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CURRENT STATUS AND FUTURE PROSPECTIVE OF USE OF BIOTECHNOLOGY IN PLANT DISEASE MANAGEMENT

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Abstract

This paper attempts to review on the works undertaken in the field of biotechnology to combat the disease occurring in the major crops of agricultural importance. All possible relevant papers that were published in this sector were critically reviewed and major accomplishment of the study were summarized in the form of successful technological tools to mitigate the emerging problems of diseases in the context of climate change. Several biotechnological tools were developed and implemented in the scientific world that encompasses tissue culture techniques which was the most widely accepted and applied in many economically important crops. Biotechnology has opened the avenues for many other options to mitigate the plant disease problems. More recently new biotechnological tools have been arising including candidate gene approaches, gene pyramiding for durable resistance in many important crops and RNA interference techniques as highly useful and eco-friendly tool for the management of disease in a ecological manner. In summary, advances in the biotechnology research has offered remarkable and enormous opportunity to the present day plant breeders and pathologist to works in consolidated and consorted way to overcome the issues posed by the new dynamics of disease and pest problems.

Keywords: Biotechnology, Candidate gene, durable resistance, RNA interference

BACKGROUND

Biotechnology is the genetic manipulation, and multiplication of any living organism through novel techniques and technologies such as tissue culture and genetic engineering resulting in the production of improved or new organism and products that can be used in variety of ways (Agrios, 1988). Genetic engineering is also part of biotechnology by which it is possible to isolate particular gene from one organism, insert them into the genome of another organism and make them to express at right time. Cells of plants can be cultured in special nutrient medium and whole plants can be regenerated from cultured cells. This technique of growing plants in *vitro* is called "Tissue culture". Plant diseases are a threat to world agriculture and general food security. Significant yield losses due to the attack of pathogen occur in most of the agricultural and horticultural crop species (Fagwalawa et al., 2013). Several biotechnological tools and approaches have been employed to overcome the hazardous effects of disease and pest in crop plants. Majority of the plant disease can be successfully managed by the tissue culture techniques that are products of biotechnology.

Tissue culture and Somaclonal variation for disease resistance: Somaclonal variation refers to the tissue culture derived variation- Plants regenerated from somatic cells, using tissue culture are not genetically uniform but display significant genetic variability. This variability is very high when compared to spontaneous mutation. Somaclonal variation has been demonstrated in a large number of plant species (wheat, rice, oats, maize, tobacco, potato, sugarcane, brassica, etc.) for various traits such as resistance to fungal, viral and bacterial diseases. The procedure involves growing of cell cultures for several cycles on nutrient medium without any selective agent, followed by regeneration of plants.

Meristem or shoot tip culture: Meristem and shoot tip culture are used to eliminate virus from infected germplasm. It has long been observed that the rapidly growing meristems of plant are usually free of viruses, or at least have much lower concentration of viruses than non meristems cells. This situation has been exploited for the production of virus-free plants by meristems culture. It is commonly used in cassava, potato, sweet potato and ornamental plants. The presence of viruses in the plants can reduce the yield and quality of crops. In infected plants, the apical meristems are generally either free or carry a very low concentration of the viruses. Five main possibilities have been suggested to explain the mechanisms underlying the resistance of meristems to viruses.

1. Exclusion of the viruses from the meristems by lack of suitable vascular or plasmodesmatal connections.
2. Competition for key metabolites by the rapidly dividing meristem cells.
3. The production of substances in meristems cells that result in breakdown of the virus.
4. Deficiency in some key components of the machinery of virus replication, and
5. Presence of inhibitors of virus replication

