RNAi and CRISPR Gene Editing in Plants

Approaches and Applications

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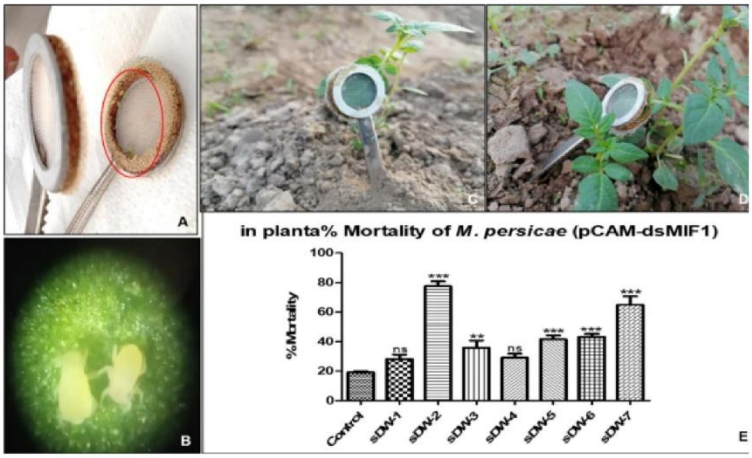

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About this Book

RNAi and CRISPR Gene Editing in Plants - Approaches and Applications examines two groundbreaking genetic modification technologies that are reshaping plant biotechnology and agriculture. This comprehensive work explains both the scientific principles and practical uses of RNA interference (RNAi) and CRISPR-based gene editing systems, giving readers the knowledge they need for both research and implementation.

The book addresses the underlying science and real-world uses of these technologies. It opens with a thorough examination of CRISPR systems, covering their essential components and mechanisms while exploring their integration with chloroplast transformation techniques. This combination creates new possibilities for advancing plant biotechnology across multiple fields, from agriculture to pharmaceutical production.

Key areas covered include:

- Clear explanation of CRISPR mechanisms and components
- In-depth analysis of chloroplast transformation vectors and their functions
- Applications in pharmaceutical production, including vaccine development and antibody bioreactors
- Agricultural stress management techniques, addressing both abiotic (temperature, drought, salinity) and biotic stressors
- RNA interference mechanisms and their applications in pest management
- Practical aspects of genetic transformation techniques, including particle bombardment and PEG-mediated transformation
- Case studies focusing on major crops like potato and maize

This volume serves both researchers in plant biotechnology, agriculture, and related fields.

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What is it and how does it work?

Genetic material that is heritable in plant cells is compartmentalized into three distinct organelles: the nucleus, plastids, and mitochondria. Fully realizing this goal of producing transgenic plants through genetic engineering has so far been substantially achieved by successfully transforming the nuclear genome to include genes of interest. Plasticomic paths do arise, however, as a great alternative that holds the potential for meaningfully contributing to sustainable food security.

Chloroplasts are the specific organelles of plant cells and eukaryotic algae, where photosynthesis and biosynthesis of a wide variety of essential compounds take place, including fatty acids, amino acids, nucleotides, phytohormones, vitamins, and secondary metabolites, as well as sulfur and nitrogen assimilation. The chloroplast has a highly polyploid, circular genome, termed the plastome, which possesses its own unique genetic machinery for transcription and translation. It is thought to have originated from endosymbiotic cyanobacteria that had been assimilated into an ancestral eukaryotic host cell, as indicated by a substantial number of lines of evidence obtained from molecular biology and comparative genomics. This plastid genome contains about 120-130 genes, which have widely been conserved among various species and possess a characteristic quadripartite structure. Two inverted repeat regions, IRA and IRB, divide the genome into small single-copy (SSC) and large single-copy (LSC) segments in the

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quadripartite structure. Whereas the general structural organization of chloroplast genomes is conserved, th Plastid transformation is a process of site-specific integration of genes at predefined sites in the plastid genome using appropriate transformation vectors designed for that purpose to enable homologous recombination. The integration involves the insertion of the transgenic cassette into one of the IR regions within the plastid genome. The presence of this integrated sequence in one IR encourages its subsequent integration into the duplicated IR region. This increases the selection pressure for acquiring homoplasmy since the replica repair system of the plastid genome works very efficiently to homogenize throughout the genome of the organelle. Besides, the highly conserved spacer regions intergenically provide a conceptual framework for designing a universal transformation vector applicable throughout the chloroplast genomes of higher plant species. This is made possible by the unique genetic machinery of plastids for the processes of transcription and translation.

However, in practice, when this is applied to unrelated species or those that have considerably lower homology from the flanking regions surrounding a target integration site, the efficiency of transformation can substantially reduce. Under such circumstances, it might be necessary to develop species-specific vectors in order for higher satisfactory levels of transformation success and expression to be observed. Biotechnology has rapidly advanced, and similar approaches have come to be used for transferring foreign genes not just to the nuclear genome but also to chloroplast genomes. Success in chloroplast transformation depends on several key factors: delivery of foreign DNA into the double membranes of the plastid, efficient selection of transplastomic plants, and stable integration of heterologous DNA into the plastid genome. Methods of Integration of Transgene into the Chloroplast Genome

Various different methods have been developed for the introduction of exogenous DNA into the chloroplast genome. Of all, the biolistic DNA delivery-more commonly referred to as particle bombardment-is probably the most used for delivering foreign DNA into the chloroplast genome. On the other hand, stable plastid transformation has also taken place through direct methods, one of which is PEG-mediated transformation. Although expensive, the particle bombardment method ensures high transformation efficiency with a fast

regeneration of transformed tissues. The PEG-mediated transformation, on the other hand, is less expensive, but requires high technical expertise, especially for the isolation of protoplasts.

Particle Bombardment is a methodology by which DNA-coated micro-projectiles are accelerated into living tissues or cells at very high speed. This is made possible with the use of various devices that may include the PDS-1000/He particle gun system or gunpowder-based systems. As one of the variations uses gunpowder, an aqueous suspension which contains DNA-coated tungsten powder is inserted into the tip of the plastic macro projectile resembling a bullet. It is the macro projectiles that are propelled through a tiny aperture. The microprojectiles are accelerated through by the explosive force generated from the gunpowder charge.

In contrast, the PDS-1000/He device employs helium gas for discharging of a microcarrier propelled by a burst of gas that is coated with millions of dried DNA-coated microcarriers. Due to the first successful application of this technique of delivering DNA into plant cells using high-density macroprojectiles in the 1980s, many improvements have been made. Members of both groups have made several modifications to limit the damage to target tissue samples, reduce cellular disruption, and increase the effectiveness of DNA delivery. The first group has proposed, in particular, an improved version of this biolistic approach - the Bio-Rad PDS-1000/He particle gun system. This equipment is designed to deliver gold particles coated with target genes along with selectable marker genes such as bar gene at rupture pressure of 650 psi, that could allow genetic transformation in immature wheat embryos. The evidential advantages of the particle bombardment technique include wide application on recalcitrant plants and a wide range of species that include both monocotyledonous and dicotyledonous plants. Also, false positives rarely occur, and the simplicity of the device makes it operational with only minimal amounts of plasmid DNA to successfully transform the plants. The technique has been especially useful in the transformation of plastids. A study has described a reproducible plastid transformation procedure in which two different tobacco-specific plastid vectors, Prrn/GFP/Trps16 and PpsbA/aadA/TpsbA, along with the plasmid pZS197, Prrn/aadA/TpsbA, have been used. Those were designed to be targeted into the large single copy chloroplast genome of potato with its inverted repeat regions.

This is a strategic design that allows successful regeneration of plants with uniformly transformed plastids, enhancing the possibility of practical applications of transplastomic technology in agricultural biotechnology and genetic engineering of plants. In a nutshell, the various technologies being utilized to integrate the transgenes into chloroplast genomes represent the cutting edge of plant biotechnology. The application of sophisticated techniques such as particle bombardment and PEG-mediated transformation gives the researcher proficient tools to enhance chloroplast genetics for the ultimate derivation of improved crops that possess improved tolerance against stress, have better nutritional value, and provide resistance against diseases. This will require continued research and optimization if the full potential of plastid transformation is ever to be realized in sustainable agriculture and food security. The genomic size varies significantly between different species, which range from 107 kb in *Cathaya argyrophylla* to 218 kb in Pelargonium. Most genomic size variation among different plastid species is due to variations within IRs, and both tRNA genes and protein-coding genes show a high level of similarity; their IRs commonly possess identical nucleotide sequences.

Indeed, from the first chloroplast genome sequencing in tobacco in the 1980s, significant progresses have been performed for other plant species. So far, approximately 800 complete sequences of chloroplast genomes are available through the organelle genomic database of NCBI. These chloroplast genomes have contributed significantly to the phylogenetic analysis of different plant families and added to our knowledge concerning evolutionary relationships across various phylogenetic clades. Chloroplast genomic sequences unveiled considerable sequence and structural diversity among plant species, both interspecifically and intraspecifically. Such variant data have been invaluable for the understanding of climate change effects on cash crops, for promoting reproduction of closely related species by homologous crosses, and for finding and conserving useful traits. Information obtained from chloroplast genomes has also been used to study and improve cultivation practices for several crops, especially legumes, which form a fundamental component in sustainable agricultural systems due to their nitrogen-fixing ability.

Recent breakthroughs have been achieved in integrating the chloroplast genome into organisms like Chlamydomonas and tobacco, thereby offering new vistas in chloroplast genome modification for a

wide range of crop species. The inherent advantages of chloroplast transformation include appendages of epigenetic influences due to the maternal inheritance pattern with plastids opposed to traditional nuclear transformation techniques. Apart from this, one additional advantage that is being offered by the expression of multiple foreign proteins from the chloroplast genome in the transplastomic plants is that they occur at very high levels. This happens mainly because every cell has a thousand copies of the chloroplast genome and also because of the absence of gene silencing mechanisms, that are usually faced by nuclear transgene expressions.

This transplastomic system also allows for higher expressions of recombinant proteins and multigene expression from operons without the complexity brought about by positional effects common in nuclear genomes. Chloroplast genes that encode for proteins involved in photosynthesis may be divided into three groups. The first class contains genes encoding components of photosystem I and II, including psaA and psaB; psbA and psbB, ATP synthase-atpA and atpB, large subunit of RubisCO-rbcL, cytochrome b6f-petA and petB, and NAD(P)H dehydrogenase genes-ndhA and ndhB. The second category of genes contains RNA genes that make up the genetic machinery of the chloroplast, such as ribosomal RNA genes: rrn5, rrn16; RNA polymerase genes, rpoA, rpoB; transfer RNA genes, trnK, trnH; and genes encoding ribosomal subunits: rps2, rps3. The third category mainly contains open reading frames for conserved plastids, ORFs, and genes for specific coding proteins, such as cemA and matK. It has been hypothesized that IR are the most suitable sites for inserting transgenes, since they are duplicated in the majority of chloroplast genomes. The integration site within IR become very important to affect the expression level of a gene and homoplasmy after selection with specific antibiotics, which guarantees stability and efficiency of the transgenic trait for generations.

Therefore, there is a bright prospect in the pursuit of transplastomic approaches to advance plant biotechnology. Given that modifications can be made by using these unique features of chloroplast genomes, we will be able to obtain crops tolerant against abiotic stresses and improve agricultural productivity and sustainability due to different global challenges regarding climate change and food security. One of the most common plastid transformation methods involves PEG transformation; this is an elaborate process initiated with protoplast

extraction from the tissue of interest in plants. Once the protoplasts are extracted, these cells are subjected to a solution that includes a cocktail of DNA, PEG, and other ions. The PEG treatment temporarily permeabilizes the plasma membrane, which allows the DNA molecules to enter the cytoplasm of the protoplasts. Then, the constructed vectors, which contain sequences homologous to a specific target region in the plastid genome, integrate into the plastid genome via homologous recombination. Selection of antibiotic is similarly done to segregate homoplastomic cells, those with uniform plastome, from cells containing a mixture of modified and unmodified plastome copies to make sure that only successful transformations are selected.

Groundbreaking application of the PEG transformation was conducted on transient expression in the unicellular red alga C. merolae using B-tubulin tagged with a hemagglutinin marker.The expression and localization of the gene product were verified by immunocytochemical analysis carried out 24 hours after PEG-mediated transformation. In addition, the Authors have shown that it is possible to obtain a very simple and low-cost protocol yielding stable plastid transformation in tobacco plants. This involves the use of transforming DNA to the leaf protoplasts via the use of PEG treatment, which further shows the power and flexibility of the PEG transformation dealing method.

Basic Elements of Chloroplast Vectors and Their Putative Functions

Standard chloroplast vectors contain at least the following elements: a promoter, a selective marker gene, a 5′ untranslated region, and the gene of interest. The 5′ UTR region flanked by two chloroplast DNA regions plays an important role in inserting the gene of interest into the host chloroplast genome through double homologous recombination.

