Measuring gene flow in barley fields under Icelandic sub-arctic conditions using closed-flowering varieties

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ABSTRACT

Genetic engineering is becoming an important tool for the improvement of plants for various forms of production. As varieties are developed for both food and non-food use different production lines must be kept separate. For good management practices of different lines an understanding of gene-flow is essential. Barley has been proposed to be an ideal plant species for genetic engineering as it has a low frequency of cross-fertilization and limited seed dispersal. In the present study, pollen-mediated exchange of genetic material between non-transgenic closed-flowering barley variants was examined in experimental plots under sub-arctic conditions in Iceland. The pollen-mediated dispersal was studied using the barley varieties Golden Promise and Ven, as pollen donor and acceptor, respectively. Only two hybrid plants were identified from a total of 700,000 screened plants giving a hybridization frequency of 0.0003%. It is concluded that adequate isolation distances and good management practices should suffice to prevent cross-fertilization between different lines of barley.

Keywords: Barley, gene-flow, hybridization, natural variants, pollen

YFIRLIT

Mælingar á genaflæði í byggökrum við íslenskar aðstæður

Eftir því sem erfðatækni vex fiskur um hrygg við kynbætur á plöntum, bæði til fæðu- og iðnaðarframleiðslu, verður sífellt mikilvægara að halda kynbótalínum aðskildum. Við ræktun á kynbótaefniviði er því nauðsynlegt að þekkja vel genaflæði í þeirri tegund sem verið er að vinna með hverju sinni. Talið er að bygg henti vel til kynbóta með erfðatækni þar sem það er að langmestu leyti sjálffrjóvga og dreifing á fræi takmörkuð. Í rannsókninni sem hér er kynnt var flutningur erfðaefnis milli byggyrkja með lokuð blóm rannsakaður í tilraunareitum við íslenskar aðstæður. Byggyrkin Golden Promise, sem þjónaði hlutverki frjógjafa, og Ven, sem þjónaði hlutverki frjóþega, voru ræktuð hlið við hlið í tilraunareitum. Skoðaðir voru 700.000 einstaklingar og fundust einungis tveir blendingar sem jafngildir 0,0003%. Því má telja að alfarið sé hægt að koma í veg fyrir flutning erfðaefnis milli byggyrkja með því að hafa lágmarksfjarlægð milli akra og viðhafa góð vinnubrögð í ræktuninni.

INTRODUCTION

Plant genetic engineering (PGE) holds great promise for the future and has been used for the improvement of traditional plant breeding characteristics as well as for the introduction of new traits. Several plants producing substances, such as oils, starches or fibres, as well as pharmaceutical compounds and edible vaccines are under development (Heyer et al. 1999, Poirier 1999, Ma et al. 2003). Despite the early promise of PGE its importance and the possible complications have been hotly debated. The ongoing debate has been between those who wish to reap the benefits of this technology, on the one hand, and those who question its safety on the other hand (Conway & Toenniessen 1999, DellaPenna 1999, Hanley et al. 2000).

Whenever varieties with novel traits are developed it is important to carefully consider all safety questions and it has been proposed that the PGE safety evaluation process should cover the production process from research to the final use of the plant variety (Wolfenbarger & Phifer 2000, Koivisto et al. 2002, Ritala et al. 2002). The general consensus is that different genetically engineered (GE) plants should be evaluated on a case-by-case basis depending on the modification and plant in question. An important part of this evaluation process is the detailed knowledge of distribution of genetic material in both time and space. The direct distribution of seeds is in most cases not a central issue as most crop plants are more or less dependent on man for their survival and are therefore not fully competitive under natural conditions. In the risk evaluation the potential of gene-flow is, on the other hand, considered crucial. This is underlined by the numerous studies on gene-flow between plant species being used or considered for GE (Ritala et al. 2002, Elliott et al. 2009, Lu & Yang 2009, McPherson et al. 2009, Song et al. 2009, Xiao et al. 2009).

The proper containment of genetic material is somewhat complicated by the fact that flow of genetic material within and between populations and species of plants is a natural process that has, among other things, contributed to the development of new cultivated plant species and varieties (Simmonds 1979). This flow should take place in equal measures in the genetically engineered plants as in their non-GE relatives, given that the genetic manipulation does not directly affect the frequency of out-crossing, for example by changing the flower structure or time of flower opening.

