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Field assessments of gene flow from transgenic to cultivated rice (*Oryza sativa* L.) using a herbicide resistance gene as tracer marker

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Abstract Development of plant genetic engineering has led to the deployment of transgenic crops and, simultaneously, to the need for a thorough assessment of the risks associated with their environmental release. This study investigated the occurrence of gene flow from transgenic rice to non-transgenic rice plants under agronomic conditions using a herbicide resistance gene as a tracer marker. Two field experiments were established in the paddy fields of two main Mediterranean rice-growing areas of Spain and Italy. In both locations analyses of phenotypic, molecular and segregation data showed that pollination of recipient plants with pollen of the transgenic source occurred at a significant frequency. A gene flow slightly lower than 0.1% was detected in a normal side-by-side plot design. Similar results were found in a circular plot when the plants were placed at 1-m distance from the transgenic central nucleus. A strong asymmetric distribution of the gene flow was detected among this circle and highest values (0.53%) were recorded following the direction of the dominant wind. A significant lowest value (0.01%) was found in the other circle (5 m from the transgenic plants) as was expected according to the characteristics of rice pollen. Such circular-field trial designs could also prove to be very useful in studying the gene flow to other commercial cultivars of rice with the aim of establishing strategies to prevent pollen dispersal from commercial transgenic fields to the neighbouring conventional fields.

Keywords Gene flow · Transgenic rice · *Oryza sativa* · Risk assessment · Herbicide resistance

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Introduction

The rapid development of plant genetic engineering has led to the deployment of transgenic crops and, simultaneously, for the need of a thorough assessment of the risks associated with their environmental release. Among the commonly listed risks is the escape of foreign genes through pollen dispersal from engineered plants to other cultivars of the same species or to weed relatives. Gene flow is a major concern in allogamous (cross-pollinated) species, like maize and sugar beet and self-pollinated crops with a high outcrossing rate such as rape seed, while it is considered negligible in autogamous (self-pollinated) crops, like rice, wheat and soybean (Wilcox 1987; McDonald and Copeland 1997). In self-pollinated crops, information about the isolation distance ensuring the containment of gene flow is often limited to data accumulated in production fields of certified or basic seeds by plant breeders. These recommend a minimal isolation distance to reduce accidental cross-pollination with foreign pollen to an acceptable level. Gene flow, the establishment of transgenes in natural populations and the possible associated risks have been investigated in several plant species in the last decade (Bhatia and Mitra 1998; Daniel 1999, and references cited) and more recently in rice (Mew et al. 1999; Oard et al. 2000).

Cultivated rice (*Oryza sativa* L) is an autogamous plant propagated through seeds by self-pollination. The floral architecture is one of the main factors that determine self-pollination. The time of day when the flower opens and the period of flowering in rice depend on the cultivar used and the prevailing environmental conditions (humidity and temperature). The lemma and palea separate and the stigmas protrude, followed rapidly by

the anther bursting, so that the stigma of a flower receives pollen from the anthers of the same flower, hence resulting in self-pollination. The flowers open from the tip of the panicle downwards, over a period of a few days. The time the flower remains open appears to depend on the humidity and is from as little as 6 min to more than 1 h (Grist 1986). Rice pollen grains lose their viability within 5 min of shedding from the anther. In exceptional cases, a few pollen grains may remain viable for up to 15 min. The horizontal movement of pollen is limited. An isolation distance of 10 m is considered sufficient in hybrid seed-production fields to avoid contamination with pollen from adjacent fields (Khush 1993).

Nevertheless, cross-pollination is possible and does indeed take place to some extent, the amount depending largely on climatic and variety differences. The degree of out-crossing is generally higher in indica cultivars and wild species than in japonica cultivars (Oka 1988). Lord (1935) found that in two climatically different districts of Sri Lanka, the amount of natural crossing-frequency ranged from 0.34 to 0.67%, while Brown (1957) in Malaysia obtained 0.41% for natural cross-pollination. Srinivasan and Subramanian (1961) in India found than natural cross-pollination ranged from 0.04 to 0.03% and the distance up to which it took place ranged from 1.8 to 2.1 m from the pollinating agent. More recently, Reano and Pharm (1998) demonstrated that cross-pollination could occur to some extent depending on the variety and the planting designs used.

The possibility of disseminating transgenes and conferring resistance to chemicals, abiotic stresses, pests and diseases in the environment through pollen is a particularly major concern when the genetically engineered cultivar is grown in areas where sexually compatible, related wild species naturally exist. Cross-pollination between wild species of the *Oryza* genus and *O. sativa* cultivars has been reported to occur in natural habitats (Oka and Chang 1961). Natural hybrid swarms between *O. sativa* and *Oryza glaberrima*, and *O. sativa* and *Oryza rufipogon,* are found in African and Asian rice-growing areas, respectively. However, zygote inviability, hybrid weakness, hybrid sterility and hybrid breakdown often reported in distant crosses, may reduce the success of hybridization and the establishment of progenies in the environment.