Flanking Sites versus Promoters Homologous recombination requires the presence of flanking DNA sequences. Such sequences should be kept to make appropriate endogenous splicing elements available for correct expression of the transgenic insert. A few flanking sites have been applied for efficient integration of the gene of interest into the plastid genome: rbcl/accD, rps12/trnV, and trnM/trnG.

On the other hand, empirical evidence shows that trnI/trnA genes within the ribosomal operon of the chloroplast genome constitute some of the most efficient sites for integrating transgenes through plastid transformation. This efficiency has been attributed to the favorable location of the insert at the trnA site, which in turn correlates with its positioning in the inverted repeat region, a replication origin, and associated intron sequences contributing to the high copy number of the transgene. This may be because, compared to the transcriptionally silent regions of rbcl/accD, when the transgene was integrated to the spacer region trnl/trnA, which is transcriptionally active, expression could be raised to over 25-fold. This could be the result of multiple promoters-endogenous or heterologous-that enhance the expression activity. Plastid transcription is mediated mainly by two types of plastid RNA polymerases: the plastid-encoded plastid RNAP (PEP) and the nucleus-encoded RNA polymerase (NEP). There are many plastid promoters, each with specific transcription start sites matching both PEP and NEP. Core subunits (rpoA, rpoB, rpoC1, and rpoC2) of PEP are encoded in the plastome. The RNA-synthesizing enzymes of the T-type bacteriophages have also been related to the single subunit, nucleus-encoded RNA polymerase NEP.

Most plastid promoters contain those consensus sequences for sigma factors at positions -35 and -10 that resemble strongly bacterial RNAP sequences, namely TTGaca and TATaaT, respectively. This only goes further to reinforce the evolutionary connection between the plastids and their cyanobacterial ancestors. Plastid promoters are widely included in chloroplast transformation vectors, usually the 16S rRNA promoter (Prrn16) or the psbA promoter (PpsbA) to enable the transcriptional insertion of transgenes into the chloroplast genome. The interesting point is that the PpsbA transcription start site has an exclusive association with PEP while Prrn allows transcription initiation to be mediated by both PEP and NEP. It is important to note that the major transcriptional products of both NEP and PEP activities are polycistronic mRNAs. Such products require extensive post-transcriptional modifications including removal of introns, processing of mRNA into monocistronic or oligocistronic forms, removal of the 5′ and 3′-ends, and RNA editing.

Untranslated Regions (5′ and 3′)

The interaction between the RNA sequences and structural elements with other functional elements has a great impact on the process of gene expression; this influence comes mainly from the stabilizing effect it confers to plastid mRNAs. This is done by stabilizing the RNA secondary structures, particularly in 5′ and 3′ UTRs. The translated proteins must be shielded from chloroplast proteases since they will easily degrade if subjected to these proteolytic enzymes. The 5′ UTR of plastid transcripts plays an especially important role in determining the ultimate protein expression level of chimeric transgenes. There are numerous 5′ UTRs-one for each transcript-and all differ with respect to the stability of the resultant transcript and the lifetime of the encoded protein in the chloroplast of a transformed plant. For instance, the coding sequence of human serum albumin was fused with 5′ UTRs regulated by the chloroplast psbA promoter upon illumination, resulting in a huge level of increment in protein expression: as high as 500-fold. Apart from evidence supporting that structural elements confer protection to foreign proteins against degradation, investigations have equally aimed at determining whether the fusion of the Chlamydomonas reinhardtii 16S promoter with its 5′ UTR regulatory elements result in increased levels of protein synthesis. It has been established that higher steady-state levels of luxCt mRNA, along with increased mRNA production via the 16S promoter, are associated with higher protein accumulation resulting from the atpA 5′ UTR.

Although RNA stability did not appear as a determining factor when trying to forecast the overall expression levels of chimeric constructs, there have indeed been reports of a remarkable increase in protein expression, namely a 200-fold improvement for those constructs containing the 5′ UTR of psbA relative to those with the rbcL 5′ UTR. Shine-Dalgarno sequences located on the 5′ UTRs of prokaryotic messenger RNAs enable the turning on of translation. These sequences act as ribosome-binding sites by recognizing a specific sequence motif at the 3′ end of the 16S ribosomal RNA of the 30S ribosomal subunit. It is interesting to point out that in some bacterial strains, mRNAs can achieve activation of translation with high efficacy without any detectable SD structural motif. This observation pinpoints the complexity of translation mechanisms and suggests that alternative pathways might exist which allow effective protein synthesis under

different conditions. In brief, the interaction of each other, the components of chloroplast vectors with their regulatory elements and mechanisms for controlling the gene expression couple to become very important in the development process of plastid transformation technologies. The mechanism of expression would amply help in devising strategies for novel approaches toward improved protein production and enhancing functional features of the transgenic plants for agricultural biotechnology and food security. The in vivo study of predicted RBS efficiency for the tobacco chloroplast atpI gene showed that upstream sequences near the putative ribosome-binding site are required for effective translation in chloroplasts. Four different SD-like sequences, each exhibiting the characteristic signature motif of GGAGG, have been identified within the 5'-untranslated regions of tobacco chloroplast mRNAs. These suggest that functional SD-like sequences about 10 nucleotides upstream from the translational initiation codon are important for efficient ribosome recruitment during translation initiation. The 5'-UTR was suggested to function as a binding platform for mRNA-specific translational activator proteins, which, in turn, supports the direct recruitment of the ribosomal 30S subunit to the initiation codon. This mechanism becomes highly relevant considering the fact that not all SD sequences retain their conservation within the range of 4 to 9 nucleotides upstream of the start codon. The SD sequences of bacteriophages have been used appropriately to increase efficiency in translation initiation of reverse genetics applications and expression of transgenes in plastids.

The 3' UTRs of chloroplast mRNAs are directly downstream of the stop codon and have great impact on the regulation of chloroplast mRNAs. Such 3' UTRs enclose inverted repeats that can fold into specific stem-loop structures. These types of structures are basically important in the maturation and stabilization of mRNA transcripts, thus effectively impeding the activity of the 3' to 5' exonucleases and obstructing polyadenylation. Importantly, ribonuclease has been used in the elimination of protective stem-loop structures together with polyadenylation sites, thereby activating plastid mRNA degradation. The 3' UTRs of rps16, rbcL, psbA, and rpl32 are considered important loci as far as the regulation of heterologous gene expression in plastids is concerned.

Keeping in mind the background of accurate and reliable detection of transformations, it is pertinent that the chloroplasts and cell lines

associated with the transformants contain only homoplasmic copies of the transformed plastomes. Therefore, the manipulation of the marker gene, in conjunction with a plastid expression system, is indispensable for carrying a plastid promoter, a 5′ UTR possessing a Shine-Dalgarno sequence for ribosome association, and a 3′ UTR containing appropriate cis-acting elements. Herbicide resistance markers such as phosphinothricin acetyltransferase pat/bar gene, Bromoxynil nitrilase bxn gene, and a range of antibiotic resistance genes have also seen wide applications in the selection of transformants. neo gene, aphA6 confer resistance to kanamycin, the gene coding for the aminoglycoside 3′-adenylyltransferase confers on the host resistance against antibiotics. Besides biosafety considerations, public concerns also arise about deploying antiseptic or herbicide resistance genes as specific markers for plant transformation.

Therefore, methods targeted at eliminating antagonist bacterial genes from cloned plants have the highest relevance. Methods developed that avoid reliance on antibiotic or herbicide resistance genes include homology-based excision mediated by directly repeated sequences, phage site specific recombinases, co-integration mediated targeted gene insertion and co-transformation segregation.

For instance, one investigation considered the noxious gene from spinach (*Spinacia oleracea*) that was transformed for its marker gene into chloroplast genomes and observed that chloroplast transformation was 25% efficient in BA compared with streptomycin. Selection is based on the enzymatic activity of the chloroplast site-specific gene-encoded enzyme BADH, which converts the toxic betaine aldehyde—a compound present only in chloroplasts—into the value-added compound Gly betaine. More recent developments have shown that the dsdA gene can serve as a specific marker for plastid and nuclear transformation systems, respectively, and its utilisation as a marker gene in the plastid transformation of monocotyledons has been proposed.

Application Overview of Chloroplast Transformation

Extensive research has been carried out in biotechnology for the exclusive and significant benefits of chloroplast transformation in developing cost-effective biopharmaceuticals, including vaccines and antibodies. The chloroplast genome has been transformed for useful

enzymes, biomaterials, and biofuels, while the enhancement of biomass production contributes to food production sustainability against adverse environmental factors that may impair agricultural output.

Pharmaceutical Production of Vaccines

Chloroplast transformation has received considerable interest from the pharmaceutical industry in view of the high copy number of plastid DNA, which makes high-level production feasible for the synthesis of vaccine proteins in large amounts.

This method relieves the financial burden that attends production and further purification chemically synthesized vaccines; thus, it promises a bright avenue toward the supply of low-cost vaccines to populations in developing countries. The basic premise is that the chloroplast genome in a plant can be genetically transformed to encode genes of bacterial and viral antigens that are responsible for various diseases. Ultimately, such genetic modifications have resulted in the secretion of such immunogenic proteins into edible plant tissues.

However, it is essential to note that only antigens that are not glycosylated can be produced within these plastids because the organelles do not have pertinent glycosylation machinery that is considered necessary in the production of glycosylated proteins. To date, there has been significant progress made in using plant chloroplasts as bioreactors, with evidence that plants may drastically enhance immunogenicity. One hypothesis supplied by the scientists themselves was that the newly integrated gene within the plants could be duplicated sans intrinsic restrictions, as was evidenced from findings that the transgenic plants produced HBsAg antigenically identical to HBsAg particles derived from recombinant yeast and human serum. Further evidence for this was the presence of HBsAg in the leaves, at a level consistent with mRNA abundance, as determined by the enzyme-linked immunoassay employing a monoclonal antibody against human serum-derived HBsAg. Similarly, the chloroplast-expressed LecA, a promising candidate to inhibit amoebiasis, showed a production rate of 6.3% of TSP or 2.3 mg LecA per gram of leaf tissue. More importantly, expression of the secretory human protein somatotropin in chloroplast resulted in levels of recombinant protein accumulation way over 300-fold higher than the level obtained by

traditional nuclear transformation. Besides, synthesis of the p24 antigen from HIV-1 has been done in a high biomass tower tobacco variety and led to a high yield of total soluble protein due to the expression of multiple additional bacterial and viral antigens. This is an application of innovative chloroplast transformation technology for promising advances in agricultural biotechnology and the pharmaceutical industry. Employing the unique capabilities of plastids that enable facilitation of large-scale production of valuable proteins contributes to enhanced food security and sustainable agriculture by helping create effective biopharmaceutical solutions to global health challenges. Application in Sustainable Food Security

Over the years, chloroplast transformation has emerged as one of the major strategies in order to achieve sufficient nutritionally valued and affordable food. Various reports clearly described the successful genetic transformation of the chloroplast genome for the value of different agriculturally important features. These have aimed at developing resistance against herbicides and insecticides, imparting tolerance to water scarcity and salinity stressors.

Chloroplast Transformation in Nutritional Enhancement

Recent breakthroughs in chloroplast transformation have directed their attention to improving nutritional biochemical pathways by adding vital nutrients lacking in non-green plant tissues. Examples include improvements in carotenoid biosynthesis in tomatoes; studies to enhance the level of carotenoids in tomatoes have shown that expression of the bacterial lycopene beta-cyclase gene greatly enhances the conversion of lycopene to beta-carotene. This has resulted in an amazing fourfold increase in pro-vitamin A accumulation in fruit.

Further, lycopene beta-cyclase genes of the *Narcissus pseudonarcissus* and the *Erwinia herbicola* expressed in the tomato plant genome enhanced enzymatic activity responsible for the conversion of lycopene to provitamin A (beta-carotene. Scientists showed that such conversion leads to the increased accumulation within the xanthophyll cycle pigments due to lycopene. The process has been able, in turn, to reduce the alpha-branch xanthophyll lutein in the green foliage of the transplastomic tomato plants. Remarkably, lycopene conversion to beta-carotene allowed the level of provitamin A to be raised to a

concentration of approximately 1 mg per dry weight in the fruit. In addition, high-level expression of γ-TMT has been described to significantly enhance the conversion efficiency from γ-tocopherol to α-tocopherol up to as high as about tenfold in the transplastomic seeds. Genetic enhancement further imparts tolerance against salt and heavy metal stress by inhibiting the production of ROS resulted in minimal ion leakage and lipid peroxidation.

Chloroplast Transformation in Agricultural Stress Management

Abiotic stresses, which include drought, salinity, extreme temperatures, cold, chilling, and heat, are some of the trials being faced in the agricultural landscape. The major threats to crop reliability are biotic stresses such as diseases and pest infestations. Biotechnology applications, especially through chloroplast transformation, have emerged as a fundamental and effective tool that has demonstrated the ability to improve agricultural yields against the impending challenges caused by contemporary climate change and the exponential growth of the human population.

