Crop Improvement through Modification of the Plant's Own Genome

Caius M. Rommens*, Jaime M. Humara, Jingsong Ye, Hua Yan, Craig Richael, Lynda Zhang, Rachel Perry, and Kathleen Swords

J.R. Simplot Company, Simplot Plant Sciences, Boise, Idaho 83706

Plant genetic engineering has, until now, relied on the incorporation of foreign DNA into plant genomes. Public concern about the extent to which transgenic crops differ from their traditionally bred counterparts has resulted in molecular strategies and gene choices that limit, but not eliminate, the introduction of foreign DNA. Here, we demonstrate that a plant-derived (P-) DNA fragment can be used to replace the universally employed Agrobacterium transfer (T-) DNA. Marker-free P-DNAs are transferred to plant cell nuclei together with conventional T-DNAs carrying a selectable marker gene. By subsequently linking a positive selection for temporary marker gene expression to a negative selection against marker gene integration, 29% of derived regeneration events contain P-DNA insertions but lack any copies of the T-DNA. Further refinements are accomplished by employing Ω-mutated *virD2* and isopentenyl transferase cytokinin genes to impair T-DNA integration and select against backbone integration, respectively. The presented methods are used to produce hundreds of marker-free and backbone-free potato (*Solanum tuberosum*) plants displaying reduced expression of a tuber-specific polyphenol oxidase gene in potato. The modified plants represent the first example of genetically engineered plants that only contain native DNA.

Both the agronomic performance and nutritional characteristics of food crops can be enhanced by genetically modifying their genomes. Given the molecular complexity of plants, it is not surprising that most early transformation experiments were related to the introduction of viral and bacterial genes. These efforts resulted in the generation of plants displaying resistance against antibiotics, herbicides, viruses, bacteria, fungi, and insects (Shah et al., 1995). High-level expression of foreign genes was often secured with foreign regulatory elements such as the 35S promoters of cauliflower mosaic virus and figwort mosaic virus (Sanger et al., 1990) and the terminator of the Agrobacterium nopaline synthase gene. Additional foreign genetic elements often include the Agrobacterium transfer (T-) DNA and adjacent vector backbone sequences, which may be inadvertently cotransferred with the T-DNA (Kononov et al., 1997). Until now, the genetic engineering of plants has always resulted in the stable incorporation of genetic material that could not have been introgressed through traditional breeding. Transgenic plants approved for commercialization contain, on average, eight genetic elements derived from viruses, bacteria, or plants that are not sexually compatible with the target crop (www.agbios.com).

Public concern about the introduction of foreign DNA into food crops is at the center of many objections to transgenic plants (Gaskell et al., 2000; Wolfenbarger and Phifer, 2000; Verhoog, 2003). A recent United States consumer survey showed that although 81% of consumers would eat a vegetable with an extra gene from the same vegetable, only 14% would eat that vegetable if it had an extra gene from a virus (Lusk and Sullivan, 2002). Recently, it was proposed to categorize genetically modified organisms into different classes on the basis of the genetic distance between target organism and the source of new genetic variation (Nielsen, 2003). Plants that contain genetic material from within the same sexual compatibility group would be termed intragenic, whereas the crossing of species barriers would create famigenic or transgenic lines. The proposed terms would permit a more precise communication of the sources of genetic variability and may prove necessary to improve the public response to engineered organisms and their products (Nielsen, 2003).

Advances in plant molecular biology have greatly facilitated efforts to isolate plant genes associated with agronomically important traits (Pereira, 2000), thus providing alternatives to approaches that rely on genetic variation evolved in unrelated species. Application of methods such as promoter trapping and RNA fingerprinting has, likewise, resulted in the isolation of native regulatory elements that can now be exploited for the precise expression of desired traits (Meissner et al., 2000; Trindade et al., 2003). Furthermore, methods were developed to remove selectable marker genes from the genomes of transgenic plants. One of these methods exploits an inducible recombination system to excise a marker gene positioned between recombination sites (Zuo et al., 2001), while a second strategy relies on the segregation of independently integrated T-DNAs (Komari et al., 1996). Unfortunately, marker-removal

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421

methods are often too immature and inefficient to allow their widespread use in product development (Konig, 2003), especially in asexually reproducing or vegetatively propagated crops.

Although it is also possible to transform plants by omitting a selection step and thus circumventing the need for marker genes, these methods are highly inefficient and demand wholesale screening of large populations of plants. In the case of potato (*Solanum tuberosum*), only 0.2% of regenerated shoots represent transgenic events under nonselective conditions, which can be boosted to a modest 4.5% using supervirulent Agrobacterium strains (De Vetten et al., 2003). Assuming the high costs associated with generating transgenic plants, the frequency of backbone vector insertion, and the large numbers of plants required to identify lines that are suitable for commercialization, low transformation frequencies may become cost-prohibitive.

Thus far, molecular strategies and gene choices may have limited, but not eliminated, the foreign DNA introduced into a plant. Here, we describe new and efficient Agrobacterium-based methods that utilize a plant-derived transfer DNA and a novel transient selection system to insert only native DNA into plants. These methods are applied to incorporate black spot bruise tolerance into potato.

RESULTS

Plant Border-Like Sequences

Methods to generate intragenic plants by transforming them with native DNA would require the use of functional plant analogs of the Agrobacterium T-DNA borders. The presence of such analogs is implied by the identification of border-like sequences in rice (*Oryza sativa*) and Arabidopsis, shown in Figure 1A. Putative transfer DNAs were isolated from potato by carrying out PCRs on pooled DNAs from 66 genetically diverse but sexually compatible potato accessions (core collection, U.S. Potato Genebank, Sturgeon Bay, WI) with a variety of border-specific degenerate primers. Amplified fragments were sequence analyzed, and the sequence of border-like regions was confirmed by performing inverse PCRs with nested primers.

One particularly interesting DNA fragment was delineated by regions that shared most homology with the left border of nopaline strains (21 of 25 bp) and the right border of octopine strains (22 of 25 bp) of Agrobacterium (Fig. 1B). Because this plant DNA (P-DNA) fragment also lacked any open reading frames and contained a high A/T content, which is believed to promote the DNA transfer process (Depicker et al., 2001), it was tested for its ability to support plant transformation.

