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Intragenic vectors for gene transfer without foreign DNA

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Abstract The intragenic vector system involves identifying functional equivalents of vector components from the genome of a specific crop species (or related species to which it can be hybridised) and using these DNA sequences to assemble vectors for transformation of that plant species. This system offers an attractive alternative to current genetic engineering strategies where vectors are based on DNA sequences that usually originate from bacteria. The construction of intragenic vectors enables the well-defined genetic improvement of plants with all transferred DNA originating from within the gene pool already available to plant breeders. In this manner genes can be introgressed into elite cultivars in a single step without linkage drag and without the incorporation of foreign DNA. The resulting plants are non-transgenic, although they are derived using the tools of molecular biology and plant transformation. The use of intragenic vectors for the transfer of genes from within the gene pools of crops may help to alleviate some of the major public concerns over the deployment of GM crops in agriculture, notably the ethical issue associated with the transfer of DNA across wide taxonomic boundaries. This paper reviews the progress toward the development and use of intragenic vectors and the implications of their use for the genetic improvement of crops.

Keywords *Agrobacterium*–mediated transformation · Cisgenics · Crop transformation · Direct DNA uptake · Intragenic vectors · Plant-derived vectors

Dedicated to the late Hans Sandbrink for his enthusiasm in helping to develop intragenic vectors

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Introduction

Over the past 20 years, scientific advances in molecular and cell biology have resulted in the development of technology to enable genetic engineering of plants. The resulting plants are usually referred to as transformed plants, transgenic plants or genetically modified (GM) plants.

GM technology offers new opportunities for the incorporation of genes into crop plants and holds the potential for the next level of genetic gain in crop breeding.

There has been considerable concern, especially among the general public and politicians, about the use of GM crops in agriculture (Conner et al. 2003; Nap et al. 2003). A main underlying issue involves transfer of genes across very wide taxonomic boundaries (Macer et al. 1991; Nuffield Council on Bioethics 1999). A key possibility of genetic engineering is the extension of the germplasm resources for crop improvement to any source of DNA (other plants, microbes, animals, or entirely synthetic genes) for transfer to plants. This is often perceived as ''playing God'' and has raised many ethical concerns, especially associated with food (Macer et al. 1991).

As the knowledge of plant genes and genomes develops, more genes are being identified from crop species which would be of benefit to agriculture and industry if they were transferred to genotypes of the same crop used in agriculture. The use of genetic engineering approaches for transferring genes between genotypes within the existing gene pools available to plant breeders has several advantages over traditional breeding (Conner and Jacobs 1999) such as:

1. The repeated transfer of new genes directly into existing cultivars or elite lines in plant breeding programmes without repeated backcrossing. This contributes to the more efficient development of new cultivars without many generations of hybridisation and selection to recover the desired plant. Gene transfer from related wild species by hybridisation may require up to 15–20 generations of additional plant breeding.
2. The transfer of single genes does not suffer from the potential linkage drag that is associated with the transfer of many undefined and possibly undesirable neighbouring genes in traditional plant breeding. The negative effects of such linkage drag are often more pronounced when chromosome fragments are introgressed from more distantly related germplasm sources.
3. The design and development of new gene configurations based on endogenous DNA sequences within plants. This may involve the new combinations of promoters and coding regions that target gene expression in plants at a specific time, location or in response to a specific environmental signal. In addition, gene silencing approaches such as antisense or RNAi can be used to down regulate or knock-out specific functions in plants.

Current research in plant genomics will deliver a new platform of knowledge for genetic improvement of crops. The annotated genome sequence of all major crops is likely to be available in the near future. It will allow for comprehensive searching in germplasm collections for novel alleles that represent variant versions of genes, or genes with altered functions. This will provide a source of DNA sequences for transfer via genetic engineering approaches from within the gene pools already utilised by plant breeders. The transfer of such genes, known as intragenic (Nielsen 2003), all-native (Rommens 2004) or cisgenic (Schouten et al. 2006a, b) transfers, will provide opportunities for highly targeted genetic changes in biochemical pathways of plants and the accumulation of specific metabolites for specific functions. Moreover, the transfer of genes between plants of the same species does not seem to raise similar ethical concerns in the GM debate as transfer of genes from unrelated species. Public opinion surveys have repeatedly found genetic engineering for gene transfer within species to be a more acceptable approach. This viewpoint has been consistent across societies throughout the world, from New Zealand (Gamble and Gunson 2002; Small 2004) to North America (Lusk and Sullivan 2002) and Europe (Schaart 2004).

However, current GM technology for transfer of genes within species still requires the use of components based on DNA from highly divergent species. Essential components of the vectors currently used are derived from bacterial systems, such as the T-DNA border regions, selectable markers genes and/or recombination sites for their subsequent removal, and the DNA into

which the gene-of-interest is cloned. To ensure public acceptance is retained when the intention is intragenic transfer of genes within plant species, gene transfer must be achieved without the presence of any DNA from 'foreign' sources. We have developed the concept of 'intragenic vectors' consisting of only plant-derived DNA fragments. It proved possible to identify DNA fragments from specific crop genomes with the functional equivalence of important vector components. This implies that for each crop amenable to GM, intragenic vector systems can be developed for plant transformation in which all the DNA destined for transfer originates from within the genome of the target crop. If 'transgenic' is defined as 'containing foreign DNA', intragenic vectors allow the development of 'non-transgenic' GM crops using all the standard tools of molecular biology and gene transfer. Such an approach allows targeted genetic improvement of crops without the introduction of foreign DNA. In this paper we review the progress toward the development and use of intragenic vectors and the implications of their use for the genetic improvement of crops.