Temperature Stress Management

Quite a good number of research works over these years have been done to explain the mechanisms of different genes involved in responses against environmental stresses. The possible genetic modification of unsaturated fatty acids in plant lipids has great importance in terms of the increased resistance of higher plants to such stress factors. For example, the changes in leaf fatty acid composition of plants obtained via transplastomics have yielded an increase in unsaturation levels in both seeds and leaves, indicating that these varieties may have an increased tolerance for cold conditions. One particular case of this is through the chloroplast genome genetic modification in Nicotiana tabacum to express the gene L-aspartate-alpha-decarboxylase (AspDC) of Escherichia coli. This enzyme catalyzes the conversion of L-aspartate into beta-alanine and carbon dioxide in a reaction that greatly alters the plant's biochemistry.

Badawi et al. demonstrated that the expression of panD from *E. coli* remarkably enhances thermal tolerance associated with photosynthetic efficiency and biomass accumulation under high-temperature stresses. Oxidative stress is considered one of the critical

factors which adversely affects plants under various environmental stressors.

Drought and Salinity Stress Management

Salinity stress is considered now one of the most important threats in agriculture, as it affects around 7% of the total land area and 5% of agricultural lands.

This condition is incurring a high economic cost due to stunted growth, poor development, and reduced yields for all crops planted on the saline soil. Various osmoprotectants are known that may involve sugars like trehalose and betaines to protect cellular mechanisms responsible for the elimination of ROS and preservation of macromolecules, which better equip the plant toward the tolerance of salinity, cold, and drought stress. Research has been able to document the somatic cell fusion of transplastomic plants, particularly in carrot cells. These transgenic plants exhibit the expression of betaine aldehyde dehydrogenase under higher concentrations of NaCl. Hence, the transformed carrot cell lines grew sevenfold faster and accumulated 50 to 54 fold more betaine than the wild-type cell cultures cultivated in liquid medium supplemented with 100 mM NaCl.

Enhancing Resilience to Biotic Stressors

Biotic stress is one of the major threats that acutely endanger food sustainability at this point in time, from a global perspective. The most important consequence of such biotic stressors may be so severe as to give rise to grave endemic disease problems affecting global food availabilities if these stresses remain unchecked. Toxic metabolite buildup due to chemical applications used in disease and pest control is another pressing reason that calls for urgent alternatives to its more viable control methods.

Pathogen Resistance in Transplastomic Plants

Plastid transformation has been one of the important methodologies in developing disease resistance to pathogenic bacteria and fungi in plants. This is especially because of the locational convenience of many target proteins released during the hypersensitive response of the plant to the infection in one area. Several works have reported on the use of transplastomic plants resistant to a wide range of pathogenic

diseases. One of the most remarkable examples is the expression of the agglutinin gene from *Pinellia ternate*, which showed resistance against insect pests and plant pathogens. The Cry9Aa2 gene from Bacillus thuringiensis has also been transferred into tobacco plant plastids to provide resistance against the potato tuber moth. CeCPI and chitinase genes have also been transformed in tomato plants for ectopic expression. In tobacco, CeCPI and sporamin showed high-level resistance against various insect pests and phytopathogens. Further experiments showed that the co-expression of several defense genes encoding protease inhibitors and chitinases contributed to the high levels of broad-spectrum resistance obtained in Nicotiana benthamiana against a wide range of pests, diseases, and abiotic stressors. This multilevel approach to pathogen resistance points to the huge prospect for transplastomic technology regarding improving plant resistance to biotic stressors.

Transplastomic Plants with Improved Insect Resistance

Emerging evidence testifies that transplastomic plants are able to show marked resistance against larvae of the pest *Helicoverpa armigera*, regardless of their developmental stages. Cry1Ia5 protein can accumulate as high as 3% of the TSP in the tissue of transplastomic plants, which is a 300-fold increase in expression when compared to the analogous protein expressed in nuclear-transformed plants. Commercial transgenic plants that express Bt crystal proteins include Cry1Ab in maize and Cry1Ac in cotton have been widely cultivated for their potential to positively affect the balance in pest management. The crystal proteins of Bacillus thuringiensis (Bt) are generally recognized as safe biological pesticides; however, substantial controversy exists over the toxic protein transfer to non-target organisms through pollen dissemination. Because this process occurs outside the nucleus and the chloroplast is maternally passed on to subsequent generations, much effort has been invested into studying the process of chloroplast transformation as a means of overcoming this limitation. A mountain of evidence supports the hypothesis that targeting a chloroplast genome may enable high-level expression necessary for effective pest management and control. More recently, RNA interference technology has also begun to be applied within the frame of transplastomic transformation for a method providing insect-specific and ecologically compatible control. This novel technology affects only the target insect specifically because of which the

beneficial insect populations are preserved, and there is a reduction in the toxic protein production within the plant as observed with traditional Bt proteins.

The hpRNAs produced inside chloroplasts do not cross the cytoplasmic compartment. It has also been reported that such siRNAs, which were targeted against these genes, were present within midgut tissues of beetles fed transplastomic potato foliage. Messenger RNA transcript levels of these genes were significantly down-regulated in response to feeding against the Colorado potato beetle on leaves of transplastomic potato cultivars. Such findings represent the potential of trans-plastomic approaches toward pest control and insect resistance in crops. The potato Solanum tuberosum L. is considered to be the fourth most consumed food crop in the world, containing primary carbohydrates, dietary fibers, vitamins, proteins, minerals, and antioxidants essential for human nutrition. It must be given that to date, a total of forty species of viruses and two species of viroids have been identified as pathogens of potatoes. Of these, the prevalence in Pakistan is attributed to particularly notable viruses. Among them come Potato mop-top virus infection-PMTV, Potato virus A infection-PVA, Potato virus M-PVM, Potato virus S-PVS, and of prime importance, Potato leaf roll virus-PLRV, Potato virus X-PVX, and Potato virus Y-PVY. In general, warm climate regions, as in Pakistan, demonstrate the incidence of disease resultant from aphid-transmitted viruses like PVY at a higher rate mainly because of increased vector pressure. The contact or mechanical transmission of PVX, among the ten most consequential plant viruses globally, shows symptom severity to be exacerbated more at higher temperatures above 28 °C, varying from mild interveinal mosaic patterns to severe leaf mottling. Therefore, the application of transplastomic technology brings great optimism in improving plant tolerance against biotic stressors like pathogens and insect pests. By using novel approaches in biotechnology, such as RNA interference and targeting gene expression, researchers are opening the doors to newer possibilities that could answer the chewy problem of biotic stress on human food security. It will be of great importance to the development of crops each day more resilient and sustainable against an increasing array of biotic threats for the continued exploration of the techniques of chloroplast transformation.

Improvement in Resilience Among Potato Cultivars Against Biotic Stressors Using RNA Silencing Mechanism

These biotic stressors, which cover the spectrum of pathogens such as viruses, bacteria, and fungi, are growing concerns in trying to address agricultural sustainability. For potato cultivation, these can cause very critical economic losses, adding woe to food insecurity. Probably among the most devastating biotic stressors on potato crops in the world are viral infections. If uncurbed, the viral infections are more likely to bring endemic disease problems, limiting food supplies by crippling crop yields. The toxic metabolite residues that build up from chemical applications in controlling the pests and diseases underline an urgent need for prudent, novel, and sustainable control measures. The co-infection of PVX with PVY in the same host plant sometimes expresses more pronounced symptoms due to a synergistic interaction of viruses. Propagation of infected seed, a common mode of transmission in many areas, especially in Pakistan, permits the unimpeded spread of these viruses to succeeding generations. Because most of the viruses are vegetatively propagated, reliance on infected seed can lead to degeneration of the seed fairly rapidly and, in turn, reduced yields in potato cultivar sensitive to the viral infection.

Several methodologies have been adopted to produce transgenic plants that express resistance against such viruses. Some of the most important strategies include movement-protein-mediated resistance, REP-protein-mediated resistance, replicase-mediated resistance, coat-protein-mediated resistance, and RNA-mediated resistance. However, despite advances in biotechnology, there remains a lack of substantial resistance achieved in most transgenic lines, which has raised concerns regarding the efficacy and reliability of these approaches. Among the emerging strategies, post-transcriptional gene silencing (PTGS) has surfaced as a particularly promising technique for establishing resistance in transgenic plants. PTGS involve the mechanism of RNA silencing, which has been hypothetically considered to prevent viral infections and regulate at the same time the expression of certain genes involved in eukaryotic organisms.

The synthesis of self-complementary hairpin RNA or dsRNA during the intermediate stages of viral replication triggers RNA silencing. This is mediated by members of the RNase III family of Dicer endoribonucleases, responsible for the processing of dsRNA into

segments of siRNA 21 to 25 nucleotides in length. The interaction of siRNA with RISC enables degradation of target RNA carrying the corresponding sequence and hence provides effective attenuation of the viral load within the plant.

Several studies have shown the attainment of effective resistance through RNA silencing in transgenic plants. Transgenic potatoes were obtained showing resistance against PVY while *Nicotiana benthamiana* was made resistant to beet necrotic yellow vein virus. Transgenic perennial plum trees showed resistance against plum pox virus and *Nicotiana benthamiana* showed resistance against cucumber green mottle mosaic virus. The genome of PVX encodes about 6,435 base pairs of positive-sense, single-stranded RNA with five ORFs encoding important elements: RNA-dependent RNA polymerase RdRp, movement proteins TGBp1, TGBp2, TGBp3 and the coat protein CP gene.

For effective RNA silencing, a short hairpin RNA was designed with a conserved segment of PVX CP, designated CP-PVX. The shRNA was further modified for stability and efficiency by adding flanking and loop sequences from miR403, a microRNA highly expressed in potato. The efficiency of the shRNA constructs was highly regarded based on the examination of mRNA expression in PVX inoculated transgenic potato plants representing the Sante variety. Indeed, Sante is among the most popular, white-skinned potato varieties in Pakistan, generally described as tolerant to PVX. However, it has been reported to be infected with PVX in Pakistan, showing typical symptomatic pheno-expressions. Although the mechanisms underlying this infection are partially unknown, it was reported that the high temperatures break down plant immunity against some viral pathogens. These findings have therefore cast doubt on the ability of Sante to effectively resist PVX at variable environmental conditions hence requiring the creation of more effective ways of resistance. In particular, RNA viruses are grounded for causing a yield reduction in potato tubers of up to 80% grown in Pakistan, although yield loss globally due to PVX alone is reported as high as up to 30%. RNA interference technology stands practical for the knockdown of target gene expression irrespective of their origins being endogenous or viral. It is not possible to belittle the involvement of dsRNA in the RNA silencing pathway, because it sees a wide application in developing virus resistance. A single promoter can be used to transcribe each of the antisense and sense strands of siRNA

duplexes in order to allow stable expression of siRNA. In other strategies, siRNAs are expressed as stem-loop precursors that are then processed by Dicer into active siRNAs.

Although antisense- and sense-mediated gene-silencing mechanisms are operative and effective in botanical systems, specific expression cassettes that generate hairpin-like molecules have been reported to confer superior silencing efficiency. In this paper, we have specifically designed a 107-bp shRNA transgene targeting a highly conserved region within the CP gene of PVX for constructing genetically modified potato lines. The CP gene in PVX plays a crucial role in translation, translocation, and uncoating during the infection cycle of the virus, and its specific use has been shown to provide high resistance to the genetically transformed plants. We are of the view that constitutive expression of shRNAs, especially in the form of the most abundant microRNA in potato, miR403, would reduce the mRNA expression of the invading PVX to a level low enough to prevent viral infection in the transgenic plant system.

This novel approach was executed in the successful cloning of the shRNA transgene into the binary vector pCAMBIA1301, aided by the CaMV35S promoter, which expressively drives the transgene. In the present work, we derived nine transgenic lines of potato cultivar Sante by Agrobacterium-mediated transformation characterized by stable shRNA expression. The methodology of plant transformation allows the introduction of traits of interest from different species into the genomic structure of the host plant. Thus, some of the characteristics hard to obtain with classical breeding methods, for example, resistance against insects and viral pathogens, can be conferred to the transgenic plant lines. mRNA-expression levels of the transgene below the threshold were observed in seven transgenic potato lines, while in two transgenic lines, mRNA-expression levels of the CP-PVX gene after PVX inoculation were significantly lower than those in the non-transgenic potato plants. There are a few factors that contribute to the differential effectiveness of resistance among the transgenic lines. First, the most probable reason might be that the shRNA transgene could integrate into a different chromosomal location, leading to positional effects that can regulate gene expression. Second, the host plant having different ploidies may express unequal amounts of siRNAs; thus, it leads to variable resistance efficiencies.

