes, such as those that provide resistance against plant pathogens and pests, have many advantages. In this review the new biotechnological methods related to plant protection are discussed.

KEY WORDS: Recombinant DNA, resistance, gene transfer

INTRODUCTION

An Engineer Bacterium

Agrobacterium tumefaciens is a plant pathogen that causes a disease in dicotyledonous (broad-leafed such as cotton) plants called crown gall. Crown gall is a tumor that usually forms on the stem of plants and consists of rapidly dividing cells. The crown gall is essentially a plant cancer. These galls do not kill the plant directly but tap resources from the plant and can sometimes grow large enough to block the vascular system of the stem. Virulent strains of *Agrobacterium* that are capable of causing tumors in plants carry large plasmids called *Ti* (tumor inducing) plasmids (Caplan *et al* 1983). When infection occurs, a portion of the Ti plasmid is transferred to the plant cell and is incorporated into the plant genome (Fig. 1). The segment of the DNA that is transferred to the plant is

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called T-DNA, which is flanked by direct repeats that are necessary for the transfer and includes several genes expressed in the plant cell host. Two of these genes encode enzymes that are involved in the production of the plant growth regulators auxin and cytokinin. These phytohormones, when present in abnormally high concentrations cause plant cells in grow into rapidly proliferating undifferentiated tissue called callus. This mass of cells forms the crown gall. In addition to these tumor-causing genes, the T-DNA also carries genes for enzymes that produce unusual amino acids, called octopines, in the plant cell (Kado 1991). While octopines, such as nopaline, cannot be used by the plant, the virulent *Agrobacterium* uses them as food source. Therefore, when *Agrobacterium* infects a plant, it causes the growth of a tissue that will supply the surrounding *Agrobacterium* with food. Therefore, *Agrobacterium* can carry out a natural form of genetic engineering; this system has been adapted by researchers to efficiently transfer foreign genes into plant tissues. To do this, it is necessary to remove the tumor-causing genes from the T-DNA of the Ti plasmid (Kado 1991, Kado 2000). Such a "disarmed" plasmid is still capable of transforming plant cells but does not cause tumors. Next, a synthetic gene that encodes an enzyme called *neomycin phosphotransferase* II (*npt II*) is introduced into the Ti plasmid. This enzyme breaks down the antibiotic kanamycin and expression of this enzyme in plant cells allows them to grow in the presence of this antibiotic. The synthetic *npt II* gene consists of coding sequences derived from a transposon from *Escherichia coli* under the control of a promoter from a plant virus called the Cauliflower Mosaic Virus (CaMV) 35S (Harpster *et al* 1988). Therefore, infection of plant tissues with *Agrobacterium* that carry the modified Ti plasmid results in plant cells that express the *npt II* gene and are capable of growth in tissue culture, on media that contains kanamycin (De Vries *et al* 1998). In this way, it is possible to transfer T-DNA from the *Agrobacterium* to plant cells and select those cells that are transformed by growing them in the presence of kanamycin (Hinchee *et al* 1994). Some plants, such as tobacco, can be easily regenerated from tissue culture. So, it is a relatively simple to transform tobacco and regenerate transformed tobacco plants. These plants are perfectly normal, except that they carry a piece of T-DNA inserted in one of their chromosomes. When these plants flower and self pollinate, they produce seeds that inherit the T-DNA insert and the kanamycin resistance gene that it carries. In addition to the *npt II* gene, virtually any other gene can be introduced into the T-DNA and transferred to plant cells. In this way it is possible to genetically engineer plants to express genes that provide them with unique characteristics (Hansen and Wright 1999). Since the Ti plasmid is large and difficult to manipulate in vitro, modified

Table 1. Techniques used in plant biotechnology (United States 1988; Nottingham 1998; National Research Council 1987; Union of Concerned Scientists, http://www.ucsusa.org/agriculture/biotech.whatis.html, 1999).