Transformation vectors and gene transfer to plants

Vectors for *Agrobacterium*-mediated transformation

Agrobacterium strains capable of inducing crown gall or hairy root development on plants do so through a highly sophisticated form of parasitism. This is achieved by the transfer to plant cells of a discrete segment of DNA (transfer DNA or T-DNA) containing genes for inducing tumour or hairy root formation and opine biosynthesis. The T-DNA resides on the Ti or Ri plasmids, as do several virulence loci with key *vir* genes responsible for the transfer process (Gheysen et al. 1998; Gelvin 2003). The combined action of the *vir* genes and several other chromosomal based genes in *Agrobacterium* results in the transfer and integration of the T-DNA into the nuclear genome of plant cells. The T-DNA region is delimited by short imperfect direct repeats of about 25 bp known as the right and left border (Gheysen et al. 1998; Gelvin, 2003).

The tumour-inducing and hairy root-inducing genes encoded by the T-DNA of Ti and Ri plasmids are well known to prevent the regeneration of plants or result in plants with abnormal phenotype (Grant et al. 1991; Christey 2001). A key step toward *Agrobacterium*-mediated gene transfer to plants was the development of ''disarmed'' *Agrobacterium* strains through either the removal of the genes responsible for tumour formation or the complete removal of the T-DNA. These approaches led to the development of co-integrate and binary vector systems, respectively. Notably the latter have revolutionised gene transfer to plants. *Agrobacterium*-mediated transformation is the preferred method for gene transfer because it is driven by biological processes and results in a high frequency of single locus insertion events without rearrangements of the transferred DNA (Gheysen et al. 1998; Gelvin 2003). Furthermore, *Agrobacterium*-mediated transformation can effect the transfer of very large fragments (150–200 kb) into plant genomes (Miranda et al. 1992; Hamilton et al. 1996).

An important breakthrough for the development of binary vectors for *Agrobacterium*-mediated gene transfer was the finding that the T-DNA and the *vir* region could be separated onto two different plasmids (Hoekema et al. 1983; de Frammond et al. 1983). The *vir* genes on a Ti or Ri plasmid with the T-DNA region deleted (the helper plasmid) could act in *trans* to effect T-DNA processing and transfer to plant cells of a T-DNA on a second plasmid that is referred to as the binary vector. The main advantages of binary vectors are their relatively small size, ease of manipulation in *Escherichia coli*, high frequency of introduction into *Agrobacterium*, and their immediate use in any *Agrobacterium* strain with *vir* genes (Grant et al. 1991).

A wide range of binary vectors with versatile T-DNA regions are available to generate transgenic plants (van Engelen et al. 1995; Hellens et al. 2000). They contain alternative selectable marker genes for plant cells and/or alternative cloning regions with different series of unique restriction endonuclease sites. As a rule, such binary vectors also contain additional DNA elements as part of

the T-DNA region that are present for convenience rather than being necessary for the desired modification. Such DNA often includes an origin of replication and bacterial selectable marker gene, a *lacZ* cloning region for easier cloning, a reporter gene, and more. For the release of transgenic plants into agricultural production, such additional DNA regions either necessitate additional risk assessment or may be unacceptable to regulatory authorities (Nap et al. 2003). For the use of transgenic plants in agriculture, this led to the concept of 'minimal T-DNA vectors' with no unnecessary DNA segments as part of the T-DNA (Düring 1994; Barrell et al. 2002; Barrell and Conner 2006). These simple binary vectors contain the minimum features necessary for efficient plant transformation. They consist of a very small T-DNA with a selectable marker gene tightly inserted between the left and right T-DNA border and a short region with a series of unique restriction sites for inserting genes-of-interest.

Mechanism of gene transfer

The preferred event resulting from *Agrobacterium*-mediated gene transfer is the integration of a single intact T-DNA. The two 25 bp T-DNA border sequences delineate the T-DNA by defining the target site for the VirD1/VirD2 border specific endonucleases that initiate T-DNA processing (Gelvin 2003). This predominantly involves a single strand nick in the double-stranded T-DNA between the third and fourth nucleotide of the lower strand. After nicking of the border, the VirD protein remains covalently linked to the 5′ end of the resulting single-stranded T-DNA molecule that is referred to as T-strand (Gheysen et al. 1998; Gelvin 2003). This single-stranded T-strand is covered by Vir proteins and transferred to plant cells. The attachment of the VirD protein to the 5′ end of the T-strand at the right border rather than at the left border sequence establishes a polarity between the borders and determines the initiation and termination sites for T-strand formation.

