

Mapping of QTL for seed dormancy in a winter oilseed rape doubled haploid population

Jörg Schatzki · Burkhard Schoo · Wolfgang Ecke ·
Cornelia Herrfurth · Ivo Feussner · Heiko C. Becker ·
Christian Möllers

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Abstract Following winter oilseed rape cultivation, considerable numbers of volunteer oilseed rape plants may occur in subsequent years in following crops. The appearance of volunteer oilseed rape plants is based on the capability of the seeds to become secondary dormant and to survive in this stage for many years in the soil. Genetic reduction of secondary seed dormancy in oilseed rape could provide a means to reduce the frequency of volunteer plants and especially the dispersal of transgenic oilseed rape. The objective of the present study was to analyse the inheritance of primary and secondary seed dormancy in a winter oilseed rape doubled haploid population derived from the cross Express 617 × R53 and to study correlations to other seed traits. Field experiments were performed in Germany for 2 years at two locations with two replicates. Seeds harvested from open pollinated plants were used for all analyses, including a laboratory test for seed dormancy. A previously developed molecular marker map of the doubled haploid population was used to map QTL of the relevant traits. For primary, secondary and total seed dormancy, the results showed significant effects of the genotypes and their interactions, with years and locations. Two, four and five QTL were detected for primary, secondary and total

seed dormancy which explained 19, 35 and 42 % of the phenotypic variance, respectively. Results show that secondary seed dormancy is a heritable trait and that selection for low secondary seed dormancy is possible.

Introduction

Primary dormancy of mature seeds is characterised by their inability to germinate under favourable conditions relating to water, light, air and temperature (Bewley 1997; Finch-Savage and Leubner-Metzger 2006). Primary dormancy before harvest is a desired trait in crop species because it prevents the early germination of mature seeds on the plant in many regions of the world during their exposure to cool moist conditions before harvest (i.e. pre-harvest sprouting or vivipary). Hence, the genetic control of pre-harvest sprouting is an important trait mainly in cereal but also in oilseed rape breeding programmes (Gubler et al. 2005; Gerjets et al. 2010; Marzougui et al. 2012; Feng et al. 2009). Secondary seed dormancy can be induced after harvest in mature non-dormant seeds by environmental conditions that do not favour germination (e.g. high temperature and anoxia, Gubler et al. 2005). The induction and the release of seed dormancy are regulated by complex interactions between environmental and genetic factors that are still poorly understood. However, the interplay of the phytohormones abscisic acid (ABA), gibberellic acid (GA), ethylene and brassinosteroids are considered to be decisive for the regulation of seed dormancy and germination (Finch-Savage and Leubner-Metzger 2006; Finkelstein et al. 2008). The capacity of seeds to become secondary dormant is of particular relevance to oilseed rape, because before and during harvest of oilseed rape considerable amounts of seeds may be lost due to silique shattering (Hossain et al.

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J. Schatzki · B. Schoo · W. Ecke · H. C. Becker · C. Möllers (✉)
Plant Breeding Division, Department of Crop Sciences,
Georg-August-Universität Göttingen, Von-Siebold-Str. 8,
37075 Göttingen, Germany
e-mail: cmoelle2@gwdg.de

C. Herrfurth · I. Feussner
Department of Plant Biochemistry, Albrecht von
Haller-Institute for Plant Sciences, Georg August University,
Justus-von-Liebig-Weg 11, 37075 Göttingen, Germany

2011; Laga et al. 2011, 2012). Under unfavourable conditions those seeds become secondary dormant in the soil and remain viable for a period of 10 years and longer (Lutman et al. 2003). To date it is not clear if there is a link between seed dormancy and seed longevity under natural conditions (Gräber et al. 2012).

Repeated cultivation of oilseed rape on the same field leads to an increase of the dormant seed bank in the soil (Gruber et al. 2010). Under favourable conditions those dormant seeds may germinate and occur as a weed or so called ‘volunteer oilseed rape’ in succeeding crops for several years (Gruber et al. 2004; Pekrun et al. 1997a, b). Secondary dormancy can be induced conveniently in vitro by incubating seeds in polyethylene glycol solution for a period of 2–4 weeks in darkness (Pekrun et al. 1997a, b; Momoh et al. 2002). Comparing the induction of secondary seed dormancy under in situ and under in vitro conditions in PEG has led to concurrent results (Gulden et al. 2003; Gruber et al. 2004). Previous work has shown that there are large and significant differences among current winter oilseed rape cultivars with respect to their capacity to produce secondary dormant seeds after artificial dormancy induction and the trait proved to have a high heritability (Schatzki et al. 2013). Genetic reduction of secondary seed dormancy in oilseed rape could provide an option to reduce the frequency of volunteer oilseed rape plants and therefore also the undesired dispersal of seeds and pollen from transgenic oilseed rape. The objective of the present study was to analyse the inheritance of seed dormancy and of ABA content in the seeds of the winter oilseed rape doubled haploid population Express 617 × R53, and to estimate correlations to other seed traits.

Materials and methods

Plant material and field experiments

A doubled haploid (DH) population consisting of 229 lines was derived from a cross between the inbred line no. 617 of the German winter oilseed rape cultivar Express (canola quality) and the resynthesised line R53, an interspecific hybrid between *Brassica oleracea* var. *sabellica* (kale) and *Brassica rapa* ssp. *pekinensis* (Chinese cabbage, see Radoev et al. 2008). R53 has high erucic acid content in the seed oil and high glucosinolate content in the seeds. Seed material of the 229 DH lines and the parental genotypes Express 617 and R53 was cultivated in observation plots in field experiments with two replicates at the two locations, Göttingen and Thüle in north-western Germany, in two consecutive seasons (2008/09 and 2009/10). The field experiments were performed as a randomized block design. Seeds were sown in double rows with a density of

50 seeds m^{-2} in Göttingen and in fourfold rows with a density of 60 seeds m^{-2} in Thüle. Final plot size was 2.1 m^2 in Göttingen and 2.75 m^2 in Thüle. Standard cultivars were sown as border rows. Average annual temperature and rainfall in 2009 were 9.3 °C and 670 mm in Göttingen and 11.2 °C and 891 mm in Thüle (Salzkotten-Oberntudorf). In 2010 average annual temperature and rainfall were 8.0 °C and 632 mm in Göttingen and 9.7 °C and 862 mm in Thüle (Salzkotten-Oberntudorf). Height above sea level was 173 m in Göttingen and 176 m in Thüle. The DH population was expected to show a considerable variation for the trait ‘secondary seed dormancy’, because the two parental lines revealed in a previous test considerable difference in their capacity to form secondary dormant seeds after artificial dormancy induction. Express 617 showed a low and R53 a high expression of this trait (data not shown). In both field years and locations, seeds were harvested at maturity from the main inflorescence of 10 open pollinated plants per genotype and replicate. Seeds from the 10 plants were bulked for further analyses. For ABA analysis 3 g of seeds from each of the two replicates of a genotype from the two locations in each year were pooled.

