Paternal inheritance of plastid-encoded transgenes in *Petunia hybrida* in the greenhouse and under field conditions

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ABSTRACT

As already demonstrated in greenhouse trials, outcrossing of transgenic plants can be drastically reduced via transgene integration into the plastid. We verified this result in the field with *Petunia*, for which the highest paternal leakage has been observed. The variety white 115 (W115) served as recipient and Pink Wave (PW) and the transplastomic variant PW T16, encoding the *uid*A reporter gene, as pollen donor. While manual pollination in the greenhouse led to over 90% hybrids for both crossings, the transgenic donor resulted only in 2% hybrids in the field. Nevertheless paternal leakage was detected in one case which proves that paternal inheritance of plastid-located transgenes is possible under artificial conditions. In the greenhouse, paternal leakage occurred in a frequency comparable to published results. As expected natural pollination reduced the hybrid formation in the field from 90 to 7.6% and the transgenic donor did not result in any hybrid.

1. Introduction

Genetically modified (GM) plants have been widely adopted in agriculture. They were grown on 179.7 billion hectare in 2015, which represents 13% of the cultivated area worldwide [1]. However, since their first commercial release in 1996, GM plants have been under debate, including concerns about outcrossing of transgene-encoded traits to conventional crops [2]. Extensive research has been conducted to determine the rate of transgene transmission via pollen mediated gene flow (PMGF) [3]. Studies with corn, wheat, rice and canola demonstrated that, depending on the crop, a distance of 20 to 50 m was sufficient to keep the fraction of GM plants below 0.9%. Nevertheless, presence of transgenes below 0.9% was detectable up to a maximum distance of 350 m [4–8]. In addition to unintended admixture with non-transgenic crops, the potential introgression of GM plants into wild relatives is discussed, particularly when the transgene might increase the plant fitness or competitiveness [9–12]. Some member states of the EU imposed extensive isolation distances, which are difficult to implement in farming practices and have high costs [13,14]. In Luxembourg, for example, 3 km distances are demanded between GM and conventional canola plots [14,15].

Integrating transgenes into the plastid genome represents a natural confinement strategy, because in most angiosperms they are mainly inherited by the female gametophyte, but not by the pollen, [16].

Plastid transformation has been established for a variety of plant species and crop plants e.g. sugar beet [17], cabbage [18,19], soybean [20], potato [21], petunia [22], tomato [23] and tobacco [24]. Occasional paternal transfer of plastids was observed in *Antirrhinum majus* [25], *Setaria italica* [26], *Nicotiana tabacum* [27,28] and *Arabidopsis thaliana* [29], even though the frequency was quiet low ranging from 10^{-4} to 10^{-6}. In petunia (*Petunia hybrida*) paternal inheritance was detected for up to 2% of the progeny [30]. However, it is unlcear to what extend these results from experiments conducted in the greenhouse under controlled conditions can be transferred to the conditions present in the field [31]. Only one study investigated the paternal leakage under field conditions in non-transgenic *A. thaliana* with a mutated endogen as marker. But comparable experiments in the greenhouse were not conducted [32].

In the present study, we assessed the paternal inheritance of plastid encoded genes by comparing outcrossing frequencies in greenhouse and field trials. *P. hybrida*, for which the highest rate of paternal leakage has been reported, was chosen as a model. The variety white 115 (W115)

Fig. 1. Schematic presentation of the T-DNA cassette of transplastomic P. hybrida PW T16. aadA: coding region of the aminoglycoside-3′-adenyltransferase gene (Escherichia coli), 5′rrnBn: promotor of 16 s rRNA gene (Brassica napus); 3′BnpsbC:Terminator of the psbC gene (B. napus), uidA: coding region of the β-glucuronidase (E. coli), 5′rrnNt: promotor of the 16 s rRNA gene (Nicotiana tabacum), 3′NtpsbA: Terminator of the psbA gene (N. tabacum). T16-fw/-rv: primer pair used for the detection of the T-DNA.

[33] was used as pollen recipient while the transplastomic event Pink Wave (PW) T16 encoding the *uid*A gene for β-glucuronidase (GUS; Fig. 2) served as pollen donor. The near isogenic line PW [22] was used for control crossings. In hybrids of W115 x PW T16, paternal transmission can be tracked by the detection of the T-DNA and/or measuring of GUS enzyme activity.

Manual pollination in the greenhouse was compared to manual and natural pollination in the field. In the greenhouse, paternal leakage was observed (5 events), which is in line with previous studies under these conditions [25–29]. However, in the field, despite emasculation and manual pollination, cross-pollination rates were extremely low for W115 x PW T16 (max. 2.0%) while those of W115 x PW (96.2%) were comparable to the greenhouse results. Nevertheless, one case of paternal leakage was found after manual pollination which proves that paternal inheritance of transgenes located in the plastids is possible in the field.

2. Material and methods

2.1. Plant material and growth conditions

The *Petunia hybrida* variety W115, forming white flowers [33,34], served as pollen recipient. Either the non-transgenic *P. hybrida* variety Pink Wave (PW) or the transplastomic event (PW T16), carrying the marker genes *aad*A and *uid*A [22] (Fig. 1), both flowering deep pink, were used as pollen donors. Hybrids can be phenotypical identified by light pink flowers (Fig. S1) and paternal leakage can be detected by GUS-staining (Fig. S2).