Another point to note is that the degradation in the folding of dsRNA due to interference from sense RNA or ribosomal scanning may lead to impaired Dicer function and consequently the whole RNA silencing process. In addition, natural variation in transgene expression among individual plants can result in variable resistance in different transgenic lines further confounded by the dose of the viral inoculum applied during experimentation. This holistic approach therefore lays the foundations for developing PVX-resistant potato cultivars that would improve productivity, further aiding sustainable agriculture considering shifting viral incidents. The gravity of the above situation is therefore gigantic, going past the issue of mere resistance against viruses into the realms of food security itself and agricultural resilience. The need for innovative solutions that protect vital crops, like potatoes, against volatile weather conditions will only become increasingly serious as global populations continue to rise and climate variability increases.

Certainly, the use of RNA silencing mechanisms in developing PVX-resistant potato varieties can be considered a proactive future strategy for reducing the impact of biotic stresses on agricultural sustainability. Applying genetic engineering can open ways for developing resistant varieties that thrive under adversities, hence ensuring food security for future generations. Refining these other strategies through continuous research and collaboration while developing effective solutions to urgent challenges facing potato cultivation and other critical agricultural sectors worldwide is paramount.

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Chapter 2: RNA interference (RNAi)

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Targeting the Impact of Spotted Stem Borer on Maize Production

Using RNA Interference Strategies *Chilo partellus*, commonly referred to as the Spotted Stem Borer, ranks as one of the most economically important pests of maize in Africa. It thus causes massive yield losses among resource-poor farmers and generally contributes to food insecurity, especially among developing countries. Because of its destructive feeding habits, this pest causes very great losses among smallholder resource-poor farmers, estimated at a cost of millions of dollars annually. Climate change is expected to favorably impact its infestation and further exacerbate this already negative impact prevalent in maize production. Various predictive models predict that changes in temperature and rainfall will further assist the Spotted Stem Borer to expand geographically and prolong its life cycle, thus exacerbating its infestation. Even though many methods are available for its control, the infestation by this pest has been on the increase.

Among the many practices, the measure of chemical control, mainly through pesticide application, remains the most favored. However, dependence on chemical pesticides has been a significant concern with respect to ecological integrity and human health. Over-application of pesticides can induce effects related to pest resurgence, development of secondary pest populations, and even toxicity to non-target organisms, hence further complicating the general performance of pest management practices. One of the core strategies toward containing insect pests and ensuring sustainable food production should, therefore, focus on reducing crop losses using ecologically and economically integrated pest management practices. Recently, over the last few decades, there has been rapid development in the area of RNA interference technology as a potential strategy for the

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management of insect pests, especially the Spotted Stem Borer population. This new use of RNAi provides a fresh alternative to the conventional controls in a non-chemical sense.

The RNAi pathway is initiated under the presence of exogenous dsRNA, which consequently induces degradation of mRNA sequences complementary to the guide strand of siRNA. This is initiated through RNase III-like enzymes, which cleave dsRNAs into siRNA duplexes with about 20-25 nucleotides in length. Resulting siRNAs give rise to guide strands and passenger strands with divergent thermodynamic properties due to the asymmetry rule. The guide strands contain a less stable 5' end and more often associate with the Argonaute protein in the active RNP complex termed the RNA-induced silencing complex - or RISC. The guide strand, after incorporation into the RISC, now interacts with the targeted complementary regions of mRNA. Cleavage to the phosphodiester bond at specific nucleotides occurs and, in turn, a downregulation of gene expression is achieved. Modern agricultural practices have utilized this mechanism for combating various agricultural pests very effectively.

Recent advances with the RNAi technology have considerably improved our knowledge concerning the mechanism of RNAi and gene knockdown in many insect species. Recently, active development of mechanisms for delivering dsRNA is yet under way, since effective cellular internalization of dsRNA is one of the key factors for successful application of the RNAi technology in insects. Silencing signals triggered by dsRNA or siRNA spread genetic regulatory information between cells and act via both cell-autonomous and non-cell-autonomous pathways. In cell-autonomous RNAi response, the silencing effect remains restricted to the cell of origin or where it occurred, while in the non-cell-autonomous event, the silencing phenomenon can manifest itself in all cells capable of taking up dsRNA through environmental RNAi. In this context, systemic RNAi is defined as the movement of the silencing signal from the originating cell to adjacent cells or tissues. Among the impressive numbers of mechanisms worked out for dsRNA uptake, SID-1 protein is one of the most studied along with endocytosis. Several SID mutants, viz. SID 1, SID 2, SID 3 and SID 5 have been studied in Caenorhabditis elegans in order to improve our knowledge about systemic RNAi pathways. Interestingly, it has been shown that even insect orthologues of the SID-1 protein are present in a great number of insect species,

indicating a conserved mechanism of dsRNA uptake across different taxa. By contrast, whereas sid-1-like genes follow a broad distribution in insects, the presence of such insects does not systematically ensure a powerful systemic RNAi response, Insect research findings have gone on to show.

Thus far, the endocytic pathway has generally been considered a major method of dsRNA uptake among most insect species, with clathrin-mediated endocytosis being investigated as a mechanism of dsRNA uptake crosses numerous insect taxes. Most often, the effectiveness of RNAi in insects has been related to the amount of uptake of dsRNA, and that again depends on a few factors: physiological pH, the genes being targeted, and the occurrence of nucleases that might degrade dsRNA. The truly effective identification of target genes through RNAi-mediated biopesticides for insect pests is a big challenge. The most effective target gene of *Chilo partellus* and its optimal dsRNA form, which can be utilized to achieve maximum RNAi silencing, were determined as part of a potential control strategy. The stability of four types of dsRNA and their effectiveness in inducing the RNAi pathway in *Chilo partellus*. Bacterial-expressed dsRNA and column-purified dsRNA fragments corresponding to genes like V-ATPases, Acetylcholinesterase, Chymotrypsin, and Arginine kinase were studied, which in the past were tested within *Lepidoptera*, were fed to *Chilo partellus*. The gene expression was carefully monitored at several time points in order to determine optimal dsRNA formulation. The results indicated that oral delivery of bacterially expressed dsRNA elicited tremendous RNAi knockdown and hence presents a promising platform for the management of pests.

It is one of the strategies for a different approach in which the knockdown of an essential gene in agricultural pests can help manage insect populations. Whereas some *lepidopterans* are resistant to RNAi, there have been poor silencing results obtained in some cases. Relevant target gene identification for RNAi in insects represents one major bottleneck yet to be overcome for the advance of RNAi-based biopesticides. The study specifically evaluated, through RNAi, the effectiveness in *Chilo partellus* of targeting four candidate genes-V-ATPases, Acetylcholinesterase, Chymotrypsin, and Arginine kinase-endowing a variety of biological functions to the pest.

V-ATPase is a multi-subunit proton pump that, by its proper performance, energizes transport processes across plasma membranes in insect cells and epithelial tissues, thus playing an important role in the maintenance of cellular homeostasis and the mediation of various metabolic processes. Acetylcholinesterase is a key insect central nervous system enzyme whose job is to terminate nerve impulse transmission by hydrolysis of neurotransmitter acetylcholine at synaptic junctions of cholinergic neurons. The insects always express high activities of Acetylcholinesterase, which makes the fast cessation of the spread of nerve impulses possible; therefore, even a slight depression of its activity may result in disturbance in the transmission of nerve impulses, eventually leading to the loss of insect viability. Chymotrypsin is a member of the serine protease family, which bears great importance in food digestion, immune defense mechanisms, and activation of zymogens. The involvement of chymotrypsin in digestive processes underlines its importance for the overall fitness and survival of the pest. Finally, Arginine kinase is an important enzyme in energy metabolism, participating in ATP recycling, further underlining its importance in insect physiology. The study will, therefore, be in a position to provide details concerning the use of RNA interference as a strategy in the management of the Spotted Stem Borer and its impact on maize production by identifying effective target genes and optimization of dsRNA formulations in the development of a novel, environmentally sustainable approach to control this pest and contribute toward improved food security and agricultural sustainability. Further research and collaboration will also be necessary in refining these RNAi strategies and overcoming some problems associated with the practical application of RNAi-mediated biopesticides under field conditions.

Improvement of Delivery Mechanisms for dsRNA as Interfering Agent in Insect Pest Management

The methodology in double-stranded RNA delivery has evolved to include oral administration, microinjection, soaking, transfection, and delivery through the host plant. Among these, oral administration has been observed to be an efficient method of RNAi delivery for Lepidoptera. In a study, an oral delivery approach was used to deliver both bacterial expressed dsRNA and purified dsRNA into the gut microenvironment of *Chilo partellus*, with the objective of assaying the knockdown efficiency of certain targeted genes. The degradation rate

of dsRNA by haemolymph and gut extracellular ribonucleases is an important determinant affecting the efficiency of RNAi. For efficient RNAi induction, it is important that dsRNA remains intact and is taken in by insect cells without degradation. In our studies, we paid adequate attention to find out the stability of purified dsRNA under a lot of environmental conditions. Researchers wanted to check whether the insectary environment would have any adverse impact on the stability of dsRNA. Their observations revealed that within the insectary environment, dsRNA remained intact for approximately 72 hours. In contrast, when dsRNA was applied to the artificial diet, it remained stable for about 36 h and started degradation when exposed to 48 h. Their results were in accordance with other studies reporting a 48-84 h degradation time associated with nucleases in artificial diets. Furthermore, it was found that haemolymph and gut content of C. partellus degrades dsRNA very rapidly. Specifically, degradation of dsRNA in the haemolymph started within about one minute and was completed within thirty minutes. Instability of dsRNA in the gut contents and haemolymph severely limits the potency of RNAi in *C. partellus*. These findings agreed with previous studies that showed high degradation of dsRNA in the gut content and haemolymph of *Ostrinia nubilalis*, as determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). This was also further supported by the established correlation between the rapid degradation of dsRNA within the gut contents and greatly lowered RNAi efficiency in various insect models.

Several studies are documented on the induction of a proper RNAi response through feeding-based RNAi in agricultural insect species. The success of RNAi relies on the sufficient dosage of the dsRNA or siRNA required for the triggering of the RNAi pathway. However, in oral feeding assays, higher concentrations are usually applied to ensure sufficiently high uptake. Extended exposure of *C. partellus* larvae to dsRNA would not lead to increased silencing of the majority of the targeted genes studied. On the contrary, extended exposure appears to counteract the silencing effects and might point toward possible induction of another pathway, although mechanisms for such an event are unknown. To identify the cause of such low RNAi success in *C. partellus*, several detailed investigations will be required. In this respect, it is envisaged that siRNA and shRNA targeting such genes, with involvement in stress response pathways, would nonspecifically respond to lower the apparent gene silencing of target gene

transcripts. A comparison of transcript knockdown in larvae treated with bacterial expressed dsRNA versus purified dsRNA demonstrated an effective knockdown for all four of our targeted genes. Also, most of the targeted genes had no significant knockdown when larvae were fed with only pure dsRNA; this may be due to the fact that pure dsRNA is degraded by nucleases in the midgut. The knockdown effects observed in the mentioned study were documented, showing that the efficiency of RNAi for target genes varies within *C. partellus*. Recent studies have also mentioned that REase competes with Dicer-2 for access to targeted dsRNA, affecting the total reads to siRNAs corresponding to the target genes and eventually affecting the RNAi efficiency. Particularly, the different physiological conditions of the tissues influence the activity of the enzymes, and diverse species produce a range of dsRNA-degrading enzymes at different concentrations.

These findings further support that the rapid degradation of dsRNAs reduces their ability to induce the RNAi activity, thereby affecting its stability. In fact, this finding is quite pertinent since bacterial expressed dsRNAs showed consistent high knockdown efficiency against the respective target genes when compared to their purified counterpart. Instability and rapid degradation of dsRNA thus result in resolving challenges to improve RNAi efficiency against C. partellus. Protection strategies against nuclease activity offer a promising approach, in that way ensuring maximum gene knockdown and further improvement of RNAi-based methods of pest control. Attempts toward the protection of dsRNA against nucleolytic breakdown, for example silencing of nucleases, have resulted in increased intake of dsRNA in agricultural pests, leading to RNAi efficiency. The hypothesis that nanoparticle-mediated delivery can hence overcome the twin disadvantages of low cellular internalization and nucleolytic degradation of dsRNAs-processing events required for the induction of a potent RNAi response in insect cells-is supported by several empirical evidence.