The well-defined nature of T-strand initiation from the right border results in most instances in only 3–4 nucleotides of the right border being transferred to the transformed plant. Alignment of T-DNA border sequences from a diverse range of *Agrobacterium* strains reveals two strongly conserved motifs of 12–13 bp and 6–7 bp that flank a variable region of 5 bp (Table 1). The importance of these two conserved motifs for T-strand transfer has been confirmed by mutational analysis (van Haaren et al. 1988, 1989). By contrast, the end point of the T-DNA sequence transferred at the left border is far less precise and may occur at or about the left border, or even well beyond this sequence. This is shown by DNA sequencing across the junctions of T-DNA integration into plant genomes (Gheysen et al. 1998). As a consequence of the less precise end at the left border, vector backbone sequences integrate into plant genomes relatively frequently (Gelvin 2003). The frequency of such events has been reported as high as 46% (*Arabidopsis thaliana*; de Buck et al. 2000), 48% (barley; Lange et al. 2006), 75% (tobacco; Kononov et al. 1997), 90% (potato; Heeres et al. 2002) and 93% (rice; Kuraya et al. 2004). It can involve the entire binary vector (Wenck et al. 1997). Backbone sequences may integrate as a consequence of either read-through at the left border or from initiation of T-strand formation at the left border (Kuraya et al. 2004). Efficient transformation is possible with only a single border in the right border orientation. Deletion of the left border has minimal effect on T-DNA transfer, whereas deletion of the right border abolishes T-DNA transfer (Gheysen et al. 1998). Retaining two borders flanking the T-DNA helps to define both the initiation and end points of transfer, thereby facilitating the recovery of transformation events without vector backbone sequences.

The transfer of vector backbone sequences is considered to be an unavoidable consequence of the mechanism of *Agrobacterium*-mediated gene transfer (Gelvin 2003). However, it is possible to select against transformation events with such additional DNA sequences, either by identification following transformation (e.g. by PCR) or by targeted selection strategies. Inserting the barnase suicide gene into the vector backbone eliminates the recovery of plants expressing this gene and markedly reduces the frequency of transformed plants with unwanted vector backbone sequences (Hanson et al. 1999). Negative selection markers such as the cytosine deaminase

Table 1 Examples of *Agrobacterium* T-DNA border sequences

T-DNA border sequence	Source	Reference/Accession number
5′ttTGACAGGATATATTGGCGGGTAAACct3′	pTiC58 (RB)	AJ237588
5′ggTGGCAGGATATATTGTGGTGTAAACaa3′	pTiC58 (LB)	AJ237588
5′gaTGGCAGGATATATGCGGTTGTAATTca3′	pTi15955 (TR RB)	Barker et al. (1983); X00493
5′ggTGGCAGGATATATCGAGGTGTAAAAta3′	pTi15955 (TR LB)	Barker et al. (1983); X00493
5′acTGGCAGGATATATACCGTTGTAATTtg3′	pTi15955 (TL RB)	Barker et al. (1983); X00493
5′ggCGGCAGGATATATTCAATTGTAAATgg3′	pTi15955 (TL LB)	Barker et al. (1983); X00493
5′ttTGACAGGATATATTGGCGGGTAAACct3′	pTiT37 (RB)	Yadav et al. (1982)
5′ggTGGCAGGATATATTGTGGTGTAAACaa3′	pTiT37 (LB)	Yadav et al. (1982)
5′acTGGCAGGATATATACCGTTGTAATTtg3′	pTiAch5 (TL RB)	Holsters et al. (1983)
5′ggCGGCAGGATATATTCAATTGTAAATgg3′	pTiAch5 (TL LB)	Holsters et al. (1983)
5′ggCGGCAGGATATATTCAATTGTAAATgg3′	pTiA6 (LB)	Simpson et al. (1982)
5′ttTGACAGGATATATTGGCGGGTAAACct3′	pTi-SAKURA (RB)	Suzuki et al. (2000); AB016260
5′ggTGGCAGGATATATTGTGGTGTAAACaa3′	pTi-SAKURA (LB)	Suzuki et al. (2000); AB016260
5′taTGACAGGATTTATCGTTATGTCATGnn3′	pTiS4 (T1 RB)	Canaday et al. (1992)
5′ggCGGCAGGATATATTTAGTTGTAAAAnn3′	pTiS4 (T1 LB)	Canaday et al. (1992)
5′ctTGACAGGATATATGGTGATGTCACGnn3′	pTiS4 (T2 RB)	Canaday et al. (1992)
5′ggTGGCAGGATGTATTGTCATGTAAACnn3′	pTiS4 (T2 LB)	Canaday et al. (1992)
5′gtTGGCAGGATTTATTGCTAAGTCATCnn3′	pTiS4 (T3 RB)	Canaday et al. (1992)
5′gaTGGCAGGATATATCAAAGTGTAAGTnn3′	pTiS4 (T3 LB)	Canaday et al. (1992)
5′gcTGACAGGATATATACCGTTGTAATTcg3′	pTiTm4 (RB)	U83987
5′ggCGGCAGGATATATTGAATTGTAAATgt3′	pTiTm4 (LB)	U83987
5′acTGACAGGATATATGTTCCTGTCATGtt3′	pRiA4 (TL RB)	Slightom et al. (1986); K03313
5′ggTGGCAGGATATATTGTGATGTAAACag3′	pRiA4 (TL LB)	Slightom et al. (1986); K03313
5′tgTGACAGGATATATCTTGTGGTCAGGta3′	pRiA4 (TR RB)	Bouchez and Tourneur (1991); X51338
5′gcTGACAGGATATATTCCGTTGTCGGCta3′	pRi8196 (RB)	Hansen et al. (1991); M60490
5′ttTGACAGGATATATTCTAAAGTAATGtg3′	pRi1724 (RB)	Moriguchi et al. (2001); AP002086
5′ggTGGCAGGATATATTGTGGTGTAAACga3′	pRi1724 (LB)	Moriguchi et al. (2001); AP002086
5′ttTGACAGGATATATCCCCTTGTCTAGtt3′	pRi2659 (RB)	AJ271050
5′ggTGGCAGGATATATTGTGGTGTAAACga3′	pRi2659 (LB)	AJ271050

