Genetic variation and inheritance of secondary seed dormancy in winter oilseed rape (*Brassica napus* L.)

Genetische Variation und Vererbung von sekundärer Dormanz bei Samen im Winterraps (*Brassica napus* L.)

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"We are what we repeatedly do.
Excellence, therefore, is not an act but a habit."
- Aristotle -

TO
MY FAMILY & FRIENDS

TABLE OF CONTENTS

AIM OF THE PROJECT

The work in this thesis was realised as part of the cooperation project "Confinement strategies for oilseed rape" and was implemented in the research program "Biosafety Research On Genetically Modified Plants" funded by the Federal Ministry of Education and Research (BMBF) in Germany. The occurrence of volunteer oilseed rape emerging from germinating seeds poses a common threat in subsequently cultivated crops due to outcrossing of the unknown genetic quality of the pollen from volunteer oilseed rape. Shed seeds during or at end of oilseed rape cultivation are considerably increasing the soil seed bank and can remain viable in the soil for many years by induction of secondary seed dormancy. A selection for genotypes with a low potential to evolve secondary seed dormancy would therefore represent a most practical and sustainable method to decrease the amounts of volunteer oilseed rape. Furthermore, it limits the risk for unwanted gene dispersal by pollen flow and by seed admixture originating from genetically modified plants.

The major research objectives therefore were:

- to investigate inheritance of secondary seed dormancy in winter oilseed rape
- to discover the importance of genotype, environment & genotype x environmental impact referring to the trait-development of secondary seed dormancy
- to reveal correlations between the trait secondary seed dormancy & other seed traits
- to identify and supply genotypes with low secondary seed dormancy as basic breeding material
- to identify and supply trait-linked molecular markers suitable for further verification and marker assisted selection for low secondary seed dormancy

Since the in-vitro laboratory test had to be established in our laboratory, we took advantage of the possibility to analyse a set of European modern oilseed rape cultivars (see chapter 2) comparable with material tested for secondary seed dormancy by other research groups. A biparental doubled haploid population segregating for secondary seed dormancy was analysed at two different locations with two replicates in two subsequent years to gather the phenotypic data needed for analysis of quantitative trait loci (see chapter 3). The storage protein composition of *Brassica napus* has been associated with germination regulation processes and was therefore examined in connection with the secondary seed dormancy potential (chapter 4).

Chapter 1 - General Background

AGRONOMICAL RELEVANCE OF OILSEED RAPE

In world agriculture, the oil crops sector has been one of the most vivacious in the recent decades. Just in these past 20 years the sector grew at 4.3 per cent per year compared with an average of 2.3 per cent per year for all agriculture (FAO, Statistical yearbook 2012). The most important reason for that increase has been the growth of food consumption in developing countries, mostly in the form of vegetable oil as well as direct consumption of soybeans, groundnuts, etc., but also in the form of derived products other than oil. Additionally, the vast growth in demand for protein products for animal feed has been a great supporting factor in the optimism of the oil crops sector. Moreover, support policies of the European Union (EU) helped shift the world production of oilseeds in favour of oilseed rape and sunflower seed as well. All in all, 168 million tonnes of oilseeds and oil-bearing crops were gathered in 2010 (FAOSTAT 2012, faostat.fao.org) and whereas palm oil fruit is the most important oil-bearing crop in the tropics also increasingly cultivated in Southeast Asia, soybeans, oilseed rape and sunflower are the major oil crops in temperate zones. With 59 million tonnes of worldwide production in 2010 oilseed rape ranges at forths place after soybean, oil palm fruit and coconut while its production in the European Union with an amount of 20 million tonnes is leading amongst the oilcrops (FAOSTAT 2012). For oil production itself it even ranges at third place after palm and soybean oil worldwide and at first place in Europe (FAOSTAT 2012). This enormous development of oilseed rape production and its subsequently processed products was not simply gained through an increase of cultivated area but a huge achievement of quality improvements by breeding throughout the past few decades.

Oilseed rape (*Brassica napus* L.) belongs to the family of Brassicaceae and is originating in a spontaneous bastardisation between cabbage (*Brassica oleracea*) (2n=18) and turnip (*Brassica rapa*) (2n=20) resulting in an allopolyploid hybrid with combined chromosomes from both species (2n=38). The so called Triangle of U, first published by the Japanese Morinaga (1934) and the Korean U (1935) demonstrates the relationship between the three *Brassica* genomes: the A genome from turnip, the B genome from black mustard (*Brassica nigra*, 2n=16) and the C genome of cabbage. Morinaga and U showed by interspecific crosses the possibility of re-synthesising the three amphidiploid species oilseed rape (*Brassica napus*, AACC), Indian mustard (*Brassica juncea*, AABB, 2n=36) and Abyssinian mustard (*Brassica carinata*,

BBCC, 2n=34). The creation of these resynthesised species initially explained their origin on the morphological level but was later also supported by Palmer et al. (1983) in form of molecular biological methods.

Nowadays, Germany is leading in oilseed rape oil production in the European Union with nearly 3 million tonnes in 2010, before France and Poland (FAOSTAT 2012). Although appreciated for its multiple uses as resource for pharmaceuticals, paints and varnishes, industrial oil or recently biofuel, until the early 1970s the value of oilseed rape cultivation for food supply was assessed limited due to its high amounts of erucic acid and glucosinolates (Becker 2011) in oil and meal, respectively. A great success was accomplished by the breeding of so called double low (00, canola) quality oilseed rape first established as standard quality in Germany in 1987 (Röbbelen 1999) and the creation of high oleic acid oilseed rape, based on mutants identified by Rücker and Röbbelen (1995), and low linolenic acid (HOLL). Canola research was concentrated so far on enhancing the seed oil content and improving the oil quality as well as reducing the concentration of undesirable compounds in the meal like e.g. sinapic acid esters (Zum Felde et al. 2006), phytic acid (Lickfett et al. 1999) and fibre content (Dimov et al. 2012). Protein amount and protein composition was only of minor importance in the last decades (Hougen and Stefansson 1983). But since discussions over genetic modified soybeans aroused natively produced European protein sources e.g. oil extracted oilseed rape meal became more and more interesting (Lühs et al. 2001) for it is a valuable feedstuff for animals and a potential protein source for human nutrition (Wanasundara 2011). Therefore not only increasing the total oil and protein amount but also the protein composition of oilseed rape might be an interesting goal for future breeding programs.

STORAGE PROTEINS IN *BRASSICA NAPUS*

The amount of oilseed rape protein besides polysaccharides and lipids ranges from 20 to 25% in the seed. These proteins mainly consist of albumins and globulins (Bhatty et al. 1968, Dalgalarrondo et al. 1986) in oilseed rape most prominently represented by 2 S and 12 S proteins classified due to their sedimentation performance (Raab and Schwenke 1986, Schmidt et al. 2004), the napin and cruciferin, respectively. These two major components of the storage protein amount to 60% and 20-40% of the total protein in mature seeds (Crouch and Sussex 1981, Höglund et al. 1992) respectively.

Cruciferin is a relatively large (~300 kDa) neutral oligomeric globulin which belongs to the cupin (small β-barrel) superfamily (Withana-Gamage 2011). It is a hexameric protein which is similar to 11 to 12 S seed proteins of other species (Rödin et al. 1992). This hexameric pro-

tein is assembled of two trimers, each including three heterogeneous subunits. Each subunit contains two polypeptides, the heavy α- (acidic, 254 to 296 amino acids) and the light β- (basic, 189-191 amino acid residues) chains which are linked by one disulfide bond (Rödin et al. 1992, Withana-Gamage et al. 2011). Three major groups of cruciferin subunits exist (cru1, cru2/3 and cru4), although one of the groups (cru2/3) comprises of two very similar subtypes (cru2 and cru3). By Southern blot analysis the gene copy number was estimated to be 3 to 4 for the cru1 and cru2/3 precursors and 2 for the cru4 precursors (Rödin et al. 1992). Whereas cruciferin functions are limitedly suggested to storage purposes for the reserve accumulation during embryogenesis, it is also known for its great foaming and emulsifying properties (Wu and Muir 2008).

On the contrary napin is a 13 kDa low molecular weight basic 2 S albumin; it consists of a large (9 kDa) and a small (4 kDa) subunit, which are connected via disulfide bonds. Napins are encoded by a multigene-family and the reported copy number varies between 10 (Josefsson et al. 1987) und 16 (Scofield and Crouch 1987) as determined via Southern blot analyses. The remaining proteins are mainly represented by oil body proteins (oleosins, Jolivet et al. 2009) and lipid transfer proteins (Uppström 1995, Malabat et al. 2003). Despite cruciferin, napin with its low molecular weight is associated with antimycotic and antibacterial properties (Polya 2003), ABA interaction (Finkelstein et al. 2002) and calmodulin inhibitory functions (Neumann et al. 1996) as well as a possible role in allergenic response (Monsalve et al. 1997, Müntz 1998, Teuber et al. 1998).

The amino acid composition is in a good balance since its amounts of essential amino acid cope well with the recommendations of the FAO and WHO (Rozan et al. 1997). Bos et al. (2007) pointed out that additional to its nutrient value for human consumption oilseed rape protein also shows a digestibility similar to milk protein. In other plant protein fractions used for feedstuff purposes cereals are lacking lysin and in small amounts threonine and thryptophan whereas in legumes the sulphur consisting amino acids are found to be insufficiently (Habben and Larkins 1995, Schwenke 1982), whereas oilseed rape protein has an considerable amount of lysine and is rich in sulphur containing amino acids like cysteine and methionine (Downey and Bell 1990, Schöne 1993). But besides these storage proteins also other complex building or reactive secondary compounds are found in the seeds mostly with inhibitory functions e.g. lectins, trypsin inhibitors, tannins, sinapins, glucosinolates or phenolic compounds (Fenwick et al. 1983, Kroll et al. 2007). Especially the isothiocyanates originating from degradation of glucosinolates are extremely reactive with proteins resulting in new protein functionality (Kroll et al. 2007). Therefore under perspective of a proper utilisation of

oilseed rape protein not only the amounts apparent in the seed had to be taken into account, but also the subsequently processing steps of oil extraction and meal processing to protein concentrates or isolates. In this context also seed hull and fibre content and therefore also the breeding of yellow seeded oilseed rape could be beneficial for the use of protein rich oilseed rape meal (Dimov et al. 2012)

DORMANCY

Although quality properties of the oilseed rape have been the most aimed-at breeding goals over the last decades, sustainability and biosafety matters shifted the field of research towards other not so prominent properties of the crop. One of these more recently important features is the secondary seed dormancy (in the following chapters also secondary dormancy, SD if not otherwise indicated) potential of oilseed rape. Seed dormancy is defined as the failure of viable seeds to germinate, even under conditions that favour the normal growth and development of the seedling (Baskin and Baskin 2004). On the contrary, the failure of viable seeds to germinate under conditions that are unfavourable for normal growth and development of the seedling, usually because of low moisture contents is defined as quiescence (also known as enforced dormancy, Harper 1957; pseudodormancy, Karssen 1995). So quiescent seeds simply lack some environmental factors necessary for successful germination whereas viable seeds that are in an environment optimal for germination (including optimal water, temperature, light and oxygen) and nonetheless fail to complete germination are characterised as dormant seeds and the phenomenon is named dormancy.

Categories of Dormancy

A simple classification for seed dormancy was defined by Harper (1957), Nikolaeva (1977) and Baskin and Baskin (1985) based on the time at which dormancy occurs. Primary dormancy is the state where germination of the progeny is prevented while maturing on the mother plant and for a certain time after the seed has separated from the plant (Hilhorst and Toorop 1997). To relieve primary dormancy an “after-ripening” phase in form of seed desiccation is usually required (Baskin and Baskin 1998). Therefore secondary dormancy is defined as the prevention of germination at any time after the drying of the seed, in some cases also prior to the alleviation of primary dormancy.Whether primary or secondary seed dormancy are regulated by the same mechanisms and simply represent the same issue, hitherto remains unclear. Baskin and Baskin (2004) suggested due to complexity of the phenomenon, it is of utmost importance for seed scientist to agree on an internationally acceptable hierarchical classification system for seed dormancy. In the past lots of publications lacked the indication on which

kind of dormancy the results explain for and so Baskin and Baskin (2004) suggested a modified version of the scheme by Nikolaeva (1977 and 1999) in three hierarchical categories: class, level and type. Their system included five classes of dormancy: physiological dormancy (PD), morphological dormancy (MD), morpho-physiological dormancy (MPD), physical dormancy (PY) and combinational dormancy (PY + PD). *Brassica napus* seeds are endospermless (Schopfer and Plachy 1984, Schopfer et al. 2001) and testa rupture and initial embryo development are therefore visual markers for completion of germination (Finch-Savage and Leubner-Metzger 2006). Since MD is evidentially dependent on the embryo to seed ratio (E : S, Forbis et al. 2002) and small E : S with therefore small embryo sizes determine the potential for MD it is most unlikely, that oilseed rape should be affected by MD or MPD. PY and therefore also PY + PD are characterised by water impermeability of the seed or fruit coat and believed to be an adaptation of the plant to specialised life habitats (Baskin and Baskin 2004). For that reason we hereby focus on the PD which is also the most common kind of seed dormancy (Baskin and Baskin 2004). PD can be divided into the levels: deep, intermediate and non-deep mainly depending on their needs for stratification. The great majority of seeds have non-deep PD. However, in seeds of many species, dormancy is not an all or nothing stage in the plant's life cycle. Seeds of most species with non-deep physiological dormancy undergo a series of temperature-driven changes in their capacities for physiological responses to various factors between dormancy and non-dormancy (Baskin et al. 1998, Probert 2000) Baskin and Baskin (2004) therefore propose 5 stages of a cycle from primary dormant to non-dormant. A reversed change from a non-dormant seed stage therefore consequently leads to a stage of secondary dormancy. But although the aim for a superior classification system for seed dormancy could prove useful, dormancy regulations in certain species yet remain partly unknown.