The basic principles of RNA silencing had first been worked out through the mechanism of mRNA-interfering complementary RNA-micRNA, in the 1980s. Initial studies dealing with key structural genes of Escherichia coli, namely the outer membrane proteins OmpF and OmpC, provided important insight. From these studies, it could be derived that the translation inhibition of OmpF mRNA was due to interaction of a complementary extending sequence located upstream

of OmpC with the 5′ terminal region of OmpF mRNA. The expression of antisense RNA against the mRNAs of lipoprotein and OmpC similarly resulted in repression in the synthesis of both lipoproteins and OmpC proteins, respectively. By the 1990s, solid evidence for RNA-mediated inhibition of protein production in plant systems had been presented, and the process is now known as post-transcriptional gene silencing. The term co-suppression was coined after the transformation of chalcone synthase gene into *petunia* plants by scientists led to an unexpected suppression of homologous chalcone synthase gene and a resultant color break in the petunia flower. A similar phenomenon of transgene-induced gene silencing, referred to as quelling, was reported in *Neurospora crassa*. In 2006, the Nobel Prize in Physiology or Medicine was awarded to Andrew Fire and Craig Mello for their important contributions to the elucidation of RNA interference mechanisms. For the purpose of this paper, RNA silencing is defined as a nucleotide sequence-specific process which catalyzes mRNA degradation and inhibits translation of proteins. According to their genesis, processing factors, and Argonaute-binding partners, the small RNAs that elicit silencing include microRNAs, endogenous siRNAs, and PIWI-interacting RNAs. Although their precursors have diverged, to some degree, the pathways of small RNAs intersect, and they are all actively involved in controlling gene expression, cellular metabolism, and even defense responses against these types of biological insults. The complex network of RNA pathways underlies the importance of RNAi as one of the most powerful tools in regulating all aspects of gene expression and provides an excellent avenue for improving pest management practices due to targeted gene silencing.

Biogenesis of siRNA and Advances in RNA Interference for Agricultural Pest Management

The siRNA biogenesis involves an RNase-III enzyme known as Dicer, which has been implicated in the cleavage of double-stranded RNA into small interfering RNA duplexes of approximately 20 to 25 nucleotides in length. These siRNA duplexes possess a specific structure that includes 2′,3′ hydroxyl groups, 2-nucleotide overhangs at the 3′ end, and 5′ monophosphates exhibiting specific thermodynamic characteristics. This less stable 5′ end allows the guide strand to have a higher binding affinity to the Argonaute protein, leading to RISC complexing in the process. It directs its activity against complementary messenger RNA transcripts through Watson-Crick base pairing and

then cleaves the phosphodiester bonds of the target mRNA post-transcriptionally, a process effective in silencing gene expression.

The rapid development of resistance to insecticides, most notably Bt proteins, in agricultural settings; the proliferation of nontarget pests; and environmental concerns all stress the need for new approaches in integrated pest management. Still, RNAi-based pest control is gaining attention as a probably sustainable and eco-friendly method that would concurrently provide an unprecedented level of precision for targeting pest species while limiting collateral damage to beneficial organisms. Unfortunately, the huge diversity among insect species translated into a wide spectrum regarding RNAi responses and susceptibility profiles, which complicates the plausible efficient delivery of dsRNA molecules to insect populations. The present review focuses on the elucidation of the genetic machinery involved in dsRNA uptake within insect pests and discusses recent advances in the development of exogenous RNA delivery modalities designed to facilitate effective RNAi-mediated control of insect pests. The characteristics of a promising novel approach for enhancing insect pest management strategies are highlighted by the dsRNA-nanoparticle complex.

RNAi: Future Perspectives in Pest Management

Although huge amounts of money have been spent to control these agricultural problems, insect pests and phytopathogens continue to spread unchecked. Given this situation, there is an ever-increasing number of emerging issues pertaining to global food security. RNAi technology has considerable potential in overcoming many such global emerging issues regarding food security. Huge investments made in the past decade have resulted in remarkable progress on the application of RNA interference technology in insect pest management. Importantly, RNAi technology has revolutionized the understanding of genetic functions and insect biology through various genomic and transcriptomic studies that are improving the identification of essential genes that may act as potential targets for RNAi-mediated control of insect pests. This novel strategy also acts very importantly in the management of insect pest populations that have developed resistance to conventional insecticides. This technology does not have significant biosafety risks because RNAi is a safer method of managing pests and does not translate proteins in

plants if their nucleotides are dsRNA or siRNA, unlike the allergens expressed in Bt proteins.

A commercially available variant of dsRNA maize has recently been developed by Monsanto in collaboration with Dow AgroSciences, denoted SMARTSTAX PRO. This will be a widely reviewed and approved variant by three of the principal regulatory agencies in the United States: the EPA, the FDA, and the USDA. It was found safe for human consumption, and unhealthy to environmental health. This is a major milestone and precursor to numerous other RNA suppression products working their way through the system of regulation.

Besides this, high expressions of dsRNA in the chloroplast genome are another novelty in developing dsRNA delivery strategy for efficient protection of crops from insect pests. Although some successes have been realized, several challenges are yet to be overcome before the general use of RNAi-mediated management of pests. The problems regarding off-target or non-target effects of dsRNA, the development of RNAi-resistant insect populations, inconsistencies in the efficiency of targeted genes, and the effective delivery of dsRNA significantly affect the viability of RNAi approaches in the realm of pest control. Delivering dsRNA as nanoparticle complexes opens new exciting vistas toward enhanced crop protection against pests, in particular those that have gained RNAi resistance. The future topical delivery of modified dsRNA and dsRNA / nanoparticle complexes is expected to be the critical future direction for RNAi-based control of pests that eliminates the need for genetic transformation of agriculturally important crops. This revolutionary delivery approach has the potential to accelerate the commercial translation of RNAi biotechnologies. Whereas the current regulatory frameworks for GMOs currently provide the structure for assessing dsRNA-based products, there is a need to establish appropriate science-informed risk assessment protocols concerning topical RNAi applications. The development of guidelines that are transparent for RNA-based biocontrol technology will help give way to the commercialization of this promising methodology. To effectively convey these benefits to regional agricultural practitioners, in particular to those in developing countries, scientists and policy makers should increase educational programs and extension services addressing RNAi technologies targeting the public at large, as well as government regulatory agencies.

The Potato Crop and its Importance

The potato has the status of the most important non-grain food crop in the world and is ranked the fourth among all crops that are widely consumable, after wheat, rice, and maize. With over 100 countries in Europe, Asia, and other continents growing potatoes, this tuber is a rich source of carbohydrates and also a good supplier of considerable amounts of vitamins B6 and C, iron, carotenoids, phenolic acid, magnesium, potassium, and dietary fiber. Due to its high yield regarding protein and energy per area, potato is considered one of the vital crops that contribute much to food security globally. It is important due to its relative ease of cultivation, furthered by the positive effects it has on human nutrition. Further still, it is currently estimated that there are about 5,000 distinct varieties available in the world.

In Pakistan, potatoes are grown in different terrains, from hilly to flat ground, during summer, autumn, and spring. The crop is grown without much ado, as the required labor for its cultivation is minimal and its harvesting is done in less than 90 days after planting. However, the crops of potatoes are susceptible to over 40 diseases caused by bacterial, fungal, viral, and nematode pathogens that attack all morphological parts of Solanum tuberosum. In developing countries, the yield losses due to potato diseases have been estimated to be as high as 50%, while in Western countries, the estimated yield loss is 25%.

The most destructive potato diseases are of fungal and viral etiology and are largely resistant to cure, with many of them surviving in subsequent tuber generations. Soil-borne fungi such as Phytophthora, Pythium, Verticillium, Rosellinia, Sclerotinia, Fusarium, and Rhizoctonia survive as spores or propagules in the soil in a quiescent stage until the environment becomes suitable for infection. Around 40 virus species have been reported infecting potato plants throughout the world. Notable examples include the Potato virus X, Potato virus A, Potato virus S, Potato virus Y, Potato virus M, Potato aucuba mosaic virus, Tobacco rattle virus, Tobacco necrosis virus, and Potato mop-top virus. Infection with a single PVX virus has precipitated yield losses ranging from 10% to 40%, whereas co-infection of PVY may heighten up to 55%. The main Potato virus Y transmission is by winged aphids within potato crops or mechanically, the virus remains infectious for only a

short period, from a few minutes to several hours. It causes losses in yields of about 10% up to 80%. Because of the importance of potatoes as a food staple, there is a need for a prompt and efficient disease management approach. A wider understanding of the involved pathogens is necessary so as to reduce the disease incidence. In managing virus diseases in potatoes, one feasible approach is handling virus-free tubers. The different potato disease management methodologies involve the application of chemical, biological, and integrated resistant cultivar approaches that are important for enhancing the productivity of the crop. The genomic alteration of crops in modern agriculture moves to use advantages from the green revolution technologies in order to address global issues that have been brought forth by population increases. As agriculture continues to shift and change, the utilization of new approaches, such as RNAi pest control, will continue to play a great role in crop protection for the future of food security. Interaction between Host-Pathogen and Development of Disease-Resistant Varieties of Crops

The physiological, cytological, and molecular events of host-pathogen interactions have played a significant role in developing disease-resistant varieties of crops. Among the plant defense mechanisms, there exists a group of proteins abundantly induced and known as pathogen-related proteins, which are divided into five subclasses: PR1-PR5, each having different and important roles against different pathogens. Among these PR proteins, there are two important enzymes, chitinases (PR-3) and 1,3-glucanases (PR-2), which are vital in the defence of plants because they hydrolyse the crucial cell-wall polysaccharides of chitin and glucans, respectively. Chitin, a linear polymer of N-acetyl glucosamine, is one of the most abundant polysaccharides in nature and also one of the major components of the fungal cell wall.

Hydrolyzing activities by enzymes such as β-1,3-glucanase are important inducers of chitinase; hence, this can enhance the plant response against fungal attacks. RNAi is a potent biological defense mechanism employed by plants to protect against viral replication. This involves two different RNA-mediated gene silencing approaches: RNA-directed DNA methylation and RNA-directed RNA degradation-these having been developed as highly effective ways for gene suppression in plant systems. Most specific work was done in the context of resistance to viral infections, where resistance to the conserved coat

protein gene of Potato virus Y (PVY) was assessed by introducing shRNA driven by the Cauliflower mosaic virus (CaMV) 35S promoter in a binary vector flanked by a nopaline synthase (NOS) terminator.

This in vivo shRNA contains sequences such as stem and loop that are derived from microRNA, namely miR304, which confer stability and structural integrity. Transgenesis of the potato cultivar Cardinal with this construct of Agrobacterium resulted in reduced mRNA expression of the coat protein of PVY, thus showing successful RNAi-mediated suppression. In this work, a transgenic potato AGB-R line has been produced through RNA interference against both PVX and PVY, along with the PR gene encoding chitinase for resistance to fungal pathogens. Genetic transformation was carried out by binary plant vector pCAMBIA2301 using Agrobacterium-mediated transformation.

Nowadays, in intensively developed contemporary agriculture, a whole gamut of plant diseases is being managed by the spraying of various synthetic agrochemicals. However, heavy reliance on these chemical agents has caused serious biosafety concern regarding care for environmental conservation, public health, and continuous development of fungicide-resistant pathogen strains. Thus, there is a great need to develop efficient and safe alternative approaches to the management of diseases in crop plants. Notably, fungi and viruses are indeed the highest risks to crops through induction of disease conditions that have adverse effects on yields. Tubers are one of the most critical food crops in the entire world; the crop is attacked by numerous fungal, bacterial, and viral pathogens, which induce widespread infection and can cause drastic yield reductions in virtually all regions where potatoes grow. The major fungal pathogens involved in causing diseases in potato tubers include Fusarium solani, *Fusarium oxysporum*, *Alternaria solani*, *Pythium spp*. *Rhizoctonia solani*, and *Phytophthora spp*. Of the major diseases, *Fusarium oxysporum* is involved in causing vascular wilt disease of members belonging to the *Solanaceae* family. Chitin and glucan are major components of the cell wall in a fungal cell; enzymatic hydrolysis of chitin and glucan may become necessary for efficient disease management. The critical issue in this respect is the expression of chitinase; for example, increased root system chitinase levels may hinder or reduce the development of fungi and bacteria soil carriers. The plant chitinases that were expressed in transgenic plants had the potential to induce resistance against all types of fungal pathogens.

A high chitinase production has been related to a drastic reduction of pathogenic fungi and nematodes, thus reducing infectivity and crop losses. Chitinases play an essential role in the reduction of populations of bacteria, fungi, and actinomycetes. It has been mentioned that rice (*Oryza sativa*) chitinase is capable of stopping lesions caused by Botrytis cinerea. In another work, the production of chitinase in beans increased both the rate and overall seedling mortality associated with *Rhizoctonia solani*. Chitinase has also been found to be very effective against diseases relating to tobacco such as *Botrytis cinerea, Rhizoctonia solani*, and *Sclerotium rolfsii*. Also, the chitinase from tomatoes was also found to reduce plant diseases relating to *Sclerotinia sclerotiorum*. The transformation of barley chitinase in potato var. Desiree demonstrated a marked inhibitive effect on the early blight pathogen *Alternaria solani*. At the global scale, it is estimated that RNA viruses are responsible for losses in yields of potato crops totaling 30%, though this can be as high as 80% in some regions of the world, such as Pakistan. The yield losses from both these viruses range in the measure of 30% by PVX and 50% by PVY when applied individually. However, if the plant is infected by both viruses together, the net yield loss can go up as high as 90%. Potatoes are generally vegetatively propagated; hence, once any plant gets infected, flow of virus till successive generations causes progressive deterioration in health of the plants. As a result, secondary infections have greater tendencies for causing damage than primary infections.