Based on the results of traditional breeding there is considerable information available on gene-flow, reflected, for example, in the isolation distances used in breeding of cultivated species. Despite the amount of knowledge available it is important to validate those results under different environmental conditions, considering the importance of climatic factors in the gene-flow. Important factors that warrant consideration are, for example, gene-flow between fields, survival of seeds in the field after harvest, occurrence of established natural populations, and possible exchange of genetic material with weeds or wild species (Giddings 2000, Saeglitz et al. 2000, Ritala et al. 2002).

Barley (*Hordeum vulgare* L.) was first genetically engineered through particle bombardment (Ritala et al. 1994, Wan & Lemaux 1994, Hagio et al. 1995) and later with other techniques (Funatsuki et al. 1995, Tingay et al. 1997, Zhang et al. 1999, Nobre et al. 2000). Barley has been proposed to be an ideal plant for genetic engineering as it has been shown to be largely self-fertilizing and therefore less likely to pose a containment problem for transgenes introduced into the plant genome (Ritala et al. 2002, Gatford et al. 2006). This is particularly important as gene-flow from GE plants to their non-GE relatives is a major factor in risk assessment of GE farming.

In addition to being mostly self-pollinating, barley has no wild relatives in Iceland with which transgenic barley could hybridize in nature. Nevertheless, there is always the possibility of cross-pollination between plants in adjacent barley fields and the results from other countries suggest that pollen dispersal and cross-fertilization between barley plants is possible over a distance up to 50 m (Ritala et al. 2002, Gatford et al. 2006).

Iceland lies on the border of the barley growing zone and is characterized by a temperate maritime climate. The aim of the present study was to evaluate the potential for gene-flow via cross-fertilization of barley in the Icelandic environment.

MATERIALS AND METHODS

Crossing of Golden Promise and Ven

Golden Promise (GP), an old two-row (2R) barley cultivar from Scotland, was used as a pollen donor and Ven, a six-row (6R) variety from Norway, as a pollen recipient. The two varieties were selected based on floral morphologies and time of flowering. They both have a closed type of flowering, a characteristic common to all varieties cultivated in Iceland. Experimental results have shown that these two varieties have similar developmental patterns and flower at the same time under Icelandic conditions (unpublished observation). Hybrids were assessed visually and hybridization was subsequently confirmed by sowing seeds from each plant likely to be a hybrid. Those cases, where both two- and six-row offspring grew up from seeds of a single plant, were taken as a confirmation of a cross-fertilization event between GP and Ven.

Experimental set-up for field trials

The field trials were carried out at Gunnarsholt, southern Iceland (63°51'N, 20°12'W) and Modruvellir, northern Iceland (65°46'N, 18°14'W).

Interchanging rows, 1.3 metres wide, of donor and receptor plants were sown side by side, making the distance between donor and receptor plants 0-0.65 metres. The experiment was sown at Gunnarsholt in May 2003, 2004 and 2005 and at Modruvellir in May 2004. Six rows of donor and receptor plants were sown at Gunnarsholt in 2003 and twelve rows in 2004 and 2005. Eight rows of donor/receptor plants were sown at Modruvellir in 2004.

All receptor plots were harvested in full in autumn and 20% of the harvested seed from each plot was sown the following year. Based on values for seed weight, seed rate and germination this equalled around 120,000, 240,000 and 240,000 plants at Gunnarsholt in 2004, 2005 and 2006 respectively, and around 100,000 plants in Modruvellir in 2005, or a total of 700,000 plants.

The spikes of the plants sown the subsequent spring were visually inspected in autumn for the '2R × 6R' phenotype and the spikes resembling such a cross were collected. The seeds from each of these spikes were sown in individual pots in a greenhouse and the phenotype of each offspring recorded. Plants that gave a mix of two-row and six-row offspring were considered true hybrids.

Weather information was obtained from Modruvellir (65°46'N, 18°14'W) and Samsstadir (63°43'N, 20°7'W), the weather station closest to Gunnarsholt (Figure 2).