Relevance of seed dormancy in oilseed rape

Seed dormancy is described as a survival strategy of wild type species to resist unfavourable environmental conditions (Radosevich et al. 1997). Since domestication of crop plants mainly lead to changes in traits related to floral and seed morphology an impact on seed dormancy was to be expected (Alonso-Blanco et al. 2009). Since genetic variation among modern winter oilseed rape cultivars is considered to be narrow (Bus et al. 2011) and a previous, indirect selection for high germination rates can be suspected, a decrease in seed dormancy potential of modern cultivars would have seemed likely. Nevertheless the emergence of unwanted oilseed rape poses a common threat due to outcrossing of unwanted pollen and as a to the cul-

tivar competitive weed. Before and during harvest pod shattering of oilseed rape leads to considerable amounts of dispersed seeds. In adverse harvest conditions seed losses can reach up to 10.000 seeds per m^2 (Lutman 1993, Gruber et al. 2005, Lutman et al. 2005). Under favourable conditions shed seeds germinate directly, but under unfavourable conditions those seeds may become secondary dormant. These seeds can remain viable in the soil for a period of 10 years and longer (Lutman et al. 2003) and consequently lead to an increase of the soil seed bank (Gruber et al. 2010). Under subsequently favourable conditions those dormant seeds may germinate and occur as a weed or so called volunteer oilseed rape in succeeding crops for several years (Pekrun et al. 1997ab, Gruber et al. 2004). Primary dormancy in oilseed rape is low (Lutman 1993, Momoh et al. 2002) whereas secondary dormancy might be of bigger agronomical interest especially under the consideration of persistence of genetically modified oilseed rape. Regulation of seed dormancy is influenced by several different abiotic factors like light, temperature, oxygen, water potential and additionally seed age and storage conditions. Furthermore growth regulating phyto hormones, seed size and importance of the genetic component are discussed.

Factors affecting dormancy

LIGHT

Light of a specific wavelength tend to be a relevant factor for secondary dormancy induction in seeds of many species (Casal and Sánchez 1998). Already imbibed *Brassica napus* seeds can develop light sensitivity when they are exposed to environments with a low osmotic potential under simultaneous exclusion of red light (660 nm) or darkness (Pekrun et al. 1997a). This principle is used to artificially induce and break seed dormancy in the laboratory (Pekrun et al. 1997a, Gruber et al. 2004), since the developed light sensitivity by involvement of phytochromes is reversible (Casal and Sanchez 1998) by application of either dormancy inducing far-red light or dormancy releasing red light. Nevertheless germination itself in *Brassica napus* does not seem to require light (Schopfer and Plachy 1984).

TEMPERATURE

The effect of temperature interacts significantly with other environmental and soil conditions and therefore cannot be treated as an independent factor. Increasing the temperature benefits after-ripening of air-dry seeds and also affects the secondary dormancy induction rate in imbibed seeds (Baskin and Baskin 1984). Pekrun et al. (1997ab) observed the induction of secondary dormancy at low temperature whereas in contrast, Landbo and Jorgensen (1977) found little or no dormancy at all in some cultivars of *Brassica napus* and *B. rapa* at low tempera-

tures. These inconsistencies were explained by the large cultivar differences in the development of secondary dormancy (Squire 1999). At high temperature in the soil, seedlings may die from the heat or enter into dormancy at low soil water potential (Zhou and Kristiansson 2000, Momoh et al. 2002).

Whereas the influence of temperature on induction of dormancy might be contrary the breaking of secondary dormancy by alternating temperature has been shown in many studies (Pekrun et al. 1997a, Baskin and Baskin 1998, Momoh et al. 2002).

ANOXIA

In the soil anoxia and water deficiency are partly related, since water sufficiency can easily lead to anoxia in the soil. Nevertheless in deeper soil layers seeds might face both anoxia and water deficiency from resulting osmotic pressure. Several publications revealed the increased induction of secondary dormancy via anoxia caused by light sensitivity in imbibed seeds in darkness (Pekrun et al. 1997b, Momoh et al. 2002). The induction of secondary dormancy is most likely a survival mechanism to overcome anaerobic conditions (Honěk and Martinkova 1992) and prolonged anaerobic conditions can lead to loss in viability.

WATER POTENTIAL

Under field conditions the soil water potential determines the final seed water potential since all seeds, except those with water impermeable seed coat, imbibe when apparent to water. Several studies have reported on the effect of low soil water potential on seed germination and the following influence on induction of secondary dormancy (Pekrun et al. 1997a, Zhou and Kristiansson 2000, Momoh et al. 2002). As previously described, temperature and soil water potential appears to be the predominant factors that regulate the annual cycling of dormancy. Osmotical stress combined with darkness greatly decreased germination and was reported to efficiently induce secondary dormancy, which led to an establishment of a widely used in vitro test for the estimation of secondary dormancy (Pekrun et al. 1997ab, Momoh et al. 2002, Dongus et al. 2003, Gulden et al. 2004c).

PHYTO HORMONES

The influence of phyto hormones especially gibbelleric acid (GA) and abscisic acid (ABA) on seed dormancy has been widely studied and proven for several species (Schopfer and Plachy 1984, Koorneef et al. 2002). Gulden et al. (2004a) reported that a high dormant *Brassica napus* genotype contained more endogenous ABA compared to a low dormant genotype after induction of secondary dormancy. In addition to ABA and GA, a third plant hormone, ethylene, is involved in the regulation of seed dormancy and germination. Ethylene breaks dor-

mancy and/or stimulates germination in the seeds of many species (Kępczyński and Kępczyńska 1997), apparently by lessening the sensitivity of the seed to endogenous ABA. Thus, ethylene may support germination by interfering with the action of ABA (Beaudoin et al. 2000).

SEED SIZE

In *Brassica* an influence to seed dormancy by seed size has also been reported. (Dongus et al. 2003, Gulden et al. 2004a) however the general idea is that large seeds present some advantage over small ones due to viability and rate of germination (Hampton et al. 2004). On the contrary Komba et al. (2007) recently reported that seeds of kale (*Brassica oleracea* L.) graded in large, medium, small and very small by different size screens and tested for germination and vigour did not support the hypothesis that large seeds had superior performance compared to small seeds.

GENOTYPIC DIFFERENCES IN THE DEVELOPMENT OF DORMANCY

Despite domestication of oilseed rape, a lot of variation could be detected for secondary seed dormancy by several studies for instance with ranges from 3 to 76% (Gruber et al. 2004), 0 to 80% (Momoh et al. 2002) or 44 to 82% (Gulden et al. 2004). Nevertheless these studies had its limitations in small genotype numbers or few testing locations or years. An influence of the genotype therefore is likely but has not been sufficiently proven. The reason for that is mostly owed to the fact that the in-vitro test for the estimation of secondary seed dormancy is extremely time consuming. Most recently Weber et al. (2010) therefore optimised the procedure of the in-vitro test which could prove extremely beneficial for further investigations on that topic.

Additional key information about the control of germination may come from the study of natural allelic variation at loci linked to dormancy and germination. Quantitative trait loci (QTL) mapping approaches for *A. thaliana* (Alonso-Blanco et al. 2003, Koornneef et al. 2004), *Brassica oleracea* (Finch-Savage et al. 2005) and cereals (Koornneef et al. 2002) are being used to identify germination and dormancy-related genes. With the DOG-mutants (delay of germination) identified in *Arabidopsis* ecotypes (Tonsor et al. 2005, Bentsink et al. 2007) QTL analysis including the deep dormant ecotype Cvi (Cape Verde Islands) and the low dormant ecotype Ler (Landsberg erecta) led to identification of at least seven QTL for seed dormancy (DOG1-7). For DOG1 the Cvi-allel increases dormancy and explains 12% of the phenotypic variation in the RIL (recombinant inbred line) population. The DOG1-gene is the first cloned dormancy QTL and codes for an essential dormancy gene with yet unknown function. DOG1-

homolog sequences are known from a *Brassica napus* EST (expressed sequence tag; CN827162, amino acid-identity globally 37.8%, locally (206 amino acids) 53.4%) and, with lower homology, from wheat (Leubner-Metzger 2007). No QTL identification for secondary seed dormancy in *Brassica napus* has been reported so far.

QTL MAPPING

Understanding the factors responsible for secondary dormancy induction might contribute to practices to avoid them but are of less practical use when they are not completely understood. QTL analyses of cereal crop dormancy is one way to identify genes that underlie these physiological problems (Koornneef et al. 2002, Gubler et al. 2005) and enables the understanding of trait correlations on the genetic level.

The regions within genomes that contain genes associated with a certain quantitative or complex trait are known as quantitative trait loci. QTL mapping is only possible by creation of a map of previously identified polymorphic markers that have been screened across the entire mapping population, as well as the parents. By analysing the segregation of markers, the relative order and distances between markers can be defined. This implies that a low recombination frequency between two markers results from a close placement on a chromosome. This marker segregation analysis results in coding data for each DNA marker on each individual of a population, called marker map. Including this marker map and the phenotypic data of the individual genotypes QTL mapping can be executed by use of special mapping programs, calculating the most likely position, whereas the distance along a linkage group or chromosome is defined in terms of the recombination frequency between genetic markers (Paterson 1996). Widely used methods for identifying QTL are single marker analysis, simple interval mapping (SIM) and composite interval mapping (CIM) (Liu 1998, Tanksley 1993). SIM is defined by simultaneous interval analysis of neighbouring pairs of linked markers along chromosomes and is considered statistically more powerful than single marker analysis (Lander and Botstein 1989). Nevertheless, CIM has become more popular for mapping QTL in combining interval mapping with linear regression and statistically including additional markers to the neighbouring pair of linked markers (Zeng 1994). When positions for QTL are estimated, the resulting maximum or peak has to surpass a specified significance threshold. The estimation of the thresholds is most commonly performed by including permutation tests (Churchill and Doerge 1994). These tests involve a certain number of repeats (e.g. 500 or 1000) where the false positive marker-trait associations are estimated by randomisation of the phenotypic data with simultaneously constant marker values of the genotypes (Hackett 2002).

QTL mapping is considered a useful tool for identification of trait linked markers and their inclusion in marker assisted breeding (also marker assisted selection, MAS) programs, although reliability of markers and costs-effectiveness might be limiting and lead to consideration on a case by case basis (Dreher et al. 2003).

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Chapter 2 - Genetic variation for secondary seed dormancy in a set of current European winter oilseed rape cultivars

2.1 ABSTRACT

Secondary seed dormancy in oilseed rape is a phenomenon that allows seeds to survive in the soil for many years without germination. Following soil cultivation, dormant seeds may germinate in subsequent years and they are the reason for the occurrence of volunteer oilseed rape plants in successive crops. Genetic reduction of secondary dormancy in oilseed rape could provide a mean to reduce the frequency of volunteer plants and the dispersal of pollen from emerging volunteer oilseed rape. However, little is known about the genetic variation for and the environmental influence on secondary seed dormancy of current winter oilseed rape cultivars. The aim of the present study was to analyse secondary seed dormancy in a set of 28 current winter oilseed rape cultivars tested in 2008/2009 in field experiments at six different locations in Germany. Bulked seeds samples obtained from open pollinated plants from 4 replicates were used for the analysis of secondary seed dormancy, applying an in vitro laboratory test. In the ANOVA highly significant effects were found for the influence of the locations and for the variation among the genotypes. Among the genotypes, secondary seed dormancy ranged from 8% to 56% and its heritability was high with 0.97. The overall means for secondary dormancy of the cultivars at the six locations ranged from 12% to 38%. The large genetic variation and the high heritability indicate that an effective breeding for reduced secondary seed dormancy could be performed.

2.2 INTRODUCTION

Before and during harvest of oilseed rape considerable amounts of seeds may be lost due to pod shattering. In adverse harvest conditions seed losses can reach up to 10.000 seeds per m^{-2} (Lutman 1993, Gruber 2004, Lutman et al. 2005). Shed seeds germinate directly under favourable conditions, but under unfavourable conditions those seeds may become secondary dormant. Secondary dormant seeds remain viable in the soil for a period of 10 years and longer (Lutman et al. 2003) and lead to an increase of the soil seed bank (Gruber et al. 2010 and references therein). 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, Pekrun et al. 1997b). Secondary seed dormancy occurs in spring oilseed rape under Canadian growing conditions (Gulden et al. 2003, Fei et al. 2009) and as well in winter oilseed rape under European growing conditions (Gruber et al. 2010). Volunteer oilseed rape plants may occur in such high numbers that herbicide application is required to prevent yield losses. They also represent a problem, if the seed quality is changed e.g. from high erucic acid to low erucic acid or from 00-quality to high oleic acid low linolenic acid quality (HOLL). In this case quality problems may occur due to the admixture with seeds harvested from those volunteer plants but also due to cross pollination during the flowering period. The longevity of oilseed rape seeds in the soil also is a problem if dispersal of the transgenic crop via seeds and pollen is an issue (Begg et al. 2006, Colbach et al. 2008). In the literature several factors are mentioned to possibly trigger secondary seed dormancy, e.g. temperature (Pekrun et al. 1997a), light (Bazanska and Lewak 1986, López-Granados and Lutman 1998), moisture (Bewley 1997, Pons 1991), seed age and anaerobic conditions (Momoh et al. 2002). However, Gulden et al. (2004) reported that the total variation for secondary seed dormancy detected among 16 genotypes was caused predominantly by the genotype, followed by seed size and different harvest regimes. In the laboratory, secondary seed dormancy can be conveniently induced by incubating the seeds in a polyethylene glycol (PEG) solution for two to four weeks in darkness (Gruber et al. 2004 and 2009, Gulden et al. 2004). 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).