Rice cultivars cross easily with their related weed form (red rice) found in direct-seeded paddy fields and produce viable and fertile hybrids. Hybridisation rates ranging from 1.08% to 52.18% were found by collecting 12,000 seeds from red rice plants in fields of six different rice varieties, and by determining the incidence of hybrid progenies by isozyme analyses (Langevin et al. 1990). Differences among varieties were thought to be due to the differential flowering times of red rice and cultivated rice. More recently, using RFLP analysis, 24 strains of Korean weedy rice were classified into two groups identical to the short- and long-grain types by morphological and physiological characters. It was deduced that the short-grain strains of Korean weedy rice belonged to Japonica, while the long-grain strains were closer to the Indica type (Cho et al. 1995). Similarly, based on morpho-physiology, isozymes and RAPD markers, some weedy rice types have been classified into japonica- or indica-type, demonstrating that some of these weedy rices might have originated at least partly from gene flow between indica, japonica or both types (Suh et al. 1997). Moreover, in the case of red rice, gene flow from the weedy rice to transgenic rice has also to be considered, because of the inheritance of the dominant wild traits (Langevin 1990; Noldin et al. 1999), which may lead to the conversion of transgenic rice to a transgenic red rice hybrid weed. In the case of oilseed rape, for instance, it has been shown that the frequency of pollination from wild radish (*Raphanus raphanistrum* L.) to transgenic oilseed rape under agronomic conditions is higher than that of the reciprocal cross (Chèvre et al. 2000). Recent analyses of the segregation of the *bar* gene and the evaluation of the traits in individuals produced from controlled crosses between ammonium glufosinate-resistant transgenic rice lines and red rice has shown that the transfer of the *bar* gene did not alter fitness values for traits such as dormancy or seed production which were associated with the reproductive success of the hybrids (Oard et al. 2000).

A diverse range of genetically engineered genes has already been introduced into rice (recently reviewed in Tyagi and Mohanty 2000). Although no commercial release has been reported, it is expected in the next few years, and numerous field trials have already been conducted (e.g. Oard et al. 1996, 2000; Tu et al. 2000 a,b). Given the ecological importance of the rice crop grown on 140 million hectares world wide, it is important to determine the extent of pollen dissemination from transgenic rice to other cultivars and wild relatives in various environmental conditions.

Transgenic plants with a marker gene, such as herbicide resistance, are specifically useful for investigating pollen dissemination in controlled experimental field trials. Among the marker genes available, the *bar* and *pat* genes, both encoding phosphinothricin acetyl transferase and conferring resistance to the broad spectrum herbicide ammonium glufosinate, are particularly suitable and have been extensively used to determine the hybridisation rate between cultivated crops and their wild relatives, notably in rapeseed (*Brassica napus* L.) (Baranger et al. 1995; Metz et al. 1995; Scheffler et al. 1995; Chèvre et al. 1998, 2000).

The objective of the present study was to assess the frequency of gene flow from transgenic plants to non-transgenic plants of the Mediterranean *japonica* rice varieties. Of particular interest was the evaluation of pollen dispersal and flight distance in open-field conditions to nearby cultivated rice fields. Another objective was to evaluate the influence of the wind on pollen dispersal and cross-pollination under field conditions.

Materials and methods

Plant material

Transgenic lines T1506–5-6 and A2504–1-7 of the commercial rice mediterranean cultivars Thaibonnet and Ariete used in this study were both obtained by polyethylene glycol-mediated transformation of protoplasts isolated from immature embryo callus-derived cell-suspension cultures, essentially as described by Chair et al. (1996).

Protoplasts were co-transformed with a mix of pUC18 derivatives bearing cassettes p35S:*pat:*CaMVt (p35SAc plasmid kindly supplied by P. Eckès, AgrEvo, Germany) and either pΔ1176:*gusA*: nost (pΔ1176 plasmid)(for the T1506–5-6 event) or pEMU-*cry1Ac*-nost (pEmu-*cry1Ac*) (for the A2504–1-7 event).

The p35S:*pat:*CaMVt cassette contained a synthetic sequence of the *pat* gene derived from *Streptomyces viridochromogenes*, encoding phosphinotricin-acetyl-transferase that confers resistance to the herbicide ammonium glufosinate via de-toxification through acetylation, under the control of the promoter CaMV35S.

The pΔ1176:*gusA*:nost construct contained the *gusA* encoding β-glucuronidase under the control of the full-length promoter of the rice *ltp1* gene (Vignols, unpublished), whereas the pEMU:*cry1Ac:*nost cassette consisted of the synthetic sequence of the *cry1Ac* gene (Sardana et al. 1996) directed by the composite pEmu promoter (Last et al. 1991).

Detailed Southern-blot analyses of the T1506–5-6 and A2507–1-7 transgenic lines using appropriate restriction enzymes and probes consisting of *pat*, *gusA* or *cry1Ac* coding sequences revealed that T1506–5-6 contained both a complete and a rearranged cassette of the p35SAc plasmid and a truncated cassette of the pΔ1176 plasmid, whereas the A2504–1-7 event had integrated a single complete copy of the p35SAc cassette and at least three copies of pEmu-*cry1Ac*, two of which were rearranged (data not shown). Histochemical and fluorimetrical GUS assays revealed that, as expected, the *gusA* gene was not expressed in the T1506–5-6 event. Immunoblot analyses of protein extracts of leaf tissues of A2504–1-7 using polyclonal antibodies raised against cry1Ac did not allow detection of the toxin, suggesting that the *cry1Ac* gene was not expressed, or only at a very low level, in the transgenic line.

