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ADVANCES IN PLANT TRANSFORMATION TECHNOLOGY: 2. VECTOR DESIGNING FOR EFFICIENT TRANSGENE INTEGERATION AND EXPRESSION

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Vector designing over the years has played a key role in optimising DN Adelivery methods for plant transformation aimed at more efficient, stable and biosafe GM crop production as also plant functional genomics. From initially designed binary vectors and the 'binary vector system' have evolved several types as modular, superbinary, Gateway and their derivatives. The main objectives have been simple stable transgene insertion, site specific clean integration, transgene stacking, marker free progeny and facilitation of high-throughput analysis in reverse genetic studies. Breakthrough advances have also been made in the intragenic approach for biosafe GM crops with plant based gene regulatory elements. The review briefly covers also Genome editing a recently emerging, efficient tool for non-transgenic gene modification.The main aim of the review is to highlight the untiring efforts of the recombinant-DNA scientists engaged in the production of biosafe GM crops acceptable in both the regulatory framework and the public domain.

Key words: Binary vector, superbinary vector, pBIBAC, pORE modular vectors, Gateway vectors, pANIC, pGreen, pSoup, pCLEAN-G, pCLEAN-S, P-DNA, Intragenic vectors, ZFN, CRISPR-CAS SYSTEMS

The inevitable hazards of climate change will certainly affect crop and vegetable farming with consequent risks to food security. Even in our main area of operation, the Malwa region of MP, a part of the drylands of India (www.samprag.org), major crops as soyabean of *khareef* season, wheat and Bengal gram of *rabi* season have got ruined, due to untimely heavy rains and hailstorms, in the past two years. Further, investigations on the impact of induced environmental stresses (as, heat, drought and cold) on plants, have shown the male reproductive system of plants to be highly susceptible to such stresses and can lead to male sterility (De Storme and Geelen 2014).The impairments can be due to deleterious morphological and molecular effects during the meiotic male sporogenesis and /or microspore forming gametogenesis phases. Besides traditional practices, GM technology, with wider gene pool, will certainly be able to help in overcoming the challenges through necessary adaptive improvements in crops and vegetables. However, it is essential that the GM crops released be ecofriendly, biosafe and meet the requirements of the regulatory framework. This can be achieved through advances in functional genomics in the context of plant transformation for input and output traits. The findings can have positive and viable applications in commercial farming. Crucial to these objectives is development of more efficient vectors, especially for high-throughput analyses. In an earlier review major efforts of recombinant DNA scientists towards efficient transfer and integration of the transgene were covered (Banerji 2013). In this review advances in vector designing have been described, with main focus on binary vectors for *Agrobacterium* mediated plant transformation.

In the early seventies it was demonstrated that the gram negative soil bacterium,*Agrobacterium tumefaciens* induces tumour formation by transfer of the T-DNA of its Ti plasmid to wounded sites of many dicot plants (Van Larebek *et al.* 1974).The bacterium reaches a wound site of the host plant and transfers the T-DNA (transfer DNA) of its Ti plasmid to the plant cell. In the cell, the T-DNA moves to the nucleus and integrates with the plant chromosomal DNA. The T-DNA is delimited by two imperfect direct repeats, the right border (RB) and the left border (LB). The T-DNA enters the plant cell with its RB first and the transfer ends near the LB. Between, the LB and RB are the phyto-oncogenes which encode enzymes for synthesis of tumour inducing phytohormones and genes for synthesis of opines, the nutrients for *Agrobacterium*.The virulence (*vir*) genes of the Ti plasmid conduct this transfer by encoding proteins for excision and transfer of T-DNA.

The initiation of plant transformation technology was based on simulation of this mechanism of the natural genetic engineer. The ability of the *Agrobacterium* Ti plasmid to cross species barriers opened up possibilities of using it as a vehicle or vector for transferring

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genes of interest (*goi*) from any organism to plants. *A. tumefaciens* strains, containing disarmed Ti plasmids, with genes for genetic parasitism (phytohormone and opine genes) removed, were developed. Such disarmed strains could transfer the residual T-DNA, containing mainly the left and right borders and the virulence (*vir*) genes, to dicot plant cells and such transformed cells could regenerate (Zambryski *et al.* 1983). This initiated the use of disarmed Ti plasmids as vehicles or vectors for the transfer of any DNA fragment of interest for plant cell transformation.

