*Assessment of the pollen-mediated transgene flow from the plants of herbicide resistant wheat to conventional wheat (*Triticum aestivum *L.)*

Dmitry Miroshnichenko, Alexander Pushin & Sergey Dolgov

Euphytica
International Journal of Plant Breeding

ISSN 0014-2336

Euphytica
DOI 10.1007/s10681-016-1637-y

Springer

Euphytica
DOI 10.1007/s10681-016-1637-y

Assessment of the pollen-mediated transgene flow from the plants of herbicide resistant wheat to conventional wheat (*Triticum aestivum* L.)

Dmitry Miroshnichenko · Alexander Pushin · Sergey Dolgov

Received: 11 September 2015 / Accepted: 6 January 2016

Abstract Three-year field assessment of gene flow from the genetically modified herbicide-resistant spring wheat variety 'Andros' to its non-transgenic counterpart has been conducted. A circular field trial design where the wheat plants containing the *bar* and *gfp* genes were planted in a central plot, while the recipient non-transgenic plants were grown in eight compass sectors at a distance of 1–5 m from the pollen source, has been developed. Gene flow was analyzed by testing the glufosinate-based herbicide resistance in seedling progeny. The phenotypic and molecular examination of more than 712,000 germinated seeds allowed us to draw a conclusion that the pollen-mediated transgene flow might occur at a low frequency (<0.8 %) even if the nontransgenic wheat was located in the proximity to its transgenic counterpart. The strong asymmetric distribution of gene flow and the maximum outcrossing rate were detected in compass sectors following the direction of the dominant wind. The gene flow rate averaged over all wind direction varied in different years from 0.134 to 0.416 %. The dramatic reduction in transgene flow frequencies was observed while increasing the distance between transgenic and non-transgenic plots. The rise of average gene flow due to significant increase of the transgene pollen area was not confirmed statistically. These results predict that the pollen-mediated transgene flow in spring wheat can be maintained at negligible levels while the existence of a short isolation distance in order to prevent contamination of adjacent non-GM wheat.

Keywords *bar* and *gfp* genes · Genetically modified (GM) · Isolation distance · Risk assessment · Transgenes · Wheat

D. Miroshnichenko (✉) · A. Pushin · S. Dolgov
Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Science Ave 6, Pushchino, Moscow Region, Russian Federation 142290
e-mail: miroshnichenko@bibch.ru

D. Miroshnichenko · A. Pushin · S. Dolgov
All Russian Research Institute of Agricultural Biotechnology, Timiryazevskaja 42, Moscow, Russian Federation 127550

Introduction

The last decade the commercial production of GM crops has been notably increased worldwide. According to ISAAA in 2014 more than 18 million small and large farmers from 28 countries planted about 181 million hectares of biotech crops, such as maize, soybean, canola, cotton, sugarbeet, alfalfa, papaya, potato (James 2014). Although no biotech wheat has yet been officially approved for commercial cultivation, it is expected that GM wheat varieties could be released within the near future. Experimental lines of transgenic wheat with such traits as herbicide

Published online: 18 January 2016

resistance, disease and insect resistance, salt and heat tolerance have been already developed and tested in the fields of different countries (Dunwell 2014). Given the commercial and nutritional importance of the wheat crop (grown on 220 million hectares worldwide) (FAOSTAT 2014), it's important to determine the potential risk associated with GM-wheat release.

A possible consequence of release of GM crops can be unintended transgene flow to non-GM crop counterparts (crop-to-crop) and wild/weedy relative species (crop-to-wild) (Ellstrand 2003). Transgene flow can mainly occur by two ways: hybridization through pollen movement and direct movement of seeds (Chandler and Dunwell 2008). Wheat is primarily self-pollinated crop, so it's classified as an inbreeding species. However, some cross-pollination can occur due to transferring by the wind of viable wheat pollen. Some factors which can influence the frequency of gene transfer include spatial overlap or proximal geographic location between pollen receptors and pollen donor plants, pollen longevity and dispersal, asynchronous flowering of GM plants and their non-transgenic counterparts (Devos et al. 2005; Lu and Snow 2005; Loureiro et al. 2011). Pollen-mediated gene flow is not unique for genetically-modified crops; however, the process has received renewed attention within two last decades because of the new legislation on cultivars to ensure commercial co-existence of non-GM and GM grain production.

The pollen of bread wheat is relatively heavy, for this reason near 90 % of wheat pollen fell down within 3–8 m from its source (Jensen 1968; Hucl and Matus-Cádiz 2001). Under field condition wheat pollen lost its fertilizing capacity after 15–20 min, however in some circumstances the period of viability could be prolonged up to three hours (D'Souza 1970). Wheat pollen is produced in a relatively small amount (10,000 grains per anther), nevertheless a significant portion of pollen can be shed from floret and transferred by the wind as far as 1 km from the source (De Vries 1971; Khan et al. 1973; Virmani and Edwards 1983).

To date there are very few field experiments with the view of measuring the pollen mediated transgene flow from GM wheat to conventional wheat varieties. In 1996–1998 transgenic wheat plants contained *bar* gene conferring the resistance to glufosinate-based herbicides were grown in South Australia and Australian Capital Territory (Gatford et al. 2006). Several field trials involved transgenic wheat lines expressed two pathogenesis-related genes (*chi*, *glu*) and herbicide resistance gene (*bar*) were planted in Switzerland in 2008–2010 (Riebien et al. 2011; Foetzki et al. 2012). In cereal biotechnology *bar* (*pat*) gene conferring the resistance to phosphinotricin (PPT), the active ingredient of glufosinate ammonium based commercial herbicides, is commonly used for transgenic plants generation (Jones and Sparks 2009). GM wheat plants expressing *bar* gene successfully survive the spraying with a high herbicide concentration up to 100 mg PPT/L (Cannell et al. 1999; Barro et al. 2002; Becker et al. 2004). Generally the same level of herbicide tolerance exists between homozygous and heterozygous T_1–T_n transgenic wheat progenies (Miroshnichenko et al. 2007a; Foetzki et al. 2012).

Due to regulatory constrains the measurement of gene flow in the most experiments was carried out without the use of GM wheat by planting non transgenic wheat varieties possessing homozygous dominant gene markers, such as the blue-aleurone trait (Matus-Cádiz et al. 2004; Hanson et al. 2005), imidazolinone resistance trait (Gaines et al. 2007; Willenborg et al. 2009; Beckie et al. 2011) or chlortoluron tolerance trait (Loureiro et al. 2012). Usually both circular and rectangular field experimental designs are adopted to monitor gene flow (Mallory-Smith et al. 2015). In the circular design the pollen donor is located at the center, while the recipients are sown at various distances to form concentric circles or compass plots. In the rectangular design, the pollinator and recipient blocks are located side by side and the recipient blocks are planted at varying distances.