Although the amount of oilseed rape seeds becoming secondary dormant can be reduced by applying the correct tillage system after harvest (Gruber et al. 2010 and Gruber and Claupein 2006, 2009), genetically reducing the capacity of oilseed rape cultivars to produce secondary dormant seeds appears attractive. Both efforts the appropriate tillage system and the use of

cultivars with a low capacity to produce secondary dormant seeds together could provide a mean to effectively reduce soil seed banks and hence seed and pollen dispersal of transgenic modified and conventionally bred oilseed rape cultivars. In a previous study, a large genotypic variation for seed oil and protein content as well as for seed size, seed hull proportion and seed fibre content was detected in a set of current European winter oilseed rape cultivars tested in field experiments at six different locations in Germany (Dimov et al. 2012). The objective of the present study was to analyse the genetic variation and inheritance for germination rate and secondary seed dormancy in the same set of seed samples from these cultivars and to study correlations to the previously recorded seed traits.

2.3 MATERIALS AND METHODS

2.3.1 Plant material and field experiments

The seed material consisted of 28 double low quality winter oilseed rape cultivars (Tab. 3). The material was tested in 2008/09 at 15 locations in Germany (Bundes- und EU-Sortenversuch 1 Winterraps, Gronow et al. 2009). Field experiments were conducted as a randomised complete block design with 4 replicates for each cultivar at each location. Seed samples were taken after combined harvesting of the yield plots. Samples from the 4 replicates of each cultivar at each location were equally mixed and used for Near-Infrared-Reflectance-Spectroscopy (NIRS) analysis. Based on the mean oil content of the seed samples of the locations, seed samples from locations with a low oil content (Langenstein, Ihinger Hof), an intermediate oil content (Hohenschulen, Futterkamp) and a high oil content (Mollenfelde, Sophienhof) were chosen for the analysis of secondary dormancy. For more details about the cultivars and locations see Gronow et al. (2009).

2.3.2 Seed germination rate

The germination rate (GR) was determined on 2x 100 seeds per genotype and location. Therefore filter papers (MN618 with 85 mm radius, 0.32 mm thickness and 140 g/m^2 weight (Macherey-Nagel GmbH, Düren, Germany) were put in plastic petri dishes (92x 16 mm, Sarstedt AG & Co., Nürnbrecht, Germany) and 6 mL of deionised water was added. 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 rate was determined after 1, 5 and 14 days and number of germinated seeds was summed up to calculate the germination rate in per cent (%).

Then, for testing the viability (data not shown) 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 seven days under alternating light and temperature conditions (12 hours darkness at 5 °C and 12 hours light at 25 °C). Germinated seeds were counted after 3 and 7 days and number of completely germinated seeds was summed up as viable seeds.

2.3.3 Induction of secondary seed dormancy

The test for secondary seed dormancy (SD) was performed in December 2009 essentially following the protocol described by (Gruber et al. 2004). Before initiating the laboratory test, impurities and broken seeds were removed from the seed lots. The dormancy test was performed with 2x 100 seeds per genotype and location. For dormancy induction the same filter papers as indicated above were put in plastic petri dishes and 8 mL of a polyethylenglycol (PEG) solution with a concentration of 354.37 g/L (AppliChem GmbH, Darmstadt, Germany) were added. The freshly prepared PEG 6000 solution had an osmotic potential of -15 bar (Gruber et al. 2004) equal to the permanent wilting point. 100 seeds per petri dish were equally dispersed on the soaked filter paper and petri dishes were closed with the corresponding lid. All treatments were performed in a climate chamber at a temperature of 18 °C and under green light. Green light filters were obtained from the Göttinger Farbfilter GmbH (Bovenden-Lenglern, Germany). Care was taken to prevent any other light entering the growth chamber. Petri dishes were collected and stored for two weeks in the same climate chamber in cardboard boxes, carefully wrapped with black plastic foil to protect them from light.

2.3.4 Viability testing of secondary dormancy induced seeds

Viability of the secondary dormancy induced seeds was determined in three consecutive steps. At first seeds were rinsed in the petri dishes with 6 mL distilled water to dilute the PEG solution. Then the seeds were dispersed in a new petri dish onto a new filter paper soaked with 6 mL deionised water. After two days incubation in darkness, germinated seeds with a radicle longer than 2 millimeter were counted as viable and were discarded. The remaining seeds were incubated again and two days later germinated seeds were counted and discarded again. The remaining seeds were rinsed in 6 mL deionised water and were dispersed in a new petri dish onto a new filter paper soaked with 6 mL of deionised water. Ten days later the number of germinated seeds was finally counted. Counting, rinsing and transfer of seeds to new petri dishes were performed under green light. In between, petri dishes with seeds were stored in the cardboard boxes in darkness.

Then, 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 seven days under alternating light and temperature conditions (12 hours darkness at 5 °C and 12 hours light at 25 °C). Germinated seeds were counted after 3 and 7 days and number of germinated seeds was summed up. Finally, seeds that at this point still had not germinated (although in contrast to the DH population

these numbers were inconsiderably low) 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 consisted of the added number of viable seeds determined in the three steps.

2.3.5 Calculation of the secondary dormancy rate

The viable seeds that did not germinate in deionised water in darkness after the dormancy induction were considered to be secondary dormant, i.e. the sum of viable seeds minus the number of seeds germinated in the first step of the viability test. The frequency of dormant seeds (SD) was calculated as: SD (%) = (viable seeds - germinated seeds) * 100 / viable seeds.

2.3.6 Thousand kernel weight

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

2.3.7 Near Infrared Reflectance Spectroscopy

Seed samples of about 3 g were scanned with a NIRS monochromator model 6500 (NIRSystems, Inc., Silversprings, MD, USA). Spectra were recorded between 400 and 2498 nm, registering the absorbance values log (1/R) at 2 nm intervals for each sample. Oil, protein and moisture content were determined using the calibration raps2009.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel). Oil and protein content are expressed in % at seed dry matter basis. Protein content of the oil-extracted meal (% at seed dry matter) was calculated by using the seed oil and protein content data obtained from NIRS prediction.

2.3.8 Statistics

Analysis of variance (ANOVA) and calculation of heritabilities (h^2) were performed by using PLABSTAT software (Utz 2011) considering the locations as random. For secondary dormancy and germination rates ArcSin-transformed data were used due not being normally distributed. Mean values of the genotypes across the locations were used to calculate Spearman's rank correlation coefficients between traits.

2.4 RESULTS

The analysis of variance showed highly significant effects of the locations and the genotypes on secondary seed dormancy of 28 winter oilseed rape cultivars as determined by the in vitro test (Tab. 1). Highly significant effects of the locations and the genotypes were also found on thousand kernel weight (TKW), seed oil and protein content (Tab. 1), and protein content of the defatted meal. Comparatively large variance components were detected for the effect of the genotypes on secondary dormancy and thousand kernel weight, whereas large effects of the locations on oil and protein content were observed. A large effect for the genotype x location interaction on germination rate was found. Heritability was high for all traits investigated, except for germination rate.

Tab. 1: Variance components and heritabilities for secondary seed dormancy (SD, %), germination rate (GR, %), thousand kernel weight (TKW, g), for oil and protein content of the seed (in % seed dry matter) and for protein content in the defatted meal (prot idM, in % seed dry matter) of 28 current winter oilseed rape cultivars tested in field experiments at 6 locations

Source of vaiance	SD	GR	TKW†	oil†	protein†	protein idM†
location (L)	49.2**	0.43+	0.05**	6.9**	5.22**	6.21**
genotype (G)	125.5**	4.22**	0.10**	0.9**	0.36**	1.14**
G x L	25.6	10.83	0.04	0.6	0.33	0.53
heritability	0.97	0.70	0.94	0.90	0.87	0.93

**, + Significant at P = 1%, 10% (F-test, ANOVA), idM in defatted meal,

† data taken from Dimov et al. 2012

The secondary seed dormancy rate and the oil content of the seeds averaged over the genotypes varied considerably between the locations, ranging from 12% for Langenstein up to 38% for Sophienhof (Tab. 2). There was a positive relation between seed dormancy rate and oil content and a negative relation to protein content. In contrast to the secondary dormancy rate there were only minor differences in the germination rate between the locations.

Tab. 2: Mean values for secondary seed dormancy (SD, %), germination rate (GR, %), thousand kernel weight (TKW, g), for oil and protein content of the seed (in % seed dry matter) and for protein content in the defatted meal (prot idM, in % seed dry matter) of 28 winter oilseed rape cultivars tested in field experiments in 2008/2009 at six different locations.

Location	SD	GR	TKW†	oil†	protein†	protein idM
Langenstein	12.3	98.5	4.3	44.5	22.6	40.6
Ihinger Hof	20.8	98.3	4.4	46.0	22.0	40.7
Hohenschulen	23.4	97.8	5.0	49.3	18.7	36.8
Futterkamp	30.6	98.2	4.6	49.7	18.1	35.9
Mollenfelde	32.8	98.8	4.7	50.6	18.0	36.3
Sophienhof	37.7	97.5	4.6	50.9	17.1	34.8
LSD5%	4.0	1.0	0.1	0.41	0.30	0.39

LSD5% least significant difference at P=5%, idM in defatted meal,

† data taken from Dimov et al. 2012

Among the 28 cultivars there was a large variation for secondary dormancy which ranged from 8% for cultivar 'Iwan' to 56% for cultivar 'DK Secure' (Tab. 3). Compared to this the variation in the germination rate was with 94.0 to 99.6% rather narrow. A large variation was also found for thousand kernel weight, seed oil and protein content and protein content in the defatted meal.

Among the different traits recorded there was no significant correlation between secondary seed dormancy of the genotypes and their germination rate, thousand kernel weight, oil-, protein- and protein in the defatted meal content (see Tab. 4). There was also no correlation to the seed fibre content (NDF, ADF and ADL, seed hull proportion) of those cultivars (data taken from Dimov et al. 2012). For germination rate the only significant positive correlations were observed towards the protein- and the protein in the defatted meal content (0.45^{*} and 0.50^{**} respectively). Oil and protein content of the seeds were significantly negative correlated (-0.42^{*}).

Tab. 3: Secondary seed dormancy (SD, %), germination rate (GR, %), thousand kernel weight (TKW, g), oil and protein content of the seed (in % seed dry matter) and protein content in the defatted meal (prot idM, in % seed dry matter) of 28 European winter oilseed rape cultivars tested at 6 locations in 2008/2009

cultivar	type	SD	GR	TKW†	oil†	protein†	protein idM
'DK Secure'	Hzk	55.5	99.4	4.2	46.8	20.1	37.6
'NK Caravel'	H	54.8	98.6	4.5	46.8	19.8	37.2
'PR45D01'	Hzk	54.1	97.5	4.5	47.8	19.0	36.3
'Zeppelin'	H	51.2	98.8	4.4	49.6	19.3	38.3
'Arcadia'	L	49.4	98.8	4.1	48.0	19.4	37.2
'Monolit'	L	48.6	97.1	5.0	49.5	18.1	35.7
'Cuillin'	H	41.2	97.7	4.5	48.6	19.9	38.6
'Tassilo'	H	35.3	99.1	4.5	47.9	20.0	38.4
'Limone'	H	33.2	98.6	4.7	48.5	19.2	37.1
'PR44W22'	H	32.9	98.6	4.5	49.1	19.2	37.6
'Lorenz'	L	28.2	97.3	4.4	50.4	18.4	37.0
'Elektra'	H	27.8	98.8	4.9	48.0	19.4	38.0
'PR44W18'	H	26.6	98.8	4.7	48.3	19.3	37.4
'Azur'	L	25.9	98.3	4.7	49.1	19.1	37.4
'NK Pegaz'	L	17.9	95.6	4.7	48.3	18.5	35.7
'Bellevue'	L	17.1	99.6	5.2	48.6	19.9	38.8
'NK Morse'	L	16.5	98.3	4.6	48.6	18.1	35.1
'Visby'	H	13.9	97.1	5.0	47.6	19.3	36.8
'NK Aviator'	H	13.5	99.1	4.0	47.3	19.9	37.7
'Adriana'	L	11.5	99.0	5.2	49.7	19.2	38.0
'Katabatic'	L	11.4	99.0	4.5	50.0	19.2	38.3
'Hybrisurf'	H	11.0	99.3	4.4	48.8	19.9	38.8
'Loveli CS'	L	10.4	94.0	4.5	49.8	20.2	40.1
'ES Alienor'	L	10.4	97.2	5.2	47.8	19.5	37.2
'Exotic'	H	10.2	99.6	5.1	46.6	20.9	39.0
'DK Cabernet'	L	9.9	98.3	4.2	48.5	18.7	36.2
'Safran'	H	9.2	98.1	4.4	47.4	19.9	37.7
'Iwan'	L	8.1	97.8	4.7	48.9	19.7	38.5
mean	-	26.3	98.2	4.6	48.5	19.4	37.5
min	-	8.1	94.0	4.0	46.8	18.1	35.1
max	-	55.5	99.6	5.2	50.4	20.9	40.1
LSD5%	-	8.6	2.1	0.2	0.9	0.7	0.8

H, Hybrid cultivars; L, Line cultivars; Hzk, semidwarf hybrid cultivars; idM in defatted meal; LSD5% least significant difference at P=5%; † data taken from Dimov et al. 2012

Tab. 4: Spearman-rank correlation coefficients for Secondary seed dormancy (SD), germination rate (GR), thousand kernel weight (TKW), oil- and protein content of the seed, protein content in the defatted meal (prot idM), seed hull proportion (SH, in % of seed dry matter), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL, all in % of defatted meal)

	SD	GR	TKW†	oil†	protein†	protein idM	SH†	NDF†	ADF†
GR	0.07								
TKW†	-0.16	-0.09							
oil†	-0.10	-0.20	0.09						
protein†	-0.17	0.45*	-0.14	-0.42*					
protein idM	-0.27	0.50**	-0.00	0.16	0.77**				
SH†	0.25	-0.01	-0.16	-0.35	-0.24	-0.44*			
NDF†	0.19	-0.01	-0.30	-0.13	-0.33	-0.35	0.77**		
ADF†	0.12	0.06	-0.23	-0.00	-0.26	-0.18	0.66**	0.81**	
ADL†	0.00	0.14	-0.05	-0.07	-0.14	-0.12	0.56**	0.60**	0.84**

**, * significant at P=1%, 5%;

†Original data were taken from Dimov et al. (2012) to calculate Spearman's rank correlation

2.5 Discussion and Conclusion

This study revealed a large and significant variation for secondary seed dormancy among current winter oilseed rape cultivars ranging from less than 10% to well over 50%. This range confirms results obtained in earlier studies for winter oilseed rape (Pekrun et al. 1997b, Momoh et al. 2002, Gulden et al. 2003, Gruber et al. 2004, Gulden et al. 2004). In the study of Gruber et al. (2004) a variation from 3 to 76% were detected in a set of 32 winter oilseed genotypes. The secondary dormancy rate of hybrid cultivar 'Elektra' was analysed by Gruber et al. (2004) and in the present study and was found with 26.1%, respectively 27.8% to have an intermediate capacity to form secondary dormant seeds. Although there was no difference between inbred line cultivars and hybrid cultivars with respect to their secondary dormancy level, it is interesting to note that both semi-dwarf hybrids were among the genotypes with the highest secondary dormancy level. Taken into account that a selection for secondary seed dormancy has never been done in oilseed rape breeding and that genetic variation among modern winter oilseed rape cultivars is considered to be narrow (Becker et al. 1995, Hasan et al. 2006, Bus et al. 2011) the detected large variation is rather surprising. The high heritability of 0.97 indicated that the variation was caused predominantly by the genotypes. A predominant contribution of the genotypes to the overall variation in secondary seed dormancy was also reported by Gulden et al. (2004) for a set of 16 winter/spring oilseed rape genotypes.