BINARY VECTORS:

Initially,cointegrate vectors were developed through homologous recombination between a fragment of an *Eshcerichia coli* plasmid containing the *goi* and a disarmed Ti plasmid.However, due to large inconvenient size, the cointegrate vectors were discontinued and gave way to the Binary vector systems. These two-vector systems, are based on the phenomenon, that *vir* genes can act in the *trans*mode. Thus, in such 'binary vector systems', the agrobacterial cells used for transgene transfer through cocultivation with host tissue, contain two plasmids, a *helper* plasmid with only *vir* genes and another smaller disarmed *binary* vector with the *goi/DNA fragment-T-DNA cassette.* In such agrobacterial cells eg., strain LBA4404, the *vir* genes,although present on a separate helper plasmid pAL4404, can engineer the transfer of the T-DNA-*goi* fragment to a plant cell (Hoekema *et al.* 1983).The binary vector, a shuttle vector, with two different origins of replication (*ori*) for replicating in both *E.coli* and *Agrobacterium*, is cloned in *E. coli* and then transferred to an *Agrobacterium* cell containing a *helper* plasmid.

Earlier major objectives of binary vector designing were, improvements in bacterial and plant selection markers, high copy number in *E. coli*, stability in the helper containing *Agrobacterium* strain and choice of multiple cloning sites (MCS). These led to the constructions of basic binary vector series as, pBIN (Bevan 1984), pGA (An *et al.* 1985), pBI (Jeffersen *et al.* 1987), pCB (Xiang *et al.* 1999).pBIN19and pBI121 with reporter gene added came in common use for plant transformation. Then the pZP series was developed with features as MCS with more options for DNA fragment insertion sites, ColE1 *ori* for higher yield in *E.coli* and elements for enhanced stability in *Agrobacterium* (Hajdukiewicz *et al.* 1994). Improvements in pZP vectors further led to the pCAMBIA series (www.cambia.org).Some of these are still used in commercial GM crop production. Many have been extensively used in plant functional genomic studies and in providing the backbone for improved binary vectors. Further on, developed the superbinary vectors.

Binary vector composition – basic features:

All binary vectors have two common structural features, the disarmed T-DNA with left and right borders (LB, RB) and the vector backbone. The T-DNA mainly has MCS and a plant selection marker and/ or a reporter gene with promoters, terminators and other sequences for expression of the inserted genes. The vector backbone contains *ori* for *E. coli* and *Agrobacterium*, a selectable marker for bacteria and other components as elements for transfer of the vector from *E. coli* to *Agrobacterium*.The LB and RB are imperfect direct repeats with limited sequence variation across strains, act in the *cis* mode for T-DNA transfer and have enhancer or attenuation elements near them (Yadav *et al.* 1982, Slightom *et al.* 1985 and Wang *et al.* 1987). As usually, the RB end of T-DNA enters the plant cells first, the plant selectable marker is placed near the LB after the *goi* sequence, to avoid false selections (Hellens *et al.* 2000a).The selectable marker genes mainly confer resistance to antibiotics or herbicides. While Kanamycin resistance gene (*kan*) is more common in dicots, the herbicide resistance genes conferring resistance to Hygromycin (*hyg*) and Phosphoinothricin (*bar*) have been more frequently used in monocots. An important consideration for proper selection of transformed plant material with any marker is the concentration of the selecting agent, antibiotic or herbicide, in the medium, as, species and even cultivar specific variations in sensitivity exist. The bacterial selection markers are mainly antibiotic resistance genes as, *kan, cm* (chloramphenicol), *sp* (spectinomycin) and tetracycline (*tc*). If the promoter of a plant selection marker, like *nos* (nopaline

synthase gene promoter), works also in bacteria, then additional marker for bacteria may be avoided. As penicillin derived antibiotics like carbenicillin is used to remove bacteria after the required cocultivation, ampicillin (also penicillin derived) resistance genes should not be used as a bacterial marker.