For the greenhouse experiments, seeds of Petunia were germinated in planting trays on peat soil, optimized for Petunia (F900 with Cocopor, Stender AG, Schermberg, Germany). Seedlings were decollated and transferred into pots (7 × 7 × 8 cm) with the same substrate and fertilized once a week using 0.2% Hakaphos blue (Hermann Meyer KG, Rellingen Germany). W115 x PW and W115 x PW T16 crosses were achieved via manual pollination. Shortly before flowering, W115 buds were opened, emasculated and pollinated with the corresponding donor pollen using a brush. Mature capsules were harvested once per week.

For field trials, seeds were surface-sterilized and germinated on Murashige and Skoog (MS) medium (4.g g/L MS medium including vitamins supplemented with 30 g/L sucrose, 6.5 g/L plant agar (Duchefa, Haarlem, Netherlands) and adjusted to pH 5.7) with a 16 h/8 h 24 °C/22 °C light/dark photoperiod. Seedlings were transferred from *in vitro* culture into the same peat soil as above in multi-pot-trays (each pot: top Ø 4.6 cm, bottom Ø 3.2 cm) and cultivated in the greenhouse. For propagation, Petunia plants were cut up to 10 times per plant every two to three weeks and transferred into another multi-pot-plate. Plants were directly transferred from the pots into the field. The field trials were conducted from 08/03 to 10/25 in 2009 and 05/25 to 10/11 in 2010 in Thulendorf in Mecklenburg-Pomerania, Germany, which lies in the climatic region of the Baltic Sea. The predominant wind direction is from west to east, which was taken into account for the setup of the plots (Fig. 2). The soil type is loamy soil. Two experimental setups were used: (i) one row of the recipient W115 surrounded by three rows of the transgenic pollen donor PW T16 (plot A; high pressure = HP), and (ii) alternating rows of W115 and PW T16 (plot B; low pressure = LP), corresponding to a ratio of 1:6 and 1:1, respectively (Table 1; Fig. 2). Control experiments with the non-transgenic PW were done for the first variant (plot C). In addition to natural pollination, some W115 buds were randomly selected within each plot, manually emasculated and pollinated as described above. In 2010 growth distance between individual

plants and rows was shortened from 25 × 25 cm in 2009 to 15 × 17 cm and more plants were grown (Table 1). In 2009 the highest temperatures were measured between 08/16 till 09/13 with heat maxima ranging from 22 – 32 °C, minimal temperatures ranging from 8 – 18 °C, total precipitation of nearly 50 mm and humidity ranging from 52 – 84%. In 2010 highest temperatures were measured between 06/20 till 07/18 with heat maxima ranging from 19 – 36 °C, minimal temperatures ranging from 9 – 21 °C, total precipitation of nearly 7 mm and humidity ranging from 50 – 76%. Meteorological data for the time periods of the field trials were derived from http://www.wetteronline.de for Laage, which is located close to Thulendorf.

2.2. Determination of thousand grain weight (TGW) and seed number per capsule

The thousand grain weight (TGW) was determined by weighing 50 to 600 seeds (at least 10 different samples) and using a linear regression. The number of seeds per capsule was calculated with help of the TGW.

2.3. GUS staining and DNA analysis of seedlings

Seeds from natural and manual pollination were germinated on perlite (Sunshine Seeds, Ahlen/Germany), soaked with Hoagland medium [35], and grown at 20 °C in the dark for two weeks. The number of sowed seeds was calculated, based on the TGW, while the number of germinated seedlings was counted. Seedlings were either transferred to peat soil and grown to maturity or directly used to determine the activity of the β-glucuronidase (GUS).

The GUS activity was analysed by histochemical staining according to [36]. Seedlings were examined with a stereomicroscope for GUS staining, and positive ones were flash frozen in liquid nitrogen and used for DNA analysis.

Total DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method [37]. DNA extraction was confirmed by amplification of the endogenous *ef*1α gene as described by Hippauf et al. (2010, Table 2) [38]. Hybrids were verified using the PM150 primer pair, which was designed by the Institute of Plant Science of the University of Bern for *P. axillaris axillaris* N. (Switzerland; http://www.botany.unibe.ch/deve/caps/accessions.html). With the help of these primers, single sequence repeat (SSR) elements of the nuclear genome, which differ in their size in each *Petunia* variety (Table 2; Fig. S3), were amplified. To detect paternal plastid DNA transmission, the *uid*A gene was amplified using the primer pair T16-fw/-rv.

3. Results

3.1. Determination of the detection limit of plastid-encoded transgenes

Paternal plastid transfer leads to heteroplasmy in the hybrid zygotes, but during subsequent cell proliferation only one plastid type is maintained. The plastid discrimination occurs in each cell independently and the molecular mechanism has not yet been elucidated [27,30]. Since only a few sectors of the embryo might contain the paternal plastids, the content of paternal plastid DNA is expected to be low when isolating total DNA from the entire hybrids. Thus, we determined the detection limit of the marker gene *uid*A which is located on the T-DNA of transplastomic PW T16 by a serial dilution, where the DNA-containing extracts of PW T16 were diluted in DNA extracts of W115. Using PCR, the *uid*A gene was detectable in dilutions up to 10^{-5} (Fig. 3).

plot A / C = HP
(high pollen pressure)

