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Evaluation of pollen dispersal and cross pollination using transgenic grapevine plants

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Public debate about the possible risk of genetically modified plants often concerns putative effects of pollen dispersal and out-crossing into conventional fields in the neighborhood of transgenic plants. Though *Vitis vinifera* (grapevine) is generally considered to be self-pollinating, it cannot be excluded that vertical gene transfer might occur. For monitoring pollen flow and out-crossing events, transgenic plants of *Vitis vinifera* cv. 'Dornfelder' harboring the *gus-int* gene were planted in the center of a field experiment in Southwest Germany in 1999. The rate of pollen dispersal was determined by pollen traps placed at radial distances of 5–150 m from the pollen-donor plants, at 1.00 and 1.80 m above ground. Transgenic pollen was evaluated by GUS staining, and could clearly be distinguished from pollen originating from non-transgenic grapevine plants. Transgenic pollen was observed up to 150 m from the pollen donors. The rate of out-crossing was determined by sampling seeds of selected grapevines at a distance of 10 m to the pollen source, and of a sector at 20 m distance, respectively, followed by GUS analysis of seedlings. The average cross-pollination rate during the experiment (2002–2004) was 2.7% at a distance of 20 m. The results of this first pilot study present a good base for further assessment under the conditions of normal viticulture practice.

Keywords: cross pollination / field release / genetically modified plants / grapevine / pollen dispersal / *Vitis vinifera*

INTRODUCTION

For many plant species used in agriculture, genetically modified (GM) plants have been developed or will be processed all over the world (125 million hectares in 2008, www.transgen.de) in order to encourage the sustainable supply of farmers with plant material with improved features, *e.g.* disease resistance or quality traits. But many producers and consumers are afraid of possible risks derived from transgenic plants or their products. Many of these fears are undefined, but some are explicitly addressed to dissemination of transgenic pollen, and the consequences of pollination of nearby non-transgenic plants. The future co-existence of different kinds of farming, such as conventional, organic or GM, has to take into account the issues raised by out-crossing of transgenic crops.

In German agriculture, the most important crops are cereals, maize and oilseed rape, with an overall cultivation area of 6.2 million ha, 1.8 million ha, and 1.5 million ha, respectively (2007 – source: www.bmelv-statistik.de/tabellen/f1760.1.xls/). In comparison, viticulture represents only slightly more than 100 000 ha, but for some regions the cultivation of grapevine is the basic source of income for many people. At present, it is logical that many endeavors are underway to develop strategies for monitoring concepts concerning possible risks for farming of GM crops with major economical importance. But nevertheless, for grapevine, which is a long-lasting perennial culture, the potential ecological impact of gene flow from GM grapevines in the environment has yet to be analyzed.

Since Sartorius made his first observations about pollen dispersal in 1926, the pollination of grapevine has been the subject of many investigations. The studies concerned different aspects of pollen morphology (Ahmedullah, 1983; Kozma and Scheuring, 1968; Linder and Linskens, 1978; Lombardo et al., 1976) and size (Ben Slimane and Ahri, 1989; Martens et al., 1989), or examinations of pollen distribution and fertility for an early forecast of grape and wine production (Besselat, 1994; Besselat and Cour, 1990; Cunha et al., 2003; Fornaciari and Romano, 1995; Panigai and Moncomble, 1992). The mode of grapevine pollen distribution has been discussed by numerous authors. Almost all of the commercially grown grapevine cultivars are hermaphroditic, and are generally considered to be primarily self-pollinating. The discovery of *Vitis* pollen

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in isolated locations, such as a peat bog in northern England (Barber, 1981) and an arctic ice core (Andreev et al., 1997), are evidence for long-distance transport, despite the lack of special morphological features that would facilitate wind distribution. The small size of the tricolpate *Vitis* pollen of 17–28 µm in diameter (Linder and Linskens, 1978) is typical of wind-transmitted a pollen, since Hyde and Adams (1958) observed an average diameter of 18 to 60 µm in many species in which pollen is airborne. Di Collalto et al. (1982), Kevan et al. (1985), and Bronner and Wagner (1997) favored the fertilization of grapevine plants with airborne pollen, whereas pollination by insects like bees seems to be a rare event. Nevertheless, within a winegrowing region, it is difficult to determine the origin of grapevine pollen. Numerous cultivars are grown side by side, and pollen clouds consist of pollen of various cultivars. Within cultivars of one species (*Vitis vinifera*) the variation of pollen morphology is rather small (Ahmedulla, 1986), and possible cross-pollination is difficult to monitor. However, a sophisticated approach to solve this problem is the use of GM grapevine. Questions concerning gene dispersal and co-existence with non-GM-farming can easily be addressed. For grapevine, an important crop worldwide (Aigrain, 2006), potential risks of releasing GM plants have to be assessed before future cultivation.

This paper presents the first preliminary quantification of pollen dispersal and out-crossing events of transgenic grapevine plants in a landscape that is mainly characterized by viticulture. The transgenic plants were part of the first field release (RKI Az. No. 6786-01-0100) of GM grapevine plants in Germany. Transgenic plants of cv. 'Dornfelder' transformed with the *gus-int* gene can easily be monitored by a histological GUS assay (Jefferson et al., 1987). Pollen dispersal from these plants was visualized in pollen traps, and fertilization of grapevine plants with transgenic pollen could be traced back by the blue staining of the developed seedlings.