Border regions are presented in upper case letters with shaded sequences representing conserved motifs

(*cod*A) gene (Stougaard 1993) could accomplish the same result. Alternatively, a reporter gene such as *β*-glucuronidase placed outside the T-DNA can be used to allow the convenient recognition of plants in which vector backbone sequences have been integrated (Kuraya et al. 2004). An alternative approach has involved the use of an isopentenyl transferase gene for cytokinin production, which results in the regeneration of shoots with a typical stunted, pale green phenotype that fail to initiate roots (Rommens et al. 2004). However, these targeted selection strategies do not necessarily identify all partial backbone sequence integrations.

The intragenic vector system

The intragenic vector system is a major extension of the earlier minimal T-DNA vector system: it aims to present T-DNA vectors capable of effecting gene transfer to a given host plant, but using vectors that consist of only DNA that originates from the same crop species (or related species to which it can be hybridised). Meeting this aim involves identifying functional equivalents of vector components in plant genomes and using these DNA sequences to assemble vectors for plant transformation.

The three components minimally needed to assemble effective plant transformation vectors, apart from the gene-of-interest to accomplish the desired modification, are (1) a plant-derived T-DNA-like region that should contain two (or at least one) T-DNA border-like sequences in the correct orientation and a series of restriction sites suitable for cloning the gene(s)-of-interest intended for transfer; (2) an origin of replication and (3) a selectable element (usually an antibiotic

resistance gene), the latter two are both required to maintain the vector in both *E. coli* and *A. tumefaciens*. Approaches to obtain these essential components from a given crop genome are described below.

The P-DNA approach

The P-DNA (acronym for plant-DNA) method involves replacing the *Agrobacterium* T-DNA by plant-derived transfer DNA (P-DNA) (Rommens 2004). Using a series of border-specific degenerate primers, putative P-DNAs were isolated from pooled DNAs of 66 genetically diverse potato accessions by PCR (Rommens et al. 2004). The amplified fragments were sequenced and this information was used for inverse PCR with nested primers to determine the sequence of the border-like regions. This approach allowed the identification of a 391 bp fragment flanked by sequences with sufficient similarity to *Agrobacterium* T-DNA border sequences (Rommens et al. 2004). Following the insertion of a plant-expressed *npt*II gene into the P-DNA region placed on a binary vector backbone for proof of principle, the P-DNA region was effective for *Agrobacterium*-mediated transformation of potato.

Although this P-DNA is effective for potato transformation, the general presence of such P-DNA within the genomes of plants remains to be established. It requires the presence of a DNA fragment within the genome of the target crop species which has preferably two T-DNA border-like sequences oriented as direct repeats. Ideally these border-like sequences should be less than about 1–2 kb apart and span a sequence with restriction enzyme sites suitable for cloning the genes intended for transfer. The probability of finding such features on a single relatively short fragment in a plant genome is extremely small. The P-DNA strategy will therefore often require relaxing the sequence similarity to authentic T-DNA borders. This potentially compromises functionality, since many T-DNA border-like sequences found in plant genomes show reduced frequencies of gene transfer (Rommens et al. 2005).

Assembly of plant-derived T-DNA-like regions

An alternative approach for constructing plant-derived T-DNA regions involves adjoining two or more fragments from the same species (Baldwin et al. 2006). The shorter motifs of the T-DNA border sequences (Table 1) obviously occur in much higher frequency than a full length T-DNA-like border sequence. For example, non-exhaustive searches of plant EST databases revealed the presence of the longer conserved motif (5′GRCAGGATATAT3′) in numerous ESTs from over 80 species from diverse plant families, with the shorter motif (5′KSTMAWS3') being considerably more abundant (Baldwin et al. 2006). Consequently, plant genomes can be searched for DNA sequences containing these motifs and plant-derived T-DNA-like regions can be assembled by adjoining these sequences. In this manner we have assembled in silico vectors with plant-derived T-DNA-like regions for a wide range of plants. These include dicotyledonous species such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), petunia (*Petunia hybrida*), *Nicotiana benthamiana*, *Medicago truncatula*, and apple (*Malus x domestica*); monocotyledonous species such as rice (*Oryza sativa*) and onion (*Allium cepa*); and gymnosperms such as loblolly pine (*Pinus taeda*).

