

# Pollen-mediated gene flow in maize in real situations of coexistence

Joaquima Messeguer<sup>1,\*</sup>, Gisela Peñas<sup>1</sup>, Jordi Ballester<sup>2</sup>, Marta Bas<sup>2</sup>, Joan Serra<sup>3</sup>, Jordi Salvia<sup>3</sup>, Montserrat Palaudelmàs<sup>1</sup> and Enric Melé<sup>1</sup>

<sup>1</sup>*Consorci Laboratori CSIC-IRTA de Genètica Molecular Vegetal, Departament de Genètica Vegetal, Centre de Cabriels, Carretera de Cabriels s/n, Cabriels 08348 (Barcelona), Spain*

<sup>2</sup>*Applus + Agroalimentario Análisis Genéticos, Ctra. d'Accés a la Facultat de Medicina s/n, Campus UAB, Bellaterra 08193 (Barcelona), Spain*

<sup>3</sup>*IRTA-Estació Experimental Agrícola Mas Badia, Mas Badia la Tallada d'Empordà, 17134 (Girona), Spain*

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\*Correspondence (fax 34 93 753 39 54;

e-mail joaquima.messeguer@irta.es)

## Summary

We present the first study on cross-fertilization between *Bt* and conventional maize in real situations of coexistence in two regions in which *Bt* and conventional maize were cultivated. A map was designed and the different crops were identified, as were the sowing and flowering dates, in *Bt* and conventional maize fields. These data were used to choose the non-transgenic fields for sampling and analysis by the real-time quantification system-polymerase chain reaction (RTQ-PCR) technique. In general, the rate of cross-fertilization was higher in the borders and, in most of the fields, decreased towards the centre of the field. Nine fields had values of genetically modified organism DNA to total DNA of much lower than 0.9%, whereas in three the rate was higher. Some differences were found when comparing our results with those of common field trials. In real conditions of coexistence and in cropping areas with smaller fields, the main factors that determined cross-pollination were the synchronicity of flowering and the distances between the donor and receptor fields. By establishing an index based on these two variables, the rate of the adventitious presence of genetically modified maize could be predicted, as well as the influence of other factors. By applying this index, and in the case of a fully synchronous flowering time, a security distance between transgenic and conventional fields of about 20 m should be sufficient to maintain the adventitious presence of genetically modified organisms as a result of pollen flow below the 0.9% threshold in the total yield of the field.

**Keywords:** coexistence, maize, pollen gene flow.

## Introduction

Commercial cultivation of genetically modified (GM) maize in Europe has been well legislated since 2003 (Directive, 2001/18/CE; Regulation (EC), 1829/2003, 1830/2003). In these regulations, the concept of coexistence has been established as 'the principle that farmers should be able to cultivate freely the agricultural crops they choose, be it GM crops, conventional or organic crops'. All European countries need to develop national strategies to ensure coexistence (Commission Recommendation, 2003), taking into account that the threshold value of 0.9% for labelling GM maize food and feed has been established.

Coexistence can be affected by the adventitious presence of one crop with another, which can arise for a variety of reasons. These include seed impurities, cross-pollination, volunteer presence, harvesting and storage practices on the farm, transport, storage and post-farm processing. The adventitious presence of genetically modified organisms (GMOs) as a result of cross-pollination is one of the factors that needs to be evaluated in different cropping areas, as local climatic conditions may influence the extent of pollen-mediated gene flow. The maize plant is monoecious and diclinous, with male and female flowers borne separately on the same plant. Maize is protandrous, with pollen being shed before the silks are receptive, but, as there is some

overlap, up to 5% self-pollination can occur (Purseglove, 1972).

Numerous trials have been conducted on maize pollen dispersal, because of its economic importance (for reviews, see Emberlin *et al.*, 1999; Treu and Emberlin, 2000; Brookes *et al.*, 2004). These studies clearly show that, although maize pollen is relatively large and heavy, it can travel long distances on the airflow, when suitable meteorological conditions occur, and therefore cross-pollination will take place to some extent. The rate of cross-pollination between fields depends on pollen viability, synchronization of flowering and the relative concentrations of pollen in the donor and receptor plots. In traditional plant breeding programmes, the recommended separation distances within fields of 2 ha or more are 200 m to maintain 99% grain purity and 300 m to maintain 99.5% grain purity (Ingram, 2000). The potential impact of cross-pollination increases notably with the size and number of fields planted (Treu and Emberlin, 2000). Moreover, Jones and Brooks (1950) found that the percentage of outcrossing within a field was related to the depth of the field in the direction of the source of contamination. These authors also observed that the percentage of outcrosses occurring in successive rows at different isolation distances indicated that the first five rows adjacent to the source of contamination functioned as a barrier to the dispersal of contaminating pollen.

A farm-scale evaluation of gene flow from GM herbicide-resistant maize to non-GM maize has been performed by Henry *et al.* (2003) in the UK, where 55 fields were tested over a 3-year period. Analysis of kernel samples using real-time polymerase chain reaction (PCR) showed that there was a rapid decrease in the rate of cross-pollination within the first 20 m from the donor crop, and beyond this distance the rate of decrease was much slower. There was significant variation in the levels of GMO–non-GMO cross-pollination between sites in each year ( $P < 0.01$ ), although the variation between years across all sites was not significant ( $P > 0.05$ ). Results from individual fields could be correlated with the wind direction during the flowering period, synchronicity of flowering between the two (GM and conventional) crops and the separation distance between the crops. The authors concluded that an isolation distance of 24.4 m was required to meet the 0.9% threshold recommended by the European Union for food and feed in the UK.

More recently, Ma *et al.* (2004) have conducted field experiments at three sites in Ottawa, Canada, over a 3-year period, using yellow kernel *Bt* maize and white kernel maize to detect cross-fertilization. In this study, it was concluded that it was possible to produce non-GM maize grains by removing the outer rows of non-GM maize plants (about

30 m) neighbouring the GM maize field concerned, if the acceptance threshold was set at 1% or less, but that the generally recommended 200-m distance between two genotypes appeared to be appropriate for *Bt* and other GM maize.

The results obtained in a field trial carried out in Spain (Brookes *et al.*, 2004; Melé *et al.*, 2004) were used to estimate the likely levels of adventitious presence of GMO in non-GM maize fields of different sizes and different distances downwind from a GM emitter crop. The level of adventitious presence of GMO likely to be found in non-GM maize crops (1 ha) planted adjacent to a GM plot (0.25 ha) was, on average, 0.83% (measured for the total harvest in the 1-ha plot). The level of adventitious presence of GMO likely to be found in non-GM maize crops in a same-sized plot as the transgenic nucleus (0.25 ha) planted adjacent to a GM plot was, on average, 1.77%, but this decreased to 0.77% when a 6-m buffer zone was maintained between the GM and non-GM crops.