RESULTS

Characterization of transgenic 'Dornfelder'-plants

Phenotypically, no differences were observed between greenhouse- and vineyard-grown transgenic and control plants, considering traits like vigor, morphology, fertility, yield, typicity, and wine quality (tested sensorily and analytically). However, Southern analysis revealed differences in the copy number, with 1 to 4 copies of the *gus-int* gene having been inserted (not shown). Analysis of GUS expression in pollen showed differences between the lines, but without any correlation to the copy number of the *gus-int* gene. In two lines (Do1, Do3; 2–3 copies of the *gus* gene), about 1% of the pollen could be stained blue, whereas two other lines (Do2, Do4; 1–2 copies) showed a GUS activity in 34% and 23% of the pollen, respectively. From the fifth line (Do5; 4 copies), 65% of the pollen could be stained and identified as transgenic. For a hemizygous *gus* transformant carrying a single-copy integration, it is expected that 50% of the pollen would be GUS positive. For multiple-copy insertions, however, a higher proportion of pollen should be positive for GUS expression. The unexpected low rates of blue-stained pollen in plants carrying multiple *gus* copies can be explained by silencing effects (De Buck and Depicker, 2001; Muskens et al., 2000) and limitations of the sensitivity of the histochemical GUS assay. Thus the number of GUS-positive pollen grains probably underestimates the proportion of the pollen that was GM.

Determination of pollen dispersal

The rate of pollen flow was investigated during the flowering period of 2002 and 2003, according to the experimental design shown in Figure 1. Pollen dispersal was recorded as the percentage of grapevine pollen in the pollen traps that could be stained blue by the GUS assay (Fig. 2A), thus originating from the transgenic pollen-donor grapevines. In 2002, the weather conditions during grapevine flowering were mainly characterized by sunny days with an average temperature of 21.0 °C (daily min./max.:min. = 14.9 °C; max. = 26.7 °C, 2 m above ground) and some rain showers during the night, even at the end of the flowering period. In the second year, a slightly higher daily average temperature of 22.3 °C (15.7 °C min.; 29.1 °C max.), and continuously sunny days were predominant during the flowering period. In both years, wind velocity was recorded within a range of 2.8 $m.s^{-1}$ as 24 hours mean (daily min. = 2.3; max. = 3.7) in 2002, and 2.5 $m.s^{-1}$ as 24 hours mean (min. = 1.9; max. = 2.9) in 2003. In 2002, many omni-directional winds were detected, whereas winds from the north-west prevailed in 2003.

In summary, 9127 grapevine pollen grains (1.00 m trap 6085 grains, 1.80 m trap 3042 grains) stuck on 480 pollen traps in 2002 (radii 5, 10, 20, 50 m). In 2003, the same 480 pollen traps on the same radii caught 8639 grapevine pollen grains (1.00 m trap with 6325 grains, 1.80 m trap with 2314 grains). These traps captured 66 transgenic pollen grains in 2002 (39 in 1.00 m and 27 in 1.80 m traps) and 62 transgenic pollen grains in 2003 (41 in 1.00 m and 21 in 1.80 m traps), respectively. As transgenic pollen could be detected at the largest distance of 50 m in 2002; additional pollen traps were placed at 100 m and 150 m distance in 2003. These additional pollen traps, which were partly located outside the vineyards, contained 1521 pollen grains (1.00 m trap

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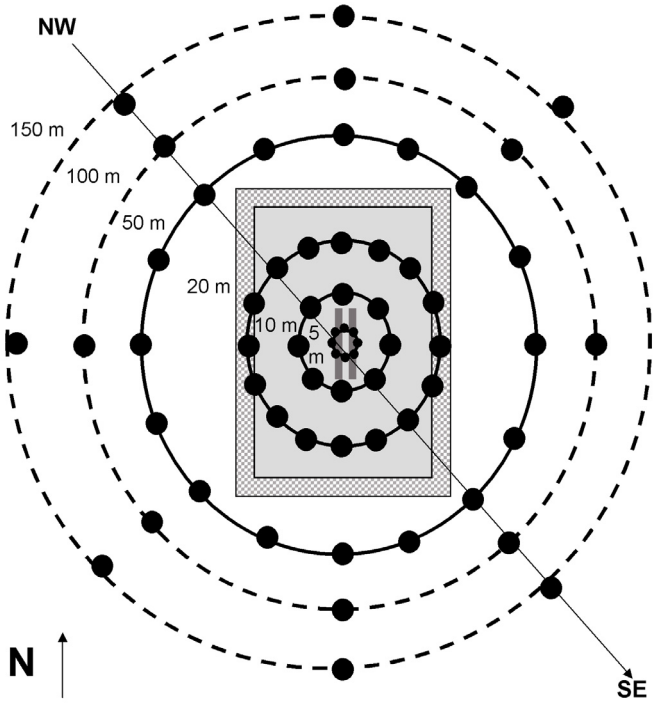

Figure 1. Schematic representation of the experimental design for evaluation of pollen dispersal from transgenic pollen donor plants of the grapevine cv. 'Dornfelder' in the field release of the Institute for Grapevine Breeding Geilweilerhof.

Different constituent parts of the field release:

30 transgenic pollen donor plants of grapevine cv. 'Dornfelder' with *gus-int* gene

Transgenic grapevine plants carrying fungal resistance genes (non-GUS plants)

Protection plantation with non-transgenic grapevine plants

Locations with pollen traps

Distances of radii to pollen donor plants in 2002 and 2003

Additional distances to pollen donor plants in 2003

Prevailing wind direction

with 1175 grains, 1.80 m trap with 346 grains). The percentage of the trapped grapevine pollen that was GUS-positive was evaluated on odd-numbered flowering days (Tab. 1). However, the data must be regarded as an underestimate of the percent GM pollen, since the expression of GUS in the donor plants varied as indicated above.