Further, the choice of constitutive promoters for the expression of the marker gene is also crucial. In dicots the main promoters used have been CaMV 35S which expresses the 35S transcript gene of *Cauliflower mosaic virus* and *nos*, needed for nopaline synthase gene expression (Depicker *et al.* 1982, Odell *et al.* 1985). Popular promoters in monocots, are *Ubi/ ZmUbi1*, the promoter of ubiquitin gene of maize including its intron and *Act/ OsAct1*, promoter of actin gene plus intron in rice (Zhang *et al.* 1991, Christensen *et al.* 1992, Himmelbach *et al.* 2007, Kim *et al.* 2009).

Reporter genes have been extensively used in vectors for selection of transformed plant material, because of the ease of visual tracking of their products. Thus, such genes,as, genes for β-glucuronidase (*gusA* or *uidA*, Jefferson 1987), green fluorescent protein (*gfp*, Pang *et al.* 1996) and luciferase (*luc*, Ow*et al.*1986), have been widely used for assessment of several molecular processes as, role of regulatory elements in promoter efficiency, intranuclear transport of T-*goi*-DNA and its integration into host plant DNA. Further, reporter genes after fusion with a *goi*, aid in tracing the subcellular location of the latter (Tzfira *et al.* 2005, Chakrabarty *et al.* 2007). These are also regularly used as components of gateway vectors (Nakagawa *et al.* 2007).

For successful transfer of smaller DNA fragments eg. Bt gene or herbicide resistance genes, efficient replication of their binary vectors is necessary in both *E. coli* and *Agrobacterium*.Therefore, the type of replication origin (*ori*) incorporated in the vector plays a crucial role. The earlier constructed binary vectors as pBin19 contained the replication functions of incompatibility group P (IncP, Bevan 1984) which yielded low copy numbers. Improvements were induced by using better promoters. For instance, pGA482 was developed by retaining IncP for replication in *Agrobacterium* but adding ColE1 for replication in *E. coli* (An *et al.* 1985). Similarly, for higher copy number in both pZP and pCAMBIA series vectors, the *ori* used have been pVS1 and ColE1 for replication in *Agrobacterium* and *E. coli* respectively (Hajdukiewicz *et al.* 1994, www.cambia.org). Likewise, in pGreen series of vectors derived from plasmids with replication elements of the incompatibility group W(IncW) the replication in *Agrobacterium* is through the native IncW*ori*, but, for replication in *E. coli*pUC*ori* was incorporated (Hellens *et al.* 2000b).Besides the above components, the binary vectors need to have also plasmid mobilization elements when their passage from *E. coli* to *Agrobacterium* is designed through conjugation.

ADVANCES IN VECTOR DESIGN FOR *AGROBACTERIUM* MEDIATED TRANSFORMATION:

In the following sections, further advances in vector designing have been discussed.

Transgene transfer using binary vectors: For construction of the binary vector, the *goi* DNA fragment of interest is cloned in the pUCor pBluescript type donor vectors. Thereafter, it is transferred to the T-DNA of a binary vector eg., a pZP vector. This is typically achieved by digestion with a restriction enzyme, the site for which is present in the T-DNAs of both the donor and the binary vector, followed by ligation of the compatible ends. The existence of similar restriction sites in the MCS of T-DNAs of both the donor vector and the acceptor binary vector is necessary. However, the key point is, such a restriction site must not occur in the *goi*/DNA fragment. The restriction site has to be unique. With increasing number of *goi* or DNA fragment to be examined and used, explorations for restriction enzymes with unique sites has become a challenge. Restriction enzymes with recognition sites of 8bp or longer (eg.,*Not*I, *Sfi*I, *Pac*I), the rare cutters, have come in vogue. Modular vector systems, pORE, have been developed with binary vectors sharing common unique restriction sites(Goderis *et al.* 2002, Coutu *et al.* 2007, Himmelbach *et al.* 2007). Three major pORE vectors are available, the *Open* series for gen-