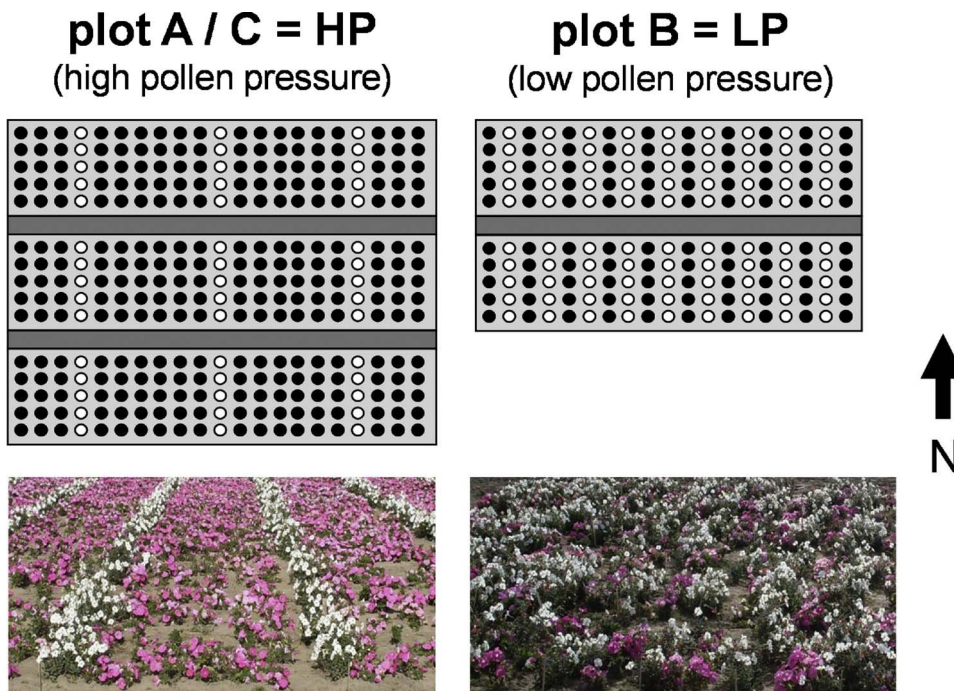# plot B = LP
(low pollen pressure)

Fig. 2. Schematic structure of field trial plots. Dots represent the position of *Petunia hybrida* W115 (white) as pollen recipient and either non-transgenic *P. hybrida* PW or transplastomic PW T16 (black) as pollen donor.

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Table 1
Absolute numbers of plants per plot and field trial.

Plot	2009		2010	
	W115	PW/PW T16	W115	PW/PW T16
A/C	540	3240	756	4536
B	540	570	756	784

W115: *Petunia hybrida* W115 as pollen recipient; PW: non-transgenic *P. hybrida* as pollen donor; PW T16: transplastomic *P. hybrida* as pollen donor.

Table 2
Primer used in the study.

Primer	Sequence (5′-3′)	Fragment size	Reference
EF1α-fw	CTTGGTGGTATTGACAAGCGTG	500 bp	[38]
EF1α-rv	ATTTCATCGTACCTAGCCTTG		
PM150-fw	CGTCGAATGCCTTAACTGC	100 bp (*P. hybrida* W115)	Institute of Plant Science
PM150-rv	GGAACAACACAGAAACTGTC	120 bp (*P. hybrida* PW/PW T16)	University of Bern
T16-fw	AGGAGCAATAGCACCCTCTTG	387 bp	This work
T16-rv	AATACGGCGTGGATACGTTAG		

Fig. 3. Determination of the uidA gene detection limit. uidA: fragment of coding region of the β-glucuronidase amplified by the primers T16-fw/T16-rv; (1, 10) GeneRuler DNA Ladder Mix (Thermo Fisher Scientific; Darmstadt, Germany), (2–7) Total DNA of Petunia hybrida PW T16 diluted in total DNA of *P. hybrida* W115 up to 10^{-2} (2), 10^{-3} (3), 10^{-4} (4), 10^{-5} (5), 10^{-6} (6), and 10^{-7} (7). (8) Genomic DNA of *P. hybrida* W115, (9) control PCR without template.

3.2. Paternal plastid DNA transmission after manual pollination in the greenhouse

In order to test paternal leakage in the greenhouse, 1820 and 2084 flowers of *Petunia* W115 were emasculated and manually pollinated with pollen of PW (W115 x PW) or PW T16 (W115 x PW T16), respectively. In 2010, however, the amount of PW T16 pollen was drastically reduced. Capsule setting of pollinated flowers was equal (66% vs. 62%), but W115 x PW T16 crossings resulted in a higher percentage of small capsules compared to W115 x PW (Fig. S4). Capsules from W115 x PW T16 produced 60% fewer seeds and averaged a third of the seed weight compared to the control crossing (Table 3).

While the viability of seeds of the control crossing W115 x PW was only moderately affected (66.8%, Tab 3), the germination rate of seeds from the crossing W115 x PW T16 was severely comprised (5.1%, Table 3).
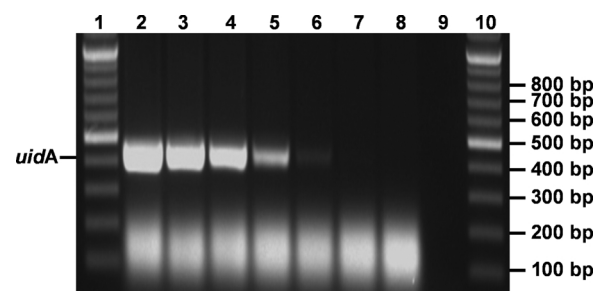
For analysis of the hybrid frequency, two week old seedlings were randomly selected either to be fully grown to maturity where the hybrid status was detected by their flower color and confirmed via SSR-PCR or via direct SSR-PCR of the seedling DNA (Table 3). Hybrid frequencies were similar for both crossings with 96.8% (274/283 for W115 x PW) and 91.3% (94/103 for W115 x PW T16).