Beside the strong genetic component, the analysis of variance (Tab. 1) showed also a large and significant effect of the location on secondary seed dormancy. The mean values over the locations ranged from 12.3 to 37.7% (Tab. 2). This was not surprising since the 6 locations were selected among 15 locations based on large differences in mean seed oil content (Tab. 2). Seeds harvested from locations with high oil contents and with high yield levels (cf. Dimov et al. 2012) clearly had higher secondary seed dormancy rates. This could indicate that optimal conditions during plant growth and maturation do not only increase seed yield and oil content but also the capacity to form secondary dormant seeds. On the other hand, suboptimal conditions during maturation or a too early harvest may not only affect seed yield and oil content but may lead to a reduced capacity to form secondary dormant seeds. Fei et al. (2007) studied gene expression in maturing seeds of different cultivars in relation to their potential for induction of secondary dormancy and found few differences at the mature stage but a significant number at the transition stage from full-size embryo to mature seed. In this context, it is noteworthy that the seed samples analysed in the present study were derived from yield plots of field experiments and represent a mixture of seeds derived from the main inflorescence as well as from secondary, tertiary etc. racemes. At harvest those seeds may be in

different maturation stages and hence may show differences in their secondary dormancy capacity.

Although there was a clear positive relation between seed oil content and the secondary seed dormancy rate of the seeds harvested at different locations (Tab. 2) no correlation between the oil content and the secondary dormancy rate of the genotypes was found (Tab. 4). There was also no significant correlation to the other recorded traits, indicating that an indirect selection for a low secondary seed dormancy capacity will not be feasible. The results are in contrast to those obtained by Gulden et al. (2004), who reported an influence of seed size on the secondary seed dormancy rate. For the seed samples analysed in the present study Dimov et al. (2012) reported large and significant genotypic differences for seed hull proportion and seed fibre content (NDF, ADF, ADL). The results from this study are also not supporting evidence from *Arabidopsis* (Debeaujon 2000) that the seed coat may influence the capacity to form secondary dormant seeds. However, a lack of correlation to those traits may simply be caused by the composition of the plant material used in this study. The analysis of a segregation population derived from a cross between lines with different seed hull and fibre content would yield more meaningful results.

The large differences in the capacity to form secondary dormant seeds raises the question whether these differences might be related to differences in seed longevity found among genotypes stored under ambient or cold conditions in the genebank (Nagel and Börner 2010). Seeds from *Brassica* spp. typically lose 50% of their viability within 7.3 years of storage at 20 °C and 50% relative humidity (Nagel et al. 2011), and within 23 years under standard low temperature (-18 °C) storage conditions (Walters et al. 2005). Artificial ageing tests may be applied to the seeds (Nagel et al. 2011) to determine differences among genotypes. Work is currently in progress to determine the differences in seed longevity of the seed samples analysed in this study by applying an artificial ageing test (A. Börner, personal communication).

In conclusion, applying an in vitro test the present study revealed large differences between winter oilseed rape genotypes in the capacity to form secondary dormant seeds and a high heritability for the trait. Hence selection of genotypes with low seed dormancy should be effective in a breeding programme. However, performing the in vitro test for secondary dormancy is laborious and time consuming and it is unlikely that this test will be integrated in a practical breeding program. So far, results do not indicate that an indirect selection for low seed dormancy can be performed applying simpler tests for other traits.

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Chapter 3 - Mapping of QTL for secondary seed dormancy in a winter oilseed rape doubled haploid population

3.1 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 seed 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 mean to reduce the frequency of volunteer plants and the dispersal of pollen from emerging volunteer oilseed rape. The objective of the present study was to analyze the inheritance of secondary seed dormancy in a winter oilseed rape doubled haploid population consisting of 229 genotypes derived from a cross 'Express 617' x 'R53' and to study correlations to other seed traits. Field experiments were performed in Germany in two years at two locations with two replicates. Seeds harvested from open pollinated plants were used for all analyses, including a laboratory test for secondary seed dormancy. A previously developed molecular marker map of the doubled haploid population was used to map QTL of the relevant traits. The results showed significant effects of the genotypes and their interactions with years and locations, respectively on secondary seed dormancy. Heritability was 0.85. Five QTL for secondary seed dormancy were detected which together explained 42% of the phenotypic variance. Results show that secondary seed dormancy is a heritable trait and that selection for low secondary seed dormancy is possible.

3.2 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 at harvest is a desired trait in crop species because it prevents the early germination of mature grains 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 in cereal breeding programs (Gubler et al. 2005, Gerjets et al. 2010, Marzougui et al. 2012) and in some regions of the world also in oilseed rape breeding programs (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 phyto hormones 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 the oilseed rape crop, because before and during harvest of oilseed rape considerable amounts of seeds may be lost due to pod shattering (Hossain et al. 2011, Laga et al. 2011 and 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). 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. 1997ab). Secondary dormancy can be conveniently induced in vitro by incubating seeds in polyethylene glycol solution for a period of 2 to 4 weeks in darkness (Pekrun et al. 1997ab, Momoh et al. 2002). 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 (see chapter 2 and references therein). Genetic reduction of secondary seed dormancy in oilseed rape could provide a mean to reduce the frequency of volunteer oilseed rape plants and especially the undesired dispersal of seeds and pollen from transgenic oilseed rape. The objective of the present study was to analyze the inheritance of secondary seed

dormancy and of the abscisic acid content in the seed of the winter oilseed rape doubled haploid population 'Express 617' x 'R53', and to estimate correlations to other seed traits.

3.3 MATERIAL AND METHODS

3.3.1 Plant material and field experiments

The seed material was obtained from 229 doubled haploid (DH) lines, which were cultivated in field experiments with two replicates in the two consecutive years 2008/09 and 2009/10 at the two locations Göttingen and Thüle in northwestern Germany. The DH population 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 *B. oleracea* var. *sabellica* (kale) and *B. rapa* ssp. *pekinensis* (chinese cabbage, see Radoev et al. 2008). 'R53' had high erucic acid content in the seed oil and high glucosinolate content in the seed. The parental genotypes 'Express 617' and 'R53' were cultivated together with the DH population in the field. The DH population was expected to show a great variation for the trait ‘secondary seed dormancy’, because the two parental lines revealed in a previous test a distinct 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 years 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 analysis. For abscisic acid (ABA), erucic acid and Dumas based combustion method analysis 3 g of seeds from each of the four replicates of a genotype and field year were pooled.

3.3.2 Test for secondary seed dormancy and germination rate

The test for secondary seed dormancy (SD) and the estimation of the germination rates were performed as previously described in Chapter 2.3 essentially following a protocol described by Gruber et al. (2004). For germination rate calculation three countings were conducted on 2x 100 seeds per genotype whereas seeds evolving a radicle greater than 2 mm within 14 days were rated and summed up as germinated seeds. The seed samples were analysed replicate-wise starting with the two replicates from Göttingen. The dormancy induction tests (DI) were conducted 3, 4, 7 and 8 weeks after harvest in 2009 and 6, 7, 10 and 11 weeks after harvest in 2010. A tetrazolium test for viability was added to the protocol as described in Chapter 2.3.4 for germination test without induction and dormancy induction test, respectively.

3.3.3 Thousand kernel weight

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

3.3.4 Determination of abscisic acid (ABA) by HPLC-MS/MS

10 g from each of the mixed seed samples (see 3.3.1) were ground in a coffee mill model Krups F203 for 6 seconds (3 times 2 seconds 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 the internal ABA standard. Each sample was extracted with 0.75 mL methanol, 2.5 mL methyl-tert-butyl ether and 20 ng D_6-ABA (CDN Isotopes Inc., Quebec, Canada as internal standard) and covered with a layer of argon. After one hour 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. All three combined extracts were dried and the pellet was re-suspended in 100 µL acetonitrile and dried again. The final pellet was put back into suspension with 100 µL acetonitrile/water/acetic acid (20:80:0.1, v/v/v). Analysis of constituents was performed mainly following a protocol by (Pan et al. 2008) 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). Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (type TriVersa NanoMate, Advion BioSciences, Ithaca, USA). Reversed-phase HPLC separation was performed on EC 50/2 Nucleodure C18 gravity 1.8 µm column (50 x 2 mm, 1.8 µm particle size, Macherey and Nagel, Düren, Germany). ABA values were expressed in pmol/g fresh matter.

3.3.5 Other seed quality traits

Oil content and protein content (% at 91% DM), glucosinolate content (GSL, µmol/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 oil-extracted meal (% at 91% DM) was calculated by using the seed oil and protein content data obtained from NIRS prediction. For reference Erucic acid content was determined by gas chromatography after harvest in 2009 and 2010 following a standard protocol resulting in a Spearman's rank correlation coefficient of 0.89^{**} towards the NIRS-values. NIRS based

predictions for seed protein content showed a high Spearman rank correlation of 0.93** with the N-determined protein contents analysed by the Dumas based combustion method (Jung et al. 2003, Kirsten and Grunbaum 1955) using a CN Elemental analyzer, type vario EL by Elementar Analysensysteme GmbH (Donaustraße 7, D-63452 Hanau; http://www.elementar.de) as described in the manual (Anonymous 1992). Factor 5.7 was used to calculate the protein content from the nitrogen content (Kroll et al. 2007 and references therein). For all 22:1 and protein contents shown only NIRS-values were used.

3.3.6 Statistics

Analysis of variance and calculation of heritabilities (h^2) were performed by using PLABSTAT software (Utz 2011) considering the years, locations and replicates as random. For ABA-values originating only of mixed seed samples each year only the years were considered as random. For secondary seed dormancy and germination rates 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 significant at P=10, 5 and 1%, respectively

3.3.7 Molecular marker 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 genetic distance of all markers covering 21 linkage groups was 9.5 cM calculated with Kosambi equation. QTL mapping was performed using the freely accessible software QTL Network 2.1 (Yang et al. 2009) with a significance level of P=5% for the QTL. A permutation test was performed (1000 permutations) to determine the critical F value threshold for each trait. Epistatic interactions among the loci were also assessed by using QTL Network 2.1 whereas the estimated effect represents the additive × additive genetic interaction. Major QTL are defined with explaining more than 10% of the phenotypic variation.

3.4 RESULTS

The analysis of variance for the trait secondary seed dormancy resulted in significant effects of the genotypes but not for the locations and the years (Tab. 1). However, interactions between genotypes and years, genotypes and locations as well as the threefold interaction were significant. Variance components were comparatively large for the genotypes and heritability was high. Large and significant effects of the genotypes were also found for the germination rate, thousand kernel weight, oil, protein and ABA content of the seeds as well as for protein content of the defatted meal. The effects of the year were high for secondary seed dormancy, germination rate and ABA content but low for thousand kernel weight (TKW), oil and protein content. High heritabilities were also determined for germination rate, TKW, oil and protein content of the seeds as well as for the protein content in the defatted meal. Low and intermediate heritabilities were detected for germination rate and for ABA content of the seeds. 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 secondary seed dormancy was detected among the genotypes which ranged from 5 to 96% with a mean of 60% (Tab. 2). The parental genotype 'Express 617' showed as expected with 30.5% a comparatively much lower secondary seed dormancy rate than 'R53' with 64.4%. The mean of the secondary dormancy rate of the DH population was nearly as high as the dormancy rate of 'R53'. Thereby transgressive segregation was observed with 108 genotypes having a higher secondary dormancy rate than 'R53' (64%) and 30 genotypes having a lower secondary dormancy rate than 'Express 617' (31%, Fig. 1). Large variations were also found for all other traits (Tab. 2). Although the two parental genotypes showed only a minor difference in the seed germination rate, a large and transgressive segregation was observed for this trait in the DH population. A large and transgressive segregation was also found for ABA content of the seeds. Figures 1 and 2 show the frequency distribution for the traits secondary seed dormancy and germination rate of the DH population. Both frequency distributions were clearly skewed towards higher values. In addition, the frequency distribution of the secondary dormancy rate indicated at 65-60% a possible separation of the genotypes in a group with lower dormancy and a group with higher dormancy. This indication of a class separation remained even when the class limits were shifted by 1 to 2% (not shown).