Test for primary seed dormancy (PSD)

The seed germination (%) in darkness was determined on 2 × 100 seeds per genotype, replicate, location and year. Filter papers (MN618 with 85 mm radius, 0.32 mm thickness and 140 g/m² weight (Macherey–Nagel GmbH, Düren, Germany) were put in plastic Petri dishes (92 × 16 mm, Sarstedt AG & Co., Nürnbrecht, Germany) and 6 mL of deionised water was added. Under green light (Göttinger Farbfilter GmbH, Bovenden-Lenglern, Germany) 100 seeds per Petri dish were equally dispersed on the soaked filter paper and Petri dishes were closed with the corresponding lid. Petri dishes were then stored in cardboard boxes in complete darkness in a climate chamber at 18 °C. Germination was determined after 1, 5 and 14 days and number of germinated seeds was summed up to calculate the germination percentage (%) in darkness. For testing the viability of the remaining seeds that had not germinated within the 14 days incubation on moist filter paper, Petri dishes were put together in transparent plastic bags and were incubated in a climate chamber for 7 days under alternating light and temperature conditions (12 h darkness at 5 °C and 12 h light at 25 °C). Germinated seeds were counted after 3 and 7 days and number of germinated seeds was summed up (Range 83–100 %; mean 98.6 %). Finally, seeds that at this point still had not germinated were incubated in a 0.2 % (w/v) solution of 2, 3, 5-triphenyl tetrazolium chloride (Peters 2000) and red stained seeds were counted as viable. The number of viable seeds (Range 98–100 %; mean 99.6 %) consisted of the total number of germinated

seeds plus the number of viable seeds determined in the tetrazolium test. Primary seed dormancy (PSD, %) was calculated in the following way: (total viable seeds – germinated seeds in darkness) \times 100/total viable seeds. The test for primary seed dormancy was performed in parallel to the test for seed dormancy.

Test for total seed dormancy (TSD) and secondary seed dormancy (SSD)

The test for TSD was performed essentially following a protocol described by Gruber et al. (2004). In brief, total seed dormancy was induced by incubating seeds in Petri dishes on filter paper soaked with PEG 6000 solution (354.37 g/L) and seeds were stored in complete darkness for 2 weeks. Thereafter, seeds were washed and transferred to Petri dishes with deionised water and seeds germinating during a period of 2 weeks were counted. All treatments and counting of germinated seeds were performed in a climate chamber at a temperature of 18 °C and under green light. For testing the viability of the remaining seeds that had not germinated, Petri dishes with seeds were put together in transparent plastic bags and were incubated in a climate chamber for 7 days under alternating light and temperature conditions (12 h darkness at 5 °C and 12 h light at 25 °C). Germinated seeds were counted and number of germinated seeds was summed up to give the total number of germinated seeds. Then, as described above a tetrazolium test was performed with the seeds that still had not germinated to determine the total number of viable seeds. The seed samples were analysed replicate-wise with 2 \times 100 seeds per replicate. The dormancy induction tests were performed once per year, location, genotype and replicate. Because of the large number of samples the tests could not be performed for all genotypes at the same time. Dormancy induction tests were conducted 3, 4, 7 and 8 weeks after harvest in 2009 and 6, 7, 10 and 11 weeks after harvest in 2010. The viable seeds that did not germinate during the first 2 weeks in deionised water in darkness after the dormancy induction were considered to be dormant, i.e. the sum of viable seeds minus the number of seeds germinated in the first step of the viability test. TSD was calculated as $TSD (\%) = (\text{viable seeds} - \text{seeds germinated within 2 weeks in deionised water}) \times 100/\text{viable seeds}$. SSD was determined as $SSD (\%) = TSD - PSD$.

Thousand kernel weight

Thousand kernel weight (TKW) in grams was determined from 500 seeds using a seed counter (model Contador, Pfeuffer GmbH, D-97318 Kitzingen, <http://www.pfeuffer.com>).

Determination of abscisic acid (ABA) by HPLC–MS/MS

10 g from each of the mixed seed samples were ground in a coffee mill model Krups F203 for 6 s (3 times 2 s with in between mixing of the meal). Extraction was essentially performed as described earlier by Matyash et al. (2008) with some modifications. Two times 200 mg meal were used for duplicate analysis of each genotype. The extraction was performed with optically opaque vials to impede photo isomerisation of *cis*-ABA to *trans*-ABA. Each sample was extracted with 0.75 mL methanol, 2.5 mL methyl-tert-butyl ether and 20 ng D₆-ABA (CDN Isotopes Inc., Quebec, Canada) as internal standard and covered with a layer of argon. After 1 h of extraction at 5 °C, 0.625 mL of water was added and mixed. Vials were incubated to allow the formation of two phases. The upper organic phase was dried under streaming nitrogen and due to the high oil content in oilseed rape meal was extracted with acetonitrile thrice. The three extracts were combined, dried and the pellet was re-suspended in 100 μ L acetonitrile and dried again. The final pellet was put back into suspension with 100 μ L acetonitrile/water/acetic acid (20:80:0.1, v/v/v). Analysis of constituents was performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (MDS Sciex, Ontario, Canada). Nano electrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate, Advion BioSciences, Ithaca, USA). Reversed-phase HPLC separation was performed on an EC50/2 Nucleodure C18 gravity 1.8 μ m column (50 \times 2 mm, 1.8 μ m particle size, Macherey and Nagel, Düren, Germany). ABA values were expressed in pmol/g fresh weight. The binary gradient system consisted of solvent A, acetonitrile/water/acetic acid (20:80:0.1, v/v/v) and solvent B, acetonitrile/acetic acid (100:0.1, v/v) with the following gradient programme: 28 % solvent B for 2 min, followed by a linear increase of solvent B up to 52 % within 3 min, a further linear increase of solvent B up to 92 % within 1 min and an isocratic run at 92 % solvent B for 8 min. The flow rate was 0.3 ml min⁻¹. For stable nanoESI, 100 μ l min⁻¹ of 2-propanol/acetonitrile/water/formic acid (70:20:10:0.1, v/v/v/v) delivered by a 2150 HPLC pump (LKB, Bromma, Sweden) were added just after the column via a mixing tee valve. By using another post column splitter 740 nL min⁻¹ of the eluent were directed to the nanoESI chip. Ionization voltage was set to –1.7 kV. ABA was ionized in a negative mode and determined in multiple reactions monitoring mode. Mass transitions were as follows: 269/159 [declustering potential (DP) –55 V, entrance potential (EP) –9 V, collision energy (CE) –16 V] for D₆-ABA and 263/153 (DP –55 V, EP –9 V, CE –16 V) for ABA. The mass analysers were adjusted to a resolution of 0.7 amu full width at

half-height. The ion source temperature was 40 °C, and the curtain gas was set at 10 (given in arbitrary units). Quantification was carried out using a calibration curve of intensity (m/z) ratios of ABA/ D_6 -ABA vs. molar amounts of ABA (0.3–1,000 pmol).

