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TECHNICAL ADVANCE

Modifying fatty acid profiles through a new cytokinin-based plastid transformation system

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SUMMARY

The widespread use of herbicides and antibiotics for selection of transgenic plants has not been very successful with regard to commercialization and public acceptance. Hence, alternative selection systems are required. In this study, we describe the use of *ipt*, the bacterial gene encoding the enzyme isopentenyl transferase from *Agrobacterium tumefaciens*, as a positive selectable marker for plastid transformation. A comparison between the traditional spectinomycin-based *aadA* selection system and the *ipt* selection system demonstrated that selection of transplastomic plants on medium lacking cytokinin was as effective as selection on medium containing spectinomycin. Proof of principle was demonstrated by transformation of the *kasIII* gene encoding 3-ketoacyl acyl carrier protein synthase III into tobacco plastids. Transplastomic tobacco plants were readily obtained using the *ipt* selection system, and were phenotypically normal despite over-expression of isopentenyl transferase. Over-expression of KASIII resulted in a significant increase in 16:0 fatty acid levels, and a significant decrease in the levels of 18:0 and 18:1 fatty acids. Our study demonstrates use of a novel positive plastid transformation system that may be used for selection of transplastomic plants without affecting the expression of transgenes within the integrated vector cassette or the resulting activity of the encoded protein. This system has the potential to be applied to monocots, which are typically not amenable to traditional antibiotic-based selection systems, and may be used in combination with a negative selectable marker as part of a two-step selection system to obtain homoplasmic plant lines.

Keywords: plastid transformation, cytokinin, fatty acids, *Nicotiana tabacum*, technical advance.

INTRODUCTION

Plastid genetic engineering has become an attractive tool in plant biotechnology, offering several distinct advantages over more conventional nuclear transformation. These advantages include the potential for very high transgene expression (Oey *et al.*, 2009), a lack of epigenetic effects, and simultaneous expression of multiple genes as polycistronic units (De Cosa *et al.*, 2001; Verma and Daniell, 2007). Furthermore, plastids have the ability to correctly fold complex proteins in the stroma, and form disulfide bonds and other post-translational modifications required for protein maturation (Cardi *et al.*, 2010). Plastid transformation also offers increased transgene containment due to the maternal inheritance of plastids, minimizing the risk of cross-pollination with non-genetically modified plants or their relatives in the wild (Ruf *et al.*, 2007; Thyssen *et al.*, 2012). Despite these advantages, current plastid transformation methods are limited to a small range of plant species.

To facilitate efficient recovery of transgenic plants from transformed cells, the use of selectable marker genes conferring antibiotic or herbicide resistance is generally

required for plastid transformation. The *Escherichia coli* aminoglycoside 3′-adenylyltransferase (*aadA*) gene is the most commonly used selectable marker gene for plastid transformation (Day and Goldschmidt-Clermont, 2011) due to its high specificity and limited side-effects (Li *et al.*, 2011). Both spectinomycin and streptomycin are inactivated by *aadA*, through adenylation, preventing the antibiotics from binding to plastid ribosomes. In addition to *aadA*, a number of alternative selectable marker genes have been used for plastid transformation purposes, including *nptII*, encoding neomycin phosphotransferase, and *aphA-6*, encoding aminoglycoside phosphotransferase, both of which confer kanamycin resistance (Carrer *et al.*, 1993; Huang *et al.*, 2002), and *CAT*, encoding chloramphenicol acetyltransferase (Li *et al.*, 2011). However, the use of antibiotic resistance genes as selectable markers has several disadvantages. First, excretion of necrotic substances from dying untransformed tissue into the medium may result in reduced transformation efficiency (Endo *et al.*, 2001). Second, plastid genome engineering in major economically important crops has been hindered due to the lack of available antibiotic resistance marker genes that are functional in crops. Cereals are endogenously resistant to spectinomycin, rendering the most preferred antibiotic for plastid transformation ineffective (Fromm *et al.*, 1986). Other aminoglycoside antibiotics, commonly used in dicots for selection of transplastomic plants, are also becoming less effective in cereals (Ahmadabadi *et al.*, 2007). Furthermore, there is a lack of public acceptance to the continuous presence of antibiotic and herbicide selectable marker genes in transgenic plants due to issues such as increased numbers of resistant pathogens via transfer of antibiotic resistance genes into microorganisms by horizontal gene transfer and the emergence of 'super weeds' resulting from crossing out of herbicide-resistant genes into wild relatives (Khan *et al.*, 2011).