In general, the field trials described above were designed by planting a nucleus of maize (GM or a cultivar with a special phenotypic trait) and then studying the cross-fertilization in an adjacent field. In most trials, both genotypes had been sown at the same time to increase the synchronicity of flowering, in order to detect cross-fertilization in the worst situation that could be found in an area in which GM and non-GM maize coexist. However, more data are needed to elucidate to what extent the results encountered in these field trials can be applied to real situations of coexistence, in which GM and non-GM maize fields are sown with different cultivars, with different sowing dates, mixed with other crops, and with different barriers that may influence pollen dissemination.

In this study, bearing in mind the real situation of coexistence that we have in Spain, we evaluated the rate of cross-fertilization in several non-GM maize fields in two different regions in which *Bt* and conventional maize are commonly used. The results obtained in this study may be useful for determining how, and to what extent, the different sowing dates, cultivars and natural and artificial barriers that influence pollen dissemination affect the adventitious presence of *Bt* maize. The data may also be useful for the validation of models predicting pollen flow at the landscape level under different cropping systems.

## Results

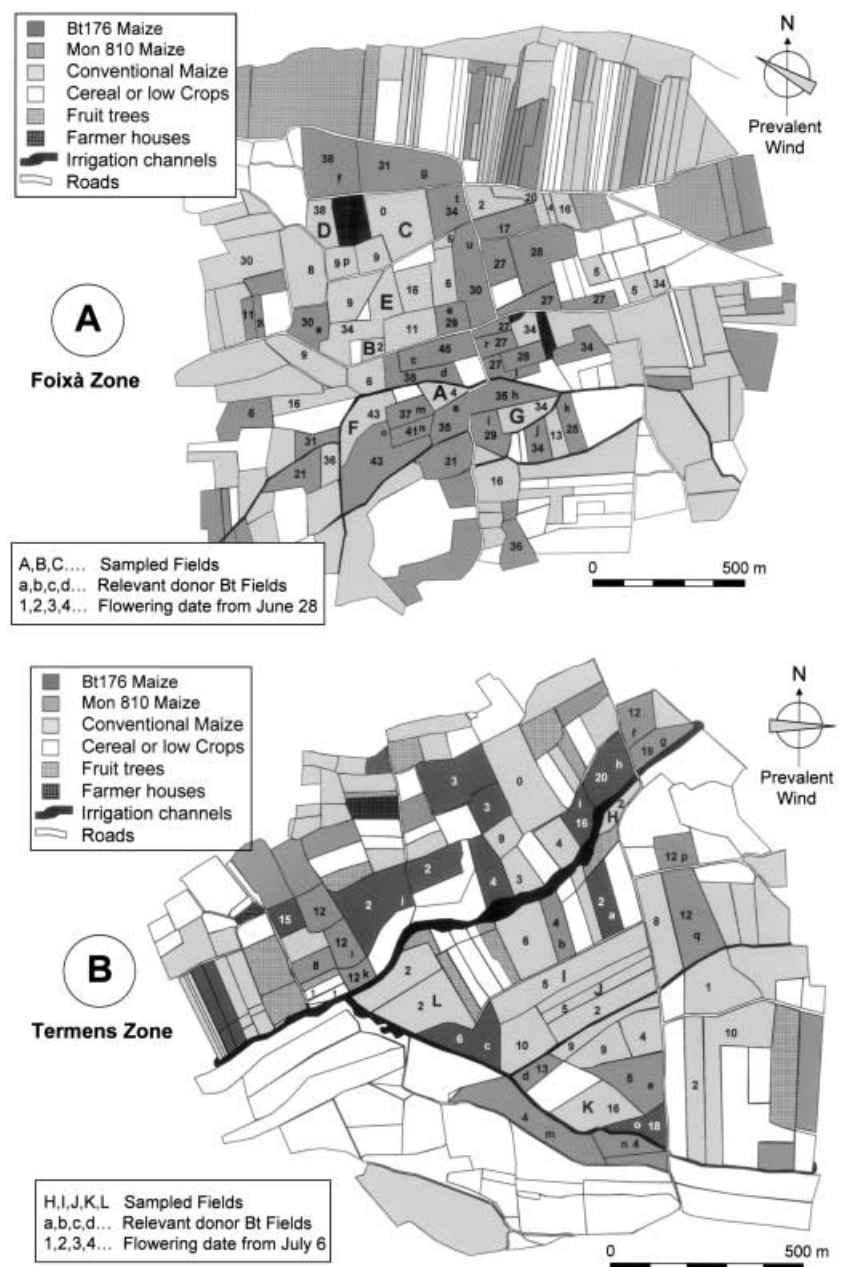
Both areas studied contained *Bt* and conventional maize fields distributed at random and coexisting with cereals, fruit trees or non-cultivated fields. During the 2004 growing season, there was a wide range of conventional maize cultivars and

16 *Bt* cultivars on the market. In the T ermens area, the most cultivated *Bt* maize was Compa CB (*Bt*176) from Syngenta Seeds Co. (Basilea, Switzerland), whereas, in the Foix a area, *Bt* cultivars with the Mon810 event from Pioneer Hi Bred (Des Moines, IA) were the most commonly used (mainly PR33P67). The geographical position of the *Bt* transgenic maize fields, conventional maize fields, fruit trees, cereals and non-cultivated fields, as well as the geographical and physical barriers that may disturb pollen flow, are shown in Figure 1A (Foix a) and 1B (T ermens).

In the T ermens region, the sowing period was quite similar for transgenic and conventional maize, and nearly all fields

were sown early, between 18 March and 28 April. In the Foix a region, there were two different sowing periods (from 15 March to 20 April and from 10 May to 30 May) because heavy rainfall flooded the fields in the middle of April and growers had to wait at least 3 weeks until the fields dried. Bearing in mind that the attack of corn borers is more severe as the temperature increases, most of the growers that had to sow their crops later decided to use *Bt* maize, whereas those that sowed earlier used conventional maize.