A 1066 nucleotide sequence for a T-DNA-like region derived from three petunia ESTs is illustrated in Fig. 1. We have constructed this T-DNA region and inserted it onto the backbone of the binary vector pART27 (Gleave 1992). The final binary vector contains two T-DNA border sequences, at least nine unique restriction sites between the borders, and petunia sequences that extend beyond both borders. The effectiveness of this intragenic binary vector for plant transformation was tested using marker-free *Agrobacterium*-mediated transformation (de Vetten et al. 2003) of petunia. Transformation was confirmed by PCR showing that the petunia plants resulted from the effective transformation with the petunia intragenic T-DNA-like region (Cooper et al. unpublished data).

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1     GTCGACTTTA TGATCCTGGC TATCTCAACA CAGCGCCTGT TCGGTCATCA ATATGTTATA
61    TAGATGGTGA TGCCGGGATC CTTAGGTATC GAGGTTACCC TATTGAAGAG CTGGCTGAGG
121   GAAGCTCCTT CTTGGAAGTG GCTTATCTTT TATTGTACGG TAATTTGCCA TCTGAGAACC
181   AGTTGGCAGA CTGTGAGTTC ACAGTTTCAC AACATTCAGC AGTTCCACAA GGACTCCTTG
241   GATATCATAC AGTCAATGCC CCATGATGCT CATCCGATGG GTGTTCTTGT CAGTGCAATG
301   AGCGCTCTTT CTGTCTTTCA CCCTGATGCC AATCCAGCTC TTAGGGGACA GGATATATAC
361   AAGTCTAAAC AAATGAGAGA TAAACAAATA GTCCGGATCG ATACGTGAAG ATCAAAATGA
421   AAAGGGGAGG CGATAGATTA GCAGCATGAG CCTATATTTC TCTCACAAAA ATTCCCAGAT
481   ATTCGACACA ATAGCTCTAA CAACACTGAG CTTTTGATTA CTTGGGTCAC TTCTTCATTT
541   CTCTATCGTC TGTTCAGTCT TTTCCTCTGA TTTAGTTTCT GCATCATAAG TTTTGCCAAA
601   GCCAAGTTCT GACATGTCTT GCTTTGCCAT CAAATTCTTC TCCATACGAC ACTCCAGGTA
661   CTTCCTAGAG AGGTGTCTAC ACTGCTCAGA TTTATGCCCA GCGGATTTTA GACAACTAAG
721   GTATTCCTTC TTCTCCACGT CACATAAATG CATGTGATCC AAAGGGAAAA CTCCTTTTTC
781   TGGTGGAACC GGTCTCAATC CTCTATTTCC ACCAAATGCT CCCCCTGCAC TCATTACGGA
841   GATGGCAGGA TATATGTTCT TGTCATGGAA TAGGCCACTG CTTTCAGCTG TCTGGAGACC
901   GTGAAGTGTA CGTTGAGCCA CAGCCCATTG TGCTTCCCTC TCACCTTTTC CGTAATCCTT
961   CTTGGTTGTG AAGGCAGTCT TATTCTGCAT CATTGATTGC CAGGCGTCAC CACTCAACGT
1021  GTAACGGCTG ATGAATTTAA GAATATCAAG AGGGAAATAG GTGATAATTG TCGAC
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Fig. 1 A T-DNA-like region assembled from *Petunia hybrida* (petunia) ESTs. All nucleotides are from the petunia genome, except those in italics added to both ends to complete a *Sal*I site (underlined). The sequence is composed of: the complete sequence of the 394 nucleotide fragment from sgn-e521144 (positions 6–399), the reverse complement of nucleotides 85–540 from sgn-e534315 (positions 400–855), and the reverse complement of nucleotides 121–336 from sgn-u207691 (positions 856–1071). The T-DNA border-like sequences are shown in bold. The left border is nucleotides 347–370 and the right border is nucleotides 844–867

A chimeric right T-DNA border

A more specific approach involves the creation of a chimeric right T-DNA border. The initiation of *Agrobacterium*-mediated transformation involves a nick between the 3rd and 4th nucleotide of the bottom strand of the right border, with the T-strand synthesised from the DNA 3′ to this nick. Consequently, the majority of the right border is not transferred to the recipient plant genome. In the context of the intragenics concept, only the first 3–4 nucleotides of the right border need to be of plant origin, whereas the remainder of the right border can be made identical to the authentic *Agrobacterium* borders.