Direct DNA update	Certain methods or electrical treatments allow direct uptake or incorporation of DNA into plant cells. Because hundreds of cells can be simultaneously treated, it is a relatively easy technique. Cells expressing the desired trait can be generated and tested further.
Vector-mediated transfer	Uses vectors (plasmid or virus) to transfer genes into plant cells. Vectors can generally improve gene transfer because they permit genes to be stably maintained and easy to track. Different species and cells may require different types of vectors, and often, much work must go into creating an appropriate vector system before genes can be transferred into a specific organism.
DNA mic.injection	DNA is directly introduced into individual cells using special apparatus. Although fewer cells can be injected with DNA, a higher frequency of successful uptake and incorporation of the foreign genetic material can be achieved (up to 14 percent of injected cells).
Plant tissue culture	Cells are grown on an artificial solid or liquid medium by taking a sterile sample of young, actively growing tissues and transforming them using bacterial vector or gene gun techniques. Allows for a large number of small clonal plants to be produced. The process of producing intact, viable organisms from undifferentiated tissue is called plant regeneration.
Cell fusion	Combines the entire genetic contents of two cells, producing hybrid cells that often express certain traits from both parents. This method is useful for transferring multigenic traits or for using cells from plants that cannot be crossed sexually, thus permitting the exchange of genetic material beyond natural breeding barriers.
Gene guns	Developed in the 1980s, the so-called projectile method uses metal slivers to deliver genetic material into the interior of the cell. One projectile method, known as bioballistics or the biolistic method, propels the coated silver into the cell using a special shotgun. A perforated metal stops the shell cartridge but allows the slivers to pass through and into the living cells on the other side.
Gene silencing	Silences an organism's own gene to prevent it from being expressed. Gene silencing was first used to create tomatoes with a higher solid content and a longer shelf life by preventing the synthesis of an enzyme involved in the ripening process.

systems have been developed that use smaller, more easily manipulated T-DNA containing plasmids that can replicate in both *E. coli* and *Agrobacterium* (Hansen and Wright 1999). These are called binary vectors because the mobilization of the T-DNA to plant cells is catalyzed by the *vir* genes of a Ti plasmid from which the T-DNA has been deleted (Matzke and Chilton, 1981). The *vir* genes produce the enzymes responsible for cutting the T-DNA, copying it and transporting it to the plant cell (Gelvin 1998).

Exempt for the engineer bacterium *A. tumefaciens* the other methods (Table 1) are summarized as;

Viral Vectors

These methods allow vector-mediated transfer of DNA. As infectious particles, viruses contain genetic material to which a new gene can be added. The virus can carry the new gene into a recipient cell in the process of infecting that cell. At the same time, the virus can be disabled so that while it can carry a new gene into a cell, the cell will not necessarily respond to the virus by making thousands of copies of it.

Plasmids work differently. They are small DNA circular structures of genetic material found in bacteria that have the ability to cross species boundaries. These circles can be broken and receive new genetic material. Once augmented with new genetic material and placed to move across microbial cell boundaries, plasmids add the new genetic material to the bacterium's own genes. Viruses are ideal for transferring genes since the virus is spread to every cell in the plant. Viral vectors also have a wide host range and have many advantages. Therefore viral vectors are being replaced. For instance; in recent years cauliflower mosaic virus has been used to stimulating of the lipid transfer protein gene resulted in resistance against bacterial and fungal infection (Sohal *et al* 1999). This type of virus known as a promoter gene is placed in the transfer vectors along with the genes, and the foreign genes have a greater chance of appearing.

Protoplasts

Protoplasts are plant cells from that the cell walls have been removed; this makes it easier to get DNA into them, with treatment with particular chemicals or by the use of high-voltage electric shocks (Fromm *et al* 1986).

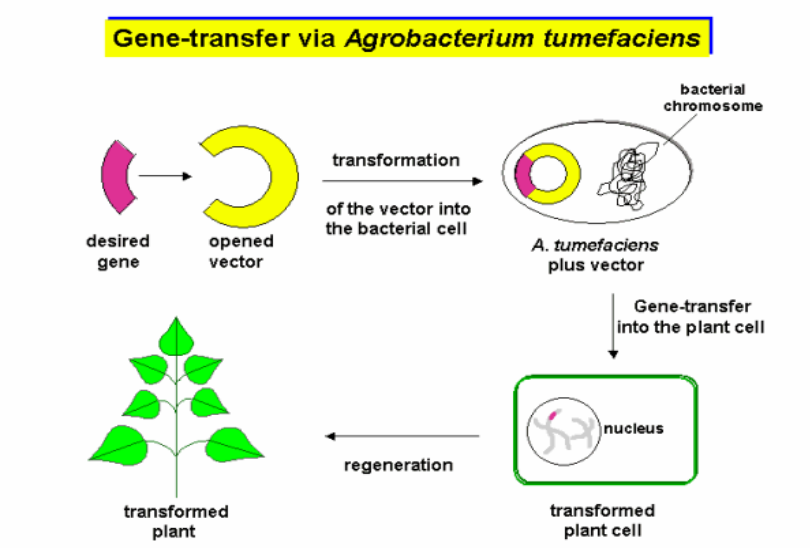

Figure 1. Basic steps in transformation of plant cells by *Agrobacterium tumefaciens*. The T-DNA transfer is represented according to updated knowledge on this process