Pollen mediated gene flow experiments conducted by different authors, showed that the outcrossing rates can greatly vary even at the same isolation distance due to the tendency for some cultivars to possess higher cross-pollination rate. In most studies, at the distance below 1 m the average gene flow frequencies were reported to be under 1 % (Matus-Cádiz et al. 2004; Hanson et al. 2005; Gaines et al. 2007; Willenborg et al. 2009; Loureiro et al. 2012). In close proximity (0–30 cm) higher gene flow rates up to 10.6 % have been reported (Hucl and Matus-Cádiz 2001; Matus-Cádiz et al. 2004; Lawrie et al. 2006). With increasing distances to the pollen source the gene flow frequency declined rapidly even for commercial-scale fields. Matus-Cádiz et al. (2004) reported the

reduction of maximal gene flow rate in Canadian wheat varieties from 0.44 % at a distance of 0.2 m to 0.01 % at a distance of 60–100 m. The similar result was obtained by Loureiro et al. (2012) for Spanish wheat cultivars. In their experiments the average gene flow rate fell from 0.02 to 0.62 % at 0 m to 0.00–0.02 % at 100 m. Hanson et al. (2005) found only single outcrossing events at 29 and 42 m from the pollen source, while the maximum gene flow (0.45 %) was detected only when plants were grown closest to the blue aleurone pollen source. Beckie et al. (2011) reported that the imidazoline herbicide-resistant gene flow frequency was higher at the common border between pollen donor and receptor (0.2 %) than at 10 m distance (0.06 %). Using a field-sized pollen source from 16 ha, they found outcrosses at the maximum distance of 80 m. A higher numbers of outcrosses at more remote distances could be found when commercial fields represent a large pollen source. For example, Matus-Cádiz et al. (2007) detected pollen-mediated gene flow at the maximum distance of 2.75 km from a 33 ha pollen source.

It's usually assumed that GM wheat would behave similar to conventional varieties, however the results from scant experiments are somewhat contradict this suggestion. Rieben et al. (2011) found that the rate of pollen mediated gene flow to neighboring GM-wheat lines was significantly higher (0.8–8.5 %) than to corresponding non-GM recipients lines (0.5–1.9 %). They supposed that unexpected variation of outcrossing rate among GM lines and original variety was evidently due to the different transgene insertion events. On the other hand Gatford et al. (2006) reported extremely low transgene flow frequency from 0.0037 to 0.012 % in adjacent field plots. Despite the previous observation that single outcrosses could be found at considerable distance from the pollen donor, Foetzki et al. (2012) found only a limited number of outcrosses within the border crop surrounded the GM wheat plot, however the experimental field was set up at a distance of 200 m.

Though the most of the previous studies were conducted under similar semi-arid conditions (Australia, Canada, USA and Spain) the results obtained at experimental fields in specific region did not necessarily represent the situation in another region. The environmental conditions in those geographical locations together with the individual biology of wheat varieties clearly influenced the extent of outcrossing. This indicates the need to assess the potential risks of gene escape on the regional, seasonable and genotypic basis for successful introduction of biotech crops into the modern agriculture.

Currently wheat is the most important cereal crop in Russian agriculture with the overall cultivation area of 23 million ha (FAOSTAT 2014). There are no wild relatives capable of hybridization with transgenic wheat in industrial wheat growing regions. However, the possibility of cross-pollination between adjacent wheat fields still exists. In the present study we have analyzed the frequency of pollen mediated transgene flow from the herbicide resistant GM wheat to its non-transgenic counterpart by performing a circular field experimental design in the temperate climate environmental conditions of Russia's Central European part.

Materials and methods

Plant material

Transgenic herbicide resistant wheat (line A-20) and its non-transgenic counterpart 'Andros' were involved in the experiments. A dual screening/selection approach based on the combination of *gfp* gene as a vital reporter gene and *bar* gene as a transgenes selection gene has been used for generation of primary transgenic wheat plant A-20 (Miroshnichenko et al. 2007b). Segregation studies carried out with T_1 progeny obtained from self-pollinated A-20 plant primary revealed Mendelian segregation (3/4 resistant and ¼ sensitive). Homozygous T_1 sub-lines were identified by spraying of T_2 seedlings with the herbicide Basta and through analysis of segregation using *gfp* fluorescence. Further seeds were propagated in the greenhouse and field plots (Miroshnichenko et al. 2007a) to obtain sufficient homozygous T_3–T_5 seed material for further experiments.

Experimental field design and data collection

The transgene flow experiments were carried out within the isolated area, owned by the Institute of Horticultural Crops Breeding, Oryol Region, Central Federal District of Russia, in 2004, 2005 and 2008. There was a small opportunity for designing the plots since a very few permits concerning field trials were