To avoid these problems, a number of strategies have been explored: (i) removal of selectable marker genes from transformed plants once homoplasmy has been achieved, (ii) elimination of the use of antibiotic and herbicide genes for selection of transformed cells, and (iii) a combination of these strategies. One such strategy is use of a positive selection system to obtain genetically transformed plants. Positive selection systems utilize non-toxic compounds to exploit the auxotrophies of the transformed tissue, rendering them unable to regenerate and develop without the supply of specific exogenous compounds. This is achieved by introducing gene(s) into transformed cells that allow them to either produce the essential substance themselves, or to use an exogenous substrate that confers a metabolic advantage over non-transformed cells (Wenck and Hansen, 2005). One such gene is the *ipt* gene of *Agrobacterium tumefaciens*. The *ipt* gene encodes the enzyme isopentenyl transferase, which catalyzes one of the early steps in cytokinin biosynthesis, allowing cells to proliferate and differentiate into shoots without the use of exogenous cytokinin. The *ipt* gene has been successfully used as a positive selectable marker for nuclear transformation of numerous plants including tobacco (*Nicotiana tabacum*) (Kunkel *et al.*, 1999; Endo *et al.*, 2001), rice (*Oryza sativa*) (Endo *et al.*, 2002), potato (*Solanum tuberosum*) (Khan *et al.*, 2011) and citrus (*Citrus sinensis*) (Zou *et al.*, 2013). However, use of *ipt* as a positive selection marker for plastid transformation has not been reported.

Here we report a novel plastid transformation technology using the *ipt* gene as a positive selectable marker to generate transplastomic tobacco plants with altered fatty acid profiles. Our data demonstrate that the *ipt* selection system does not interfere with the expression of transgenes or the resulting activity of the encoded protein, and suggest the applicability of this new plastid transformation system for crops of agricultural and economic importance that are not amenable to currently used selection systems.

RESULTS AND DISCUSSION

Assessment of *ipt* as a selectable marker gene in plastid transformation

To assess the possibility of using the *ipt* gene as a selectable marker for plastid transformation, a chimeric marker gene expression vector, pP1016-control (Figure 1a), was designed. The pP1016-control vector contained both the *ipt* gene and the *aadA* gene arranged as an operon, which allows direct comparison between the *ipt*-based selection system and the more commonly used *aadA*-based selection system (Day and Goldschmidt-Clermont, 2011). Both the *ipt* and the *aadA* genes were placed under the control of the strong constitutive plastid rRNA operon promoter (P*rrn*). The vector also contained the *rbcL* 3′ UTR (T*rbcL*) and plastid genome flanking sequences, allowing targeted integration of the vector into *orf*131 in the inverted repeat region of the tobacco chloroplast genome.

The pP1016-control vector was introduced into tobacco (*Nicotiana tabacum* cv. Petit Havana) leaves by biolistic bombardment (Svab and Maliga, 1993; Hogg *et al.*, 2010). In order to directly compare the *ipt* selection system and the *aadA* selection system, the bombarded leaves were cut into 10 mm^2 pieces, and half were placed onto regeneration medium containing 500 mg l^{-1} spectinomycin, while the other half were placed onto regeneration medium lacking both spectinomycin and the synthetic cytokinin 6-benzylaminopurine. It has been reported that, when the *ipt* gene has been used as a selectable marker in nuclear transformation, regenerating shoots exhibit an abnormal shoot morphology called the 'extreme shooty' phenotype. This is thought to be due to the over-production of cytokinin by the *ipt* gene, which leaks out of cells causing abnormal phenotypes (Endo *et al.*, 2002), and the inability

