Measuring Gene Flow in the Cultivation of Transgenic Barley

A. Ritala,* A. M. Nuutila, R. Aikasalo, V. Kauppinen, and J. Tammisola

ABSTRACT

Genetic engineering is becoming a useful tool in the improvement of plants and plant-based raw materials. Varieties with value-added traits are developed for nonfood use in industrial and medical production, and different production lines must be kept separate. For good management practices, knowledge of relevant gene flow parameters is required. In the present study, pollen-mediated dispersal of transgenes via cross-fertilization was examined. A transgenic barley (*Hordeum vulgare* L.) line carrying a marker gene coding for neomycin phosphotransferase II (*nptII*) was used as a pollen donor. For maximum resolution, a cytoplasmically male-sterile barley line was utilized as recipient and the flow of *nptII* transgene was monitored at distances of 1, 2, 3, 6, 12, 25, 50, and 100 m from the donor plots of 225 and 2000 m^2. Male-fertile plots at a distance of 1 m were included to measure the transgene flow in normal barley. The number of seeds obtained from male-sterile heads diminished rapidly with distance and only a few seeds were found at distances of 50 and 100 m. Molecular genetic analysis (polymerase chain reaction—PCR) revealed that all seeds obtained from male-sterile heads at a distance of 1 m were transgenic, as anticipated. However, only 3% of the distant seeds (50 m) actually carried the transgene, whereas most of them resulted from fertilization with nontransgenic background pollen. This background pollen was mainly due to pollen leakage in some male-sterile heads. In normal male-fertile barley, the cross-fertilization frequency with transgenic pollen varied from 0 to 7% at a distance of 1 m, depending on weather conditions on the heading day. We conclude that, because of competing self-produced and nontransgenic background pollen, the possibility of cross-pollination is very low between a transgenic barley field and an adjacent field cultivated with normal barley. However, adequate isolation distances and best management practices are needed for cultivation of transgenic barley.

Since the early 1980s, the development of plant genetic engineering has given rise to a number of practical applications (e.g., Conway and Toenniessen, 1999; DellaPenna, 1999; Hanley et al., 2000; Mahon et al., 1998). Genetic engineering can be used for precise and efficient improvement of traditional plant breeding characteristics. It can also be applied for introducing essentially new traits to plants, thus offering great prospects for the generation of value-added new plant varieties for the production of high-quality raw materials specially designed for certain industrial sectors. Plant varieties producing special oils, starches or fibers, as well as drugs, medicinal proteins, and edible vaccines are under development or have been bred by means of modern biotechnological methods (e.g., Heyer et al., 1999; Lassner, 1997; Mahon et al., 1998; Poirier, 1999). Good management practices of such special varieties must be based on adequate knowledge of their population genetic parameters, especially gene flow between different populations in cultivation.

The first transgenic barley plants were produced by particle bombardment (Hagio et al., 1995; Jähne et al., 1994; Ritala et al., 1994; Wan and Lemaux, 1994). Later on other techniques have also resulted in transgenic barley plants (Funatsuki et al., 1995; Matthews et al., 1997; Nobre et al., 2000; Salmenkallio-Marttila et al., 1995; Tingay et al., 1997; Zhang et al., 1999). The most reproducible gene transfer method in barley has been the bombardment of embryos of the variety Golden Promise (Wan and Lemaux, 1994). The majority of gene transfers aiming at commercial applications have been carried out by this method (e.g., Jensen et al., 1996; Nuutila et al., 1999).

When varieties with novel traits are being developed, safety questions should also be considered. In the release of transgenic plants, the effect of the gene is of prime importance, not the way in which it was introduced into the genome (EUCARPIA, 1989). The safety assessment should cover the whole chain from research and production of transgenic plants to cultivation, processing, and the final use of the product (e.g., Koivisto et al., 2001; Wolfenbarger and Phifer, 2000). General ecological factors deserve attention in the first stage. Breeding can be broadly defined as the modification of a cultivated organism's genetic material for human needs (OECD, 2000). Many crop plants are fully dependent on man for their existence (EUCARPIA, 1989). During the millenia of agriculture and plant breeding, the cultivated crops have been adapted to special cultivation conditions provided by man. The cultivated crops have been bred for higher yield and for lower production of naturally occurring toxic compounds harmful to man (Ames et al., 1990; Ames and Gold, 2000; Simmonds, 1979). Thus they would usually not be competitive in nature. Most traits enhancing product quality or yield would not be beneficial for a plant in nature, and therefore such traits tend not to occur in natural populations. The majority of genetic engineering projects involve the introduction of nonadaptive traits into ecologically incompetent hosts, and belong to low-risk categories (Regal, 1994). Nevertheless, the impacts of different genetically modified plants should always be considered on a case-by-case basis according to the actual trait and plant in question.

Gene flow between plants has occurred ever since the emergence of the plant kingdom, and it has contributed to the evolution of new cultivated plant species, varieties, and weeds during the millenia of the agricultural era (e.g., Simmonds, 1979). Provided that the gene in consideration does not change the flower structure

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Abbreviations: *nptII*, gene coding for neomycin phosphotransferase II (NPTII); GM, genetically modified.

and thereby the frequency of outcrossing, gene flow of transgenes does not differ from that of other genes and common population genetic principles apply in all cases. In the risk assessment, the potential of gene flow is crucial (EUCARPIA, 1989). Even if the trait were adaptive, the lack of related plants may block its spread in the nature. Much knowledge on relevant phenomena is already available in the literature and has accumulated from the practice of traditional breeding. For example, practical isolation distances applied in breeding of cultivated species have been composed on the basis of these early studies (e.g., Bateman, 1947). However, to confirm with the earlier results and gain more detailed estimates of gene flow in local cultivation conditions, further studies using modern methods with high resolution are considered desirable. Essential factors that deserve study in the cultivation of novel varieties are gene flow between fields, survival of seeds in the field after cultivation, occurrence of established natural populations, and potential exchange of genetic material with weeds or wild species through hybridization (e.g., Giddings, 2000; Saeglitz et al., 2000).