More detailed results of the distribution of transgenic grapevine pollen are given by the daily evaluation of all traps during the ten-day flowering period in 2003 (Fig. 3) on the axis of the prevailing wind direction of NW → SE (see Fig. 1). Nearly constant weather conditions with only some minor rain showers and a moderate wind velocity characterized the flowering period. The number of pollen grains collected, especially of transgenic pollen, did not show a normal curve of distribution, with an increasing number at the beginning and a decreasing number at the end of the flowering period. The highest number of grapevine pollen grains was collected on day 8 of flowering, and corresponded with a high number of transgenic pollen grains on the same day. A surprisingly high number of GM pollen grains at a distance of 100 m (NW-orientation) to the pollen source at the inflorescence level was collected on day 3. This was caused by only a single trap which carried about 50% (33 of 67)

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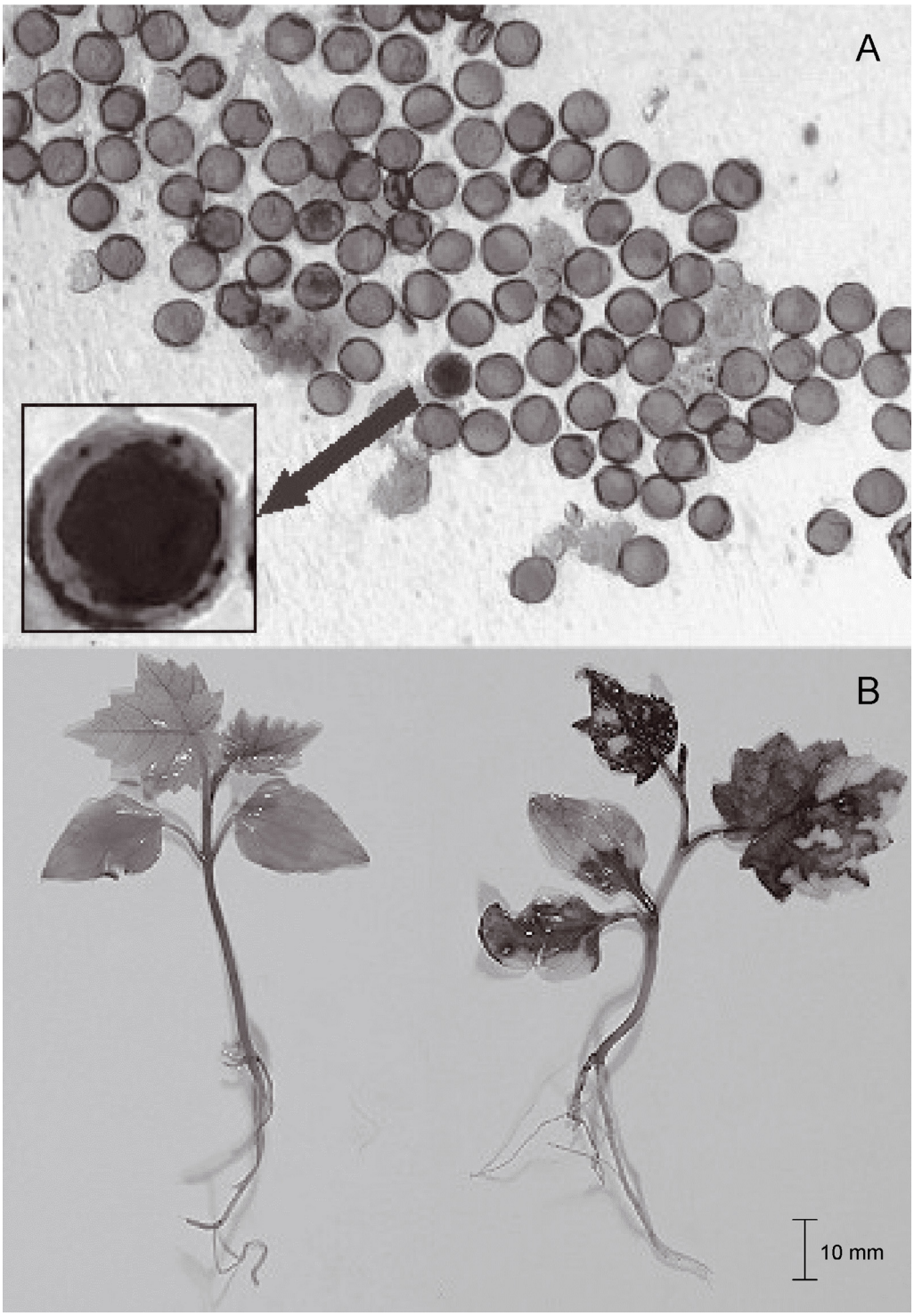

Figure 2. GUS staining of pollen and seedlings of the grapevine cv. 'Dornfelder'.
A. Evaluation of transgenic pollen (magnified inset) on pollen traps under the light miscroscope.
B. Identification of non-transgenic (left) and transgenic (right) F1-seedlings.

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Table 1. Percentage of pollen dispersal at defined radii, and as a function of the position of pollen traps in 2002 and 2003 (ratio of GUS-positive grapevine pollen grains to the total grapevine pollen grains collected on odd-numbered days).

Radius	Number of pollen traps per radius at both heights (*n*)	Transgenic pollen in 2002 (%)		Transgenic pollen in 2003 (%)	
		Height above ground		Height above ground	
		1.00 m	1.80 m	1.00 m	1.80 m
5 m	16	1.1	0.7	1.3	2.1
10 m	16	0.4	1.2	0.5	0.7
20 m	32	0	1.1	0.2	0.2
50 m	32	0.6	0.4	0.9	0.5
100 m	16	n.t.	n.t.	8.6	0
150 m	16	n.t.	n.t.	0	0

n.t.: Not tested.

transgenic pollen. This phenomenon might be explained by an artifact that occurred during sampling, or by local thermal turbulences and updrifts caused by the weather conditions, although no special weather conditions were recorded on that day.