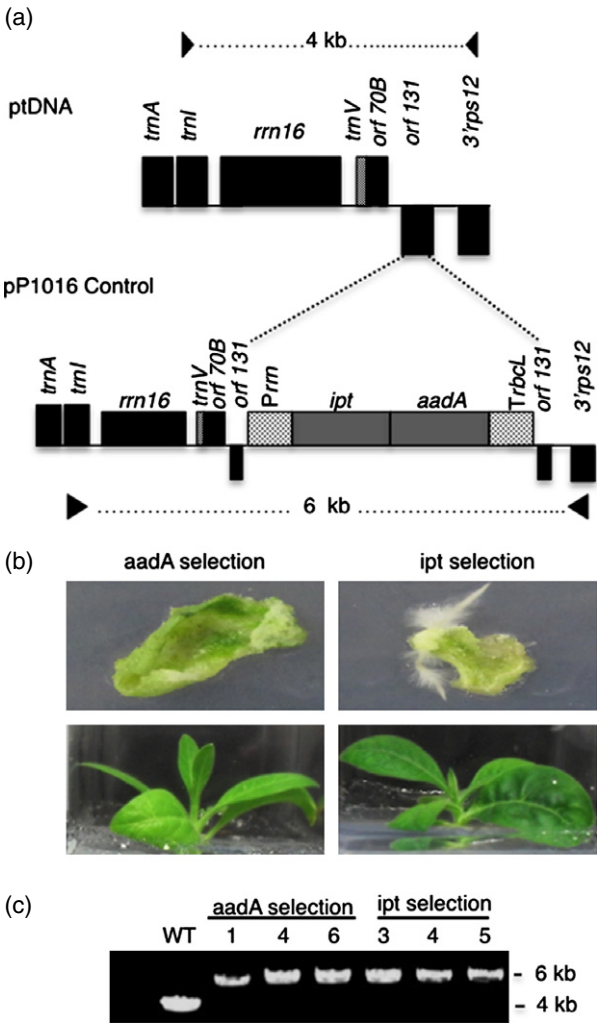

Figure 1. Generation of transplastomic plants using the pP1016-control vector.

(a) Physical map of the targeting region in the plastid genome. The pP1016-control vector contains the *ipt* and *aadA* genes under the control of the *rrn* promoter (P*rrn*) and the *rbcL* terminator (T*rbcL*). The transgenes are targeted to the *orf131* region in the tobacco genome.

(b) Recovery of transplastomic tobacco plants after bombardment with the pP1016-control vector using either the *ipt* or *aadA* selection system. The root formation observed on some explants selected using the *ipt* system did not affect shoot regeneration, which resulted in phenotypically normal shoots (plants were 2–3 weeks old).

(c) Confirmation of transgene integration into the tobacco plastid genome by PCR. PCR analysis of putative pP1016-control vector-transformed plants using primers flanking the transgene insertion site. A 4 kb product was amplified from the wild-type plastid genome, whereas a 6 kb product was amplified from the transplastomic genome in all plants selected using the *ipt* or *aadA* selection system. PCR confirmed homoplasmy in these plants. The positions of primer binding sites and expected product size for the PCR reaction are indicated by the arrows in (a).

of *ipt* shoots to root (Khan *et al.*, 2006). In our study, although a number of leaf explants exposed to the *ipt* selection system displayed root formation prior to shoot formation 2 weeks after bombardment, all newly regenerated shoots appeared phenotypically normal at the early stages of development, and did not display any lack of apical dominance that may result in the 'extreme shooty' phenotype (Figure 1b). Furthermore, all transplastomic plants displayed normal root formation. Both the *ipt*- and the *aadA*-based selection systems generated equivalent numbers of putative transformed plants, and three independently generated *aadA* lines and three independently generated *ipt* lines were selected for further characterization. Unlike the *aadA* selection system, in which the antibiotic kills non-transformed cells, the *ipt* selection system is a positive selection system and hence does not deplete the existing pool of non-transformed cells. Transformed cells grow out of the mass of untransformed cells, resulting in a heterogeneous population. Thus, the plants produced by the *ipt*- and the *aadA*-based selection systems were brought to homoplasmy by performing additional regeneration cycles on regeneration medium (RMOP) supplemented with 500 mg l^{-1} spectinomycin. Correct transgene integration and homoplasmy were assessed by PCR analysis using primers flanking the transgene integration site. All transplastomic lines obtained using both selection systems showed the presence of the expected 6 kb transgenic product and the absence of the 4 kb wild-type product, confirming the homoplasmic nature of the plants (Figure 1c).

Together, these results demonstrate that this new *ipt* plastid transformation system is fully functional in terms of selection of transplastomic tissue, and that the presence of the *ipt* gene does not interfere with plant regeneration.

Plastid transformation using *ipt* as a positive selectable marker

To demonstrate proof of principle, two additional chloroplast expression vectors, pP1016-KasIII (Figure 2a) and pPS59a-KasIII (Figure 2b), were designed to determine whether the *ipt* selection system may be used for selection of transplastomic plants without affecting the expression of transgenes within the integrated vector cassette or the resulting activity of the encoded protein. The *kasIII* gene encoding 3-ketoacyl acyl carrier protein synthase III was chosen as the transgene of interest as (i) fatty acid biosynthesis is catalyzed by a set of enzymes located in plastids in higher plants, (ii) kasIII is responsible for initiating both straight- and branched-chain fatty acid biosynthesis (Li *et al.*, 2005), and (iii) previous studies have shown that over-expression of KASIII results in altered fatty acid profiles (Dehesh *et al.*, 2001). Therefore, any changes in fatty acid profiles may be traced back due to the activity of the transgene rather than the selectable marker genes. The pP1016-KasIII vector, a modified version of the pP1016-control vector, included an additional expression cassette containing a FLAG-tagged *kasIII* gene linked to the P*rrn* with the *rbcL* 5′ UTR and the *psbA* 3′ UTR (T*psbA*)

