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Molecular Biology Essay Question:
Give an example of gene cloning in agriculture.

Papaya Resistant to Ringspot Virus.

Introduction

Agriculture is the domestication of plants for human benefits; plants are chosen among others for their nutritional values, their resistance to different environments and ease to be grown. Before the era of molecular biology, the selection of the best candidates occurred unconsciously and phenotypically; nowadays, thanks to the advent of cloning techniques, plant breeding can occur consciously and genotypically. New organisms can be designed in order to fulfil human or environmental needs; a toxin encoding gene from *Bacillus thuringiensis*, as a classic example, can be cloned in the genome of corn, so that the plant can produce its own pesticide. In this essay, I will focus on the cloning of the *cp* gene from the Papaya Ringspot Virus (PRSV) into Papaya cells, in order to make the plant resistant to the virus itself. This technique, which was first developed in the late 1980s by the University of Hawaii and Cornell, allowed Hawaiian farmers to tackle the big problem of the Ringspot Virus, which yearly infected and killed 70% of papayas, causing losses of circa 40 million dollars every season (Tecson Mendoza, Laurena & Botella, 2008). The genetically modified "Rainbow" and "SunUP" papaya strains are examples of cloning, as in their genome they contain one copy (Rainbow strain is hemizygous for the *cp* gene) or two copies (SunUP is homozygous) of an alien gene. This allowed the Hawaiian agricultural industry to resist the crisis due to loss of papayas, which in Hawaii constitutes 80% of agricultural industry (Gonsalves et al, 2004).

Papaya Ringspot Virus and the Concept of Pathogen-Derived Resistance

Ringspot Virus is a pathogenic plant virus of the genus *Potyvirus,* it consists of a single stranded RNA filament of 10 kb (Souza et al, 2005). When plants are infected, they develop spots on the leaves and ultimately die without producing any edible fruit. Rainbow and SunUp transgenic papayas are resistant to the virus because they contain in their genome a copy of the coat protein gene (*cp*) derived from the virus itself. This concept of a pathogen-derived resistance was first described by Sanford and Johnston from Cornell and Duke University in 1984, who showed that GM *E. coli* with a cloned coat protein gene from QB bacteriophage was resistant to the virus (Sanford and Johnston, 1985). The principle behind this idea is that if the host itself can produce proteins needed for the assembly of the viral capsids, these translated proteins will interfere with the transcription of the viral RNA. In this case, the *cp* gene from PRSV cloned in the papaya genome is translated into coat proteins, which bind to the regulatory sites of viral RNA and block the transcription, as if signalling to the viral RNA that coat proteins have been already produced and there is no need to transcribe any more RNA. Another possible mechanism responsible for the viral resistance conferred by the *cp* gene has been proposed by Gonsalves: PRSV resistance could be mediated by RNA posttranscriptional gene silencing. *Cp* is transcribed into RNA but not further translated into a polypeptide, consequently the expressed RNA could bind to the homologous sequence of the viral RNA and block its transcription, as in the process of RNA interference. In both cases, the viral RNA cannot be transcribed and so is unable to assemble a functional viral capsid capable of infecting and lysing the host cell. Transgenic papayas which contain the *cp* gene are thus resistant to the virus.