Tab. 1: Variance components and heritabilities for secondary seed dormancy (SD, %), germination rate (GR, %), thousand kernel weight (TKW, g), for oil and protein content of the seed and for protein content in the defatted meal (prot idM, in % at 91% DM), for glucosinolate content (µmol/g FM seeds), for erucic acid content (22:1, % of the oil) and for ABA content (pmol/g FM seed meal) of the DH population tested in field experiments in 2 years at two locations with two replicates

source of variance	SD†	GR†	TKW	oil	protein	prot idM	GSL	22:1	ABA††
year (Y)	221.6	29.2^{+}	0.01	-1.36	-1.21	-1.42	13.99	1.86^{+}	434.0**
location (L)	82.6	11.5	0.00	-1.36	-1.26	-1.55	-3.69	1.00	-
genotype (G)	208.3**	15.0**	0.18**	2.31**	0.73**	1.45**	235.20**	79.48**	302.9**
G x Y	33.6**	12.4**	0.02**	0.29**	0.16**	0.18**	4.36**	1.92**	243.9††
G x L	13.6**	4.6**	0.00	0.00	0.01	0.05	-0.16	-0.03	-
G x Y x L	22.6**	5.5**	0.03**	0.04	0.09*	0.24**	3.04*	0.58	-
residual	51.9	21.2	0.09	1.22	0.81	1.05	29.47	11.09	-
h^2	0.85	0.54	0.86	0.88	0.78	0.84	0.97	0.97	0.71

$^{+, *, **}$ denotes significant at P=10, 5 and 1%;

† ArcSin transformed data were used

†† data for each genotype and year were obtained from pooled samples of the 2 locations, G x Y includes residual

Tab. 2: Min, max and mean values for secondary seed dormancy (%), germination rate (%), thousand kernel weight (TKW, g), for oil and protein content of the seed and for protein content of the defatted meal (prot idM, in % at 91% DM), for erucic acid content (22:1, % of the oil) and for ABA content (pmol/g FM) of the DH population and the parental lines tested in field experiments in 2 years at two locations with two replicates

	SD	GR	TKW	oil	protein	prot idM	GSL	22:1	ABA[†]
min	4.7	73.3	3.8	39.3	17.7	32.9	13.9	0.0	25.7
max	96.2	99.9	6.4	47.7	23.5	40.1	74.7	31.1	149.0
mean	59.9	96.0	4.9	43.5	20.6	36.3	37.4	13.3	62.6
LSD5% (G)	25.7	10.3	0.5	1.6	1.3	1.5	7.2	4.4	30.8
'Express 617'	30.5	99.7	5.4	45.7	18.0	33.1	26.7	0.0	47.2
'R53'	64.4	96.8	4.7	42.6	20.6	35.9	41.8	15.9	102.1

LSD5% = least significant difference at P=5%

[†] data for each genotype and year were obtained from pooled samples of the 2 locations

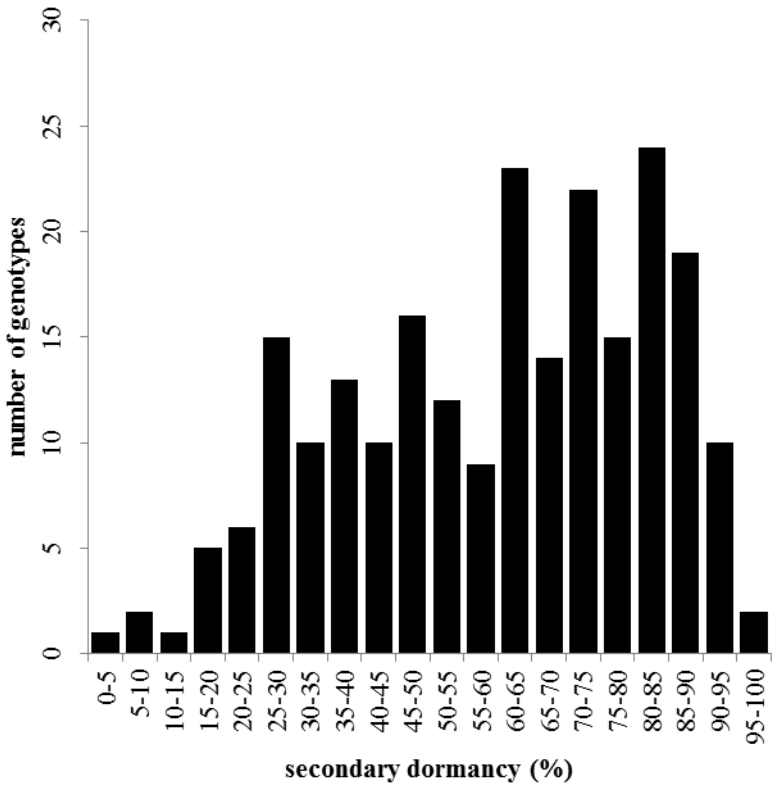

Fig. 1: Frequency distribution of 229 doubled haploid winter oilseed rape lines for secondary seed dormancy (means of 2 years, 2 locations and 2 replicates; non-transformed data)

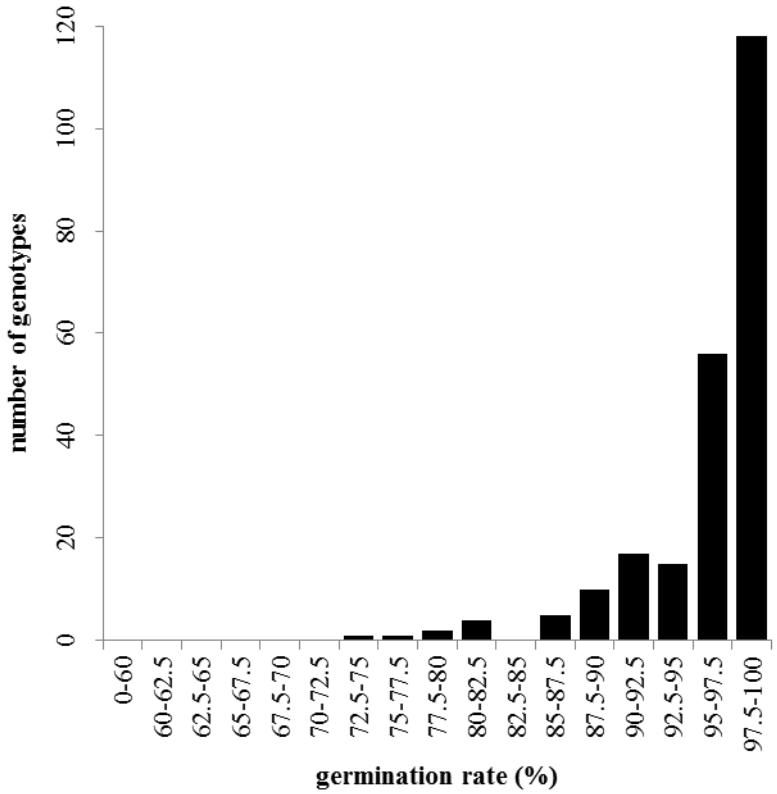

Fig. 2: Frequency distribution of 229 doubled haploid winter oilseed rape lines for germination rate (means of 2 years, 2 locations and 2 replicates; non-transformed data)

Secondary seed dormancy was significantly negative correlated with germination rate and glucosinolate content and positive with ABA content (Tab. 3), but only the correlation to the germination rate was close. However, the scatter plot (Fig. 3) revealed an unusual distribution, which indicated that selection for a high germination rate would not have an effect on the selection of genotypes with a low secondary dormancy rate. Vice versa a selection for low germination rate would be effective for selection of genotypes with a high secondary seed dormancy rate. There was no close correlation of secondary dormancy rate to the other seed quality traits.

Tab. 3: Spearman-rank correlation coefficients for secondary seed dormancy, germination rate and other seed traits

GR	-0.71**	-	-	-	-	-	-	-
TKW	-0.08	0.06	-	-	-	-	-	-
oil	0.10	0.02	-0.13*	-	-	-	-	-
protein	-0.13	0.03	0.03	-0.66**	-	-	-	-
prot idM	-0.10	0.08	-0.08	-0.06	0.77**	-	-	-
GSL	-0.14*	0.10	0.16*	-0.39**	0.36**	0.13*	-	-
22:1	0.09	-0.02	-0.16*	0.63**	-0.12	0.38**	-0.09	-
ABA	0.14*	0.02	-0.09	0.00	-0.09	-0.12	-0.02	-0.01
	SD	GR	TKW	oil	protein	prot idM	GSL	22:1

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

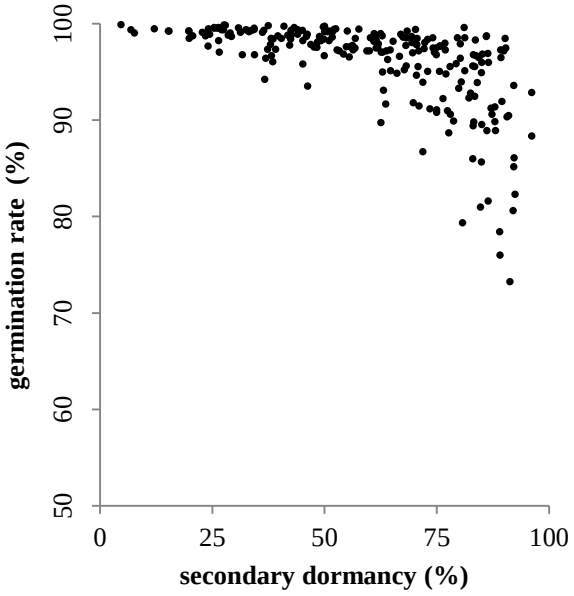

Figure 1: Scatter plot of secondary seed dormancy rates associated with germination rates of 229 doubled haploid winter oilseed rape lines (means of 2 years, 2 locations and 2 replicates)

QTL mapping led to the identification of 5 QTL for secondary seed dormancy, which together explained 42% of the phenotypic variance (Tab. 4). The QTL for SD 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 SD-1 of linkage group N5 the substitution of the 'R53' allele by an 'Express 617' allele led to a reduction of the secondary seed dormancy rate of 6.13%. Hence, the results show that in three out of five cases, the 'Express' allele led to a reduction in secondary seed dormancy. Only in the case of QTL SD-3 and SD-5 the 'Express 617' allele led to an increase of the secondary seed dormancy rate. QTL for germination rate, thousand kernel weight, and abscisic acid content were also identified (Tab. 4 and Fig. 4 and 5) some of whose positions and confidence intervals overlapped with the confidence intervals of the QTL for secondary seed dormancy (Tab. 4, Fig. 4 and 5). However, the proportions of the explained phenotypic variance of the QTL were comparatively low and ranged from 15% for ABA content to 19% for germination rate. An epistatic effect between QTL with main effects was observed only for QTL for thousand kernel weight.

Tab. 4: Mapped QTL and their most likely positions for secondary seed dormancy (SD), germination rate (GR), thousand kernel weight (TKW) and abscisic acid (ABA)

QTL	LG	position [cM]	CI [cM]	a	h² (a)	V(A)/V(P)	V(I)/V(P)	V(G)/V(P)
SD-1	N5	65.4	41.5-79.7	-6.13	0.08	0.42	-	0.42
SD-2	N13	196.4	190.4-205.1	-8.90	0.11			
SD-3	N15	89.5	82.3-97.2	6.14	0.10			
SD-4	N18	89.7	79.3-101.4	-6.41	0.09			
SD-5	N19	78.5	72.5-83.5	4.68	0.06			
GR-1	N13	194.4	173.6-214.1	1.11	0.05	0.19	-	0.19
GR-2	N18	97.4	85.7-103.4	1.80	0.14			
TKW-1	N5	92.3	76.7-97.7	0.21	0.11	0.18	0.03	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; the substitution of the 'R53' allele by an 'Express 617' allele leads to an increase of the trait when positive, 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,

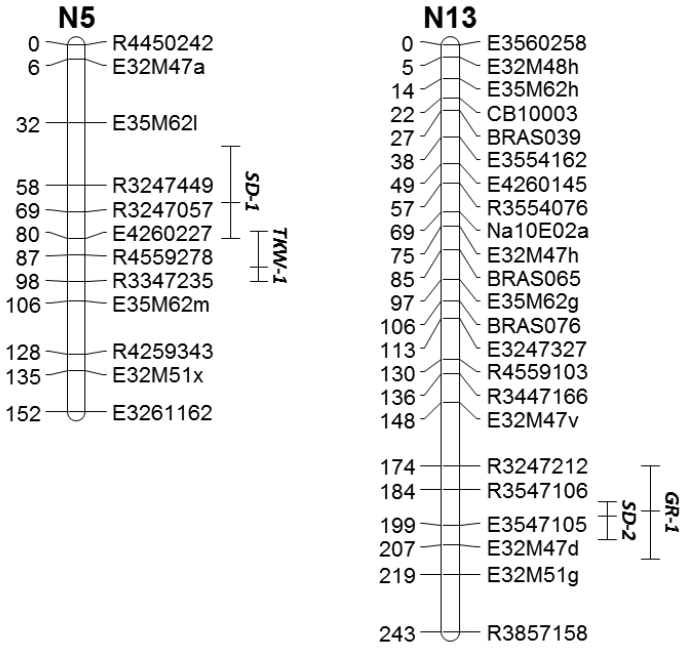

Fig. 4: Linkage groups N5 and N13 showing relevant QTL for secondary seed dormancy (SD), germination rate (GR) and thousand kernel weight (TKW)

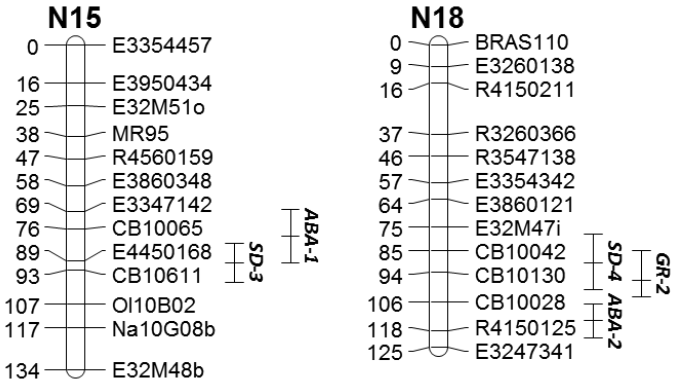

Fig. 5: Linkage groups N15 and N18 showing relevant QTL for secondary seed dormancy (SD), germination rate (GR) and abscisic acid (ABA) content