The remaining seedlings were GUS-stained and GUS positive events subsequently analyzed for the presence of the *uid*A-gene. In the case of W115 x PW T16 crossings, GUS-staining occurred in 190 of 6022 seedlings (3.2%, Table 3), the *uid*A-gene could be detected in 5 (Table 3, Fig. 4a), and all proved to be hybrids in the SSR-PCR (Fig. 4b). In the non-transgenic control W115 x PW, 42 out of 2368 seedlings displayed GUS-activity (1.8%). As expected the *uid*A-gene was not detected in any of them (Table 3). Hence this is assumed to be an endogenous activity in Petunia seeds, which − to our knowledge − has not yet been reported for Petunia, but for various other seedlings [39–43]. Thus, GUS-staining could not be used as reliable marker for plastid transmission but only for preselection of events. The PCR-based tracking of transgene proved to be more reliable and highly sensitive (Fig. 3).

Table 3
Manual pollination in the greenhouse and under field conditions in 2009 and 2010.

place	year	crossing	no. of pollinated flowers	capsule setting	seeds per capsule	TGW of seeds	germinated plants	hybrid plants	GUS-positive seedlings	PCR positive seedlings (uidA-gene)
green house		W115 x PW	1820	66%	255 (± 13)	100 mg(± 6 mg)	2640/3952(66.8%)	274/283(96.8%)	42/2368(1.8%)	0
		W115 x PW T16	2084	62%	102 (± 11)	33 mg(± 4 mg)	6092/119,037(5.1%)	94/103(91.3%)	190/6022(3.2%)	5
field 2009		W115 x PW	3077	45%	212 (± 13)	123 mg(± 4 mg)	7535/10,465(72.0%)	763/793(96.2%)	125/6756(1.9%)	0
		W115 x PW T16	9336	21%	346 (± 12)	58 mg(± 4 mg)	8093/61,193(13.2%)	19/932(2.0%)	183/7222(2.5%)	1
field 2010		W115 x PW T16	3155	14%	111 (± 10)	42 mg(± 5 mg)	5234/52,358(10.0%)	8/615(1.3%)	214/4921(4.3%)	0

TGW: thousand grain weight.

3.3. Paternal plastid transfer after manual pollination under field conditions

In the field study, cross pollination was studied both via manual and natural pollination. Both experiments were conducted within the same plots and under the same conditions in order to allow comparison. Petunia W115 seeds were planted in the same plot with PW or PW T16. W115 and PW were cultivated in a ratio of 1:6 (plot C, Fig. 2), while W115 and PW T16 were planted in ratio 1:1 (plot B, Fig. 2) and 1: 6 (plot A, Fig. 2). In the three plots, randomly selected W115 flowers were manually emasculated, pollinated and labelled.

In 2009, plants were released to the field at the beginning of August due to the late approval of the field trial. In 2010, however, plants were released at the end of May. In both years, plants were grown and seeds harvested until mid-October.

In 2009, manual pollination was conducted both for W115 x PW and W115 x PW T16. Capsule setting of W115 x PW was more than twice as high compared to W115 x PW T16 (45% vs 21%, respectively; Table 3), and seed capsules from W115 x PW T16 crossings were on average smaller in size (Fig. S4). W115 x PW produced fewer seeds per capsule (212 vs 346), but the TGW of the seeds was twice as high (123 mg vs 58 mg). Similar to the manual pollination in the greenhouse, the viability of seeds from the crossing W115 x PW T16 was severely impacted (13.2%, Tab 3). In contrast, 72.0% of the seeds from the control crossing W115 x PW germinated (Table 3).

As in the greenhouse experiment, the hybrid status of randomly selected seedlings was determined either via the flower color or SSR-PCR. In contrast to the 96.2% hybrids identified for W115 x PW (763/793), only 19 of 932 W115 x PW T16 plants (2.0%) were hybrids.

Out of the 7222 W115 x PW T16 seedlings obtained in 2009, 183 showed GUS staining, and the uidA gene was detected in 1 descendant, which also proved to be a hybrid in the SSR-PCR (Fig. 5).

In case of the control crossing W115 x PW, 125 out of 4,362 descendants displayed GUS-activity, but the uidA gene was not detected in any of them (Table 3).

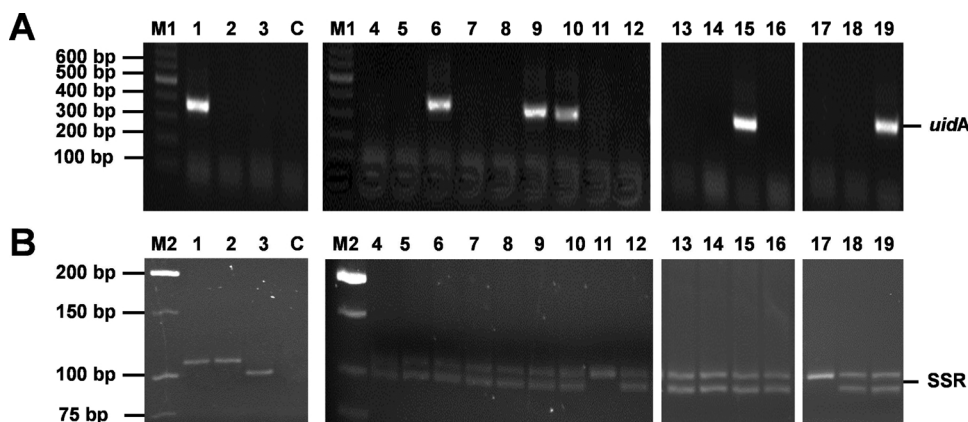Since so few seed capsules and seeds were obtained for W115 x PW