Transgenesis in disease resistance: Tissue culture in conjunction with recombinant DNA technology is becoming increasingly important to insert foreign genes and produce transgenic plants. For successful infection of virus particles, the coat protein should be removed from viral RNA. If the host is made to synthesize coat proteins in large amount, naked viral RNA formation will be negligible. The host coat protein will encapsulate the RNA of the virus and prevent its multiplication. This will result in reduction and delay in symptom development. E.g. transgenic tobacco plants expressing the tobacco mosaic virus coat protein protected the plants against this virus. The expression of the viral genome in transgenic plants also conferred resistance to virus infection. These regions include portion of the viral replicas as well as, antisense RNA to coat protein. Transgenic tobacco plants transformed with a DNA copy of the satellite RNA of cucumber mosaic virus (CMV) were shown to produce large amounts of satellite RNA following inoculation with CMV and symptom development was greatly reduced. The constitutive expression of the groundnut stilbene synthase gene in transgenic tobacco plants results in the synthesis of resveratrol (phytoalexin) and the transgenic plants show resistance to *Botrytis cinerea*. Transgenic tobacco plants expressing acetyltransferase which detoxifies the tabtoxin, show resistance to *Pseudomonas syringae* pv. *tabaci.* More recently, chitinase gene from *Manduca sexta,* tobacco horn worm, has been cloned into *P.fluorescens* to increase their antagonistic potential against *R.solani*.

Role of Biotechnology in Plant Disease Control:

Recent advances in plant genetic engineering strategies for the management of bacterial diseases of plants are now available. The use of tissue culture and genetic engineering for controlling plant diseases has been described very concisely (Fagwalawa et al., 2013), while the role of biotechnology in controlling plant disease has been discussed by (Mandahar and Khurana, 1998). Plant biotechnology impinges or helps plant pathology in many ways;
1. To obtain pathogen-free mother plants through rapid clonal propagation.
2. New plants to which genes have been incorporated through genetic engineering
3. The main vehicle for transferring genes from donor to recipient, in plant pathogens, particularly the bacterium Agrobacterium tumefaciens and the cauliflower mosaic virus.
4. Control of plant diseases by inserting resistance genes into plants by genetic engineering techniques.
5. The study of plants genes for resistance to disease and of pathogen genes for virulence to pathogen has already added considerably by genetic engineering techniques.

Tissue Culture Techniques: Almost all tissue culture techniques are used in plant pathology. Some of the importance tissue culture techniques and their importance to plant pathology are briefly described;

Protoplast Fusion: Disease resistance in breeding program may come either from closely related species or from more distantly related species. Problems are generally encountered if an effort is made in crossing distantly related species. Protoplast fusion is one of the methods that can be used to circumvent problems in introgression genes for resistance. By this method, factors that contribute to crossing barriers between species can be avoided and viable hybrids (Cybrids) have been recovered even between distantly related species (Harms, 2018). Examples of disease resistant plants, produced from protoplast fusion are shown in Table 1.

Table 1. Disease Resistant Plants Produced from Protoplast Fusion

Species used for fusion	Diseases	Reference
Lactusa sativa	Dowmy mildew (*Bremia lactucae*)	Maloy, (2005)
Brassila oleracea and *Raphanus sativus*	Club root (*Plasmodiophora brassical*)	Maloy, (2005)
Brassica napus and *Brassica nigra*	Black leg (*Phoma lingum*) club root.	Maloy, (2005)
Solanum brevidens and *Solanum tuberosum*	Bacterial soft rot (*Erwinia* spp*.*)	Maloy, (2005)

Source: (Eck and Smith, 1996).

Chemically induced fusion: Isolated protoplasts are sticky, tend to aggregate in suspension and show fusion spontaneously during incubation. Chemicals tend to increase the fusion frequency. Fusion can occur in the presence of high CA^{2+} and high pH (9-10) but a commonly used chemical (Fusogen) is poly ethylene glycol (PEG). Due to the addition of PEG there is adhesion of protoplast to their neighbors which can be assessed by microscope. Subsequent dilution of stabilized PEG, either stepwise or at once results in fusion and mixing of the cytoplasm. It has been possible to inoculate the protoplast of plant with

viruses and study their replication and physiology. The bacterium *Agrobacterium tumefaciens* or its modified T-plasmid and the double-stranded DNA virus cauliflower mosaic virus have been used to introduce foreign genetic material into plant cell.