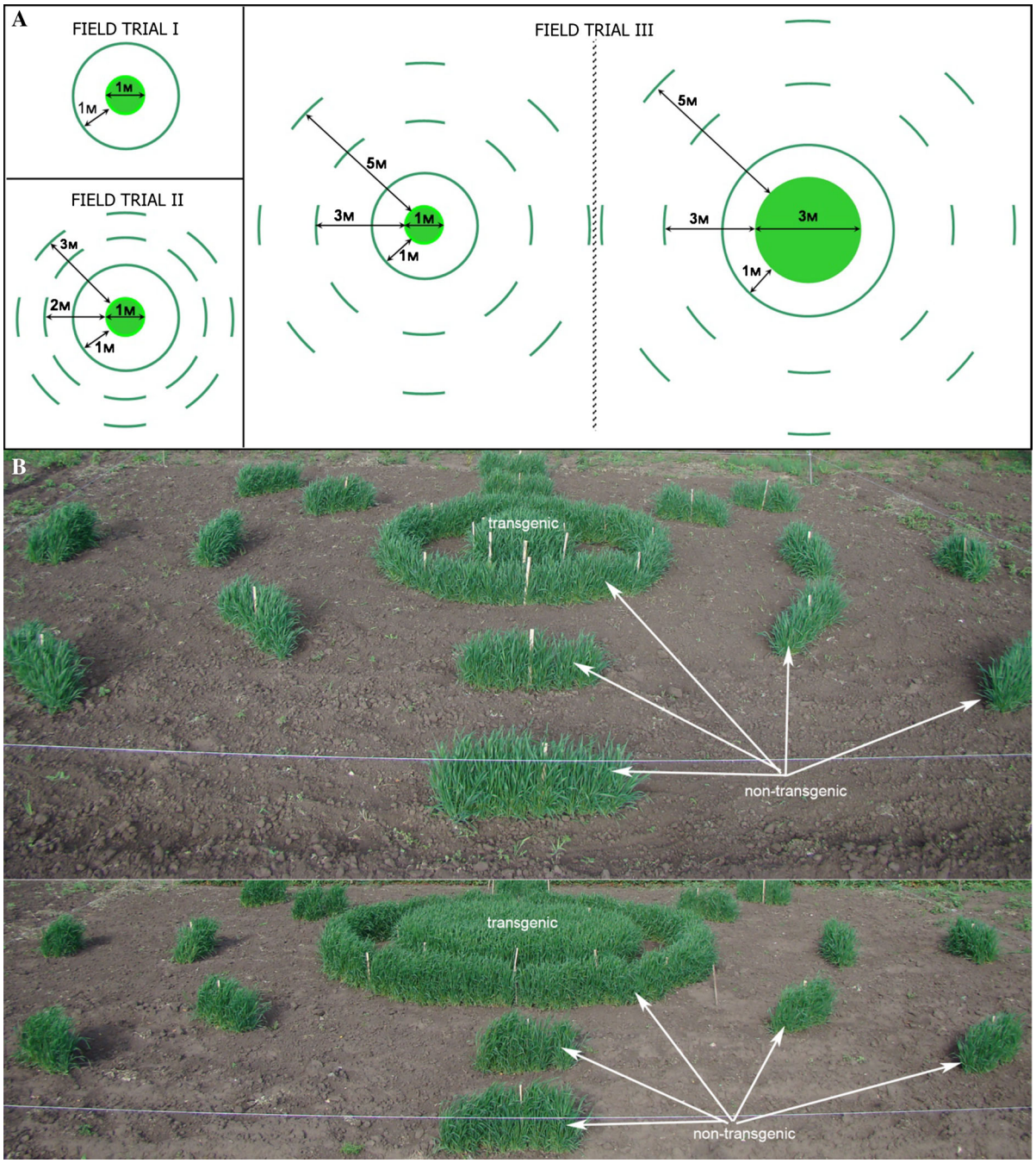

Fig. 1 Experimental field trials set-up for gene flow measuring from transgenic wheat. **a** The experimental design consists of a central circle with homozygous transgenic wheat line A-20 (Ø 1 or 3 m), carrying *bar* and *gfp* genes; transgenic wheat line A-20 is surrounded by concentric circle or plots of non-transgenic wheat plants of the same variety 'Andros'. Receptor plots are planted in accordance with the geographical orientation (N, NE, E, SE, S, SW, W and NW). **b** The general view of the field Trial III consisted of two experimental fields where non-transgenic plants were planted around homozygous transgenic wheat plants grown in two circular plots of 1 m diameter (*upper*) and 3 m diameter (*lower*)

issued by Russian Interagency Committee for GMO activity. A circular design was used to evaluate gene flow from transgenic to non-transgenic wheat plants (Fig. 1). The circular area of homozygous transgenic wheat plants (300 seeds m^2) with a diameter of 1 m was located in the center of the field. Non transgenic plants were planted at different distances in order to evaluate the gene flow frequency. Field tested non-transgenic plants (Field Trial I, conducted in 2004) were planted in the concentric circle at 1 m distance from the border of the transgenic plot. In Field Trial II (conducted in 2005) non-transgenic wheat plants were planted both in the concentric circle at 1 m distance and in eight plots at the distances 2 and 3 m, following the eight compass directions. Field Trial III (conducted in 2008) consisted of two experimental fields where non-transgenic plants were planted at a distance of 1, 3 and 5 m around two circular plots (∅ 1 or 3 m) of homozygous transgenic wheat plants (Fig. 1b). The minimal distance between the borders of two experimental fields in Field Trial III was 300 m. In all experiments the length of individual compass plot was 1 m. The trial was conducted under standard seed production practices without irrigation.

Determinations of air temperature, wind speed and its direction were performed with 3-h intervals from 6:00 a.m. to 9:00 p.m. during the flowering period (data were provided by the Meteorological Service of the Institute of Horticultural Crops Breeding). The average daytime temperature during pollination ranged from 18.1 to 19.6 °C (Table 1). In the first field trial the highest relative humidity of 74.4 % and the highest wind speed of 3.32 m/s were recorded. The lowest relative humidity (67.3 %), the lowest wind speed (2.47 m/s) and the lowest total wind run (2083 km) were observed in the Field Trial II. During the flowering period the average relative humidity was 72.6 % at the Field Trial III; average wind speed was 3.28 m/s; the total wind run was 3262 km. Within the first two years the prevailing wind direction was from N, NW and W during the pollination (Table 1). In the Field Trial III the main direction of winds was more variable and the prevailing winds ranged from NW (24 %), N (18 %) and S (17 %) during the period of pollination.

At seed maturity all seeds from non-transgenic plants were harvested. The concentric circle of non-transgenic plants was divided into eight compass sectors equally (N, NE, E, SE, S, SW, W, NW). All spikes of non-transgenic plants were harvested manually, and their localization in regard to the geographic orientation on the field was recorded. Later on the collected seeds were counted individually in laboratory. To avoid the occasional contamination of seedlings by transgenic seeds, the spikes of transgenic plants were collected 2 weeks before the harvest of non-transgenic plants.

Hybrids identification among non-GM and GM wheat

After storage at a room temperature all the seeds were sown in trays of a peat-based substrate in a greenhouse. At 3–4 leaf stage seedlings were treated with 1 % solution of Basta herbicide (Bayer, Germany). Each herbicide-resistant seedling was considered as an independent transgene flow event and was transferred into the individual pot for subsequent growth and final harvest. All of the survivors were screened for the presence of *bar* and *gfp* genes by PCR and Southern blot analysis. Total genomic DNA was extracted from leaf tissue of the plantlets in accordance with the method described by Rogers and Bendich (1994). PCR analysis was carried out with the use of specific primer pairs designed to amplify the 310-bp fragment of *bar* gene (forward 5′-TGC ACC ATC GTC AAC CAC TA-3′; reverse 5′-ACA GCG ACC ACG CTC TTG AA-3′) and 600-bp fragment of *gfp* gene (forward 5′-GCG ACG TAA ACG GCC ACA AG-3′; reverse 5′-CCA

Table 1 Daily weather summary (06:00–21:00) during the pollination in three experiments on gene flow

Field trial	Pollination duration (days)	Average temperature (°C)	Average relative humidity (%)	Wind speed (m/s)	Total wind run (km)[a]	Wind run from each vector, %							
						N	NE	E	SE	S	SW	W	NW
I	18	18.1	74.4	3.32	3231	18.7	2.4	1.6	4.9	5.7	10.6	26.8	29.3
II	16	18.5	67.3	2.47	2083	29.5	4.5	6.8	2.3	2.3	9.1	26.1	19.3
III	17	19.6	72.6	3.28	3012	18.2	4.0	2.0	5.1	17.2	15.2	14.1	24.2

[a] Total wind run determined as the total distance of the traveled wind over a flowering period

GCA GGA CCA TGT GTG ATC G-3′). Wheat genomic DNA (30 μg) was digested overnight at 37 °C with 60U *HindIII* for Southern blot analysis. The fragments were separated in 0.9 % agarose gel and then transferred to a positive-charged nylon membrane Hybond N+ (GE Healthcare, UK) by capillary blotting following the manufacturer's instructions. The DNA probe was constructed by means of PCR using plasmid psGFP-BAR (Richards et al. 2001) as the template, and primers described above. DNA probe was labeled with alkaline phosphatase using Amersham Gene Image AlkPhos Direct Labelling and Detection System (GE Healthcare, UK). Prehybridization, hybridization (overnight at 60 °C) with the alkaline phosphatase-labeled probe, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling System protocol. Detection was performed with the use of CDP-Star detection reagent in accordance with the manufacturer's directions (Amersham CDP-Star Detection reagent, GE Healthcare, UK).

