An Overview of Genetic Transformation of Soybean

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1. Introduction

Soybean (*Glycine max* (L.) Merrill) is a model legume crop, widely grown in the world for human consumption or animal fodder. Moreover, soybeans have gained worldwide research interest in many public laboratories and industrial sectors. Soybean seeds contain protein, oil, carbohydrates, dietary fibers, vitamins, and minerals. For the last few decades the majority of research laboratories have been investigating genetic traits to improve the yield of protein or oil in soybean seeds through genetic engineering, thereby achieving improved quantity and quality of soybean seeds. Until now, most of the transformation experiments have implemented a single functional gene not multiple genes. Those agronomically and economically important traits affect the enhancement of grain quantity and quality [1]. However, the majority of agronomic and genetic traits such as complex metabolic, biological, and pharmaceutical pathways are polygenetic traits and are produced in a complex pathway. Therefore, those traits are encoded and regulated by a number of genes. In an attempt to study and manipulate those pathways, the transfer of multigene or large inserts into plants have been developed by multigene engineering technology and have also been involved in metabolic engineering. Several examples of multigene or large insert transfers have been reported such as the application of carotenogenic genes in rice, canola, and maize [2-4], and of polyunsaturated fatty acid and vitamin E genes in soybean and *Arabidopsis* [5-7]. Therefore, reliable systems for transforming large DNA fragments into plants make it feasible to introduce a natural gene cluster or a series of previously unlinked foreign genes into a single locus.

Over the last two decades, the transfer of DNA into plant cells has been achieved by using several methods. In soybeans, the most frequently employed plant genetic engineering methods are *Agrobacterium*-mediated transformation and particle bombardment. Both systems have successfully been used in genetic transformation of soybean. Since the initial reports of fertile transgenic soybean production [8-9], various efforts have been made to improve the transformation efficiency and to produce transgenic soybean. Particularly, the

preferred and reproducible transformation is the use of the cotyledonary node as a plant material, which is based on *Agrobacterium*-mediated gene transfer [10-12]. Nevertheless, new methods have been developed for more efficient soybean transformation. There still remain, however, many challenges for genotype- and tissue- specific independent transformation of soybean. This review provides an overview of historical efforts in developing and advancing soybean regeneration and transformation systems. In addition, recent advances and challenges in soybean transformation are discussed.

2. Different approaches for soybean transformation

In soybean transformation, two major methods are now widely utilized: *Agrobacterium-mediated* transformation of different explant tissues and particle bombardment. The *Agrobacterium*-mediated method, as a simple protocol, does not require any specific or expensive equipment. Moreover, this method usually produces single or low copy numbers of insertions with relatively rare rearrangement [13]. On the other hand, bombardment technique directly introduces desired genes into the target plant cell with small tungsten or gold particles [9]. The success of this approach critically depends upon the ability of the target tissue to proliferate as well as proper pre-cultures to make a target plant.
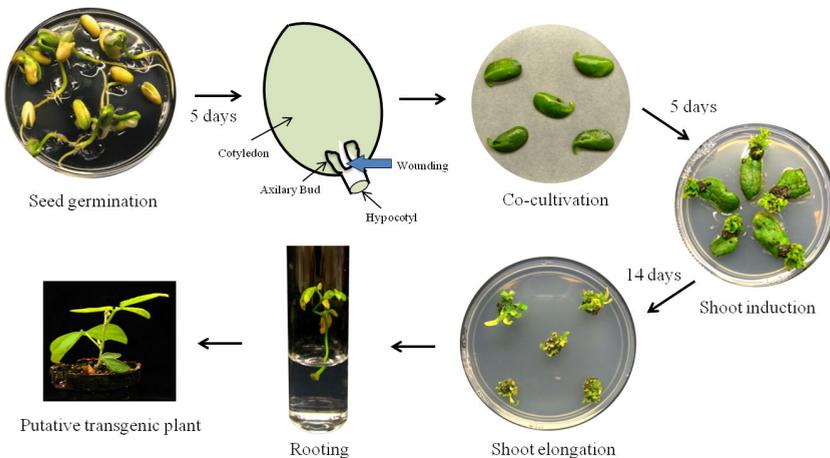
2.1. Cotyledonary-node-based transformation