Fig. 5. Analysis of *Petunia* W115x PW T16 from manual pollination in the field via (A) *uid*A- and (B) SSR-PCR. (M1): GeneRuler DNA Ladder Mix (ThermoFisherScientifc; Darmstadt, Germany); (M3) GeneRuler Low Range DNA Ladder (ThermoFisherScientific, Darmstadt, Germany); (1) *Petunia hybrida* W115 (2) PW; (3) PW T16; (C) Control-PCR without template (4–10) Hybrids of W115 x PW T16;.

T16 in 2009 and so few of those seeds germinated, in 2010, all efforts focused on the manual pollination of W115 x PW T16 and W115 x PW was not conducted. Similarly to 2009, capsule setting of W115 x PW T16 was low (14%), the TGW reduced (42 mg), and only few seeds germinated (10.0% Table 3). Only 8 of 615 plants were hybrids (1.3%, Table 3). In the GUS assay, out of 4921 seedlings, 214 displayed GUS-staining, though the uidA gene was not detected in any of them.

3.4. Paternal plastid DNA transmission by natural pollination under field conditions

In 2009 and 2010, paternal inheritance of plastids via natural pollination was studied in the field using the experimental plot setups

Fig. 4. Analysis of Petunia W115 x PW T16 offspring from manual pollination in the greenhouse via (A) *uid*A- and (B) SSR-PCR. (M1): GeneRulerDNA Ladder Mix (ThermoFisherScientifc; Darmstadt, Germany); (M2) Low Molecular Weight DNA Ladder (New England Biolabs GmbH, Frankfurt am Main, Germany); (1) *P. hybrida* PW T16; (2) PW; (3) W115; (C) Control-PCR without template; (4–19) Hybrids of W115 x PW T16.
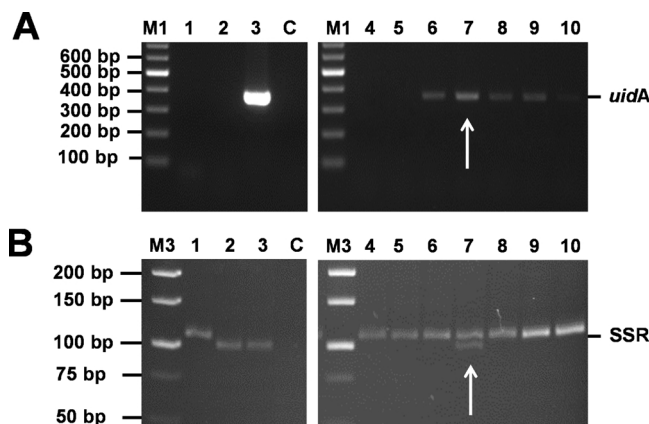
Table 4
natural pollination under field conditions in 2009 and 2010.

year	crossing	seeds per capsule	TGW of seeds	germinated plants	hybrid plants
2009	W115 x PW HP	187 (± 13)	81 mg (± 4mg)	378/1400 (27.0%)	14/185 (7.6%)
	W115 x PW T16 HP	201 (± 14)	90 mg (± 4 mg)	822/3073 (26.7%)	0/566 (0%)
	W115 x PW T16 LP	194 (± 14)	91 mg (± 6 mg)	737/2000 (36.9%)	0/282 (0%)
2010	W115 x PW HP	228 (± 12)	89 mg (± 4 mg)	175/240 (72.9%)	2/82 (2.4%)
	W115 x PW T16 HP	207 (± 11)	85 mg (± 5 mg)	124/240 (51.7%)	0/53 (0%)
	W115 x PW T16 LP	200 (± 13)	88 mg (± 4 mg)	155/240 (64.6%)	0/66 (0%)

HP: high pressure: W115 x PW/PW T16 planted in a ratio of 1:6; LP: low pressure: W115 x PW T16 planted in a ratio 1:1; n.d.: not determined; TGW: thousand grain weight.

described for manual pollination.

Comparing the two independent field trials 2009 and 2010, capsule size (Fig. S4), number of seeds per capsule and TGW of the different crossings did not differ (Table 4). However, independent of the crossing partners and scheme, the germination rate was drastically lower in 2009, ranging from 26.7–36.9%, compared to 51.7%–72.9% in 2010 (Table 4).

In 2009, only 14 of 185 (7.6%) W115 x PW seedlings were hybrids, and for W115 x PW T16 no hybrids were detected out of 566 (plot HP; Fig. 2) and 282 (plot LP, Fig. 2) plants tested.

Therefore the main question to be asked in 2010 was whether the low frequency of hybrids was due to the environmental conditions or to somaclonal variation in PW T16 resulting in low pollen quality. Hence the number of W115 x PW T16 seedlings analyzed for their hybrid status was restricted to a size where a pronounced increase in hybrid formation would become obvious. As in 2009, in 2010 no W115 x PW T16 hybrids could be identified. The amount of W115 x PW hybrids was in roughly the same range as 2009 (Table 4). We therefore assume that the PW T16 pollen quality is responsible for the failure of hybrid formation. Since none of the W115 x PW T16 seedlings were hybrids pollen mediated flow of the *uidA* gene was impossible and hence not analyzed.