Transgene flow frequency was determined as the ratio of transgenic seedlings number to the total number of examined seedlings. The statistical analysis (ANOVA) of transgene flow frequencies at different distances and pollen donor areas was conducted by means of Statistica10 software. The correlation coefficient (r^2, Pearson) between the wind direction and transgene flow was calculated additionally.

Segregation studies

To clarify the presence of *bar* and *gfp* at the hemizygous stage in survived seedlings the segregation of *gfp* fluorescence was analyzed in all of the herbicide tolerant PCR positive plants progenies. With this goal the immature embryos from young kernels of each survived plant were isolated, cultured in vitro and analyzed for GFP fluorescence as it was described by Miroshnichenko et al. (2011). Chi square analysis was applied to test the 3:1 ratio hypothesis ($p < 0.05$) expected for GFP segregation for heterozygous transgene origin.

Results

Field observation and weather conditions

In all field experiments the agronomic behavior of transgenic and non-transgenic plants was similar to the standard culture with the exception to the duration of flowering time. The estimated duration of flowering samples was 16–18 days (Table 1), whereas the standard flowering period for spring wheat is usually 7–10 days. That prolongation was caused by specific morphological characteristic of non-commercial wheat variety 'Andros'. Unlike many other varieties the spikes of 'Andros' are less aligned providing some flowering asynchrony of the individual plant. In the field experiments both transgenic and nontransgenic 'Andros' wheat plants had demonstrated synchronous flowering, thereby providing the maximum opportunity for pollen transfer.

Seasonal weather differences affected the seed production among the experiments (Table 2). By the reason of poor weather conditions (insufficient rainfall) the average number of seeds was lower in the Field Trial II. On average we collected 6000 seeds per one compass sector in the Field Trial II, while in the Field Trial III (favorable season) the average seed production reached more than 9700 seeds per one compass sector. The average germination rates of seeds collected from all non-GM plots was 96–98 %, ensuring the obtainment of the reliable data with the use of a sufficient number of seedlings. A total of 712 thousands of seeds harvested from non GM recipient wheat plants were screened including more than 62 thousands seeds collected in the Field Trail I, about 125 thousands seeds collected in the Field Trial II and more than 525 thousands seeds collected in the Field Trial III.

Field Trial I

Gene flow was analyzed by testing the glufosinate-based herbicide resistance in seedling progeny (Fig. 2). A total of 259 herbicide resistant seedlings were found among germinated seeds harvested at a distance of 1 m from the pollen donor. The transgene specific PCR examination confirmed that all survived seedlings were true hybrids acquired the resistance from transgene flow (Fig. 3a). The herbicide resistant seedlings exhibited the same integration pattern of *bar* gene as the homozygous pollen donor plant A-20 used in the field trial (Fig. 3b). No false-positive escapes were found. The average frequency of transgene flow for all directions was 0.424 ± 0.108 %. The localization of transgene flow according the eight compass directions is shown in Table 2. Most of herbicide

Table 2 Transgene flow frequencies detected in non-GM wheat at different distances from the GM pollen plot at eight compass directions

Filed trial/transgenic plot size	Distance between transgenic plants and recipient plot	Number of herbicide resistant PCR+ seedlings	Gene flow in accordance with compass sectors[a]								Average gene flow frequency (%) ± standard error	Correlation coefficient (r^2, Pearson)
			N	NE	E	SE	S	SW	W	NW		
I/0.75 m^2 (∅ 1 m)	1 m	259	0.094 (8468)	0.611 (8020)	0.796 (6660)	0.797 (7404)	0.564 (7980)	0.265 (9071)	0.214 (8400)	0.047 (8446)	0.424 ± 0.108	0.883**
II/0.75 m^2 (∅ 1 m)	1 m	64	0.090 (5539)	0.206 (6312)	0.232 (5604)	0.305 (5571)	0.213 (5161)	0.015 (6613)	0.063 (6352)	0.000 (6617)	0.141 ± 0.040	0.684
	2 m	13	0.000 (3902)	0.067 (5967)	0.030 (3382)	0.017 (5945)	0.023 (4398)	0.042 (4748)	0.085 (4712)	0.000 (3837)	0.033 ± 0.011	0.042
	3 m	1	0.000 (3056)	0.020 (4930)	0.000 (5839)	0.000 (5311)	0.000 (5565)	0.000 (7860)	0.000 (3500)	0.000 (4398)	0.003 ± 0.003	0.295
III/0.75 m^2 (∅ 1 m)	1 m	174	0.140 (8567)	0.453 (11,041)	0.420 (10,962)	0.357 (9236)	0.139 (7923)	0.018 (11,133)	0.097 (10,347)	0.118 (8476)	0.218 ± 0.059	0.857**
	3 m	17	0.022 (9203)	0.051 (11,810)	0.062 (11,317)	0.018 (11,029)	0.000 (8852)	0.000 (10,177)	0.000 (8936)	0.000 (9533)	0.019 ± 0.009	0.793**
	5 m	8	0.008 (12,948)	0.000 (11,108)	0.040 (9973)	0.020 (10,210)	0.000 (8716)	0.000 (11,747)	0.000 (9533)	0.010 (10,491)	0.010 ± 0.005	0.516
III/7.00 m^2 (∅ 3 m)	1 m	413	0.236 (12,304)	0.333 (18,603)	0.203 (18,243)	0.610 (16,553)	0.558 (15,424)	0.270 (17,777)	0.094 (19,092)	0.188 (16,979)	0.312 ± 0.064	0.241
	3 m	24	0.101 (5943)	0.097 (9306)	0.020 (10,124)	0.009 (10,542)	0.042 (7163)	0.000 (8543)	0.032 (6219)	0.013 (7959)	0.039 ± 0.014	0.058
	5 m	7	0.013 (7552)	0.019 (10,739)	0.034 (11,758)	0.000 (10,562)	0.000 (8946)	0.000 (9390)	0.000 (9784)	0.000 (7739)	0.008 ± 0.005	0.628

[a] Data in parentheses indicate the number of examined germinated seeds

** The critical value of Pearson coefficient is proved for $p < 0.05$

Fig. 2 Non-transgenic wheat seedlings 10 days after the herbicide treatment (1 % Basta). *Arrows* indicate the herbicide-resistant seedlings