Other seed quality traits

Oil content and protein content (% at 91 % DM), glucosinolate content (GSL, $\mu\text{mol/g}$ seed at 91 % DM) and erucic acid content (22:1, % of the total fatty acid content) as well as moisture content were determined by NIRS using the calibration raps2009.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel). Protein content of the defatted meal (% at 91 % DM) was calculated by using the seed oil and protein content data obtained from NIRS prediction.

Statistics

Analysis of variance and calculation of heritabilities (h^2) were performed by using PLABSTAT software (Utz 2011) using the following model: $Y_{ijkl} = \mu + g_i + y_j + l_k + r_{l(jk)} + gy_{ij} + gl_{ik} + yl_{jk} + gyl_{ijk} + \varepsilon_{ijkl}$. Where Y_{ijkl} is observation of genotype i in year j in location k and in replicate l ; μ is general mean; g_i , y_j , l_k , and $r_{l(jk)}$ are effects of genotype i , year j , location k , and replicate l within year j and location k ; gy_{ij} , gl_{ik} , yl_{jk} , gyl_{ijk} are the interactions and ε_{ijkl} is the residual error. Years, locations and replicates were considered as random variables. For ABA-values originating from mixed seed samples of each year only the years were considered as random. For total seed dormancy, secondary seed dormancy and primary seed dormancy ArcSin-transformed

data were used. Mean values of the genotypes across the locations were used to calculate Spearman's rank correlation coefficients between traits. +, *, ** denotes significance at $P = 10, 5$ and 1% , respectively.

Genetic map and QTL mapping

The framework map developed by Radoev et al. (2008) was improved and extended and finally consisted of 229 markers including 80 SSR and 149 AFLP markers. The mean distance of all markers covering 21 linkage groups was 9.5 cM, the standard deviation was 5.7 cM and the maximum distance was 26.6 cM (Kosambi). Total map size was 2,283 cM. Quantitative trait loci (QTL) mapping was performed using the freely available software QTL Network 2.1 (Yang et al. 2009) with a significance level of $P = 5\%$ for declaring a QTL. Permutation analyses were performed (1,000 permutations) to determine the critical F value threshold for each trait. Additive \times additive epistatic interactions between loci were also evaluated by using QTL Network 2.1. Linkage groups N1 to N10 correspond to linkage groups A1 to A10 of *B. rapa* and linkage groups N11 to N19 correspond to linkage groups C1 to C9 of *B. oleracea* (Parkin et al. 1995).

Results

The analysis of variance showed for primary, secondary and total seed dormancy highly significant effects of the genotypes (Table 1). Effects of the year and the locations were either not significant or only weakly significant. However, interactions between genotypes and years, genotypes

Table 1 Variance components and heritabilities (h^2) for total seed dormancy (TSD, %), primary seed dormancy (PSD, %), secondary seed dormancy (SSD, %), thousand kernel weight (TKW, g), oil and protein content of the seed, protein content of the defatted meal (prot odM, in % at 91 % DM), glucosinolate content (GSL, $\mu\text{mol/g}$

FM seeds), erucic acid content (22:1, % of the oil) and ABA content (pmol/g FM seed meal) of the DH population and the parental lines tested in field experiments in 2 years at two locations with each two replicates

Source of variance	TSD ^a	PSD ^a	SSD ^a	TKW	Oil	Protein	Prot odM	GSL	22:1	ABA ^b
year (Y)	221.6	26.5	139.0 ⁺	0.01	−0.93	−0.63	−0.44	−0.73	0.27	434.0 ^{**}
Location (L)	82.6	10.8	50.9 ⁺	0.00	0.22 [*]	−0.30	−0.43	0.22	14.68 ^{**}	−
Genotype (G)	208.3 ^{**}	17.1 ^{**}	128.1 ^{**}	0.18 ^{**}	2.82 ^{**}	0.95 ^{**}	1.81 ^{**}	269.3 ^{**}	91.46 ^{**}	302.9 ^{**}
G \times Y	33.6 ^{**}	12.5 ^{**}	60.0 ^{**}	0.02 ^{**}	0.28 ^{**}	0.18 ^{**}	0.22 ^{**}	5.15 ^{**}	3.14 ^{**}	243.9 ^b
G \times L	13.6 ^{**}	4.8 ^{**}	21.5 ^{**}	0.00	0.16 ^{**}	0.10 ^{**}	0.18 ^{**}	3.05 ^{**}	1.64 ^{**}	−
G \times Y \times L	22.6 ^{**}	7.5 ^{**}	29.1 ^{**}	0.03 ^{**}	0.30 ^{**}	0.21 ^{**}	0.34 ^{**}	5.59 ^{**}	1.91 ^{**}	−
Residual	51.9	20.0	51.4	0.09	1.29	0.86	1.07	32.15	11.31	−
h^2	0.85	0.57	0.70	0.86	0.92	0.86	0.89	0.98	0.98	0.71

+ , * , ** denotes significant at $P = 10, 5$ and 1%

^a ArcSin transformed data were used

^b Data for each genotype and year were obtained from pooled samples of the 2 locations, G \times Y includes residual

Table 2 Minimum, maximum and mean values for total seed dormancy (TSD, %), primary seed dormancy (PSD, %), secondary seed dormancy (SSD, %), thousand kernel weight (TKW, g), oil and protein content of the seed, protein content of the defatted meal (protodM, in % at 91 % DM), glucosinolate content (GSL, $\mu\text{mol/g}$ FM seeds), erucic acid content (22:1, % of the oil) and ABA content (pmol/g FM) of the DH population and the parental lines tested in field experiments

	TSD	PSD	SSD	TKW	Oil	Protein	Prot odM	GSL	22:1	ABA ^a
Minimum	4.7	0.0	4.7	3.8	39.6	18.1	34.2	16.8	1.3	25.7
Maximum	96.2	26.4	89.0	6.4	48.3	24.3	41.6	81.5	36.7	149.0
Mean	59.9	3.6	56.2	4.9	44.1	21.1	37.8	42.6	17.9	62.6
LSD5 % (G)	25.7	10.1	20.6	0.5	1.4	1.1	1.3	6.6	4.2	30.8
Express 617	30.5	0.1	30.4	5.4	45.7	18.0	33.1	16.7	0.0	47.2
R53	64.4	2.7	61.7	4.7	42.6	20.6	35.9	41.8	15.9	102.1