The routine regeneration system was first reported by using the mature cotyledonary-node [14]. The multiple adventitious buds and shoots from explant tissues were proliferated and regenerated on culture media containing cytokinin by organogenesis. The transgenic soybean plants have been successfully and reproducibly produced using mature or immature cotyledon explants via *Agrobacterium*-mediated transformation. Hinchee et al. [8] for the first time reported the production of fertile transgenic soybean plants using mature cotyledonary-node by *Agrobacterium*-mediated transformation, but transformation efficiency was very low. The system employed the neomycin phosphotransferase II (*NPT* II) gene as a selectable marker and combined kanamycin as a selective agent. However, this selection was addressed with a problem of regeneration of non-transgenic or chimeric shoots at the shoot formation stage. Moreover, the system was highly genotype-dependent. To overcome the high genotype-dependency and high chimerism problems by the *NPT* II selection and develop a new selection system for soybean transformation, Zhang et al. [10] developed the selection system employing herbicide bialaphos resistance (*bar*) gene as a selectable marker coupled with glufosinate as a selective agent. This system enabled to transform many soybean genotypes with stable transgene inheritance, albeit transformation efficiency remained to be improved. Meanwhile, to solve the escape problem caused by kanamycin selection, Clemete et al. [15] deployed the herbicide glyphosate as a selective agent, leading to high stringent selection and good transgene inheritance. It was discovered later that addition of various thiol compounds in the co-cultivation medium significantly increased the transformation efficiency [11, 16-17]. These thiol compounds, as antioxidants, reduce the oxidative burst that caused tissue browning or necrosis and also promote organogenesis and shoot growth from buds [18].

Recently, an alternative cotyledonary explant derived from mature soybean seed for *Agrobacterium* transformation has been reported by Paz et al. [19]. The term half-seed explants were used as an experiment material and fertile transgenic plants were attained.

In fact, several laboratories have contributed to enhanced soybean transformation using a cotyledonary-node explant. To overcome the low transfer of *Agrobacterium* into plant cell, the infection media were first amended with the phenolic compound, 4'-Hydroxy-3',5'-dimethoxyacetophenone (acetosyringone), to induce expression of the virulence (*Vir*) genes [20-21]. To increase the infection sites, Trick and Finer [22] evaluated cotyledonary node transformation efficiency using a developed sonication assisted *Agrobacterium*-mediated transformation (SAAT) protocol. Although this treatment was not able to obtain fertile transgenic plants, the increase of *Agrobacterium* transfer was shown. Olhoft et al. [16-17] discovered that thiol compounds enhanced *Agrobacterium* infection in soybean. At the same time, however, these compounds caused counter-selection effect when glufosinate was used as a selective agent under previously published selection conditions. To solve this problem, Olhoft et al. [11] developed Hygromycin phosphotransferase (*HPT* II) selection system using hygromycin B as a selective agent. This has led to a substantial increase in transformation frequency. Transformation efficiency with thiol compounds was increased 5-fold by using refined glufosinate selection [12].

Since the transformation process by use of kanamycin or hygromycin B as selection agent has been proven to be genotype-dependent, the most widely used selection system has been the combination of *bar* gene with the herbicide phosphinothricin (glufosinate) [10, 12]. In this selection system, the concentration of agent glufosinate greatly affects the transformation frequency [12], so the appropriate selection schemes can be varied among genotypes, seed vigor and other *in vitro* culture conditions.

Figure 1. Scheme for genetic transformation of soybean (*Glycine max* (L.) Merrill) cotyledonary nodes.