Selection for Disease Resistance: In-vitro selection has a distinct advantage over other selection systems since it allows significant saving of space, time and money. For plant diseases that cause damage through toxins, cell selection for toxin resistance in cultures and regeneration of plants from descendants of the selected cell lines can give disease-resistant genotype. For example, disease resistant crop plants have been produced through in vitro selection in potato against *Phytophtora infestans* (late blight of potato), in tobacco, (*Nicotiana tabacum*) against *Pseudomonas tabli*.

Recombinant DNA Technology: Advances in molecular biology have opened up enormous potentiality of identifying and isolating any gene for an organism, and mobilizing and expressing it in a different organism of one's choice.

Engineering Plants for Resistance to Disease: A landmark achievement has been made with regard to viral diseases following use of r-DNA technology. For example, a major achievement has been the transfer and expression of coat protein genes of tobacco mosaic virus (TMV) and alfalfa mosaic virus (AMV) in tobacco, resulting in protection against or delay of disease development in the transgenic plants (Beachy et al., 1990). The purpose of introducing coat protein genes to give resistance against the virus is that the multiplication of infecting viral RNA is somehow checked by coat protein synthesized in the plant cells. Engineered plants synthesized chitinase which breaks down the fungal cell wall and this kills the soil borne pathogen, *Rhizoctonia solani* (Maloy and Powrie, 2005).

Monoclonal Antibodies Technique: The hybridoma technique discovery led to the production of monoclonal antibodies (Fagwalawa et al., 2013). In this technique there is the fusion of mycloma cells (cancer cells) with antibody-body producing while blood cell (B- lymphocytes). The resulting hybrid celled is called a hybridoma. In the last few years, techniques have been developed to produce large quantities of identical antibodies. These new antibody-forming hybrid cells, hybridomas, can now be grown culture indefinitely. Each hybridoma clone produces only one type of antibody. But via selection techniques the clone that produces the desired antibody can be chosen. Monoclonal antibodies can be obtained from the liquid of hybridoma cultures and can be used to detect, identify and measure the antigens that induced their production (Nagarajan et al., 1992). Monoclonal antibodies are however, very specific and may not detect strains of the same virus. It is for this reason that mixtures of several monoclonal antibodies are often used in the detection of viruses and in screening test.

Uses and Applications of Monoclonal Antibodies:

1. The most effective application of monoclonal antibodies has been with plant pathogenic viruses. This technique has be used for accurate identification of viruses for tracing the viruses during epidemiological studies, to distinguish between virus strains and in isolation and purification of viruses.
2. These procedures are now being used to study bacteria and fungi and will probably be used in investigation of various populations of rhizosphere organism. This technology is also very helpful in attempts to identify bacteria in mixed population. Application of this technology to fungi is in its infancy, but the potential is great.

Transgenic Plant Disease Management: Diseased resistance genes could be sourced from plant pathogens themselves, as was possible with coat protein-mediated plant viral resistance and with toxin in activating protein-mediated bacterial resistance (Agrios, 1988). Host plants also contribute an enormous number of disease resistance genes such as those encoding pathogenesis-related (PR) proteins, which have been used against fungal disease (Schippers, 1988).

Candidate Genes against Viral Pathogens: The use of transgenic resistance against plant disease is that was accomplished in the management of papaya ring spot virus (PRSV) in Hawaii (Jain, 1993). Under these circumstances, coat- protein-mediated resistance using coat protein genes sourced from a Hawaiian strain of PRSV was attempted. One transgenic line was found to be completely resistant to PRSV (Fagwalawa et al., 2013). Recently, a gene silencing mechanism has been put to productive use in obtaining rice yellow mottle virus. An open reading frame of the virus itself is expressed in rice in order to stop the viral spread in an effective manner. Similar attempts also have been made in obtaining multiple viral infections (tomato spotted wilt virus and turnip mosaic virus) in plant.

