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

Patricia Horna, Henrik Nauscha, Susanne Baars, Jörg Schmidtke, Kerstin Schmidt, Inge Broer

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 uidA 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 unclear 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) [33] was used as pollen recipient while the transplastomic event Pink Wave (PW) T16 encoding the uidA 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.

Corresponding author.

E-mail address: henrik.nausch@uni-rostock.de (H. Nausch).
Manual pollination in the field 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 × PW T16 (max. 2.0%) while those of W115 × 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 hybrid 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 aadA and uidA [22] (Fig. 1), both flowering deep pink, were used as pollen donors. Hybrids can be phenotypically 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 (P900 with Cocopor, Stender AG, Schermberg, Germany). Seedlings were decolated 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 (4.6 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 eFla 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/vega/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 uidA 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 uidA 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 uidA gene was detectable in dilutions up to 10⁻⁵ (Fig. 3).
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%, 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  | Sequence (5′-3′)                              | Fragment size | Reference          |
|---------|-----------------------------------------------|---------------|--------------------|
| EF1α-fw| CTTGGGTGATATTGACAAGGCTG                       | 500 bp        | [38]               |
| EF1α-rv| ATTTCATCGTACTAGCGCTG                          |               |                    |
| PM150-fw| CGTCGAATGCTTAACTGC                           | 100 bp (P. hybrida W115) | Institute of Plant Science |
| PM150-rv| GGAAACAACAGAAACTGTGC                       | 120 bp (P. hybrida PW/PW T16) | University of Bern |
| T16-fw  | AGGAGCAATAGCAATTCTGTG                        | 387 bp        | This work          |
| T16-rv  | AATAGGCGTGTAGTTAG                             |               |                    |

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.

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 uidA-gene. In the case of W115 x PW T16 crossings, GUS-staining occurred in 190 of 6022 seedlings (3.2%, Table 3), 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 uidA-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).

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%, 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  | Sequence (5′-3′)                              | Fragment size | Reference          |
|---------|-----------------------------------------------|---------------|--------------------|
| EF1α-fw| CTTGGGTGATATTGACAAGGCTG                       | 500 bp        | [38]               |
| EF1α-rv| ATTTCATCGTACTAGCGCTG                          |               |                    |
| PM150-fw| CGTCGAATGCTTAACTGC                           | 100 bp (P. hybrida W115) | Institute of Plant Science |
| PM150-rv| GGAAACAACAGAAACTGTGC                       | 120 bp (P. hybrida PW/PW T16) | University of Bern |
| T16-fw  | AGGAGCAATAGCAATTCTGTG                        | 387 bp        | This work          |
| T16-rv  | AATAGGCGTGTAGTTAG                             |               |                    |
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 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...
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 T16 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 uidA-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 uidA-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 uidA-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 uidA-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 uidA 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 uidA 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.

### Funding

The project was financed by the Federal Ministry of Education and Research (BMBF, Germany, https://www.bmbf.de/; Funding number: 0315212A). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### 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

We acknowledge Prof. Dr. Leister and co-workers (department of molecular biology of the plant of the Ludwig-Maximilians-University Munich, Germany), who introduced us in the manual pollination of petunia.

We thank Alex Rajewski for critical reading of the manuscript.

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.

[2] J. Ding, H. Duan, Z. Deng, D. Zhao, G. Y. Li, Molecular strategies for addressing gene flow problems in GM-crop production in China, J. Appl. Genet. 117 (2008) 75–96.

[3] S.H. Wani, S.K. Sah, L. Sági, K. Solymosi, Transplastomic plants for innovations in plant biotechnology, Biotechnol. Adv. 37 (2016) 1–10.

[4] A.K. Azhagiri, P. Maliga, Exceptional paternal inheritance of plastids in Arabidopsis suggests that long-distance leakage of plastids via pollen may be universal in plants, Plant J. 52 (2008) 817–823.

[5] A. Cornu, H. Duleu, Pollen transmission of petal–DNA under genotypic control in the canna hybrid taxa, J. Hered. 79 (1988) 40–44.

[6] T. D’Hertefeldt, R.B. Jørgensen, L.B. Pettersson, Long-term persistence of GM oilseed rape in field conditions in Spain, Ind. Crops Prod. 85 (2016) 22–28.