4. Discussion

This is the first study which aimed at measuring the pollen mediated gene flow of plastid located transgenes in the field. Unexpectedly, hybrid production with the transgenic pollen donor was much lower − both for natural conditions and manual pollination − compared to the manual pollination in the greenhouse. The reduction in hybrid formation was observed in both years and was accompanied by a strong reduction of pollen in PW T16 and − since even high amounts of pollen applied manually to the stigma did not lead to a hybrid formation comparable to the PW pollen − a reduction in pollen quality. Pollen quality can be affected by adverse environmental conditions like heat or drought [44]. In the field, high temperatures were accompanied by extensive drought in both years, while in the greenhouse the plants were well watered. Therefore it might be assumed that the integration of the transgene into the chloroplast genome or somaclonal variations − that might have occurred during the transformation process − led to a higher sensitivity of the pollen to drought. It is also possible that the expression of the *uid*A-gene in the plastids have a negative impact under field conditions, since retarded growth, delayed flowering, reduced fertility and seed production was reported for transgenic tobacco and potato plants with a nuclear-encoded *uid*A-gene under field conditions, while plants with other transgenes were unaffected [45–47]. This phenomenon cannot be assigned to somaclonal variation because all events carrying the *uid*A-gene showed a similar phenotype [45]. Nevertheless, GUS expression did not affect the agronomic traits in nuclear-transformed papaya, banana, eucalyptus, spruce and poplar in the field [48–51]. Thus the effect might be species-specific and cannot be directly related to *Petunia*. Although the potato and tobacco studies [45–47] did not analyze the events in the greenhouse in parallel, this phenomenon might be restricted to the field, since other tobacco plants carrying the nuclear-encoded *uid*A-gene were completely unaffected under greenhouse conditions [52–54]. Hence we assume that the reduction in pollen quantity and possibly quality in the field might be due to the *uid*A expression.

The reduced pollen quality might also be the connected to the reduced hybrid seed viability and the capsule size in the presence of the PW T16 genome.

Nevertheless, when *Petunia* W115 was manually pollinated by PW T16, paternal leakage of the plastid-encoded *uid*A transgene could be detected both in the greenhouse (5 events) and in the field (1 event). In the greenhouse paternal leakage was also observed for *Petunia* varieties [30,55] and other transplastomic plants [25–29]. Paternal leakage in the field was described for plastid-located genes in non-transgenic *A. thaliana* [32]. Unfortunately, due to the low number of hybrids a frequency cannot be calculated and compared with previous studies. Nevertheless, the fact that pollen-mediated transgene flow was measured after manual pollination in the field proves that this is possible. Under natural conditions, no hybrids were formed and hence no paternal pollen transfer was possible. It remains to be ascertained whether similar results can be obtained in other cultivars, namely in agriculturally relevant crops, but it has to be assumed that although paternal transfer of plastid located genes is possible, the frequencies should be drastically lower than the hybrid formation. In addition, the paternal plastids are only maintained in few cells of the developing hybrid embryo, as demonstrated by Cornu and Dulieu [30] and Ruf et al. [27], but only transmission into the germline is relevant. In fact, Ruf et al. [27] observed in F1 seedlings of *N. tabacum* an extremely lower frequency of paternal plastid transmission into the shoot apical meristem ($2.86 * 10^{-6}$) compared to the transmission into the cotyledons ($1.58 *10^{-5}$) and assumed that the frequency can be reduced down to 10^{-8} in the field when keeping a distance of 10 m. Hence, although not ensuring absolute confinement, plastid transformation can facilitate a practically applicable and economic feasible co-existence of GM and non-GM crops.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2017.11.001.

References

[1] C.C. James, Global Status of Commercialized Biotech/GM Crops, ISAAA, 2015 (2015).

[2] A. Nicolia, A. Manzo, F. Veronesi, D. Rosellini, An overview of the last 10 years of genetically engineered crop safety research, Crit. Rev. Biotechnol. 34 (2015) 77–88.

[3] J. Ding, H. Duan, Z. Deng, D. Zhao, G. Yi, Y. Li, Molecular strategies for addressing gene flow problems and their potential applications in abiotic stress tolerant transgenic plants, Crit. Rev. Plant Sci. 33 (2014) 190–204.

[4] S. Dong, Y. Liu, C. Yu, Z. Zhang, M. Chen, C. Wang, Investigating pollen and gene flow of WYMV-resistant transgenic wheat N12-1 using a dwarf male-sterile line as the pollen receptor, PLoS One 11 (2016).

[5] Y. Liu, F. Chen, X. Guan, J. Li, High crop barrier reduces gene flow from transgenic to conventional maize in large fields, Eur. J. Agron. 71 (2015) 135–140.

[6] Y. Liu, F. Ge, Y. Liang, G. Wu, J. Li, Characterization of competitive interactions in the coexistence of Bt-transgenic and conventional rice, BMC Biotechnol. 15 (2015) 1–12.

[7] I. Loureiro, E. García-Ruiz, E. Gutiérrez, P. Gómez, M.C. Escorial, M.C. Chueca, Pollen-mediated gene flow in the cultivation of transgenic cotton under experimental field conditions in Spain, Ind. Crops Prod. 85 (2016) 22–28.