On the axis of the prevailing wind direction (NW → SE, see Fig. 5) across the experimental field, 14 813 grapevine pollen grains were detected (7832 grains in the NW direction, 6981 grains in SE-direction) during the 10 days of the flowering period in 2003 (Fig. 4A). In the NW orientation, 43 transgenic pollen grains were observed, whereas downwind (SE), 31 GMO pollen grains were detected (Fig. 4B). On the NW-SE axis, the overall percentage of transgenic pollen dissemination was 0.5%, up to a distance of 150 m from the pollen donor plants. Transgenic pollen was found even in the most distant trap (150 m). In general, there was no clear correlation between pollen dispersal and the position of the pollen traps.

Evaluation of cross-pollination

As postulated by the European Commission, one of the most important parameters for a risk assessment is the evaluation of cross-pollination from transgenic plants into neighboring ones. The transgenic 'Dornfelder' grapevine plants are an adequate tool for the determination of both pollen dispersal and the fertilization of recipient plants in order to evaluate the distribution of out-crossing events. In 2002/2003, 23 grapevine plants located on the 10 m radius were used as pollen recipients. In 2003/2004 the experimental design was extended by 11 acceptor plants on a 20 m radial sector east of the pollen donors. In autumn, the seeds of each bunch of a plant were collected, stratified for four months during winter, and germinated in the greenhouse. The number of seeds as well as the number of seedlings derived from acceptor plants at 10 m increased from 2002 to 2004 as a consequence of the increasing yield of older and thus more vigorous plants. At 20 m, the seed number decreased as a result of poor fruit set of some acceptor plants in 2004 (Tab. 2). The average germination rate of the F1 seeds was higher than 50%, which is quite good for grapevine. The resulting 109 652 seedlings (from 2002–2004) were subjected to GUS staining (Fig. 2B) to determine the out-crossing rate (Tab. 2). Finally 2375 transgenic F1-hybrids in the progeny were confirmed by GUS staining. With respect to each measuring point, the out-crossing rates revealed some exceptional peak values of up to more than 18% on the 10 m radius and of 13% at 20 m distance from the pollen donor plants (Fig. 5).

The average out-crossing rate observed during three years was 2.0% on the radius of 10 m. On the sectors of 10 m and 20 m in the prevailing wind direction, the level of gene flow was 2.0% and 2.7%, respectively.

DISCUSSION

Since April 2004, food and feed originating from genetically modified plants or containing GM material, as well as food products obtained from these GMOs, are regulated in the European Community under Regulations (EC) No. 1829/2003 and No. 1830/2003. In order to be labeled non-GM, a threshold of less than 0.9% GM in food and feed is required. As a consequence, this presetting requires improved information about pollen dissemination from GM plants to neighboring conventional plantations and about subsequent cross-pollination events, which is by no means available for grapevines.

Experimental design

Over long periods, numerous discussions have been raised about self- and cross-pollination of *Vitis vinifera*

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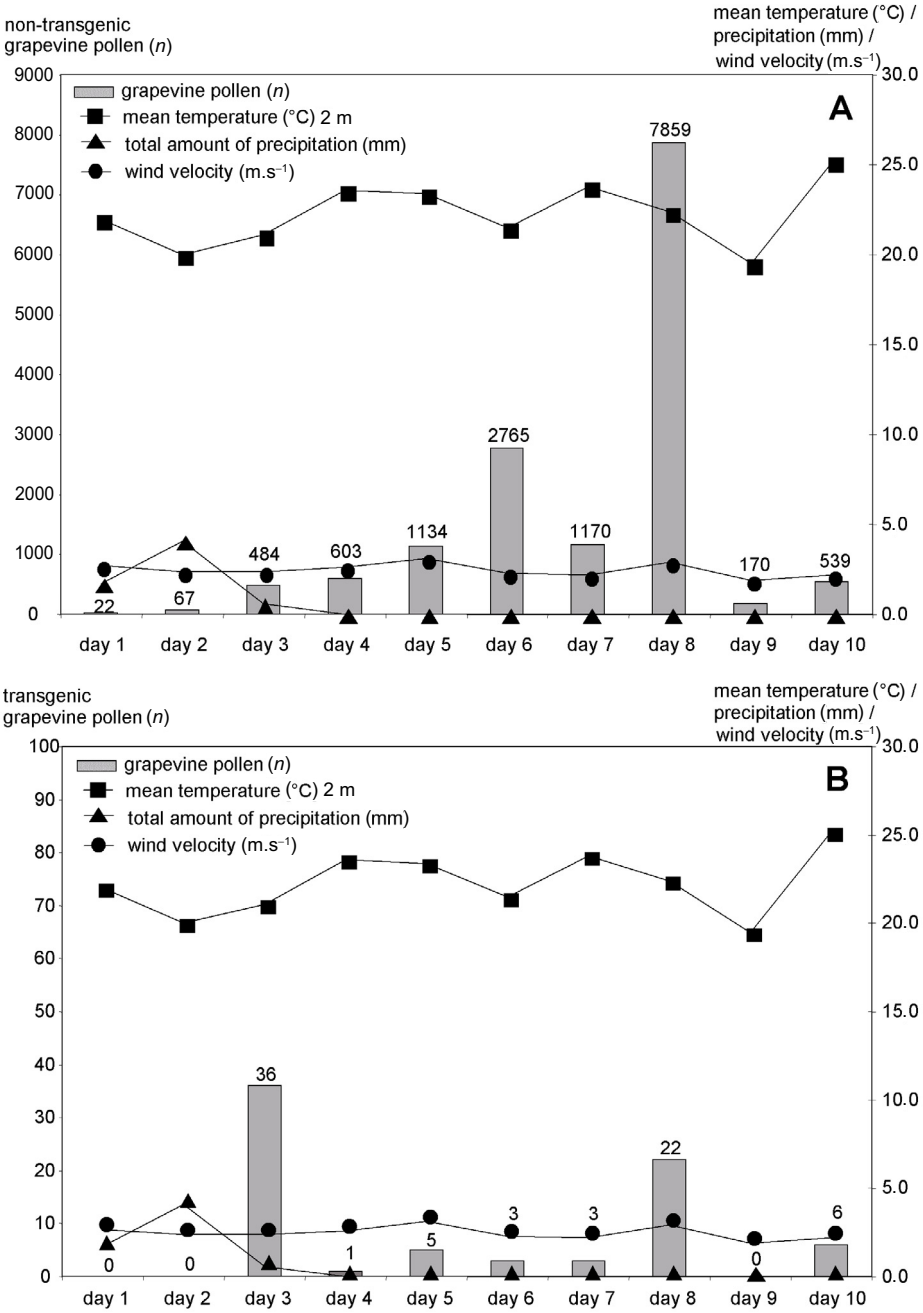