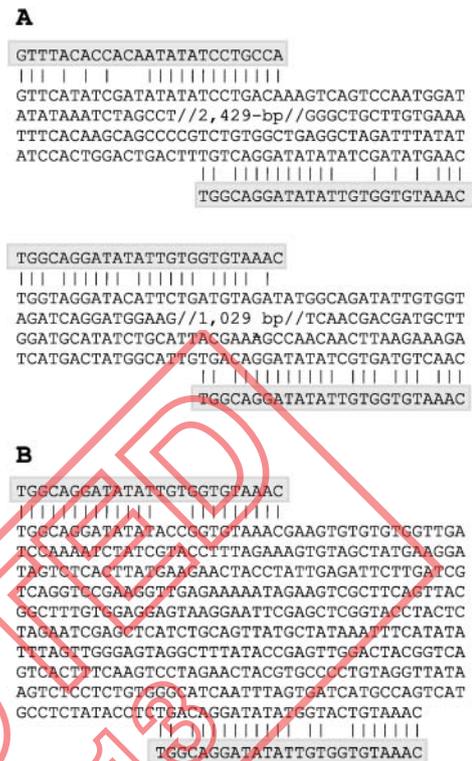To facilitate P-DNA functional tests, an expression cassette for the neomycin phosphotransferase (*nptII*)

Figure 1. Putative plant transfer DNAs. A, Both the inverted repeats flanking a 2.6-kb rice DNA fragment (GenBank accession no. AC097279) and the imperfect repeats delineating a 1.2-kb DNA fragment from Arabidopsis (NM114337) share homology with T-DNA borders, especially with the highlighted left border of Agrobacterium nopaline strains (shaded). B, Sequence of a 0.4-kb potato DNA fragment bordered by sequences that resemble the left border of Agrobacterium nopaline strains (shaded).

gene was placed within the P-DNA, and the resulting DNA segment inserted into a plasmid that can be maintained in both *Escherichia coli* and *Agrobacterium tumefaciens* (pSIM108). The conventional T-DNA vector pBI121, containing the identical *nptII* gene expression cassette, functioned as a transformation benchmark.

Agrobacterium LBA4404 strains carrying the two different vectors were used to infect tobacco (*Nicotiana tabacum*) and potato explants. Figure 2A shows that the average number of calli that developed on tobacco explants infected with pSIM108 was significantly greater than that for pBI121-infected explants. Likewise, the infection of potato stem explants with the P-DNA strain resulted in a greater frequency of regenerating shoots than with the strain carrying the T-DNA vector (Fig. 2B). Thus, the P-DNA supports an effective transfer of DNA from Agrobacterium binary plasmids to the genome of individual plant cells.

Development of a Transient Selection Method to Generate Marker-Free Plants

Given the low transformation frequencies of currently available marker-free transformation methods
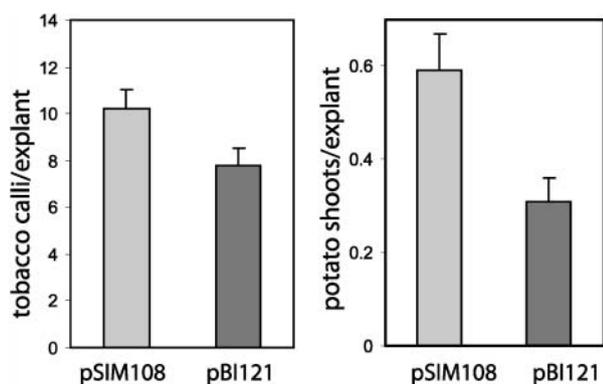
Figure 2. P-DNA mediated transformation. Left, Average number of calli that developed within 3 weeks of infection per tobacco stem explant. Right, Average number of shoots that arose from infected potato stem explants within 3 months of infection. Data are the mean ± SE of three experiments.

that omit a selection step, we hypothesized that intragenic plants might be produced more efficiently by developing a transient selection method. This hypothesis is based on the phenomenon that the co-transfer of two different DNA molecules from Agrobacterium to a single plant cell nucleus is not necessarily followed by the cointegration of both (De Buck et al., 2000). We assumed, as modeled in Figure 3, that plant cells could survive a temporary selection phase by virtue of the transient expression of a marker gene present in a conventional T-DNA. When selection is relieved, some cells would have the opportunity to integrate the P-DNA independently from the T-DNA molecule, thus offering marker-free P-DNA integration events.

Ideally, the transient selection phase would irreversibly arrest plant cell proliferation and regeneration to limit the frequency of escape. A suitable selection agent was identified by subjecting tobacco and potato explants for 5 d to a panel of phytotoxic chemicals, used at standard working concentrations for stable transformation procedures. Although explants that had been exposed to hygromycin, mannose, or cyanamide rapidly developed calli and shoots upon transfer to selection-free media, parallel explants did not recover from 5 d of kanamycin treatment (data not shown). Based on this result, the notion of a two-binary, marker-free P-DNA transformation system was tested with a conventional T-DNA vector carrying the *nptII* gene (Fig. 4A). This vector functioned as a life support (LifeSupport-1) for the cotransferred P-DNA. In all optimization experiments, the P-DNA vector contained all potato-derived DNA cassettes for both a modified polyphenol oxidase and a vacuolar invertase inhibitor gene.

Potato stem explants were simultaneously infected with two Agrobacterium strains, one carrying the marker-free P-DNA vector and the other containing the LifeSupport T-DNA vector. After a 2-d cocultivation period, the infected explants were subjected to a

5-d kanamycin selection period and transferred to selection-free media to promote plant cell proliferation and regeneration. As a control, explants were only infected with the strain carrying the P-DNA vector. Two months after initiation of the experiment, treated explants were analyzed for the presence of calli and shoots.

Verifying our pilot transient selection experiment, control explants infected with only the P-DNA vector failed to regenerate shoots. By contrast, explants infected with both the life support and P-DNA strains proliferated extensively and contained multiple shoots. A total of 500 shoots were transferred to hormone-free media to allow further growth and induce root formation. Three weeks later, the resulting plants were analyzed by PCR to determine the frequencies of the four different genotypes shown in Figure 3. This analysis resulted in the identification of 1% marker-free P-DNA integration events (Table I). Twenty-nine percent of the shoots lacked any stably integrated new DNA. Based on the results from the control experiment, these shoots were most likely not derived from plant cells that escaped the selection step but from individual cells that temporarily expressed, and then lost, a transferred T-DNA selectable marker. A much greater percentage (53%) only contained the T-DNA, whereas insertions of both elements were found in 17% of shoots.