To quantify the synchronicity of flowering between the fields, the value zero was given to the fields that flowered first, and each field was then assigned a number that



**Figure 1** Maps from the Foix a (A) and T ermens (B) areas. Analysed fields are indicated by capital letters. Flowering dates are indicated by a number, with 0 being that of the field that flowered first (day 0) and an appropriate number being assigned to each field. *Bt* fields are indicated by lower case letters.

expressed the flowering date with reference to day 0. As shown in Figure 1A, there were many fields in Foixà in which the delay in flowering date was more than 20 days (with reference to the first field that flowered), whereas, in Tèrmens (Figure 1B), the flowering dates were more concentrated. From the data collected on sowing and flowering dates in the particular situation studied, it was observed that a delay of 1 month in sowing date produced a delay in flowering of approximately 15 days.

#### Detection and quantification of the cross-fertilization rate

Five conventional fields in the Tèrmens area and seven in the Foixà area were chosen to detect and quantify the rate of cross-fertilization (Figure 1A,B). These fields were chosen according to their position with respect to the surrounding *Bt* fields, and taking into account the flowering dates, in order to study a wide range of different real situations of coexistence. The number of analysed samples differed from one field to another (from 18 to 37) depending on the area and the shape of the field. In the Tèrmens area, fields H, I, J and K (Figure 1B) were analysed for both the Mon810 and *Bt176* events, whereas, in the Foixà area, the analysis was performed for the Mon810 event only, because neither the fields of the selected zone nor the fields of the surrounding zone had been sown with *Bt176* maize. The amplification of the *Zea mays* invertase gene and the Mon810 and *Bt176* events were unique and were resolved as single bands on gels. Four hundred and eighty-eight DNA quantifications by real-time quantification system-polymerase chain reaction (RTQ-PCR) were performed.

Figure 2 shows the adventitious presence of GMO for each of the analysed fields at each sampling point, expressed as a percentage of target/transgenic DNA sequences per target taxon-specific sequence, and calculated in terms of haploid genomes (percentage of GMO-DNA to total DNA), as recommended in Directive 2004/787/EC. The spatial distribution of the GMO content in the field was also represented by colouring the delimited areas according to the average of their GM values found in their vertices. This distribution was not uniform in the field, showing some affected areas and providing useful information about the putative pollen donors. In general, the rate of cross-fertilization was higher in the borders and decreased towards the centre of the field.

The stratified sampling allowed for a weighted average from the different strata and for an estimate of the adventitious presence of GMO in the total yield production of each field (Table 1). Nine of the 12 analysed fields gave values much

**Table 1** Estimated percentage of genetically modified (GM) DNA/total DNA for *Bt176* and Mon810 events in all analysed fields with the standard error of the weighted areas

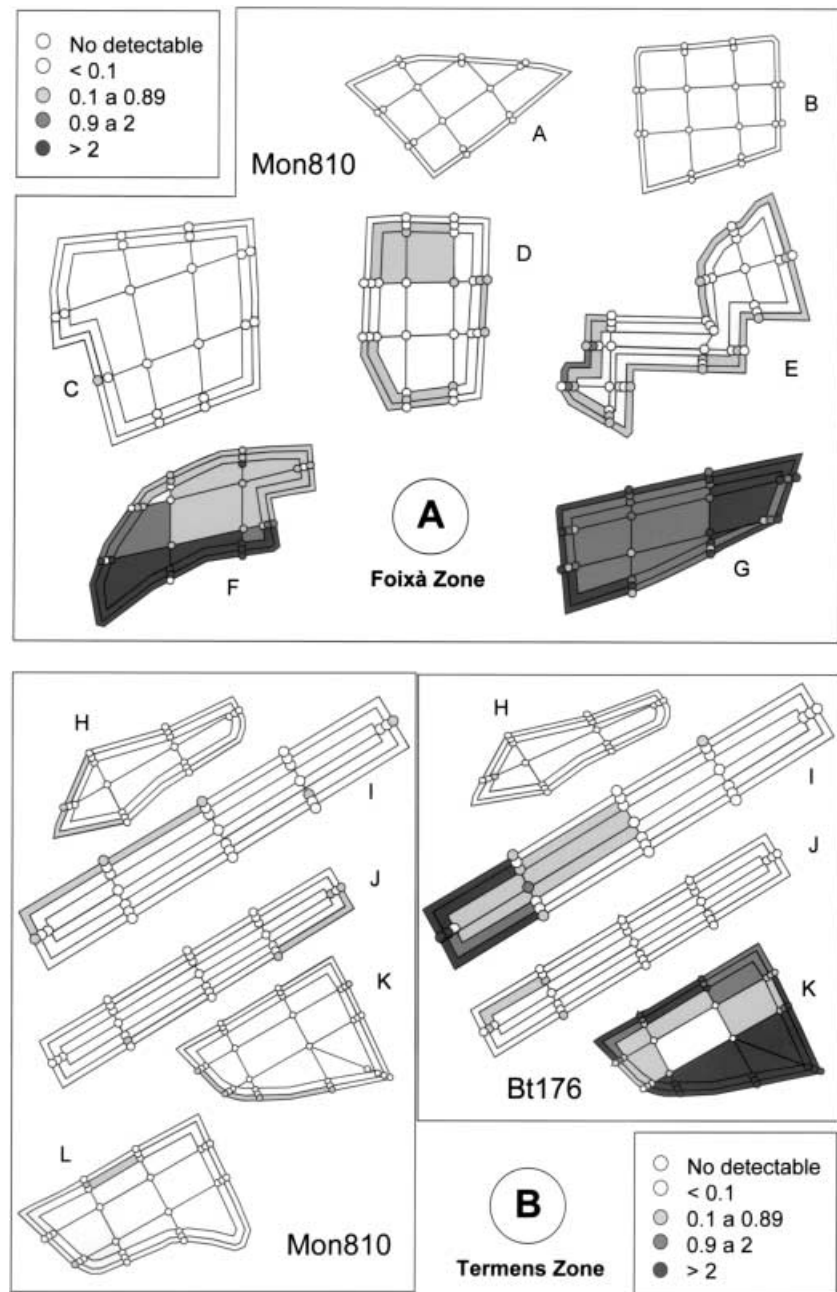
Conventional field			% GM DNA/total DNA $\pm$ standard error		
Zone	Field	Area (ha)	Mon810	<i>Bt176</i>	Total
Foixà	A	1.07	0.00	–	0.00
Foixà	B	0.58	0.00	–	0.00
Foixà	C	4.63	0.00	–	0.00
Foixà	D	1.89	0.05 $\pm$ 0.03	–	0.05 $\pm$ 0.03
Foixà	E	3.56	0.11 $\pm$ 0.05	–	0.11 $\pm$ 0.05
Foixà	F	1.10	1.22 $\pm$ 0.16	–	1.22 $\pm$ 0.16
Foixà	G	1.5	1.89 $\pm$ 0.23	–	1.89 $\pm$ 0.23
Tèrmens	H	0.5	0.03 $\pm$ 0.02	0.01 $\pm$ 0.01	0.04 $\pm$ 0.03
Tèrmens	I	3.08	0.02 $\pm$ 0.01	0.51 $\pm$ 0.13	0.53 $\pm$ 0.14
Tèrmens	J	0.97	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.07 $\pm$ 0.02
Tèrmens	K	1.89	0.01 $\pm$ 0.01	2.28 $\pm$ 1.01	2.29 $\pm$ 1.02
Tèrmens	L	2.55	0.01 $\pm$ 0.01	–	0.01 $\pm$ 0.01