In the present study, we carried out a safety assessment of the cultivation of transgenic barley. We concentrated on pollen-mediated dispersal of genes via cross-fertilization in the cultivation of transgenic barley. The spread of transgenes through pollen flight involves crossing with cultivated barley or with wild relatives. Barley is considered to be a self-pollinating species and in Finland there are no wild relatives with which transgenic barley could hybridize. Nevertheless, there is a slight possibility of cross-pollination between adjacent barley fields. Our aim was to evaluate the distribution of viable pollen and the consequent potential for transgene flow via cross-fertilization in cultivation scale. The occurrence of transgenic volunteers in the field was followed but seed dispersal through animal feeding, seeding, harvest, transportation, handling, or storage technology was not estimated. Transgenic barley carrying the *nptII* gene was used as a pollen donor and the paternity was confirmed by molecular analyses. For a maximum resolution in catching the cross pollen, a male sterile line was utilized as the recipient.

MATERIALS AND METHODS

Plant Material

Transgenic Donor Barley

Microspore culture-derived protoplasts of barley (*H. vulgare* cv. Kymppi) were transformed by electroporation with the construct pHTT303 (provided by T.H. Teeri, University of Helsinki; Ritala et al., 1993) carrying the gene coding for neomycin phosphotransferase II (*nptII*). The regenerated plants and their progeny were analyzed by NPTII activity assays and Southern blot hybridization. These confirmed that the transferred gene was integrated in the genome and that the new trait was inherited by the progeny (Salmenkallio-Marttila et al., 1995). Because of its microspore culture origin, the transgenic barley line proved to be homozygous with respect to the *nptII* gene. The donor barley represented a relatively closed flowering type characteristic of two-row varieties (Hammer, 1975).

Male-Sterile Recipient Barley

A cytoplasmically male-sterile barley line (*H. vulgare* L. cv. Agneta, provided by G. Persson and L. Lehmann, Svalöf Weibull) was sown as the male-sterile recipient. For best resolution in pollen capture, the recipient line was chosen to represent an openly flowering type of barley. From previous experiments it was known that male-sterility is high in the selected recipient line. Nevertheless, the male-sterility is not absolute and rare heads with pollen-producing stamens do occur. Such escaped heads usually produce a full seed set via inbreeding, as in ordinary barley (Lehmann, 1988).

Male-Fertile Recipient Barley

The maintainer line of the male-sterile barley (maintainer of *H. vulgare* cv. Agneta, also provided by G. Persson and L. Lehmann, Svalöf Weibull) was used as male-fertile control recipient. It was equivalent to the male-sterile line including the open-flowering habit, except for pollen production. The earlier cultivation experiments revealed that flowering of the donor barley occurred 4 to 6 d later than that of the recipient lines. Thus the flowering time of the recipient lines was synchronized with the transgenic donor barley by adjusting the sowing times appropriately.

Wild Barley

Two wild barleys from a Nordic collection representing species of *H. murinum* L. and *H. jubatum* L. (provided by R. von Bothmer, Swedish Agricultural University) were used in forced crossing experiments. Attempts to cross these wild barleys with the transgenic donor cultivar Kymppi were carried out by highly competent personnel in greenhouse conditions.

Experimental Set-Up for Field Trials

The field trials with transgenic barley were arranged in 1996 and 1997 at Boreal Plant Breeding Ltd, Jokioinen, Finland, according to the scheme presented in Fig. 1. The small scale donor area of homozygous transgenic (*nptII*) pollen was 225 m^2. In addition to the small scale donor plots, a cultivation scale donor area of 2000 m^2 was used in 1997.

The male-sterile recipient plots were placed at distances of 1, 2, 3, 6, 12, 25, and 50 m, and in the 1997 cultivation scale trial also at 100 m, from the border of the donor plot in the four directions of monitoring. To compensate for the diminution of the cross-pollination frequencies anticipated with distance, the area of the recipient male-sterile plots was increased accordingly with distance. At a distance of 1 m, a 1-m barley row was sown, whereas at a distance of 100 m two rows of 3 m were used. The areas surrounding the whole trials and areas between recipient lines were planted with other plants than barley: In 1996, spring oats (*Avena sativa* L.) was used and in 1997, alfalfa (*Medicago sativa* L.) was planted in the small scale trial and winter rye (*Secale cereale* L.) in the cultivation scale trial.

Male-fertile recipient plots were only included at distance of 1 m, because the amount of pollen produced outside the actual donor plot had to be kept at a minimum. Furthermore, the male-fertile plots, two in each border, were placed symmetrically at distance of 3.5 and 11 m from the male-sterile sectors in the small and cultivation scale experiments, respectively, in order to reduce the consequent contamination in the male-sterile recipient plots.

During the anthesis, heads reaching the opening stage were marked daily with colored tapes and, in the autumn, the heads

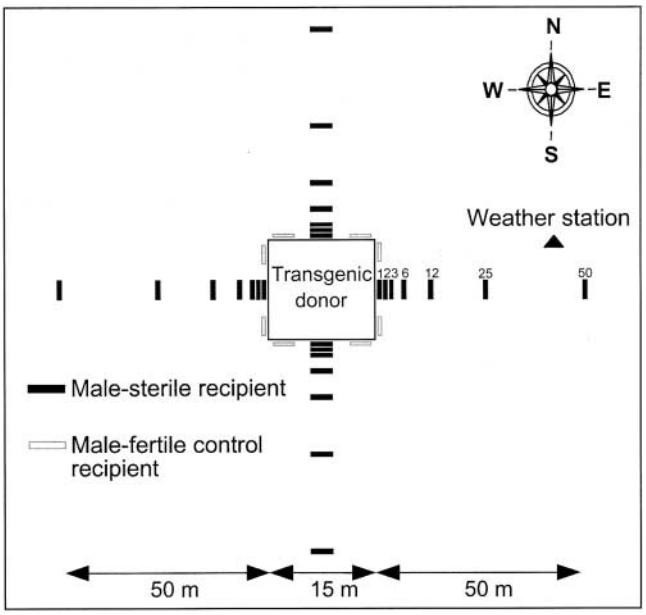

Fig. 1. Experimental set-up of the field trials to measure gene flow from the transgenic barley at the Boreal Plant Breeding, Jokioinen, Finland, in 1996 and 1997. In the cultivation scale experiment, the distance of 100 m for the male-sterile recipient plots was included. The areas between the recipient lines and also the surrounding areas were planted with other plants than barley (see text).