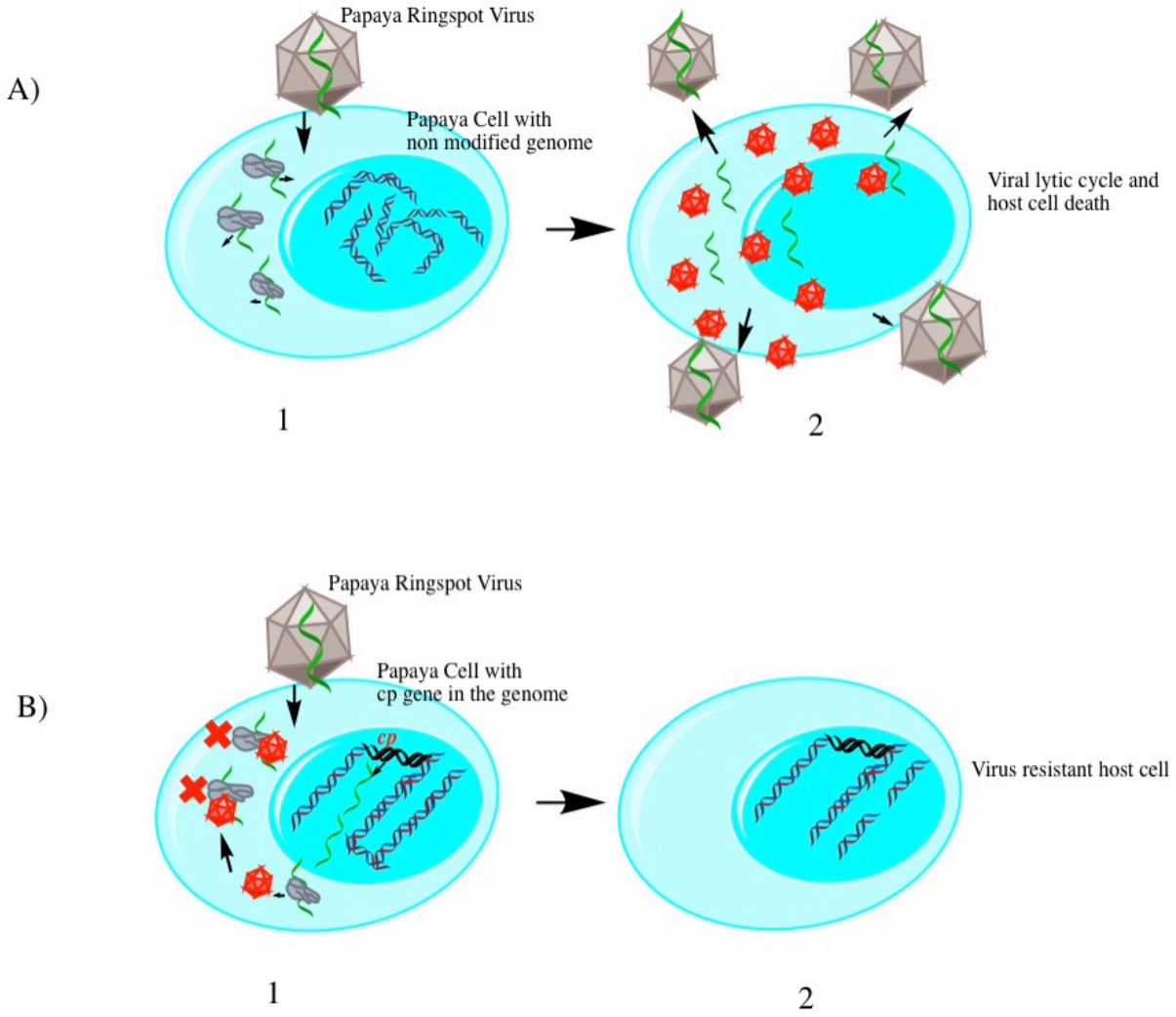

Fig.1) Personal diagram of the concept of PDR applied to Papaya.
A: PRSV transfers its RNA to the host cell, the RNA is transcribed and translated to proteins that assemble into new viral capsids. The infected cell lyses, releases the viral progeny and dies.
B: Transgenic papaya contains the *cp* gene in the genome, which is transcribed and translated into cp protein by the host cell machinery (as suggested by Sanford and Johnston). Coat proteins translated in this way bind to the regulatory sites in the viral RNA and block its transcription. These OGM papaya strains are resistant to the virus. Gonsalves proposed that *cp* is not translated into protein but its RNA transcript binds and blocks the transcription of viral RNA (not diagrammed here).