2.2. Immature embryos-based transformation

The regeneration using immature embryos via somatic embryogenesis was first reported by Christianson et al. [23]. The immature embryos excised from soybean pods were suspended on semi-solid media or liquid media containing high concentration of auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D), and the whole plantlets were recovered [24-25]. After immature embryos were developed as an alternative plant material, transgenic plants were first obtained from this explant tissue via particle bombardment [26]. This system has been exclusively used to produce transgenic soybean such as glyphosate tolerant, hygromycin resistance, and *Bacillus thuringiensis* (BT) transgenic soybean [27-29]. As the formation of proliferative embryogenic tissue depends on genotype, the use of immature embryos for transformation has been limited to few genotypes cultivars including "Jack" and "Williams 82."

The use of particle bombardment with immature embryos tends to be highly variable, and multiple copies of the introduced DNAs are commons. Moreover, this problem has compounded with aged embryogenic suspension cultures from which a high percentage of regenerated plants lost their fertility [29]. In spite of this limitation, the embryogenic cultures have several advantages, one of which is its relatively high transformation efficiency and less chimeric plants recovered.

2.3. Embryogenic shoot tips-based transformation

The embryonic shoot tip explant is another source of explant which has been used for soybean transformation. McCabe et al., [30] first reported the stable transformation using meristemic cell, shoot apex, by particle acceleration. The shoot derived from these meristems via organogenesis has been produced to form multiple shoots prior to mature plants. However, all of the primary transgenic plants were chimeric. Martinell et al., [31] described the successful method using meristemic shoot tip from germinated seedling by *Agrobacterium*-mediated transformation. This system has provided rapid and efficient soybean transformation. Liu et al. [32] also reported the regeneration system using embryonic shoot tips by shoot organogenesis. The explants have been shown the high regeneration and the transformation efficiencies using *Agrobacterium*-mediated with up to 15.8%.

2.4. Immature cotyledonary-nodes

The regeneration capacity of immature cotyledonary-node was found by Parrott et al [33]. Based on this regeneration system, first transgenic soybean plants have been developed by *Agrobacterium tumefaciens* [34]. This system was tested using two different *Agrobacterium* strains, LBA4404 and EHA101 and deploying kanamycin selection. The system utilized auxin 1-Naphthaleneacetic acid (NAA) for plant regeneration. Although these systems allowed development of transgenic plants from the explants, no fertile transgenic plants were recovered. Recently, Ko et al [35] described the efficient transformation system using immature cotyledonary-nodes by *Agrobacterium*-mediated transformation, but transformation efficiency was still very low.

2.5. Hypocotyl based transformation

Another type of explant tissue, hypocotyl, was also investigated with 13 different soybean genotypes. Most of the genotypes initiated shoots from this type of explant [36]. This method was reported to be genotype-independent regeneration protocol via organogenesis and utilized the acropetal end of a hypocotyl section from a 7-day old seedling. Despite inducing adventitious shoots from the explant, most recovered shoot did not matured in the soil. Wang et al [37] reported successful production of fertile transgenic plants using hypocotyl-based *Agrobacterium*-mediated transformation. To improve the transformation system, two different chemicals, cytokinin hormone 6-Benzylaminopurine (BAP) and silver nitrate, were added to the shoot formation media. In spite of the term "hypocotyl" used in the above transformation system, the true tissues responsible for regeneration are actually the preexisting meristem tissues located at the nodal area of the cotyledon, essentially the same source of tissue as cotyledonary-nodes [17] except that cotyledons were removed [45, 46].

2.6. Leaf tissue-based transformation

The reproducible regeneration methods for whole plants from primary leaf tissue or epicotyls were first reported by Wright et al [38]. The multiple shoots from those explants were continually initiated and proliferated with cytokinin BAP hormone. Rajasckaren et al [39] described regeneration of several varieties of soybean by embryogenesis from epicotyls and primary leaf tissues, thereby inducing fertile plants from those explants. Kan et al [40] first tested transformation efficiency using epicotyls and leaf tissues by *Agrobacterium tumefaciens*. To find out proper transformation condition for those explants, they investigated different *Agrobacterium* strains, EHA101 and LBA4404, but also different treatments on inoculation stage, sucrose and mannose.