eral plant transformation, *Reporter* series for promoter analysis and *Expression* series for transgene expression in plants. The T-DNA in all the three has some common elements. Thus, in the *Expression* series the *sequence*of elements in T-DNA from RB to LB is: --RB--Promoter for *goi*-- MCS--T_{NOS}--FRT--Promoter for Plant selectable marker --Plant selectable marker--T_{NOS} -- FRT-- LB--. The interesting feature is the FRT sequence, flanking the plant selectable marker cassette. This sequence is recognized by the FLP recombinase , encoded by the *flp* gene of the yeast 2 µm plasmid. Thus, after plant transformation, the marker gene and unnecessary sections can be removed by using the pORE-*Excision* plasmid which carries the *flp*gene in its T-DNA region (Gidoni *et al.* 2001). The MCS of pORE series contain around 10 to 20 unique restriction sites, providing a wide range for *goi*/DNA fragment insertion. The promoters, besides Cauliflower Mosaic Virus 35S gene promoter (CaMV35S) are from both dicot and monocot plants eg., P_{HPL}, the *Arabidopsis thaliana* hydroperoxidelyase gene promoter and P_{TAPADH}, a *Triticum aestivum*, modified lipid transfer protein gene promoter. These promoters and the T_{NOS} sequence (transcription termination and polyadenylation signal from nopaline synthase gene) ensure optimal transgene expression in transformed plants.

Gateway system of Invitrogen has made the task simpler. Instead of the lengthy digestion-ligation protocol, this system is based on site specific DNA recombination mechanism of lambda (λ) phage. The Gateway technology is a simulation of the λ–phage lysogeny-lysis cycle. During lysogeny, integration of the phage DNA into *E. coli* DNA occurs through recombination between common homologous *att* sites (referred to as *attP* in phage and *attB* in bacteria) mediated by a mix of *att*- site recognizing λ–derived Integrase (Int) and *E.coli* derived Integration Host Factor (IHF), the BP clonase system. The lytic cycle, the excision of phage DNA from *E.coli* DNA, is mediated by, again an *att*-site recognizing λ–derived Excisionase (Xis), the LR clonase. For vector construction modified *att* sequences and clonase systems are used. A *goi*/DNA fragment flanked by *att* sequences is engineered into a pDoner vector with *att* sequence-flanked selectable markers by BP clonase mediated recombination. The resulting recombinants are cloned to make pEntry libraries. From a pEntry recombinant, the *goi*/DNA fragment is transferred to another vector, pDest (destination vector), again with *att*segment flanked selectable markers by LR clonase catalyzed recombination to yield Exp (expression clones). The *goi*/DNA fragment containing Exp recombinant clones are used mainly for functional plant genomic studies. The Gateway cassette usually consists of the sequence *attR1-cm-ccdB-attR2*(*attR1* and *attR2* = recombination sites, *cm*= Chloramphenicol resistance, *ccdB* = additional selectable marker to kill *E. coli* by inducing double stranded DNA breakage). The *goi*/DNA fragment of interest and the vectors T-DNA integration site/s are designed to be flanked by such homologous sequences. This technology, as mentioned, enables rapid and easy transfer of a *goi*/DNA fragment from an "entry" cloning vector to a "destination" binary vector sharing the common Gateway cassette sequence (Hartley *et al.* 2000, Curtis and Grossniklaus 2003, Karimi *et al.* 2007). This system has also been used for transgene stacking (Tzfira *et al.* 2005, Chen *et al.* 2006). The gateway system is a breakthrough for high-throughput screening in an era of a gradual shift from forward genetics to reverse genetics investigations.While forward genetics seeks to find the genetic basis of a phenotype or trait, reverse genetics seeks to find what phenotypes arise as a result of particular genetic sequences. Recently a set of pANIC(after *Panicum virgatum*, switchgrass) gateway"destination" vectors have been designed for functional genomic studies in monocots. The main uses are in overexpression of a target gene or its down regulation (withRNAi). All such pANIC binary vectors have a pZP derivative skeleton with Gateway compatible cassette, plant selectable marker (eg., *OsAct1*/*bar*cassette,) and reporter gene cassette (eg., *PvUbi1*/*gus*Plus) besides, suitable *ori*, monocot promoters (eg.,*ZmUb1*) and other necessary elements. These vectors are highly suitable for high-throughput screening of transgenes in monocots.