Co-segregation of the inserted copies of the *pat* gene and resistance to spraying with an aqueous solution of ammonium glufosinate equivalent to 750 g/ha was observed through analyses of T1 progeny plants of the two events. The two genomic fragments hybridizing to the *pat* probe in T1506–5-6 segregated in a linked manner in the T1 progeny, indicating that the two copies of the plasmid had integrated at a single genetic locus. Homozygous lines of both events were identified through spraying of T2 seedlings at the 4–5 leaf stage with ammonium glufosinate, and further seed was increased in the greenhouse to obtain sufficient T4 seed material for the field trial.

Field designs used

Transgenic T4 (T1506–5-6 and A2504–1-7) and certified non-transgenic seeds of Thaibonnet and Ariete were sown on peat-vermiculite substrate under greenhouse conditions and transplanted in the paddy field when they reached the 4–5 leaf stage in June 1999. To evaluate the gene flow from transgenic to non-transgenic plants two different designs were used in two separate field trials: a normal side by side plot design in Mortara (Italy) to investigate the influence of distance from the margins (borders) of the non-transgenic rice field on the frequency of pollination of a recipient plant by transgenic pollen, and a circular design conducted in Amposta (Spain) aimed at evaluating the influence of the wind on pollen dispersal and flight distance in open field conditions to nearby cultivated rice fields. In Amposta, 176 transgenic plants were transplanted in the centre (seven concentric circles) and 226 non-transgenic plants were placed in three concentric circles in such a way that the inner circle was 1 m distant from the outer transgenic plants (Assay A). In assay B, 528 non-transgenic plants were also planted in three concentric circles but the inner one was situated 5 m from the outer transgenic plants (Fig. 1a). In both assays the distance between plants and between circles was 25 cm. All panicles of the non-transgenic plants were individually harvested manually, and their localisation (in relation to the geographic orientation) on the field was recorded. In Mortara, each individual 2.4×3m plot involved 12 rows of 15 plants with spaces of 20 cm between them, within the rows and between rows (Fig. 1b). The rows of transgenic and non-transgenic seedlings were transplanted side by side so that the recipient non-transgenic plot plants were downwind. Consequently the non-transgenic Ariete "recipient" plants were situated at a distance of 20 to 240 cm from the transgenic pollinator, but only rows at a distance of 20, 40, 80, 160 and 240 cm from the 3-m row of the transgenic "source" were harvested. Five plots (replicates) were made and each plot was used to collect the panicles. To facilitate cross-pollination, the orientation of the rows was perpendicular to the prevalent wind direction. At harvesting the seeds were collected separately for each non-transgenic plant and the remaining plant material collected in bulk and destroyed by incineration.

In both locations, the trials were conducted under standard seed-production practices and covered with an anti-hail net to avoid bird damage and seed dissemination at the end of the flowering.

The trials were approved by the National and Regional Spanish and Italian Biosafety Authorities under files B/ES/99/16 and B/IT/99/12 respectively.

Gene-flow evaluation

Samples of seeds harvested in Amposta from each non-transgenic Thaibonnet plant were sown in 48×28×7-cm trays containing peat-vermiculite substrate in greenhouse conditions. In previous experiments, the germination rate was determined and seed samples were weighted in order to obtain an average of 500 seedlings per tray. For every 12 trays a sample of 50 seeds of the transgenic T1506–5-6 line were also sown to use as a positive control. Seedlings at the 3–4-leaf stage were treated with commercial herbicide (Finale from AgrEvo Co.). All of these plants were treated with a lower dosage (equivalent to 200 g of active ingredient/ha) than commercially recommended and can sustain the T1506–5-6 line to avoid accidental losses of the putative transgenic seedlings at a hemizygous stage. After 3–4 weeks, all surviving seedlings were transferred to individual pots for further development and final harvest. A total of 105,000 seedlings from assay A and 110,000 seedlings from assay B were analysed. To determine the influence of the field localisation of the recipient plant on the presence of herbicide-resistant plants in its progeny, a statistical analysis was carried out by using the SAS GLM procedure for the agronomic data. The Watson one-sample U^2 test (Watson 1962) specifically appropriated for circular distributions was used to test the null hypothesis of uniformity among the circles.

In Mortara, all of the 57,000 seeds harvested from the non-transgenic Ariete plants were analysed according to the distance from the transgenic pollinator A 2504–1-7. The seeds were sown in trays (55×40×9 cm) containing a peat-sand substrate in the greenhouse with 28°C during the day and 22°C at night and 12 hours light. The germination rate was calculated by counting the sown seeds with a seed-counting machine and scoring the germinated plants in each tray. Given the differences in the germination level of the seeds collected from single plants, the percent of gene flow was calculated with respect to the number of germinated seeds. The level of herbicide resistance resulting from expression of the *pat* gene in the transgenic line A 2504–1-7 was checked before starting the screening and, considering the high level of resistance of the transgenic line in our greenhouse conditions, the plants were treated with the equivalent of 840 g of active ingredient per ha of glufosinate ammonium present in the formulate Basta (Agrevo). The seedlings were sprayed at a 4–5 leaf stage using 50 ml of aqueous solution per square meter. The material was scored at 5 and 10 days and the Ariete-resistant seedling plants transplanted to pots for further development and harvest.