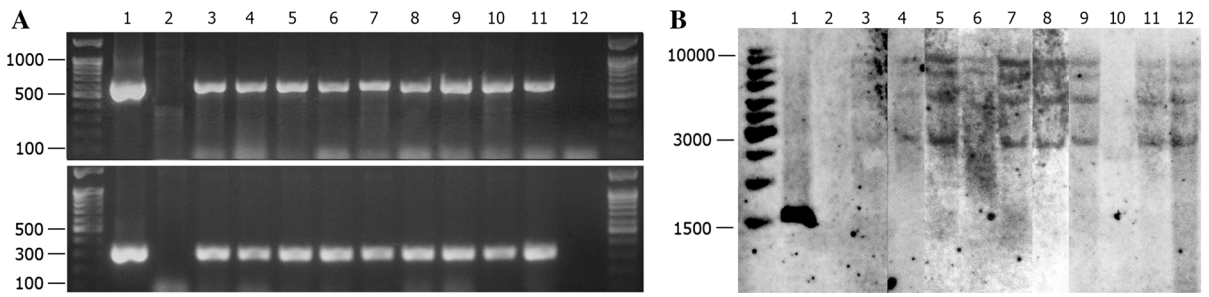

Fig. 3 Example determination of the transgenes in genomic DNA of herbicide-resistant hybrids recovered from non-transgenic recipient plants 'Andros' in the Field Trial I. **a** PCR amplification products for the presence of *gfp* (*upper panel*, a 600-bp DNA fragment is amplified), and *bar* (*lower panel*, a 310-bp DNA fragment is amplified) genes in the plasmid and genomic DNA; *lane 1* psGFP-BAR plasmid; *lane 2* non-transgenic 'Andros' plant; *lanes 3–11* herbicide-resistant hybrids; *lane 12* no DNA added to the PCR reaction. **b** Southern blot analysis; the genomic DNA was digested by *HindIII* and hybridized with a *bar* probe; *lane 1* psGFP-BAR plasmid digested by EcoRI; *lane 2* non-transgenic 'Andros' plant; *lanes 3–8* and *11–12* herbicide-resistant hybrids; *lane 9* transgenic homozygous T_3 plant of the pollen donor line A-20; *lane 10* transgenic homozygous T_3 plant of line A-37 as positive control with insertion pattern differs from that of line A-20; A-37 was not used in gene flow experiments

resistant seedlings (80 %) were found in the seeds collected from the sectors located in NE, E, SE and S of the transgenic plot. The maximum frequency of transgene flow (0.98 %) was detected in E and SE sectors, which were downstream prevailing W and NW wind (Table 1). On the contrary the lowest gene flow of 0.047 % was observed in NW sector of circular plot, which was upstream the prevailing winds. Variation in transgene flow was compared with the prevailing wind direction pattern, and correlation coefficient (r^2, Pearson) was calculated (Table 2). A reasonable correlation was found between gene flow frequency and the wind run from the appropriate compass direction ($r^2 = 0.882$, $p < 0.01$) on the basis of wind data.

Field Trial II

A total of 78 survived seedlings were identified among progenies of recipient 'Andros' plants located at different distances both in concentric circle and compass sectors (Table 2). The frequency of pollen-mediated gene flow was three times lower as compared with the previous experiment (0.134 ± 0.034 %) at a

distance of 1 m for all directions. However the character of the herbicide resistant seedlings distribution was similar. The most of PCR positive germinated seeds (54 out of 64) were collected from the sectors located in NE, E, SE and S of the transgenic plot with the maximum frequency of 0.305 % detected in SE sector. The remaining survived germinated seeds were found in N, SW and W sectors. Since no resistant seedlings were detected in NW sector it indicated the minimum of gene flow (Table 1). Asymmetric distribution of the herbicide-resistant seedlings correlated with the prevailing wind direction pattern ($r^2 = 0.684$). However the subsequent significance testing showed that the observed correlation was less than the critical value for $p = 0.05$ ($r^2_{tabl} = 0.707$).

The increase of the distance from the transgenic circle plot to recipient wheat plants declined the average frequency of pollen-mediated gene flow to 0.035 ± 0.011 % (distance of 2 m) and to 0.002 ± 0.003 % (distance of 3 m). The maximum of 4 herbicide-resistance seedlings obtained from the seeds collected at a distance of 2 m were detected both at NE and W plots. At two sector plots (N and NW) the recipient plants did not form hybrid seeds. At a distance of 3 m the only one survived seedling was found among the seeds collected from NE plot, so the frequency of transgene flow reached 0.020 %. There was no correlation between gene flow frequency and the wind run from the appropriate compass direction at both distances (Table 2).

Field Trial III

The average transgene flow obtained in the Field Trial III was influenced both by the size of transgenic central plot and the distance to the recipient plants. In the case of 1 m diameter for central transgenic plot the average transgene flow was 0.218 ± 0.059 % at a distance of 1 m, 0.019 ± 0.009 % at a distance of 3 m, and 0.010 ± 0.005 % at a distance of 5 m. When non-transgenic receptor plants were grown around the larger transgenic central plot (7.00 m^2, ∅ 3 m) the extent of gene flow increased to 0.312 ± 0.064 % and 0.039 ± 0.014 at the distances of 1 and 3 m respectively, but it was equal at a distance of 5 m (0.008 ± 0.005 %). Despite the significant increase in the transgene pollen area, statistical analysis did not confirm the rise of average gene flow. ANOVA analysis demonstrated non significant differences between the experiments involving different transgenic plot sizes if the recipient plants were located at the same distances ($df = 2$, MS = 0.013, $F = 1.23$, $p = 0.3019$). At the same time the distance significantly affected the transgene flow in both cases ($df = 2$, MS = 0,297, $F = 28.13$, $p < 0.0001$).

In the case of larger transgenic central plot the distribution of the herbicide tolerant seedlings was partially associated with the prevailing wind directions. At a distance of 1 m the maximum gene flow detected in S (0.610 %) and SE (0.558 %) sectors correlated with the prevailing NW and N winds during the flowering period. At a distance of 3 m the maximum rate of gene flow (0.101 %, N sector) was lower than the minimum gene flow frequency at a distance of 1 m (0.188 %, NW sector). Only seven PCR positive hybrid seeds were found at a distance of 5 m in three compass sectors (N, NE and E) with a peak of 0.020 % gene flow at E sector. Analysis between the variation of transgene flow and the wind run from the appropriate compass direction showed that the values of correlation coefficient r^2 did not exceed the critical value for $p > 0.05$.

On the other hand, the gene flow rates detected at the distances of 1 and 3 m from the central plot 1 m in diameter revealed a statistically significant relationship associated with the prevailing winds, with r^2 values ranging from 0.793 to 0.857. At 5 m distance the critical value of correlation coefficient was less than expected for $p = 0.05$ level ($r^2 = 0.516 < r^2_{tabl} = 0.707$). The variability of gene flow frequencies ranged from 0.453 % (SE sector) to 0.097 % (W sector) at a distance of 1 m, from 0.062 % (E sector) to 0.000 % (S, SE, N, NW sectors) at a distance of 3 m, and from 0.040 % (E sector) to 0.000 % (NE, S, SE, N sectors) at a distance of 5 m.

Segregation studies

In order to confirm that the herbicide resistant seedlings identified among the progeny seeds of the recipient non-transgenic plants had happened as a result of hybridization with the pollen of A-20, rather than with contaminated seeds, we examined the segregation of the *gfp* fluorescence in the offspring of all seedlings. The 3:1 ratio expected for the Mendelian segregation of transgene at a hemizygous

status was accepted ($p < 0.05$) for the most progenies (Table 3). The percentage of progenies with 3:1 ratio was 92.3 % in the Field Trial I, 87.2 % in the Field Trial II and 97.4 % in the Field Trial III. Since no homozygous seedling was found, we might conclude that the origin of all the individuals was the result of pollination by the pollen from transgenic wheat plants.