Depicted are means over 2 years, two locations with each two replicates

LSD5 % = least significant difference at $P = 5\%$

^a Data for each genotype and year were obtained from pooled samples of the 2 locations

and locations as well as the threefold interaction were highly significant for all three seed dormancy traits. Heritability was high for total seed dormancy, somewhat lower for secondary seed dormancy and moderate for primary seed dormancy. Large and significant effects of the genotypes were also found for TKW, oil, protein, glucosinolate and ABA content of the seeds as well as for protein content of the defatted meal. The effects of the year were large for all three seed dormancy traits and ABA content but small for TKW, oil and protein content. High heritabilities were also determined for TKW, oil and protein content of the seeds as well as for the protein content of the defatted meal. Since the segregating DH population was derived from a cross between the Canola-type cultivar Express 617 and the resynthesised line R53 with high erucic acid (22:1) content of the oil and high glucosinolate content of the seed, very high variance components and heritabilities were found for the genotypic effects of these traits.

A large and significant variation for total seed dormancy was detected among the genotypes which ranged from 5 to 96 % with a mean of 60 % (Table 2). The parental genotype Express 617 showed as expected with 31 % a much lower total seed dormancy than R53 with 64 %. The mean of the total seed dormancy level of the DH population (60 %) was nearly as high as the dormancy level of R53 due to a transgressive segregation of this trait. With 108 genotypes showing higher total seed dormancy than R53 and only 30 genotypes with a lower dormancy than Express 617 (Fig. 1) the segregation resulted in a high average total seed dormancy level. Large variations were also found for the primary and secondary seed dormancy as well as for most of the other traits (Table 2). Figure 1 shows the frequency distribution for primary, secondary and total seed dormancy of the DH population. The frequency distributions for total and secondary seed dormancy were skewed towards higher values, whereas the frequency distribution of the primary

dormancy was clearly skewed towards lower values. In addition, the frequency distributions of the secondary and total seed dormancy indicated a bimodal distribution in the DH population, with the two dormancy classes separated at the 60 % line. This indication of a bimodal distribution remained evident even when the class sizes were shifted by 1 or 2 % (not shown).

Primary, secondary and total seed dormancy was significantly positive correlated to each other (Table 3). However, the scatter plots (Fig. 2) revealed unusual distributions, which indicated that low primary seed dormancy is not necessarily linked to low secondary and total seed dormancy. Vice versa, genotypes with a high primary dormancy showed also a high secondary and total seed dormancy. A loose but significantly positive correlation was found between ABA content and secondary seed dormancy as well as total seed dormancy. There was no close correlation of seed dormancy traits to other seed quality traits.

QTL mapping led to the identification of 5 QTL for total seed dormancy (TSD), which together explained 42 % of the phenotypic variance (Table 4). The QTL for total seed dormancy were located on linkage groups N5, N13, N15, N18 and N19. For the mapped QTL, the estimated additive effect (a) is shown as the substitution of an R53 allele by an Express 617 allele. For example, at the QTL TSD-1 of linkage group N5, the substitution of the R53 allele by an Express 617 allele led to a reduction of the total seed dormancy of 6.13 %. Hence, the results show that in three out of five cases, the Express allele led to a reduction in total seed dormancy. Only in the case of QTL TSD-3 and TSD-5 the Express 617 allele increased total seed dormancy. QTL for primary seed dormancy (PSD), SSD, TKW, and ABA content were also identified (Table 4; Fig. 3) some of whose positions and confidence intervals overlapped with the confidence intervals of the QTL for total seed dormancy

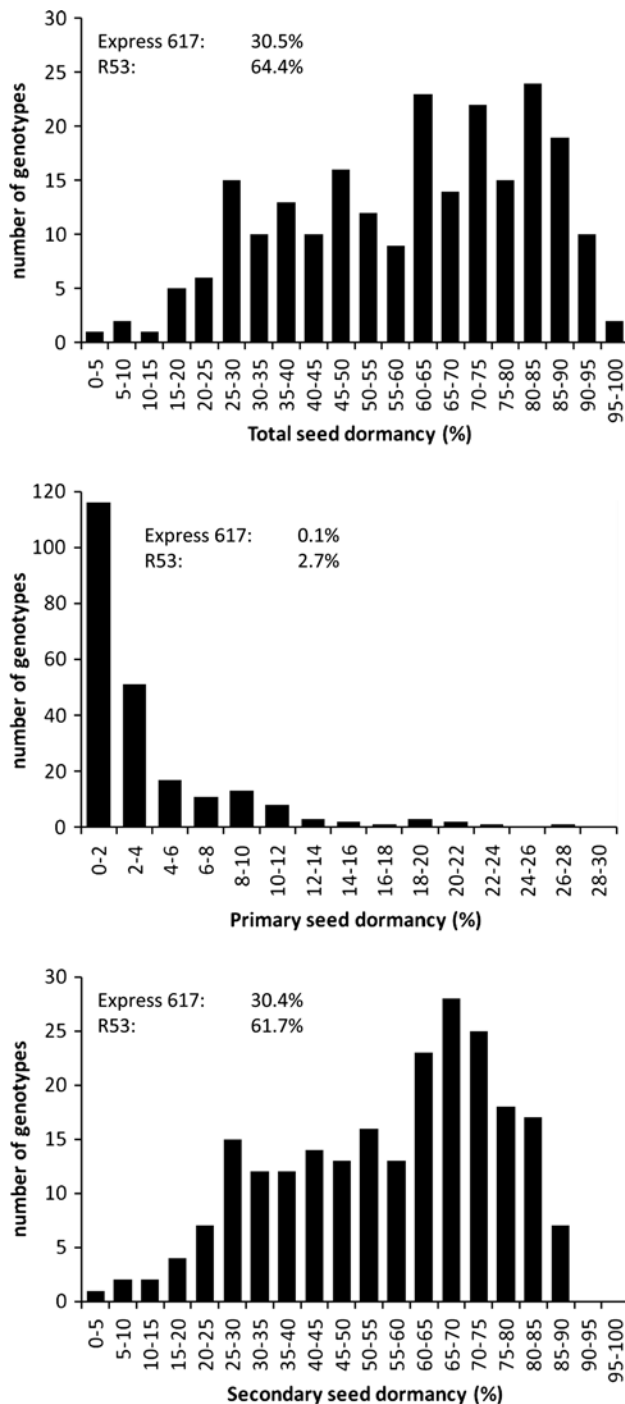


Fig. 1 Frequency distribution of 229 doubled haploid winter oilseed rape lines for total seed dormancy (TSD), primary seed dormancy (PSD) and secondary seed dormancy (SSD); means of 2 years, 2 locations and 2 replicates; non-transformed data)

(Table 4; Fig. 3). The proportions of the explained phenotypic variance of the QTL together were somewhat lower, ranging from 15 % for ABA content to 35 % for secondary seed dormancy. An epistatic effect between QTL with main effects was observed only for QTL for TKW.