PCR and Southern analysis

Genomic DNA from leaves of the resistant plants was extracted by the CTAB method (Doyle and Doyle 1990) and the presence of the *pat* transgene verified first by PCR analysis. For the resistant plants collected in Amposta a pair of primers (pat F: ccggagaggagaccagttg, and pat R: ctggccttggaggagctgg) was designed to amplify a DNA fragment of 523 bp within the *pat* gene. For the resistant plants collected in Mortara, two 35 S promoter-specific primers (IGP264-F: gctcctacaaatgccatcattgcg, and IGP265-R: gcgtcatcccttacgtcagtggag) amplified a 181-bp fragment of the regulatory region of the transgene. The PCR reaction mixture contained 50 ng of template DNA, 1×LAB buffer, 0.4°µM of each primer, 0.4 mM of dNTPs, 1 mM of $MgCl_2$ and 2.5 units of *Taq* DNA polymerase in a total volume of 25°µl. The template DNA was initially denatured at 94°C for 5 min, followed by 35 cycles of PCR amplification with the following parameters: 20 s at 94°C, 20 s at 65°C and 30 s extension at 72°C. The amplified products were resolved by electrophoresis on a 2% agarose gel in a 0.25× NEB buffer.

Genomic DNA isolated from leaves (10 ng) was separately digested with the enzymes HindIII and EcoRI which respectively cut once, releasing the gene cassette in p35Ac. The DNA fragments were separated by electrophoresis in a 1% agarose gel and then transferred to a nylon membrane (Hybond). The probe used was the 523-bp fragment of the *pat* gene amplified by PCR using the same conditions as indicated above.

Segregation studies

To ascertain that the *pat* gene was at the hemizygous stage in the ammonium glufosinate-resistant seedlings, segregation of the herbicide resistance trait was analysed in progenies of 20 herbicide-tolerant, PCR and Southern-positive Thaibonnet plants. Twenty four seeds from each plant were disinfected with commercial bleach (1.6% active chloride) for 20 min, rinsed three times with sterile distilled water, and inoculated in culture tubes containing MS medium (Murashige and Skoog 1962). Following 5 days incubation in a culture chamber at 25±2°C with a 16-h photoperiod provided by fluorescent lamps (Sylvania cool white) developing plantlets were transferred to MS medium supplemented with 2 mg/l of phosphinothricin (PPT Sigma). Segregation was determined by counting the number of dead seedlings after 3 weeks in culture.

Segregation analysis was also performed in progeny plants of the ammonium glufosinate-resistant Ariete plants with a panicle-to-row scheme on a tray of the same type and at the same conditions of gene-flow evaluation. The seedlings were sprayed at a 840 g/ha active ingredient concentration, and the segregation of resistant to sensitive plants was scored after 15 days.

Results

Field observations

The agronomic behaviour of transgenic and non-transgenic plants was normal and similar to a standard culture in the field trial established in Amposta (Fig. 1a) where 50% of both transgenic and non-transgenic plants formed their first spikes 7 weeks after the plants were planted in soil conditions, and 50% of the grain ripening occurred approximately 3 months after planting. The number of panicles/plant was 19.79±0.81 (transgenic) and 19.79±0.51 (non-transgenic), whereas the plant height was 93.63±0.53 for transgenic and 93.30±0.43 for non-transgenic plants. These values for the main parameters of plant growth perfectly matched the standard values of the Thaibonnet crop. In Mortara the flowering was from August 21 to 26 for both Ariete and A2504–1-7, 8 weeks after transplanting (Fig. 1b). The number of panicles/plant was 9.57±0.32 (transgenic) and 11.28± 0.43 (non-transgenic) whereas the plant height was 70.21±0.63 (transgenic) and 73.70±0.54 for non-transgenic plants.

In both locations, flowering was synchronous between the non-transgenic and transgenic plots throughout the flowering period, thereby providing maximum opportunity for pollen transfer.