Because the introduced P-DNA is comprised of genetic elements that are represented multiple times in the potato genome, the number of integration events was determined by repeating the experiment with a P-DNA carrying the β-glucuronidase (*gus*) gene.
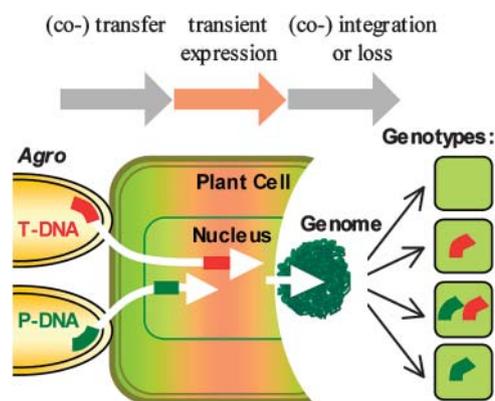

Figure 3. Hypothetical model for the use of markers to generate marker-free plants. By infecting explants with two different Agrobacterium strains, one containing a T-DNA vector with a selectable marker gene and the other one carrying a P-DNA vector, some plant cells may receive both a T-DNA (red bar) and a P-DNA (green bar). Only plant cells that at least temporarily express the marker gene will survive a subsequent transient selection step. Whereas some of these surviving cells contain a stably integrated T-DNA, others may have lost that T-DNA. Some of the latter group of plant cells may contain stable insertions of the P-DNA. The four different genotypes predicted to result from this coinfection are: P-DNA⁻/T-DNA⁻, P-DNA⁻/T-DNA⁺, P-DNA⁺/T-DNA⁺, and P-DNA⁺/T-DNA⁻.
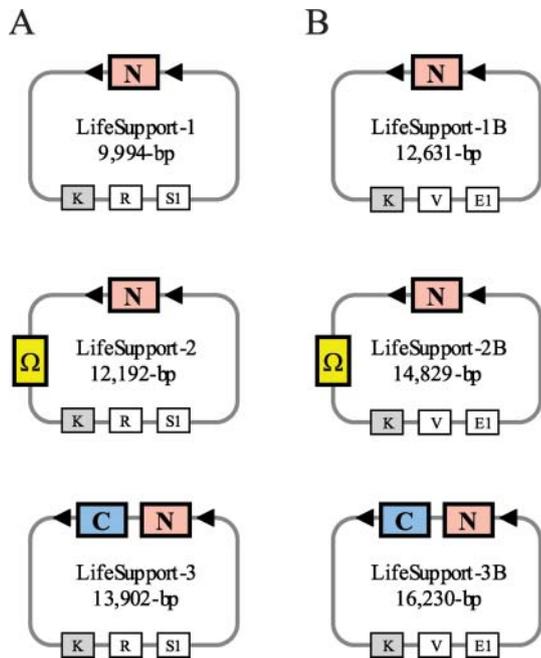
Figure 4. Schematic representation of LifeSupport vectors. A, Vectors used for two-strain approaches. B, Vectors used for the one-strain approach. Colored large boxes indicate expression cassettes for the *nptII* gene (N), Ω-mutated *virD2* gene (Ω), and *codA* gene (C). Small white boxes represent replication origins from pBR322 (R), pSV1 (S1), ori V (V), and ColE1 (E1). The small gray box indicates the *nptIII* kanamycin resistance gene.

DNA gel blot analysis of five transformed plants revealed the presence of one to three independent insertions (Fig. 5). Collectively, our data demonstrate that a transient selection system can be used to generate marker-free P-DNA plants. The relatively low P/− and high P/T genotype frequencies imply a strong linkage between cotransfer and cointegration.

Optimization of Two-Strain Marker-Free Transformation Procedures in Potato

Because an Ω-mutated virD2 protein had been shown to impair the integration of transferred T-DNAs (Shurvinton et al., 1992), we inserted an expression cassette for this modified gene into the backbone of the original LifeSupport vector. A strain carrying the resulting LifeSupport-2 (Fig. 4A) was used, together with the P-DNA vector, to coinfect potato explants. Genotype analysis of shoots demonstrated that 5% of events represented marker-free P-DNA plants (Table I). This 5-fold increase in desirable transformation events coincided, as expected, with a reduction in total T-DNA integration events (−/T plus P/T) from 70% for LifeSupport-1 to 40% in this experiment. Our results indicate the utility of the mutant virD2 protein in severing the link between cotransfer and cointegration of T-DNAs and P-DNAs.

An alternative strategy to generate marker-free P-DNAs more efficiently was based on the incorporation of a negative selection step for T-DNA integration. For this purpose, the cytosine deaminase (*codA*) negative selectable marker (Perera et al., 1993) was inserted next to the *nptII* gene between T-DNA borders, creating LifeSupport-3 (Fig. 4A). The standard transformation procedure was modified by transferring explants, 1 month after infection, to shoot induction media containing 5-fluorocytosine. PCR analysis of shoots demonstrated a dramatic decrease in overall T-DNA integration frequency to 20% (Table I). More importantly, addition of the negative selection step resulted in a 29-fold boost in the recovery of marker-free P-DNA plants.

Sequential Transformation

Until now, stem explants had been simultaneously infected with two Agrobacterium strains. We hypothesized that a sequential infection of explants might enhance the efficiency of marker-free transformation. To test this idea, explants were first infected with a P-DNA strain, incubated for 4 h (a 4-h window) on one-tenth Murashige and Skoog (MS) medium, infected again with a strain containing LifeSupport-1, and then treated in the same way as described above. Genotype analysis demonstrated that five of 55 shoots (9%) represented marker-free P-DNA shoots, whereas 18 shoots (33%) contained a T-DNA insertion. The frequency of T-DNA integration events was approximately the same using a 6-h window, but no shoots could be generated by extending the window to 24 h (data not shown). Our results demonstrate that a sequential infection with a short time window can aid the recovery of marker-free plants but also suggests that extending the time lag inhibits LifeSupport T-DNA transfer.

Table I. *Genotypes of shoots derived from coinfected potato explants*

T, *nptII*-containing T-DNA; P, P-DNA. Data are the mean ± SE of three independent experiments.