Candidate Genes against Bacterial Pathogens: A wide-spectrum bacterium bacterial blight resistance gene Xa21, sourced from an African rice, *Oryza longistaminata* was backcrossed into cultivated variety. The resistance gene was cloned using molecular means by Pam Ronald of University of California and distributed to labs all over the world, so that the gene could be put into rice cultivars of local importance (OSWALD, 1951).

Wild Fire disease of tobacco caused *Pseudomonas syringae PV. tabaci* is a serious disease. A phytotoxin secreted by the pathogen drastically modifies the amino acid metabolism of the plant Interestingly, the pathogen that synthesizes the phytotoxin remains unaffected by the toxin. This formed the basis for a search of the candidate gene from the pathogen itself. A toxin-inactivating gene, which was named 'ttr' was successfully isolated from the pathogen and the same was cloned into tobacco cultivars, which showed excellent wildfire resistance (Agrios, 1988).

Candidate Genes against Fungal Pathogens: PR protein genes appear to be a very effective source for candidate genes for fungal resistance. These proteins

may play a direct role in defense by attacking and degrading pathogen cell wall components. Typical candidate genes are that encoding chitinases and B – 1, 3 glucanases (Fuchs and Gonsalves, 2007) increasing expression of individual and multiple PR proteins in various crops have demonstrated some success in enhancing disease resistance in particular pathogens (e.g. in rice against (Rhizoctonia solani, the shealth blight pathogen). A result of a research shows a chitianase gene from an anti-fungal bio control fungus species (*Trichoderma viridae*) confers transgenic resistance against the rice sheath blight pathogen.

Pathogen derived resistance: The pathogen derived resistance can be protected from diseases with transgenes (genes that are inserted into plants) that are derived from the pathogens themselves, a concept referred to as pathogen-derived resistance. For example, plant viral transgenes can protect plants from infection by the virus from which the transgene was derived.

Genetic engineering: A novel and powerful tool to combat plant virus diseases: The concept of pathogen derived resistance has stimulated research on obtaining virus resistance through genetic engineering. Pathogen-derived resistance is mediated either by the protein encoded by the transgene (protein mediated) or by the transcript produced from the transgene (RNA-mediated). Transgenic tobacco expressing the coat protein gene of tobacco mosaic virus (TMV) was resistant to TMV and that the resistance was due to the expressed coat protein as explained by (Abel et al., 1986). Recent research indicates that pathogen-derived resistance to viruses is mediated by an RNA-based post-transcriptional gene silencing mechanism. This plant defense system results in degradation of mRNA produced both by the transgene and the virus. In general, protein mediated resistance provides moderate protection against a broad range of related viruses while RNA-mediated resistance offers high levels of protection only against closely related strains of a virus (Dawson, 1996). Coat protein-mediated protection has been reported for tobacco mosaic virus, (TMV) papaya leaf curl virus (PLCV), tomato mosaic virus (ToMV), cucumber mosaic virus (CMV), alfalfa mosaic virus (AlMV), potato virus X (PVX), potato virus Y (PVY), and potato leaf roll virus (PLRV). Compared to conventional breeding for virus resistance, genetic engineering provides a quicker and more precise technology to obtain plants that are resistant to viruses.

Antimicrobial proteins to enhance plant resistance:

Plants have both structural and biochemical defense strategies against pathogens. The plant pathogens have counter strategies to ensure successful infection. Plant disease results when interactions between plants and pathogens lead to abnormal growth and yield components. Plants grown for food, fiber, forage and ornamental purposes may be severely damaged and killed by diseases caused by pathogens. Chemical and biological treatments, cultural practices, and resistant cultivars are used to control plant diseases and prevent severe crop losses.

The production of active oxygen species (ROS) like superoxide anions, hydroxy radicals and hydrogen peroxide, have been observed in many plant pathogen interactions and are known to play an important role in plant defense. Plants have been engineered to continuously produce active oxygen species. In transgenic potatoes containing a H_2O_2-generating glucose oxidase gene from the fungus *Aspergillus niger*, the resulting apoplastic accumulation of peroxide ions enhanced the plants resistance to *Phytophtora infestans*, late blight; *Verticillium dahliae*, Verticillium wilt; and *Alternaria solani*, early blight (Wu et al., 1997).