Gene-gun

The gene-gun (biolistics) method can be applied on all plant species. In this method gold or tungsten micro particles, coated with transgenic DNA, are used and fired into the target tissue by an explosive discharge or pressurized helium (Kikkert 1993). This method allows to penetrating of DNA to the nucleus of the plant cell and may be incorporated among the plant's own genes (Finer *et al* 1999).

New Approaches in Plant Biotechnology and Plant Protection

The ability to introduce foreign gene constructs into plant tissues and to regenerate whole fertile plants has been developed over the past twenty

years. Using these procedures, crop plants have been developed with improved resistance against certain herbicides (Roundup ready), to insects (Bt gene) or diseases, or improved characteristics like enhanced vitamin A level (golden rice). These improvements are important but they represent only the beginning of a revolution in plant breeding.

Herbicide resistance

Herbicide resistance is genetically engineering crops to achieve immunity from herbicides, which are weed killing chemicals. Applying of herbicides only affects and killed the weeds and the crops are remained and crop yield increases and the crops are being much healthier. In addition containing of the soil from previous sprays with herbicide can also damage crops. Herbicide resistance prevents the occurring of these cases. Herbicide resistance is achieved by taking detoxifying enzymes to plant body and inserting them into the plant gene (Lyon *et al* 1989). The genes detoxify the herbicide or alter the quantity or sensitivity of the enzymes and it resulted in killing of the plant. Most of the time, herbicide resistance is limited to a certain kind of herbicide. Resistance to most of the herbicidal groups has already been obtained. The herbicide *Roundup TM* contains the active ingredient glyphosate. Glyphosate kill plants by binding to the active site of an enzyme called enolpyruvalshikimate phosphate synthase (EPSP synthase). This enzyme is critical for the synthesis of aromatic amino acids. Since plants must synthesize all of the amino acids that they need for protein production, inhibition of EPSP synthase by glyphosate causes the plant cells to starve for amino acids. Roundup is an extremely effective herbicide but kills almost all plant species, including most crop plants. However, Roundup is undamaging to human beings and animals because they do not have EPSP synthase. Therefore, Roundup can be used for spot weed control but not for general application on crops. If crop varieties are developed that are resistant to glyphosate, then Roundup could be used as an "over the top" herbicide for weed control. To do this, researchers have developed modified EPSP synthase genes that produce enzymes that are still functional but are not inhibited by glyphosate. This glyphosate-resistant EPSP synthase gene is used to develop a gene construct controlled by a strong CaMV 35S promoter. When introduced into plants, using Agrobacterium-mediated transformation, the regenerated plants are found highly resistant to treatment with Roundup. Using this technology, varieties of crop plants including cotton and soybeans etc. have been developed and released to producers. Genes that provide resistance to other herbicides have also been developed. These include genes for resistance to BuctrilTM, Sulfonyl ureas, gluphosinates, and 2,4-D. Transgenic plants that carry these genes have been developed and tested and some are either on the market or will soon be.

Insect resistance

Bt toxins are a group of crystalline protein that are produced by a bacterium of the species *Bacillus thuringiensis* (Bt). When these proteins are eaten by larvae of certain species of Lepidopter insects (moths), they bind to the cells that line the insect digestive tract and cause the cells to breakdown with quick death of the larva. Pesticide sprays and powders that contain Bt toxin have been used for many years and, since the toxic effects are limited to a few species of insects, these pesticides are safe. Since the Bt toxins are proteins, the genes that encode them have been cloned and characterized (Maagd *et al*. 1999). The development of effective gene constructs that could express Bt toxin in plant tissues at very high levels to kill predatory insects was difficult but has been accomplished. The reason for the difficulty is based mostly on the sequence characteristics of the bacterial Bt toxin gene which produces mRNAs that are not easily translated in plant cells. Since the genetic code is redundant, it was possible to redesign the Bt toxin gene so that it produces mRNA that encodes the same protein as the native Bt toxin gene but is much more easily translated in plant cells. Transgenic crop plants, including cotton and maize, that express Bt toxin genes have been developed and commercialized. Bt toxin-producing cotton plants were sold during the 1996 growing season by Delta and Pineland Co. under the variety name Bollgard TM. These varieties have increased resistance to boll worms and producers expect to be able to save production costs by reducing the use of traditional pesticides. One of the major concerns with wide spread use of Bt toxin expressing plants is the possibility of selection of insects that are resistant to the toxin. This will almost surely happen but it can be delayed by providing insects with areas of nonresistant plants for reproduction. Ultimately, a wide variety of different Bt toxins that target different receptors in the insect gut will be used to reduce the likelihood of the development of resistant insects (Perlak *et al* 1990). Also, since Bt toxins are only effective against a narrow range of insect species, research to identify and develop other insecticidal gene constructs is underway.

