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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)

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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).

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