Plant resistant (*R*) genes and Corresponding Avirulent (Avr) genes for disease resistance: R genes encode putative receptors that respond to the products of 'Avr genes' (Avr, avirulence) expressed by the pathogen during infection. In many cases, a single R gene can provide complete resistance to one or more strains of particular pathogen, when transferred to a previously susceptible plant of the same. The strong phenotypes and natural variability at R loci have also been exploited by molecular geneticists to clone the R genes and investigate their molecular modes of action. R gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently halt pathogen growth with minimal collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, R genes are often quickly defeated by co-evolving pathogens. Many *R* genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies (McDowell and Woffenden, 2003).

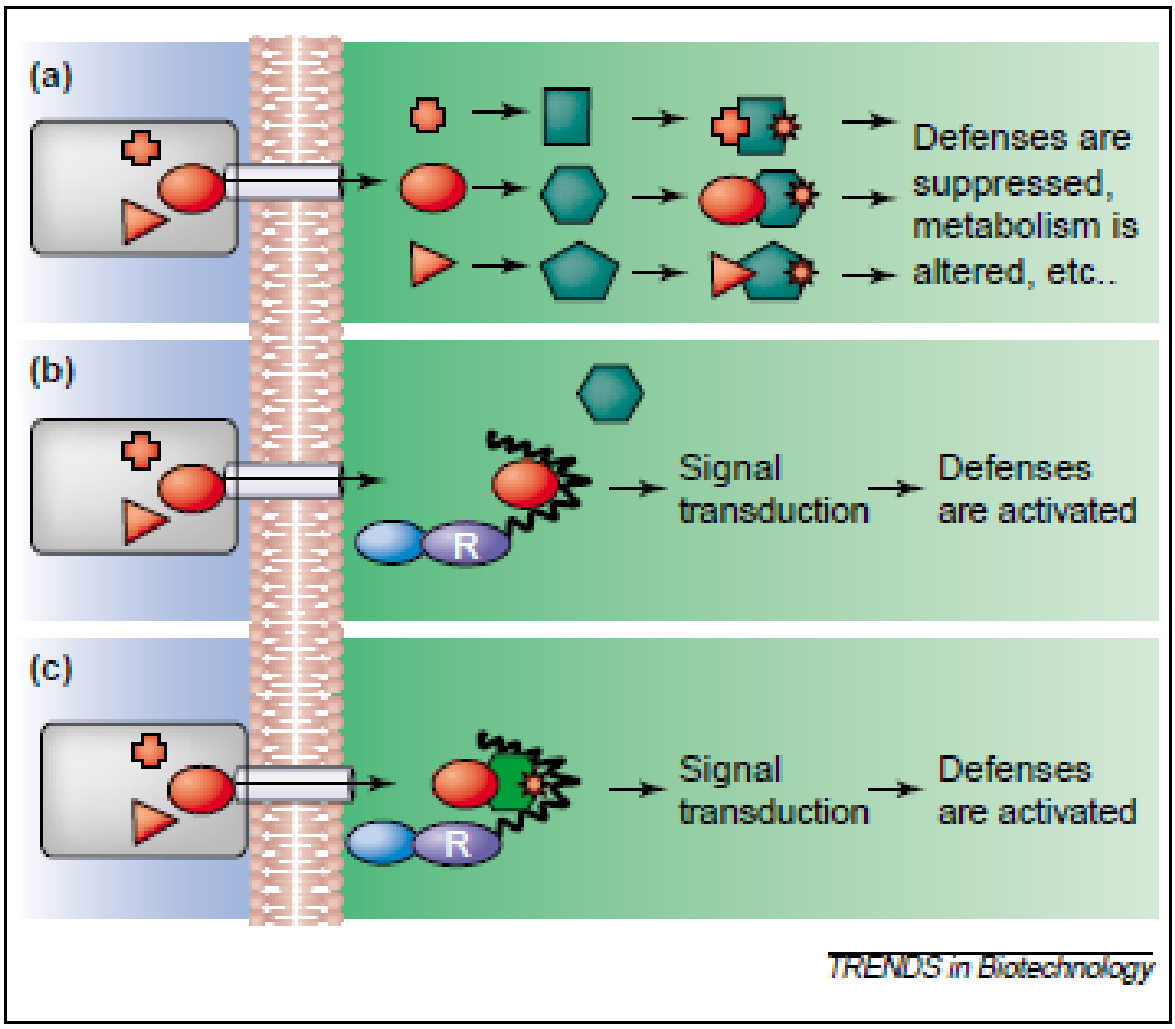

Figure 1. Interaction between pathogen Avr proteins and plant R proteins. A hypothetical pathogen (grey) has attached to a plant cell and expressing a suite of virulence proteins (red). These proteins are translocated into plant cells via Type III secretion (bacteria) or other unknown mechanisms (fungi and oomycetes). Once inside, they target host proteins (green) that control defense responses, metabolism or other plant process that affect pathogen virulence. (a) In this panel, the plant cell does not express an R protein that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and defenses are at best only weakly induced. Diseases then results from the collective action of the virulence proteins. (b) This panel depicts the classic receptor-elicitor hypothesis. In which an R protein directly binds a virulence proteins. This recognition event activates a complex signal transduction network, which in turn triggers defense responses. (c) This panel depicts the guard hypothesis, in which an R protein detects a modified host protein (red star), perhaps as a complex with the attacking virulence protein.