RESULTS

Crosses of Golden Promise and Ven were produced in a greenhouse to study the resulting phenotype and to verify whether or not the phenotype could be used to reliably identify '2R × 6R' crosses. The resulting phenotype is an intermediate between the two parent phenotypes, with slightly more obvious residual seeds than what is seen in the two rowed variety (Figure 1). The crosses show that the intermediate phenotype is stable, i.e., all crosses led to a plant with a clear intermediate phenotype. This approach should therefore give a good idea of the true gene-flow frequency without

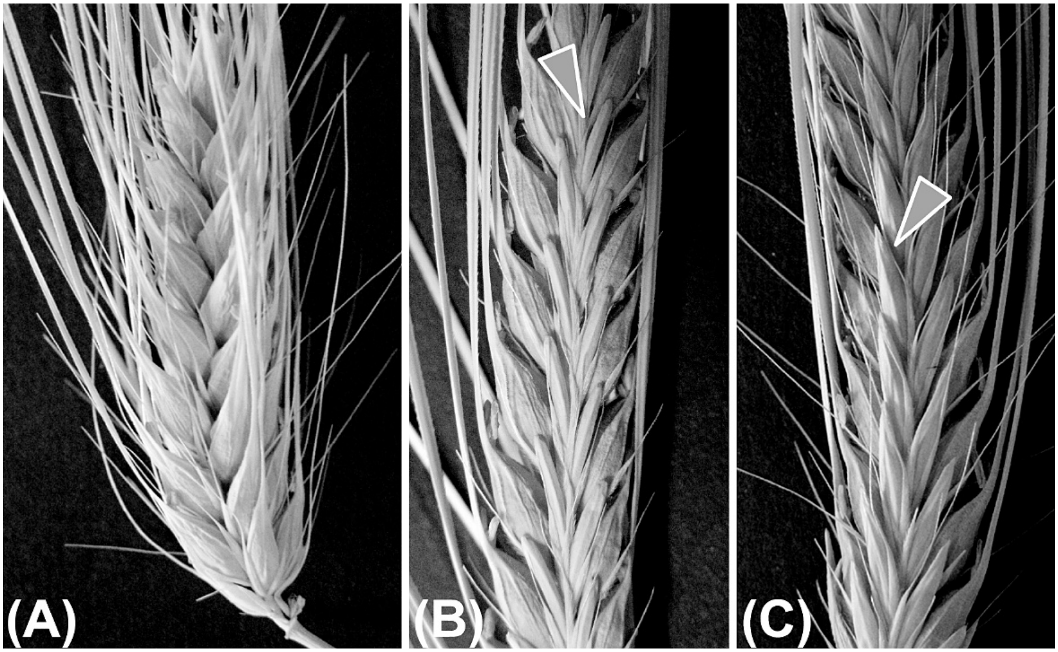

Figure 1. The phenotypes of the six-rowed barley variety Ven (A) and the two-rowed barley variety Golden Promise (B) compared to the intermediate phenotype of the '2R × 6R' hybrid (C). The arrowheads in (B) and (C) point to differently developed residual seeds.

false negatives, '2R × 6R' crosses showing the 6R phenotype, biasing the results.

Not a single '2R × 6R' hybrid was detected in the experiments at Gunnarsholt. From a screening of approximately 100,000 plants from Modruvellir harvested in 2004, two plants were identified that phenotypically resembled the outcome of a cross between GP and Ven. No other potential hybrids were identified. The seeds collected from these two plants gave a mix of six- and two-rowed plants, 2 and 19 plants respectively, which was taken as verification of a hybridization event between Golden Promise and Ven in the experimental plots. Two '2R × 6R' plants from a total of 100,000 screened plants gives a hybridization frequency of 0.002% under the conditions at Modruvellir in 2004, but only about 0.0003% when calculations are based on all four plots inspected.

The weather is known to affect the rate of cross-fertilization and so information on temperature, humidity, and wind for the two experimental locations was compared (Figure 2 and data not shown). The weather was relatively warm around the flowering time in all years or around 12°C (11.5-12.6°C). In 2004, the humidity at Samsstadir was around or above normal, but at Modruvellir the weather was especially warm and sunny during flowering time. The two varieties used here have been shown to flower at the end of July, the 24^{th} - 28^{th}, taking into consideration the time of sowing (unpublished observation). In this period no difference was observed for wind speed but the average temperature was considerably higher at Modruvellir or 13.4°C compared to 12.1°C at Samsstadir (Figure 2). This might have contributed to the differences seen in cross-fertilization frequencies at the two locations although that can not be positively confirmed.