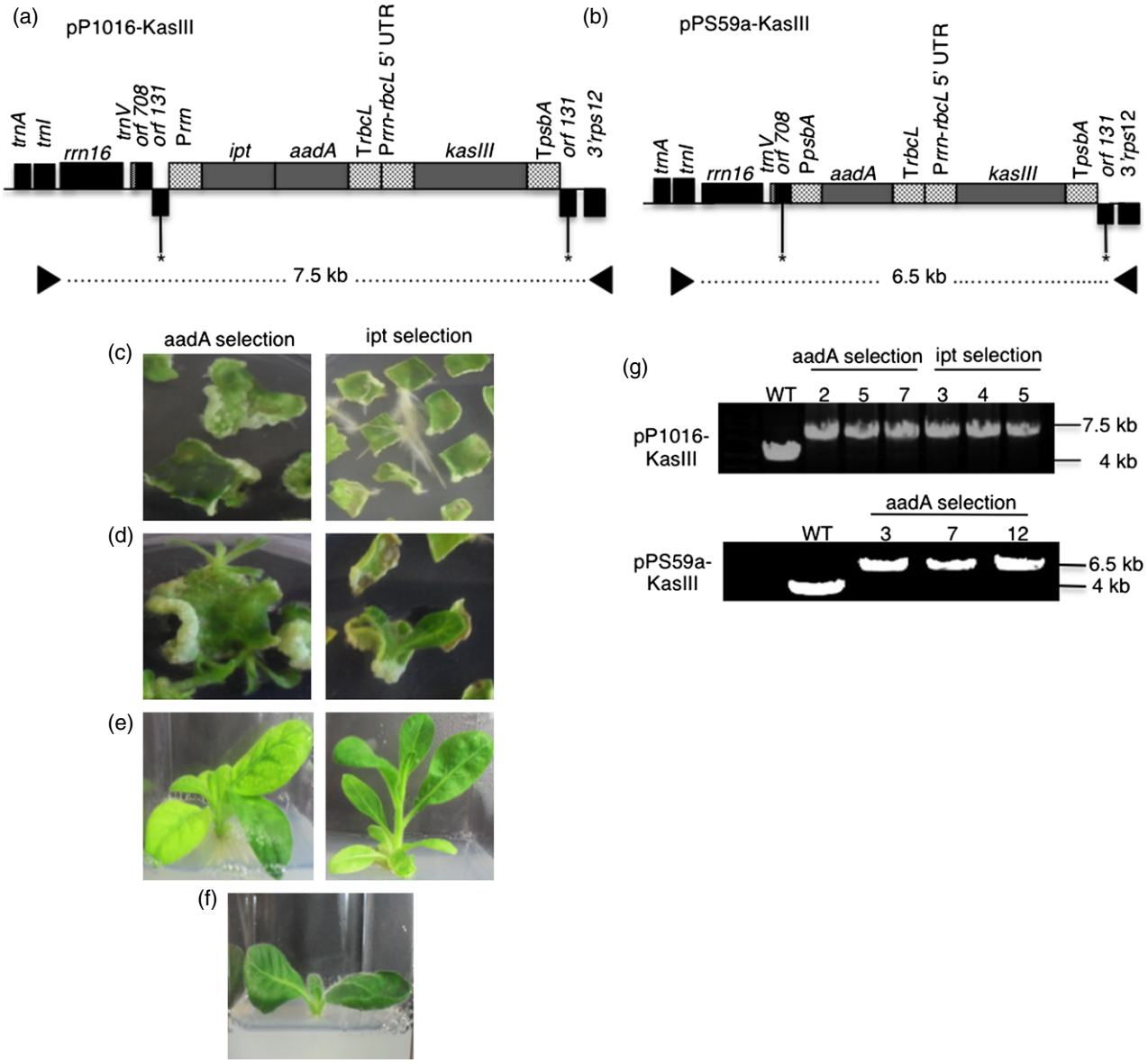

Figure 2. Characterization of pP1016-KasIII and pPS59a-KasIII transplastomic tobacco lines using *aadA* or *ipt* selection.
(a,b) Maps of the plastid transformation vectors (a) pP1016-KasIII and (b) pPS59a-KasIII. Both vectors contain the *kasIII* gene under the control of the *Prrn* linked to the 5′ UTR of *rbcL* and the T*psbA*. The selectable marker gene cassettes contain the *ipt* and *aadA* genes under the control of the *Prrn* and the T*rbcL* (pP1016-KasIII), and the *aadA* gene under the control of the P*psbA* and the T*rbcL* (pPS59a-KasIII). The positions of the transgene integration sites are indicted by asterisks. Primer binding sites and expected product sizes used to confirm plastid integration of the vectors and homoplasmy are indicated by arrows.
(c) Leaf explants 2 weeks after bombardment following *aadA* or *ipt* selection. Root growth was observed for some leaf explants on *ipt* selection medium, but did not affect shoot regeneration.
(d) Shoot regeneration 3–4 weeks after bombardment following *aadA* or *ipt* selection.
(e) Rooting of transplastomic tobacco plants after transfer to Magenta boxes containing spectinomycin selection medium or *ipt* selection medium.
(f) Transplastomic tobacco plant initially selected using the *ipt* selection system and brought through a round of regeneration using spectinomycin selection.
(g) PCR analysis of putative pP1016-KasIII and pPS59a-KasIII plants using primers flanking the transgene insertion site. A 4 kb product was amplified from the wild-type plastid genome, whereas 7.5 kb (pP1016-KasIII) and 6.5 kb (pPS59a-KasIII) products were amplified from the transplastomic genome. PCR confirmed homoplasmy in these plants.