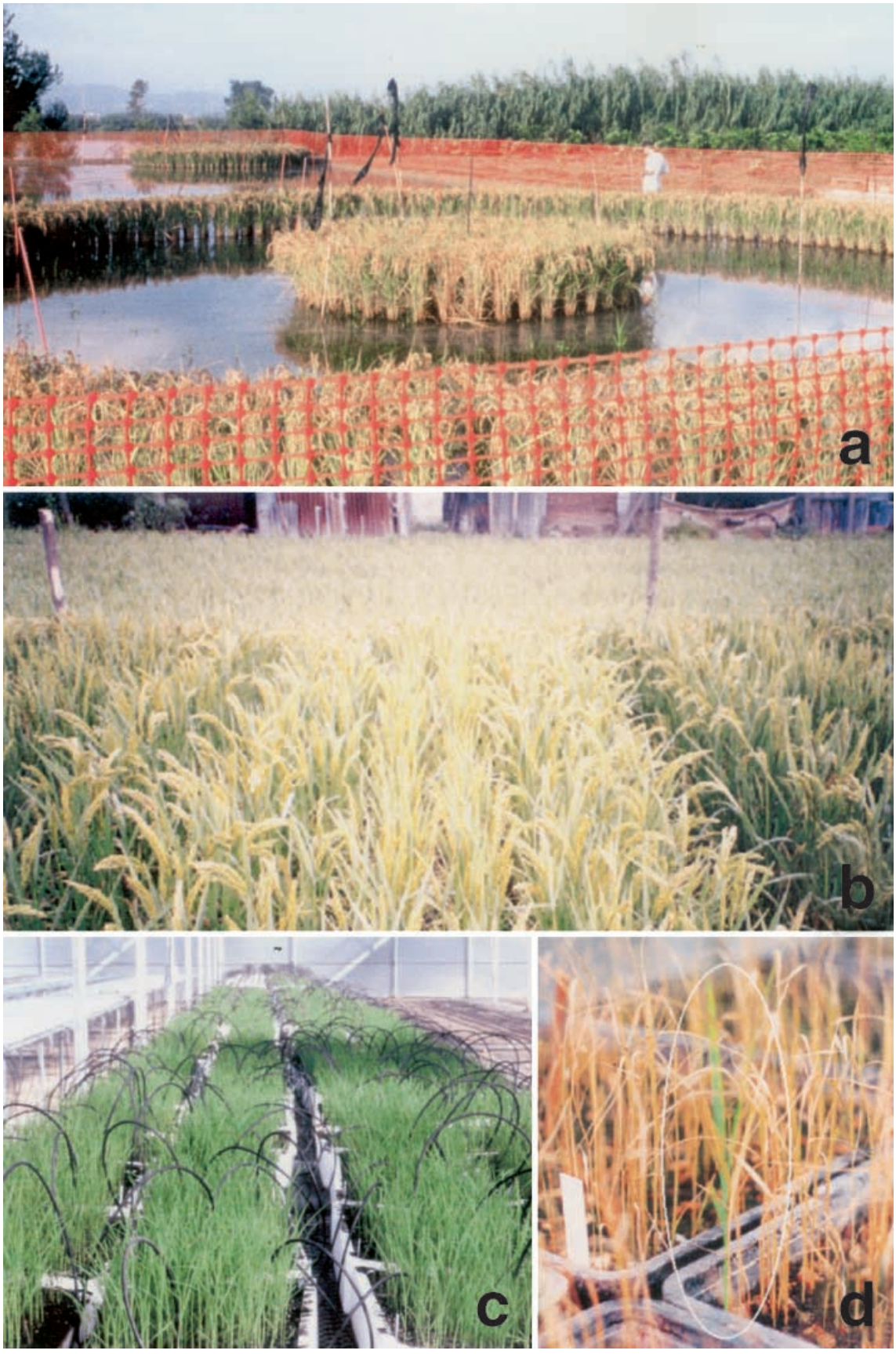
Assessment of gene flow frequencies

For the seeds harvested from recipient Thaibonnet plants grown in the external circular plots, the average number of seedlings per tray (estimated from 20 trays) was 503±12 (Fig. 1c). Three to four weeks after spraying with ammonium glufosinate equivalent to 200 g/ha, 398 and 587 plants survived among seeds collected from recipient plants of the assay A and B, respectively. The surviving plants were transferred to individual pots for further development, leaf DNA isolation, and subsequent PCR and Southern-blot analyses. One hundred plants were PCR-positive in circle A and 13 were PCR-positive in circle B (Fig. 2a). Integration of the *pat* gene was confirmed by Southern analysis in a sample of PCR-positive seedlings. As shown in Fig. 3, the PCR-positive seedlings exhibited the same pattern of integration of the *pat* gene as the source plants (T1506–5-6) used in the field trial. Taking into account that 105,000 and 110,000 seedlings from circle A and B have been screened respectively, we can conclude that the gene flow from transgenic to non-transgenic Thaibonnet plants occurred at a 0.091% and a 0.010% frequency for plants grown at a 1- and 5-m distance from the transgenic plot, respectively.

In the Mortara trial, the frequency of Ariete seedlings resistant to ammonium glufosinate treatment (Fig. 1d) ranged from 0.08 in the first row of recipient plants, situated 0.2 m adjacent to the transgenic source plot, to 0 at a distance of 2.4 m, decreasing rapidly while the distance increased. The presence of the *pat* transgene was also confirmed by PCR (Fig. 2b).

To confirm that the herbicide-resistant seedlings identified in the progeny seeds of recipient non-transgenic Thaibonnet and Ariete plants were the result of hybridisation with the pollen of the transgenic plants, rather than from a contaminating seed, we examined the segregation of the herbicide resistance trait in progenies of a sub-sample of 20 Thaibonnet and of the 14 Ariete plants