Transformation efficiency and superbinary vec-

tors : Superbinary vectors were developed to enhance the efficiency of plant transformation and expand the host range for *goi*. These binary vectors, have certain *vir* genes as, *virB, virE* and *virG* added. These *vir* genes enhance transfer efficiency in higher doses. Superbinary vectors are constructed by homologous recombination between an acceptor vector containing the mentioned *vir* genes and an intermediate vector containing the T-DNA with the *goi*, with both sharing a homologous sequence eg., *-Cos-ColE1-Bom-* sequence (*Cos* = *cos* site for lambda phage, *ColE1*= replication origin of *E. coli, Bom* = plasmid transfer of *ColE1*) for recombination (Jin *et al.* 1987, Srivatanakul *et al.* 2000, Wu *et al.* 2008)

As mentioned above, replication origins as IncP which yield low copy numbers, are not suitable for transfer of the usual *goi*/DNA fragments of short length. The wider interest in genomics and applications of plant transformation technology however, demand capabilities for transfer of large DNA fragments (50 kb or more) and multigene transfers. In such cases, use of low copy replication functions of for instance,IncP, F factor and R1 plasmid in binary vectors aseg.,pBIBAC have shown some success (Hamilton 1997).

Removal of unnecessary selectable genes and vector DNA after transformation: After transgene transfer, certain regions of the vector (selectable and reporter genes) become unnecessary and can cause disruptions in the native plant genes. These also include, rearranged or extra T-DNA sections and certain vector backbone sections. It is preferable to remove the selectable markers also for cleaner transformation. This can be achieved by co-transformation (Komari *et al.* 1996), site specific recombinases(Ow 2001) or placing additional LB sequences near the native LB sequence (Kuraya *et al.* 2004). The pGreen vector system was developed with this objective (Hellens *et al.* 2000b). In this vector series, selectable marker genes or reporter genes can be located in the LB or RB sequences without affecting the efficient MCS derived from pBluescript series. pGreen replicates in *E coli* by itself , but, in *Agrobacterium* needs pSoup vector which provides replication function in *trans*mode, constituting a pGreen/pSoup dual binary system. By reducing further unnecessary elements of T-DNA and vector backbone of pGreen and pSoup vectors, pCLEAN-G and pCLEAN-S vectors were developed and used in various combinations of dual binary system, for stable and transient transformations in tobacco (*Nicotiana benthamiana*) and rice (*Oryza sativa*) (Thole *et al.* 2007). The pCLEAN series has advantages of shorter T-DNA, allowing multi transgene delivery and marker removal from the progeny.

INTRAGENIC VECTORS WITH PLANT DERIVED REGULATORY SEQUENCES: Crop improvement through plant genetic engineering, utilizing vectors with mainly plant derived genetic elements; the Intragenic approach is a recent milestone effort for mainly modification of native genes of a plant.DNA sequence elements of plant origin have been used as LB and RB of T-DNA to develop plant transfer DNA, the P-DNA. Such P-DNA is transferred into host plants by a suitable marker free system or with plant derived markers. For instance, a P-DNA was constructed from a 1.6 kb fragment isolated from a gene pool of wild potato, reduced to a 0.4 kb, containing several restriction sites and St01 borders flanking the strand (Rommens *et al.* 2004). A common intagenic vector pSIM371, ca. 13kb long has P-DNA and, in its backbone contains *ipt* (isopentenyl phosphor transferase, the backbone integration marker gene), *aadA*(spectinomycin resistance gene), pBR322 *ori* and pVS1 *ori*. Vector construct scan be designed with plant promoters as potato *Ubiquitin3* and *Ubi7*and terminators as of potato *Ubi3*gene (Garbarino and Belknap 1994). Similarly, selectable markers of plant origin are also available as, *Arabidopsis* Acetohydroxy acid synthase gene conferring resistance to chlorsulfuron (Conner *et al.*2007), *Arabidopsis* chloroplasticprotoporphyrinogen I oxidase gene for acifluorfen herbicide resistance (Lermontova and Grimm 2000), spinach betaine aldehyde dehydrogenase gene (Daniell *et al.* 2001). With such plant derived expression/ supression cassettes activation of useful genes and down regulation of undesirable genes have been achieved followed by even marker free transformation protocols. Much work has