(Figure 2a). pPS59a-KasIII contained the *kasIII* gene under the control of the P*rrn-rbcL* 5′ UTR and T*psbA*, and the *aadA* gene regulated by the *psbA* promoter (P*psbA*) and the T*rbcL* (Figure 2b). The absence of the *ipt* gene in the pPS59a-KasIII vector facilitated analysis of any effects resulting from expression of the *ipt* gene on phenotypic abnormalities as well as on KASIII protein production and enzymatic activity in the resulting transplastomic plants. Both the pP1016-KasIII and pPS59a-KasIII vectors target the expression cassette between the tRNA-Val and ribosomal

protein S12 genes in the orf70b and orf131 *B* regions within the inverted repeat region of the tobacco chloroplast genome.

Both expression vectors were introduced into tobacco plastids by biolistic transformation (Svab and Maliga, 1993; Hogg *et al.*, 2010). To select for primary transformants, leaves bombarded with pPS59a-KasIII were cut into 10 mm^2 pieces and placed on RMOP containing 500 mg l^{-1} spectinomycin. Leaves bombarded with pP1016-KasIII were cut into 10 mm^2 pieces, and half were placed on RMOP containing 500 mg l^{-1} spectinomycin and the other half on RMOP lacking 6-benzylaminopurine and spectinomycin. The development of transgenic events was monitored, and 2 weeks after bombardment, root growth was observed before shoot formation for some leaf explants that had been placed on medium lacking 6-benzylaminopurine and spectinomycin (*ipt* selection) (Figure 2c). As before, this did not affect shoot regeneration, which was observed for both selection systems (*aadA* and *ipt* selection) at 3–4 weeks after bombardment (Figure 2d). Although the P*psbA* used to drive *aadA* expression in the pPS59a-KasIII vector is considered to be a weaker promoter compared to the P*rrn* used to drive both *aadA* and *ipt* expression in the pP1016-KasIII vector (Newell *et al.*, 2003), no significant differences in plant growth or development were observed between pPS59a-KasIII- and pP1016-KasIII-transformed plants selected using *aadA* and *ipt*. The majority of pP1016-KasIII-transformed plants selected using the *ipt* selection system displayed a wild-type phenotype (Figure 2e), although a yellow/pale green phenotype was observed in approximately 1% of the plants (Figure 3a). A possible explanation for the observed yellow/pale green phenotype may be the occurrence of a flip-flop recombination event between the two *rrn* promoters. This type of rearrangement event may occur between a strong full-length P*rrn* and a shorter and weakened P*rrn*, resulting in the strong full-length P*rrn* being placed in front of the transgene, causing higher accumulation of transgene protein. This in turn may interfere with photosynthesis or some other chloroplast function, resulting in the yellow/pale green phenotype. Flip-flop rearrangements have previously been observed between two P*rrn* promoter copies in pRB95-derived plastid transformation vectors (McCabe *et al.*, 2008; Zhou *et al.*, 2008); however, further investigation is warranted to prove this theory in the current transformation experiments. To demonstrate the flexibility of the use of multiple selection marker genes within the same construct, transplastomic plants selected initially using the *ipt* selection system were subjected to a round of regeneration using spectinomycin selection, and subsequently rooted in Magenta boxes containing MS medium with 500 mg l^{-1} spectinomycin without any ill effects (Figure 2f). Correct transgene integration and homoplasmy were confirmed by PCR analysis based on the presence of a 7.5 kb band (pP1016-KasIII) and a 6.5 kb band (pPS59a-KasIII), and the absence of the 4 kb wild-type band in all transgenic lines selected using both selection systems (Figure 2g).