3. *Agrobacterium*-mediated transformation of soybean

3.1. *Agrobacterium*-mediated transformation mechanism

Agrobacterium is a unique organism to generate transgenic plants and in natural conditions [41]. It allows introduction of a single stranded copy of the bacterial transferred DNA (T-DNA) into a host cell and integration of the genomic DNA of interest, resulting in genetic manipulation of the host. Since the development of disarmed tumour-inducing (Ti) plasmid [42-43], *Agrobacterium* has been used to transform various major crops for genetic modification [44-46].

Agrobacterium recognizes wounded host plant cells which produce penolic compounds such as acetosyringone as inducers of *vir* gene expression [47], and attach to the plant cells to export the T-DNA after virulence (Vir) protein activation. Acetosyringone is now routinely used for improving transformation efficiency. After *vir* gene activation, a single stranded T-DNA copy (T-strand) is transferred into the plant by type IV secretion system (T4SS) which is related to VirB complex [48]. The VirB complex is composed of at least 12 proteins (VirB1-11 and VirD4) which form a multisubunit envelope-spanning structure [49]. Various

Agrobacterium proteins, such as VirD2-T-DNA, VirE2, VirE3, VirF, and VirD5, pass though VirB complex to transfer into plant cells [50-51]. VirE2 and VirD2 interact with cytosolic T-DNA in the plant cells and form a complex which is later imported into the nucleus when it is bound to VIP1 plant protein [52-55]. Recently, Gelvin et al., hypothesized that T-complex (T-DNA, VirE2, VirD2 and VIP1) is imported into the nucleus through actin cytoskeleton and thus myosin may be involved in *Agrobacterium*-mediated transformation [56]. However, the specific mechanism of T-DNA movement through myosin is still unknown.

The T-complex is imported into the nucleus by the phosphorylation of VirE2 Interacting Protein 1 (VIP1), induced by mitogen-activated protein kinase (MAPK), such as MPK3 [55]. After T-complex is imported into the host nucleus, VirE2 and VIP1 need to be degraded before T-DNA integration by a subunit of the SCF (SKP-CUL1-F-box protein) ubiquitin E3 ligase complex. Not only *Agrobacterium* protein VirF but also protein VBF can mark VIP1 protein for the degradation. Furthermore, binding of VIP1-binding F-box (VBF) to T-complex can induce the degradation of VIP1 and VirE2 by the 26S proteosome, and at the end T-strand is integrated into plant genomic DNA and expressed in the host plants [57-58].

3.2. History of *Agrobacterium*-mediated soybean transformation research

Among various transformation technologies, *Agrobacterium*-mediated transformation method has shown to be effective for the production of transgenic soybeans because of straightforward methodology, familiarity to researchers, minimal equipment cost, and reliable insertion of a single transgene or a low copy number [13]. Till now, a number of reports have been published related to the optimum condition to achieve a high yield of soybean transformation; such as *Agrobacterium* inoculation conditions, regeneration media components, etc. For *Agrobacterium*-mediated transformation methods, the susceptibility of soybean to *Agrobacterium* and various *Agrobacterium* strains have been tested to improve the transformation efficiency (Table 1). Also, *Agrobacterium* strains and growth conditions which affect the soybean transformation efficiency have been published [8, 59-62]. After Pederson et al., [46] and Owens et al., [59] showed the susceptibility of certain soybean genotypes against tumor induction, *Agrobacterium* biology study has been advanced to enhance transformation efficiency. In addition to *Agrobacterium* biology study, chemical contents for inoculation have been studied such as varying acetosyringone and syringaldehyde concentrations [63]. For high inoculation efficiency, Mauro et al., [64] tested various *Agrobacterium* biotypes (nopaline, agropine and octopine) to identify the most effective *Agrobacterium* biotype for soybean transformation.