3.5 DISCUSSION AND CONCLUSION

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 solution (Pekrun et al. 1997ab, Momoh et al. 2002, Gulden et al. 2003 and 2004). Gruber et al. (2004) found that in a set of 32 winter oilseed rape genotypes secondary seed dormancy ranged from 3 to 76%. In another previous study (see chapter 2) a variation from 8% to well over 50% in a set of 28 winter oilseed rape cultivars tested in field experiments in six contrasting locations was reported. The parental genotypes used in this study for the development of the DH population showed with 31% ('Express 617') and 64% ('R53') rather intermediate secondary dormancy levels (see Tab. 2). However, a large transgressive segregation was found in the DH population derived from F1 plants of the cross 'Express 617' x 'R53'. Secondary seed dormancy levels as low as 5% and as high as 96% 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 rate of the DH population (see Tab. 3) may be explained by epistatic interactions leading to lower secondary dormancy in either one or both parental lines. The dormancy values appear quite reliable, because they represent mean values from two years of field experiments at two locations with each two replicates. The heritability for secondary dormancy was with 0.85 at a similar high level as the heritability for oil and protein content. A hitherto not reported unexpected large variation was also found for the seed germination rate (Tab. 2). Spearman rank correlations revealed a close negative correlation between secondary seed dormancy and germination rate (-0.71^{**}, Tab. 3), which is in contrast to the results from the previous study (see previous chapter 2). However, the xy-scatterplot of the data revealed that a selection for high seed germination rate would not be effective for the identification of genotypes with low secondary seed dormancy. It is likely that the genotypes with a low seed germination rate may still have high primary seed dormancy, since the mean viability for the genotypes as determined with the tetrazolium test ranged from 98-100% (data not shown). This is contrary to the marginally primary dormancy rates reported by Gruber et al. (2004) in 32 freshly harvested oilseed rape cultivars but might be owed to the plant material used in this study. However similar results were obtained in a set of 28 current winter oilseed rape cultivars (see chapter 2) with a germination rate range of 94-100% indicating low primary dormancy. No correlation was found between thousand kernel weight (TKW) and second-

ary seed dormancy rate and between TKW and germination rate respectively, which is in agreement with the results from the previous study (see chapter 2).

Surprisingly, only a loose positive correlation between ABA content of the seeds and their capacity to produce secondary dormant seeds was found (Tab. 3). This finding is partly in contrast to the bunch of published literature which indicates a role of ABA in seed dormancy induction and maintenance (Finch-Savage and Leubner-Metzger 2006, Finkelstein et al. 2008 and references therein). The lack of a closer relationship between ABA content and secondary seed dormancy may be explained by the fact that in the present study ABA content of mature dry seeds was determined. However, ABA synthesis may be induced during the incubation 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 or a high dormancy potential genotype. In their study they also examined the influence of applications of exogenous ABA and the herbicide fluridon. And whereas application of ABA had no significant effect on the genotypes germination after two weeks of osmotic stress, the application of fluridon vastly increased the germination of the high dormant genotype. Fluridone prevents the biosynthesis of ABA by inhibiting the production of a carotenoid which is a precursor for ABA synthesis (Bartels and Watson 1978). Nevertheless, this data is supported by experiments between wild-type and ABA-deficient mutants of *Arabidopsis* (Karssen et al. 1983, Koornneef and Karssen 1994, Nambara and Marion-Poll 2003), tomato (Groot and Karssen 1992, Hilhorst 1995) and *Nicotiana plumbaginifolia* (Frey et al. 1999) reporting that only ABA produced by the embryo itself throughout seed development is needed to enforce an enduring dormancy. 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. It is not clear whether maternal ABA can penetrate the embryonic axis, but it is known to affect aspects of seed development other than dormancy (Finkelstein 1994, Koornneef and Karssen 1994). Since seeds of oilseed rape are endospermless the function of ABA in germination and dormancy processes might differ from other species. So ABA does not inhibit testa rupture but the subsequent radicle growth (Schopfer and Plachy 1984) in *Brassica napus*. 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 which together explained 42% of the phenotypic variance (Tab. 4). At three of the five QTL, the 'Express 617' allele led to a reduction in secondary seed dormancy and only at QTL SD-3 and SD-5 the 'Express 617' alleles led to an enhancement of secondary seed dormancy. This finding is consistent with the large transgressive segregation observed for this trait in the DH population. The positive sign of the additive effect of the QTL ABA-1 is consistent with the positive sign of the QTL SD-3 which again is consistent with the general assumption that a higher ABA content leads to a higher secondary dormancy rate. However, in the case of linkage group N18 the sign of the additive effect of the QTL ABA-2 is different from that of the QTL SD-4 for secondary seed dormancy, which is not in agreement with the above mentioned general idea. But in this case the confidence intervals of the QTL ABA-2 and SD-4 did not overlap, suggesting that different loci are involved in the expression of those two traits.

Supporting to the close negative correlation found between secondary dormancy rate and germination rate QTL SD-2 on linkage group N13 and QTL SD-4 on linkage group N18 showed well overlapping confidence intervals with the QTL for germination rate GR-1 and GR-2, respectively (see Fig. 4 and 5). Together these QTL explained phenotypic variance of 20% for dormancy and 19% for germination of a totally explained phenotypic variance of 42% and 19% for all QTL (see Tab.4), clarifying the strong negative correlation on the QTL level. Furthermore, on linkage group N15 the confidence interval of the QTL SD-3 overlapped with the confidence interval of the QTL ABA-1 and on linkage group N18 the QTL ABA-2 mapped nearby the QTL SD-4. Overlapping confidence intervals of different QTL indicate that either a gene located in this region has a pleiotropic effect on two or more traits or that more than one gene is located in the QTL region, each affecting a different trait.

Despite germination rates in modern oilseed rape cultivars prove to be already sufficiently high (Chapter 2) the results in this study might partly explain why on the other hand variation for secondary dormancy in modern oilseed rape remains surprisingly high. We detected QTL for secondary dormancy that are putatively unlinked from germination rate loci and are responsible for a relatively high secondary seed dormancy rate in the parental cultivar 'Express 617'. The markers identified in the corresponding confidence intervals might be valuable for marker assisted selection for future breeding programs aiming for low dormancy potential in *Brassica napus*.

Further work is in progress to analyse the seed samples from this field experiments for their seed fibre content, i.e. NDF, ADF and ADL (Suprianto et al. 2011). Results from the analysis of the seed samples from the first year field experiment showed that there are significant dif-

ferences for NDF, ADF and ADL content of the genotypes of the DH population. After completion, results will show to which extent the different fibre fractions are involved in the inheritance of secondary seed dormancy.

3.6 ACKNOWLEDGEMENTS

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Chapter 4 - Mapping of QTL for the seed storage proteins cruciferin and napin in a winter oilseed rape doubled haploid population and their inheritance in relation to other seed traits

4.1 ABSTRACT

Canola-type oilseed rape (*Brassica napus* L.) is an economically important oilseed crop in temperate zones. Due to its oil composition of the seed the canola protein shows potential as a value-added food and nutraceutical ingredient. The two major storage protein groups occurring in oilseed rape are the 2 S napins and 12 S cruciferins. The aim of the present study was to analyse the genetic variation and the inheritance of napin and cruciferin content of the oilseed rape seed protein in the winter oilseed rape DH population 'Express 617' x 'R53' and to determine correlations to other seed traits previously determined in this population. Seed samples obtained from open pollinated plants from two locations in Germany with two replicates were bulked for the napin and cruciferin analysis resulting in data for 229 genotypes at two consecutive years (2009 and 2010). A previously developed molecular marker map of the DH population was used to map QTL of the relevant traits. The results indicated highly significant effects of the year and the genotype on napin and cruciferin content as well as on the ratio of napin to cruciferin. Heritabilities were comparatively high with 0.79 for napin and 0.77 for cruciferin. Napin and cruciferin showed a significant negative correlation (-0.36^{**}). A negative correlation between secondary dormancy and napin contents (-0.19^{**}) was also detected although the QTL of these traits showed no overlapping confidence intervals. Three QTL for napin and two QTL for cruciferin were detected together explaining for 47% and 35% of the phenotypic variance of their corresponding trait respectively. We also observed a close genetic relation between some of these QTL and the major QTL for glucosinolates content explaining 72% of its phenotypic variance. So that despite napin and cruciferin seem to be heritable traits and selection for low napin or cruciferin is possible the breeding value for feed purposes might be lessened due to this close relation with glucosinolate levels in the seed.

4.2 INTRODUCTION

The meal of Canola-type oilseed rape (*Brassica napus* L.) with a low glucosinolate content in the seed and a low erucic acid content of the seed oil is a valuable feedstuff for animals and a potential protein source for human nutrition (Leckband et al. 2002, Wanasundara 2011) as well as for other non-food uses (Malabat et al. 2001). The oil extracted meal contains about 35 to 40% protein (Dimov et al. 2012). Canola research was focused so far on enhancing the seed oil content and improving the oil quality as well as reducing the concentration of undesirable compounds in the meal like e.g. sinapic acid esters (Hüsken et al. 2005, Zum Felde et al. 2006), phytic acid (Lickfett et al. 1999) and fibre content (Bell 1993, Wittkop et al. 2009, Dimov et al. 2012).

The seed storage protein of oilseed rape is composed mainly of cruciferin and napin which account for 60% and 20%, respectively, of the total protein in mature seeds (Crouch and Sussex 1981, Höglund et al. 1992). The remaining proteins consist mainly of oil body proteins (oleosins, Jolivet et al. 2009) and lipid transfer proteins (Uppström 1995, Malabat et al. 2003). Napin is a 13 kDa low molecular weight basic 2 S albumin, it consists of a large (9 kDa) and a small (4 kDa) subunit, which are connected via disulfide bonds. Napins are encoded by a multigene-family and the reported copy number varies between 10 (Josefsson et al. 1987) und 16 (Scofield and Crouch 1987) as determined via Southern blot analyses. Cruciferin is a comparatively large (300 kDa), neutral oligomeric 12 S-Globulin which belongs to the cupin (small β-barrel) superfamily (Withana-Gamage 2011). It is a hexameric protein which is similar to 11 to 12 S seed proteins of other species (Rödin et al. 1992 and references therein). The hexameric protein is assembled of two trimers, each comprising three heterogeneous subunits. Each subunit consists of two polypeptides, the heavy α- (acidic, 254 to 296 amino acids) and the light β- (basic, 189-191 amino acid residues) chains that are linked by one disulfide bond (Rödin et al. 1992, Withana-Gamage et al. 2011). Three major groups of cruciferin subunits exist (cru1, cru2/3 and cru4), although one of the groups (cru2/3) consists of two very similar subtypes (cru2 and cru3). By Southern blot analysis the gene copy number was estimated to be 3 to 4 for the cru1 and cru2/3 precursors and 2 for the cru4 precursors (Rödin et al. 1992).

The amino acid composition of the oilseed rape protein is excellently balanced (Sosulski 1979) with perhaps only a slightly limited amount of lysine (Kohno-Murase 1995). With 3.0 to 4.0% of sulphur-containing amino acids, the oilseed rape protein is closer to FAO recommendations for humans than any other available vegetable protein (Ohlson and Anjou 1979). Napin has a higher content of the sulphur amino acids cysteine and methionine and of lysine than cruciferin. Hence, reported differences in the amino acid content between oilseed rape

genotypes may be indicative for different contents of napin and cruciferin (Malabat et al. 2003). The amino acid composition of the seed storage protein is relevant when the meal is used in animal feeding diets (Kohno-Murase et al. 1995). Genetically modifying the composition of the seed storage proteins therefore should directly affect the amino acid composition. In a transgenic antisense approach oilseed rape plants were obtained that totally lacked napin as determined by polyacrylamide gel electrophoresis. In those plants reduced napin synthesis was counterbalanced by an increased cruciferin synthesis so that the total protein content of the seeds remained unchanged (Kohno-Murase et al. 1994). In a follow up complementary antisense approach the level of cruciferin was reduced which led to an increased napin level and enhanced contents of cysteine, methionine and lysine. As before, the total seed protein content did not change. Malabat et al. (2003) investigated to what extend the conversion from glucosinolate and erucic acid containing cultivars to Canola quality affected the seed storage protein composition. They observed that the former varieties tended to have higher contents of napin and reduced contents of cruciferin. The implications of the changed protein composition of the seeds on traits like germination, primary and secondary dormancy (see chapter 2 and 3), seed longevity in the soil (Nagel et al. 2011) and fungal disease resistance (Terras et al. 1992, Barciszewski et al. 2000) has not been investigated.

There is an increasing commercial interest to investigate and to control the genetic variation of the seed storage protein composition of oilseed rape, because napin and cruciferin have quite different functional properties which makes them attractive for a number of applications in food and non-food production (Wu et al. 2008, Wanasundara 2011).

The aim of the present study was to analyse the genetic variation and the inheritance of napin and cruciferin content of the oilseed rape seed protein in a winter oilseed rape doubled haploid population and to determine correlations to other seed traits previously determined in this population (see chapter 3).

4.3 MATERIAL AND METHODS

4.3.1 Plant material and field experiments

The seed material was obtained from 229 doubled haploid (DH) lines, which were tested in field experiments with two replicates in 2008/09 and 2009/10 at two locations (Göttingen and Thüle) as described in chapter 3.3.1. Seed samples obtained from open pollinated plants from the two locations and the two replicates were bulked for the napin and cruciferin analysis (see also Chapter 3.3.1).