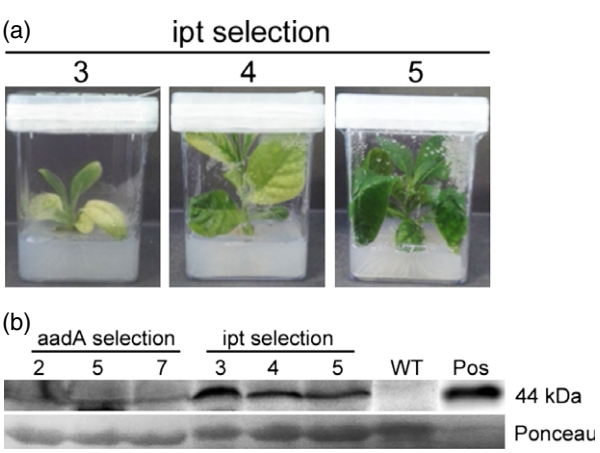

Figure 3. Immunodetection of the KASIII protein in transplastomic pP1016-KasIII plants.
(a) Protein was extracted from plants selected using the *aadA* selection system, and from three phenotypically different plants selected using the *ipt* selection system.
(b) The 44 kDa KASIII protein was detected using a FLAG antibody in all plant samples. The membrane was stained with Ponceau stain to assess protein loading; approximately 20 μg of total plant protein was loaded per lane.

This method of using both positive selection followed by negative selection may be valuable for recalcitrant plant species such as cereals and grasses. The presence of point mutations within some monocot species renders them endogenously resistant to spectinomycin (Fromm *et al.*, 1986). Aminoglycoside antibiotics, e.g. kanamycin, spectinomycin and streptomycin, which are commonly used in dicots for the selection of transplastomic plants, are also becoming less effective in cereals, and, in some cases, e.g. maize (*Zea mays*), they are completely ineffective when the selection of transgenic cells requires dark conditions (Ahmadabadi *et al.*, 2007). Using a positive selection system such as the *ipt* system in combination with a negative selection system may prove invaluable for selection of plastid transformants in monocot species by allowing selection pressure to initially be applied in the dark followed by antibiotic pressure once putative transformed cells are moved into the light for shoot regeneration.

KASIII over-expression in transgenic plastids leads to altered fatty acid profiles

KASIII protein levels were assessed in three independent pP1016-KasIII lines selected using the *aadA* system (plant lines 2, 5 and 7), and in three phenotypically different plants generated using the *ipt* selection system (plant lines 3, 4 and 5) (Figure 3a). All samples were taken from 5-week-old plants grown *in vitro.* Western blot analysis of

total protein extracts, using a polyclonal anti-FLAG antibody demonstrated the presence of KASIII (44 kDa) in all lines tested (Figure 3b). Expression of the pP1016-KasIII transformation vector in *E. coli* was used as a positive control.

We next wished to determine whether insertion of *kasIII* into the plastid genome has an effect on fatty acid profiles. We analyzed fatty acid profiles by one-step extraction of total lipids from leaf material and generation of fatty acid methyl esters. Fatty acid methyl esters were identified by comparison with known standard mixtures (retention time) and separate GC/MS analysis, whilst quantification was performed via inclusion of a 17:0 standard in each sample run. We observed a moderate but significant increase in the levels of 16:0 fatty acids (from 13 to 15%), and a significant decrease in the levels of 18:0 fatty acids (from 3 to 1%) and 18:1 fatty acids (from 3 to 1%) compared to the controls (Figure 4). Previous studies have reported that over-expression of KASIII in *Nicotiana tabacum*, *Arabidopsis thaliana* and *Brassica napus* increased the amounts of C16:0 fatty acids and decreased the amounts of C18 fatty acids. Dehesh *et al.* (2001) observed an increase from 18 to 24% in C16:0 fatty acid levels in tobacco leaves, compensated for by a decrease from 55 to 50% in the levels of C18:3 fatty acids following nuclear transformation (Dehesh *et al.*, 2001). An increase in the levels of 16:0 was also observed in *Arabidopsis thaliana and Brassica napus* seeds over expressing KASIII (Dehesh *et al.*, 2001). Similarly, expression of the plastid-targeted *kasIII* gene affected the fatty acid profile of storage lipids in *B. napus*, resulting in decreased amounts of C18:1 fatty acids compared to control plants (Verwoert *et al.*, 1995). Our data demonstrates that the *ipt* gene does not interfere with the expression or the activity of transgenes of interest.

In summary, we describe a novel selection and regeneration plastid transformation system based on the hormone cytokinin. We demonstrate that the *ipt*-based positive plastid transformation system may be used in isolation or in combination with a negative *aadA*-based selection system, yielding the same results. We also show that expression of IPT does not affect the expression of transgenes within the integrated vector cassette or the resulting activity of the encoded protein. This new plastid transformation system allows increased flexibility in terms of the selection process for crops, and may circumvent the need for selectable marker recycling as part of multiple gene knockout or site-directed mutagenesis strategies for plastid-encoded genes.

