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Biological Confinement Strategies for Seed- and Pollen-Mediated Gene Flow of GM Canola (*Brassica napus* L.)

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Canola (*Brassica napus*) varieties containing inherent restrictions on gene flow may address problems of seed- and pollen-mediated gene flow. The aim of this study was to explore the effectiveness of 1) low-dormancy varieties in avoiding GM soil seedbanks and volunteers, and 2) cleistogamous (clg) genotypes to avoid outcrossing.

GM and non-GM varieties with altered seed ingredients had a wide dormancy range that turned out to be genetically determined. Quantitative trait locus (QTL) mapping identified four QTLs, which together explained 35% of the phenotypic variance. The fiber content of the seeds appeared to additionally affect seed dormancy. A qualitative PCR assay was developed for clg canola by cloning and sequencing of *clg1*-specific DNA fragments. Detection of characteristic bands in 0.1% clg spiked samples illustrated the sensitivity of this method.

The inherent traits of low dormancy and cleistogamy may be feasible tools to minimize gene flow and may be considered for coexistence of GM and non-GM canola.

Key words: cleistogamy, modelling, new traits, PCR, pre-harvest sprouting, QTL, seed color, seed dormancy, soil seed bank, soil type.

Introduction

Situation of Canola (Oilseed Rape) in Europe

Canola (oilseed rape, *Brassica napus* L.) is the major food oil crop in central Europe. The importance of canola has increased during the last decades due to the increasing demand for renewable energy sources. Additionally, the material use of specific seed ingredients (for instance, polyunsaturated fatty acids) has found a market. Winter canola, as it is usually grown in central Europe, has benefits for agro-ecosystems because of its long growing period (providing autumn and over-winter soil cover) and preventing soil from erosion and nitrogen from being leached. Canola can also break infection cycles of diseases, particularly in cereal-dominated crop rotations. The economic relevance of canola has led to the employment of innovative approaches in plant breeding and crop science. New traits have been introduced in canola by genetic modifications (GM) such as herbicide tolerance. Additionally, in a second generation of GM crops, altered seed ingredients have become more interesting. GM canola varieties are not yet launched in the European Union (EU). In the public debate about GM crops, canola is often considered "not suited for coexistence" by the critics of GM crops, both because of the potential for cross-pollination and its capacity for seed persistence in the soil (which may lead to volunteers over many years). Research on confinement strategies for GM canola is therefore stringently required.

State of the Art

Harvesting losses of canola can account for several thousand seeds m^{-2} (Gruber et al., 2007). These seeds

drop onto the soil and can acquire dormancy (secondary dormancy, in the following referred to as dormancy) if they are buried in a dry soil without access to sunlight (Pekrun, Lutman, & Baeumer, 1997). Once dormancy has been acquired, seeds remain non-germinated even if the conditions for germination seem suitable. In the stage of dormancy, seeds often persist in the soil seed bank for a long time, and they are often not released from dormancy before more than 10 years (Lutman, Freeman, & Pekrun, 2003), emerging as volunteer plants in following crops (Gruber & Claupein, 2007a). If they germinate and develop to volunteers, these plants can contribute to unwanted gene dispersal (for instance if GM volunteers emerge) by pollen flow and by seed admixture. This is even more likely as crop rotations become shorter and the share of canola in the rotation increases because of the increasing demand for canola oil.

Tillage (both stubble tillage and primary tillage) is a major issue determining the size of the soil seed bank (Gruber, Pekrun, & Claupein, 2005; Pekrun, Hewitt, & Lutman, 1998), but another crucial effect on the soil seed bank is the genotypic disposition of varieties to acquire dormancy. Differences in seed dormancy among varieties are known (Gruber, Pekrun, & Claupein, 2004a; Momoh, Zhou, & Kristiansson, 2002); these also significantly mirror differences in the size of the soil seed bank (Gruber et al., 2004b, 2005; Gruber, Emrich, & Claupein, 2008). Selection for low dormancy is generally possible (Gruber & Claupein, 2004, 2007b). Various studies have shown a wide variation in seed dormancy among conventional and GM varieties (Gruber et al., 2004a). A meta-study from several surveys confirmed this effect over several years and locations (Gruber, Emrich, & Claupein, 2009). Non-dormant seeds are not expected to persist in the soil and, thus, they would not be the source for volunteer canola. As a consequence, gene dispersal and GM admixture by volunteers would be largely reduced if varieties with low or no seed dormancy were grown. Empirical data about fitness of canola volunteers in different crops and under different environmental conditions are rare, but they are essential to assess biosafety and the potential for coexistence. Similarly, information about the inheritance of the trait "dormancy" is almost non-existent. The detection of quantitative trait loci (QTL) would help to advance the breeding of low-dormancy GM varieties, facilitating coexistence.