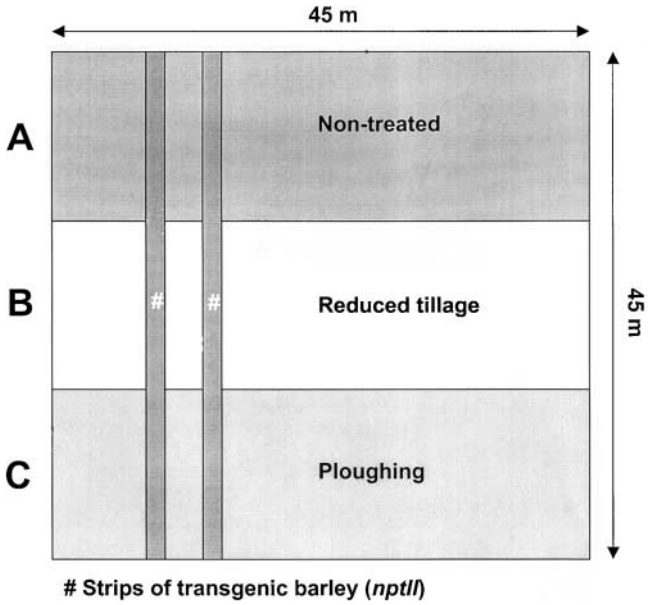

Fig. 2. Monitoring of transgenic barley volunteers the year after the field trial. The experimental set-up in the 1997 field trial plot.

were collected separately. The collected seeds were counted individually in the laboratory and the presence of the transgene was analyzed by PCR. Detailed weather measurements were continuously recorded at the plots during the experiments.

Seed Germination and DNA Extraction

Seeds were surface sterilized with 70% (v/v) ethanol for 5 min and with sodium hypochlorite (4% available chlorine) for 10 min, and then rinsed thoroughly with sterile water. The surface-sterilized seeds were germinated on 0.7% (w/v) water-agar, which was supplemented with 2.4 mg L^{-1} of the seed disinfectant Baytan [Bayer AG, Germany; active ingredient triadimenol—1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl) butanone]. After germination, total genomic DNA was isolated from leaf tissue of the plantlets by CTAB (hexadecyltrimethylammonium bromide) method (Murray and Thompson, 1980).

PCR Analysis

The plantlets were screened for the presence of *nptII* by PCR reactions containing 100 ng of plant DNA, 100 μ*M* dNTPs, 2.5 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 1× Taq DNA polymerase buffer (Perkin-Elmer, Norwalk, CT) and 1.25 U Taq DNA polymerase (Perkin-Elmer) in a final volume of 50 μL. The primers 5′-ACA CGC TGA AAT CAC CAG TCT C and 5′-TCG CCC AAT AGC AGC CAG TC were designed for the *nptII* gene and amplify a 420-bp fragment (Ritala et al., 1994). Thirty-five cycles were performed under the following conditions: 30 s denaturation at 95°C, 1 min annealing at 60°C, and 1.5 min extension at 72°C. The amplified products were analyzed by electrophoresis on nondenaturing 5% (w/v) acrylamide gels and stained with ethidium bromide.

Monitoring of Transgenic Volunteers

The donor plot areas of transgenic barley were monitored during subsequent years for the occurrence of transgenic volunteers. The transgenic barley materials from donor plots were harvested in the autumn with an experimental scale harvester. Consequently, less seeds were scattered on the soil than in normal cultivation.

In 1996, the straw residue was collected and taken away from the trial area after harvesting. Half of the donor plot area was left untreated, whereas ploughing of soil was carried out for the other half. The occurrence of volunteers was studied in the following summer. The observed volunteers were dug up and planted in a greenhouse for seed production. The obtained seeds were germinated and the presence of the transgene (*nptII*) in the progeny was analyzed by PCR as described above.

In the 1997 trials, the donor plot area (225 m^2) of the small scale trial was left untreated after harvesting except that the straw residue was collected and removed from the area. In the cultivation scale trial, after careful harvesting, the shredded straw residue was left on the soil. The donor plot area (2000 m^2) was then divided into three parts (Fig. 2). Part A was left untreated, for part B a reduced tillage was applied the following spring, and part C was ploughed in a normal way in the autumn. To provide better comparison of treatment effects, two 5-row strips with controlled amounts of *nptII*-barley seeds (10 seeds per meter) were sown after harvesting across all three parts (Fig. 2). The occurrence of volunteers and germination of transgenic seeds were studied in the following summer.