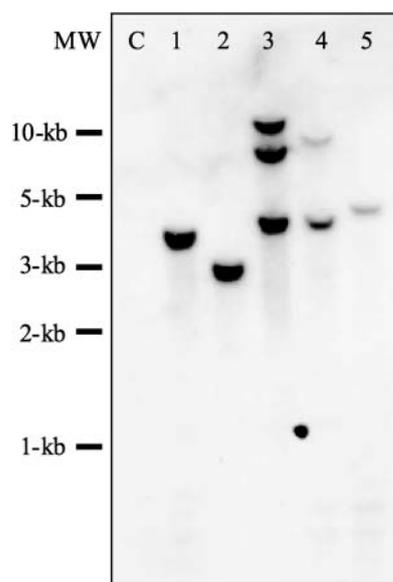| LifeSupport	T-DNA Gene(s)	Backbone Gene	Total Shoots	Genotypes			
				−/−	−/T	P/T	P/−
				%			
1	*nptII*	−	500	29 ± 4	53 ± 8	17 ± 2	1 ± 1
2	*nptII*	Ω*virD2*	105	55 ± 7	34 ± 4	6 ± 1	5 ± 1
3	*nptII* + *codA*	−	105	51 ± 5	10 ± 4	10 ± 1	29 ± 6

Figure 5. DNA gel blot analysis of marker-free P-DNA potato plants. Autoradiogram of a gel blot containing 10 μg EcoRI-digested DNA of an untransformed control plant (C) and five P-DNA plants, hybridized with a gus probe.

One-Strain Approach

Reasoning that the use of a single Agrobacterium strain would enhance cotransfer frequencies, we modified LifeSupport-1 so that it could be maintained with the P-DNA vector in the same Agrobacterium strain (see "Materials and Methods").

As shown in Table II, the infection of potato stems with a single strain carrying both the P-DNA vector and the resulting LifeSupport-1B yielded a high frequency (18%) of marker-free P-DNA shoots. Thus, the efficiency of generating marker-free P-DNA plants using a basic one-strain approach with LifeSupport-1B is about 18-fold higher than the efficiency that was accomplished with the corresponding LifeSupport-1 vector in a two-strain approach. By contrast, expression of the mutated virD2 protein in the strain containing a modified LifeSupport-2, designated Life-Support-2B (Fig. 4B), suppressed this frequency to only 2% (Table II). This is consistent with the prediction that expression of the virD2 mutant protein in a strain carrying both a T-DNA and a P-DNA impairs the integration of both DNAs and is therefore disadvantageous in a one-strain approach.

Marker-Free Transformation in Tobacco

Both two-strain and one-strain transformation approaches were also tested in tobacco. In these studies, LifeSupport-1 was used for the two-strain approach and LifeSupport-1B for the one-strain approach. Table III shows the genotypes of shoots as determined 2 months after transformation. Although marker-free transformation frequencies for the one-strain approach were similar to those determined for potato (19% versus 18%), we found that the two-strain approach was much more effective in tobacco (18% versus 1%). These results demonstrate that (1) the potato P-DNA is functionally active in tobacco and that (2) the two different approaches are about equally successful in generating marker-free tobacco plants.

Selection against Backbone Integration

Given the high levels of backbone integration in Solanaceous plants, we sought to develop a method to actively select against plants containing vector sequences. For this purpose, the Agrobacterium isopentenyl transferase (*ipt*) cytokinin gene was inserted into the backbone portion of pSIM108. Transformation of potato cells with the resulting vector yielded transgenic shoots that were grouped into two different classes. The first class of shoots was phenotypically indistinguishable from those transformed with an Agrobacterium strain carrying pSIM108, whereas the second class of shoots displayed an ipt phenotype, typified by plant stunting, small leaves, a light-green to yellow color, and an inability to root upon transfer to hormone-free media (Fig. 6). To confirm that transgenic shoots with an ipt phenotype contained the *ipt* gene, DNA was isolated from 193 shoots and used for a PCR with *ipt*-specific primers. Both genotypic and phenotypic analysis demonstrated that 72% of transgenic shoots contained the *ipt* gene. Analysis of 300 shoots derived from a tobacco transformation experiment showed a lower backbone integration of 59% in that crop. Thus, the *ipt* gene is a useful marker for the identification of undesirable backbone integration events. Our results also indicate that backbone integration frequencies for P-DNA vectors are similar to those for conventional T-DNA vectors (data not shown).

Table II. *Efficiencies of one-strain transformation approaches in potato*
Data are the mean ± SE of three independent experiments.

LifeSupport	T-DNA Gene(s)	Backbone Gene	Total Shoots	Genotypes			
				−/−	−/T	P/T	P/−
				%			
1B	*nptII*	−	91	52 ± 9	10 ± 3	20 ± 4	18 ± 1
2B	*nptII*	Ω*virD2*	105	85 ± 6	13 ± 4	0	2 ± 1

Table III. *P-DNA transformation efficiencies in tobacco*

Data are the mean ± SE of three independent experiments.

Approach	LifeSupport	T-DNA Gene(s)	Total Shoots	Genotypes			
				−/−	−/T	P/T	P/−
				%			
2-Strain	1	*nptII*	105	36 ± 9	26 ± 6	20 ± 4	18 ± 2
1-Strain	1B	*nptII*	105	16 ± 5	15 ± 4	50 ± 2	19 ± 6

Application of Marker-Free P-DNA Transformation Methods

Polyphenol oxidases (PPOs) play a role in the activation of defenses against pests and pathogens (Thipyapong and Steffens, 1997) but also trigger enzymatic browning, which negatively impacts potato storage and processing quality (Stevens and Davelaar, 1997). To control enzymatic browning without impairing inducible defense mechanisms, we isolated clones from a variety of potato cDNA libraries and selected the gene that is predominantly expressed in mature tubers, weakly expressed in microtubers, not expressed in leaves, and not induced upon infection (data not shown). Both a sense and an antisense copy of the unique 154-bp untranscribed trailer of this gene were placed between a GBSS promoter and the Ubi-3 terminator. The resulting all-potato expression cassette was placed within the P-DNA and introduced into a vector carrying the *ipt* backbone marker (Fig. 7A).

Potato explants were infected with a single strain carrying both this modified P-DNA vector and Life-Support-3B, which contains the *nptII* and *codA* genes inserted within the T-DNA (Fig. 4B). PCR analysis of 3,620 phenotype-negative (ipt⁻) plantlets obtained after application of the positive-negative selection system described above demonstrated that 221 individuals (6.1%) carried the P-DNA and lacked both the *nptII* and *ipt* genes. Subsequent DNA gel blot analysis

Figure 6. In vitro-grown transgenic potato shoots expressing the *ipt* gene. The phenotype of a pSIM108 control plant (left) is compared to those of four transgenic plants displaying an ipt-positive phenotype (right).

with probes immediately adjacent to the P-DNA revealed that about 12% of these plants still contained parts of the backbone (Fig. 7B; data not shown). The remaining 195 intragenic plants were transferred to soil and grown for 1 month in a plant growth chamber to allow tuber set.