Fig. 1A–D Field trials and analysis of collected seedlings from non-transgenic plants. **A** General view of field trials in Amposta (Spain). **B** Field plot in Montara (Italy). **C** Seedlings from non-transgenic plants before the herbicide treatment (500 seedlings/flat). **D** Detail of a glufosinate-resistant green plant in the Ariete population harvested in the field ▶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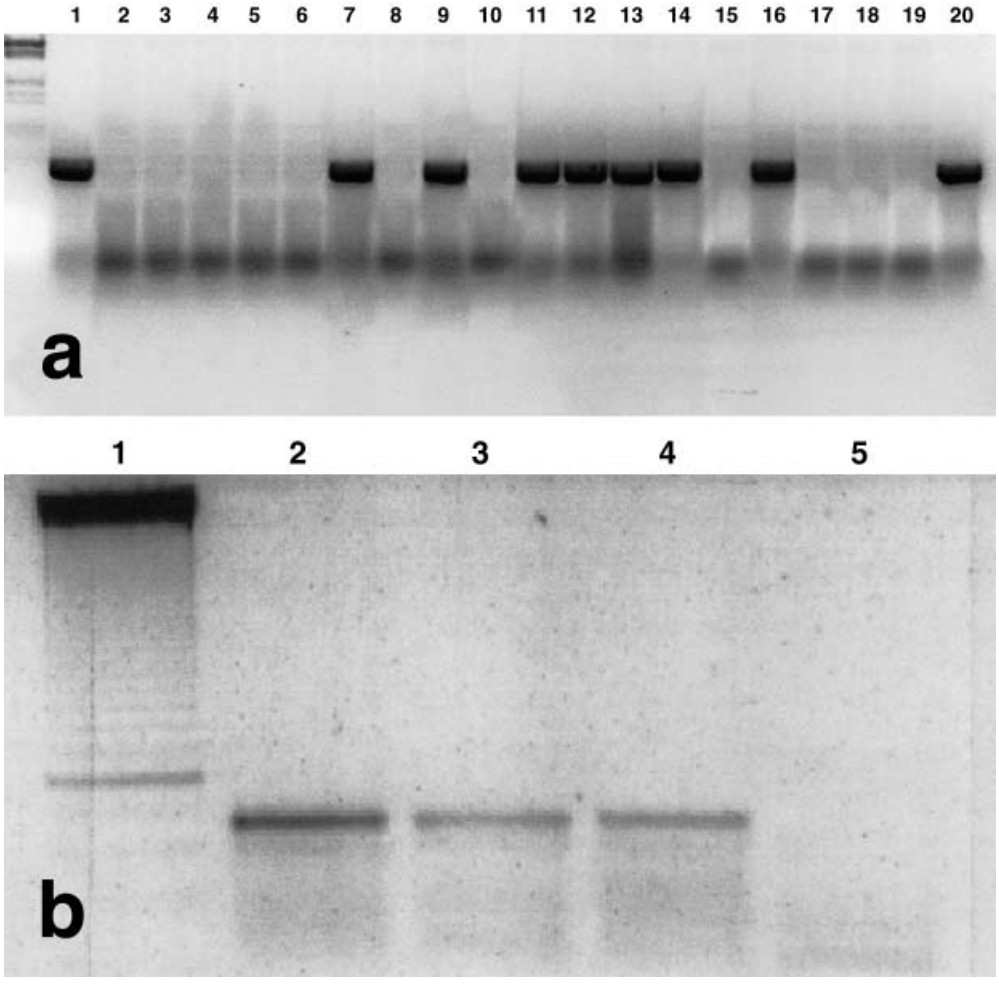

Fig. 2a, b PCR amplification of resistant seedlings. **a** Amplification of a 523-bp DNA fragment of the *pat* gene. *Lane 2* Non-transgenic control plant. *Lanes 1 and 20* Transgenic T506 plants with the *pat* gene. *Lanes 3 to 19* Progeny of non-transgenic plants cultivated in the field around the transgenic plants. **b** PCR test used to verify the presence of the transgene in the Ariete glufosinate resistant plants identified in the greenhouse. *Lane 1* Molecular marker; *lane 2* transgenic line A 2504–1-7; *lanes 3 and 4* resistant plants found in the seed population

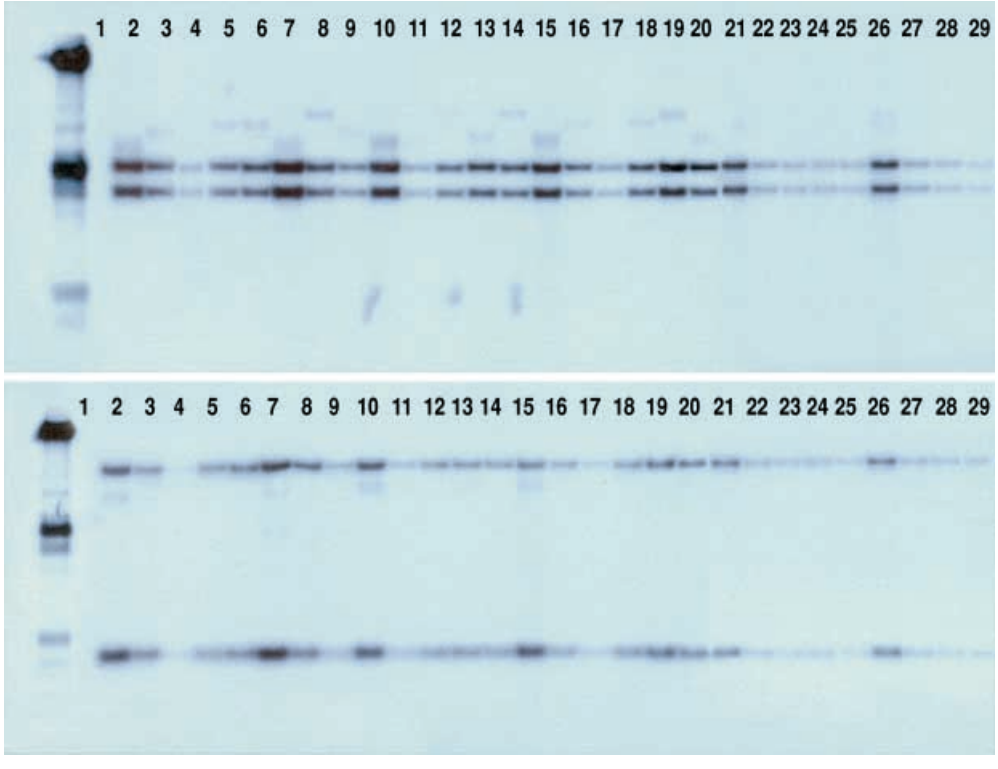

Fig. 3 Southern analysis of the Thaibonnet field trial rice plants. Genomic DNA from leaves was digested with the enzymes *Hin*dIII (above) and *Eco*RI (below). All the samples were hybridised with a probe of 523-bp fragment of the *pat* gene amplified by PCR. *Lane 1* Non-transformed plant. *Lane 2* Transgenic T506 plant. *Lane 3* progeny of T506. *Lanes 4 to 29* Progeny of non-transgenic plants cultivated around the transgenic plants in the circle A