Statistical Analyses

The obtained data was processed with the variance analysis using the Statgraphics program package(Statistical Graphics Corporation, Princeton, NJ). Confidence interval estimates for probabilities (e.g., relative numbers of seeds, and cross-fertilization frequencies; Fig. 3 and 4) were obtained from the exact formula for binomial probabilities (e.g., Spiegel, 1961) as follows. The *r* (%) confidence limits for the probability *p* were determined by finding the two values of *p* satisfying the Eq. [1].

$$(k/n - p)^2 = (g^2/n)p(p - 1) \qquad [1]$$

Here k/n is the observed proportion, and g is the number

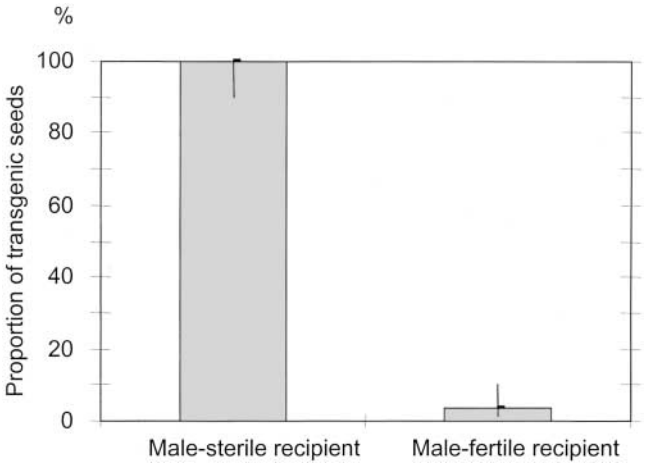

Fig. 3. The cross-fertilization frequencies of transgenic barley with male-sterile and male-fertile recipient plots at a distance of 1 m from the donor area in the cultivation scale experiment in 1997. The 95% confidence interval is indicated with line segments.

satisfying the equation $\Phi(g) - \Phi(-g) = r/100$, in which $\Phi()$ denotes the cumulative standardized normal distribution.

The standard errors for the mean number of seeds for each distance class in Fig. 5 were obtained by dividing the square root of the variance in the class in question by the square root of its number of observations.

RESULTS

Seed-Set in Male-Sterile Recipients

Preliminary results from small scale trials of 1996 suggested that transgenic pollen dispersal and cross-fertilization was possible up to 50 m into the dominant wind direction (Ritala et al., 1999). The experimental scale was increased and the applicability of the preliminary findings was studied in a cultivation scale trial with molecular confirmation in 1997. The 1997 experiments confirmed that the frequency of cross-fertilization in barley was low in Finnish conditions. The seed set in the male-sterile recipient heads surrounding the transgenic plots was in general low. In the male-sterile recipient plots at a distance of 1 m, on average one seed per head was obtained in a preliminary trial in 1996 (data not shown) and less than half a seed in 1997 (Fig. 5). Furthermore, the seed set in the male-sterile recipients diminished dramatically with distance. Only a few seeds were found at distances of 50 and 100 m (Fig. 5). A major proportion of these seeds had originated from contaminating nontransgenic background pollen as confirmed by PCR analyses.

Cross-Fertilization Frequency

The germination of the seeds obtained from male-sterile heads was low. This was considered to be due to the poor weather conditions of the summer of 1997 and consequent high microbial contamination present in the seeds. Without seed disinfectant, no germination occurred, and addition of 2.4 mg L^{-1} Baytan to the water-agar was needed. With this treatment some of the seeds germinated. The germination frequencies for seeds from male-sterile heads remained clearly lower than for seeds

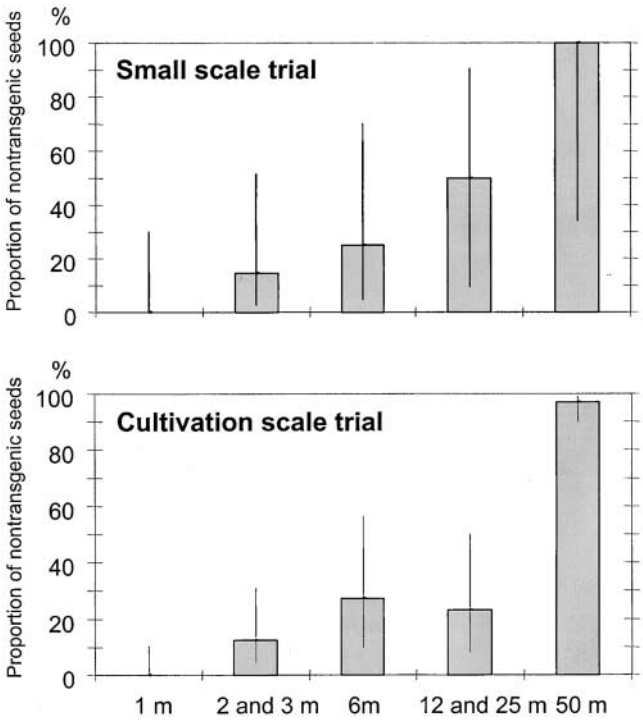

Fig. 4. The proportion of nontransgenic seeds in male-sterile plots increased with distance. The flowers of male-sterile heads were mainly pollinated by nontransgenic background pollen. Such background pollen is to be expected from pollen leakage, i.e., because of occasional formation of pollen reported to occur in some male-sterile heads. The 95% confidence interval is indicated with line segments.

from male-fertile heads (Table 1). After germination all the plantlets were analyzed by PCR (Fig. 6).

Molecular Confirmation of the Seed Paternity

PCR analyses revealed that all seeds produced in the male sterile recipient heads at a distance of 1 m were transgenic (Fig. 3 and 4). In the corresponding male-fertile recipient plots at a distance of 1 m, the great majority of the seeds produced were nontransgenic and only a few originated from cross-fertilization with transgenic donor pollen. The cross-fertilization frequency varied from 0 to 7%, depending on how favorable the weather conditions of the heading day were for cross-pollination (Fig. 3). Furthermore, the PCR analyses revealed that only 0 to 3% of the distant seeds (50 m) were transgenic (Fig. 4). The few seeds obtained at 100 m did not germinate and therefore could not be analyzed by PCR.