Discussion

The herbicide resistance trait conferred either by transgenic or non-transgenic gene markers are considered to be the most useful tool to quantify the gene flow between transgenic and not transgenic cross- and self-pollinated crops (Chandler and Dunwell 2008; Mallory-Smith et al. 2015). Nevertheless in the previous reports the herbicide spray tests of wheat seedlings germinated from the seeds collected in field trials did not give a clear distinction between transgenic and non-transgenic plants. Gatford et al. (2006) reported only one transgenic hybrid out of 58 seedlings that survived the spraying with 0,3 % Basta (15 mg/L PPT). The most of 900 survived wheat seedling found by Foetzki et al. (2012) during several years of field trials by application of the similar herbicide concentration (10 mg/L PPT) were also false-positive. Messeguer et al. (2004) reported the same problem of transgenic hybrids identification among herbicide resistant seedlings in rice transgene flow experiment. The lowering of the herbicide concentrations was forced by the inability of hybrid wheat plants to resist the dosage recommended for agricultural use (25–50 mg/L PPT), presumably due to the insufficient level of *bar* gene expression in transgenic pollen donor plants. Our previous results showed that the low expression of *bar* gene can lead to leaf damage and the loss of overall yield after spraying of field grown transgenic wheat plants with the recommended herbicide dosage (Miroshnichenko et al. 2007b).

The homozygous transgenic progenies of wheat line A-20 that previously exhibited no damage symptoms after spraying with 1 % Basta at field trials (Miroshnichenko et al. 2007b) were used in the present experiments to overcome such identification problems. Since A-20 plants were easily recovered after the spraying with the dosage of 150 mg/L PPT in preliminary greenhouse tests, all seedlings germinated from the seeds collected out of recipient plants were treated once at the concentration recommended for agricultural use (50 mg/L PPT). 7–15 days after spraying the herbicide-resistant seedlings could be easily identified. Analysis of molecular (Fig. 3) and phenotypic data (Table 3) did not reveal any escapes among the seedlings survived after the herbicide treatment. Those data showed that the method of screening for herbicide-resistant hybrids applied in the present study was much more reliable than those used in the previous experiments (Messeguer et al. 2004; Gatford et al. 2006; Foetzki et al. 2012).

In our experiments the average frequencies of pollen-mediated transgene flow from the herbicide resistant GM wheat plants vary within different seasons. Generally, gene flow rates observed in the Field Trial II were lower than in the Field Trials I and III. This could be associated with the differences in pollen dispersal and its viability during various seasons. According to De Vries (1972) the highest concentration of pollen dispersal appeared to be

Table 3 The segregation of transgenes in the progenies of herbicide-resistant wheat seedlings resulted from three gene flow experiments

Filed trial/transgenic plot size	Number of seedlings (*bar*+/*gfp*+) tested for segregation	Number of embryos (progenies) tested for *gfp* segregation	Seedlings (*bar*+/*gfp*+) shown *gfp* segregation 3:1 (according χ^2 test*)		Seedlings (*bar*+/*gfp*+) without *gfp* segregation	
			The number	The ratio, %	The number	The ratio, %
I/0.75 m^2 (∅ 1 m)	259	9970	239	92.3	0	0.0
II/0.75 m^2 (∅ 1 m)	78	4721	68	87.2	0	0.0
III/0.75 m^2 (∅ 1 m)	199	8518	194	97.5	0	0.0
III/7.00 m^2 (∅ 3 m)	444	18,360	432	97.3	0	0.0

* $p < 0.05$

released at a temperature of 16–20 °C and relative humidity of 70–75 %. Along with this there is a report that the number of pollen grains per spike can be reduced up to 70 % in drought seasons (Westgate et al. 1996). In the present study the weather conditions during the Field trials I and III were closer to the optimum for plant flowering, while in the Field Trial II the extent of the humidity and the total wind run were lower during flowering time. These factors along with the insufficient rainfall in the Field Trial II during the period of spike development apparently were responsible for the differences observed in hybrid formation.

In the earlier experiments on wheat transgene flow measurements the rectangular layouts were adopted (Gatford et al. 2006; Rieben et al. 2011; Foetzki et al. 2012). This design may save the land and labour, providing data on the maximal frequency and maximal distance of gene flow for determination of safety isolation distance (Yuan et al. 2007; Mallory-Smith et al. 2015). Circular layouts adopted in the current study, were represented by transgenic plants surrounded by recipient plants in order to allow more informative capturing of gene flow events in relation to the wind direction. Our results had demonstrated that the extent of transgene gene flow was associated with the wind direction. It was evident that 75–85 % of the cumulative gene flow events occurred in several sectors located downstream the prevailing wind, especially at a close distance, while in the sectors located at upstream direction only 0–15 % of events were scored. Triennial reproducibility of these observations indicates that the spatial location (upstream or downstream the prevailing wind) is one of the main factors affecting the transgenic pollen dispersal in wheat over different distances. These data correspond to those reported for other self- and cross-pollinated grain species such as rice, barley, and sorghum obtained in transgene flow field trials (Ritala et al. 2002; Schmidt and Bothma 2006; Yuan et al. 2007).

The results obtained in our field experiments revealed low frequency of transgene flow from GM wheat A-20 to its closeup non-GM counterpart spring wheat 'Andros', though the frequencies varied significantly in different compass sectors (0.000–0.797 %). Even in the worst case the pollen mediated transgene flow rates were always below the value of 1 %, which is the strictest threshold adopted to determine GMO admixture for feed and food in the international trade (EC No 1828/2003). This result is in accordance with the results of previous studies conducted by different authors to ensure seed purity in which the range of outcrossing frequencies averaged around 1 % (Matus-Cádiz et al. 2004; Hanson et al. 2005; Gaines et al. 2007; Willenborg et al. 2009; Loureiro et al. 2012). Undoubtedly when donor and recipient wheat plant are bagged together or grown in adjacent rows at a 'zero distance' the highest possible outcrossing could be rather high, up to 8.5–10.6 % (Lawrie et al. 2006; Rieben et al. 2011). Nevertheless it is important to point out that transgene flow frequencies reduced dramatically with the increase of the distance from pollen source and up to recipient plants (Matus-Cádiz et al. 2004; Hanson et al. 2005; Matus-Cádiz et al. 2007; Beckie et al. 2011; Loureiro et al. 2012). In the present study the considerable decrease of transgene flow frequencies (up to <0.01) was observed when the distance was increased up to 5 m.