Harvested minitubers were screened for PPO activity by pipetting catechol on cut surfaces. Compared to controls, visual browning of the tuber regions was reduced in about 90% of intragenic minitubers, with 75 lines appearing almost colorless (Fig. 8A). Some residual PPO activity, especially in the skin, indicates that nontargeted *PPO* genes still function effectively during this early stage of tuber development. Seven lines containing the PPO silencing construct were propagated and planted in the field. As expected, both the catechol assay and a spectrophotometric assay demonstrated greater than 85% control of enzymatic browning in the harvested mature field-grown tubers (Fig. 8, B and C; data not shown). This result was confirmed by abrading tubers and evaluating them after 24 h for discoloration. Our ability to generate black spot bruise-tolerant potato lines demonstrates the feasibility of genetic engineering approaches that rely on the transfer of only native DNA for crop improvement.
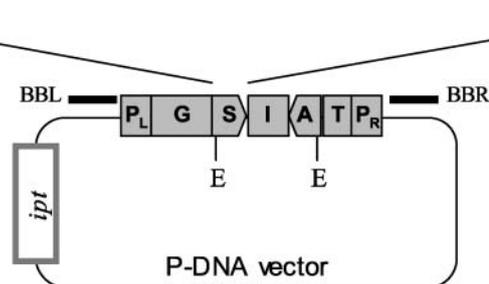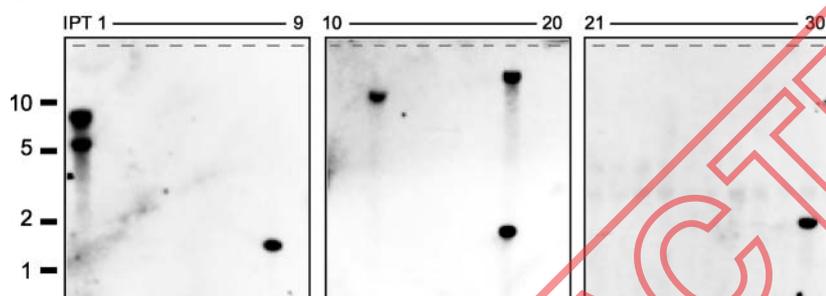
DISCUSSION

Plant-Derived Transfer DNAs

Replacement of the Agrobacterium T-DNA by a P-DNA fragment is a key aspect of the native DNA transformation approach described in this study. Because P-DNA transformation frequencies are higher than those for the nopaline strain-derived T-DNA of pBI121 in plants such as tobacco and potato, the unique borders of the P-DNA may be more effectively spliced by the endonuclease virD2. Alternatively, it is possible that the higher A/T content of the P-DNA in the regions adjacent to the borders (58% versus 50% for T-DNA) may promote the DNA transfer process (Depicker et al., 2001). We anticipate that new P-DNA-like sequences will be identified through public and private plant genome sequencing efforts. Functional analyses of these candidates may further define sequence requirements and local nucleotide context important in Agrobacterium P-DNA mobilization. A previous study explored the requirements of

A

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TTAGTCTCTATTGAATCTGCTGAGATTACACTTTGATGGATGATGCTCTGTTTTTGTTTTCTT
GTTCTGTTTTTTCCTCTGTTGAAATCAGCTTTGTTGCTTGATTTCATTGAAGTTGTTATTCAA
GAATAAATCAGTTACAATTATGTTTGGG
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B

Figure 7. DNA gel blot analysis of intragenic potato plants. A, Schematic representation of the P-DNA vector. Expression cassette for a sense (S) and an antisense (A) copy of the trailer of a tuber-expressed *PPO* gene, separated by the intron (I) of the Ubi-7 gene, placed between the GBSS promoter (G) and Ubi-3 terminator (T), and inserted between the left (P_L) and right (P_R) part of the P-DNA. *ipt, ipt* gene expression cassette. The probes used for hybridization were derived from the vector backbone adjacent to the right P-DNA border (backbone-right; BBR) and left border (backbone-left; BBL). B, Autoradiogram of a gel blot containing 10 μg *Eco*RI-digested DNA of a ipt-positive control plant (IPT) and 30 intragenic P-DNA plants, hybridized with a mixed BBR/BBL probe.

T-DNA borders for DNA transfer, but results were inconclusive due to ambiguity of negative controls and lack of molecular confirmation (Van Haaren et al., 1989). While we show the sequence requirements for recognition by virD2 are not extremely tight, a comparison between P-DNA and T-DNA sequences indicates conservation of a 12-bp motif, GRCAG-GAT[A/G][T/G][A/G]T, which is implicated as a nick site for bacterial conjugative DNA transfer (Waters et al., 1991). It is not clear whether border-like sequences are involved in recombination in plants. Sequences flanking P-DNA and putative P-DNA borders described here do not share homology with known bacterial DNA (data not shown), suggesting that analyzed border-like elements do not represent ancient Agrobacterium infections.

Efficient Marker-Free Transformation

The described transformation methods make it possible to generate marker-free transgenic plants with the high frequencies that are required for commercial production. These methods rely on the discovery that a short kanamycin selection phase is as effective as a constant selection regime in arresting the development of wild-type tobacco or potato cells. The presented methods should be generally applicable to plant species amendable to Agrobacterium transformation, especially those not requiring somatic embryogenesis.

While use of transient marker gene expression in transformation is a novel strategy, transient expression of other kinds of genes has long facilitated protein

functional screening and promoter analysis (Tai et al., 1999; Thirkettle-Watts et al., 2003). We found a 5-d period adequate for temporary kanamycin selection in Solanaceous species but expect that shorter or longer periods may be equally effective. Modified procedures must neither promote the proliferation of uninfected plant cells nor force too much stable integration of the marker gene. A 1-month selection period, for instance, failed to consistently produce transgenic events due to a heavy bias toward the stable integration of the marker gene, which is subsequently lost if subjected to codA negative selection against the T-DNA (J.M. Humara, unpublished data). Given the known period of transient marker and reporter gene expression (Mysore et al., 1998), we believe that the temporary selection step should not last longer than about 8 d.