Occurrence of Transgenic Volunteers

The transgenic donor areas were monitored during the following summers. After the 1996 preliminary trial, four barley volunteers were found from the ploughed area. The four volunteers were dug up and planted in a greenhouse for seed production. Each of them produced a normal yield of grains. The seeds obtained were germinated and the seedlings were analyzed by PCR for the presence of the *nptII* gene. The PCR analyses

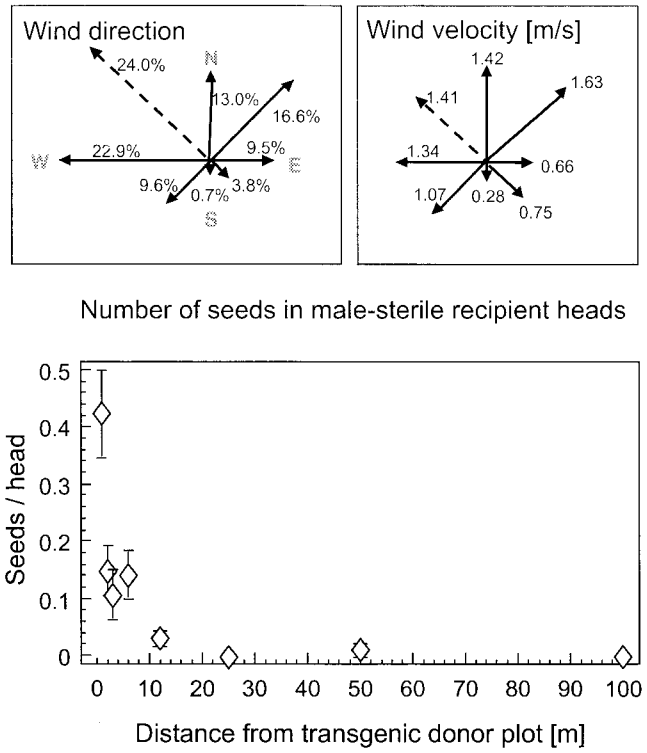

Fig. 5. The seed-set in recipient male-sterile barley plots in the dominant wind direction of the cultivation scale experiment (2000 m^2) at Boreal Plant Breeding, Jokioinen, Finland, in 1997. Full heads originating from escaped individuals with full seed-sets are not included in the numbers, because they constitute a strong biasing disorder in the male-sterile recipient line (see text). The bars indicate the mean plus and minus one standard error.

revealed that each of these four barley plants represented true transgenic volunteers. After the 1997 trials, not a single volunteer was found in 1998, not even from the arranged experiment with purposely sown seeds.

DISCUSSION

Gene flow has traditionally been studied on the basis of pollen capture records (e.g., Raynor et al., 1972). Giddings et al. (1997a,b) showed that the amount of pollen flow did not decrease uniformly with distance. Furthermore, they stated that the weather conditions, especially wind speed and turbulence, made the pollen flight models complex and prediction difficult. We also carried out some preliminary experiments with pollen collectors in 1994. The flight of barley pollen was studied in different directions and at different distances from a barley field. In practice, the lack of reliable genetic and molecular analyses hindered confirmation of the true origin of the captured pollen. Thus, the origin of the pollen remained obscure. It also proved impossible to

Table 1. Variation in germination frequencies in the 1997 cultivation scale trial.

Seed source	Germination frequency
Male sterile	46.2%
Male fertile	59.9%
(Day 206, high cross-poll.)	(63.6%)
(Day 216, low cross-poll.)	(56.3%)

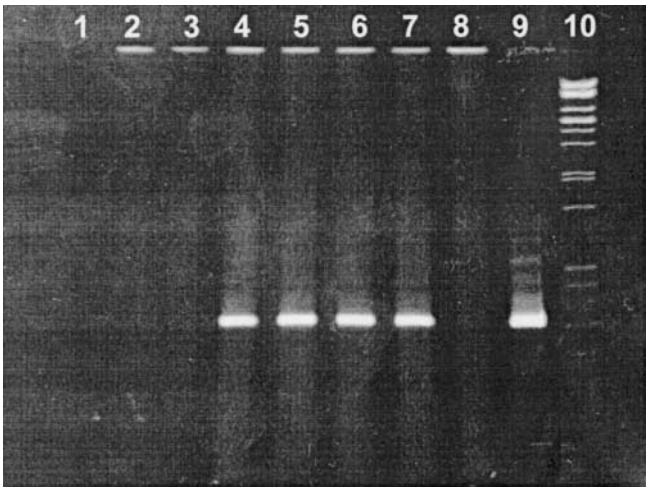

Fig. 6. PCR analysis of the seeds obtained from male-sterile heads. Lane 1, reagent blank; Lanes 2 and 3, nontransgenic seeds; Lanes 4 through 7, transgenic (*nptII*) seeds obtained from male-sterile heads; Lane 8, negative control (seed); Lane 9, positive control (*nptII* plasmid); Lane 10, molecular size standard (λ DNA, digested with *Pst*I).

discriminate reliably barley pollen from pollen of several open-pollinating grass species. Furthermore, this study provided no information on the viability of the collected pollen. More important, it was shown that pollen flow and gene flow are two different parameters. Thus, the true gene flow should be measured by analyzing the actual seeds produced (Tonsor, 1985).

Transgene Flow

Transgene flow is species specific and depends also on genotype, place, and season. Attention should be paid to differences in pollination types when estimating the pollen-mediated dispersal of transgenes. According to the results of this study, in barley transgene flow was low. However, cross-fertilization with very low frequencies was recorded with enhanced experimental systems up to a distance of 50 m (Fig. 5). Rare gene transfer over the same distance has earlier been reported in wild barley on the basis of isoenzyme studies (Wagner and Allard, 1991). In normal male-fertile barley, the cross-pollination frequency was between 0 to 7% at the distance of 1 m from the transgenic donor area. In corn (*Zea mays* L.), a wind pollinated species, a cross pollination frequency of 1% between GM and non-GM corn at a distance of 30 m from the transgenic donor area was reported by Jemison and Vayda (2000). However, according to their results the cross pollination frequency dramatically decreased to 0.1 and 0.04% with distances of 37 and 43 m from the donor area, respectively. At a distance of 350 m, no cross pollination was observed.