This is mainly because the RNAi technology has a great potential to downregulate the expression of target genes of any origins, including endogenous and viral. Transgenic plants resistant to viral pathogens are achieved mainly either by an antisense sequence or a hairpin configuration that produces dsRNA upon transcription. One of the other main requirements and features of RNA silencing mechanisms is the presence of dsRNA. Encoding transgenes for viral dsRNA, which subsequently allow the plant to recognize the invading viral entities and respond promptly, has extended its major utilities in enhancing virus resistance. The dsRNA, supplied either by a transgene or by replicating virus, is processed into fragments of about 21 nucleotides in length by the Dicer enzyme. Transgenic plants showing resistance to virus infection have been obtained that exploit the RNAi pathway to express hpRNAs or miRNAs of synthetic, virus-specific derivation. Its goal was to design transgenic plants resistant both to fungal pathogens and viruses, PVX and PVY, respectively. This will be done through the

addition of the chitinase gene for conferring fungal resistance and the shRNA transgenes targeting the coat protein genes of PVX and PVY into the binary vector pCAMBIA2301. The construct was driven by the CaMV35S promoter, followed by the terminator sequence NOS. As a result, Agrobacterium-mediated transformations led to the development of approximately 30 lines of stable transgenic potato, named AGB-R.

Further, the antifungal efficacy of the transformed potato variety AGB-R against *Fusarium oxysporum* was assayed. Protein crude extracts from both control and transgenic plants were assayed for activity against *F.oxysporum*; the highest inhibition rate being around 65%, recorded in line SP-21, while the least inhibition rate was about 14%, observed in line SP-89. Notably, chitinase activity with respect to *A. solani* showed the highest inhibition of 60.5% and the lowest inhibition rate of 39.5%. Inhibition in fungal growth ranged from 40% to 56% against *C. Falcatalum* in sugarcane expressing the barley chitinase II gene. In tobacco plants, inhibition of fungal growth ranged from 56.4 to 65.5% in different transgenic lines.

The enzyme activities of endochitinase were done utilizing specific assay kits from the company Merck KGaA, Darmstadt, Germany. This assay determines the quantity of chitinase in crude protein from transformed plants required to inhibit p-nitrophenyl-N,N',N''-triacetylchitotriose. From the said assay, the highest activity determined was 1.19 U for SP-21, while the lowest activity determined was for SP-89 at 0.23 U. The positive control being provided in the kit gave an activity level of 5.67 U.

This value is comparable to an assay done by another research work comprising 0.46 U/mL maximum endochitinase activity. Activity in the Trichoderma strains was reported to be between 0.014 and 0.051 U/mL while it was 1.08-1.40 U/mL for the transgenic plants. Similarly, endochitinase activity in sugarcane with barley chitinase II has also been detected within a range of 0.58 to 0.72 U/mL. The detached leaf assay further demonstrated resistance to the targeted fungal pathogen, *F. oxysporum*, in addition to the overexpression of barley chitinase in the leaves of the transgenic plants. Leaves from non-transgenic (control) plants showed necrosis and significant chlorosis after seven days of inoculation, while the leaves of transgenic plants showed minimal or no symptoms. A similar study showed a significant

difference in necrosis when leaves were challenged with infection by *Rhizoctonia solani*, such that a maximum of 41% of necrotic lesions were manifested by the transformed plants, while non transformed plants had 90% necrotic area of lesions. Furthermore, nine out of 16 plants showed high resistance in detached leaf assays, with a resistance value scale over 2 excess. These findings demonstrate, therefore, that RNAi technology coupled with the expression of PR proteins, especially chitinases, has great potential to improve disease resistance in important crops like potatoes. The integration of these molecular technologies is an eco-friendly approach to disease management, and the resultant increased crop yield will ensure food security.

Improvement of Resistance against Viruses in Potato Plants by Genetic Engineering

Potato plants have been genetically modified to show better resistance against viral infections by expressing shRNA-a specific hairpin-like RNA whose design aims at silencing those targeted genes that confer susceptibility to viruses. qRT-PCR was performed as the investigation tool for determining PVX and PVY thirty days post-inoculation in both transgenic and non-transgenic potato plants. The results showed lower expression levels of CP mRNA for PVY in the transgenic plants compared to the non-transgenic plants. Notably, out of the 21 plants that were inoculated with both PVX and PVY-positive plants, only 2 yielded positive for the presence of both viruses, highlyрот evidencing their new genetics. The highest mRNA level knockdown observed for PVX and PVY was in SP-21 at 89% and 85%, respectively. In contrast, SP-148 transgenic line showed PVX mRNA-level knockdown at 68% and PVY mRNA-level knockdown at 70%. It was also in agreement with previous reports of variable expression of resistance, some as low as 21.973%, while other works realized more than 90% resistance against multiple virus attacks under similar techniques. Another independent work also reported a full 100% PVY resistance in potato lines using the same approach.

It was also reported in a study that the expression of dsRNA derived from prokaryotic sources which gave an efficacy rate of 71.68% against CP-PVY in tobacco plants, whereas genetic transformation with siRNA has obtained a tolerance level of 40% and a resistance level of 20% against the coat proteins of PLRV, PVX and PVY in potato plants. The

phenotypic evaluation of transgenic potato plants manifested mild yellowing symptoms and mosaic appearance against the control manifestation of intensive symptoms of viral infection, which culminated in the death of the plants. The inoculation of the transgenic plants with the mixture of PVX and PVY demonstrated resistance, while the non-transgenic plants were showing severe symptoms, hence confirming previous research studies on virus resistance. In plant cells, heritable genetic materials are ordered systematically into three cellular compartments: the nucleus, plastids, and mitochondria. The major successes achieved in transgenic plants have been a result of the successful insertion of genes of interest into the nuclear genome. However, transplastomic approaches have appeared as one of the great potentially promising alternatives to secure food sustainability. Chloroplasts are organelles of plant cells and eukaryotic algae, in which important functions concerning photosynthesis, biosynthesis of fatty acids, amino acids, nucleotides, phytohormones, vitamins, secondary metabolites, as well as the assimilation of sulfur and nitrogen take place. The chloroplast has a highly polyploid, circular genome-plastome-with specific genetic machinery for transcription and translation. This genetic machinery is thought to originate from endosymbiotic cyanobacteria integrated into an ancestral eukaryotic host cell. The genes in plastid genomes are around 120 to 130 highly conserved and arranged as a quadripartite composition. That includes two inverted repeat regions, IRA and IRB, dividing the genome into SSCs and LSCs.

With this overall structural conservation, there is variation in the genomic size among different chloroplast species, which ranges from 107 kb in *Cathaya argyrophylla* to 218 kb in Pelargonium. These variations within the inverted repeat regions are partly responsible for the various genomic sizes in the different plastid species; however, the tRNA genes and protein-coding genes show a high degree of similarity, since the IRs usually bear identical nucleotide sequences. Starting from the early 1980s, the time when the chloroplast genome of tobacco (Nicotiana tabacum) was sequenced, up to now, there is visible development in the sequencing of the chloroplast genomes of other plant species. There are approximately 800 complete sequences of chloroplasts in the Organelle genomic database of NCBI at present. Chloroplast genomes have contributed much to the phylogenetic analysis of many plant families and have therefore improved knowledge on the various phylogenetic relationships within different

phylogenetic clades. The sequencing of chloroplast genomes has further revealed considerable variation in sequences and structures among the different plant species studied, both interspecifically and intraspecifically.

Such variant data have been of special value in studying the effects of climate on cash crops, encouraging the reproduction of closely related species, and facilitating the search for and conservation of immensely useful features. Then again, information coming from chloroplast genomes has also been utilized concerning the domestication of some crops, especially legumes. Recent advances in chloroplast genome transformation in Chlamydomonas and tobacco introduced new avenues for chloroplast genome manipulation in higher plants. Lack of epigenetic silencing and maternal transmission attributed to plastids acts as an added advantage over traditional nuclear transformation. Unlike the nuclear transgenes, a single transplastomic plant can express multiple foreign proteins from the chloroplast genome at extremely high expression levels. This is so because of the presence of several thousand copies of the chloroplast genome in each cell, and secondly, because of the lack of any gene silencing mechanisms. Moreover, this allows the production of ultra-high levels of recombinant protein and allows multiple genes from operons to be expressed while position effects are eliminated.

Chloroplast genes that have been implicated in photosynthesis can be divided into three distinct classes. The genes that code for proteins of photosystems I and II, namely psaA and psaB for photosystem I and psbA and psbB for photosystem II; genes coding for the subunits of ATP synthase are represented by atpA, atpB; genes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is rbcL; genes for cytochrome b6f are petA, petB; genes for NAD(P)H dehydrogenase are represented by ndhA, ndhB.

In the second group, a suite of RNA genes, such as ribosomal RNA (rrn5, rrn16), involved with the chloroplast genetic machinery, RNA polymerase genes (rpoA, rpoB), tRNA genes (trnK, trnH), genes encoding ribosomal subunits (rps2, rps3). One major group consists of open reading frames assigned for conserved plastids and genes encoding proteins, like cemA, matK. It was postulated that the IR regions provide an advantageous environment for inserting transgenes because of the duplication in the majority of chloroplast genomes. The

site of integration is crucial for modifying the levels of gene expression and enabling the enhancement of homoplasmy through selective antibiotic conditions. Chloroplast transformation is efficiently achieved by the accurate integration of genes into the pre-determined site of the plastid genome through homologous recombination of the transformation vector. Insertion of the transgene cassette into one copy of the inverted repeat thus aids in facilitating its integration into the other copies and thereby applying a much higher selection pressure to attain homoplasmy via the replication and repair mechanisms of the plastid genome. The intergenic spacer region is very conserved; therefore it offers the basic principle of a universal vector for higher plant chloroplast genomes. This, therefore, forms a basis for the utilization of this strategy in transforming the plastomes belonging to a wide range of phylogenetically related plant taxa because of the distinct genetic machinery for transcription and translation special to plastids. Conversely, the non-related taxa, or those showing considerably reduced homology to the gene of interest flanking region, may give less than optimum expression and may require the construction of species-specific vectors for the effective transformation of each particular species.

With the progressive advance in biotechnology, similar methodologies have been worked out for the introduction of exogenous genes to both nuclear and chloroplast genomes. Thus, effective passage of foreign DNA across the double membrane of the plastid, along with efficient selection for the resulting transplastomes and integration of heterologous DNA, indicates chloroplast transformation. Such developments in genetic engineering, together with further clarification of the functionality of chloroplasts, are likely to offer sustainable solutions to increase disease resistance in key crops such as potatoes, toward further progress in agricultural productivity and food security. Methods of Integration of Transgene within Chloroplast Genome

Different methods have been reported for the introduction of foreign DNA into the chloroplast genome. Of the various methods so far reported to deliver foreign DNA into the chloroplast genome, biolistic or particle bombardment DNA transfer is widely recognized as the most prevalent method.

There are, however, direct methods through which successful stable plastid transformation has been achieved, including the use of polyethylene glycol. The particle bombardment technique is characterized by high costs, although it has an advantage in ensuring high transformation efficiency and fast regeneration of transformed tissues. The PEG-based method is economically viable, although it requires more skill, especially in the process of isolating protoplasts.

Particle Bombardment

The biolistics methodology is based on a basic principle in which DNA-encapsulated microprojectiles reach living cells or tissues rapidly, directly. Devices such as the PDS-1000/He™ apparatus or a gunpowder-based model are used for this purpose. In the gunpowder type of device, a small aqueous suspension of DNA-coated tungsten powder is placed at the tip of plastic macro projectile resembling a bullet. These macro projectiles are forced out through a small aperture while micro projectiles by the force of the detonation of the gunpowder charge propels. In the PDS-1000/He™ apparatus, a microcarrier is to be propelled in a burst of helium gas molecules coated with millions of desiccated DNA-coated microcarriers.

Since the first successful transfer of DNA into a plant species in the 1980s, several refinements have occurred in the methodology of DNA delivery into cells by high-density macro projectiles. These refinements have been to minimize target tissue sample and cellular structure damage with increased efficiency of delivery. One of the notable advancements that came upon this technique was one proposed by the researchers who developed the Bio-Rad PDS-1000/He particle gun system, wherein it facilitates the transportation of gold particles coated with a target gene and a selectable marker gene at rupture pressure of 650 psi, that induces alterations on wheat immature embryo.

With this method, the advantages are many and range from wide application in the genetic transformation of some plant species, which are tough, both monocotyledons and dicotyledons. Most noticeably, the rate at which the occurrence of false positives is very small, and the use of the apparatus is relatively simple and requires only a tiny amount of plasmid DNA to achieve successful transformation. Particle bombardment also found its application in the transformation of

plastids. Scientists have, for example, reported on a reproducible method of plastid transformation using two different tobacco-specific plastid vectors, which shall allow for the targeted integration into the large single copy potato chloroplast genome with inverted repeat regions, thereby making the regeneration of plants with homogenously transformed plastids possible.