Because T-DNA integration can occur as soon as 2 d after explant infection (Mysore et al., 1998), some level of undesirable T-DNA integration may be inevitable. The application of a negative selection system for T-DNA integration is, therefore, highly advantageous. Here, we employed the bacterial *codA* gene, which had been shown previously to support the excision of transformation markers (Gleave et al., 1999). We also assessed the efficacy of a fusion between *codA* and the uracil phosphoribosyltransferase (*upp*) gene, which is known from mammalian cell studies to be more efficient than *codA* in converting 5-fluorocytosine into 5-fluorouracil (Tiraby et al., 1998). Although *codA::upp* enabled a tighter negative selection in our system, it did not promote higher marker-
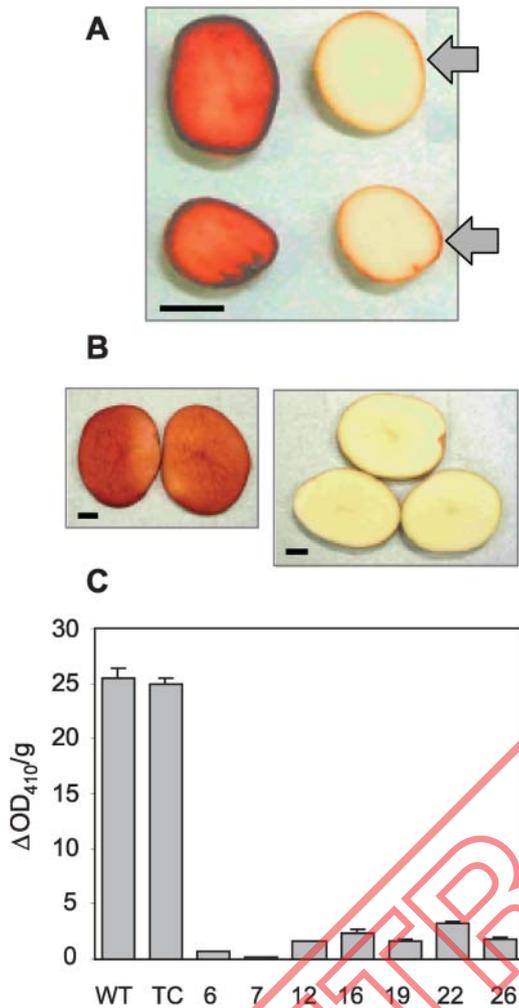
Figure 8. PPO activity in tubers. (A) phenotypes of sliced wild type (left) and intragenic (right) mini-tubers after catechol treatment. (B) phenotypes of treated wild type (left) and intragenic (right) field-grown tubers. Arrows show coloration of the epidermal region. Bar = 1 cm. (C) PPO enzyme activity in field-grown wild type (WT), transgenic control (TC), and seven silenced lines.

free transformation frequencies (data not shown). Alternative negative selection markers may include a bacterial cytochrome P450 monooxygenase gene (Koprek et al., 1999), the Xanthobacter *dhlA* gene (Naested et al., 1999), and various genes involved in the activation of apoptotic cell death.

In the two-strain method, the frequency of independent P-DNA integration in potato was also boosted by impeding T-DNA integration. This was accomplished by using a LifeSupport vector containing an Ω-mutated *virD2* gene, whereby Agrobacterium strains containing the vector would express both the mutant and wild-type virD2 protein. Association of the mutant protein with T-DNAs appears to have a negligible effect on T-DNA nicking and nuclear targeting but greatly impairs T-DNA integration into the plant genome (Shurvinton et al., 1992; Mysore et al., 1998), precisely the effect we needed. As expected, this

approach offered a lower level of T-DNA integration while increasing recovery of marker-free P-DNA plants 5-fold over the basic method. Use of the Ω mutation was only effective when the T-DNA was delivered in a separate Agrobacterium from the P-DNA. Adding the Ω-mutated *virD2* T-DNA with the desired P-DNA in a one-stain system caused dramatic reduction of P-DNA frequency to only 2%, presumably due to the presence of mutant virD2 protein interfering with all transfer DNA integration.

Efficacy of transformation in potato was further improved with delivery of the two binary vectors, the P-DNA and T-DNA LifeSupport, by a single strain of Agrobacterium. By introducing both the P-DNA and T-DNA vectors together, we saw an 18-fold increase in P-DNA integration frequency, without significantly increasing the frequency of cointegration. Interestingly, tobacco appears relatively indifferent to how DNA is delivered, offering high levels of P-DNA integration using either the one- and two-strain approach. This species difference suggests the importance of testing both methods for efficacy in new crops.

Alternative Transformation Systems

Instead of marker-free transformation methods described here, it may be possible to generate intragenic plants by exploiting native markers. For instance, a Na+/H+ antiporter gene that was used to develop salt tolerance in transgenic plants (Shi et al., 2003) may be domesticated for transformation by selecting transformed cells on media containing high concentrations of sodium chloride. It may also be possible to use a betaine aldehyde dehydrogenase gene for conversion of betaine aldehyde to glycine betaine (Daniell et al., 2001) or optimize the functional activity of plant 5-enolpyruvylshikimate-3-phosphate synthase genes to provide glyphosate tolerance (Padgette et al., 1987). Plant-derived cytokinin genes, such as *PGA22*, *CKI1*, and *ESR1*, have been proposed as native screenable markers, but due to undesirable shooty phenotypes, these genes require excision or silencing in the final transgenic plant (Kakimoto, 1996; Banno et al., 2001; Sun et al., 2003).

In sexually reproducing plants, constant selection schemes may be applied for the stable integration of both P-DNAs and T-DNAs because the latter can be segregated out in subsequent progenies (Komari et al., 1996). However, tight linkage between most cointegrated DNAs limits the efficiency of this method, thus increasing the resources required to develop crops with new advantageous traits (Konig, 2003).

Backbone Markers

Because of the frequent infidelity of DNA transfer and our desire to limit introduction of foreign DNA, it was important to develop a method to select against P-DNA plants containing vector backbone sequences. We therefore inserted an expression cassette for the

bacterial *ipt* cytokinin gene into the backbone of our P-DNA vector. Other cytokinin genes, such as the Agrobacterium transzeatine synthase gene (Krall et al., 2002), could serve this same purpose. In our hands, this strategy has proven extremely useful in potato and tobacco, which have high backbone transfer rates. Rather than eliminating plants containing backbone, as is the case with the barnase suicide gene (Hanson et al., 1999), we identify the subpopulation containing ipt, which can be verified molecularly. In other plant systems, backbone integration is a significant issue, with frequencies of 45% to 67% reported in rice, tomato, and grape (Hanson et al., 1999; Kim et al., 2003). We believe the ipt system may provide a useful tool to monitor transfer frequencies in different crops, evaluate new P-DNA border-like sequences, and develop methods aimed at restricting vector backbone transfer.