Pollen can only spread if the flower has opened and releases pollen to the environment. Pollen dispersal by wind or insects is strongly limited in genotypes with closed flowers (cleistogamous genotypes [CLG]), so the plants are mainly self-pollinated. Cleistogamy was induced in canola by chemical mutation recently (Renard & Tanguy, 1997). The specific gene for cleistogamy (*clg1* gene) was then identified and patented, and the existing plant material was monitored. By targeted selection for stability of the trait "cleistogamy," further genotypes have been developed and tested. The most stable breeding line showed a proportion of closed flowers between 90% and 99% (Leflon et al., 2010). In addition, the efficiency of cleistogamy in limiting deposition of allo-pollen was tested. Analyses showed that allo-pollination rates of cleistogamous plants were lower than those generally observed in oilseed rape: they averaged 10.1% on isolated plants that are grown under a high pressure of allo-pollen, reaching the maximum 14% immediately adjacent to the allo-pollen block and decreasing exponentially at greater distances (Leflon, Hüsken, Pendergast, & Pinochet, 2011). It is likely that cleistogamy highly reduces pollen emission; this characteristic would be interesting in rapeseed production by decreasing the risk of adventitious presence through cross pollination when diversifying seed quality of rapeseed production. Finally, detection methods and field experiments are needed to estimate the effect of cleistogamy on pollen emission and the resulting cross-pollination rate.

Both aspects—seed dormancy and cross pollination—were the basic approaches used to develop strategies for biological confinement to minimize gene dispersal in time (by volunteers from dormant seeds) and in space (by pollen dispersal). The aim of this study was to explore the effectiveness of choosing 1) low-dormancy varieties to avoid GM soil seedbanks and volunteers, and 2) cleistogamous (*clg*) genotypes to validate the causality chains.

a. Low dormancy – low seed persistence – low number of volunteers – low seed admixture
b. Cleistogamy – low cross-pollination – low pollen-mediated gene flow

This article comprises results of a joint project funded by the German Federal Ministry of Education and Research with the following experimental questions.

1. Is seed dormancy a useful confinement strategy to minimize gene dispersal by volunteers?

Table 1. Canola genotypes used for dormancy tests and burial experiments. Traits: seed coat color[a], NDF (neutral detergent fiber), ADF (acid detergent fiber), ADL (acid detergent lignin), and major fatty acids, referred to the dry weight. No significant differences for dormancy values with same letters at P<0.05.

Variety/genotype	Color	NDF %	ADF %	ADL %	Lignin/cellulose/ hemicellulose	Dormancy %	Harvest
SinRed (GM)	n.d.	n.d.	n.d.	n.d.	n.d.	42.9 d	n.d.
Drakkar (isogen)	n.d.	n.d.	n.d.	n.d.	n.d.	19.4 c	2008
Resveratrol (GM)	n.d.	n.d.	n.d.	n.d.	n.d.	24.1 c	n.d.
Lisora (isogen)	n.d.	n.d.	n.d.	n.d.	n.d.	4.9 b	2008
Yellow 1	4.4	14.6	10.3	3.4	low/med./med.	0.9 a	2008
Yellow 2	2.0	n.d.	10.5	3.4	low/n.d./high	5.9 b	2008
Black	9.0	n.d.	17.6	10.4	high/n.d./high	62.3	2008
Thin	9.0	14.1	12.0	6.6	high/low/low	9.5 b	2008
Thick	9.0	21.0	14.9	7.8	high/high/high	59.4 e	2008
Holli 1 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	26.9 c	2008
Holli 2 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	39.0 d	2008
Express [c]	n.d.	n.d.	n.d.	n.d.	n.d.	8.9 b	2008
Smart [c]	n.d.	n.d.	n.d.	n.d.	n.d.	93.0 f	2008

[a] Color determined according to a rating scheme from 1-9 (1-yellow, 9-black)
[b] 79% C 18:1, 2.8-3.4% C 18:3
[c] Standard varieties with low/high seed dormancy

   a. Is low dormancy already present in new canola varieties?
   b. Is dormancy correlated to another wanted/ unwanted trait?
   c. Is dormancy heritable?
   d. Is the trait easy to handle?
   e. Do low-dormancy varieties result in lower volunteer numbers in agricultural canola fields?
2. Is cleistogamy a useful confinement strategy to minimize gene dispersal by pollen?
   a. Can we develop a *clg1*-specific polymerase chain reaction (PCR) detection method?
   b. To what extent are pollen dispersal and cross pollination reduced by cleistogamous genotypes?

Materials and Methods

The general structure and aims of the joint approach included the following additive experimental steps.