Discussion

Previous experiments have shown that there are large differences among spring and winter oilseed rape genotypes for their capacity to produce secondary dormant seeds after induction in hyperosmotic polyethylene glycol solutions (Pekrun et al. 1997a, b; Momoh et al. 2002; Gulden et al. 2003, 2004). Gruber et al. (2004) found in a set of 32 winter oilseed rape genotypes secondary seed dormancy ranging from 3 to 76 %. In a more recent study (Schatzki et al. 2013) a variation from 8 % to over 50 % was reported for a set of 28 winter oilseed rape cultivars tested in field experiments at six contrasting locations. The parents of the F1 derived DH population used in the present study showed with 30 % (Express 617) and 62 % (R53) rather intermediate secondary dormancy levels (see Table 2). However, a large transgressive segregation was found in the DH population. Secondary seed dormancy levels as low as 5 % and as high as 89 % were observed among the DH lines. Transgressive segregation in the DH population can be explained by the recombination of parental alleles in DH lines that either enhance or reduce the capacity to produce secondary dormant seeds. The comparatively high mean value for the secondary seed dormancy of the DH population (see Table 3) may be explained by epistatic interactions leading to lower secondary dormancy in either one or both parental lines. The dormancy values for the DH population and the parental genotypes should be quite reliable, because they represent mean values from 2 years of field experiments at two locations with two replicates each. The heritability for secondary dormancy was with 0.70 lower than the heritability for oil and protein content and lower than reported before for a set of 28 cultivars (Schatzki et al. 2013).

A hitherto not reported unexpected large variation for primary seed dormancy ranging from 0 to 26 % was found (Table 2). However, the mean primary seed dormancy was with 3.6 % comparatively low. This may be explained by the fact that the analysis of the seeds for dormancy was performed between 3 to 11 weeks after harvest (c.f. “Materials and methods”). The frequency distribution (Fig. 1) indicated that only few genotypes had higher primary seed dormancy rates. The low mean primary seed dormancy explains the very similar results obtained for secondary and total seed dormancy. Spearman rank correlations revealed close positive correlations between the three seed dormancy types (Table 3). However, the scatter plots of the data revealed no direct association between primary seed dormancy and secondary seed dormancy and total seed dormancy, respectively. The mean viability for the genotypes as determined with the tetrazolium test after the secondary seed dormancy test and the alternating light and temperature treatment ranged from 98 to 100 % (c.f. “Materials and methods”). Genotypes that after the alternating light

Table 3 Spearman-rank correlation coefficients for total seed dormancy (TSD), primary seed dormancy (PSD), secondary seed dormancy (SSD), thousand kernel weight (TKW, g), oil and protein content of the seed, protein content of the defatted meal (prot odM), glucosinolate (GSL), erucic acid (22:1) and ABA content

PSD	0.74**	–	–	–	–	–	–	–	–
SSD	0.97**	0.62**	–	–	–	–	–	–	–
TKW	–0.08	–0.08	–0.06	–	–	–	–	–	–
Oil	0.10	–0.01	0.09	–0.13*	–	–	–	–	–
Protein	–0.13	–0.04	–0.12	0.03	–0.66**	–	–	–	–
Prot odM	–0.10	–0.07	–0.09	–0.08	–0.06	0.77**	–	–	–
GSL	–0.14*	–0.08	–0.12	0.16*	–0.39**	0.36**	0.13*	–	–
22:1	0.09	0.03	0.07	–0.16*	0.63**	–0.12	0.38**	–0.09	–
ABA	0.14*	0.00	0.18**	–0.09	0.00	–0.09	–0.12	–0.02	–0.01
	TSD	PSD	SSD	TKW	Oil	Protein	Prot odM	GSL	22:1

*, ** Denotes significance at $P = 10, 5$ and 1%

and temperature treatment did not germinate (0–17 %; c.f. “Materials and methods”) may either still have a high primary dormancy or a dormancy state caused by interaction between primary seed dormancy and the induced secondary seed dormancy. Hence, seeds that did not germinate could be still dormant or alternatively may carry alleles that somehow affect germination. This seems unlikely since both parental lines were homozygous and showed germination percentages of 97 % (data not shown). Unfortunately, seeds were not analysed for their germination percentage 6 months after harvest, a point of time when primary dormancy can be expected to be nil. The reasons for the low germination and concurrent high viability of some genotypes of this population are yet unknown and require further investigation. The observed high primary dormancy of some genotypes is contrary to the marginally primary dormancy percentage of 32 freshly harvested oilseed rape cultivars as reported by Gruber et al. (2004). However, cultivars have gone through a long selection process and genotypes with a high primary dormancy unlikely become a cultivar. No correlation was found between TKW and secondary seed dormancy which is in agreement with the results from the previous study (Schatzki et al. 2013).

Surprisingly, no or only a loose positive correlation between ABA content of the mature dry seeds and their primary and secondary dormancy was found (Table 3). This finding is partly in contrast to published literature which indicates a role of ABA in primary seed dormancy induction and maintenance (Martínez Andújar et al. 2011; Finch-Savage and Leubner-Metzger 2006; Finkelstein et al. 2008). However, the role of ABA as dormancy factor seems controversial since no clear relationship between the ABA content of mature dry seeds and dormancy intensity has been reported for a number of species (Gräber et al. 2012), which is in accordance with the results of the present study. Nevertheless, ABA synthesis may be induced

during incubation of seeds in the PEG solution and this may have much larger influence on secondary seed dormancy. The results from Gulden et al. (2004) further corroborate this hypothesis since they showed that the ABA content of the seeds during and at the end of the imbibition period in PEG solution changed differently for a low and a high dormancy potential genotype. In their study they also examined the influence of applications of exogenous ABA and of the ABA biosynthesis inhibitor fluridone. The application of ABA had no significant effect on the germination of both genotypes after 2 weeks of osmotic stress treatment whereas the application of fluridone (Bartels and Watson 1978) vastly increased the germination of the high dormant genotype. These results are supported by data obtained from a number of species which show that only ABA produced during seed development by the embryo is effectively causing dormancy (Nambara and Marion-Poll 2003). Maternal ABA naturally occurring in the seed covering layers, or ABA application (resembling maternal ABA) during seed development, both fail to induce seed dormancy for yet unknown reasons. Since seeds of oilseed rape lack an endosperm, the function of ABA in germination and dormancy processes might differ from other species. ABA does not inhibit testa rupture but the subsequent radicle growth in *Brassica napus* (Schopfer and Plachy 1984). Also, one could have assumed a role of gibberellic acid in seed dormancy induction and maintenance, however, in preliminary analyses gibberellic acid content in dry mature seeds proved to be below the detection limit (data not shown). These results are in accordance with the results obtained by Zhang (2008) but in contrast to those found by Gulden et al. (2004).