4.3.2. Protein extraction

10 g from each of the mixed seed samples were ground in a coffee mill model Krups F203 for 6 seconds (3 times 2 seconds with in between mixing of the meal). From each sample 100 mg of oilseed rape meal was weighed in a 1.5 mL tube and mixed with 1000 µL ultra-pure water for 10 seconds and incubated for 3 minutes in a thermo mixer (model Thermomixer 5436, Eppendorf AG, Barkhausenweg 1, D-22339 Hamburg) with 1000 rpm at 30°C and finally sonicated for 2 minutes at 30 °C. Subsequently the samples were centrifuged for 10 minutes at 14000 rpm (model Micro 200, Andreas Hettich GmbH & Co.KG, Föhrenstr.12, D-78532 Tuttlingen) at 4 °C in the cooling chamber and 500 µL of the interphase was transferred to a new 1.5 mL tube and centrifuged again. 400 µL of the interphase was transferred to a new 1.5 mL tube and was frozen for further use. 2 weeks before conducting the gel electrophoresis the protein extracts were centrifuged with 14000 rpm for 5 minutes at 4 °C and mixed (1:1) with a Laemmli sample buffer (125 mM Tris/HCl, pH 6.8; 20% (v/v) Glycerol; 4% (w/v) SDS; 0.1% (w/v) bromphenol blue; 5% (v/v) ß-mercaptoethanol) and aliquots of 10 µL were frozen for final use. A lyophilisate of purified napin provided by Dr. Klaus Duering (Axara Consulting, Auf dem Rotental 47, D-50226 Frechen) was solved in ultra-pure water, mixed (1:1) with Laemmli sample buffer and frozen until final use.

4.3.3. SDS-PAGE

Frozen protein samples, purified napin standard (0.5 µg/µL) and an unstained molecular weight marker (14.4-116 kDa, Lot No.: 00068297, expiry date: May 2012) (Fermentas GmbH**,** Opelstraße 9, D-68789 St. Leon-Rot) were gently thawed on ice and subsequently boiled at 95°C in the thermo mixer with 1000 rpm and centrifuged at 14000 rpm for 5 minutes. From each sample 2 µL were put onto precast 4-15% gradient gels (model Mini-PROTEAN© TGX™, Bio-Rad Laboratories GmbH, Heidemannstr. 164, D-80901 München) with 15 wells running with constant 60V for 140 minutes with Laemmli buffer system in a

Mini-PROTEAN© chamber. Afterwards the gel was stained with a 0.04% Coomassie blue (PlusOne Coomassie tablet PhastGel™ Blue R-350, GE Healthcare Deutschland, Oskar-Schlemmer-Str. 11, D-80807 München), 40% (v/v) ethanol, 10% (v/v) acetic acid solution for 30 minutes and washed twice (2x1 minute) with distilled water. De-staining was performed for 30 minutes with 20% (v/v) ethanol and 10% (v/v) acetic acid and washed once with distilled water for one minute before the gels were put into de-staining solution with 10% (v/v) ethanol and 5% (v/v) acetic acid over night for 15 hours.

4.3.4. Evaluation of protein amounts

The protein gels were washed in distilled water for 30 minutes and subsequently scanned with a dual lens scanner (model Epson Perfection V700 Photo, Seiko Epson Corporation, Tokyo, Japan) with following settings (scan mode: film / positive film, type: 48-Bit color, quality: optimal, resolution: 800 dpi, no adjustment and processing was performed). The freely accessible program ImageJ version 1.45 (Rasband 2011) was used with standard settings for gel analysis to integrate peaks of protein bands. Protein amounts were calculated in relation to the mean of two assisting purified napin standards on each gel. No absolute protein amounts were determined therefore the given unit for napin and cruciferin is µg per µg napin standard (STD). For identification of the napin protein we used the given napin standard (see lane G and H in Figure 1) and for identification of cruciferin we used the molecular weights published in the literature (Schwenke et al. 2000) indicated by the lanes A, B, C, D in Figure 1.

4.3.5 Sulphur

Sulphur content (% seed dry matter) was determined by NIRS using the calibration raps2009.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel).

4.3.6 Statistics

Analysis of variance and calculation of heritabilities (h^2) were performed by using PLABSTAT software (Utz 2011) considering the years as random. Mean values of the genotypes over the years were used to calculate Spearman's rank correlation coefficients between traits. $^{+}$, *, ** denotes significant at P=10, 5 and 1%, respectively

4.3.7 Molecular marker 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 genetic distance of all markers covering 21 linkage groups was 9.5 cM calculated with Kosambi equa-

tion. QTL mapping was performed using the freely accessible software QTL Network 2.1 (Yang et al. 2009) with a significance level of P=5% for the QTL. A Permutation test was performed (1000 permutations) to determine the critical F value threshold for each trait. Epistatic interactions among the loci were also valued by using QTL Network 2.1 whereas the estimated effect represents the additive × additive genetic interaction. To simplify comparisons with previous publications the chromosome nomenclature follows the N1-N19 nomenclature for *Brassica napus*, alike the A and C genome nomenclature suggested by the Multinational *Brassica* Genome Project (see www.brassica.info). N1-N10 equals A1-A10 and N11-N19 equals C1-C9.

4.4 RESULTS

The separation of seed proteins extracted from the oilseed rape meals on denaturing polyacrylamide gels (SDS-PAGE) with a gradient of 4-15% worked quite satisfactorily (Fig. 1). In routine analysis only those gels were evaluated which showed a clear separation of the protein bands. The lane with the napin standard (S in Fig. 1) showed clear separation of the two napin bands, which represent after SDS treatment and under reducing conditions the large and small subunit (cf. introduction). There were also two additional protein bands with a larger molecular weight (14-18 kDa) visible, indicating that the napin standard was not 100% pure. The band of the smaller protein was also detectable in the extract samples of the DH population. The cruciferin bands A to E in Fig. 1 were identified according to Schwenke et al. (2000). Under reducing conditions 5 cruciferin bands were identified, consisting of α-chains (27-31 kDa) and β-chains (18-21 kDa). Since it was not clear if oleosins (19 kDa) were present in the extract, protein band E was excluded from the calculation of the total cruciferin amount.

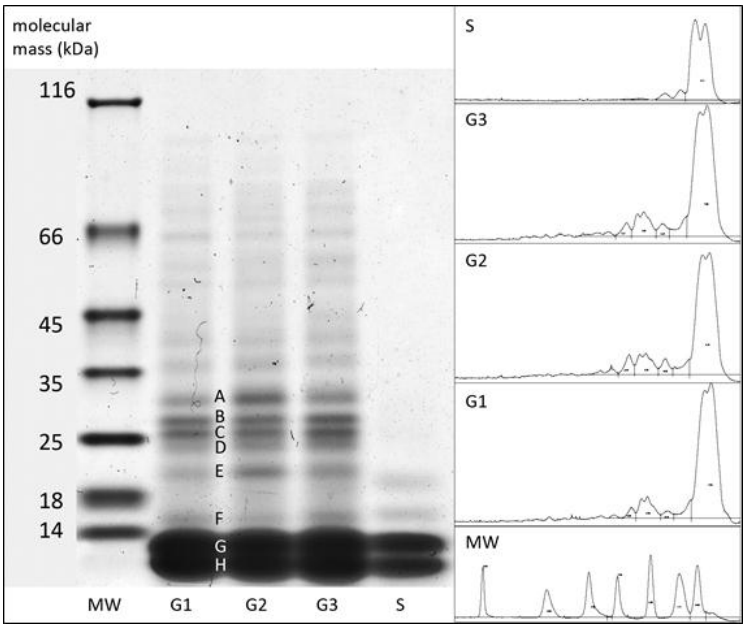


Fig. 1, left: separation of molecular weight marker and oilseed rape seed proteins on a 4-15% polyacrylamide gel (SDS-PAGE) in presence of ß-mercaptoethanol. Proteins were stained with Coomassie Blue and gels were scanned. Right: corresponding lanes as ImageJ scan. MW: molecular weight marker, G1-3: canola protein extract from seed of 3 different genotypes, S: purified napin standard

The analyses of variance indicated highly significant effects of the year and the genotype on napin and cruciferin content as well as on the ratio of cruciferin to napin (cru/nap ratio, see Tab. 1). The variance components of the genotype by year interaction for these traits include the residual error and were about half the size of the genotypic effects. The genotype by year interaction was comparatively much lower for the cruciferin to napin ratio and the heritability was surprisingly high with 0.92. Highly significant effects of the genotype were also found for all other traits. Genotype by year interaction was significant for glucosinolate-, sulphur-, protein-, oil-, protein in the defatted meal- and erucic acid content. Heritabilities were high for glucosinolate-, sulphur- and oil content of the seeds as well as for the erucic acid content of the oil.

Tab. 1: Variance components and heritability for napin-, cruciferin content (µg/µg napin standard), cru/nap ratio, glucosinolate- (µmol/g seed at 91%), protein-, oil-, protein in defatted meal- (% at 91% DM), sulphur- (% seed DM) and erucic acid content (22:1, % of the oil).

Source of variance	napin	cruciferin	cru/nap ratio	GSL†	sulphur	protein†	oil†	protein idM†	22:1†
year (Y)	0.008**	0.009**	0.002**	13.99	-0.0003	-1.21	-1.36	-1.42	1.86^{+}
location (L)	-	-	-	-3.69	-0.0005	-1.26	-1.36	-1.55	1.00
genotype (G)	0.065**	0.020**	0.026**	235.20**	0.0144**	0.73**	2.31**	1.45**	79.48**
G x Y	0.034	0.012	0.005	4.36**	0.0003**	0.16**	0.29**	0.18**	1.92**
G x L	-	-	-	-0.16	0.0000	0.01	0.00	0.05	-0.03
G x Y x L	-	-	-	3.04*	0.0001	0.09*	0.04	0.24**	0.58
residual	-	-	-	29.47	0.0025	0.81	1.22	1.05	11.09
h^2	0.79	0.77	0.92	0.97	0.96	0.78	0.88	0.84	0.97

$^{*, **}$ denotes significant at P=5 and 1%, idM in defatted meal
†data taken from Chapter 3

A large variation among the 229 genotypes was detected for napin- and cruciferin content ranging from 0.8 to 2.2 and from 0.2 to 1.3 µg/µg napin standard, respectively (see Tab.2). The cruciferin to napin ratio ranged from 0.13 to 1.05. Both parental genotypes had a much higher napin than cruciferin content as apparent from the low cruciferin to napin ratio. The cru/nap ratio mean of the DH population was as high as the value of the higher parent 'Express 617'. Large variations were also observed for all other traits.

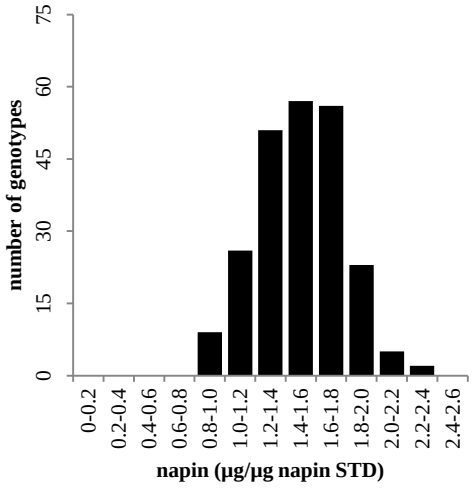
Tab. 2: Min, max and mean values for napin-, cruciferin content (µg/µg napin standard), cruciferin/napin ratio, glucosinolate- (µmol/g seed at 91%), protein-, oil- , protein in defatted meal-(% at 91% DM), sulphur- (% seed DM) and erucic acid content (22:1, % of the oil) of the DH population and the parental lines tested in field experiments in 2 years at two locations with two replicates.

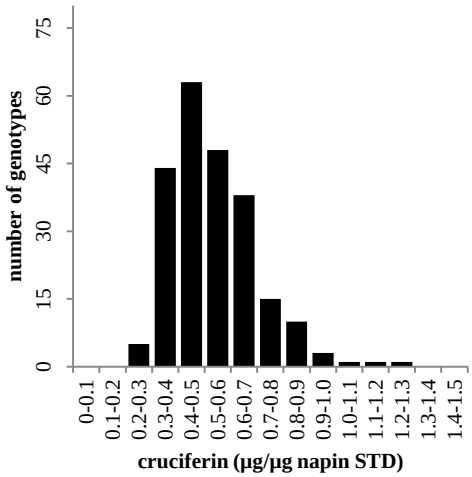
	napin	cruciferin	cru/nap ratio	GSL†	sulphur	protein†	oil†	protein idM†	22:1†
min	0.80	0.23	0.13	13.9	0.30	17.7	39.3	32.9	0.0
max	2.22	1.26	1.05	74.7	0.83	23.5	47.7	40.1	31.1
mean	1.50	0.53	0.38	37.4	0.52	20.6	43.5	36.3	13.3
LSD5%	0.36	0.22	0.13	7.2	0.06	1.3	1.6	1.5	4.4
'Express 617'	1.38	0.53	0.38	26.7	0.41	18.0	45.7	33.1	0.0
'R53'	1.60	0.35	0.22	41.8	0.58	20.6	42.6	35.9	15.9

LSD5% least significant difference at P=5%, idM in defatted meal

†data taken from chapter 3

The frequency distribution for napin showed a normal distribution (Fig. 2a). For cruciferin and the cruciferin to napin ratio the frequency distribution was skewed towards lower values (Fig. 2b, c).