1. Laboratory (a) and burial (b) experiments for canola seeds to characterize dormancy and seed survival in new trait/GM genotypes and standard varieties
2. QTL mapping of the trait "dormancy" for a DH population
3. Development of a test to determine the potential for pre-harvest sprouting of low- and high-dormancy varieties to better assess the confinement strategy "low dormancy"
4. Derivation of a prediction model from field data on volunteer abundance in canola fields by determining the long-term factors for volunteer emergence under on-farm conditions (including variety effects)
5. Establishment of an applied model to predict the number of volunteers
6. Cleistogamy: extraction of canola seed DNA (a), and development of a PCR assay (b).

Dormancy Induction

The basic method for determination of dormancy was the incubation of 4 × 100 seeds per treatment on polyethylene glycol for 14 days in darkness, with a following germination test in darkness on water, both at 20°C (Gruber et al., 2004a). This method was refined and a test (Hohenheim Standard Dormancy Test) was developed that was then used for the following experiments (Weber, Frick, Gruber, & Claupein, 2010). Seeds of winter canola genotypes with new traits were analyzed for dormancy: two GM genotypes (SinRed: sinapin reduced; Resveratrol: resveratrol enhanced) and their near-isogenic counterparts; three genotypes with different seed coat color and fiber content; two genotypes with different seed coat thickness; two high-oleic/low-linolenic (Holli) genotypes; and one low- or high-dormancy standard variety (Table 1).

Seed Burial

Canola seeds with new traits (same genotypes as for dormancy induction) were enclosed in fabric mesh bags (500 seeds each) and buried in an open-air S1-facility from September 2008 to March 2009 at the experimental station Ihinger Hof of the University of Hohenheim, SW Germany. The bags were placed in plots with silty clay, clayey loam, and loamy sand at a depth of 10 cm. A canopy excluded the experimental area from rain for the first three weeks after burial. All seeds remaining after the burial period were tested for germinability, and, after successful germination, were counted for survival. Seed survival was calculated as percentage of survived seeds/buried seeds. Data were analyzed using the procedure GLIMMIX in the statistical software package SAS 9.2.

QTL Mapping of Dormancy

Doubled haploid (DH) lines were produced by crossing an inbred line (No. 617) of the *B. napus* 00 winter variety Express with a resynthesized line R53 (Radoev, Becker, & Ecke, 2008). Seed material was obtained from 230 DH lines and grown in the 2008/09 season in field plots (two locations, two replicates) in North Germany. Ten open-pollinated plants/line*replicate were bulked for further analysis. Dormancy was determined according to Weber et al. (2010). Fiber analysis was performed for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL; Schatzki, Schoo, Ecke, Becker, & Möllers, 2011; Suprianto et al., 2011). The framework map for molecular markers (Radoev et al., 2008) was used and refined. It consisted finally of 229 markers including 80 SSR and 149 AFLP markers. Considering all markers covering 21 linkage groups, a mean map distance of 9.5 cM was computed in accordance with Kosambi's function. The software QTL Network 2.1 (Yang et al., 2009) was used for QTL mapping with P=5% for QTL detection. A permutation test (1,000 permutations) determined the critical F-value threshold for each trait. PLABSTAT software (Utz, 2008) was used for ANOVA and heritability calculations.

Rapid Test for Pre-harvest Sprouting

Five pods of five winter canola varieties (high and low dormancy) from a similar insertion were taken weekly until harvest (2010, experimental station Ihinger Hof), beginning 10 weeks after full flowering, when the pod color has turned to yellow and the seed color to brown. Four replications of the pods were rolled into filter paper and placed upright in a tray with water so that the pods were permanently wet. Incubation at 20°C under light for 7 or 14 days allowed the seeds to sprout inside the pods (Stockmann, Weber, Frick, Gruber, & Claupein, 2011).

Regression Tree for Volunteer Abundance in Canola Fields

Canola volunteers were surveyed in more than 100 German canola fields in the 2009/10 and 2010/11 seasons (Thöle, Dietz-Pfeilstetter, & Hüsken, 2011). As a prediction model, a regression tree was built (De'ath & Fabricius, 2000). For each field, the median number of volunteers represented the response. Considered as potentially relevant for the variation in volunteer abundance were: soil climatic region, frequency of canola in the rotation, succession of primary tillage, time between canola harvest and first tillage, seed loss at harvest, timing of herbicide application in subsequent cereal crops, and canola varieties. Locations were represented by soil-climate regions developed as strata to concentrate and simplify multi-environment variety trials in Germany (Graf, Michel, Rossberg, & Neukampf, 2009). The share of canola in the crop rotation was classified as low (<20%), medium (20-25%), or high (>25%). A regression tree analysis was performed to handle the complexity of multiple variables, their interactions, and lack of balance. For this analysis, the software package rpart (version 3.46, recursive partitioning) was used within the statistical system R (version 2.11.1). Tree building was checked by cross validation. Data exploration revealed that the factor "variety" could not be stratified because of the individual cropping history of each field. In an alternative approach, leaf samples of canola volunteers were collected in order to investigate the genotypic origin of the plants. Using Inter Simple Sequence Repeat (ISSR)-PCR (Andersen, Rasmussen, & Jørgensen, 2010; Charters, Robertson, Wilkinson, & Ramsay, 1996), DNA fingerprints of volunteers were produced and compared to fingerprints of reference varieties.