Table 1 Number of glufosinate resistant plants found in the 1999 field trials of Mortara (Italy) designed to measure the level of gene flow trough pollen in the self-pollinated rice cultivar Ariete

Pollinator receiver distance (cm)	Number of seeds tested	Number of resistant plants	Percent of gene flow	Number of PCR+plants	Number of progenies segregating in a 3:1 ratio
20	11,540	9	0.08	9	9
40	10,300	2	0.02	2	2
80	11,650	0	0.00	–	–
160	11,200	3	0.03	3	3
240	12,500	0	0.00	–	–

Table 2 Transgenic seedlings found in each circle, grouped giving their orientation in the circle. The eight groups were chosen according to the wind compass card. The average of the PCR+ is expressed as a ratio of the 1,000 seedlings tested

Orientation degrees	Wind to	Circle A		Circle B	
		n plants tested	Seedlings PCR+/1,000	*n* plants tested	Seedlings PCR+/1,000
67.50–112.50	E	9	0.07±0.07	22	0.14±0.16
112.50–157.50	SE	8	0.15±0.10	21	0.06±0.11
157.50–202.5	S	9	0.07±0.07	22	0.0
202.50–247.50	SW	9	0.22±0.11	22	0.22±0.19
247.50–292.50	W	9	0.29±0.23	22	0.06±0.11
292.50–337.50	NW	9	5.26±3.44	21	0.14±0.16
337.50–22.50	N	9	1.41±0.23	22	0.22±0.19
22.50–67.50	NE	8	0.15±0.10	22	0.06±0.11

surviving the ammonium glufosinate spraying. The 3:1 ratio expected for segregation of a transgene at a hemizygous status was accepted ($\chi^2<3.5$) in the progenies of all the Thaibonnet plants tested through in vitro culture on medium supplemented with 2 mg/l of PPT. The progenies of the 14 herbicide-resistant Ariete plants also segregated in a 3:1 ratio (Table 1). This demonstrates that the mother plants are the result of cross-pollination with the pollen from the transgenic plants.

Influence of the wind on pollen movement from transgenic plants

Concerning the influence of the wind, Table 2 shows the localisation of transgenic seedlings in the circular assays, grouped by the eight principal orientations on the compass card. The frequency of transgenic seeds in circle B was very low ranging from 0 to 0.022% with a global average of 0.01%, whereas in circle A, great variability, ranging from 0.526% at the NW to 0.007%, was found with similar low values in the other groups. Figure 4 shows a more accurate representation of the results of the field trial because the plants are not grouped in artificial sectors but every analysed plant is represented exactly in its position and orientation on the circle. Although the two circles in the field were separated, they are represented in a concentric plot to compare the results more easily. Analysing the circular distribution of the plants on circle B with PCR-positive seedlings, and applying the Watson one sample U^2 test, the null hypothesis cannot be rejected. The sample data is from a population uniformly or randomly distributed with no vectorial effect.

Applying the same test to circle A, the null hypothesis ($p<0.0001$) must be rejected, demonstrating the strong influence of wind on the direction of transgenic pollen flow. When non-transgenic plants were placed at a 1-m distance from transgenic plants, 90% of transgenic seedlings were harvested from a narrow range of plants situated in the NWN direction. This effect is probably due to the local wind coming from the SSE, called “Vent de Baix”, which is a dominant wind during the central hours of summer days. Data collected from the meteorological station 500 m away from the assay trial confirm this hypothesis. This wind results from the differential heating between the ground and the sea, and is consistent in its direction due to the flat orography of the Delta de l’Ebre region.

This influence of the dominant wind was not detected in circle B, where non-transgenic plants were growing at a 5-m distance from the transgenic central nucleus. PCR-positive seedlings found in this circle came from plants randomly distributed among the circle and have a certain similarity to the distribution on circle A if data coming from plants placed at the NWN direction are not considered. In fact, when the Watson one-sample U^2 test is applied to data from circle A without the points ranging from NW to N, the H_0 would not be rejected at the $p=0.05$ level.

Another factor in addition to the wind that produces this residual effect could be insects or culture management. There is insufficient data to characterise these effects. A radial pattern decreasing with the distance from the centre could suggest that movement of insects is the cause, whereas a lineal pattern suggests accidental or occasional activity, such as human activity, as a cause of this detected flow.