Figure 3. Weather conditions and distribution of non-transgenic (A) and transgenic grapevine pollen (B) on the axis of the prevailing wind direction (NW → SE) on each day of the flowering period of pollen-donor plants in 2003 (sum of all distances, and of both heights).

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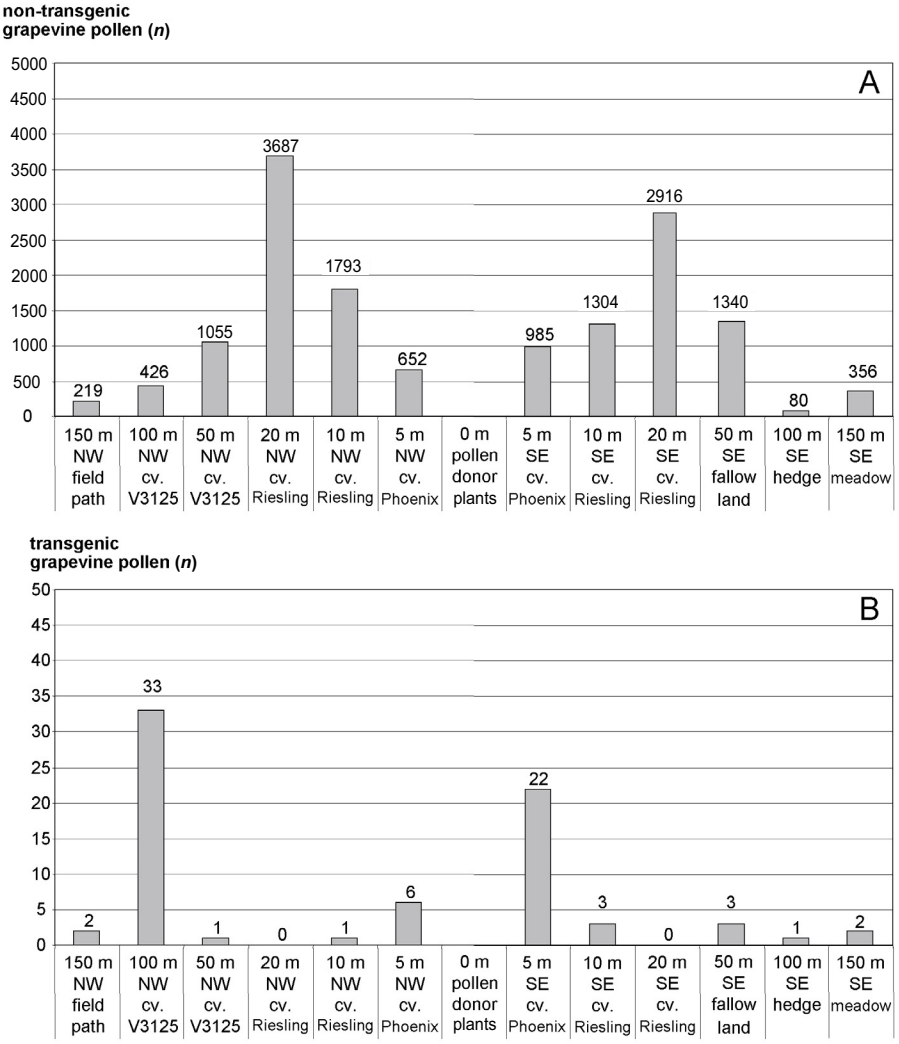

Figure 4. Collected grapevine pollen (A) and distribution of GMO pollen from transgenic 'Dornfelder' grapevine plants (B) in the prevailing wind direction (NW → SE) in 2003 (see axis in Fig. 1) depending on distance and location of defined pollen traps (sum of 10 days of the total flowering period of pollen donor plants, and of both heights).

(Heazlewood and Wilson, 2004; Koblet and Vetsch, 1968; Müller-Thurgau, 1884, 1888; Sartorius, 1926; Staudt, 1999), demonstrating that knowledge about anthesis, pollen distribution and pollen fertility in this species is still incomplete. Due to this ongoing interest in pollination and fertilization, especially in the case of transgenic grapevine pollen, essential basic studies are required.