Gene Pyramiding for durable resistance: Many R genes lack durability because they can be defeated by a single loss-of-function mutation in the corresponding Avr gene. Because individual Avr genes often make only incremental contributions to virulence, pathogens can afford to alter or discard an Avr gene with little or no fitness penalty. Traditional breeding strategies have used R genes 'one at time' in crop monocultures. Such homogeneous host populations exert strong selection for mutation of the relevant Avr gene, and then become extremely vulnerable to the emergent pathogen. As an alternative to single-gene deployment, multiple R genes ('pyramids') can be bred into individual plant lines. There are also prospects for transgenic use of single R genes that have previously been proven durable. For example, the pepper gene Bs2 has provided long-standing resistance against bacterial spot disease, caused by the bacterium *Xanthomonas campestris*. Bs2 has been cloned from pepper and shown to encode a NB-LRR protein. *X. campestris* is also a significant pathogen of tomato and a pepper. Bs2 transgene works effectively in tomato against *X. campestris*. Recently cloned R genes with potential use against fungal pathogens include the barley Rpg1 gene and the tomato Ve1 and Ve2 genes (McDowell and Woffenden, 2003). Rpg1 has provided remarkably durable resistance to stem rust for decades while Ve1 and Ve2 target *Verticillium* species that cause wilt in many different crops. The Ve genes can provide resistance to different *Verticillium* species and are functional in potato when expressed as transgenes.

Increasing the broad of resistance: Many R genes have a narrow range of resistance; on the other hand some R genes do provide a wider spectrum. An broad-spectrum resistance, based on coordinate expression of an R gene and a corresponding Avr

transgene, controlled by a pathogen-inducible promoter has been illustrated in Figure 2. This tactic enables induction of defense by multiple pathogens without pyramiding numerous R transgenes. One crucial aspect of this strategy lies in selecting the right promoter to drive the Avr gene. An ideal promoter would respond rapidly to a wide variety of pathogens and thereby provide broad-spectrum resistance. The promoter must be inactive under disease free conditions to ensure that the plant does not sustain collateral damage from spurious defense responses triggered by leaky expression of the Avr transgene (McDowell and Woffenden, 2003).