DISCUSSION

For the successful integration of GE crops into mainstream agriculture it is essential to understand the risk of gene flow in the environment where the crops are to be grown. This can only

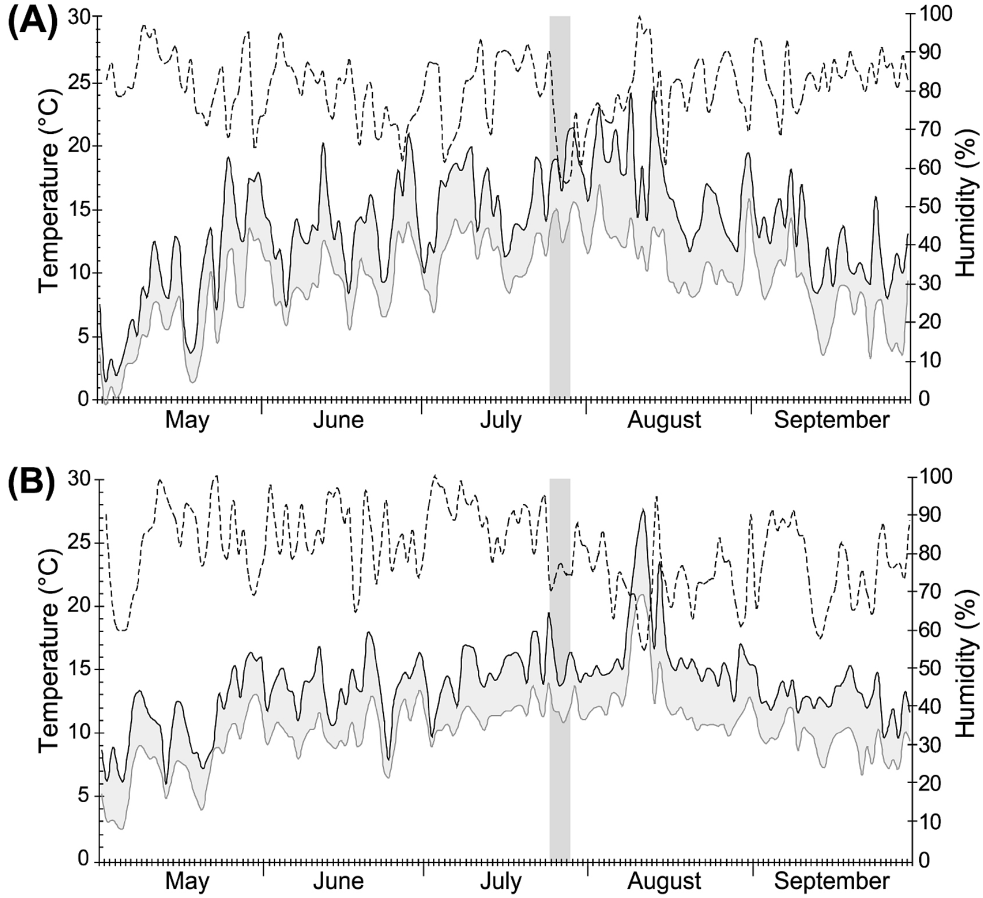

Figure 2. Climatic conditions during the 2004 growing season, from beginning of May to end of September at Modruvellir (A) and Samstadir (B), the weather station closest to Gunnarsholt. On the left is shown the temperature, as mean and max for each day (lower and upper lines of shaded area, respectively). On the right axis is shown the average humidity (dashed line). The approximate flowering time, 24-28 July, is indicated in the grey box.

be done by measuring gene flow under local field conditions and should ideally rely on plant varieties that behave in a similar way to the plants to be used (Bartsch & Schuphan 2002, Waines & Hegde 2003).

Gene flow can occur in different ways, but cross-fertilization is of greatest interest as it mostly drives the need for segregation of GE and non-GE plant varieties. This problem is highlighted with out-breeding species, such as canola (*Brassica napus* L.), where gene flow has been observed between fields as well as in hybrids between GE canola and related species, including wild radish (Rieger et al. 2002).

Importantly, gene flow has been shown to be mostly species specific and to depend on both genetic and environmental factors. For example, there is considerable variation in the proportion of pollen shedding outside the floret between different wheat varieties, ranging from 3% to 80% of the produced pollen (Beri & Anand 1971). As expected, frequencies of cross-fertilization differ between studies and have been shown to vary considerably between years, most likely due to changing environmental factors (Ritala et al. 2002, Matus-Cádiz et al. 2004, Gatford et al. 2006).