After Hinchee et al., [8] developed *Agrobacterium*-mediated soybean transformation methods, many *Agrobacterium* strains have been tested and employed, such as EHA101, EHA105, LBA4404 and AGL1. Parrott et al., [33] showed that EHA101 was highly potent to transform immature soybean cotyledons, especially PI283332, and had higher recovery of transformed plants over LBA4404. Dang and Wei [65] tested transformation efficiency using embryonic tips instead of cotyledonary explants and somatic embryos, and when embryogenic tips were infected for 20 hours, hypervirulent strain KYRT1 showed increased efficiency over EHA105 and LBA4404.

Recently, *A. tumefaciens* KAT23 (AT96-6) which has an ability to efficiently transfer the T-DNA into soybean, was isolated from peach root. After 20 stains were confirmed by common bean and soybean transformation, Yukawa et al. [66] tested their potential availability as legume super virulent *A. tumefaciens* in various soybean cultivars (Peking, Suzuyutaka, Fayette, Enrei, Mikawashima, WaseMidori, Jack, Leculus, Morocco, Serena, Kentucky Wonder and Minidoka). Without modifying vectors or *vir* function, they showed that KAT23 (AT96-6) has a high potential to function as a common strain to increase soybean transformation efficiency. Therefore, this study identified a novel soybean super virulent *A. tumefaciens* strain which transferred not only the T-DNA of the Ti-plasmid but also introduced T-DNA of the binary vector efficiently. These results indicate that KAT23 (AT96-6) has the ability to transform soybeans at high efficiency.

There has been a significant improvement in soybean transformation over the past two decades. However, the efficiency of soybean transformation is not great enough for practical needs and shows high variation. Thus, considering the potential application of soybean transformation, the importance of *Agrobacterium* can't be over-emphasized.

Strain of *A. tumefacients*	Soybean genotype	Selection		Reference
		Marker	Agent	
A208	Peking, Maple Prest	*npt* II	kanamycin	(8)
AGL1	Bert	*bar*	phosphinothricin	(67)
EHA101	Williams 82	bar	glufosinate	(12)
EHA101	Williams, Williams 79, Peking, Thorne	bar	glufosinate or bialaphos	(68)
EHA101	Thorne, Williams, Williams 79, Williams 82	*bar*	glufosinate	(19)
EHA105	AC Colibri	*npt* II	kanamycin	(69)
EHA105	Hefeng 25, Dongnong 42, Heinong 37, Jilin 39, Jiyu 58	*hpt*	hygromycin	(70)
EHA105	A3237	*bar*	glufosinate	(10)
LBA4404	Jungery	*bar*	phosphinothricin	(71)
LBA4404, EHA105	Bert	*hpt*	hygromycin	(11)

Table 1. Summary of cotyledonary-node transformation system.

4. New directions of soybean genetic engineering, skills and vectors

To date, the *Agrobacterium*- and biolistic-mediated transformation methods remain the very successful methods in soybean transformation, whereas other available transformation technologies have not been practical in soybean, which include electroporation-mediated transformation [72], PEG/liposome-mediated transformation [73], silicon carbide-mediated

transformation [74], microinjection [75] and chloroplast-mediated transformation [76]. Of these two, *Agrobacterium*-mediated transformation has become more adapted in public laboratories worldwide. On the other hand, there are unintended insertions such as unwanted antibiotic markers and promoters, which can be inserted during transformation. This problem has raised potential biosafety issues related to environmental concerns and human health risks. To overcome these potential risks, methods of developing marker free transgenic plants have been developed, such as cotransformation [77], transposon-mediated transformation [78] and site-specific recombination [79].