lower than 0.9%, whereas, in the other three (F, G and K), the values were higher than 0.9%.

For each analysed non-GMO field, Table 2 shows a list of the surrounding transgenic fields, with an evaluation of their possible effect using the estimated cross-pollination index (ECP index). This ECP index (see 'Experimental procedures') was applied to the GMO fields, and takes into account the effect of the distance and the synchronicity of flowering. High values of this index indicate a significant contribution to the adventitious presence of GMO detected in the non-transgenic field and, of course, must be accompanied by a visible effect over the proximal edges (compare, as an example, the high values of the west part of field E in Figure 2A and the ECP index of 6 of field 'a' at the west side in Figure 1A). Similar relations could be detected by applying the ECP index to the other fields.

For each individual field studied, a global index (GI) was calculated by the addition of the ECP indices of the surrounding transgenic fields, giving an estimation of the global gene flow produced considering only the distance and the synchronicity of flowering (Table 2).

For each studied field, Figure 3 shows the average percentage of GMO obtained by RTQ-PCR analysis of the samples vs. the GI of the ECP indices. A good correlation ( $R^2 = 0.9491$ ,  $n = 15$ ) was found, showing that GI may be useful for estimating the percentage of GMO in the fields. For this calculation, the field K for *Bt176* was excluded because, as discussed later, the high GMO content could not be a result of cross-pollination only. Nevertheless, when this field was included, the correlation changed to  $R^2 = 0.618$  ( $n = 16$ ), and the slope of the regression of the percentage of GMO vs. GI changed from 0.0689 to 0.081.



**Figure 2** Adventitious presence of genetically modified organisms (GMO) (% GM DNA/total DNA) at each sampling point, and an estimation of the spatial distribution of these rates in the analysed fields: (A) Foixà; (B) Térmenes.

## Discussion

Several factors influenced the increase in *Bt* maize cultivation in 2004 in both regions studied, despite consumer concerns as in other European Union countries. One of the factors was the availability of more numerous and better commercial *Bt* varieties. In Foixà, new *Bt* cultivars with the Mon810 event were grown, but, in Térmenes, many growers continued to use the *Bt*176 Compa CB that they had sown in previous years. Another factor was the possibility of maize fields

becoming infested by corn borers (*Sesamia nonagroides* Lefebvre and *Ostrinia nubilalis* Hübner). In central Europe, there was a single generation (Bohn *et al.*, 1999), whereas in southern Europe, including the areas studied, there were two to three generations (Kergoat, 1999; Farinós *et al.*, 2004). Damage and yield losses are higher when corn borer attack occurs during the early stages of plant development. When farmers can sow very early (middle of March to the beginning of April), the number of insects able to infest plants is lower than that with later sowings (end of April to May), when the

**Table 2** Empirical index estimating the influence of the surrounding transgenic fields. The estimated cross-pollination (ECP) index was calculated by dividing the flowering synchronicity expressed in days by the squared distance expressed in decametres (1 for distances between 0 and 9 m, 2 for distances between 10 and 19 m, etc.). We only considered fields nearer than 150 m and with a flowering synchronicity of almost 1 day. The global index (GI) for each field was calculated by totalling the ECPs

Field	Event	Donor	Flowering synchronicity	Distance (m)	ECP
<b>Foixà</b>					
<b>A</b>	Mon810	–	–	–	<b>GI = 0</b>
<b>B</b>	Mon810	–	–	–	<b>GI = 0</b>
<b>C</b>	Mon810	–	–	–	<b>GI = 0</b>
<b>D</b>	Mon810	f	10	10	2.5
		g	3	118	0.02
<b>GI = 2.52</b>					
<b>E</b>	Mon810	a	6	2	6
		d	6	65	0.12
		e	5	128	0.03
<b>GI = 6.15</b>					
<b>F</b>	Mon810	m	5	3	5
		n	8	32	0.5
		o	10	3	10
		d	5	10	1.25
		c	8	64	0.16
<b>GI = 6.15</b>					
<b>G</b>	Mon810	h	9	3	9
		i	5	6	5
		j	10	5	10
		k	1	5	1
		l	3	121	0.02
		s	9	140	0.04
<b>GI = 25.06</b>					
<b>Térmens</b>					
<b>H</b>	Mon810	–	–	–	<b>GI = 0</b>
<b>H</b>	Bt176	a	10	66	<b>GI = 0.2</b>
<b>I</b>	Mon810	b	9	10	2.25
		d	2	126	0.01
		q	3	71	0.05
		p	3	142	0.01
		m	9	145	0.04
<b>GI = 2.36</b>					
<b>I</b>	Bt176	a	7	10	1.75
		c	9	5	9
<b>GI = 10.75</b>					
<b>J</b>	Mon810	b	9	99	0.09
		d	2	72	0.03
		e	10	150	0.04
		q	3	74	0.05
<b>GI = 0.21</b>					
<b>J</b>	Bt176	a	7	95	0.07
		c	9	112	0.06
<b>GI = 0.13</b>					
<b>K</b>	Mon810	d	7	92	<b>GI = 0.07</b>
<b>K</b>	Bt176	o	8	2	<b>GI = 8</b>
<b>L</b>	Mon810	k	1	72	0.02
		i	1	130	0.01
		m	8	144	0.04
<b>GI = 0.07</b>					