Resistance to plant pathogens

Plants are susceptible to diseases caused by infection of viruses, bacteria and fungi. Most progress has been made with plant disease resistance. Researchs on expression of a gene that encodes the coat protein of tobacco mosaic virus (TMV) in transgenic tobacco plants caused the plants to resist TMV infection. It was found that the protection was specific and did not protect against other virus strains or pathogens. Engineering of crop plants for resistance to fungal and bacterial infections has been more difficult. However, by studying the defensive genes that are expressed in naturally disease-

resistant plants important progress has been made (Rommens and Kishore 2000). The proteins encoded by these so called pathogenesis related genes (PR) can, in some cases, provide some disease protection in transgenic plants. Crops are often affected by viruses. Viruses cause a large number of diseases and a large amount of economic damage. Genetic modification could be achieved so that the crop becomes immune to a certain virus (Timmerman 1993). A weakened or dead virus would be inserted into the DNA of the crop so that it would be ready to fight the real virus if encountered. Tobacco and tomato plants have already been developed with resistance to the tobacco mosaic virus.

Fungi, like viruses, cause destruction to crops. Antifungal sprays are used to controlling of plant pathogens. Antifungal proteins, however, are present in plants such as tobacco. Genetically modified plants could be created that express fungi resistance (Broekaert *et al* 1997, Bolar *et al* 1999).

Nematode resistance

Chemicals and soil fumigation, the traditional methods used for control, are both expensive and toxic. Some fumigants even have damage to the ozone layer. In recent studies the scientists have found a gene isolated from a wild beet which is resistant to nematodes. Nematodes only eat one part of the plant. Due to the presence of protease inhibitors in rice, nematodes can not eat the actual grain. Scientists have transferred the genes containing these protease inhibitors to the root of the plant to create nematode resistance (Narayanana *et al* 1999). Mi and Hero from tomato, cre3 from wheat (Seah *et al* 1998) isolation of nematode controlling Bt genes etc. The best results have been recorded on Arabidopsis in which transgenes producing protease inhibitors give increased tolerance to a range of nematodes. Furthermore, using of antisense aquaporin genes are promised in tobacco and cotton to give enhanced tolerance to root-knot nematode.

Tolerance to drought and frost

Scientists were created tolerance to poor soil conditions with using of tissue culture. Some clones will alter their genes while clones are made and subject to different conditions. This is called somaclonal variation. Plants can also be made tolerant to high salt content in the soil by adding salt to the tissue culture clones. The surviving plants can be grown as salt tolerant (Zhu 2000, Zhang *et al* 2000; Apse and Blumwald 2002). Genetic modifications are made it possible to ability to alter the salt content in their cells on yield roots which penetrate deeper into the soil, which thicker cuticles to retain water. These methods are used to potentially create drought resistant plants. Water could be conserved, growing seasons could increase, and of course growing crops in desert areas would be possible.

Another problem is frost and is responsible for damage in agricultural production. A specific bacteria has been found that doesn't provide a surface for ice to form. Crops could be engineered to express this anti-frost surface. Longer growing seasons would result and food could be stored at subzero temperatures. Anti-freezing proteins are another option. A gene from the winter flounder, an arctic fish, has been found to express anti-freezing proteins. This gene could be incorporated into plants (Zhang *et al* 2000). Another way would be to alter the fat composition. Unsaturated fats contain more fluid at low temperatures, therefore preventing freezing.

CONCLUSION

Scientists are involved in finding ways to make plants more resistant to plant disease resistance and environmental stresses such as extreme temperatures, water deficit. While these techniques provide exciting new tools for the development of crop plants with new characteristics, they will not replace traditional plant breeding however, transgenic plants and traditional breeding techniques will be necessary to effectively incorporate these new technologies into useful varieties. It is important to remember that even the best transgenic cannot make a poor variety successful.

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