The QTL analysis resulted in the detection of 5 QTL for total seed dormancy which together explained 42 % of the phenotypic variance (Table 4). At three of the five QTL, the Express 617 allele led to a reduction in total seed

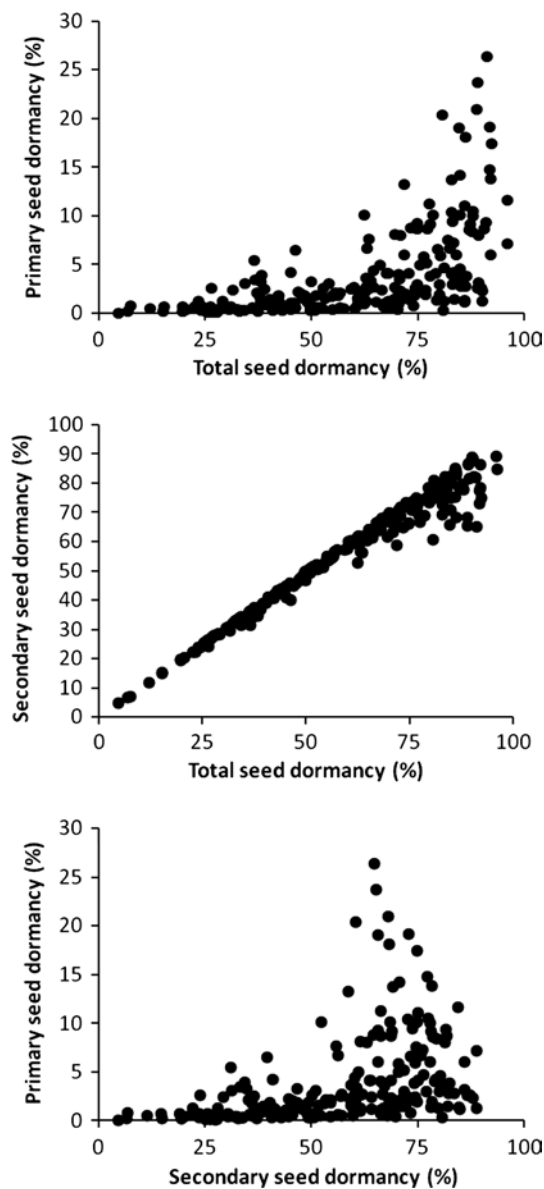


Fig. 2 Scatter plot of primary, secondary and total seed dormancy of 229 doubled haploid winter oilseed rape lines (means of 2 years, 2 locations and 2 replicates)

dormancy and only at QTL TSD-3 and TSD-5 the Express 617 alleles increased total seed dormancy. This finding is consistent with the large transgressive segregation observed for this trait in the DH population. The additive effects of the QTL TSD-3 and ABA-1, which are both located on N15 with overlapping confidence intervals, show the same sign. This is consistent with the general assumption that a higher ABA content leads to a higher dormancy. However, in the case of linkage group N18 the direction of the additive effect of the QTL ABA-2 is different from that of the QTL TSD-4, PSD-2 and SSD-4, which is not in agreement with the above mentioned general idea. But in this case the

confidence intervals of the QTL ABA-2 and TSD-4, PSD-2 and SSD-4 did not overlap, suggesting that different loci are involved in the expression of those two traits. Linkage between QTL for seed dormancy (TSD, PSD and SSD) and ABA content probably contributed to the low correlation between those traits.

In agreement with the close positive correlations found between secondary and total seed dormancy, four of the five QTL for total seed dormancy showed similar positions and overlapping confidence intervals with the QTL for secondary seed dormancy (see Fig. 3). On linkage group N19 at position 78.5 cM there was also a QTL for secondary seed dormancy, which however was not significant. The two QTL for primary seed dormancy PSD-1 on N13 and PSD-2 at N18 were at similar positions as QTL for total and secondary seed dormancy. Furthermore, the effects of the QTL for primary, secondary and total seed dormancy on the same linkage groups had the same direction. Together this indicates that there are common mechanisms involved in the expression of primary and secondary seed dormancy. On linkage group N15 the confidence intervals of the QTL TSD-3 and SSD-3 overlapped with the confidence interval of the QTL ABA-1 and on linkage group N18 the QTL ABA-2 mapped nearby the QTL for seed dormancy. Overlapping confidence intervals of different QTL may indicate that a gene located in this region has a pleiotropic effect on two or more traits, although the alternative explanation, that more than one gene is located in the QTL region, each affecting a different trait, cannot be excluded by QTL mapping alone.

A possible link between seed dormancy and seed longevity has been discussed for some time (Gräber et al. 2012). Further experimental work has to show if the present DH population is segregating for seed longevity and pre-harvest sprouting. In that case it will be interesting to see whether confidence intervals of QTL for seed longevity and pre-harvest sprouting overlap with those of QTL for primary and secondary dormancy. In *Arabidopsis*, the gene *DELAY OF GERMINATION1* (*DOG1*) has been identified as major QTL for seed dormancy. *DOG1* mutants are completely non-dormant and do not show any obvious pleiotropic effects, apart from a reduced seed longevity (Alonso-Blanco et al. 2003; Bentsink et al. 2006). In a recent paper, Nguyen et al. (2012) reported a negative correlation between seed dormancy and seed longevity in six *Arabidopsis* recombinant inbred line populations. QTL for germination ability after storage (GAAS) collocated with *DOG* loci previously identified in those populations. The *DOG1*-gene was recently shown to be conserved in other plant species, amongst others in *B. rapa* (Gräber et al. 2012, 2010). Genetic evidence has been provided to show that *DOG1* functions independent from ABA and that the presence of both *DOG1* and ABA is required for seed

Table 4 Mapped QTL and their most likely positions for total seed dormancy (TSD), primary seed dormancy (PSD) and secondary seed dormancy (SSD), thousand kernel weight (TKW) and abscisic acid content (ABA)