With this chimeric right border approach, we constructed an intragenic T-DNA region from the genome of *A. thaliana* (Conner et al. 2004). BLAST searches identified a single T-DNA border-like sequence in the *A. thaliana* genome. This sequence, 5′GACAGGATATATCGTG-ATGTCAAC3′ (AL138652, nucleotides 60629–60606), is from chromosome 3 and is very similar to authentic T-DNA borders from *Agrobacterium* Ti or Ri plasmids, with all nucleotide substitutions occurring in variable regions (see Table 1). Using specific PCR primers, we fused an additional 23 nucleotides of the authentic right border of pTiT37 at the point where the inner 4 nucleotides of a T-DNA border already existed in *A. thaliana* (see Fig. 2) and ligated the fragment to the backbone of the binary vector pART27 (Gleave 1992). Four unique restriction sites (*Eco*RI, *Pvu*II, *Xba*I, *Nco*I), suitable for subsequent cloning of genes, exist between the right and left T-DNA borders in the resulting vector. A 5.8 kb *Xba*I fragment of *A. thaliana* DNA from pGH1, containing an acetohydroxyacid synthase gene conferring tolerance to chlorsulfuron (Haughn et al. 1988), was then inserted to allow selection with an endogenous *A. thaliana* gene (Fig. 3). The resulting vector was shown to generate transformed, chlorsulfuron-tolerant, intragenic *A. thaliana* using established transformation methods. As all DNA introduced in the *A. thaliana* genome is of plant origin, extra care has to be given to demonstrate actual transformation. Demonstrating the presence of the expected new junctions between DNA fragments not present in the original plant genome is the only way to definitively confirm effective transformation using an intragenic vector (Fig. 3).

Whole plant-derived vectors

Further vector components can be derived from intragenic sources to further minimise foreign DNA on vector backbones. We have constructed complete vectors from entirely plant DNA-derived sequences. Transformed plants derived from such vectors contain no foreign DNA regardless of whether transformation events

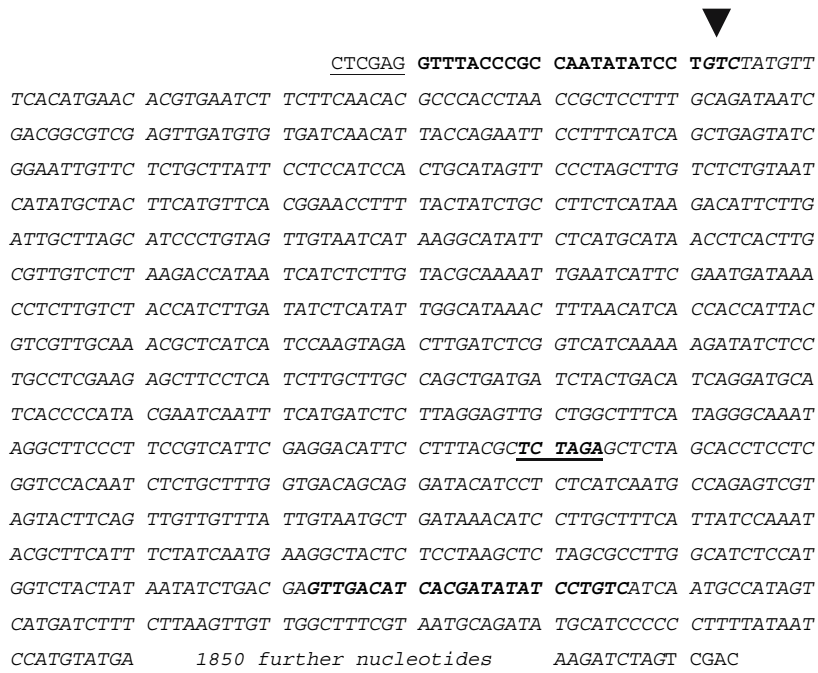

Fig. 2 An intragenic binary vector for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The 2838 nucleotide fragment from *A. thaliana* is illustrated in italics (nucleotides 59735–62572 of Genbank accession AL138652), with the T-DNA borders in bold and the unique *Xba*I site underlined in bold. The arrowhead at the top indicates the site of T-strand initiation for T-DNA transfer to plants. The 2864 nucleotide fragment bound by the *Xho*I and *Sal*I sites (underlined sites at each end) was ligated onto the 8004 nucleotide *Sal*I backbone of the binary vector pART27 (Gleave 1992) to give pTC1

extend beyond the T-DNA region. Plasmids derived entirely from plant sequences would also provide vectors suitable for direct DNA uptake using transformation approaches such as electroporation or biolistics.

Important vector components required for intragenic vectors to be derived from plant DNA sequences are (1) a bacterial origin of replication and (2) a selectable element to maintain the vector in bacterial systems. Functional equivalents of these components have been assembled from plant genomes by adjoining two or more fragments from the same species in a manner identical to the strategy as described above for T-DNA borders (Conner et al. 2005).

The smallest known prokaryotic origins of replication are the 32–33 bp ColE2 and ColE3 from the Colicin E2 and E3 plasmids found in *E. coli* and *Shigella* sp. (Del Solar et al. 1998). These replication origins require only one specific factor for replication (*Rep*) which can be provided from a helper plasmid (Yasueda et al. 1989; Shinohara and Itoh, 1996). The ColE origins of replication are characterised by two direct repeat sequences of 7–9 bp separated by 5–8 bp. BLAST searches of plant ESTs with sequences similar to ColE2 or ColE3 identified numerous ESTs from a diverse taxonomic range of species (Lokerse et al. 2006). Adjoining two sequences allows plant-derived bacterial origins of replication to be assembled for the propagation of plasmids in bacteria (Lokerse et al. 2006).