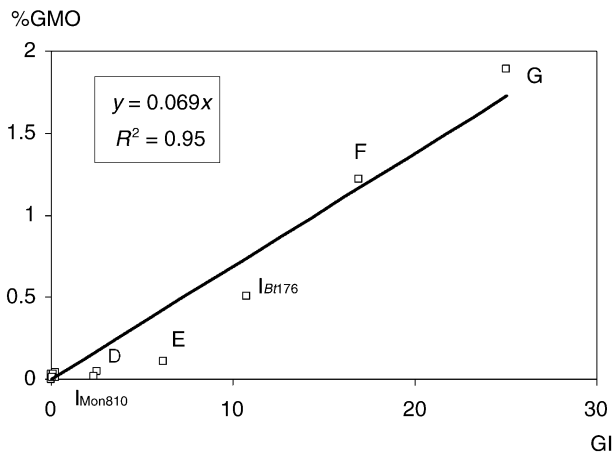
number of corn larvae can completely destroy the young plant. In the Foixà area, nearly all growers that sowed early used conventional maize, whereas those that had to sow very late used *Bt* maize. This situation produced a delay in the flowering period between many *Bt* and some conventional maize fields, giving a very low cross-pollination rate. This was not the case in the Térmens area, where most of the fields were sown approximately in the same period of time, with the synchronicity of flowering between *Bt* fields and conventional fields being higher.

Although there is no official legislation in Spain to regulate the coexistence between transgenic and conventional maize, growers and growers associations have managed to grow both crops. About 80% of cultivated maize in Spain is for feed production, whereas 20% is for food production (Alcalde and Pelaez, 2004), with all *Bt* maize used only for feed production (on the farm or sale to feed manufacturers). Most feed manufacturers import transgenic soybean to add to the feed to increase the protein content, so that, until now, *Bt* maize has been sold through normal marketing channels, without any requirement for on-farm or post-farm segregation from conventional maize (Brookes and Barfoot, 2004). A more complex situation is found when maize is used for food consumption, because the manufacturing companies refuse *Bt* maize. In this case, arrangements are agreed between farmers and companies before the start of the sowing season. Frequently, in some areas, such as Foixà, growers also agree on sowing dates.

#### GMO content and distribution in the fields

As shown in Table 1, only three fields gave values higher than the 0.9% threshold established for labelling. The selected fields were not a random sample, but were chosen to cover the widest range possible of real situations. In this way, it was possible to gain an idea of the global flow produced without any measure of contention. As expected, fields F and G gave a high content of GMO, whereas fields A, B and C, that had been chosen to confirm the effect of the delay in flowering, gave zero content.

An unexpected result was found in the case of field K for *Bt176*. The GMO content (2.28% GMO DNA/total DNA) cannot be explained by cross-pollination only. Moreover, some samples 10 m inside the field gave extremely high values (24.75%) that were higher than those found in the other fields and also in our other field trials. The high values found in the northern and western parts of the field are very difficult to explain because the nearest field with the Compa CB (*Bt176*) variety was 200 m away, suggesting that other



**Figure 3** Correlation ( $y = 0.069x$ ;  $R^2 = 0.9499$ ) between the global index (GI) and the percentage of genetically modified organisms (GMO) from each of the analysed fields.

factors, such as seed mixing during the sowing period, could have occurred. This possibility cannot be neglected because growers sometimes use the same sowing machine for different fields, and it is possible that a *Bt176* maize field could have been sown first, with some seeds remaining in the machine. Another factor could be the presence of volunteers in the field. In the areas studied, we detected some fields in which, depending on the climatic conditions and crop management, some kernels from the previous year germinated and flowered. At the time, we did not quantify the extent of this phenomenon, but we are studying it this year to determine to what degree the presence of volunteers could influence the adventitious rate of GMO in maize.

Studies performed recently to evaluate gene flow in maize (Henry *et al.*, 2003; Ma *et al.*, 2004; Bannert and Stamp, 2005; Melé *et al.*, 2005; Weber *et al.*, 2005) confirmed the high interception of pollen by the first few maize rows, and suggested that, by removing 10–20 m from the borders, the 0.9% GMO allowed would almost never be exceeded in the harvested grain. In our particular situation, in which the size of the fields was small (0.5–4 ha), most fields had an accumulation of GMO content in the borders, decreasing towards the centre of the field (Figure 2). Consequently, a large decrease in the GMO content should be obtained by removing 10 m from the borders. Three of the fields (F, G and K) had an estimated percentage of GMO higher than 0.9%, but, in one, field F from Foixà, the estimated content decreased from 1.22% to 0.62% when 12 rows (10 m) from the borders were theoretically removed. However, this approach was not sufficient to decrease the percentage of GMO below 0.9% in the other two fields. Asynchronicity in

flowering, found in the non-irrigated field G, could be the reason why there was more pollination with *Bt* pollen from neighbouring fields in this field.

In several of the analysed fields, for example fields D and J for *Bt176*, the values taken at 3 and 10 m within the fields were higher than those found in the borders. In planned field trials, this situation was not found. Gene flow decreased progressively from the borders to the centre of the field. In contrast, in a cropping area, other factors can disturb pollen flow, such as the accumulation of tall weeds or physical barriers that protect the borders. A similar effect can be produced by deficient plant growth because of the border effect.

### Effect of the distance and synchronicity of flowering

In this complex situation, we attempted to elucidate the main factors that influenced the adventitious presence of GMO produced by cross-pollination in the fields studied, firstly by quantifying the effect of the synchronicity of flowering and the distance between donor *Bt* fields and the receptor field. Although maize pollen can travel long distances (Jarosz *et al.*, 2003), it has a high settling speed and rapid deposition (Di-Giovanni *et al.*, 1995; Aylor *et al.*, 2003). The pollen concentration and, consequently, successful fertilization decrease rapidly with the distance from the source, following a leptokurtic pattern with a long tail. At distances greater than 30–50 m, the level of pollen dispersion is very low (Bassetti and Westgate, 1994; Sears and Stanley-Horn, 2000; Pleasants *et al.*, 2001; Jarosz *et al.*, 2003, 2005).

In our study, this effect of distance can be confirmed by examining the spatial distribution of the receptor fields in relation to the donor field distances (Figure 1A,B). The effects of fields i, j, k and h surrounding field G in the Foixà zone and of field c (*Bt176*) on field I in the Tèrmens zone are very clear. In these cases, the fields are close and flower at the same time. Particular information can be extracted from field E, for which various fields with an acceptable synchronicity of flowering and placed at different distances produced adventitious pollination proportional to their proximity that could be identified over the edges of this irregular field. Taking into account these data, we consider that only those *Bt* donor fields within 0–150 m can significantly influence the rate of cross-pollination.