Cloning Strategy

There are several ways to clone a gene from an organism (in this case, a virus) into another one; in this section, I will describe the cloning method adopted in 1984 by Gonsalves and his team to produce transgenic "Rainbow" and "SunUp" papayas (Fitch et al, 1992). The complete sequence of PRSV RNA is known, together with the position of the genes. First of all, the *cp* gene has to be isolated and amplified; in their experiment the researchers from the University of Hawaii and Cornell used a RT-PCR reaction.
RT-PCR stands for *reverse transcriptase polymerase chain reaction:* analogously to normal PCR, this technique can amplify exponentially a genetic sequence marked by two oligonucleotide primers, but instead of amplifying DNA, RT-PCR can amplify RNA and convert it to DNA at the same time. The key to this method is an enzyme called *Reverse*

Transcriptase, which is the reverse of RNA polymerase, as it is a RNA-directed DNA polymerase: it catalyses the polymerization of a DNA chain complementary to an RNA template. Oligonucleotide primers have been designed in order to hybridize to the initiation and termination sites of the viral *cp* gene. The enzyme binds to the primer and catalyses the nucleophilic attack of the 3' hydroxyl group of the last ribonucleotide to the α Phosphorus of a free deoxynucleotide triphosphate, which were added to the solution; the free deoxynucleotide triphosphate attached to the growing chain is complementary to the ribonucleotide of the RNA strand. The complementarity is due to the fact that it has to form Watson-Crick base pairing in order to associate with the polymerase. Reverse transcriptase from a virus (Maloney Murine Leukaemia virus in this case) has been added to the solution, and after incubation, the cDNA produced was treated with RNAse in order to degrade the RNA strand of the RNA/DNA hybrid. The cDNA was then amplified in a PCR reaction, using Taq DNA polymerase and the remaining RNA fragments digested by the RNAse that acted as primers. After PCR, the number of copies of the *cp* gene are 2^n (where n= number of cycles= 31 in this case). The amplified *cp* clones, in order to be incorporated in the papaya genome, are ligated in a plasmid vector, which was engineered *ad hoc*. The plasmid vector contained the same restriction sites found also in the terminal sides of the *cp* genes (*Nco*I). Clones and vectors cut with the same restriction enzymes have complementary "sticky ends", which due to Watson-Crick base pairing, anneal together as a result of complementary nitrogenous bases. DNA ligase is then added to the tube containing digested clones and plasmids; DNA ligase catalyses the formation of a phosphodiester bond between the 3'C hydroxyl group at the 3' end and the α Phosphorus at the 5' end of the interrupted sequence, using the free energy derived from ATP hydrolysis; ATP thus is added to the solution so that DNA ligase can join together the digested fragments that have annealed together to produce a ligated continuous circular DNA, comprising both the vector and the cloned *cp* gene.