The smallest known bacterial selectable elements are based on repressor titration which requires the presence of a short non-expressed *lac* operator sequence on a plasmid to enable its selection and maintenance (Williams et al. 1998; Cranenburgh et al. 2001, 2004). This operator repressor titration (ORT) system utilises *E. coli* strain DH1*lacdapD*, which has a chromosomal conditionally essential gene (*dapD*) under the control of the *lac* operator/promoter system. Bacterial growth is only possible in either the presence of an inducer (e.g. IPTG) or of a

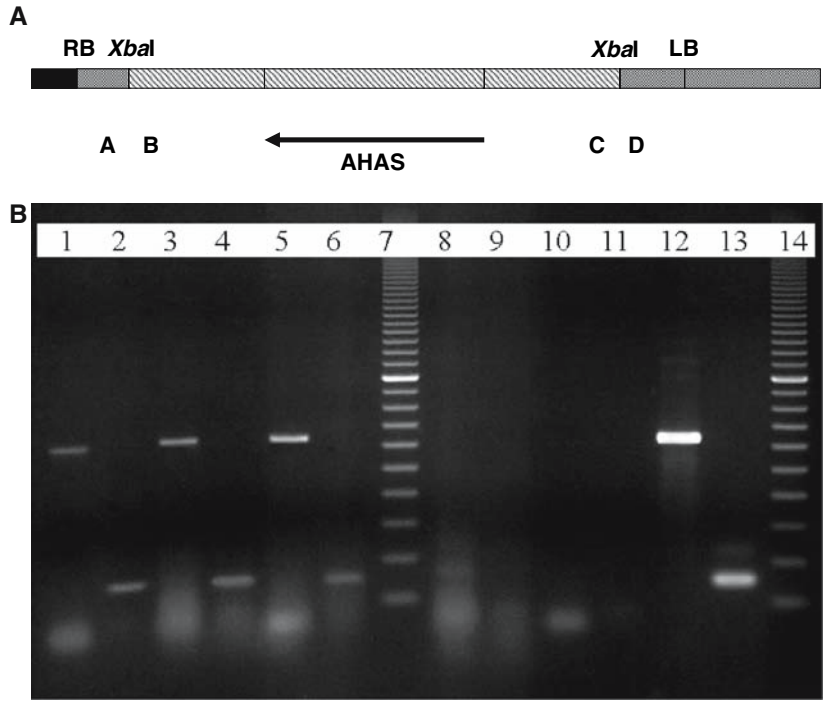

Fig. 3 *Arabidopsis thaliana* 'Columbia' transformed with the intragenic vector pTCAHAS. (**A**)The intragenic T-DNA region of the binary vector pTCAHAS schematically showing the T-DNA borders (RB and LB), *Xba*I sites, the AHAS gene, and the primer positions (A, B, C, and D). The black region represents part of the vector backbone and the two cross-hatched regions represent two different fragments of *A. thaliana* DNA. (**B**)PCR products using primers A + B and C + D. Lanes 1–2, 3–4, and 5–6 are from three independently derived *A. thaliana* lines transformed with the intragenic vector pTCAHAS; lanes 8–9 are from non-transformed *A. thaliana*; lanes 10–11 are no template controls; lanes 12–13 are the intragenic vector pTCAHAS. Lanes 1, 3, 5, 8, 10, and 12 are from using primers A + B and designed to amplify a 643 bp product from the intragenic vector T-DNA. Lanes 2, 4, 6, 9, 11, and 13 are from using primers C + D and designed to amplify a 149 bp product from the intragenic vector T-DNA. Lanes 7 and 14 are the 100 bp molecular ruler (170-8206, Bio-Rad Laboratories USA)

plasmid with a *lac* operator sequence. The higher copy number of the plasmid operator sequence titrates the repressor protein from the chromosomal operator and thereby allows *dapD* expression and bacterial growth. BLAST searches of plant ESTs with *lac* operator-like sequences identified numerous ESTs in a diverse taxonomic range of species (Lokerse et al. 2006). Plant-derived selectable elements based on the ORT system for the selection and maintenance of plasmids in bacteria can be assembled by adjoining two sequences.

Considerations for the proper design of intragenic vectors

When designing intragenic vectors based on sequences from plant genomes, it is important to consider which sequences should be preferred and which should be avoided. The DNA fragment making up the T-DNA should preferably not involve known regulatory elements such as promoters. The presence of such elements may have an unintended influence on expression of the inserted target genes. Furthermore, the DNA fragment on which the T-DNA is based should not be derived from heterochromatic regions (non-coding, non-expressed, condensed DNA) as this may suppress activity of the genes intended for transfer. Both of these limitations can be circumvented by assembling intragenic vectors from exons (coding regions) as found in EST repositories. For example, in the intragenic vector constructed from *A. thaliana* DNA, the T-DNA region (Fig. 2) is from an open reading frame for a putative protein of unknown function. There-

fore, it will not contain promoter elements and presumably is not from a heterochromatic region. Avoiding such DNA regions is conveniently achieved by selecting sequences resembling vector components from EST databases. Fortunately, the vast majority of DNA sequences for most crops are currently of EST origin.