Applied Model for Prediction of Canola Volunteers

Based on data from pre-existing studies of the last decade about the population dynamics of canola volunteers, a simple linear and deterministic model was developed in a spreadsheet. Data included harvesting seed loss, mode and depth of tillage, crop sequence,

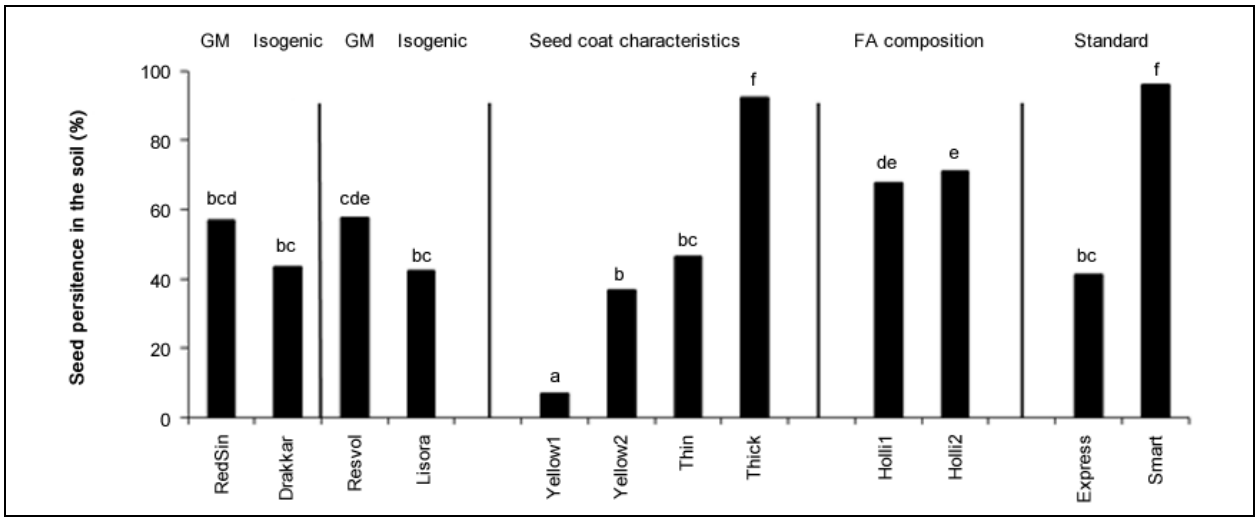

Figure 1. Seed survival (% viable seeds/buried seeds) after burial over 6 months (September 2008 - March 2009) in 10 cm soil depth.

Same letters indicate no significant differences (P<0.05).

occurrence of flowering volunteers, and dormancy level of the volunteer source variety.

Cleistogamy—Extraction of DNA. Seeds were ground by a hand-held blender (UNOLD AG, Hockenheim, Germany) for 1 min at maximum speed (19,000 Rpm). DNA was extracted and purified by combining a lysis step of a CTAB extraction protocol followed by a DNA purification step using a commercial kit (NucleoSpin Plant II Kit, Macherey & Nagel, Germany). Two g of ground seed were re-suspended in 10 ml CTAB-extraction buffer (1.4 M NaCl; 0.1 M Tris/HCl; pH 8.0; 20 mM EDTA; pH 8.0; 20 g/l CTAB). Twenty µl Proteinase K (10 mg/ml) was added, and the homogenates were incubated for 2 hours at 65°C in a hybridization oven under slow rotation. The homogenates were centrifuged for 10 minutes at 4,000 × g to pellet seed debris. A volume of 1 ml lysate was transferred to a 1.5 ml Eppendorf tube, 10 µl RNase A was added and incubated for 15 minutes at 37°C. A 600 µl volume of the lysates then was transferred to a new 1.5 ml Eppendorf tube and 675 µl PC buffer was added. Seed DNA was extracted and purified according to NucleoSpin Plant II Kit and eluted in 120 µl of PE buffer.