Polyethylene Glycol (PEG)

In the case of the PEG transformation technique, the necessary protoplasts need to be extracted and further treated with a solution that contains DNA, PEG, and various ions. At this moment, the plasma membrane becomes permeable, and DNA passes inside the cytoplasm. Constructed vectors contain homology sequences corresponding to a preselected target area within the plastid genome for integration onto the target plastid genome.

After integration, antibiotic selection is utilized to segregate homoplastomic cells from cells that have segregating copies of their modified plastome. For example, HA-tagged B-tubulin has been transiently expressed in Canidioschyzon merolae. The expression and localization of the gene product were confirmed by immunocytochemistry 24 h after PEG-mediated transformation. Somewhat simpler and inexpensive stable plastid transformation in tobacco has been shown by the treatment of leaf protoplasts with transforming DNA using PEG.

Essential Components of Chloroplast Vector and Putative Function A typical conventional chloroplast vector generally contains several elements: a promoter, a selective marker gene, a 5′-untranslated region (UTR), and the gene of interest. This 5′ untranslated region, flanked by two chloroplast DNA flanking regions, enables insertion by double homologous recombination into the chloroplast genome in a relatively easy manner.

Flanking Sites vs. Promoters

Flanking DNA sequences should be used effectively in order to facilitate homologous recombination events and thus ensure that endogenous regulatory sequences are present, which are important for targeted transgenic expression. Several flanking sites have been tried in order to ensure that the gene of interest is integrated

successfully in the plastid genome. The major flanking sites are rbcl/accD, rps12/trnV, and trnM/trnG. This is because studies have demonstrated that the trnI/trnA genes, which flank the ribosomal operon in the chloroplast genome, serve as highly efficient sites for the integration of transgenes during plastid transformation. This efficiency has been related to the trnA site, given the fact that it associates the positioning of the transgene in the inverted repeat region with a replication origin and with intron sequences, thus increasing its copy number. The empirical studies showed that the integration of transgenes in the spacer region, which is transcriptionally active, induced an expression level that surpasses by 25 times the spacer region that is transcriptionally silent, trnl/trnA versus rbcl/accD. This is explained through multiple heterologous or endogenous promoters amplifying the reaction of transcription. Transcription processes in the plastids are mostly mediated by two kinds of plastid RNA polymerases, namely, the plastid-encoded plastid RNAP (PEP) and the nucleus-encoded RNA polymerase (NEP).

A large number of plastid promoters exists, including different transcriptional start sites for the two RNA polymerases PEP and NEP. The genes for the core subunits of PEP are carried in the plastome as rpoA, rpoB, rpoC1, and rpoC2. It has a relation between the RNA-synthesizing enzymes of T-type bacteriophages and the single-subunit, nucleus-encoded RNA polymerase NEP. Most plastid promoters include consensus sequences matching sigma factors at the -35 and -10 positions of the form, respectively, TTGaca and TATaaT, that demonstrate extreme similarity to those in bacterial RNAP where this fact provides proof for plastids of cyanobacterial origin. The plastid transformation vector usually contains plastid promoters, often the 16S rRNA promoter (Prrn16) or psbA promoter, respectively, which enable the transcriptional integration of the transgenes into the chloroplast genome. Indeed, when speaking about this, it should be mentioned that the initiation site of PpsbA is associated exclusively with PEP, whereas the initiation site of Prrn involves both PEP and NEP. It should be noted that the major transcriptional products of both NEP and PEP are polycistronic. Thereby, such transcripts need extensive post-transcriptional processing: splicing of introns, processing of mRNA into monocistronic or oligocistronic forms, 5' and 3' trimming, with accompanying RNA editing. All these components and processes interact in a very complicated manner to ensure effective expression of the transgenes within the chloroplast genome. This technology was

developed mainly to enable the advancement of plant biotechnology, and the methods involved in attempting to improve crops. Untranslated Regions (5' and 3')

The interplay is crucial in the stabilization of plastid mRNA due to the intricate interaction between the RNA sequences, structures, and other active elements within the process of gene expression. These stabilizations are also greatly enabled by the RNA secondary structures coupled with their respective 5' and 3' UTRs. The proteins produced in chloroplasts must be protected from chloroplast proteases, which are capable of degrading such proteins within an extremely short period. The 5' untranslated region of plastid transcripts is, for example, influential in affecting the proteins formed and their level of expression, particularly when chimeric transgenes are expressed. There are numerous 5' UTRs, which each differ in their potential in terms of the stability of transcripts and protein within chloroplasts of transgenic organisms.

For example, the coding sequence of human serum albumin fused with 5' UTRs and under the regulation of the chloroplast psbA gene in light conditions showed a fantastically high enhancement of protein expression up to a factor of 500. This salient expression may be afforded by its structural configuration protecting the exogenous protein from catabolism and hence may imply a straight correlation between UTR design and protein stability. The studies done to explain what happens due to the integration of the promoter with its 5' UTR regulatory elements in Chlamydomonas reinhardtii 16S have presented an increase in the number of protein synthesis. Empirically, it has been found that increased steady-state concentrations of luxCt mRNA, that is the marker gene, increase mRNA production through the 16S promoter and further give rise to enhanced protein accumulation provided by atpA 5' UTR. When overall expression levels within the chimeric constructs were compared, it was observed that RNA stability played no significant role. A 200-fold increase was noted in protein expressions within the constructs containing the 5' UTR of psbA as opposed to those with the rbcL 5' UTR. Shine-Dalgarno (SD) motifs in the 5' untranslated region of prokaryotic messenger RNAs are important to ensure efficient translation.

These motifs bind ribosomes, and their action depends on the recognition of a specific pattern at the 3' end of the 16S ribosomal RNA

within the 30S ribosomal subunit. Evidence is emerging to indicate that translation of mRNAs from certain bacterial species lacking an identifiable SD motif can, nevertheless, occur with ready efficiency in those systems that naturally lack an SD sequence. Further studies have also identified modes of translation activation independent of a classic ribosome-binding site. In contrast, an in vivo test of the predicted ribosome-binding site activity of the tobacco chloroplast atpI gene indicates that upstream elements of the putative ribosome-binding site are needed for efficient translation initiation in chloroplasts. SD-like sequences, which can be summarized as GGAGG, were found, for most tobacco chloroplast mRNAs, within a region located about 10 nucleotides upstream from the translational initiation codon, suggesting that functional SD-like sequences do exist. The 5'-UTR was suggested to be the binding site for mRNA-specific translational activator proteins and thus facilitates attachment of the ribosomal 30S subunit to the initiation codon. This is a very important recruitment, considering that not all SD sequences are conserved in a region of 4–9 nucleotides upstream of the start codon. SD sequences from bacteriophages have been used in an effective manner to regulate translation initiation efficiency in reverse genetics and for the expression of transgenes within plastids.

The 3' UTRs are exclusive features of chloroplast mRNAs immediately downstream of the stop codon. These sequences contain IRs, which may form secondary stem-loop structures that contribute to the maturation and stability of the transcript and suppress the activity of 3' to 5' exonucleases, thus blocking polyadenylation. The use of ribonuclease led to the elimination of polyadenylation sites and protective stem-loop structures and thus enabled the start of plastid mRNA degradation. Loci like the 3' UTRs of genes such as rps16, rbcL, psbA and rpl32 are the most commonly used for controlling expression of foreign genes in plastids.

Marker Genes

To detect transformation properly and reliably, chloroplasts and the cell lines carrying the modified plastomes must be homoplasmic-that is, carry identical transgene copies. Thus, this involves the construction of a marker gene to be integrated within a plastid expression framework comprising a plastid promoter, a 5' UTR containing a Shine-

Dalgarno sequence for ribosome interaction, and a 3' UTR with all the required cis-acting elements.

Other herbicide resistance markers which have been used to select transformants include phosphinothricin acetyltransferase pat/bar gene, Bromoxynil nitrilase bxn gene and various antibiotic resistance genes. Antibiotic resistance markers neo gene, aphA6, are examples of genes conferring resistance against kanamycin, whereas aminoglycoside 3'-adenylyltransferase is amongst the most commonly deployed antibiotic resistance genes imparting resistance against a wide number of antibacterial agents. The application of antibiotic or herbicide resistance genes as specific markers in the transformation of plants has public safety and biosafety concerns. For this reason, the elimination of antagonistic bacterial genes from cloned plants is very essential. Some of the strategies developed to avoid the inclusion of antibiotic or herbicide resistance genes include homology-based excision due to directly repeated sequences; excision mediated by phage site-specific recombinases, co-integration of the specific gene, and separation after co-transformation. Research has documented that Spinacia oleracea spinach can act as a model species in carrying a unique marker gene for chloroplast genome transformation and in a recent report showing a 25% chloroplast transformation efficiency versus streptomycin with BA selection. The selection is based on the activity of the chloroplast site-specific gene product BADH enzyme involved in the oxidation of toxic betaine aldehyde-unique in the chloroplasts-to useful Gly betaine. Recent isolations have indicated that the dsdA gene may serve as a marker gene for both plastid and nuclear transformation systems. Such a gene's ability to also serve as a marker gene in plastid transformation of monocotyledons has been one of the current breakthroughs in plant biotechnology, potentially presenting new possibilities to improve transformation efficiency and further meet regulatory requirements on genetically modified crops.

Application of Chloroplast Transformation

Research in biotechnology has extensively harnessed the peculiar and important advantages provided by chloroplast transformation, for producing cost-effective biopharmaceuticals like vaccines and antibodies. Furthermore, genetic engineering of the chloroplast genome has been used very effectively in the production of important enzymes, biomaterials, and biofuels. It also contributes much to

enhancing biomass production and promoting sustainable food production in response to various challenges that might adversely impact agricultural yield.

Pharmaceutical Production of Vaccines

Chloroplast transformation has attracted the attention of the pharmaceutical industries due to large copies of plastid DNA, as it helps in large-scale production of vaccine proteins. Besides addressing the limitations imposed by the cost of chemically synthesized vaccines, the advantages mentioned here thus turn this approach into a source of low-cost vaccines for populations in developing countries. The general principle is based on the introduction of specific genes into the chloroplast genome of a plant encoding bacterial and viral antigens responsible for certain diseases. These are then expressed as immunogenic proteins in edible plant tissues.

It is important to point out that essential highlighting has been done only non-glycosylated antigens can be produced successfully in plastids because the chloroplast lacks glycosylation machinery. However, outstanding success has been realized with plant chloroplasts being used as bioreactors. Evidence obtained demonstrates that such systems can exceptionally improve immunogenicity. Transgenes introduced into plant systems have also been proven to be expressed without certain intrinsic limitations. Plants expressing the gene have been found to contain HBsAg that is antigenically similar to HBsAg particles produced through recombinant yeast and human serum.

Confirmation of the presence of HBsAg in leaf extracts has been established, and its concentrations were shown to correlate with high mRNA, as evidenced by enzyme-linked immunoassays that utilized monoclonal antibodies against HBsAg derived from human serum.

Moreover, LecA is a protein described as a promising target for the inhibition of amoebiasis, located within the chloroplasts and making up 6.3% of the TSP, translating into an incredible accumulation of 2.3 mg LecA per gram of leaf tissue. Further, during the expression of the human secretory protein somatotropin in the chloroplast, there was found over a 300-fold increase in recombinant protein accumulation as compared to nuclear transformation. Further, the antigen of the HIV-1 p24 has been produced in a high-biomass tobacco variety. The antigen

did not only result from it but also a higher amount of total soluble protein was obtained from the transformation of various bacterial and virus antigens in different plants plastids.

With these advances in plant-based pharmaceutical vaccine production, there is still a gap to be improved concerning the correlation of this method with various clinical trials. Recently, however there is a report that clinical success was achieved with the tool called ZMapp, developed by a systematic selection from antibody components, using a rapid transient expression methodology in tobacco species, and targets Ebola virus disease. Currently, adjuvants co-expressed with antigens serve the dual purpose of antigen delivery and eliciting an immune response against a target. Chloroplasts thereby function as bioreactors for hybrid protein expression. For instance, investigations on recombinant superoxide dismutase expressed by *Withania somnifera* were carried out using a chloroplast transformation vector carrying a gene encoding Cu/Zn SOD. It was observed that the transplastomic plants accumulate the recombinant SOD up to levels of 9% of TSP in the leaves and pure chloroplast-expressed recombinant SOD in an estimated specific activity of 4600 U/mg. Results showed that chloroplast transformation is effective for the industrial production of recombinant superoxide dismutase in plants.