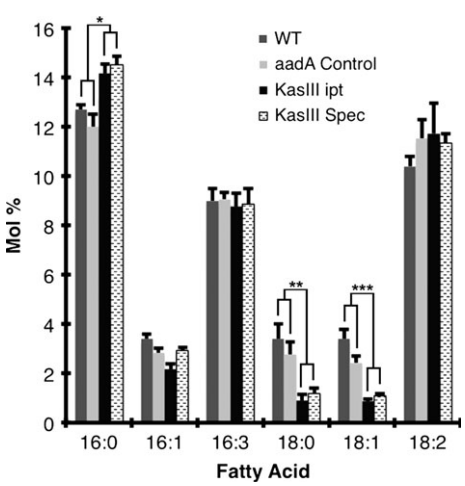

Figure 4. Relative levels of fatty acid enzyme activity in wild-type and transgenic pP1016-KasIII and pPS59a-KasIII plants.
KASIII enzyme activity is modest, but there was a significant increase in the relative levels of 16:0 fatty acid, compensated for by a decrease in the levels of 18:0 and 18:1 fatty acids in leaves. Values are means ± SD of three replicate samples. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Sterile tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown on agar-solidified Murashige and Skoog (MS) medium supplemented with 30 g l^{-1} sucrose. Young leaves from 4 to 6-week-old plants were harvested and used for all biolistic bombardment experiments.

Vector construction

For construction of the pPS59a chloroplast transformation vector, all fragments were amplified by PCR using Pwo DNA polymerase (Roche; www.lifescience.roche.com) from tobacco DNA, and cloned into pPCR-Script (Stratagene, Agilent Technologies. www.agilent.com) before sub-cloning into the final transformation vector as described below, unless otherwise stated. Nucleotide positions are given with respect to the Nicotiana tabacum chloroplast genome; GenBank accession number Z00044. First, two homologous recombination regions were amplified by PCR: the left region (nt 138 544–140 318) was flanked by *Fsp*I and *Nde*I sites, and the right region (nt 140 317–141 483) was flanked by *Not*I and *Sac*I sites. The plastidic ribosomal RNA operon promoter (P*rrn*; nt 59 034–59 303), fused to the 5′ UTR of the plastid *rbcL* gene and the downstream sequence encoding rbcL amino acids 1-14, flanked by *Nde*I, *Nhe*I and *Eco*RI sites (NdeI flanked the 5′ end of the fragment and NheI and EcoRI flanked the 3′ end), was synthesized by DNA2.0 (USA, www.dna20.com), and cloned into pPCR-Script. The left homologous fragment was cloned into the *Fsp*I and *Nde*I sites of this vector to generate an HOML-P*rrn-rbcL* cassette [HOML (left homologous recombination border repeat)]. Concurrently, the *psb*A 3′ (PacI and BsiWI were at the 5′ end and NotI at the 3′ end) UTR flanked by *Pac*I, *Bsi*WI and *Not*I sites (T*psbA*; nt 141–536) was cloned into pPCR-Script, and the right homologous fragment was cloned into the *Not*I and *Sac*I sites of this vector to generate a T*psbA*-HOMR cassette [HOMR (right homologous recombination border repeat)]. The T*psbA*-HOMR cassette was excised using *Eco*RI and *Sac*I, and cloned into the same sites of the HOML-P*rrn-rbcL* vector to generate HOML-P*rrn-rbcL*-T*psbA*-HOMR. Finally, a *PsbA-aadA*-T*rbcL* cassette flanked by *Nde*I sites was cloned into the internal *Nde*I site of this vector to generate pPS59a.

In pTI016-Control, the *aadA* selection cassette of pPS59a was replaced with the *ipt*/*aadA* selection cassette, which comprises the *ipt* gene from *Agrobacterium tumefaciens* and the *aadA* gene flanked by the *rrn* operon promoter and the ribulose-1,5-bisphosphate carboxylase/oxygenase 3′ UTR (T*rbcL;* nt 59 035–59 303). Both the *ipt* and *aadA* genes are preceded by a Shine–Dalgarno sequence (GGAGG) and a 6 bp spacer (ATCACT).

The *kasIII* sequence (At1 g62640) was obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). A FLAG tag was introduced at the C-terminus of *kasIII* by PCR using primers 5′-ATGCTAGCATGGCTAATGCATCTGGGTTC-3′ and 5′-CGTACGT CACTTGTCATCGTCATCTTTATAATCCCTCCATCGCATAATTGCTGA TCC-3′. The *kasIII*-FLAG construct was subsequently cloned into the *Nhe*I and *Bsi*WI sites of pPS59a to generate pPS59a-KasIII. The T*rbcL*-P*rrn-rbcL*- 5′ UTR *kasIII*-FLAG-T*psbA* cassette from pPS59a-KasIII was subsequently excised using *Asc*I and *Not*I, and ligated into the same sites of pTI016 to generate pP1016-KasIII.