QTL	LG	Position (cM)	CI (cM)	<i>a</i>	<i>h</i> ² (<i>a</i>)	V(A)/V(P)	V(I)/V(P)	V(G)/V(P)
TSD-1	N5	65.4	41.5–79.7	−6.13	0.08	0.42	–	0.42
TSD-2	N13	196.4	190.4–205.1	−8.90	0.11			
TSD-3	N15	89.5	82.3–97.2	6.14	0.10			
TSD-4	N18	89.7	79.3–101.4	−6.41	0.09			
TSD-5	N19	78.5	72.5–83.5	4.68	0.06			
PSD-1	N13	195.4	174.6–214.1	−1.08	0.05	0.19	–	0.19
PSD-2	N18	96.4	85.7–103.4	−1.77	0.14			
SSD-1	N5	64.4	39.5–77.7	−5.97	0.08	0.35	–	0.35
SSD-2	N13	201.1	191.4–206.1	−7.81	0.11			
SSD-3	N15	88.5	82.3–93.2	5.82	0.10			
SSD-4	N18	88.7	77.3–102.4	−4.15	0.06			
TKW-1	N5	92.3	76.7–97.7	0.21	0.11	0.18	0.03 ^a	0.20
TKW-2	N12	17.7	9.5–27.4	−0.14	0.04			
ABA-1	N15	79.3	68.1–89.5	5.19	0.05	0.15	–	0.15
ABA-2	N18	114.3	107.3–121.0	7.09	0.09			

LG linkage group, CI confidence interval, *a* additive effect, *h*² (*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

^a Epistatic effect between QTL TKW1 and TKW2. For the mapped QTL, the estimated additive effect (*a*) is shown as the substitution of an R53 allele by an Express 617 allele

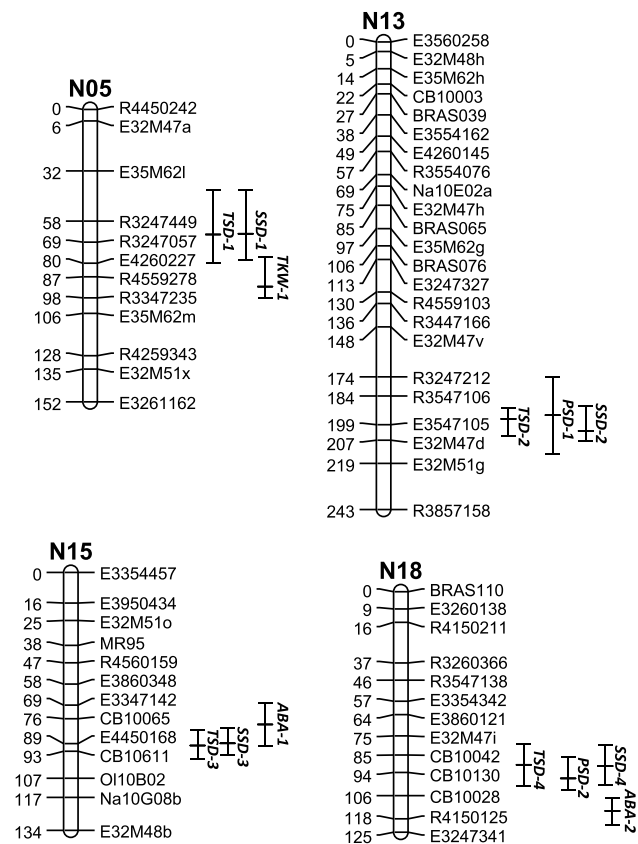


Fig. 3 Linkage groups N5, N13, N15 and N18 showing QTL for total seed dormancy (TSD), primary seed dormancy (PSD), secondary seed dormancy (SSD), thousand kernel weight (TKW) and abscisic acid (ABA) content

dormancy (Nakabayashi et al. 2012). Interestingly, a link seems to exist between the vernalisation response pathway and seed germination. The FLOWERING LOCUS C (*FLC*) is a major regulator of flowering time in dicot species. Results of Chiang et al. (2009) indicate that in *Arabidopsis* natural variation at the *FLC* locus and in *FLC* expression is associated with natural variation in temperature dependent seed germination. *FLC*-mediated germination acts through additional genes in the flowering pathway (*FT*, *SOC1*, and *API*) before involving the abscisic acid catabolic pathway and gibberellic acid biosynthetic pathway in seeds (Kendall et al. 2011). Furthermore, the gene *MFT* (MOTHER OF *FT* AND *TFL1*) has been shown in wheat to be involved in seed dormancy, with high expression leading to increased seed dormancy and reduced expression to pre-harvest sprouting (Nakamura et al. 2011). In *Arabidopsis* *MFT* and *DOG1* expression is correlated with seed dormancy at low soil temperature during winter (Footitt et al. 2011) and *MFT* has been shown to be involved in ABA signalling (Xi et al. 2010).

In conclusion, large variation and moderate to high heritabilities have been found for primary and secondary seed dormancy which indicates that selection of genotypes with low seed dormancy would be effective in a breeding programme. However, selection for low primary seed dormancy may lead to increased pre-harvest sprouting. The in vitro secondary dormancy test is laborious and time-consuming. The development of closely linked or functional molecular markers for low secondary seed dormancy could

help breeding improved cultivars and overcoming the problem of volunteer oilseed rape plants in agricultural production. The developed material and the achieved results will be useful to study in future work genetic interactions between secondary seed dormancy, seed longevity, primary dormancy (pre-harvest sprouting) and vernalisation requirement/flowering time in oilseed rape.