It is also recommended that a significant length (1–2 kb) of intragenic DNA occurs outside the left border. This permits a tolerance towards truncations beyond the borders during T-DNA transfer without interfering with the concept of gene transfer without foreign DNA. When using ESTs, only short regions beyond the left border may be possible, unless another plant-derived fragment is incorporated into the vector. When other vector components such as origins of replication and/or selectable elements to maintain plasmids in bacterial systems can be derived from plant genomes, they can be used as an extension of the plant-derived T-DNA region, especially at the left border end.

The intragenic T-DNA designed for transfer back into a host plant should preferably be composed of a small number of DNA fragments. In this manner intragenic vectors mimic natural DNA rearrangement in plant genomes. Our preferred approach for designing intragenic T-DNA regions is illustrated in Fig. 1. Although vectors constructed in this manner are generally composed from components of three different ESTs, in many transformation events only the single (middle) fragment will be integrated upon transformation. Such transformation events will be more common when larger plant-derived T-DNA regions are constructed with insertions of genomic regions containing genes-of-interest from the target crop species.

An important component of plant transformation vectors are selectable marker genes. Such markers can also be derived from plant-derived sequences. Obvious candidates are mutant forms of the endogenous genes capable of conferring resistance to specific herbicides. Overexpression of the endogenous *Atwbc19* ABC transporter gene confers kanamycin resistance (Mentewab and Stewart 2005). For easy-to-transform crops such as potato, selectable marker genes are unnecessary (de Vetten et al. 2003). Such crops can be cultivated with *Agrobacterium* carrying an intragenic vector with an inserted gene-of-interest, followed by the regeneration of plants that are screened via PCR to find the transformants.

Concluding discussion

Intragenic vectors present a gene transfer system composed of only DNA that originates from that host plant species (or related species to which it can be hybridised). The construction of such plant-derived vectors for DNA transfer involves identifying functional equivalents of vector components in plant genomes to assemble vectors for transformation. Such vectors are capable of effecting gene transfer without the introduction of foreign DNA. Using this approach it is relatively easy to assemble T-DNA-like regions with functional equivalents of the T-DNA border sequences required for *Agrobacterium*-mediated gene transfer for a wide range of plant species. In a similar manner, functional equivalents of other important vector components can be identified from plant genomes, such as an origin of replication and a selectable element to maintain plasmids in bacterial systems. This way, it becomes possible to assemble complete vectors only from plant-derived sequences.

Gene transfer using intragenic vectors will facilitate the well-defined genetic improvement of plants with all transferred DNA originating from within the gene pool already available to plant breeders. In this manner, genes can be introgressed into elite cultivars in a single step without linkage drag and, most importantly, without the incorporation of any foreign DNA. The resulting plants are non-transgenic, although they are derived using the tools of molecular biology and plant transformation. This concept offers an alternative to current genetic engineering strategies in which vector systems are based on DNA sequences that originate mostly from bacterial species. The genetic make-up of the resulting intragenic plants should be considered as a minor rearrangement of endogenous DNA sequences within the species. This is no different from the spontaneous changes known as micro-

translocations that can occur naturally in plant genomes or as a consequence of deliberate mutation breeding (van Harten 1998). During the past 70 years, over 2,250 new crop cultivars have been released either directly following mutagenic treatment or from progeny of the mutagenised lines, 60% of which have been released since 1985 (Ahloowalia et al. 2004). The majority of these new cultivars (89%) were induced through radiation treatments. Due to the random nature of genome rearrangements by radiation, it is probable that other micro-translocations occurred in the genomes of these plants in addition to the selected mutation.

The application of intragenic vectors will provide a valuable breeding tool for crops. It will be especially useful for highly heterozygous crops (clonal and open pollinated populations) where it is virtually impossible to recover an existing cultivar with genes introgressed by traditional breeding. For the transfer of genes from within the gene pools of crop species (or related species to which it can be hybridised), intragenic vectors may help to alleviate some of the public concerns over the deployment of GM crops in agriculture. This applies especially to the ethical issues associated with the transfer of DNA sequences across wide taxonomic boundaries. Plants derived using intragenic vectors only resolve issues around the origin of DNA used for plant transformation. For crop improvement it will still be necessary to derive large populations of transformed plants in order to identify individual events with the desired attributes. Characteristic features of plant transformation such as position-effects, epigenetic influences, gene disruption and gene silencing, will still remain as important issues.

With gene transfer using intragenic vectors, there is no longer a clear biological distinction between traditional plant breeding approaches and development of GM crops. A complete continuum now exists of crop improvement technologies ranging from approaches used in traditional plant breeding for the past 50–60 years, to GM plants with entirely synthetic genes (Conner and Jacobs 2006). Defining a clear point of demarcation on which to base a legal definition of genetic modification, which has biological relevance and is enforceable, becomes very difficult. Theoretically, the same plants could arise from genome rearrangements derived from either induced mutations or intragenic vectors. Furthermore, since all the DNA sequences transferred are already present in existing crops, it is no longer possible to apply routine GM testing procedures based on the presence of foreign DNA. Gene transfer using intragenic vectors is therefore likely to present challenges for regulatory agencies by raising new issues concerning the definitions, regulation and testing of GM crops (Conner and Jacobs 2006).

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