Chloroplast transformation and selection of transplastomic lines

Generation of chloroplast transformed tobacco (*Nicotiana tabacum* var. Petit Havana) was performed using the biolistic method as described by Hogg *et al.* (2010). Following bombardment, the leaves were cut into 0.5 × 0.5 cm pieces, and placed onto selective regeneration medium. For spectinomycin selection, leaf pieces were placed on RMOP containing 500 mg l^{-1} spectinomycin (Svab and Maliga, 1993). For *ipt* selection, leaf pieces were placed on modified RMOP lacking 6-benzylaminopurine. Shoot formation was observed after 2–3 weeks of selection for both *aadA* or *ipt* selection. Shoots identified as transformants were transferred to Magenta boxes, and rooted on either MS medium containing 500 mg l^{-1} spectinomycin or the modified RMOP lacking 6-benzylaminopurine (*ipt* selection). All shoots were subjected to additional rounds of regeneration on RMOP containing spectinomycin, after which shoots were screened by PCR for integration of foreign genes into the chloroplast genome and homoplasmy.

Analysis of transgenic tobacco plants

Genomic DNA was extracted from leaves of control and transformed plants using a GeneElute™ plant genomic DNA miniprep kit (Sigma, www.sigmaaldrich.com), and screened by PCR for insertion of the transgene expression cassettes into the chloroplast genome. All putative transformed plants were screened using external primers flanking the integration site (External Fw: 5′-CGTACTCTGG-CAACTTTACGTAA-3′; External Rv: 5′-CTCAGCCACATGGATAGTTC-3′) to confirm correct integration of the construct into the chloroplast genome and homoplasmy. PCR was performed using TaKaRa Taq polymerase (purchased from Clontech; www.clontech.com) according to the manufacturer's instructions. PCR conditions comprised 39 cycles of 95°C (30 sec), annealing for 1 min at a temperature determined by the T_m of the specific primer pair, followed by incubation at 68°C for 1 min per kb of expected amplification product, followed by an extension step at 72°C for 10 min.

Protein extraction and immunoblot analyses

Total protein was extracted from leaves (500 mg fresh weight) of transgenic tobacco plants. The samples were homogenized in approximately five volumes of extraction buffer (50 mM HEPES/KOH, pH 7.5, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride and 1% β-mercaptoethanol). The homogenized samples were centrifuged at 21 000 ***g*** for 15 min. The supernatant from each sample was incubated at 37°C for 10 min, and separated by SDS–PAGE (Laemmli, 1970). The proteins were transferred from the gel onto a hydrophobic poly(vinylidene difluoride) membrane (GE Healthcare, www.gehealthcare.com) using a semi-dry blotting apparatus (Bio-Rad, www.bio-rad.com). Detection of the KASIII protein was performed using a mouse anti-DYKDDDDK antibody (1:1000 v/v; Clontech) and subsequently goat-anti-mouse antibody (1:10 000 v/v; Sigma) using an ECL Western blot detection system (ThermoScientific, www.thermoscientific.com).

Analysis of fatty acids

Lipids were extracted from leaf material (50 mg) and methylated as described previously (Garcés and Mancha, 1993) with minor modifications: following methylation, the heptane fraction was concentrated and re-suspended in 40 μl solvent prior to injection of a 1 μl aliquot onto the GC column. Methyl ester derivatives of total fatty acids extracted were analyzed using an Agilent 7890A (www.agilent.com) gas chromatograph with an Agilent DB-225 column (30 m × 0.32 mm × 0.3 μm; length × ID × film). The inlet and detector temperatures were set to 250°C, and 1 μl of each sample was analyzed using splitless injection and a constant flow rate of 2 ml min^{-1}. The oven temperature cycle was as follows: a start temperature of 50°C was held for 1 min to allow vaporized samples and the solvent (hexane) to condense at the front of the column. The oven temperature was then increased rapidly to 190°C at a rate of 40°C/min, followed by a slower increase to 220°C at a rate of 1.5°C/min. The final temperature of 220°C was held for 1 min, giving a total run time of 25 min 50 sec per sample. Fatty acid methyl esters were detected using a flame ionization detector. Chromatograms were analyzed using Agilent ChemStation software. The retention time and identity of each fatty acid methyl ester peak were calibrated using Supelco 37-component FAME mix (sigma). The identifications were then confirmed using an Agilent 5975B gas chromatograph/mass spectrometer. Methyl heptadecanoate (C17:0, 1 mM; Sigma) was added to the samples as an internal standard.

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