[8] X. Zhao, T. Tang, G. Chen, F. Liu, X. Wang, C. Bu, C. Lu, Rationalizing the isolation distance needed for field trials involving genetically modified rapeseed (*Brassica napus* L.) in China, Chin. Sci. Bull. 58 (2013) 1558–1567.

[9] T. D'Hertefeldt, R.B. Jørgensen, L.B. Pettersson, Long-term persistence of GM oilseed rape in the seedbank, Biol. Lett. 4 (2008) 314–317.

[10] N.C. Ellstrand, P. Meirmans, J. Rong, D. Bartsch, A. Ghosh, T.J. De Jong, P. Haccou, B.R. Lu, A.A. Snow, C. Neal Stewart Jr, Introgression of crop alleles into wild or weedy populations, Ann, Rev. Ecol., Evol. Syst. (2013) 325–345.

[11] B.R. Lu, C. Yang, Gene flow from genetically modified rice to its wild relatives: assessing potential ecological consequences, Biotechnol. Adv. 27 (2009).

[12] S.I. Warwick, A. Légère, M.J. Simard, T. James, Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population, Mol. Ecol. 17 (2008).

[13] Y. Devos, M. Demont, K. Dillen, D. Reheul, M. Kaiser, O. Sanvido, Coexistence of genetically modified (GM) and non-GM crops in the European Union. A review, Agron. Sustainable Dev. 29 (2009) 11–30.

[14] K. Ramessar, T. Capell, R.M. Twyman, P. Christou, Going to ridiculous lengths − European coexistence regulations for GM crops, Nat. Biotech. 28 (2010) 133–136.

[15] G. Masip, M. Sabalza, E. Pérez-Massot, R. Banakar, D. Cebrian, R.M. Twyman, T. Capbell, R. Albajes, P. Chistou, Paradoxical EU agricultural policies on genetically engineered crops, Trends Plant Sci. 18 (2013) 312–324.

[16] R. Hagemann, Plastid genetics in higher plants, Cell Organelles (1992) 65–96.

[17] F. De Marchis, Y. Wang, P. Stevanato, S. Arcioni, M. Bellucci, Genetic transformation of the sugar beet plastome, Transgenic Res. 18 (2009) 17–30.

[18] C.W. Liu, C.C. Lin, J.J.W. Chen, M.J. Tseng, Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. capitata L.) by particle bombardment, Plant Cell Rep. 26 (2007) 1733–1744.

[19] C.W. Liu, C.C. Lin, J.C. Yiu, J.J.W. Chen, M.J. Tseng, Expression of a Bacillus thuringiensis toxin (cry1Ab) gene in cabbage (*Brassica oleracea L. var. capitata L.*) chloroplasts confers high insecticidal efficacy against Plutella xylostella, Theor. Appl. Genet. 117 (2008) 75–88.

[20] N. Dufourmantel, M. Dubald, M. Matringe, H. Canard, F. Garcon, C. Job, E. Kay, J.P. Wisniewski, J.M. Ferullo, B. Pelissier, A. Sailland, G. Tissot, Generation and characterization of soybean and marker-free tobacco plastid transformants overexpressing a bacterial 4-hydroxyphenylpyruvate dioxygenase which provides strong herbicide tolerance, Plant Biotechnol. J. 5 (2007) 118–133.

[21] V.T. Valkov, D. Gargano, C. Manna, G. Formisano, P.J. Dix, J.C. Jay, N. Scotti, T. Cardi, High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences, Transgenic Res. 20 (2011) 137–151.

[22] M.K. Zubko, E.I. Zubko, K.V. Zuilen, P. Meyer, A. Day, Stable transformation of petunia plastids, Transgenic Res. 13 (2004) 523–530.

[23] S. Ruf, M. Hermann, I.J. Berger, H. Carrer, R. Bock, Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit, Nat. Biotechnol. 19 (2001) 870–875.

[24] Z. Svab, P. Maliga, High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene, Proc. Natl. Acad. Sci. USA 90 (1993) 913–917.

[25] L. Diers, Übertragung von Plastiden durch den Pollen bei *Antirrhinum majus*, Mol.

[26] T. Wang, Y. Li, Y. Shi, X. Reboud, H. Darmency, J. Gressel, Low frequency transmission of a plastid-encoded trait in *Setaria italic*, Theor. Appl. Genet. 108 (2004) 315–320.

[27] S. Ruf, D. Karcher, R. Bock, Determining the transgene containment level provided by chloroplast transformation, Proc. Natl. Acad. Sci. USA 104 (2007) 6998–7002.

[28] Z. Svab, P. Maliga, Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment, Proc. Natl. Acad. Sci. USA 104 (2007) 7003–7008.

[29] A.K. Azhagiri, P. Maliga, Exceptional paternal inheritance of plastids in *Arabidopsis* suggests that low-frequency leakage of plastids via pollen may be universal in plants, Plant J. 52 (2007) 817–823.

[30] A. Cornu, H. Dulieu, Pollen transmission of pastid–DNA under genotypic control in petunia hybrida hort, J. Hered. 79 (1988) 40–44.

[31] S.H. Wani, S.K. Sah, L. Sági, K. Solymosi, Transplastomic plants for innovations in agriculture. A review, Agron. Sustainable Dev. 35 (2015) 1391–1430.

[32] A. Schneider, C. Stelljes, C. Adams, S. Kirchner, G. Burkhard, S. Jarzombski, I. Broer, P. Horn, A. Elsayed, P. Hagl, D. Leister, H.U. Koop, Low frequency paternal transmission of plastid genes in *Brassicaceae*, Transgenic Res. 24 (2014) 267–277.