In plants that are primarily self-pollinating, such as wheat and barley, the frequency of gene flow is naturally very low. For example, frequencies of 0 to 7% gene flow have been reported for barley (Wagner & Allard 1991, Parzies et al. 2000, Ritala et al. 2002, Abdel-Ghani et al. 2004). It has been demonstrated that out-crossing frequencies depend on both distance and direction from the pollen source (Ritala et al. 2002, Hamblin et al. 2005, Gatford et al. 2006). A common finding is that the rate of gene flow decreases rapidly at distances beyond a few metres (Gustafson et al. 2005), although cross-fertilization in barley has been recorded at a distance of 50 metres with similar distances being reported for wild barley (Wagner & Allard 1991). An important factor affecting the possible distance over which cross-fertilization can occur is the survival time of pollen after shedding; a shorter survival time will lead to a lower cross-fertilization frequency over long distances. This survival time is not known for barley pollen but has been shown to be in the range of 15-20 minutes for wheat pollen under normal conditions and less at higher temperatures or under conditions of low humidity (Devries 1971, Gatford et al. 2006). These observations underline the effect of environmental conditions on the rate of gene flow.

Gene flow has traditionally been studied on the basis of pollen capture records (Raynor et al. 1972) or by using GE plants as donors (Ritala et al. 2002, Gatford et al. 2006). Here a relatively simple method of screening for hybrids based on phenotypic characteristics was used. This approach relies on the fact that a hybrid between two- and six-rowed barley varieties will lead to an intermediate phenotype that is relatively easy to identify and the offspring of such plants will be both two- and six-rowed, making identification easy, reliable, and relatively inexpensive. Controlled crosses show that the intermediate phenotype is stable and should give an unbiased estimate of the true cross-fertilization frequency of barley under field conditions.

The choice of barley varieties was based on two criteria, the time of flowering and floral morphologies. The floral morphologies of recipient varieties have been suggested to be responsible, at least in part, for differences in cross-pollination and gene flow in field trials of transgenic wheat (Gatford et al. 2006). We therefore used varieties with closed types of flowering as such varieties are commonly grown in Iceland. 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 since the self-pollen has a great advantage in the closed flower and in most cases fertilization has already taken place when the flower opens.

In this study frequencies of cross-fertilization were very low or only 0.0003%, over a very short distance (0.65 metres). The difference compared to previous findings can most likely be explained by two factors. Firstly, similar studies have used an open type of flower in order to maximize the resolution in pollen capture (Ritala et al. 2002). Secondly, climatic factors, such as the low average temperature in Iceland during the growing season (Figure 2) compared to the other experimental locations (Wagner & Allard 1991, Ritala et al. 2002) may have played a significant role.

Based on these findings it can be concluded that it is highly unlikely that cross-pollination will take place between different barley varieties under field conditions in Iceland. It is only necessary to consider the consequences of low gene flow frequency to nearby fields or wild relatives if the transferred trait were to give a strong selective advantage to the hybrid plants. Furthermore, it is the experience of plant breeders that it is very difficult to cross-cultivate barley with wild barley species (Baum et al. 1992). In Iceland, there are, indeed, no wild populations of barley or its relatives and no hybridization between barley and other plants has been shown to occur under natural conditions.

Gene flow between barley plants has generally been shown to be low, even in exaggerated experimental setups, and consequently control should be adequate for all practical purposes, with good cultivation practices. These include measures such as proper isolation distances, crop rotation, and the proper control of escapees. Even where a total isolation of different barley variants is required, isolation by relatively modest distances should provide adequate dilution zones and prevent crossing between GE and non-GE plants. Importantly, however, more reliable strategies of preventing the escape of transgenes are being developed and should be considered as an important tool in the future for preventing unwanted gene flow (Kuvshinov et al. 2001, 2005). The results obtained in the present study confirm that gene flow is low in barley cultivation and that the cultivation of special varieties of barley can be easily controllable under Icelandic conditions and thus barley can be considered as a suitable option for the production of novel products.

In summary, our results show that cross-pollination of closed-flowering barley varieties occurs with a frequency of less than 0.002% within a distance of 0.65 metres under Icelandic environmental conditions. The risk of gene flow in and between barley fields is therefore highly unlikely and separation of varieties by a distance of a few metres should be an adequate isolation measure. However, to ensure that the gene flow frequencies reported here are more generally applicable to barley under Icelandic conditions, it would be desirable to test this further using more varieties than were included in the current study. Although the risks and benefits of GE plants depend mainly on the actual trait under consideration, knowledge of possible gene flow is needed to determine good cultivation practices. Our conclusion is that the cultivation of transgenic barley is completely safe under Icelandic conditions provided that suitable risk management procedures in farming are carried out. It is important to bear in mind that weather conditions play an important role in pollen-mediated gene flow and the projected rise in annual temperature worldwide may call for a re-evaluation of cultivation practices in the future.

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