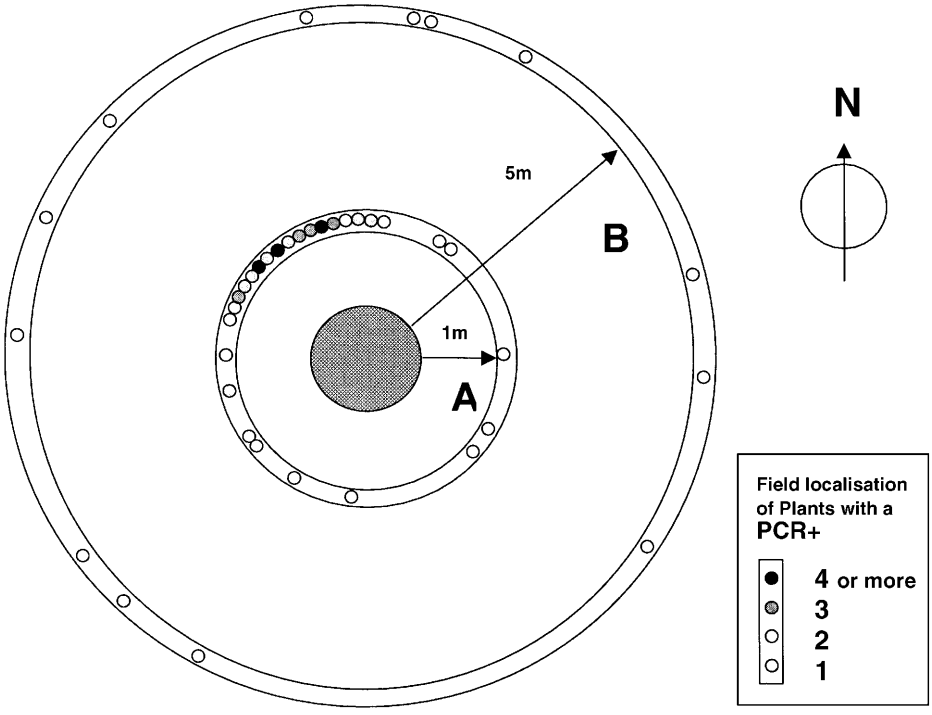

Fig. 4 Circular distribution of the plants from which PCR+ seedlings were identified

Discussion

In both locations we observed that pollination of recipient plants with pollen of the transgenic source did occur at a significant frequency, based on the analyses of phenotypic, molecular and segregation data.

Agronomic data from the Thaibonnet trial suggest that transgenic plant morphology is similar to non-transgenic plants. The transgenic plants maintain their general performance probably due to the proper integration of the transgenes, as in the case of Bt genes introduced in other commercial rice cultivars (Tu et al. 2000a). This contrasts with previous field evaluations of transgenic rice line cv Taipei 309 with the *npt*II gene. Plants were shorter, had delayed maturity and exhibited reduced fertility (Lynch et al. 1995). Also, trangenic rice lines of cvs Gulfmont and Koshihiraki with the *bar* gene exhibited significant differences in days to 50%-heading, plant height and grain yield (Oard et al. 1996). Even though transgenic Ariete plants tested in Mortara did not exhibit significant differences in days to 50%-heading, they were shorter and produced less panicles/plant than control plants.

In the two present trials, the probability of gene flow may have been favoured by using a transgenic line and its non-transgenic counterpart of the same cultivar, with a perfect match in the flowering period. In both locations, however, no climatic condition were encountered that would have decreased the fertility of the recipient plants.

The area of transgenic donor source determining the pollen load was limited (i.e. 9.62 m^2 in each one of the circular plots of Amposta) and not comparable with commercial fields. A scale-dependent effect of the donor plot on the frequency and distance of gene flow has been reported in crops such as alfalfa (St. Amand et al. 2000), with rates being ten-fold higher in field trials than in experimental plots. Therefore, gene flow may be underestimated compared to the commercial release of a transgenic rice cultivar. It seems reasonable to find these differences when the effective distance of pollen dissemination is much greater than the width of the experimental field used, because all plants can export their pollen out of the field. But if the distance of pollen dissemination is less than the field width, increasing the field size will not increase the amount of pollen found outside of the experimental field because pollen from plants placed in the inner part of the field will not reach the borders. Dissemination of rice pollen is considered low because anther dehiscence usually occurs just before, or when, the lemma and palea open; consequently, most pollen grains fall onto the stigma. For this reason self-pollination is favoured rather than cross-pollination. Taking into account the low dissemination of rice pollen, its low viability (Yoshida 1981) and the size of the experimental plots used, we conclude that the transgenic flow detected in this study correlates with values that we can expect in Senia rice fields.

Rice is a self-pollinated crop with a floral structure that theoretically limits the occurrence of gene flow. Moreover, rice pollen grains are viable for only few minutes after emerging from the anther whereas the stigma can be fertilised for 3 to 7 days (Yoshida 1981). In spite of these favourable features, our report confirms the findings reported in the literature, and common observations of breeders in selection plots that cross-pollination does occur in rice, though at very low frequencies. The frequency depends on climatic and cultivar differences (Lord 1932; Brown 1957; Srinivasan and Subramanian 1961). Reano and Pham (1998) demonstrated that the

planting design used could also influence the cross-pollination in rice.

The concentric-circle-plot layout set up in Amposta detected transgenic gene flow. Pollen dissemination was likely as the result of the influence of the wind. The influence of local winds in the pollination process has been established. Such circular field trial designs, could also prove very useful in studying the gene flow to other lines or commercial cultivars of rice exhibiting a range of vegetative and reproductive features (such as fertility, height, flowering period), with the aim of establishing strategies to prevent pollen dispersal from commercial transgenic fields to the neighbouring conventional fields. As the commercial release of transgenic rice becomes a reality, such trials should also be established to determine the reciprocal gene flow frequencies between transgenic rice and its weed relatives, such as red rice.

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