It is clear that the synchronicity of flowering between nearby fields is the main factor influencing cross-pollination, as shown by fields A, B and C in the Foixà zone, in which the synchronicity of flowering with surrounding fields was zero or very low. In other cases, as in the above-mentioned field

E, the percentage of GMO detected on the west side was lower than that identified in field F, in which the synchronicity of flowering with field o was higher. As has been demonstrated by several authors (Bassetti and Westgate, 1994; Uribealrea *et al.*, 2002; Westgate *et al.*, 2003), the synchronization of pollen dispersal and silking is a crucial factor in determining the extent of outcrossing in maize. Under our conditions, the different sowing dates resulted in a different flowering time, hence limiting cross-fertilization and demonstrating that this strategy may be effective in improving coexistence, as has been suggested previously (Brookes *et al.*, 2004). However, different sowing dates or varieties differing in development could not be chosen in the German trials carried out in 2004 to avoid overlapping flowering periods (Weber *et al.*, 2005). Moreover, in countries with a colder climate, maize cannot be planted earlier in the season, and the postponement of the sowing date may be at the expense of the yield. Therefore, a strategy based on decreasing the synchronicity of flowering is an effective tool to ensure coexistence, but its application is limited by the specific climatic conditions of each crop area.

In order to evaluate the effect of these two main variables, we established an ECP index for each pair of transgenic–non-transgenic fields. This index is very simple and considers, firstly, the number of days that the two fields are flowering at the same time. It is normally accepted that maize pollen is released over a typical period of 5–8 days, depending on the variety, for each individual panicle (Brookes *et al.*, 2004; Ma *et al.*, 2004). There is also certain variability between plants in the same field. From our measures aimed at establishing the flowering date of each field in the regions studied (data not shown), an interval of 1–2 days was detected from the first appearance of flowering until the time at which 50% of the plants bloomed. This period may be increased slightly in the case of non-irrigated fields as a result of a lack of uniformity in the crop. Only field F was non-irrigated in the studied zones. Consequently, 10 days is a good estimation for the total field pollen shedding period. We assumed that a delay in flowering dates greater than 10 days would reduce the pollen flow to a non-significant level. The number of days in which pollination takes place ranges from 0 to 10 as a maximum and, to obtain the ECP index, this value is divided by the square of the distance between the most proximal edges of the fields. Therefore, it is assumed in this ECP index that adventitious pollination is directly proportional to the number of days of synchronous flowering and inversely proportional to the square of the distance. We chose the square of the distance instead of the distance because, as described in multiple assays, the decline in flow is very large

in the first few metres but progressively decreases with distance.

The use of the GI of the ECPs for each studied field, and its correlation with the average percentage of GMO obtained by RTQ-PCR analysis of the samples, provided confirmation that a large part of the variability of the percentage GMO encountered can be explained by the degree of synchronicity of flowering and the distance between the fields (i.e. fields A, B, C, H, J and K for Mon810).

### Effect of other factors (physical barriers, wind, and size and shape of the fields)

By applying the GI to the studied fields, some had an estimated content different from the values obtained by RTQ-PCR analysis (Figure 3). These differences were used to identify other factors that could modify the effect of distance and synchronicity of flowering.

A physical barrier will produce depletion of pollen from the airflow by impact and filtering (Emberlin *et al.*, 1999; Treu and Emberlin, 2000), and it was found that a maize field or a tree barrier reduced cross-fertilization more effectively than bare ground (Jones and Brooks, 1952). In this study, the effect of physical barriers in minimizing gene flow can be seen in field D where, despite high synchronicity of flowering with *Bt* fields close by, the estimated content of adventitious presence in the yield was lower than expected. The representative point in Figure 3 was placed below the adjusted line, showing that the GI overestimated the real content. This can be attributed to the barrier effect of the road (2 m in height) with tall trees, which was designed some years ago for the containment of the river in case of flooding, and a farm building placed in between.

There were some differences between our results and those from normal field trials. In real situations of coexistence, and in cropping areas with small fields, the main factors that determine cross-pollination are the synchronicity of flowering and the distances between donor and receptor fields. Prevalent winds during the flowering period have a less definite effect than in field trials especially designed to detect their influence. In our study, we detected some wind effects, mainly elongating the flow along the edges of the fields, but any overall effect was diluted because the donor fields faced different directions.

The shape of the fields is another factor that may influence cross-pollination (Ingram, 2000; Novotny and Perchang, 2002; Meier-Bethke and Schiemann, 2003). The amount of cross-fertilization is clearly higher in elongated recipient fields than in rectangular ones of the same surface area when the

long side of the field faces the source. Although, in the Foixà area, there were some fields with these characteristics, it was not possible to analyse them, but we have studied some elongated fields (field I for example) with the donor *Bt* field on the narrow side. In this case, the GI gave a distorted value because its distance to the field edge is small, but the donor fields are in fact far from the major part of the recipient field. A similar effect was detected in field E at Foixà, which had an irregular and elongated shape. The donor field 'a' is placed on the western side but far from the rest of the field. As shown in Figure 3, the representative values of fields I (*Bt*176 and Mon810) and E are placed clearly below the adjusted line. The predicted values of the transgenic content obtained using these GI values are clearly higher than the real percentage of GMO found in the fields. However, if we divide these elongated fields into parts and calculate their GIs separately, the new averaged GI values will be 3.1 for field E (two parts) and 5.48 and 0.92 for field I (*Bt*176 and Mon810, three parts, respectively), greatly improving their line fitting.

In conclusion, the ECP index and GI are useful tools to predict the pollen flow in real conditions of coexistence. Their accuracy could be improved and validated with new experimental data. As a result of their simplicity, they could be used before harvesting to detect in advance possible conflicting fields.

### Implications for separation distances

Data obtained in several field trials especially conducted to quantify the adventitious presence of GMOs have been used to predict the separation distance between transgenic and non-transgenic fields that needs to be established in order to prevent or minimize the adventitious presence of GMOs in conventional fields (Fouellassar and Fabié, 2003; Henry *et al.*, 2003; Messeguer *et al.*, 2003; Bénétrix, 2004; Ma *et al.*, 2004; Melé *et al.*, 2004, 2005; Weber *et al.*, 2005). However, almost none of the *Bt* fields studied in the Foixà and Tèrmens areas had a separation distance or a buffer zone (recommended by seed producers only for fields larger than 5 ha). Several different situations were encountered, depending on agronomical and physical factors, and on the fact that, in the real situation of coexistence, there was competition between the pollen produced by the analysed field and pollen coming not from one field only, but from several fields both close to and at different distances away from the analysed field.