[33] A.Z. Mitchell, M.R. Hanson, R.C. Skvirsky, F.M. Ausubel, Anther culture of petunia: genotypes with high frequency of callus root, or plantlet formation, Zeitschrift für Pflanzenphysiologie 100 (1980) 131–145.

[34] T. Gerats, M. Vandenbussche, A model system for comparative research: petunia, Trends Plant Sci. 10 (2005) 251–256.

[35] D.R. Hoagland, D.I. Arnon, The water-culture method for growing plants without soil, California Agric. Exp. Station 347 (1950) 1–32.

[36] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.

[37] S.P.S. Khanuja, A.K. Shasany, M.P. Darokar, S. Kumar, Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils, Plant Mol. Biol. Rep. 17 (1999) 74–74.

[38] F. Hippauf, E. Michalsky, R. Huang, R. Preissner, T.J. Barkman, B. Piechulla, Enzymatic, expression and structural divergences among carboxyl O-methyltransferases after gene duplication and speciation in *Nicotiana*, Plant Mol. Biol. 72 (2010) 311–330.

[39] A. Alwen, R.M.B. Moreno, O. Vicente, E. Heberle-Bors, Plant endogenous β-glucuronidase activity: how to avoid interference with the use of the E. coli β-glucuronidase as a reporter gene in transgenic plants, Transgenic Res. 1 (1992) 63–70.

[40] C.Y. Hu, P.P. Chee, R.H. Chesney, J.H. Zhou, P.D. Miller, W.T. O'Brien, Intrinsic GUS-like activities in seed plants, Plant Cell Rep. 9 (1990) 1–5.

[41] L. Plegt, R.J. Bino, β-Glucuronidase activity during development of the male gametophyte from transgenic and non-transgenic plants, Mol. Gen. Genet. MGG 216 (1989) 321–327.

[42] M. Schulz, G. Weissenböck, Partial purification and characterization of a luteolin-triglucuronide-specific β-glucuronidase from rye primary leaves (*Secale cereale*), Phytochem. 26 (1987) 933–937.

[43] C. Sudan, S. Prakash, P. Bhomkar, S. Jain, N. Bhalla-Sarin, Ubiquitous presence of β-glucuronidase (GUS) in plants and its regulation in some model plants, Planta 224 (2006) 853–864.

[44] N. de Storme, D. Geelen, The impact of environmental stress on male reproductive development in plants: biological processes and molecular mechanisms Plant, Cell Environ. 37 (2014) 1–18.

[45] W.R. Belknap, D. Corsini, J.J. Pavek, G.W. Snyder, D.R. Rockhold, M.E. Vayda, Field performance of transgenic russet Burbank and lemhi russet potatoes, Am. Potato J. 71 (1994) 285–296.

[46] P.D.S. Caligari, Y.M.H.B. Yapabandara, E.M. Paul, J. Perret, P. Roger, J.M. Dunwell, Field performance of derived generations of transgenic tobacco, Theor. Appl. Genet. 86 (1993) 875–879.

[47] P.J. Dale, H.C. McPartlan, Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings, Theor. Appl. Genet. 84 (1992) 585–591.

[48] D.D. Ellis, J. Rintamaki-Strait, K. Francis, K. Kleiner, K. Raffa, B. McCown, Transgene expression in spruce and poplar: from the lab to the field, in: M.R. Ahuja, W. Boerjan, D.B. Neale (Eds.), Somatic Cell Genetics and Molecular Genetics of Trees, Springer, Dordrecht: Netherlands, 1996, pp. 159–163.

[49] S. MacRae, J. van Staden, Greenhouse/field performance and present status of transgenic eucalypt plants, in: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forstry 44: Transgenic Trees, 109 Springer-Verlag, Berlin, 2012.

[50] R.M. Manshardt, C. Mello, S.D. Lum, L. Ta, Tracking papaya pollen movement with the GUS transgene marker, HortScience 40 (2005) 1094.

[51] J.-Y. Paul, H. Khanna, J. Kleidon, P. Hoang, J. Geijskes, J. Daniells, E. Zaplin, Y. Rosenberg, A. James, B. Mlalazi, P. Deo, G. Arinaitwe, P. Namanya, D. Becker, J. Tindamanyire, W. Tushemereirwe, R. Harding, J. Dale, Golden bananas in the field: elevated fruit pro-vitamin A from the expression of a single banana transgene, Plant Biotechnol. J. 15 (2016) 520–532.

[52] J.E. Wilkinson, D. Twell, K. Lindsey, Activities of CaMV 35S and nos promoters in pollen: implications for field release of transgenic plants, J. Exp. Bot. 48 (1997) 265–275.

[53] A.J. van Tunen, L.A. Mur, G.S. Brouns, J.D. Rienstra, R.E. Koes, J.N.M. Mol, Pollen-and anther-specific chi promoters from petunia: tandem promoter regulation of the *chi*A gene, Plant Cell 2 (1990) 393–401.

[54] V. Gupta, R. Khurana, A.K. Tyagi, Promoters of two anther-specific genes confer organ-specific gene expression in a stage-specific manner in transgenic systems, Plant Cell Rep. 26 (2007) 1919–1931.

[55] A. Derepas, H. Dulieu, Inheritance of the capacity to transfer plastids by the pollen parent in *Petunia hybrida* hort, J. Hered. 83 (1992) 6–10.

Gen. Genet. MGG 100 (1967) 56–62.