Among the various methods, co-transformation system is one of the most commonly used methods to produce marker free transgenic plants. In co-transformation systems, a marker gene and genes of interest are placed on separate DNA molecules and introduced into plant genomes. Then, the non-selectable genes segregate from the marker gene in the progeny generations. Most strains of *A. tumefaciens* have the ability to contain more than one T-DNA, and crown gall tumors were often co-transformed with multiple T-DNAs [42]. As a result, there are two possibilities; Multiple T-DNAs were delivered into plant cells either from a mixture of strains ('mixture methods') or from a single strain ('single-strain methods'). Depicker et al. [80] described that a single strain method was higher in efficiency than a mixture method. For a single-strain method of co-transformation, Kamori et al., [77] tested co-transformation method to develop a suitable superbinary vector system. Using the unique plasmids which carried two T-DNA segments marker free rice and tobacco were produced and evaluated. LBA4404, a derivative of an octopine strain, were used for these co-transformation methods and they hypothesized that LBA4404 may be an important factor contributing to the high frequency of unlinked loci.

To improve plant genetic traits, many soybean research labs have developed tools for soybean functional genomics, such as several libraries containing large inserts of bacterial artificial chromosome (BAC) and plant transformation competent binary plasmids clone (BIBAC) (81). In functional genomic research, bacterial artificial chromosome (BAC) is a single copy artificial chromosome vector and is based on the *E. coli* fertility (F-factor) plasmid. They are not only stable in host cell, but also are used for large scale gene cloning and discovery [82]. However, some BAC libraries that are desirable for functional genomics are often not amenable for transformation directly into plants because of their large subclones. Therefore, binary bacterial artificial chromosome (BIBAC) libraries have been developed for *Agrobacterium*-mediated plant transformation and gene functional complementation. The BIBAC library is based on BAC vector and has both an F-factor plasmid for replication origin of *E.coli* and an Ri plasmid for replication origin of *Agrobacterium rhizogenes*. The vector also has a *sacB* gene as a positive selection for *E. coli* and a selectable marker gene for plant. Since BIBAC vectors were reported, these vectors have been used for plant transformation in some model plant species including tobacco, canola, tomato, and rice [83-86]. Although the transformation efficiency was very low, the BIBAC vectors have been successfully employed to transfer large inserts into those crops as a single locus via *Agrobacterium*-mediated transformation. The introduced T-DNA was stably maintained and inherited through several generations and no gene silencing was observed [85]. However, soybean transformation us-

ing a BIBAC vector has not been achieved to date. Moreover, plant transformation with DNA fragments below 20 kb is routine whereas the stable plant transformation with DNA fragments larger than 50 kb is challenging.

5. Zinc finger nucleases (ZFNs) and transcription activator-like effectors (TALEs)

Although many methods have been developed, soybean is considered a recalcitrant plant to transform compared to *Arabidopsis* and rice. Since full genome sequencing data has been rapidly updated in soybean, soybean transformation technology is becoming an essential approach for genomic research. For the phenotypic analysis of genes, knock-out or gene-silencing plants are used to study gene function. However in soybean, making bulk knock-out mutants through conventional mutagenesis approaches is not immediately feasible because of low transformation efficiency. Thus, development of innovative gene targeting methods is necessary to make knock-out plants in soybean.

Zinc finger nucleases (ZFNs) and meganucleases cut specific DNA target sequences *in vivo* and thus are powerful tools for genome modification. In particular zinc finger domain, which predominantly recognize nucleotide triplets, have been widely used in this research. Importantly, ZFNs modification has been reported in soybean (*Glycine max*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*) and *Arabidopsis* [87-90]. Unfortunately, Ramirez et al. [91] found a major disadvantage of ZFNs; they observed that the modular assembly method of engineering zinc-finger arrays has a higher failure rate than previously reported.