Although the reliability of this approach must be confirmed by accurate calculations using mathematical models, we can obtain some idea of the size of the security distance needed

to maintain the adventitious presence of GMO under the 0.9% threshold required by European Union regulations. The slope of the regression line of GMO content vs. GI is 0.0689 and, on the basis of a field surrounded by four transgenic crops with total synchronicity of flowering, the expected value of the GMO content is 2.76% if the distance is within the first 10 m, 0.69% within the second and 0.30% within the third. This means that a security distance between 10 and 20 m should be sufficient to maintain the GMO content below the 0.9% threshold under the worse circumstances. The studied areas must be considered as 'difficult zones' for controlling pollen flow, because they are flat and windy, with small fields (2 ha average), and with a high percentage of transgenic maize.

### General conclusions

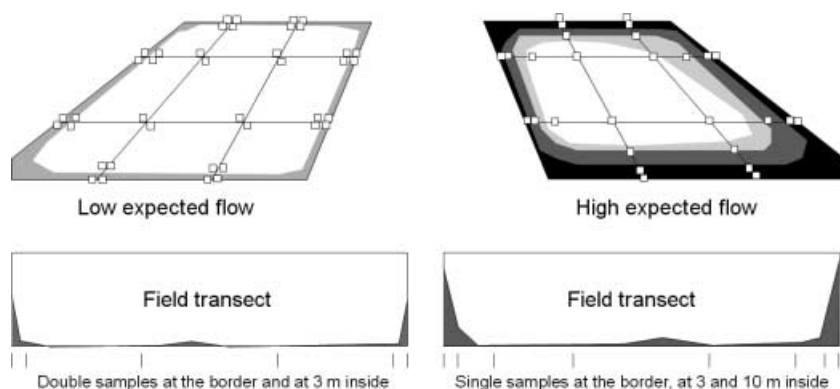
Many factors can affect the rate of adventitious presence of GMO as a result of cross-pollination in real conditions of coexistence. In the particular situation studied, in which the size of the cultivated fields was small and with no containment strategy, we found some fields with an adventitious content higher than the 0.9% threshold for pollen-mediated gene flow. However, the results obtained in this study suggest that a distance of about 20 m would be sufficient to control the adventitious presence of GMO as a result of pollen-mediated gene flow and to obtain a GMO content below the 0.9% threshold. Larger security distances, such as 30–50 m, are useful to maintain the GMO content at a very low level, and perhaps could contribute to minimize the effect of other factors, such as mixing during sowing, harvesting and storage. However, the use of this policy in zones similar to those studied here would be impossible, as can clearly be seen by observing the decrease in surface area on the map.

We have shown that coexistence between GM and conventional fields can be achieved by establishing simple rules that take into account the synchronicity of flowering and the distance between fields. The data collected in this study will be very useful for the validation of models to predict pollen flow at the landscape level with different spatial distributions (smaller and larger fields).

### Experimental procedures

#### Areas studied

Two crop regions were chosen during the growing season of 2004 in which irrigated transgenic *Bt* and conventional maize fields coexisted with other crops. The first was located in



**Figure 4** Sampling methodology for low and high expected flow.

Térmens (Lleida) and the second in Pla de Foixà (Girona), both in Catalunya, Spain. Térmens has a continental climate, whereas Foixà has a Mediterranean climate because of its proximity to the sea. Both regions are characterized by the small size of the fields (0.5–5 ha, with a mean of about 2 ha). Wind speed and wind orientation data during the flowering period were taken from a meteorological station in the crop areas. In both regions, two areas were defined: the central area in which conventional fields were selected for sampling and the surrounding area that may influence the rate of pollen-mediated gene flow. The total surface area studied in Térmens was 300 ha, with a central area of 43 ha, whereas, in Pla de Foixà, the area was 400 ha, with a central area of 100 ha.

A map was designed using the land registry and aerial photographs from the Spanish Global Information System (GIS). The different crops (cereals, fruit trees, maize, etc.) were identified. During the cropping season, all maize farmers were contacted to establish which maize cultivar was used (either *Bt* maize or a conventional maize) and its sowing and flowering dates. All of these data were used to choose the non-transgenic fields for sampling.

### Sampling methods

During the harvesting period, two sampling methods were applied depending on the synchronicity of flowering between *Bt* and conventional fields. Taking into account that the risk of cross-pollination is higher in the borders than in the centre of the field (as a result of the buffer effect of maize plants), in both cases we applied a stratified sampling system, dividing the fields into different zones according to the distance from the borders, in such a way that it was possible to estimate the presence of GMO in each zone. For those fields in which a low rate of pollen-mediated gene flow was expected (fields A, B and C from Foixà), two samples were taken for each sampling point at 0 (border) and 3 m inside

the field; moreover, eight samples were taken at four sampling points in the middle of the field (Figure 4). In those fields in which a high rate of pollen-mediated gene flow was expected (the remaining fields), only one sample was taken at 0 (border), 3 and 10 m inside the field and four more samples from the middle of the field. In both cases, the number of sampled points for each border depended on the size and the particular shape of the field. At the practical level, some transects (the number depending on the shape of the field) were determined on the map using Universal Transverse Mercator (UTM) coordinates and taking into account the direction of the crop lines to make sampling easier. In all cases, three cobs per sample were taken and stored in open paper bags in a drying chamber.

### DNA isolation and quantification

The three cobs from each sample were dried at room temperature, threshed by hand and the kernels were ground to a fine powder and mixed thoroughly before analysis.

#### DNA analysis

Genomic DNA was isolated from 0.2 g of maize powder using a Wizard-based protocol, as described in a Promega (Madison, WI) update review ([www.promega.com/pnotes/73/8235-23/8235-23.pdf](http://www.promega.com/pnotes/73/8235-23/8235-23.pdf)). The DNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington DE). DNA concentrations and quality were further checked by 1% agarose gel electrophoresis and ethidium bromide staining. Powdered Certified Reference Materials (CRM) Mon810 maize (IRMM 413) and *Bt*176 maize (IRMM 411) were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and commercialized by Fluka (Buchs, Switzerland). The DNA of CRM standards was extracted and quantified in parallel using the same protocol as for the maize samples.