Application of the marker-free transformation methods made it possible to generate introgenic plants displaying black spot bruise tolerance. As opposed to previous efforts that relied on silencing the family of homologous *PPO* genes (Bachem et al., 1994; Coetzer et al., 2001), our approach only targeted the *PPO* gene that is predominantly expressed in mature tubers. Tuber infection with the fungal pathogen *Phytophthora infestans* confirmed that this genetic modification did not increase disease susceptibility (C. Richael, unpublished data).

By applying the methods described here, we generated for the first time transformed potato plants containing only native DNA. The discovery of valuable plant genes has been expedited through extensive efforts in plant genomics. The all-native DNA transformation methods make it possible to exploit such genes without the need to incorporate foreign DNA into plants.

MATERIALS AND METHODS

BLAST Searches

Publicly available databases including those maintained by the National Center for Biotechnology Information and SANGER were searched for T-DNA border sequences using the Motif Alignment and Search Tool (Bailey and Gribskov, 1998) and advanced BLASTN (penalty for nucleotide mismatch = -1; expect $= 10^5$; Altschul et al., 1997).

Vector Construction

P-DNA Vectors

A T-DNA-free plasmid that can be maintained in *Escherichia coli* and *Agrobacterium tumefaciens* was generated by ligating a 5.9-kb *Sac*II-*Sph*I fragment of pCAMBIA1301 (CAMBIA, Canberra, Australia), carrying bacterial origins of replication from plasmids pVS1 and pBR322, and the *nptIII* gene for kanamycin resistance, with two fragments amplified from that same vector using the oligonucleotides pairs: 5′-CCG CGG TGA TCA CAG GCA GCA AC-3′ and 5′-AAG CTT CCA GCC AGC CAA CAG CTC CCC GAC-3′, and 5′-AAG CTT GGC TAC TAG TGC GAG ATC TCT AAG AGA AAA GAG CGT TTA-3′ and 5′-GCA TGC TCG AGA TAG GTG ACC ACA TAC AAA TGG ACG AAC GG-3′, respectively. A 0.4-kb potato (*Solanum tuberosum*) P-DNA fragment (deposited as GenBank accession no. AY566555) flanked by *Hin*dIII and *Spe*I sites was inserted into the corresponding sites of the resulting plasmid, and an expression cassette comprising the Agro-

bacterium *ipt* gene preceded by the Ubi-3 promoter (Garbarino and Belknap, 1994) and followed by the Ubi-3 terminator was introduced as a 2.6-kb *Sac*II fragment. The P-DNA vector pSIM108 contains the *nptII* gene, driven by the Ubi-7 promoter (Garbarino et al., 1992) and followed by the terminator of the yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase gene (GenBank accession no. V01292), positioned within the P-DNA region. Marker-free transformation procedures were optimized by using a P-DNA vector that contained both a 2.9-kb expression cassette for a nonfunctional *PPO* gene (Rommens et al., 2003) inserted between the promoter of the granule-bound starch synthase I (GBSS) gene (Rohde et al., 1990) and Ubi-3 terminator, and a 1.6-kb cassette consisting of a vacuolar invertase inhibitor gene (Rommens et al., 2003) located between the same regulatory elements. The P-DNA vector that was used to apply marker-free transformation methods contains an expression cassette for a sense and an antisense copy of the trailer of a tuber-expressed *PPO* gene, deposited as GenBank accession number AY566556.

LifeSupport Vectors

The basic LifeSupport-1 plasmid that was used for two-strain approaches was derived from pCAMBIA1301 by replacing all genetic elements within the T-DNA borders with a 3.2-kb *nptII* gene expression cassette. LifeSupport-2 contains a 2.2-kb DNA fragment from plasmid pCS45 (courtesy of Dr. Walt Ream, Oregon State University, Corvallis, OR), comprising the Ω-mutated *virD2* gene driven by the *virD* promoter and followed by the *virD* terminator, cloned into the *Sac*II site of the LifeSupport-1 backbone. LifeSupport-3 was derived from LifeSupport-1 by insertion of a 3.9-kb cassette consisting of the *codA* gene (InvivoGen, San Diego), positioned between Ubi-7 promoter and Ubi-3 terminator within T-DNA borders. The LifeSupport vectors that were used for one-strain approaches were generated by replacing the entire pCAMBIA plasmid backbone with the backbone region of the binary vector pBI121, which contains ColE1 and ori V origins of replication (GenBank accession no. AF485783).

Polymerase Chain Reaction

P-DNA was isolated by using a robust and reliable method, which is described basically elsewhere (Xin et al., 2003). This method greatly reduces the incidence of DNA cross-contamination by omitting the steps of freezing and grinding tissues in liquid nitrogen. The isolated DNA was used as a template for amplification reactions with Eppendorf HotMaster Taq DNA polymerase according to the manufacturer's recommendations (Brinkmann, NY). The quality of potato DNA was confirmed by first amplifying an endogenous *PPO* gene using the primers 5′-CGA ATT CAT GGC AAG CTT GTG CAA TAG-3′ and 5′-CGA ATT CTT AAC AAT CTG CAA GAC TGA TCG-3′. To ensure that amplified DNA was not derived from contaminating Agrobacterium cells, a second control experiment was performed with primers 5′-GAT GCG GAC ATG CTC GAT TCT C-3′ and 5′-GAT AGT AGT TGC CGA CTC CAT C-3′, which are specific for the Agrobacterium *virE2* gene.

To carry out inverse PCR, plant DNA digests (2 µg) were circularized for 1 h with DNA ligase. After removal of salts using the QIAquick PCR purification kit (Qiagen, Valencia, CA), portions of the resulting DNAs (200 ng) were used as templates for PCRs (30 cycles) with a first set of primers. The amplified products of these reactions (1 µL) were used as template in subsequent PCRs (35 cycles) with a set of nested primers.

Agrobacterium Transformation

Binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 cells as follows. Competent LB4404 cells (50 µL) were incubated for 5 min at 37°C in the presence of 1 µg of vector DNA, frozen for about 15 s in liquid nitrogen, and incubated again at 37°C for 5 min. After adding 1 mL of liquid broth, the treated cells were grown for 3 h at 28°C and plated on liquid broth/agar containing streptomycin (100 mg/L) and kanamycin (100 mg/L). The vector DNAs were then isolated from overnight cultures of individual LBA4404 colonies and examined by restriction analysis to confirm the presence of intact plasmid DNA.

Tobacco Transformation

A 10-fold dilution of an overnight-grown Agrobacterium culture was grown for 5 to 6 h, precipitated for 15 min at 2,800 rpm, washed with MS liquid medium (PhytoTechnology, Shawnee Mission, KS) supplemented with

Rommens et al.