Published data on pollen dispersal and out-crossing experiments of the most important GM crops like maize and rapeseed demonstrated that a comparison between dispersal data of different field trials is hardly possible (De Marchis et al., 2003; Devaux et al., 2005; Funk et al., 2006; Klein et al., 2006; Loos et al., 2003). Limitations are given within the same crop by the strong dependence on the shape and extent of the transgenic source and receptor plot, the strong influence of different weather conditions between years of investigation, and strong climatic variations of different geographical locations of individual field trials. Our own studies confirmed the same observations concerning the seasonal weather conditions during the flowering period in two subsequent years of investigation. They showed great variation in the course of flowering, and thus in the distribution of grapevine pollen. In contrast to our expectations, no clear dependence on the prevailing wind direction was observed in the case of out-crossing events in 2002–2004. A strong influence on the quantity of

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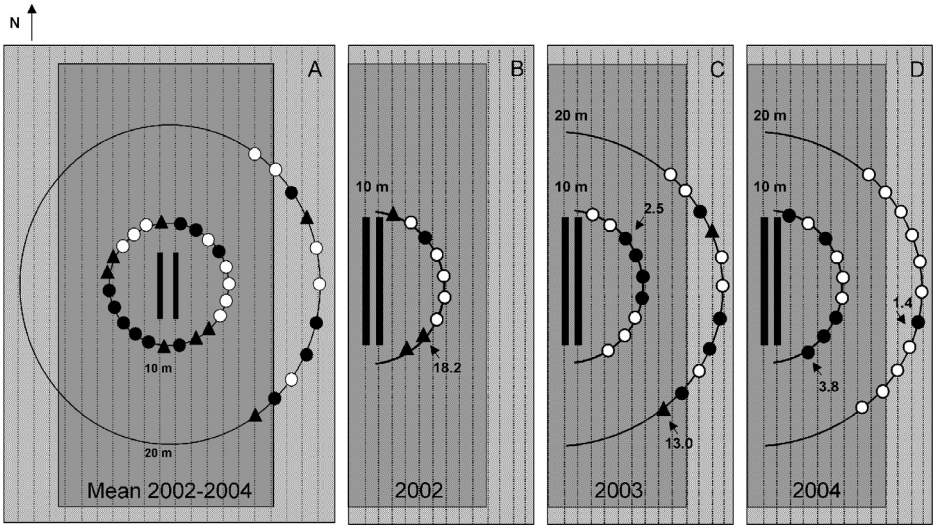

Figure 5. Out-crossing events of GM grapevines at defined pollen acceptor plants during three years of observation, shown as mean values of 2002–2004 (A) and detailed values of each year in the prevailing wind direction NW → SE (B, C, D).
Hits of out-crossing rate in a range of:

> 1.0% ○

1.0–5.0% ●

> 5.0% ▲

Peak value of out-crossing rate (%) ⟶

GM pollen and cross-pollination might be the number of transgenic donor plants, as discussed by Rognli et al. (2000) for the wind-pollinated grass *Festuca pratensis,* by De Marchis et al. (2003) on gene flow measurement of transgenic *Lotus corniculatus* and by Kuparinen et al. (2007) in different model systems for corn. Only 30 transgenic grapevine plants in the center of a field trial do not represent the situation in commercial cultivation. Generally these vineyards cover much greater areas, but the field release provided a good opportunity for a radial disposition of pollen traps at distances of 5–150 m around the pollen donor. The plot of 30 GM 'Dornfelder' grapevines has proved to be well suited to a first estimation of the spatial dissemination of transgenic grapevine pollen. The evaluation of pollen traps in 2002 gave also valuable hints for the study of cross-pollination events. The lowest distance of 5 m to the pollen donor plants could be neglected because in the direct surrounding area of the transgenic pollen donors an expected high amount of transgenic pollen were collected. Thus, acceptor plants at distances beyond 10 m were much more interesting to be analyzed. Therefore the examinations were concentrated on analysis of pollen acceptor plants in the radius of 10 m and a distance of 20 m in the prevailing wind direction. The results of the histological GUS assay of the seedlings originating from the pollen acceptor plants offered a first quantification of cross-pollination with transgenic grapevine pollen. Further studies of longer distances would have been useful, but could not be carried out due to limitations on the time and effort of evaluating the progeny of pollen acceptor plants.

Special marker genes have been used in several crops for estimation of gene flow from GM crops. Markers that produce a selectable phenotype like herbicide resistance are commonly used (Alibert et al., 2005; Eastham and Sweet, 2002; Loos et al., 2003; Messeguer et al., 2004; Rieger et al., 2002) because hybrids can easily be identified and a large number of plants can be screened. Visible markers or reporter genes like *gfp* (green flurorescent protein) as tested by Halfhill et al. (2003) in *Brassica*, or the *gus* gene were also successfully used to study pollen-mediated gene flow of transgenic crops (Alibert et al., 2005; Messeguer et al., 2004). The 'Dornfelder' plants harboring the *gus-int* gene were highly convenient for analyzing both the dispersal of grapevine pollen and subsequent cross-pollination.

Dispersal of grapevine pollen

Transgenic grapevine pollen could be detected up to a distance of 150 m from the pollen donor plants, which

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Table 2. Cultivation of F1-generation of seeds originating from all measuring points for the detection of out-crossing events by GUS staining