Determination of the isolation distance is the most effective method for preventing outcrossing and seed contamination between compatible genotypes. Wheat is considered as a species with low-risk of gene flow and seed impurity at seed production. Therefore in the majority of countries the isolation distances are very small as compared with the cross pollinated crops. In Canada and India the usual recommended isolation distance is 3 m for pedigreed seed production (Canadian regulations and procedures for pedigreed seed crop production 2015; Indian minimum seed certification standards 2013). In Australia production areas must be separated from other cereals by at least a two meter strip (Seed Services Australia 2013). Nevertheless in European Countries the isolation distances may be shorter (0.4 m) as the modern wheat varieties to be listed in national catalogs are preferably selected for closed spikelets (cleistogamy) (Foetzki et al. 2012). In USA the recommended distances for seed production is 0 m for nonhybrid wheat, however the distance should be enlarged at least to 100.6 and 201.2 m for registered and certified hybrid wheat seeds production (CFR #201.76). In Russia there is no official recommended isolation distances for bread wheat seed production, though bread wheat varieties must be isolated for a distance of 200 m from durum wheat varieties to ensure a national standard for genotypic purity (National Standard of Russian Federation GOST 52325-2005). In a view of our results we can assume that in the case of transgenic wheat the gene flow to adajacent non-GM wheat plants has the

minimum risk (less than 1 %). Due to a normal self-pollination, often favored by cleistogamic characteristics, the possibility of the unwanted GM threshold under normal standard wheat culture is below the permissible values of 0.9 % for international market. Taking into account the prevailing winds, the establishing of a security distance of 3-5 meters may completely reduce the pollen-mediated GMO admixture. From this point seed mixing during harvest, transportation, subsequent processing and storage may provide much more significant risk of GMO admixture in seeds, requiring the further intent assessment.

In present study determination of transgene flow frequencies was based on the small scale field experiments. The field size was restricted by the approval issued by the Russian Interagency Committee for GMO activity. Usually the direct measurement of gene flow from a GM crop to non-GM varieties is considered to be impractical and extremely expensive for biosafety assessment at the commercial production scale. However the main challenges for such assessment are to find out whether the gene flow frequency been measured on the basis of a small field experimental scale represents the actual scenario of transgene flow at the large scales of commercial production (Rong et al. 2012). Based on the empirical regression model Gustafson et al. (2005) predicted that the effect of source field size in the wheat pollen-mediated gene flow would be minimal (<0.1 %). On the contrary, Gaines et al. (2007) found the higher number of outcrosses at greater distances between large commercial fields as compared with those from small experimental plots. In our experiment the enlargement of the transgenic pollen donor area from 0.75 to 7.00 m^2 did not significantly affect the average frequency of gene flow, particularly at a distance of 5 m. This observation is in agreement with the results of Rong et al. (2007), who showed that transgene flow frequencies were not obviously influenced by the size of GM subplots of rice, predominantly self-pollinated cereal crop. Recent studies on rice involved four field scales ranging from 9 to 576 m^2 revealed that the highest frequencies were found in plots at the smallest scales and that the scale had a significantly negative effect, with decreased gene flow at increased scale in rice (Rong et al. 2012). When the plot size of a pollen donor becomes large, the extent of gene flow may rapidly decrease due to the exponential decrease of pollen density, in contrast the increase in the plot size of a pollen recipient would reduce of unwanted transgenic pollen as well (Rong et al. 2010). Since wheat is more strictly self-pollinated species versus rice, field size effect would be even more negligible. From this point, the frequencies of transgene flow obtained from the field experiments at a relatively small scale, such as are presented here, provide reliable information for predicting the actual level of crop-to-crop gene flow at the large field production scales.

In summary, this paper presents the first GM quantitative assessment of pollen-mediated gene flow and out-crossing events in Russian Federation for any commercial crop. Here we present the results that indicate the low risk of seed contamination at a threshold of 0.9 % for GM seeds in the total yield. Nevertheless the adequate isolation distances and the best management practices are needed to be thoroughly considered among the others risk assessments in order to approve the commercial cultivation of any GM wheat varieties with respect to the regional and genotypic particularities.

Acknowledgments This work was supported by the Ministry of Science and Education of Russian Federation, Grant No 14.M04.12.0015. Appreciation is expressed to the staff of the Institute of Horticultural Crops Breeding of Russian Federation for help with the field trials. We also thank Ekaterina Sidonskaya for her assistance with PCR analysis and Yuliya Chernykh, Grigory Poroshin and Mikhail Filippov for their technical assistance throughout the research. We appreciate Mariya Chernobrovkina (All Russian Research Institute of Agricultural Biotechnology, Russia) for English editing of the manuscript and helpful comments on the manuscript.

References

Barro F, Barceló P, Lazzeri PA, Shewry PRA, Ballesteros JM (2002) Field evaluation and agronomic performance of transgenic wheat. Theor Appl Genet 105:980–984

Becker D, Brettschneider R, Lörz H (2004) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J 5(2):299–307

Beckie HJ, Warwick SI, Sauder CA, Hall LM, Harker KN, Lozinski C (2011) Pollen-mediated gene flow in commercial fields of spring wheat in Western Canada. Crop Sci 51:306–313

Canadian regulations and procedures for pedigreed seed crop production (2015) Canadian Seed Growers' Association. Circular 6, Section 02. Cereals. Revision 1.10, 2015. http://seedgrowers.ca/seed-growers/regulations/. Accessed 1 Feb 2015

Cannell ME, Doherty A, Lazzeri PA, Barcelo P (1999) A population of wheat and tritordeum transformants showing a

high degree of marker gene stability and heritability. Theor Appl Genet 99:772–784

Chandler S, Dunwell JM (2008) Gene flow, risk assessment and the environmental release of transgenic plants. Crit Rev Plant Sci 27:25–49

Code of Federal Regulation #201.76. U.S. Code: Title 7—AGRICULTURE. § 201.76 Minimum Land, Isolation, Field, and Seed Standards. https://www.law.cornell.edu/cfr/text/7/201.76 Accessed 10 Sept 2015

D'Souza VL (1970) Investigations concerning the suitability of wheat as pollen-donor for cross-pollination by wind as compared to rye, Triticale, and Secalotricum. Z Pflzücht 63:246–269

De Vries AP (1971) Flowering biology of wheat, particularly in view of hybrid seed production—a review. Euphytica 20:152–170

De Vries AP (1972) Some aspects of cross pollination in wheat (*Triticum aestivum* L.) 1. Pollen concentration in the field as influenced by variety, diurnal pattern, weather conditions, and level as compared to the height of the pollen donor. Euphytica 21:185–203

Devos Y, Reheul D, De Schrijver A (2005) The Co-existence between transgenic and non-transgenic maize in European Union: a focus on pollen flow and cross-fertilization. Environ Biosaf Res 4:71–87

Dunwell JM (2014) Transgenic cereals: current status and future prospects. J Cereal Sci 59:419–434

Ellstrand NC (2003) Current knowledge of gene flow in plants: implications for transgene flow. Philos T Roy Soc B 358:1163–1170

FAOSTAT (2014) http://faostat3.fao.org. Accessed 01 Aug 2015

Foetzki A, Quijano CD, Moullet O, Fammartino A, Kneubuehler Y, Mascher F, Sautter C, Bigler F (2012) Surveying of pollen-mediated crop-to-crop gene flow from a wheat field trial as a biosafety measure. GM Crops Food 3(2):115–122