### GMO detection

Firstly, a 226-bp fragment of an internal unique copy gene reference of *Zea mays* invertase (Ehlers *et al.*, 1997) was amplified to validate the ability of the extracted DNA to amplify. Then, all samples were analysed for a 194-bp fragment of a specific sequence of Mon810 (Matsuoka *et al.*, 2001) and some for a 211-bp fragment of a specific sequence of *Bt176* (Hupfer *et al.*, 1998). Invertase and the *Bt176* fragment were amplified in 20 µL of a PCR mixture: 1 × buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 8.3), 2.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.5 µM of each primer, 0.5 U of FastStart Taq DNA Polymerase and DNA template. The PCR programme was as follows: 10 min at 95 °C, 35 cycles of 20 s at 95 °C, 30 s at 62.5 °C and 40 s at 72 °C, and a final extension step of 5 min at 72 °C. The Mon810 fragment was amplified in 20 µL of PCR mixture: 1 × buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM).

All qualitative reactions were carried out in a GeneAmp™ PCR System 2700 (Perkin-Elmer Cetus Instruments, Emeryville, CA, USA), and primers were purchased from TIB (TIB MOLBIOL Syntheselabor, Berlin, Germany) and MWG-Biotech Ag (Ebensburg, Germany). PCRs were performed with the FastStart Taq DNA Polymerase from Roche (Roche Diagnostics GmbH, Penzberg, Germany).

### GMO quantification

RTQ-PCR based on molecular energy transfer was only performed with Mon810- and *Bt176*-positive samples.

To measure the real quantity of Mon810, a small fragment of an endogenous unique copy gene reference of *Zea mays* invertase (Hernández *et al.*, 2004) and a small fragment of Mon810 construct were amplified in parallel. The high specificity of the method is based on the design of a TaqMan® probe containing annealing sites for both the 3' site of the transgene construct and the maize genome flanking sequences immediately adjacent. The amplicon provides an unambiguous and sensitive quantification method for the Mon810 event (Hernández *et al.*, 2003).

RTQ-PCRs were carried out in a LightCycler (Roche Diagnostics GmbH). Primers were purchased from MWG-Biotech Ag. Both TaqMan® probes INV78P and MonP were labelled at the 5' end with the fluorescent 6-carboxyfluorescein (FAM), and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) was attached to the 3' ends.

Real-time PCRs were performed with the LightCycler FastStart DNA Master Hybridization Probes. Both endogenous corn reference and Mon810 reactions used 20 µL of PCR mixture including 1 × Master Probe, 4 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.5 µM of each primer, 0.2 µM of probe and 1 U of

FastStart DNA polymerase. The following thermal cycling programme was used: 8 min at 95 °C; 40 cycles of 5 s at 95 °C and 25 s at 60 °C; and cooling for 30 s at 40 °C. The fluorescence was recorded at the end of the elongation phase.

The percentage of powdered CRM, rather than plasmid DNA material, treated as the samples, was used to quantify Mon810, because it makes the experiment totally comparable with unknown samples in all steps of the assay from DNA extraction to the final quantification.

Five different standard concentrations of Mon810 were used for the standard curves in the specific Mon810 system (containing 5%, 2%, 1%, 0.5% and 0.1%), and to normalize the reference gene maize quantity. It was realized previously that working with five different values is better than using serial dilutions of a single Mon810 standard value, as the linear correlation obtained from these five values with a valid correlation factor ensures the correct operation of the detection system.

After obtaining the data collections of the samples, the percentage of Mon810 was determined using a mathematical worksheet. The threshold cycle of samples was obtained from LightCycler software. As five different standard values of Mon810 were used for the endogenous system, the same threshold cycle was obtained in all of them, with the DNA quantity in all being adjusted to the same amount, preventing the construction of two different linear regressions. The method used in the assay allows for the construction of only one linear regression from the results of both endogenous and Mon810 systems. The parameters correlated in the linear regression were the concentration value of Mon810 standards and the anti-logarithm of the distance in cycles between threshold cycle amplification of the endogenous gene and the Mon810 system. Finally, the percentage values of the samples were obtained by extrapolating the anti-logarithm of the distance in cycles between the two systems in the linear regression.

For the quantification of *Bt176* maize, the same method was used as for Mon810. The PCR conditions differed with regard to the increase in concentration of MgCl<sub>2</sub> to 6 mM. The probe CRYtmp was also labelled at the 5' end with the fluorescent FAM, and the quencher dye TAMRA was attached to the 3' end (Studer *et al.*, 1997).

### Estimation of the total adventitious content of GMO in the non-GM fields and empirical indices

Values obtained from the RTQ-PCR analysis were used to estimate the GMO content of the studied fields. As shown in Figure 2, all the fields were divided into zones by the transects used for sampling and by two virtual lines that

follow the field perimeter at 3 or 10 m inside. The partial GMO content of each zone was calculated by averaging the four samples that delimited the surface. These values were used to make a graphical representation of the local distribution of the adventitious flow in the field. The average of these local values, weighted by their corresponding area, was used to obtain a representative estimation of the global field value.

An empirical ECP index was used as a tool to identify the major transgenic donor fields responsible for the rate of cross-pollination found in each non-GM field. This index takes into account only two scalar factors (the synchronicity of flowering and the distance) that are independent of the orientation. Other non-scalar factors, such as the relative orientation of the fields, wind direction and geographical barriers, were not considered in this first approach.

The synchronicity of flowering was calculated as 10 days minus the absolute value of the difference between the two flowering dates.  $CF = 10 - |f_1 - f_2|$ . Negative values were considered as zero.

A distance index (DI) was defined as the shortest distance calculated from the map, expressed in decametres (1 for distances between 0 and 9 m, 2 for distances between 10 and 19 m, etc.). The distances between fields on the map were not real in many cases as the crops frequently did not reach the limit, or minor separations existed, such as roads, fences, ground at different levels, etc. For this reason, we did not use a more precise measure for the distance.

For each pair of fields (donor and recipient), we calculated the ECP as the synchronicity of flowering (CF) divided by the square of the distance index ( $ECP = CF/DI^2$ ). We chose the square of the distance to emulate the rapid decrease in the flow values at short distances, according to the  $d^2$  factor that frequently appears in physical formulae for radial distances.

With this criterion, we were easily able to visualize and classify the fields most responsible for the adventitious flow detected. By totalling the ECPs affecting a non-GM field, we assigned to each studied field a GI that estimated the transgenic flow produced, and that could be correlated with the real values obtained from the RTQ-PCR analysis.

Statistical analysis of the weighted average of the sampled fields and the confidence limits of the regression slope was carried out using the MEANS and REG procedures from SAS/STAT™ software (SAS Institute Inc., Cary, NC, Release 8.2 for Windows).

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