Distances / year of observation	**2002**	**2003**	**2004**	**∑ or mean 2002–2004**
10 m radius (23 measuring points/year)				
Number of seeds (*n*)	31 569	51 852	65 984	149 405
Number of seedlings (*n*)	13 460	33 681	40 964	88 105
Germination rate (%)	42.6	65.0	62.1	58.9
Number of GUS-positive seedlings (*n*)	435	410	938	1783
Maximum out-crossing rate (%)	18.2	6.7	4.1	
Average out-crossing rate (%)♣	3.2	1.2	2.3	2.0
10 m sector in prevailing wind direction (9 measuring points/year)				
Number of seeds (*n*)	12 783	19 192	25 169	57 144
Number of seedlings (*n*)	5930	12 487	15 827	34 244
Germination rate (%)	46.5	65.1	62.9	59.9
Number of GUS-positive seedlings (*n*)	324	136	241	701
Maximum out-crossing rate (%)	18.2	2.5	3.8	
Average out-crossing rate (%)♣	5.5	1.1	1.5	2.0
20 m sector in prevailing wind direction (11 measuring points/year)				
Number of seeds (*n*)	n.t.	27 273	13 881	41 154
Number of seedlings (*n*)	n.t.	14 668	6879	21 547
Germination rate (%)	n.t.	53.8	49.6	52.4
Number of GUS-positive seedlings (*n*)	n.t.	568	24	592
Maximum out-crossing rate (%)	n.t.	13.0	1.8	
Average out-crossing rate (%)♣	n.t.	3.9	0.3	2.7

♣ Out-crossing rate is expressed as the ratio of the total number of blue-stained seedlings to the total number of seedlings.
n.t.: Not tested.

demonstrates that long-distance pollen dispersal by wind has to be considered. Non-transgenic pollen emission could be detected over distances of 50 m by Carraro et al. (1981) and was confirmed by the observations of Turner and Brown (2004), whereas Di Collalto et al. (1982) could detect grapevine pollen 240 m far from the pollen source. The concentration of dispersed pollen is influenced by the seasonal weather conditions, mainly the prevailing wind directions during grapevine flowering. Because of the impact of the experimental plot design, a forecast of transgenic pollen dispersal from grapevine plants cannot be drawn from this first study.

Extraordinary amounts of dispersed GM pollen over long distances and against the prevailing winds might not only be ascribed to special weather conditions, but might also be a result of instability in the atmosphere. With GM corn, great variations in pollen dispersal were associated with complex interactions of wind vectors (velocity, prevailing wind, etc.), but moreover to vertical updrifts (Kuparinen et al., 2007). Long-distance transport of airborne diaspores was attributed by Tackenberg (2003) mainly to thermal turbulence and updrifts, which occurred even on sunny weather conditions with a low horizontal wind velocity. The exceptional cases of pollen distribution of transgenic grapevine plants and resulting gene flow in 2003 with mainly sunny days and no conspicuous wind speeds during blossom might therefore be attributed to similar effects.

Cross-pollination events

Major attention to possible risks of GM grapevine plants is addressed to the questions of potential cross-pollination. In the present study we used 30 transgenic grapevines bearing the *gus-int* gene to follow the fertilization with transgenic pollen in seedling progeny of defined pollen-acceptor plants. An overall out-crossing rate of 2.0 and 2.7% could be detected during three years of observation, depending on distance (10 and 20 m) and wind direction (NW → SE). The majority of the single values mainly ranged between 0 to 0.9%. However, in a few cases, peak values of 18% at a distance of 10 m

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and 13% at 20 m distance from the pollen sources were observed in the prevailing wind direction. These high individual values could be due to artificial pollen dispersal during sampling or vineyard management rather than to wind distribution. Moreover, out-crossing rates above the threshold of 0.9% were observed at some rare measuring points opposite to the wind direction and at sites that differed from year to year. This phenomenon might be explained by omni-directional winds, or by extraordinary thermal updrifts (Kuparinen et al., 2007; Tackenberg, 2003). The level of gene flow might not only be influenced by the total amount of pollen dispersal, but also by additional factors such as pollen viability, pollen competition, overlapping of the flowering time, and flowering dynamics of transgenic and non-transgenic cultivars.

In general, the observed data should be considered as a detailed analysis of just a few pollen-acceptor plants within an experimental vineyard. In viticulture practice the harvest is composed of grapes from all vines of the vineyard. Due to this fact, the average values obtained will be much more significant than the consideration of some single peak values.

CONCLUSION

As an overall assessment of our investigations, three major factors influence the quantification of the out-crossing experiment: (1) the radial design, (2) sensitivity of the GUS assay, (3) the copy number and *gus*-gene expression level. These investigations of pollen flow and out-crossing should therefore be considered as a pilot study giving preliminary quantitative data, but cannot be used to define isolation distances or buffer zones.

Comprehensive and quantitative estimations of gene dispersal are therefore needed in order to discuss isolation distances or management strategies to keep GM pollination below acceptable threshold values. Based on our results, these examinations could be performed by using genotype-specific molecular markers (*e.g.* Akkurt et al., 2007) for quantitative real-time PCR. This kind of analysis will be independent from field trials with GM grapevines and can be realized within existing vineyards using a linear arrangement in the prevailing wind direction for sampling. However, it must be stressed that in principle the dispersal of transgenic pollen into neighboring vineyards would not be relevant for the common process of wine production, since the must is produced from maternal tissue (berry flesh). Only for special products like grape kernel oil would out-crossing be of importance and require consideration for cultivation of GM grapevine plants.

From this first study on spreading of transgenes at the vineyard level, the authors cannot see any potential hazard to the environment or human health from wine production with GMOs even if out-crossing in grapevine occurs.

MATERIALS AND METHODS

Plant material and field trial

In order to study and quantify pollen dispersal and out-crossing events, five transgenic grapevine lines of the cv. 'Dornfelder' (*Vitis vinifera* L.) were used. These lines (Do1-Do5; Bornhoff et al., 2000; Harst et al., 2000) carry the 35S RNA promoter of *Cauliflower mosaic virus* (CaMV) controlling the β-glucuronidase gene from *Escherichia coli* with an intron sequence (Vancanneyt et al., 1990). Copy number was analyzed by Southern blot analysis according to standard procedures. GUS staining using X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) of pollen, seedlings, and other tissues followed the protocol of Jefferson et al. (1987), and was repeated at least twice. In parallel to the release experiment, the transgenic lines were planted in a greenhouse. Each inflorescence in the greenhouse was covered with a paper bag to avoid cross-pollination. After selfing, an aliquot of pollen collected in the bags was used for pollen viability and GUS expression tests in the different lines. Pollen viability was tested by safranin staining according to recommendations of the German Pollenstiftung (www.pollenstiftung.de) and Germany's National Meteorological Service (www.dwd.de/pollenflug).