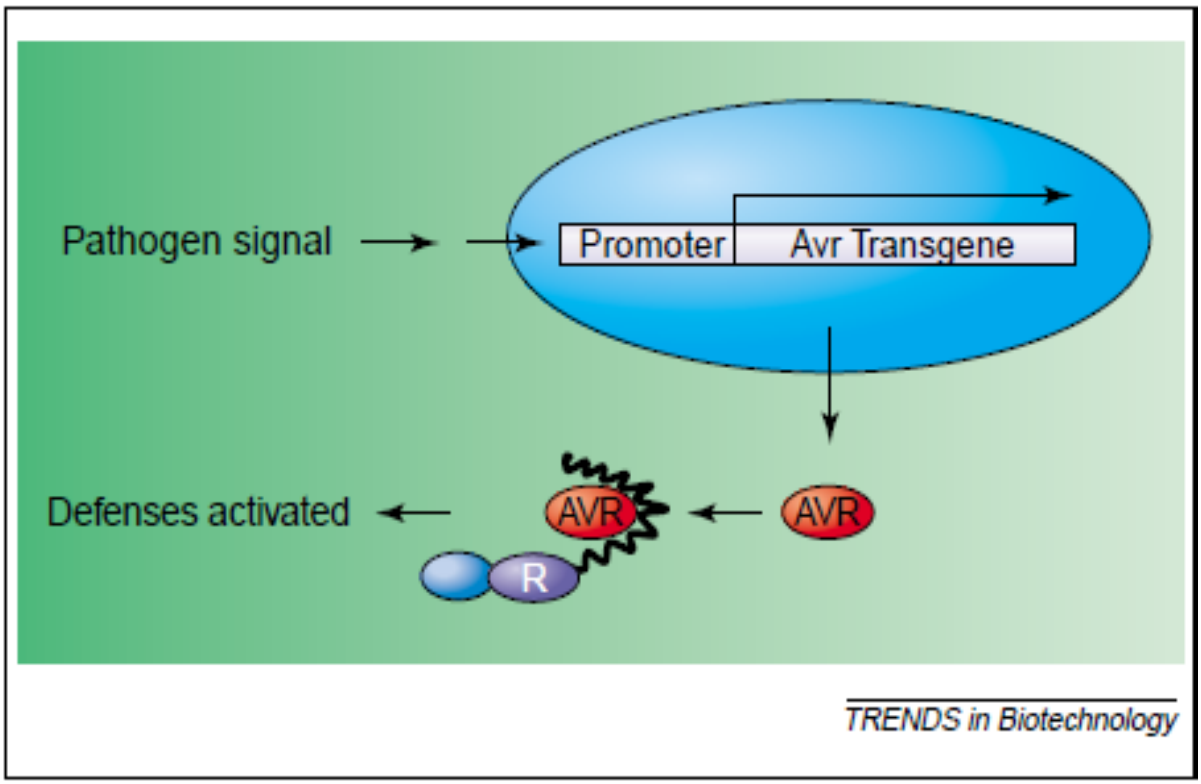

Figure 2. Strategy for engineering broad spectrum resistance by induction of Avr/R transgene combinations. A pathogen Avr gene is expressed in plant cells as a transgene, under the control of a plant promoter that is induced by a range of pathogens. A corresponding R gene (either endogenous or transgene) is also expressed. Upon pathogen attack, the pathogen-responsive promoter is activated, the Avr gene is expressed, and the Avr protein interacts with the protein to induce the HR and other defense responses. Note that this system can be activated by any pathogen (or spurious stimulus) that is capable of activating the promoter of the Avr gene.