In the present study, cross-fertilization frequency also clearly varied on a year-to-year basis because of differences in weather conditions during the growth season. The cross-fertilization frequency was lower in 1997 than in 1996. One explanation might be the lower overall wind speed in 1997, and the wind direction distribution was also different in the two summers. Furthermore,

during the flowering period some very rainy days occurred in 1997.

In Finland, there exist no established wild barley populations with which transgenic barley could cross. The few occurrences of wild barley species are ephemeral and usually situated far away from barley cultivation areas (e.g., in harbors); thus, in practice, there exists no possibility for cross-pollination with cultivated barley. In general, only if the transferred trait would give a strong selective advantage to wild relatives (provided that such wild relatives occur) is it necessary to consider the consequences of low gene flow frequency. In our study, even forced pollination failed and no seeds were produced when forced crossing experiments were carried out in greenhouse conditions between transgenic barley and two wild barley species (*H. murinum* L. and *H. jubatum.*) of the Nordic collection (data not shown). Although it is the experience of plant breeders that cultivated barley is very difficult to cross with wild barley species (Göran Persson, 1994, personal communication; Baum et al., 1992), the crossing ability depends on genotype and these results may not be generalized to other materials.

Contaminating Nontransgenic Background Pollen

PCR analyses of the seeds collected from male-sterile plots confirmed the existence of contaminating nontransgenic background pollen. The few seeds produced at distances of 50 and 100 m resulted mainly from fertilization with nontransgenic pollen. This background pollen is likely to have originated from occasional formation of fertile pollen in some male-sterile heads. Such an anomaly is fairly common in genetically male-sterile barley lines. Some pollen leakage, at a frequency of 0 to 3%, depending on the line, is also reported to occur in cytoplasmically male-sterile barley lines (Lehmann, 1988). Furthermore, some of the nontransgenic pollen may have originated from the male-fertile control plots at a distance of 1 m (see Fig. 1). Theoretically, it is also possible that some pollen might have originated from a more distant barley field. Thus, molecular analyses are necessary for estimation of the maximum resolution theoretically possible. Nevertheless, the 100% cross-fertilization in male-sterile heads at a distance of 1 m compared to the cross-fertilization frequency of approximately 4% in male-fertile heads confirmed the suitability of male-sterile lines for enhancing resolution in risk assessment studies. Consequently, more relevant gene flow estimates can be obtained by applying male-sterile recipient lines combined with molecular analyses rather than by pollen capture records.

Pollen Leakage Distorts Cross Pollination Frequencies

The prediction of pollen flow on the basis of the seed-set in male-sterile heads was an overestimation (Fig. 5). The results showing high frequency of pollination by nontransgenic background pollen (Fig. 4) confirmed our prediction on the basis of general theoretical grounds: The more rare occurrences of fertilization events are scored, the greater will be the proportion of false positives in the overall number of apparently positive results. If the frequency of the actual phenomenon under study decreases, while the frequency of its mimicking events remains considerable, an estimate based on apparent occurrence will become more and more biased until it finally represents the frequency of the mimicking events. In our study, such falsely positive cross pollination frequencies were obtained from the seeds produced by nontransgenic background pollen. For accurate results in a high-resolution experiment, it is crucial to minimize the frequency of false positive cross pollination frequencies, and to be able to discriminate the remaining false positive records from true ones with reliable analyses. One option in population studies is to complement the phenotypic observations with the confirmation of the genetic origin (paternity/maternity) of the progeny by using informative DNA markers or known DNA sequences with appropriate molecular analyses, as in the present study.

Overestimation of the Cross-Fertilization Because of Experimental Set-Up

The results of the present study apply to a transgenic donor barley representing a closed type of flowering. However, because of the characteristics of the recipient barley used, the recorded gene flow was overestimated compared with the actual situation in ordinary barley fields. First, the recipient lines (both male-sterile Agneta and its corresponding male-fertile line), represented an open type of flowering to maximize the resolution in capturing the pollen. Similarly, the results of Wagner and Allard (1991) also referred to an openly flowering barley type. However, the barley varieties commonly grown in Finland represent a much more closed type of flowering. Typically, with a closed type of flowering, the cross-fertilization frequencies are only a fraction of the values reported for the openly flowering barley types (Hammer, 1977). In the closed type of flowering, self-pollen has an advantage and the ovules are already fertilized at the time of flower opening. The second reason for overestimation is that, within a barley field with ordinary barley, the air surrounding the recipient head is crowded with local barley pollen arising from the neighboring plants. Thus, the field efficiently dilutes the concentration of the few pollen grains migrating from other fields. The border of the field is less protected, but the borders can be efficiently buffered with companion crops when necessary. The predominance of local pollen in fertilization reflects its relative abundance in the pollen cloud surrounding the recipient heads and its effect may even be accentuated by the presumably lower fertilization capacity of pollen arriving from further away, although experimental evidence for the decline of pollen fertility during flight is still missing in barley.

Occurrence of Transgenic Volunteers

The transgenic donor plots were monitored for volunteers during subsequent summers. Only four volunteers

of transgenic barley were found after the 1996 trial. Even the transgenic seeds sown deliberately failed to produce plants after the winter of 1997-1998. The barley varieties grown in Finland, including the transgenic donor used in this study, represent spring barley. Obviously, most of the seeds which had fallen from the harvester on the soil had already germinated in the autumn of the trial. At least the starch degradation had already started in the autumn and consequently hindered germination the next spring. Moistened grains will inevitably be killed by freezing during a cold winter. Probably this explanation is also valid for the seeds sown because the subsequent rainfalls may have caused starch degradation or germination in the autumn.