In 1999, the Institute for Grapevine Breeding Geilweilerhof was setting up the first field release of transgenic grapevine plants in Germany, growing six grapevine plants each of the five transgenic lines. This experimental vineyard was located in Palatinate, one of the major wine-producing regions of Germany. The landscape around the experimental plot is undulated and used for grapevine cultivation. Palatinate forest begins about 1.5 km north of the experimental planting. Generally, winds from the north-west to south-east are predominant. The vineyard was 40 m × 75 m (0.5 ha) in size, with rows in north-south orientation. Thirty transgenic 'Dornfelder' grapevine plants were placed as a plot of two rows in the center of the field release (see grey double lines in Fig. 1), which mainly consisted of GM grapevines carrying genes for fungal resistance (Bornhoff et al., 2005; Harst et al., 2000). The field trial was entirely surrounded by three rows of protection plantation of non-transgenic grapevine plants.

Investigations were carried out during the spring seasons in 2002 (first complete flowering June 10 to 20) and 2003 (June 1 to 10) in order to evaluate the pollen flow, and during the fall seasons of 2002–2004 for determining out-crossing events. Meteorological data, wind velocity and direction, temperature, and precipitation were

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recorded daily during the flowering period at a meteorological station beside the experimental field.

Evaluation of pollen flow

To sample airborne grapevine pollen, microscope slides were used as pollen traps, which were covered with a weatherproof folio (Melinexband, Winzer Laborglastechnik, Wertheim/Main) coated with Vaseline according to Carraro et al. (1981). Principally, they work similarly to conventional traps for sampling the bioaerosol composition of the atmosphere (Hirst-type samplers). The pollen traps were placed 1.00 m and 1.80 m above ground to record pollen flow directly at the level of inflorescence (1.00 m) where a higher amount of pollen can be expected than at the upper bound of the foliage zone (1.80 m). They were oriented orthogonally to the pollen donor plants for optimal collection of the dispersed pollen. The pollen traps were exchanged daily during the flowering period, and immediately after exchange of traps a histochemical GUS assay (Jefferson et al., 1987) was carried out with the pollen fixed on the folio. According to the experiences of Conner et al. (1999) with GUS staining of transgenic tobacco pollen, the samples of grapevine pollen were taken only during the flowering of the transgenic 'Dornfelder' plants to get a distinct indigo-blue staining of transgenic pollen. The folio was transferred with the Vaseline coat upside down into a buffer solution with X-gluc as substrate. After an overnight-incubation at 37 °C, the folio was embedded in safranin glycerol-gelatine (Kisser, 1935) for fixation. Safranin-staining of pollen was done as mentioned above.

Using a light microscope, GUS staining permitted a clear distinction of the blue-colored transgenic grapevine pollen from the non-transgenic pollen (Fig. 2A). The size of grapevine pollen is approximately 17 × 28 µm on average, with a tricolpate structure (Ahmedullah, 1983), which allows differentiation from pollen of other species by safranin staining.

In the first year of pollen flow evaluation, the pollen traps were placed at radial distances of 5, 10, 20, and 50 m to the pollen donor plants (Fig. 1). The radii were extended to 100 m and 150 m in the following year. Blue transgenic grapevine pollen grains and unstained ones were counted on the entire surface of a trap. The total number of pollen traps collected during the 10 days of grapevine flowering was 960 traps in 2002, and 1280 traps in 2003. However, only pollen traps of odd-numbered days were analyzed in radial distances in both years of examination in the first evaluation. The overall number of pollen traps evaluated at both heights (1.00 and 1.80 m above ground) was 480 traps in 2002 and 640 traps in 2003 at each radius. Furthermore, within the evaluation in 2003, additional 120 traps (6 traps × 10 flowering days × 2 positions) were analyzed in even-numbered flowering days in the prevailing wind direction (NW → SE).

Evaluation of cross pollination

The rate of out-crossing was determined by GUS staining on F1 seedlings that originated from open pollination of acceptor plants around the transgenic pollen donor plants. During the vintage season of three years of examination (2002–2004) all bunches of 23 defined vine plants which were located on the 10 m radius were harvested entirely (see Fig. 1). In addition to the 10 m distance, during 2003 and 2004 eleven plants located in a sector of the 20 m radius in the prevailing wind direction were also used as pollen acceptor plants. All seeds of each bunch were carefully dissected, washed in tap water to eliminate adherent pulp, and surface sterilized to reduce microbial growth during stratification. The 190 559 seeds (see Tab. 2) were placed in pools of about 400 seeds on soil in growth boxes and covered with a thin layer of soil. After stratification, the seed-containing boxes were transferred into a temperate greenhouse to accelerate germination. Seedlings at the 3–4-leaf stage were counted to determine the germination rate, and washed in tap water prior to GUS staining. Finally 88 105 seedlings of the 10 m-radius and 21 547 seedlings of the 20 m-sector were analyzed by GUS-staining (see Tab. 2). The assay was essentially performed according to Jefferson et al. (1987), incubating over night in pools of up to 400 seedlings/sample at 37 °C in 250 mL incubation buffer with 0.5 mg X-gluc.mL^{-1}. Blue stained seedlings (Fig. 2B) were counted as transgenic originating from an out-crossing event.

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