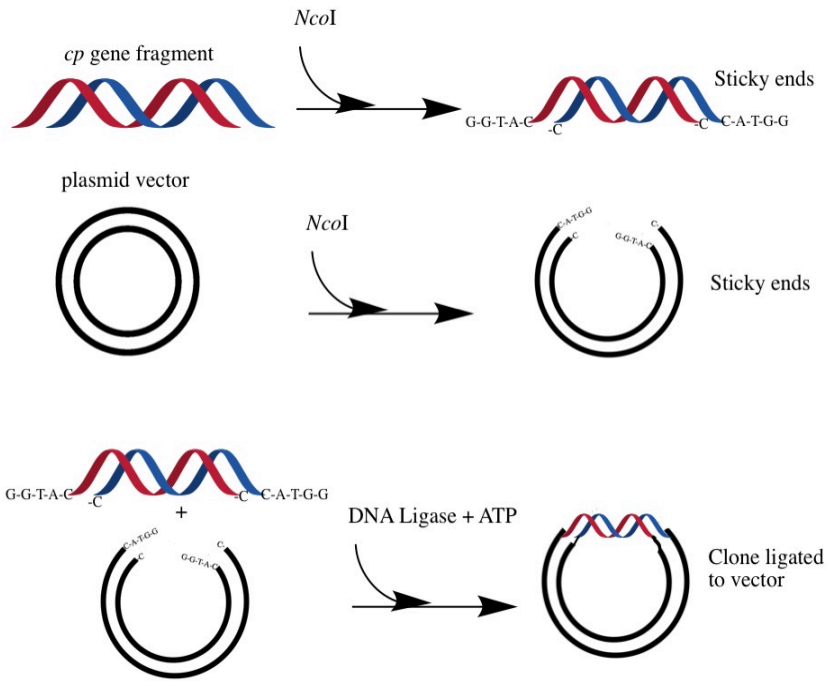

Fig.2) Personal diagram of the experiment of Gonsalves.
The amplified *cp* fragment and the plasmid vector were cut with *Nco*I restriction enzyme. Because the same restriction enzymes cut DNA at palindrome sequences, the fragments produced will have complementary sticky ends. The *Nco*I digested plasmid and insert are incubated with DNA ligase and ATP in order to be covalently joined together.

The new plasmids produced now have to be incorporated in the papaya cell. The team at the University of Hawaii transformed papaya zygotic embryos using a microprojectile bombardment method; alternatively, it is possible to transform cells using the bacterium *Agrobacterium tumefaciensis,* which infects plants by transferring its DNA in the host plant cells. For the microprojectile bombardment method, plasmid DNA are coated on the surface of 1μm diameter gold or tungsten particles (Slater et al, 2007) and are accelerated through a pressurized helium particle bombardment apparatus, which target them to the embryos, so that DNA-coated iron particles have enough momentum to penetrate the nuclear membrane of the cell.

Successfully transformed papaya embryos were selected using kanamycin as selective agent: together with the *cp* gene, the plasmid vector contained the kanamycin resistance gene as a selection marker, cells that have incorporated the plasmid will grow even in the presence of kanamycin and cells that have not been transformed will die. However, it has been observed that transgenic plants that have been selected with an antibiotic resistance gene are recovered with more difficulties; new methods of discerning transgenic plants are thus now preferred, such as visually mark them by the use of green fluorescent protein encoding plasmids (Tripathi, Suzuki and Gonsalves, 2007). The plasmid vector was designed in order to be incorporated in the genome, specific sequences similar to those of the papaya genome were present in the plasmid, so that the plasmid would have undergone homologous recombination with the nuclear genome and the genes of interested would have been integrated in the papaya genome. Papaya embryos that have integrated the plasmid in the genome are said to be "stable transfected" and are able to pass the new transgene in the germline (Alberts, 2015), thus forming a new transgenic papaya strain. The first transgenic strain produced was the SunUp strain, that is homozygous for the cp gene (CP/CP); the Rainbow strain is an F1 hybrid between SunUp and Kapoho, which was the most popular variety among farmers (Gonsalves, 1998). Rainbow papayas are therefore hemizygous for the cp gene (CP/+), because Kapoho is not transgenic and does not contain the *cp* gene and SunUp contains two copies of the cp gene (CP/CP × +/+ F1:→ CP/+).

Conclusion

OGM papaya produced with this method helped the Hawaiian farmers to tackle the crisis due to the plant viral infection. "SunUP" and "Rainbow" papaya strains were immediately commercialized and deregulated, in order not to cause any further delay in the papaya production and thus were the first transgenic fruit crop to enter the agricultural market. Nowadays OGM papayas are found all across the world, together with OGM maize (Bcorn), Golden Rice, OGM soya beans, OGM cotton and several other products. Furthermore, the production of transgenic papaya helped to better understand the mechanism of pathogen derived resistance, which is now thought to work via RNA posttranscriptional gene silencing, as proposed by Gonsalves. Molecular cloning acted as a catalyst for innovation in the agricultural world and may represent the hope for a more productive, environment-oriented and farmers-sustainable agriculture. New techniques, such as CRISPR, are being developed and are advancing at the same pace the fields of molecular biology and agriculture.

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