The location of Finland as the northernmost agricultural country in the world may not rule out entirely the possibility of survival of cereal grains through the winter. Therefore, to maintain the purity of the varieties, the same fields should not be used for the production of different varieties of the same species in consecutive years. It is known that in exceptionally dry autumns barley seeds may remain nongerminated and germinate in later years, especially if they are ploughed deep (20–25 cm) into dry soil. However, the dry conditions necessary for the survival of the seeds rarely occur because of the typical heavy rains in the autumns. Furthermore, reduced tillage is often preferred to ploughing because it causes less erosion.

Risk Management

In the study, our primary interest was in basic population science. The previous estimations of gene flow in a self-pollinating species such as barley were to be confirmed with modern, high-resolution experimental systems. In Finnish conditions, gene flow appeared low even in our exaggerated measurements. Consequently, it can be adequately controlled for nearly all practical purposes with existing good cultivation practices, e.g., isolation distances, crop rotation, and control of escapes. In most situations, there is no special need to demand extra purity, but a few percent of mixed materials are commonly allowed in the cereal trade, except in sowing seed material and in GM crops. Even if superior purity of the product were to be required in the future, e.g., for certain medical or high-tech applications, the existing control measures could probably be correspondingly enhanced on the basis of the present results. However, more general, simple and reliable strategies of blocking the escape of transgenes and transgenic plants have recently been developed and may control even with the most demanding situations with inconvenient traits, outcrossing species, or abundant wild populations (Kuvshinov et al., 2001).

CONCLUSIONS

The results obtained in this study confirm that gene flow is low in barley cultivation and that the cultivation of special varieties of barley can be sufficiently controlled in Nordic conditions. Thus, barley can be considered as a suitable host for production of novel products (e.g., value-added nonfood and medical applications). In Finland, there exist no established wild populations of barley or its wild relatives. Furthermore, no hybridization between barley and its relatives occurred, even under favorable greenhouse conditions with forced pollination. Moisture in the autumn and severe winter conditions guaranteed low survival of seeds falling from harvesters onto the soil. Only in rare dry conditions combined with deep ploughing may seeds survive through the winter. The purity of the varieties can be maintained either by intermediate buffer years or by means of different fields for the production of different varieties.

It is evident that gene transfer techniques can be of valuable assistance in barley breeding programs. As a precaution, the safety of the use of the new varieties is assessed before their release. Although the risks and benefits depend mainly on the actual trait under consideration, knowledge of transgene flow parameters is also needed for determining the good cultivation practices. On the basis of our study, cross-fertilization in male-sterile recipient barley is possible with very low frequency up to 50 m from the donor area. However, the frequency dramatically decreases with distance. Because of normal self-pollination, often favored by a closed flowering habit, the possibility of cross-fertilization remains even lower in normal, cultivated barley. Suitably designed risk management procedures implemented in farming will make it possible to transfer the benefits of genetic engineering into the production of high quality plant-based raw materials.

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REFERENCES

Ames, B.N., M. Profet, and L.S. Gold. 1990. Nature's chemicals and synthetic chemicals: Comparative toxicology. Proc. Natl. Acad. Sci. (USA) 87:7782–7786.

Ames, B.N., and L.S.Gold. 2000. Paracelsus to parascience: the environmental cancer distraction. Mutation Res. 447:3–13.

Bateman, A.J. 1947. Contamination in seed crops. III. Relation with isolation distance. Heredity 1:303–336.

Baum, M., E.S. Lagudah, and R. Appels. 1992. Wild crosses in cereals. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:117–143.

Conway, G., and G. Toenniessen. 1999. Feeding the world in the twenty-first century. Nature 402:C55–C58.

DellaPenna, D. 1999. Nutritional genomics. Manipulating plant micronutrients to improve human health. Science 285:372–375.

EUCARPIA. 1989. Risk assessment regarding the release of transgenic plants, EUCARPIA Bulletin 18:16.

Funatsuki, H., H. Kuroda, M. Kihara, P.A. Lazzeri, E. Mller, H. Lörz, and I. Kishinami. 1995. Fertile transgenic barley generated by direct gene transfer to protoplasts. Theor. Appl. Genet. 91:707–712.

Giddings, G. 2000. Modelling the spread of pollen from *Lolium perenne*. The implications for the release of wind-pollinated transgenics. Theor. Appl. Genet. 100:971–974.

Giddings, G.D., N.R. Sackville Hamilton, and M.D. Hayward. 1997a. The release of genetically modified grasses. Part 1: pollen dispersal to traps in *Lolium perenne*. Theor. Appl. Genet. 94:1000–1006.

Giddings, G.D., N.R. Sackville Hamilton, and M.D. Hayward. 1997b. The release of genetically modified grasses. Part 2: the influence of wind direction on pollen dispersal. Theor. Appl. Genet. 94:1007–1014.

Hagio, T., T. Hirabayashi, H. Machii, and H. Tomotsune. 1995. Production of fertile transgenic barley (*Hordeum vulgare* L.) plant using hygromycin-resistance marker. Plant Cell Rep. 14:329–334.

Hammer, K. 1975. Die Variabilitt einiger Komponenten der Allogamieneigung bei der Kulturgerste (*Hordeum vulgare* L.s.1.). Kulturpflanze 23:167–180.

Hammer, K. 1977. Fragen der Eignung des Pollens der Kulturgerste (*Hordeum vulgare* L.s.1.) für die Windbestubung. Kulturpflanze 25:13–23.

Hanley, Z., T. Slabas, and K.M. Elborough. 2000. The use of plant biotechnology for the production of biodegradable plastics. Trends Plant Sci. 5:45–46.

Heyer, A.G., J.R. Lloyd, and J. Kossmann. 1999. Production of modified polymeric carbohydrates. Curr. Opin. Biotechnol. 10:169–174.