been done on potato, a major vegetable. Utilizing its diverse gene pool, many problems of a US potato variety, *Solanum tuberosum* var Ranger Russet, have been reduced and quality improvements introduced. Thus, resistance to black spot bruise , developed during commercial handling, was developed by down regulation of genes of polyphenol oxidase, Phosphorylase-L and prevention of rise in cold induced sweetening, due to accumulation of reducing sugars harmful for Type II diabetes, was obtained by suppression of starch associated R1 genes (Rommens *et al.* 2006). Simlarly, increased levels of Kaempferol, a potent antioxidant, was induced by, enhancement of phenylpropanoid pathway and partial suppression of anthocyanin biosynthes is in *Solanum tuberosum* var Bintje (Rommens *et al.* 2008a). The levels of acrylamide, a neurotoxin in french fries and potato chips has been reduced by reducing the amounts of its precursor asparagine by silencing the asparagine synthetase genes with native elements based silencing cassettes (Rommens *et al.* 2008b).A classic example is remaking of FlavrSavr tomato, the first commercial transgenic crop, with increased shelf life, which had to be withdrawn. The original GM crop was developed by silencing of its Polygalacturonidase gene (*Pg* gene) with antisense RNA. By the intragenic method, a GM tomato var 'Moneymaker' with similar increased shelf life has been made.The silencing was achieved using pSIM894 with P-DNA containing modified tomato derived Leo2 left and right borders and the *Pg* gene silencing cassettes in a pSIM series binary vector backbone (Rommens *et al.* 2005).

Several systems have been developed for marker free selection. In one method the host explants were co-cultivated with *Agrobacterium* containing a vector plasmid with the P-DNA cassette and another with transiently expressing *nptII* and *codA markers*. Then the plants were subjected to Kanamycin, the survivors were further selected for about a month in media with 5-fluorocytosine (*codA* gene encodes an enzyme converting it to 5-fluorouracil). The remaining transformed plants were marker free (Rommens *et al.* 2004). In another technique plants were incubated with *Agrobacterium* containing a plasmid vector with P-DNA cassette and *ipt* gene (encodes isopentenyl adenosine, natural cytokinin production). After the required period, plantlets of wild type phenotype were selected (Richael *et al.* 2008).

VECTORS FOR PARTICLE BOMBARDMENT/ BIOLISTICS PROTOCOL

Although, *Agrobacterium*-mediated transformation is mainly used for GM crop production,biolistics protocol is widely used for transient gene expression studies and chloroplast transformation.

The vector expression cassettes for particle bombardment/biolistic protocols have several interesting features. Minimal requirement for the T-DNA cassette is, to have the goi/DNA fragment flanked by $5^{/}$ end promoter segment and $3^{/}$ end terminator and polyadenylation sequences, besides a selectable and or reporter gene with expression elements. The gene cassettes can be derived from pUC or pBluescript vectors. Plant transformations with such linear gene cassettes, with as much of the vector backbone removed as possible, has been achieved (Fu *et al.*2000, Sandhu and Altpeter 2008, Jayaraj *et al* 2008). The vector backbone can be removed by using suitable restriction enzyme sites. Further, by bombardment with particles coated with different gene cassettes, multigene transfers have been accomplished, for conferring eg., pest resistance (Maqbool *et al.* 2001, Datta *et al.*2002) and modification of metabolic pathways (Datta *et al.* 2006). Large DNA fragments aligned in suitable vector backbones can also be transferred by biolistic protocol. Thus, a tandem array of kafirin storage protein genes, inserted in a Bacterial Artificial Chromosome (BAC) was successfully inserted in maize (Song *et al.* 2004). Attempts have also been made to introduce artificial chromosome constructs of plant origin, which can exist extra-chromosomally, without disrupting the native DNA and lead to expression of some trait. For instance a 19 kb centromeric sequence could be successfully inserted in maize (Carlson *et al.* 2007). Site specific integration (SSI) of a GOI/ DNA fragment into a target genome has also been achieved by utilizing eukaryote adapted forms of recombinase-site systems as, Cre-lox of P-bacteriophage or FLP-FRT of yeast (Ow 1996, Bischof and Basler 2008). Recombinase- specific