Cleistogamy—Development of a clg1 Gene Qualitative PCR Assay. *clg1* gene sequences from a cleistogamous *B. napus* line (CLG) and the wild type were retrieved from the Patent FR 2923839 (Lu et al., 2009). Several sets of primers were designed to amplify the *clg1* gene sequence (6144 bp) from CLG and from a normally flowering variety (Marcant) seed DNA. PCR products were cloned, sequenced, and aligned to identify sequence differences between CLG and Marcant. One single nucleotide polymorphism (SNP) was identified. According to this SNP, a set of specific primers was designed to amplify the *clg1* gene of CLG. One primer was a universal primer targeting the *clg1* gene sequences of both oilseed rape lines (5'-CAAAGATCA-CAACCCCAAGG-3'), and the other targeting specifically at the SNP position (5'-CTGATGGTGCT-GAAGATGTTGT-3'). To increase the specificity of the latter primer for discriminating between the *clg1* gene sequence of CLG and the non-target Marcant, an additional base mismatch was introduced into the 3' end of the SNP primer sequence (Bui & Liu, 2009). The primer set was initially tested over a wide range of annealing temperatures to achieve specific amplification (PCR cycling conditions: 95°C for 15 minutes; 31 cycles of 94°C for 60 seconds; 54-68°C for 60 seconds; 72°C for 60 seconds; 72°C for 5 minutes). All PCR reactions were carried out in a total volume of 25 µL containing 100 ng of seed DNA, 12.5 µl Multiplex PCR Kit Mastermix (Qiagen, Hilden, Germany) containing 6 mM $MgCl_2$, and 200 µM of each primer (Eurofins MWG Operon, Ebersberg, Germany). The PCR method was tested for reliability using a dilution series of standards based on DNA from CLG seeds diluted in a background of DNA from Marcant seeds (0%, 0.1%, 0.2%, 0.3%, 0.5%, 1%, and 100% of CLG DNA in Marcant DNA, respectively).

Table 2. Mapped QTL and their most likely positions for dormancy (SD) of Canola genotypes from doubled haploid lines.

QTL	LG*	Position[cM]	CI[cM] *	a*	h² (a) *	V(A)/V(P) *	V(I)/V(P) *	V(G)/V(P) *
SD-1	N5	62.4	57.5-85.7	-5.7422	0.0823	0.3373	0.0133	0.3506
SD-2	N13	202.1	192.4-207.1	-7.0006	0.0947			
SD-3	N15	88.5	83.3-92.5	5.4468	0.0988			
SD-4	N18	90.7	73.4-94.4	-4.4749	0.0692			

* *LG = linkage group, CI = confidence interval, a = additive effect, h² (a) = heritability of additive effect, V(A)/V(P) = variance of additive effects/phenotypic variance, V(I)/V(P) = variance of epistatic effects/phenotypic variance, V(G)/V(P) = variance of genetic main effects/phenotypic variance*

Results

Dormancy of Different Genotypes and their Seed Survival in the Soil

There was a wide variation in dormancy among all genotypes tested, with a range of about 1% dormancy up to > 90% dormancy (Table 1). Both yellow-seeded genotypes, genotype "Thin," and the near-isogenic variety Lisora showed particularly low dormancy, similar to the low-dormancy standard variety Express. Highest dormancy, apart from the high-dormancy standard Smart, showed genotype "Thick" with a high fiber content; the GM genotypes were at medium dormancy level. The high-oleic acid content in combination with low linolenic acid (Holli varieties) did not appear to affect dormancy level.

Lowest seed survival in the soil (burial experiment) was exhibited by the yellow-coated, low-fiber varieties "Yellow 1" (7% survival) and "Yellow 2" (37% survival), while variety "Thick" with high crude fiber content showed a comparably high survival rate (92%), similar to the standard variety for high dormancy "Smart" (96%; Figure 1). All other cultivars ranged between 40% and 70% seed survival. Both GM genotypes showed medium survival rates of about 57% and did not differ significantly from their near-isogenic counterparts. Seed survival correlated significantly with NDF, ADF, and ADL with coefficients for determination of 0.73, 0.91, and 0.76, respectively (Weber, Frick, Stockmann, Gruber, & Claupein, 2011). Seeds survived less well in loamy sand and accounted here for about 60% of the survival rates in clayey loam and silty clay (Weber et al., 2011).