Suc (3%, pH 5.7) and resuspended in the same medium to 0.2 OD_{600} (optical density at 600 nm). The suspension was then used to infect leaf explants of 4-week-old in vitro-grown tobacco (*Nicotiana tabacum*) plants. Infected tobacco explants were incubated for 2 d on coculture medium (one-tenth MS salts, 3% Suc, pH 5.7) containing 6 g/L agar at 25°C in a Percival growth chamber (16-h-light/8-h-dark photoperiod) and subsequently transferred to M401/agar (PhytoTechnology) medium containing timentin (150 mg/L) and kanamycin (100 mg/L).

Potato Transformation

Ten-fold dilutions of overnight-grown cultures were grown for 5 to 6 h, precipitated for 15 min at 2,800 rpm, washed with MS liquid medium (PhytoTechnology) supplemented with Suc (3%, pH 5.7), and resuspended in the same medium to 0.2 OD_{600} (one-strain approach) or 0.4 (two-strain approach). The resuspended cells were mixed and used to infect 0.4- to 0.6-mm internodal segments of the potato variety Ranger Russet. Infected stems were incubated for 2 d on coculture medium (one-tenth MS salts, 3% Suc, pH 5.7) containing 6 g/L agar at 22°C in a Percival growth chamber (16 h light) and subsequently transferred to callus induction medium (CIM; MS medium supplemented with 3% Suc 3, 2.5 mg/L of zeatin riboside, 0.1 mg/L of naphthalene acetic acid, and 6 g/L of agar) containing timentin (150 mg/L) and kanamycin (100 mg/L). To test P-DNAs, after 1 month of culture on CIM, explants were transferred to shoot induction medium (SIM; MS medium supplemented with 3% Suc, 2.5 mg/L of zeatin riboside, 0.3 mg/L of GA_3, and 6 g/L of agar) containing timentin and kanamycin (150 mg/L and 100 mg/L, respectively) until shoots arose. If a transient selection was applied, the kanamycin selection was maintained only during the first 5 d of culture on CIM, and then the stems were transferred to fresh CIM containing only timentin. Shoots arising at the end of regeneration period were isolated and developed in MS medium with 3% Suc, 6 g/L of agar, and timentin (150 mg/L).

Molecular Plant Analysis

The presence of the *ipt* gene in transgenic plants was determined by performing a PCR using the *ipt*-specific oligonucleotides 5'-GTC CAA CTT GCA CAG GAA AGA C-3', and 5'-CAT GGA TGA AAT ACT CCT GAG C-3'. The primer pair used to determine the presence of pBI121 vector backbone sequences is 5'-CGG TGT AAG TGA ACT GCA GTT GCC ATG-3' and 5'-CAT CGG CCT CAC TCA TGA GCA GAT TG-3'. The primer pair used to determine the presence of pSIM108 backbone sequences is 5'-CAC GCT AAG TGC CGG CCG TCC GAG-3' and 5'-TCC TAA TCG ACG GCG CAC CGG CTG-3'. Regenerated shoots were screened for the presence of both the GBSS promoter/*PPO* gene fusion and the *nptII* gene with the primer pairs 5'-GCC ACC CGC TAT TCT CTT GAC-3' and 5'-AGC GGA TGC AGC TAA TGG TAT AGC-3', and 5'-GAG CTC TCA GAA GAA CTC GT-3' and 5'-AAG TAT CCA TCA TGG CTG AT-3', respectively.

Blotting, hybridization, and autoradiography were performed as described previously (Rommens et al., 1992). Primers used to amplify 0.5-kb DNA fragments that were used to generate probes to visualize the presence of backbone DNA flanking the P-DNA were 5'-CAC AGG AAA GAC GAC GAC CG-3' and 5'-CCT CAG CCG AGG TTC ATT CC-3' (for BBL), and 5'-CCG TTC GTC CAT TTG TAT GTG GTC-3' and 5'-CGT AGG TGG TCA AGC ATC CTG-3' (for BBR). Primers used to amplify a 0.5-kb fragment and generate a gus probe were 5'-TTG TTT GCC TCC CTG CTG CG-3' and 5'-CTG CGA CGC TCA CAC CGA TA-3', whereas primers for the 0.5-kb *nptII* gene fragment were 5'-GGG TGG AGA GGC TAT TCG GCT ATG A-3' and 5'-ATT CGG CAA GCA GGC ATC GC-3'.

PPO Assays

Potato tubers were screened for reduced PPO activity by pipetting 0.5 mL of catechol (50 mM) on cut surfaces. The levels of enzyme activity were compared with wild-type levels by mixing pulverized tubers (1 g) for 1 h in 50 mM 3-(*N*-morpholino) propane-sulfonic acid buffer at pH 6.5 (5 mL). After precipitation of the solid fraction, the change of OD_{410} was determined over time. Abrasive peel assays were performed as described previously (Coetzer et al., 2001).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY566555 and AY566556.

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RETRACTIONS

Vol. 135: 421–431, 2004

Rommens C.M., Humara J.M., Ye J., Yan H., Richael C., Zhang L., Perry R., and Swords K.
Crop Improvement through Modification of the Plant's Own Genome.

This article has been retracted at the request of the authors. Retraction is based on three inaccurate statements of facts that are associated with a plant-derived transfer DNA. Two of the inaccuracies were described on p. 422 (first paragraph of the "Results" section): the plant-derived transfer DNA was not isolated from pooled wild potato (*Solanum tuberosum*) DNA but, instead, from DNA of the commercial potato var Ranger Russet, and its sequence was not confirmed by inverse PCR. Furthermore, the sequences of the left and right border-like regions shown in Figure 1B reflect transfer DNA-like primer sequences and are not present in the Ranger Russet genome. The corresponding author, Caius M. Rommens, takes responsibility for the inaccuracies and sincerely apologizes to the readers, reviewers, and editors of *Plant Physiology*.

ADDENDA

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Plant-Derived Transfer DNAs.

The border sequences of St01 and the vector pSIM108 described in this article's findings were based on data from an article by Rommens et al. (**Rommens CM, Bougri O, Yan H, Humara JM, Owen J, Swords K, Ye J** [2004] Crop improvement through modification of the plant's own genome. Plant Physiol **135:** 421–431), which has been retracted. Specifically, these sequences, which were presented in this and the preceding article as intragenic, reflect transfer DNA-like primer sequences that are synthetic and not present in the potato (*Solanum tuberosum*) genome.

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