Jemison, J.M., and M. Vayda. 2000. Pollen transport from genetically modified corn. p. 145. *In* Agronomy Abstracts. ASA, Madison, WI.

Jensen, L.G., O. Olsen, O. Kops, N. Wolf, K.K. Thomsen, and D. von Wettstein. 1996. Transgenic barley expressing a protein-engineered, thermostable (1,3-1,4)-β-glucanase during germination. Proc. Natl. Acad. Sci. (USA) 93:3487–3491.

Jähne, A., D. Becker, R. Brettschneider, and H. Lörz. 1994. Regeneration of transgenic, microspore-derived, fertile barley. Theor. Appl. Genet. 89:525–533.

Koivisto, R.A., K.M. Törmäkangas, and V.S. Kauppinen. 2001. Hazard identification and risk assessment procedure for genetically modified plants in the field -GMHAZID. Environ. Sci. Pollut. Res. 8:1–7.

Kuvshinov, V., K. Koivu, A. Kanerva, and E. Pehu. 2001. Molecular control of transgene escape from genetically modified plants. Plant Sci. 160:517–522.

Lassner, M. 1997. Transgenic oilseed crops: a transition from basic research to product development. Lipid Technol. 9:5–9.

Lehmann, L.C. 1988. Hybrid barley research: ideas and results. J. Swedish Seed Assoc. 98:3–84.

Mahon, B.P., A. Morre, P.A. Johnson, and H.G. Kingston. 1998. Approaches to new vaccines. Crit. Rev. Biotechnol. 18:257–282.

Matthews, P., R. Brettell, S. Feig, F. Gubler, J. Jacobsen, S. Thornton, S. Tigay, M.-B. Wang, and P. Waterhouse. 1997. Transformation of barley—A progress report. p. 2:8.6–2:8.8. *In* P. Johnston (ed.) 8th Australian Barley Technical Symposium, Barley—From Conception to Consumption, Gold Coast, Queensland, Australia. 7–12 Sept. 1997.

Murray, H.G., and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321–4326.

Nobre, J., M.R. Davey, P.A. Lazzeri, and M.E. Cannell. 2000. Transformation of barley scutellum protoplasts: regeneration of fertile transgenic plants. Plant Cell Rep. 19:1000–1005.

Nuutila, A.M., A. Ritala, R. Skadsen, L. Mannonen, and V. Kauppinen. 1999. Expression of thermotolerant endo-(1,4)-β-glucanase in transgenic barley seeds during germination. Plant Mol. Biol. 41:777–783.

OECD. 2000. Report of the task force for safety of novel foods and feeds. Organisation for Economic Co-operation and Development, 17th May 2000, C(2000)86/ADD1. www.oecd.org/subject/biotec/g8_docs.htm; verified July 18, 2001).

Poirier, Y. 1999. Production of new polymeric compounds in plants. Curr. Opin. Biotechnol. 10:181–185.

Raynor, G.S., E.C. Ogden, and J.V. Hayes. 1972. Dispersion and deposition of timothy pollen from experimental sources. Agric. Meteorol. 9:347–366.

Regal, P.J. 1994. Scientific principles for ecologically based risk assessment of transgenic organisms. Mol. Ecol. 3:5–13.

Ritala, A., K. Aspegren, U. Kurtén, M. Salmenkallio-Marttila, L. Mannonen, R. Hannus, V. Kauppinen, T.H. Teeri, and T.M. Enari. 1994. Fertile transgenic barley by particle bombardment of immature embryos. Plant Mol. Biol. 24:317–325.

Ritala, A., L. Mannonen, K. Aspegren, M. Salmenkallio-Marttila, U. Kurtén, R. Hannus, J. Mendez Lozano, T.H. Teeri, and V. Kauppinen. 1993. Stable transformation of barley tissue culture by particle bombardment. Plant Cell Rep. 12:435–440.

Ritala, A., A.M. Nuutila, R. Aikasalo, J. Tammisola, R. Skadsen, V. Kauppinen, and L. Mannonen. 1999. Genetic engineering of malting barley - research and reality. p. 35–45. *In* I. Campbell (ed.) Proc. 5th Aviemore Conf. Malting, Brewing and Distilling, Aviemore. 25–28 May 1998. Institute of Brewing, London.

Saeglitz, C., M. Pohl, and D. Bartsch. 2000. Monitoring gene flow from transgenic sugar beet using cytoplasmically male-sterile bait plants. Mol. Ecol. 9:2035–2040.

Salmenkallio-Marttila, M., K. Aspegren, S. Åkerman, U. Kurtén, L. Mannonen, A. Ritala, T.H. Teeri, and V. Kauppinen. 1995. Transgenic barley (*Hordeum vulgare* L.) by electroporation of protoplasts. Plant Cell Rep. 15:301–304.

Spiegel, M.R. 1961. Theory and problems of statistics. Schaum, New York.

Simmonds, N.W. 1979. Principles of crop improvement. Longman, London.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton, and R. Brettell. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11:1369–1376.

Tonsor, S.J. 1985. Leptocurtic pollen-flow, non-leptocurtic gene-flow in a wind-pollinated herb, *Plantago lanceolata* L. Oecologia 67:442–446.

Wagner, D.B., and R.W. Allard. 1991. Pollen migration in predominantly self-fertilizing plants: barley. J. Hered. 82:302–304.

Wan, Y., and P.G. Lemaux. 1994. Generation of large number of independently transformed fertile barley plants. Plant Physiol. 104: 37–48.

Wolfenbarger, L.L., and P.R. Phifer. 2000. The ecological risks and benefits of genetically engineered plants. Science 290:2088–2093.

Zhang, S.A., M.-J. Cho, T. Koprek, R. Yun, P. Bregitzer, and P.G. Lemaux. 1999. Genetic transformation of commercial cultivars of oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) using in vitro shoot meristematic cultures derived from germinated seedlings. Plant Cell Rep. 18:959–966.