QTL Mapping

The level of dormancy from laboratory analysis of the different DH lines varied from 7.6% to 100% among the genotypes (data not shown). The factors location, genotype, and location*genotype interactions showed significant effects on seed dormancy (data not shown), with genotype contributing most to the total variance. The heritability of the trait was comparatively high with a value of 0.79. Seed dormancy was significantly positively correlated with NDF, ADF, and ADL contents with a correlation coefficient (Spearman-rank) of 0.27 for NDF and 0.32 for ADF and ADL. Four QTL for seed dormancy were found, which together explained 35% of the phenotypic variance (Table 2). A positive additive effect for the mapped QTL indicates that the 'Express 617' allele increases the value of the trait. Linkage group N5 showed overlapping QTL positions among others for the traits dormancy and seed fiber (NDF, ADF and ADL).

Pre-Harvest Sprouting

Low seed dormancy and artificial pre-harvest sprouting were significantly positively correlated at both the 7- and 14-day incubation times for nearly all sampling times. R^2 one week before harvest was 0.94** at 7 days incubation and 0.95** at 14 days incubation (data not shown).

Regression Tree for Volunteer Abundance in Canola Fields

Location and canola cropping frequency turned out to be the prevailing factors explaining the long-term development of volunteer abundance, explaining nearly 50% of the variation (Figure 2). The regression tree split into two main groups. Group 1 included fields with the highest volunteer abundance (8.5 plants m^{-2}). Two traditional canola-growing regions fell into this group: the maritime climate with loamy soils near the Baltic Sea, and the Northeast German glacially formed soils; additionally, the uplands I (Northwest Germany) were included. Group 1 further split into fields with high- and low-medium canola growing frequency. The highest number of volunteers occurred on loamy soils at the Baltic Sea, the most traditional canola region in Germany in the past. In Group 2 (2.2 volunteers m^{-2}), the tree differentiated mainly between east (lower volunteer

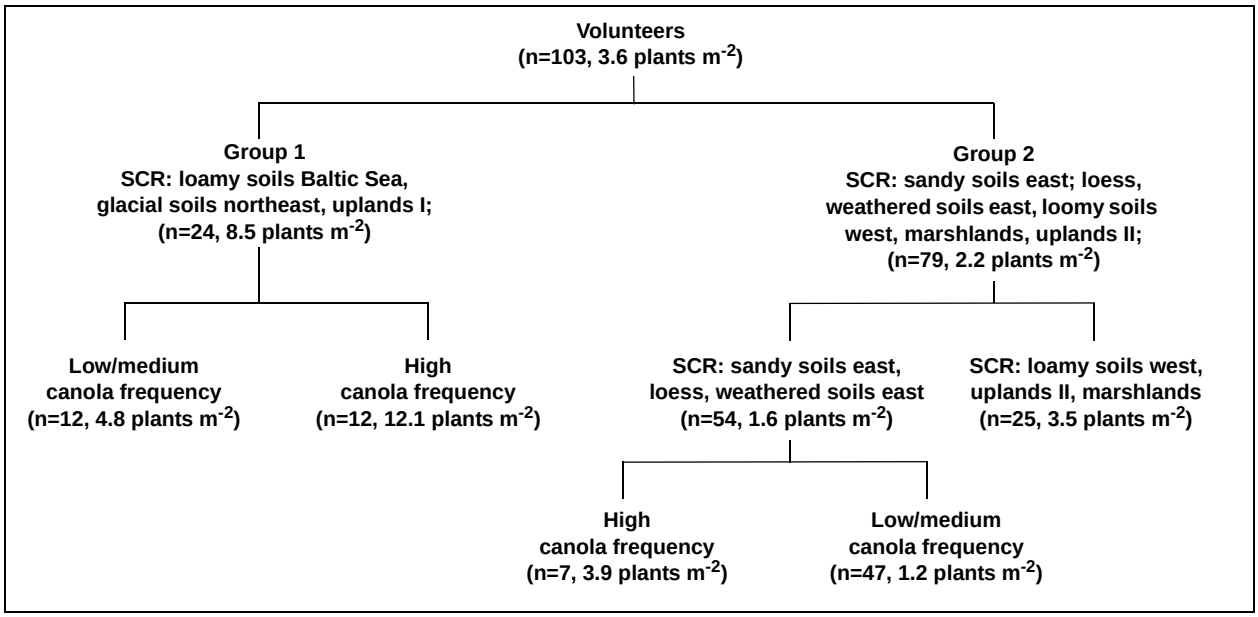

Figure 2. Regression tree for in-field canola volunteer abundance.
SCR: soil climatic region, n: number of locations surveyed.

density) and west (higher volunteer densities) German regions; the west is represented by farms with small acreage and rotations with a large share of canola, and the east is mainly represented by farms with large acreage and a small share of canola. In cropping systems with medium and low canola shares, very high proportions of volunteers could be grouped according to their genetic origin by using ISSR-PCR. These volunteers were often assigned to open pollinating varieties with high dormancy potential (data not shown).