Antibody bioreactor

A series of biotechnological advancements have made it possible to express antibodies in chloroplasts, and these pharmaceuticals are much in demand for the treatment of disorders ranging from simple to highly complex. Human monoclonal antibodies engineered to be directed against HSV glycoprotein D, as well as IgA heavy chain proteins joined with light chain proteins by peptide bonds, have also been expressed in chloroplasts. The work has gone even further to show that chloroplasts have the apparatus needed in the assembly of complex human antibodies and assembled human IgG1 monoclonal antibodies can be expressed and purified from chloroplast of *Chlamydomonas reinhardtii* for the first time. The expression of integrated proteins, such as an antibody domain against CD22-a B-cell surface epitope-and an enzyme domain from Pseudomonas aeruginosa, has already formed mono and dimeric protein toxins inside the algal chloroplast. Based on the fact that chloroplast machinery can

complete the disulfide bond formation process, there is much evidence for the production of monoclonal antibodies in plants; hence, large-scale production is possible at lower cost. Application in sustainable food security

Chloroplast transformation has been widely used for cost-effective production and nutritionally enhanced food sources. Most of the modifications in the chloroplast genome were documented in various studies, aimed at the enhancement of agronomic traits related to resistance to herbicides and insecticides and tolerance to water and salinity deficiencies.

Nutritional Enhancement Through Chloroplast Transformation

Improvements in chloroplast transformation have been investigated to further enhance nutritional biochemical pathways that allow the inclusion of vital nutrients, which are mostly deficient in non-green tissues of plants. Such that in the case of one study done to improve carotenoid biosynthesis in tomatoes, through the use of the lycopene beta-cyclase gene from bacteria, which catalyzes the conversion of lycopene into beta-carotene, it was shown through the fourfold increase in the levels of pro-vitamin A within the fruit. Likewise, lycopene beta-cyclase genes isolated from *Narcissus pseudonarcissus* and *Erwinia herbicola*, when expressed in the tomato genome, enhanced the enzymatic capacity of the plant in effectively converting lycopene into provitamin A (beta-carotene). Based on studies conducted, enhanced lycopene accumulation together with xanthophyll cycle pigments was achieved of such modifications. As a result, this conversion resulted in the depression of the alpha-branch xanthophyll lutein in the green leaves of the transplastomic tomato plants, but the lycopene was successfully converted into beta-carotene, thus accumulating the provitamin A in a concentration of about 1 mg on a dry weight basis in fruit. It has been further demonstrated that overexpression of the γ-tocopherol methyltransferase (γ-TMT) gene in transplastomic seeds enhances the conversion of γ-tocopherol to α-tocopherol by approximately tenfold. It improves the nutritional quality and enhances the tolerance against salinity and heavy metal stress by reducing the damage induced due to ROS generation, ion leakage, and lipid peroxidation. Chloroplast transformation is one of the powerful biotechnological tools with immense applications, starting from the production of

biopharmaceuticals and vaccines to improving food security and nutritional quality. Therefore, this breakthrough offers colossal hope toward solving global global health and sustainable agriculture challenges, hence making chloroplast transformation an important strategy in modern biotechnology.

Chloroplast Transformation in Agricultural Stress Management

Environmental stressors such as drought, salinity, temperature fluctuation, including extreme cold, chilling injuries, and heat shock, and biotic stresses involving pathogens and bug infestations have taken a heavy toll on agricultural production in state-of-the-art advances. These stressors not only tamper with crop yield but also threaten human food security with increased climate change and population pressure. Regarding this, the steps taken in the field of biotechnology, especially chloroplast transformation, have emerged as the counterpart to enhance agricultural resiliency against the same threats.

Temperature Stress Management

Extensive research over the years targeted the identification and characterization of multiple functions of genes associated with plant responses to environmental stresses. Alteration in the genetic material influencing unsaturated fatty acid composition in plant lipids has an appreciable influence on the temperature tolerance of higher plants. For example, transplastomic plants, whose fatty acid composition in leaves or increased unsaturated fatty acids both in seeds and leaves, indicate that these transplastomic variants may have enhanced cold tolerance.

One of the astounding works was that *Nicotiana tabacum* chloroplast genome-encoded Escherichia coli gene L-aspartate-alpha-decarboxylase (AspDC) catalyzed the decarboxylation of L-aspartate into beta-alanine and carbon dioxide as end products. These two resultant products greatly enhanced the heat tolerance of the photosynthetic machinery and accounted for a higher biomass accumulation even at higher temperature regimes. Oxidative stress is also one of the major drawbacks in plants under stressed conditions. Transgenic potato plants that overexpress superoxide dismutase and

ascorbate peroxidase have been demonstrated to be more tolerant to oxidative stress and heat stress.

Taken together, these studies demonstrate the potential of chloroplast transformation as a powerful tool for the genetic enhancement of plant temperature tolerance, able to provide varieties that are resilient enough to cope with extreme thermal conditions, hence assuring better performance of crops in the temperature fluctuation-prone climates and helping improve food security.

Management of Drought and Salinity Stress

Drought and salinity are considered two major stresses in crops. Salinity stress alone affects about 7% of the Earth's total land area and 5% of agricultural land, causing an uneconomical problem with very noticeable features of dwarfing growth, hampered development, and low crop yield. Various types of osmoprotectants, such as trehalose and betaines, have emerged as active molecules that enhance cellular mechanisms involved in the scavenging of ROS and maintenance of macromolecular integrity, hence enhancing the plant's tolerance against salinity, low temperature, and drought. Transgenic somatic carrot cells resulted in successful reconstitution of transplastomic plants in practical applications wherein the genetically transformed carrot plants expressed the BADH gene that exhibited higher concentrations of NaCl. Such carrot cell cultures from the transgenic line could grow sevenfold faster in liquid media containing 100 mM NaCl because of accumulation of 50 to 54-fold more betaine compared with untransformed cells.

Over-expression of cytosolic APX in Arabidopsis thaliana revelation studies are a reflection of its protective efficiency against active oxygen species caused by salt stress resultantly induced through NaCl. Similarly, such an important antioxidant enzyme as MnSOD over-expression in rice has a prevailing view suggesting drought tolerance capability induction in the plant. Overexpression of the BvCMO gene from beet-a halophyte species which naturally produces GlyBet-results in transgenic tobacco plants that are more tolerant to saline and drought stresses. Transformation increases photosynthetic rates and yield under salt stress at 150 mM NaCl. Recent reports further confirmed that the expression of aldehyde dehydrogenase genes in the chloroplast metabolic pathway confers significant tolerance to NaCl

stress in tobacco, again illustrating the broad versatility of chloroplast transformation in addressing multiple forms of abiotic stress.

Enhancement of Tolerance to Biotic Stressors

Another critical factor that affects the sustainability of food concerns biotic stress. If not well managed, the stressors cause severe outbreaks of diseases and further reduction in the availability of food in the world. The risk of toxic accumulation from chemical pest and disease management calls for a continued search for alternative strategies of control, especially those biotechnology based.

Chloroplast transformation has emerged as one of the key approaches towards the development of improved disease resistance against pathogenic bacteria and fungi. Many targets of resistance-related proteins are localized to subcellular compartments and get released upon plant hypersensitive response. A number of reports continue to add to the list of generated transplastomic plants with enhanced resistance against infectious agents. For example, plants expressing the *Pinellia ternata* agglutinin gene confer striking resistance to insect pests and phytopathogens. The introduction of the Cry9Aa2 gene from Bacillus thuringiensis into tobacco plant plastids has successfully attained resistance against the potato tuber moth. Also, transgenic tomatoes carrying CeCPI and chitinase have shown marked resistance against a suite of insect pests and phytopathogens underlining applicability in pest management by chloroplast transformation. The expression of different defense genes giving rise to protease inhibitors and chitinases has been ascribed as the basis for outstanding broad-spectrum resistance developed by Nicotiana benthamiana for many pests, diseases, and abiotic stressors. This innate potential for resistance can be enhanced through chloroplast transformation in order to improve food security due to emerging biotic challenges.

Transplastomic flora showing improved resistance against insects

It was reported that this transplastomic plant showed high resistance to the larvae at every development stage of *Helicoverpa armigera*. The protein Cry1Ia5 accumulated to about 3% of the total soluble protein in tissues, which represented an amazing increase of 300-fold compared to the expression in plants transformed at the nucleus. Many of the transgenic varieties have been commercialized and were

widely planted due to their efficiency in biological pesticide properties by expression of Bacillus thuringiensis crystal proteins, such as Cry1Ab in maize and Cry1Ac in cotton. While the Bt crystal proteins are recognized as safe biological pesticides, the toxic protein transfer into non-target organisms through pollen has raised concern. Extensive research related to chloroplast transformation to resolve this issue, as this transformation occurs outside the nucleus and the chloroplast is maternally inherited at subsequent generations. Several studies have confirmed that the chloroplast genome can be targeted specifically in order to increase pest insect management and control. Besides, insect-resistant transplastomic tobacco has been reported by the researchers via the up-regulation of insect pathogenic defense mechanisms. Recently, RNAi technology has been applied as an insect-specific and environmentally benign control strategy in context with the transplastomic transformation. This new technology selectively targets the populations of particular insects, hence sparing the valuable beneficial insects, besides averting the synthesis of toxic proteins in the plant which contrasts with the conventional Bt proteins. In one work, for instance, it was pointed out that the content of the foreign protein in the leaves of the mature transgenic tobacco plants expressing specific genes in *Helicoverpa armigera* amounted to 45.3% of the total soluble protein. Eventually, this caused the complete mortality of the armyworms after the consumption of the transgenic leaves. Expression of the respective gene dsRNAs expression-V-ATPase and Chi, P450 (CYP6AE14)-in the tobacco chloroplasts silenced three important genes in *Helicoverpa armigera*. These enhanced transcript levels significantly hamper the transcription of the target gene in the insect midgut, thus preventing larval development. Such sharp reductions resulted in overall larval weight, growth rate, and pupation.

In experiments aimed at determining if the intrinsic RNAi machinery of the plant was interfering with pesticide activity, the dsRNA was processed into siRNAs. Incidentally, full-length hpRNAs expressed within the transplastomic lines of *Nicotiana benthamiana* have been shown to confer high protection against *Helicoverpa armigera*, while hpRNAs derived from nuclear-transformed plants were processed into siRNAs that showed a relatively low anti-feeding effect. These observations suggest that chloroplasts contain little or no RNAi machinery or activity, and that hpRNAs made therein do not cross the cytoplasm. Further observations were that siRNAs specific to the target genes were present within midgut tissue of beetles feeding on the

leaves of transplastomic potato plants, and mRNA transcript levels of the targeted genes were lowered after this feeding by the Colorado potato beetle on transplastomic potato plants.

In conclusion, chloroplast transformation has a multi-dimensional approach in the improvement of agricultural resilience against an array of environmental and biotic stresses. Chloroplast genome targeting has the potential to confer better tolerance in transgenic plants against temperature extremes, drought, and salinity, and improved resistance against pests and pathogens. However, amidst the pressing concerns of climate change and food security that continues to beset the agricultural sector, the potential of chloroplast transformation is an increasingly powerful tool in biotechnology. Through continuous research and technological development, chloroplast transformation can make a credible contribution to agricultural sustainability that will meet the requirements of an increasing world population.

Further Readings

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About this Book

RNAi and CRISPR Gene Editing in Plants - Approaches and Applications examines two groundbreaking genetic modification technologies that are reshaping plant biotechnology and agriculture. This comprehensive work explains both the scientific principles and practical uses of RNA interference (RNAi) and CRISPR-based gene editing systems, giving readers the knowledge they need for both research and implementation.

The book addresses the underlying science and real-world uses of these technologies. It opens with a thorough examination of CRISPR systems, covering their essential components and mechanisms while exploring their integration with chloroplast transformation techniques. This combination creates new possibilities for advancing plant biotechnology across multiple fields, from agriculture to pharmaceutical production.

Key areas covered include:

- Clear explanation of CRISPR mechanisms and components
- In-depth analysis of chloroplast transformation vectors and their functions
- Applications in pharmaceutical production, including vaccine development and antibody bioreactors
- Agricultural stress management techniques, addressing both abiotic (temperature, drought, salinity) and biotic stressors
- RNA interference mechanisms and their applications in pest management
- Practical aspects of genetic transformation techniques, including particle bombardment and PEG-mediated transformation
- Case studies focusing on major crops like potato and maize

This volume serves both researchers in plant biotechnology, agriculture, and related fields.

About the Editor

Dr. Bushra Tabassum has completed her Ph.D in the subject of Molecular Biology from CEMB, University of the Punjab under the supervision of Prof. Dr. Idrees Ahmad Nasir and Prof. Dr. Tayyab Husnain. She has served CEMB for 15 years. At SBS, she is working on protection of plants from pathogenic viruses, fungi, and insect pests through the use of RNA Interference technology (siRNA, hpRNA & dsRNA). She has managed seven research projects funded by PARB, HEC, and IFS as Co-PI/PI and has published several research articles in peer-reviewed international journals. Her research interests include: Crop Protection from insect pests, fungi and viruses for which her group make use of all available molecular biology tools including RNAi, Gene Pyramiding, genetic engineering and transgenic strategies to develop a crop product with sustainable yield output.

Research Interests: Plant protection from various biotic (insect pests, viruses, fungi) and abiotic stresses (heat, frost) by employing Molecular Biology techniques like RNAi, transgenic technology, and gene pyramiding.

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