sites can be introduced into a target plant DNA by some transformation method. In such sites a DNA of interest, linked to the recombinase, can be inserted by recombination through another transformation step. Thus, *lox* sites can be introduced into a host plant DNA first, followed by retransformation with Cre-*goi* cassettes for insertion of the *goi* at the *lox* sites. There are several examples of the use of Cre-lox and FLP-FRT systems for plant transformation through biolistics (Choi *et al.* 2000, Radhakrishnan and Srivastava 2005, Djukanovic *et al.* 2006). Gateway compatible vectors have also been used in several cases.

Particle bombardment has been the major protocol for chloroplast transformation. This has been primarily done through biolistics, using cassettes with *goi* flanked by sequences homologous with some plastome sequences, for insertion of the *goi* by homologous recombination into the plastome (Maliga 2004, Grevich and Daniell 2005, Bock 2007, Zouhair *et al.* 2011).

GENOME EDITING – ZFNs AND CRISPR-CAS SYSTEMS:

In recent times successful site directed transformations have been achieved using zinc finger nucleases (ZFNs) and some other tools. Zinc fingers proteins (ZFPs) are protein transcription factors with zinc ion bound to their cysteine and histidine residues and bind to specific triplets in the DNA sequence. Thus ZFP arrays can identify a specific sequence of triplets in a chromosome. In a unique innovation of protein engineering, ZFP dimers were ligated to endonuclease domains to constitute the ZFN fusion proteins for inducing site specific recombinogenic double stranded breaks (DSBs) and repair (Puchta 2005). ZFNs can be designed to recognize a wide range of target sequences, and can be used for targeted gene addition, replacement and stacking in plants.

Thus, using a T-DNA construct with endochitinase recognizing ZFN and *pat* herbicide resistance gene, the latter could be integrated into an endochitinase sequence flanked section in some plants (Cai *et al.* 2008). Similarly, Shukla *et al.*(2005) enabled insertion of the *pat*, herbicide resistance gene into the IKP1 gene in maize by using a ZFN, with the ZF domain engineered to recognize partial IKP1 sequences. This led to the twin advantage of IKP1 promoter driven herbicide resistance and knockout of IKP1 which encodes inositol-1,3,4,5,6-penta- kisphosphate 2-kinase, an enzyme that catalyses the final step in phytate biosynthesis in maize seeds, an antinutrient and source of pollution. Using ZFN mediation, the acetolactate synthase genes (ALS SuRA and SuRB) of tobacco were modified to confer resistance to imidazolinone and sulphonylurea herbicides (Townsend *et al.*2009).

A major advance has been the use of CRISPR (prokaryotic clustered, regularly inter- spaced, short palindromic repeats) and CRISPR-associated protein (Cas) system as an effective tool for genome editing in various organisms including plants (Cong *et al.* 2013,Shan *et al.* 2013).Upadhyay and coworkers (Upadhyay*et al*.2013) used successfully the CRISPR-Cas system for editing the *inox* and *pds* genes of wheat (*Triticum aestivum*) and the pds gene of tobacco (*Nicotiana benthamiana*) to demonstrate RNA-guided genome editing in plants. Details of genome editing are beyond the scope of this review.

CONCLUSIONS

For meeting the challenges of climate change-induced vagaries of weather, improved plants which can withstand the environmental stresses are needed. Genetic engineering can certainly be of help in this effort (Banerji 2010).In this context,vector designing for efficient, preferably site specific and clean transfer of single and multigene cassettes is essential for production of acceptable biosafe GM crops. Development of intragenic vectors for plant transformation, and cisgenic systems, utilizing plant based sequences is an encouraging direction. Genome editing is an emerging field of non-transgenic gene modification. These innovations, however, depend also on availability of suitable genes for transfer as also necessary regulatory and other elements. India, with rich agro- and bio-diversity, is ideal for researches on screening of such genes and regulatory sequences.

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