Applied Model

A crop rotation: canola – winter wheat – winter barley was simulated for a high-dormancy variety, with a seed rain during harvesting of 4,000 seeds m^{-2} (Figure 3). The calculated number of seeds in position for germination (0-10 cm soil depth) before sowing the next canola crop was 208 seeds m^{-2} if the tillage management was immediate stubble tillage and a following deep inversion tillage, compared to 41 seeds m^{-2} after no till under the same rotation. A simulation for a low-dormancy variety (10%) resulted in a scenario with 23 seeds m^{-2} in 0-10 cm under stubble tillage + deep inversion tillage, and 5 seeds m^{-2} under no-till.

Cleistogamy

A qualitative PCR method was shown to be specific at an annealing temperature of 62°C for the detection of

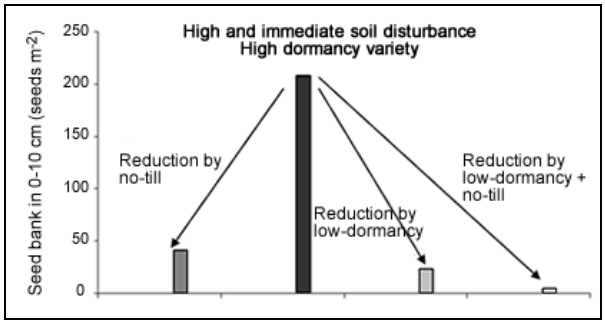

Figure 3. Canola seed bank in a depth of 0-10 cm as effect of genotype (high/low-dormancy variety: 90/10% dormancy) and of tillage (high soil disturbance: immediate stubble tillage plus deep inversion primary tillage; or no-till) at the end of a canola-winter wheat-winter barley rotation.

mutated *clg1* gene sequences of CLG seed DNA in a background of non-target canola DNA (Marcant). The primer set yielded in an amplification of a specific product of the expected size of 218 bp *clg1* gene sequences were detected, even in samples with < 0.1% of CLG seed DNA in a background of Marcant seed DNA, while the negative control (100% Marcant seed DNA) showed no amplification (Figure 4).

Discussion

The assortment of genotypes with altered seed ingredients (crude fiber, fatty acids, or novel trait: resveratrol, reduced sinapine) showed a similar distribution of dor-

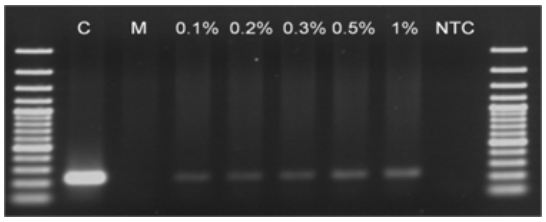

Figure 4. Detection of the mutated *clg1* gene in spiked DNA samples by PCR.

C: 100 ng CLG seed DNA; M: 100 ng Marcant seed DNA; 0.1-1%: CLG seed DNA diluted in Marcant seed DNA; NTC: no template control; size standard: Generuler 100 bp plus Ladder (Fermentas).

mancy levels as shown in previous studies (Gruber et al., 2009) and did not reveal any abnormalities. The introduction of a new GM trait (resveratrol) and the reduction of another one (sinapine) did not lead to dormancy or seed survival levels remarkably deviant from the rest of the collection. This means that the confinement strategy "low dormancy" is available for many genotypes including GM varieties with different traits. An altered fatty acid composition might affect seed dormancy and survival, as can be deduced from the experiments of Linder and Schmitt (1995) and Walker, Booth, and Walker (2000), but if so, this result did not appear in a similar way in our experiments. Crude fiber content, however, seemed to affect dormancy and seed survival in the soil, with low fiber content leading to low dormancy and survival and vice versa. Fiber content, which is a criterion for the feed quality of canola meal, might be a useful indicator for dormancy and therefore used for a simple selection. The results from the analysis of several genotypes with altered seed ingredients from Experiment 1 correspond well with the results from Experiment 2 with doubled haploid lines. Here, the positive correlation of dormancy and NDF, ADF, and ADL content also indicates that seed dormancy may be related to fiber and lignin content in the tests. It remains to be investigated whether or not the tendency of larger seeds to lower NDF, ADF, and ADL content compared to smaller seeds is correlated with decreased dormancy. The QTL studies partly corroborate the results from the dormancy tests, as they showed overlapping confidence intervals of QTL for dormancy and fiber content. All in all, a selection of low fiber (and other traits; Schatzki et al., 2011; Suprianto et al., 2011) seems to be a feasible approach to select for low-dormancy genotypes (for instance, for future GM varieties).