[7] I. Loureiro, E. García-Ruiz, E. Gutiérrez, P. Gómez, M.C. Escorial, M.C. Chueca, High crop barrier reduces gene flow from transgenic maize to non-defomed non-transgenic maize, Field Crops Res. 117 (2010) 232–237.

[8] F. De Marchis, Y. Wang, P. Stevanato, S. Arcioni, M. Bellucci, Genetic transformation of the sugar beet plastome, Transgenic Res. 18 (2009) 17–26.

[9] T. Capell, R. Albajes, P. Chistou, Paradoxical EU agricultural policies on genetically engineered crop safety research, Crit. Rev. Biotechnol. 34 (2015) 77–90.

[10] Y. Liu, F. Chen, X. Guan, J. Li, Characterization of competitive interactions in the coexistence of Bt-transgenic and conventional rice, BMC Biotechnol. 15 (2015) 1–12.

[11] N. Dufourmantel, M. Dubald, M. Matringe, H. Canard, F. Garcon, C. Job, E. Kay, G. Masip, M. Sabalza, E. Pérez-Massot, R. Banakar, D. Cebrian, R.M. Twyman, J. Tindamanyire, W. Tushemereirwe, R. Harding, J. Dale, Golden bananas in the field, in: M.R. Ahuja, T. Capell, R.M. Twyman, P. Christou, Going to ridiculous lengths – A gene, Proc. Natl. Acad. Sci. USA 90 (1993) 913–917.

[12] S.I. Warwick, A. Légère, M.J. Simard, T. James, Do escaped transgenes persist in the environment? The case of an herbicide resistance transgene in a weedy thuringiensis toxin (cry1Ab) gene in cabbage (Brassica oleracea var. capitata L.) by particle bombardment, Plant Cell Rep. 26 (2007) 173–177.

[13] S. Schulz, G. Weissenböck, Partial purification and characterization of a luteolin-7-glucuronide-specific β-glucuronidase from rye primary leaves (Secale cereale), J. Chromatogr. A 1041 (2004) 123–136.

[14] C. Neef, A. Knott, J. Cejpek, B. Piechulla, Plants as potential gene reservoirs: a review of studies on plants and seeds, Euphytica 179 (2011) 151–166.

[15] T. D’Hertefeldt, R.B. Jørgensen, L.B. Pettersson, Long-term persistence of GM oilseed rape in field conditions in Spain, Ind. Crops Prod. 85 (2016) 22–28.

[16] I. Loureiro, E. García-Ruiz, E. Gutiérrez, P. Gómez, M.C. Escorial, M.C. Chueca, High crop barrier reduces gene flow from transgenic maize to non-defomed non-transgenic maize, Field Crops Res. 117 (2010) 232–237.

[17] F. De Marchis, Y. Wang, P. Stevanato, S. Arcioni, M. Bellucci, Genetic transformation of the sugar beet plastome, Transgenic Res. 18 (2009) 17–26.

[18] T. Capell, R. Albajes, P. Chistou, Paradoxical EU agricultural policies on genetically engineered crop safety research, Crit. Rev. Biotechnol. 34 (2015) 77–90.

[19] Y. Liu, F. Chen, X. Guan, J. Li, Characterization of competitive interactions in the coexistence of Bt-transgenic and conventional rice, BMC Biotechnol. 15 (2015) 1–12.

[20] N. Dufourmantel, M. Dubald, M. Matringe, H. Canard, F. Garcon, C. Job, E. Kay, G. Masip, M. Sabalza, E. Pérez-Massot, R. Banakar, D. Cebrian, R.M. Twyman, J. Tindamanyire, W. Tushemereirwe, R. Harding, J. Dale, Golden bananas in the field, in: M.R. Ahuja, T. Capell, R.M. Twyman, P. Christou, Going to ridiculous lengths – A gene, Proc. Natl. Acad. Sci. USA 90 (1993) 913–917.

[21] S.I. Warwick, A. Légère, M.J. Simard, T. James, Do escaped transgenes persist in the environment? The case of an herbicide resistance transgene in a weedy thuringiensis toxin (cry1Ab) gene in cabbage (Brassica oleracea var. capitata L.) by particle bombardment, Plant Cell Rep. 26 (2007) 173–177.