Biotechnology and RNA Interference (RNAi):

RNAi operates in both plants and animals, and uses double stranded RNA (dsRNA) as a trigger that targets homologous mRNAs for inhibiting its transcription or translation whereby susceptible genes can be silenced. This RNA-mediated gene control technology has provided new platforms for developing eco-friendly molecular tools for crop improvement by suppressing the specific genes which are responsible for various stresses and improving novel traits in plants including disease resistance (Wani et al., 2010). RNAi technology of gene silencing is newly developed genomics tools that have great advantages over antisense and co-suppression due to their higher silencing efficiency and shorter time requirements for screening. RNAi technology can be considered an eco-friendly, bio-safe and ever green technology as it eliminates even certain risks associated with development of transgenic plants carrying first generation constructs (binary vectors and sense and antisense genes). Since RNAi triggers the formation of dsRNA molecules that target and facilitate the degradation of the gene of interest as well as the transgene itself to avoid problems arising from the synthesis of gene sequences as well as non coding regions of gene, thus limiting undesirable recombination events (Wani et al., 2010).

RNAi in Plant Disease Management: This changed scenario forced us to respond more efficiently and effectively to increasing disease and pest problems. RNAi technology has emerged as one of the most potential and promising strategies for enhancing the building of resistance in plants to combat various fungal, bacterial, viral and nematode diseases causing huge losses in important agricultural crops. Many of the examples listed below illustrate the possibilities for commercial exploitation of this inherent biological mechanism to generate disease-resistant plants in the future by taking advantage of this approach.

Management of Plant Pathogenic Fungi: RNA-mediated gene silencing (RNA silencing) is used as a reverse tool for gene targeting in fungi. Homology based gene silencing induced by transgenes (co-suppression), antisense, or dsRNA has been demonstrated in many plant pathogenic fungi. Role of RNAi in the management of some of fungal diseases have been presented in Table 2.

Table 2. RNAi effects on some targeted region in some fungal plant pathogens

Pathogen	Targeted region	Result
Magnaporthae oryzae	*eGFP*	Sequence specific degradation of mRNA
Cladosporium falvum	*cgl* 1 and *cgl* 2	Blocking disease infection spread
Venturia inaequalis	*Multiple inverted repeats*	-
Fusarium graminearum	-	-
Blumeria graminis	*Mlo*	Immunity

(Source Wani et al., 2010)

Management of Plant Pathogenic Viruses: Antiviral RNAi technology has been used for viral disease management in human cell lines. Such silencing mechanisms (RNAi) can also be exploited to protect and manage viral infections in plants. The effectiveness of the technology in generating virus resistant plants was first reported to PVY in potato, harboring vectors for simultaneous expression of both sense and antisense transcripts of the helper-component *proteinase* (*HC-Pro*) gene. The utilization of RANi technology has resulted in inducing immunity reaction against several other viruses in different plant-virus systems (**Table 3**).

Table 3. Effects of targeted regions of RNAi in various plant virus system.

Host system	Virus	Targeted region
N. benthamiana, M. esculenta	*African cassava mosaic virus*	*pds, su, cyp79d2*
Barley, wheat	*Barley stripe mosaic virus*	*pds*
Soybean	*Bean pod mottle virus*	*Pds Actin*
Barley, rice, maize	*Brome mosaic virus*	*pds, actin 1, rubisco activase*
Arabidopsis	*Cabbage leaf curl virus*	*gfp, CH42, pds*
P. sativum	*Pea early browning virus*	*pspds, uni, kor*
N. benthamiana	*Poplar mosaic virus*	*gfp*
N. benthamiana, S. tuberosum	*Potato virus X*	*pds, gfp*
Nicotiana tabacum	*Satellite tobacco mosaic virus*	*Several genes*
N. benthamiana, N. tabacum	*Tobacco mosaic virus*	*pds, psy*

(Source Wani et al., 2010)

CONCLUSION

Several biotechnological tools and approaches have been emerging in the recent years which are becoming very effective in disease management. Among these successful biotechnology tools tissue culture techniques were widely adopted by many countries in the early stage for producing disease free plants. Later on with the advent of new approaches of biotechnology and molecular tools disease management has become simpler and quick in application. Some of the highly acceptable modern techniques candidate gene approach, durable disease resistance via gene pyramiding for broad spectrum disease management as well as more recently RNAi (Gene Silencing) technology has proven itself as viable technology for disease management. These technologies are economical, eco-friendly and requires less time in development compared to conventional breeding techniques.

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