Gaines TA, Preston C, Byrne P, Henry WB, Westra P (2007) Adventitious presence of herbicide resistant wheat in certified and farm-saved seed lots. Crop Sci 47:749–754

Gatford KT, Zainuddin B, Edlington J, Lloyd J, Qureshi JA, Brettell R, Fincher GB (2006) Gene flow from transgenic wheat and barley under field conditions. Euphytica 151:383–391

Gustafson DI, Horak MJ, Rempel CB, Metz SG, Gigax DR, Hucl P (2005) An empirical model for pollen-mediated gene flow in wheat. Crop Sci 45:1286–1294

Hanson BD, Mallory-Smith CA, Shafii B, Thill DC, Zemetra RS (2005) Pollen mediated gene flow from blue aleurone wheat to other wheat cultivars. Crop Sci 45:1610–1617

Hucl P, Matus-Cádiz M (2001) Isolation distances for minimizing out-crossing in spring wheat. Crop Sci 41: 1348–1351

Indian Minimum Seed Certification Standards (2013) The Central Seed Certification Board Department of Agriculture & Cooperation Ministry of Agriculture Government of India New Delhi. http://agricoop.nic.in/imagedefault/seed/INDIAN_MINIMUM_SEED_CERTIFICATION_STANDARDS.pdf

James C (2014) Global status of commercialized biotech/GM crops: 2014. ISAAA Brief 49-2014. ISAAA, Ithaca

Jensen NF (1968) Results of a survey on isolation requirements for wheat. Ann Wheat Newsl 15:26–28

Jones HD, Sparks CA (2009) Selection of transformed plants, Chapter 2. In: Jones HD, Shewry PR (eds) Transgenic wheat, barley and oats; production and characterisation protocols. Methods in Biotechnology. Humana Press, Totowa, pp 23–37

Khan MN, Heyne EG, Arp AL (1973) Pollen distribution and seedset on *Triticum aestivum* L. Crop Sci 13:223–226

Lawrie RG, Matus-Cádiz MA, Hucl P (2006) Estimating outcrossing rates in spring wheat cultivars using the contact method. Crop Sci 46:247–249

Loureiro I, Escorial C, Santin I, Chueca C (2011) Pollen mediated gene flow in GM Crops: The use of herbicides as markers for detection. The case of wheat. In: Larramendy M (ed) Herbicides, Theory and application. InTech Europe. Croatia. doi: 10.5772/13042

Loureiro I, Escorial MC, González Á, Chueca MC (2012) Pollen-mediated gene flow in wheat (*Triticum aestivum* L.) in a semiarid field environment in Spain. Transgenic Res 21:1329–1339

Lu BR, Snow AA (2005) Gene flow from genetically modified rice and its environmental consequences. BioSci 55:669–678

Mallory-Smith C, Hall LM, Burgos NR (2015) Experimental methods to study gene flow. Weed Sci 63:12–22

Matus-Cádiz MA, Hucl P, Horak MJ, Blomquist LK (2004) Gene flow in wheat at the field scale. Crop Sci 44:718–727

Matus-Cádiz MA, Hucl P, Dupuis B (2007) Pollen-mediated gene flow in wheat at the commercial scale. Crop Sci 47:571–579

Messeguer J, Marfà V, Català MM, Guiderdoni E, Melé E (2004) A field study of pollen-mediated gene flow from Mediterranean GM rice to conventional rice and the red rice weed. Mol Breed 13:103–112

Miroshnichenko D, Filippov M, Babakov A, Dolgov S (2007a) Genetic engineering of Russian wheat genotypes for abiotic stress resistance. In: Burk HT, Nisi JE, Salomon N (eds) Developments in plant breeding, vol 12., Wheat production in stressed environmentsSpringer, Dordrecht, pp 715–721

Miroshnichenko D, Filippov M, Dolgov S (2007b) Genetic transformation of Russian wheat cultivars. Biotechnol Biotechnol Equip 4:399–402

Miroshnichenko DN, Poroshin GN, Dolgov SV (2011) Genetic transformation of wheat using mature seed tissues. Appl Biochem Microbiol 47(8):767–775

National Standard of Russian Federation, GOST 52325-2005 (2005) Seeds of agricultural crops. Varietal and sowing characteristics. General specifications (in Russian)

Richards HA, Rudas VA, Sun H, McDaniel JK, Tomaszewski Z, Conger BV (2001) Construction of a GFP-BAR plasmid and its use for switchgrass transformation. Plant Cell Rep 20:48–54

Rieben S, Kalinina O, Schmid B, Zeller S (2011) Gene flow in genetically modified wheat. PLoS One 6(12):e29730. doi:10.1371/journal.pone.0029730

Ritala A, Nuutila AM, Aikasalo R, Kauppinen V, Tammisola J (2002) Measuring gene flow in the cultivation of transgenic barley. Crop Sci 42:278–285

Rogers SO, Bendich AJ (1994) Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin SB, Schilperoort

RA (eds) Plant molecular biology manual, 2nd edn. Kluwer Academic Publishers, Dordrecht, pp 183–190

Rong J, Lu BR, Song ZP, Su J, Snow AA, Zhang XS, Sun SG, Chen R, Wang F (2007) Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. New Phytol 173:346–353

Rong J, Song ZP, de Jong TJ, Zhang XS, Sun SG, Xu X, Xia H, Liu B, Lu BR (2010) Modelling pollen-mediated gene flow in rice: risk assessment and management of transgene escape. Plant Biotechnol J 8:452–464

Rong J, Wang F, Song Z, Su J, Chen R, Lu BR (2012) Scale effect on rice pollen-mediated gene flow: implications in assessing transgene flow from genetically engineered plants. Ann Appl Biol 161(1):3–11

Schmidt M, Bothma G (2006) Risk assessment for transgenic sorghum in Africa: crop-to-crop gene flow in *Sorghum bicolor* (L.) moench. Crop Sci 46:790–798

Seed Services Australia (2013) Seed Certification Manual. 8.2. Field Crop & Other Species. Published by a business unit of the Rural Solutions SA Division of Primary Industries & Resources SA (PIRSA). http://pir.sa.gov.au/__data/assets/pdf_file/0003/148134/Seed_Certification_Manual.pdf

Virmani SS, Edwards IB (1983) Current status and prospects for breeding hybrid rice and wheat. Adv Agron 36:145–214

Westgate ME, Passioura JB, Munns R (1996) Water status and ABA content of floral organs in drought-stressed wheat. Aust J Plant Physiol 23:763–772

Willenborg CJ, Brule-Babel AL, Van Acker RC (2009) Low crop plant population densities promote pollen-mediated gene flow in spring wheat (*Triticum aestivum* L.). Transgenic Res 18:841–854

Yuan QH, Shi L, Wang F, Cao B, Qian Q, Lei XM, Liao YL, Liu WG, Cheng L, Jia SR (2007) Investigation of rice transgene flow in compass sectors by using male sterile line as a pollen detector. Theor Appl Genet 115(4):549–560