To overcome the ZFNs's weakness, in late 2009, a novel DNA binding domain was identified which was a member of the large transcription activator-like (TAL) effector family [92-93]. Transcription activator-like effectors (TALEs) are produced by plant pathogens in the genus *Xanthomonas* as virulence factors and TAL effector–mediated gene induction leads to plant developmental changes [94-95]. The type III secretion system is used by *Xanthomonas* to introduce virulence factors into plant cells [96]. Once inside the plant cell, transcription activator-like (TAL) effectors (TALEs) enter the nucleus, bind effector-specific DNA sequences, and transcriptionally activate gene expression [97-98]. For genomic engineering, two methods of TALEs were developed: TALE nucleases (or TALENs) and TALE transcription factors (or TALE-TFs). Both TALENs and TALE-TFs contain as many as 30 tandem repeats of a 33- to 35-amino-acid-sequence motif (Figure 2). The amino acids in positions 12 and 13 in each 33- to 35-amino-acid-sequence motif have the repeat variable di-residue (RVD). Using this specific ability, two pair (left and right TALENs) of repeats with different RVDs are designed by PCR and bound in the target DNA sequence [92-93]. Fok1 combined with TALE nucleases (or TALENs) make double-strand breaks (DSBs) at specific locations in the genome. These DSBs are repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ) pathways. During DSBs repair, errors in genome via insertion, deletion, or chromosomal rearrangement could be induced by HR and NHEJ (Figure 2). Unlike TALENs, TALE-TFs require only a single TALE construct for activity induction when com-

bined with VP64 activator (derived from the herpex simplex virus activation domain). VP64 binds with RNA polymerase and causes transcriptional activation of the gene of interest [99]. For making transgenic plants, the TALEs technique can be combined with the *Agrobacterium*-mediated transformation method and it is assumed that gene targeting knock-out will be applied in soybean research.
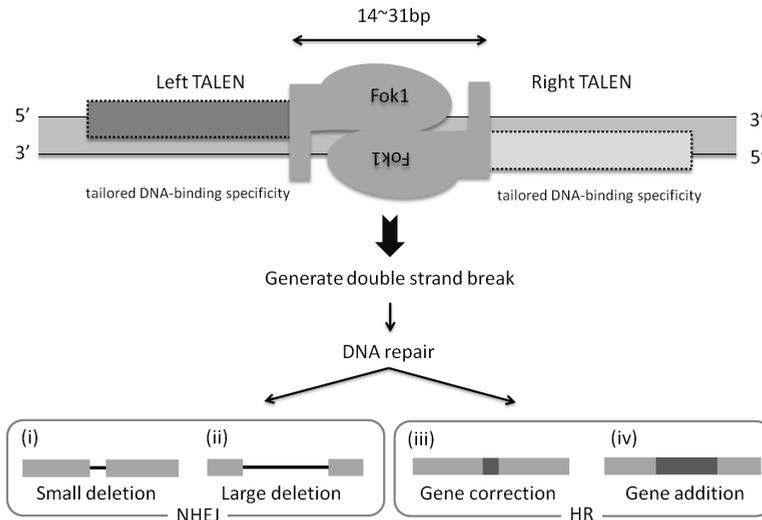
Figure 2. Summary of Transcription activator-like effectors (TALEs) nuclease.

6. Abbreviation

Acetosyringone: 4'-Hydroxy-3',5'-dimethoxyacetophenone

BAC: bacterial artificial chromosome

bar: bialaphos resistance

BAP: 6-Benzylaminopurine

2,4-D: 2,4-Dichlorophenoxyacetic acid

BIBAC: binary bacterial artificial chromosome

BT: *Bacillus thuringiensis*

DSBs: double-strand breaks

HPT II: Hygromycin phosphotransferase

HR: homologous recombination

NAA: Naphthaleneacetic acid

NHEJ: non-homologous end-joining

NPT II: neomycin phosphotransferase II

RVD: repeat variable di-residue

Ti plasmid: tumour-inducing (Ti) plasmid

T4SS: type IV secretion system

TALEs: transcription activator-like effectors

VBF: VIP1-binding F-box

VIP1: VirE2 Interacting Protein 1

Vir protein: virulence protein

ZFNs: Zinc finger nucleases

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