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References

- Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics* 164:711–729
- Bartels PG, Watson CW (1978) Inhibition of carotenoid synthesis by fluridone and norflurazon. *Weed Sci* 26:198–203
- Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. *Proc Natl Acad Sci USA* 103:17042–17047
- Bewley JD (1997) Seed germination and dormancy. *Plant Cell* 9:1055–1066
- Chiang GC, Barua D, Kramer EM, Amasino RM, Donohue K (2009) Major flowering time gene, Flowering locus C, regulates seed germination in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 106:11661–11666
- Feng F, Liu P, Hong D, Yang G (2009) A major QTL associated with preharvest sprouting in rapeseed (*Brassica napus* L.). *Euphytica* 169:57–68
- Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* 171:501–523
- Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* 59:387–415
- Footitt S, Douterelo-Soler I, Clay H, Finch-Savage WE (2011) Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways. *Proc Natl Acad Sci USA* 108:20236–20241
- Gerjts T, Scholefield D, Foulkes MJ, Lenton JR, Holdsworth MJ (2010) An analysis of dormancy, ABA responsiveness, after-ripening and pre-harvest sprouting in hexaploid wheat (*Triticum aestivum* L.) caryopses. *J Exp Bot* 61:597–607
- Gräber K, Linkies A, Müller K, Wunchova A, Rott A, Leubner-Metzger G (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae DOG1 genes. *Plant Mol Biol* 73:67–87
- Gräber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ (2012) Molecular mechanisms of seed dormancy. *Plant, Cell Environ* 35:1769–1786
- Gruber S, Pekrun C, Claupein W (2004) Seed persistence of oilseed rape (*Brassica napus*): variation in transgenic and conventionally bred cultivars. *J Agric Sci* 142:29–40
- Gruber S, Bühler A, Möhring J, Claupein W (2010) Sleepers in the soil—vertical distribution by tillage and long-term survival of oilseed rape seeds compared with plastic pellets. *Eur J Agron* 33:81–88
- Gubler F, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr Opin Plant Biol* 8:183–187
- Gulden RH, Shirliff SJ, Thomas AG (2003) Secondary seed dormancy prolongs persistence of volunteer canola in western Canada. *Weed Sci* 51:904–913
- Gulden RH, Chiwocha S, Abrams S, McGregor I, Kermod A, Shirliff SJ (2004) Response to abscisic acid application and hormone profiles in spring *Brassica napus* seed in relation to secondary dormancy. *Can J Bot* 82:1618–1624
- Hossain S, Kadkol GP, Raman R, Salisbury PA, Raman H (2011) Breeding *Brassica napus* for shatter resistance. PDF document at <http://cdn.intechweb.org/pdfs/25563.pdf> (last access 22 Oct 2012)
- Kendall SL, Hellwege A, Marriot P, Whalley C, Graham IA, Penfield S (2011) Induction of dormancy in *Arabidopsis* summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors. *Plant Cell* 23:2568–2580
- Laga B, Stevens M, Haesendonckx B, Standaert E, Crommar K (2011) A reverse genetics approach allows fine-tuning of seed shatter reduction in canola (*Brassica napus*) to optimal levels. In: Proceedings of the 13th International Rapeseed Congress, Prague, Czech Republic: 905
- Laga B, Den Boer B, Lambert B (2012) Brassica plant comprising a mutant indehiscent allele. United States Patent Application 20120023603. <http://www.freepatentsonline.com/y2012/0023603.html> (last access 22 Oct 2012)
- Lutman PJW, Freeman SE, Pekrun C (2003) The long-term persistence of seeds of oilseed rape (*Brassica napus*) in arable fields. *J Agric Sci* 141:231–240
- Martínez Andújar C, Ordiz MI, Huang Z, Nonogaki M, Beachy RN, Nonogaki H (2011) Induction of 9-cis-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. *Proc Natl Acad Sci USA* 108:17225–17229
- Marzougui S, Sugimoto K, Yamanouchi U, Shimono M, Hoshino T, Hori K, Kobayashi M, Ishiyama K, Yano M (2012) Mapping and characterization of seed dormancy QTLs using chromosome segment substitution lines in rice. *Theor Appl Genet* 124:893–902
- Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D (2008) Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lip Res* 49:1137
- Momoh EJ, Zhou WJ, Kristiansson B (2002) Variation in the development of secondary dormancy in oilseed rape genotypes under conditions of stress. *Weed Res* 42:446–455
- Nakabayashi K, Bartsch M, Xiang Y, Miatton E, Pellengahr S, Yano R, Seo M, Soppe WJJ (2012) The time required for dormancy release in *Arabidopsis* is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *Plant Cell* 24:2826–2838
- Nakamura S, Abe F, Kawahigashi H, Nakazono K, Tagiri A, Matsumoto T, Utsugi S, Ogawa T, Handa H, Ishida H, Mori M, Kawaura K, Ogihara Y, Miura H (2011) A wheat homolog of MOTHER OF FT AND TFL1 acts in the regulation of germination. *Plant Cell* 23:3215–3229
- Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. *Trends Plant Sci* 8:213–217
- Nguyen T-P, Keizer P, van Eeuwijk F, Smeeckens S, Bentsink L (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in *Arabidopsis*. *Plant Phys* 160:2083–2092
- Parkin IAP, Sharpe AG, Keith DJ, Lydiate DJ (1995) Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). *Genome* 38:1122–1131
- Pekrun C, Lutman PJW, Baeumer K (1997a) Germination behaviour of dormant oilseed rape seeds in relation to temperature. *Weed Res* 37:419–431

- Pekrun C, Lutman PJW, Baeumer K (1997b) Induction of secondary dormancy in rape seeds (*Brassica napus* L.) by prolonged imbibition under conditions of water stress or oxygen deficiency in darkness. *Eur J Agron* 6:245–255
- Peters J (2000) Tetrazolium testing handbook. Association of Official Seed Analysts: Handbook on Seed Testing, Contribution 29. <http://www.aosaseed.com>
- Radoev M, Becker HC, Ecke W (2008) Genetic analysis of heterosis for yield and yield components in rapeseed (*Brassica napus* L.) by QTL mapping. *Genetics* 179:1547–1558
- Schatzki J, Allam M, Klöppel C, Nagel M, Börner A, Möllers C (2013) Genetic variation for secondary seed dormancy and seed longevity in a set of black-seeded European winter oilseed rape cultivars. *Plant Breeding* 132:174–179
- Schopfer P, Plachy C (1984) Control of seed germination by abscisic acid. II. Effect on embryo water uptake in *Brassica napus* L. *Plant Physiol* 76:155–160
- Utz HF (2011) PLABSTAT (Version 3A), A computer program for statistical analysis of plant breeding experiments. Institute of Plant Breeding, Seed Science and Population Genetics, University of Hohenheim, Stuttgart, Germany. <https://plant-breeding.uni-hohenheim.de/software.html>
- Xi W, Liu C, Hou X, Yu H (2010) MOTHER OF FT AND TFL1 regulates seed germination through a negative feed back loop modulating ABA signaling in Arabidopsis. *Plant Cell* 22:1733–1748
- Yang J, Hu CC, Ye XZ, Zhu ZH, Zhu ZX, Zhu J (2009) QTL Network-2.1 user manual. Hangzhou: Zhejiang University, China. <http://ibi.zju.edu.cn/software/qtlnetwork/download.htm>
- Zhang W (2008) A comprehensive study on the role of hormones, seed coat and genes during the germination of canola (*Brassica napus*) seed under adverse environmental conditions. PhD thesis, Department of Plant Sciences, University of Saskatchewan, Saskatoon, Canada. <http://library.usask.ca/theses/available/etd-08112008-094956/>. Site visited March 19, 2012