According to the results of the QTL analysis, seed dormancy is clearly a heritable trait. Previous results with a similar conclusion ("dormancy is heritable") from a cluster analysis of numerous varieties, locations, and seasons (Gruber et al., 2009); from previous selection experiments (Gruber & Claupein, 2007b); and from a long-term selection for low dormancy by the authors of this study (Weber, Gruber, & Claupein, 2012) in the greenhouse and on the field are finally confirmed by a molecular biological approach. This is another reliable step on the way toward the use of dormancy as a confinement strategy. Selection for low dormancy in canola breeding material should be possible.

Under extremely wet conditions, such as provided by the artificial conditions in the laboratory test, low-dormancy varieties might tend toward higher pre-harvest sprouting. On the other hand, low-dormancy varieties are already commercially grown (Gruber et al., 2009), mostly without any known problems with pre-harvest sprouting. At least, in semiarid or continental climates with low rainfall at harvest, this risk of pre-harvest sprouting in low-dormancy varieties—if there *is* any in practical farming—can be assumed to be very low. The test developed by Stockmann et al. (2011), however, can be used to easily identify low-dormancy varieties; this is important, because at the moment breeders do not provide information about dormancy of their varieties.

The next question to answer is to what extent low-dormancy varieties contribute to the probability of volunteer abundance. The survey of more than 100 fields in Central and North Germany did not answer this question at first. Soils, climate, and cropping systems (share of canola in the rotation) turned out to be the major reasons for the occurrence of canola volunteers; but these factors only explained about 50% of the total variance in the regression tree. It is evident that interactions of the factors mentioned above with various tillage operations (Gruber, Buehler, Möhring, & Claupein, 2010; Pekrun et al., 1998) and the large number of varieties used—both in the past and today—may overlap any main effects. Variety selection can influence canola volunteer abundance through different dormancy potentials, resulting in different numbers of volunteers (Gulden, Shirtliffe, & Thomas, 2003; Gulden, Thomas, & Shirtliffe, 2004; Gulden, Warwick, & Thomas, 2008). Therefore, the genotypic origin of canola volunteers in fields was analyzed using molecular markers to further investigate this issue. More data have to be evaluated to confirm the preliminary findings that low-dormancy varieties do not persist in the soil to a relevant degree.

We learned from the applied model that low dormancy is indeed a most useful trait to reduce seeds in the soil seed bank, and thus it can be used as a confine-

ment strategy. Low-dormancy varieties are not a stand-alone solution; the effect is increased if adapted tillage strategies are also used, such as delayed first tillage, or no-till (Gruber et al., 2010). Because tillage can fail or must be adapted to current weather conditions, low-dormancy varieties provide the best pre-conditions for low gene dispersal by volunteers. In addition to the approaches mentioned, searching for varieties with shatter resistance of the pods can be included in confinement strategies.

The qualitative PCR assay was established for the specific detection of *clg1* gene sequences of cleistogamous canola as a first step to further assess cleistogamy for confinement purposes. If this method proves successful in spiked seed samples, it will be used to measure the level of cross pollination of a cleistogamous oilseed rape line at the field level.

Conclusion and Outlook

The methods developed during the course of the project can be directly used by commercial and scientific institutions for additional and further work (e.g., Hohenheim Standard Dormancy Test, QTL mapping of dormancy, PCR for cleistogamy). Growers can profit by using the pre-harvest sprouting test to assess the dormancy level of the varieties grown in the fields as a do-it-yourself rapid test. The model calculations determine the potential for gene dispersal by canola volunteers on a specific field; on this basis, decision makers and farmers have a tool which provides information about whether further actions are necessary under these conditions to limit gene dispersal (e.g., soil tillage, containment). The confinement system is useful for breeders and biotech companies as well as practical farmers. Policymakers should obtain support by reliable empirical data and model calculations.

Overall, the biological confinement system "low dormancy and cleistogamy" appears suitable for application to GM canola to facilitate and maintain coexistence. The essential basics are given: dormancy and cleistogamy are heritable, they are stable, and they can be tested under laboratory conditions. Practical relevance, at least for dormancy, is also given.

The results of this study offer the opportunity to select practice-relevant varieties for low dormancy and the chance to use low-dormancy genotypes for further transformation of particular single plants. Low dormancy and cleistogamy may become a part of the ideotype of modern GM canola varieties, and only these genotypes could be used for transformation. The route of heritability has to be further investigated by crossing experiments of homozygous lines. It is expected that our results will also be useful for conventional canola, and that they can be transferred to other crops as well.

The creation of confinement systems by non-dormant and cleistogamic genotypes may facilitate growing of GM canola in the future by limiting gene flow at the very beginning, thus avoiding a potential chain of incidents.

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