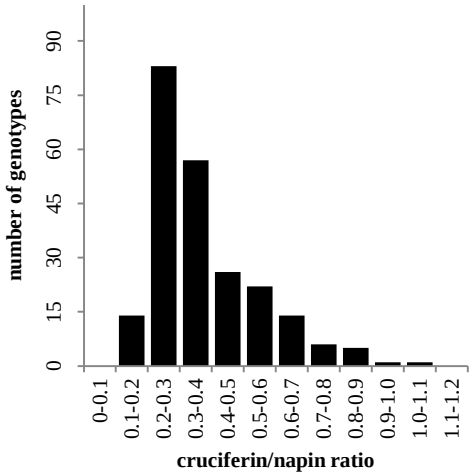


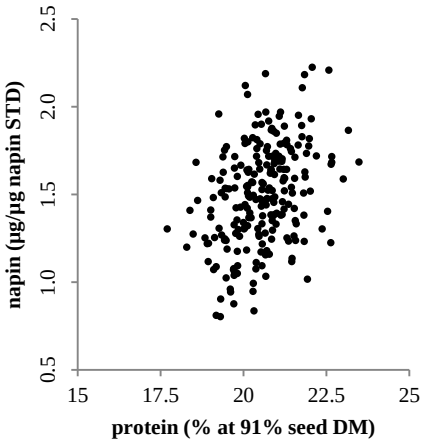
Figure 2a-c: Frequency distributions of napin- and cruciferin contents and the cruciferin to napin ratio in the DH population of 229 genotypes

In the DH population significant correlations were discovered between napin content and all other traits except for erucic acid (Tab. 3). Napin content was negatively correlated with cruciferin (Fig. 3d). Napin content was positively correlated with protein (Fig. 3a) content and negatively correlated with oil content. The opposite was observed for cruciferin content. A close positive correlation and vice versa a close negative correlation was observed between glucosinolate and napin, respectively cruciferin content (Fig. 3b, c). Exactly the same associations were found for sulphur content and the two storage protein contents. Not surprisingly, the sulphur- and glucosinolate content showed the highest positive correlation. Furthermore, napin content was positively correlated with thousand kernel weight and germination rate and negatively correlated with secondary seed dormancy.

Tab. 3: Spearman-rank correlation coefficients for napin-, cruciferin contents and other seed traits

	napin	cruciferin	cru/nap	GSL†	sulphur	protein†	oil†	prot idM†	SD†	GR†	TKW†
napin	-	-	-	-	-	-	-	-	-	-	-
cruciferin	-0.36**	-	-	-	-	-	-	-	-	-	-
cru/nap	-0.72**	0.89**	-	-	-	-	-	-	-	-	-
GSL†	0.76**	-0.62**	-0.81**	-	-	-	-	-	-	-	-
sulphur	0.79**	-0.61**	-0.83**	0.97**	-	-	-	-	-	-	-
protein†	0.36**	-0.11	-0.26**	0.36**	0.45**	-	-	-	-	-	-
oil†	-0.23**	0.23**	0.30**	-0.39**	-0.48**	-0.66**	-	-	-	-	-
prot idM†	0.28**	0.04	-0.10	0.13*	0.19**	0.77**	-0.06	-	-	-	-
SD†	-0.19**	0.06	0.13	-0.14*	-0.15*	-0.13	0.10	-0.10	-	-	-
GR†	0.17*	-0.04	-0.10	0.10	0.09	0.03	0.02	0.08	-0.71**	-	-
TKW†	0.18**	0.03	-0.06	0.16*	0.15*	0.03	-0.13*	-0.08	-0.08	0.06	-
22:1†	0.02	0.11	0.09	-0.09	-0.16*	-0.12	0.63**	0.38**	0.09	-0.02	-0.16*

†data taken from chapter 3



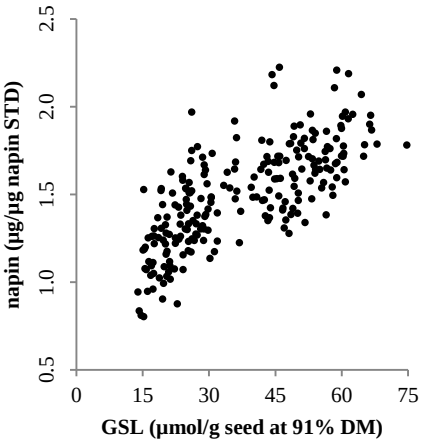
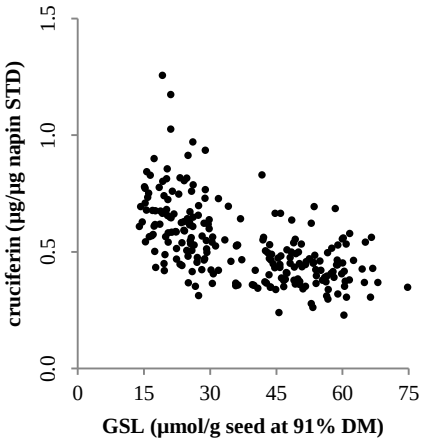


Fig. 3a-b: Scatter plots of napin content (µg/µg napin standard) associated with protein content (% at 91% seed DM) and glucosinolate content (GSL, µmol/g seed at 91% DM) respectively



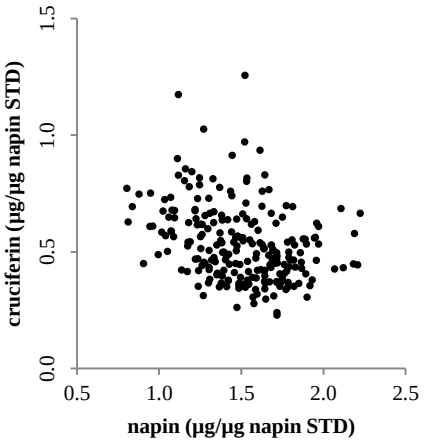


Fig. 3c-d: Scatter plots of cruciferin content (µg/µg napin standard) associated with glucosinolate content (GSL, µmol/g seed at 91% DM) and napin content (µg/µg napin standard) respectively

The QTL mapping revealed three QTL for napin content and two QTL cruciferin content (Tab. 4). The three QTL for napin content explained together 47% of the phenotypic variance. The two QTL for cruciferin content explained 35% of the phenotypic variance. Together with the epistatic effects the genetic main effects explained 39% of the phenotypic variance. In total 61% of the phenotypic variance of the cruciferin to napin ratio was explained by the variance of the additive and epistatic effects. The QTL for napin and/or cruciferin content were located on linkage groups N2, N16 and N19, whereby the QTL on N-19 had the largest effects on napin and cruciferin content.

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 Na-1 of linkage group N2 the substitution of the 'R53' allele by an 'Express 617' allele led to an increase in napin content of 6%. The results show that in two out of three cases, the 'Express 617' allele led to a reduction in napin content. This is consistent with the result that 'Express 617' has lower napin content than 'R53' (cf. Tab. 2). The QTL for napin and cruciferin content on linkage groups N2 and on N19 had clearly overlapping confidence intervals. Their signs of the additive effects had different directions, indicating that at these loci the cruciferin and napin content were affected simultaneously in different directions. On linkage group N16 there was a QTL which obviously only affected the napin content. Careful checking the data did not reveal a QTL for cruciferin content below the significance level in this region of linkage group N16. A number of QTL were also identified for glucosinolate, oil and protein content of the seeds. Interestingly, QTL for cruciferin, napin, oil, protein and glucosinolate content were located in the same region around 30 cM of linkage group 19 (see also Fig. 4). QTL GSL-2 on linkage group 19 had the largest effect on the glucosinolate content. The negative sign of the additive effect confirms that the allele from the low glucosinolate parent 'Express 617' led to a reduction in seed glucosinolate content. However, simultaneously, there is a reduction in napin content (cf. QTL Na-3) and an increase in cruciferin content (cf. QTL Cru-2).

Tab. 4: Mapped QTL and their most likely positions for napin- (Nap) and cruciferin contents (Cru) and other seed traits

QTL	LG	Position [cM]	CI [cM]	a	h^2 (a)	V(A)/V(P)	V(I)/V(P)	V(G)/V(P)
Nap-1	N2	43.5	31.0-47.4	0.06	0.06	0.47	-	0.47
Nap-2	N16	49.1	37.4-60.2	-0.07	0.05			
Nap-3	N19	31.0	27.0-38.5	-0.18	0.37			
Cru-1	N2	53.4	35.5-79.9	-0.04	0.05	0.35	0.04	0.39
Cru-2	N19	34.5	30.0-38.5	0.08	0.30			
Cru/Nap-1	N2	45.5	36.5-47.4	-0.05	0.09	0.55	0.06	0.61
Cru/Nap-2	N16	40.4	28.0-49.1	0.03	0.03			
Cru/Nap-3	N19	35.5	31.0-38.5	0.10	0.44			
GSL-1	N6	80.2	70.1-92.2	2.37	0.002	0.74	-	0.74
GSL-2	N19	33.5	32.0-35.5	-13.43	0.721			
Pro-1	N7	35.7	21.7-51.5	-0.42	0.10	0.15	-	0.15
Pro-2	N19	30.0	20.0-38.5	-0.24	0.05			
Oil-1	N12	0.0	0.0-6.0	0.45	0.07	0.47	-	0.47
Oil-2	N13	24.7	21.7-27.3	-0.98	0.28			
Oil-3	N13	131.5	123.3-138.4	0.38	0.03			
Oil-4	N19	31.0	23.0-41.5	0.43	0.08			

LG = linkage group, CI = confidence interval, a = additive effect; the substitution of the 'R53' allele by an 'Express 617' allele leads to an increase of the trait when positive, 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, †position of epistatic effects not shown

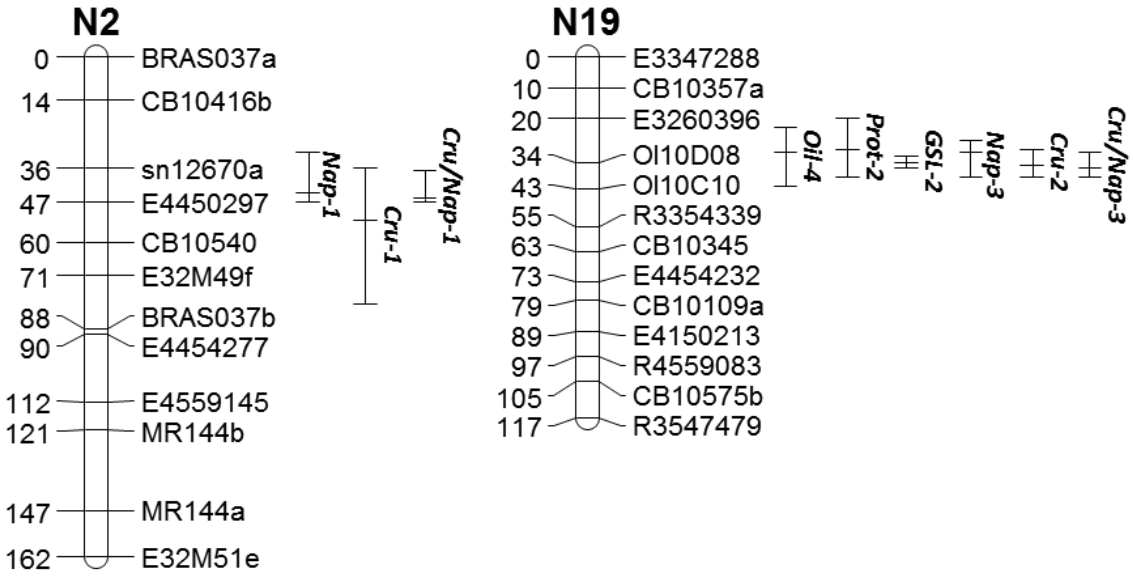


Fig. 4: Linkage groups N2 and N19 showing relevant QTL for napin- (Nap), cruciferin content (Cru), cruciferin to napin ratio (Cru/Nap), glucosinolate- (GLS), protein- (Pro) and oil content (Oil)

4.5 DISCUSSION AND CONCLUSION

The seed proteins of oilseed rape consist mainly of cruciferin, napin, oleosin and lipid transfer proteins (Raab et al. 1992, Uppström 1995, Malabat et al. 2003). Oleosins are associated with the oil bodies and are removed by defatting of the meal (Huang 1996, Hu et al. 2009, Malabat et al. 2003). After defatting of the seed meal of 64 *Brassica napus* genotypes and fractionation of the proteins according to their size on a gel filtration column, Malabat et al. (2003) identified three main peaks corresponding to cruciferin, napin and lipid transfer protein. The cruciferin and the napin content of the seed protein ranged from 32 to 53% and 25 to 45%, respectively. For most of the varieties, cruciferin proved to be the major protein and in some genotypes the cruciferin to napin ratio reached 2 (Malabat et al. 2003). However, Malabat et al. (2003) also identified genotypes with napin as predominant storage protein (cruciferin to napin ratio of around 0.7). The genotypes with a higher napin than cruciferin content mostly proved to be high in glucosinolate content. Previously Raab et al. (1992) reported for seven winter oilseed rape cultivars a cruciferin to napin ratio ranging from 0.7 to 4.9, as determined after size exclusion chromatography. In the present study, the two genotypes 'R53' and 'Express 617' proved to have a higher napin than cruciferin content with a cruciferin to napin ratio of 0.2 and 0.4 (Tab. 2) as determined after polyacrylamide gel electrophoresis. This comparatively low ratio may be specific for the genotypes but may also be explained by a suboptimal extraction of the cruciferins in the present study, which as globulins have a better solubility in a salty extraction buffer (Uppström 1995).

However, as found in previous studies (Kohno-Murase et al. 1994, Kohno-Murase et al. 1995) cruciferin and napin content were negatively correlated to each other in the doubled haploid population. Furthermore, a close positive correlation between napin content and glucosinolate content has been found (Tab. 3) which is corroborating the results of Malabat et al. (2003) obtained for the 64 *Brassica napus* genotypes. Vice versa a similar close negative correlation between cruciferin and glucosinolate content was observed (Tab. 3). This result indicates that in the presence of glucosinolates the synthesis of napins is up-regulated. Both, glucosinolates as well as napins are rich in sulphur. Hence, results also suggest that sulphur supply in relation to nitrogen availability has not been limiting during seed growth and maturation. In their review paper Tabe et al. (2002) pointed out that at a given level of nitrogen supply the availability of sulphur may influence the composition of the seed protein fraction. When N supply is sufficient, variations in S supply can result in the adjustment of the relative abundance of specific S-rich or S-poor proteins. Results from grain legumes and cereals show that at ample nitrogen supply and under limited sulphur availability the synthesis of sulphur amino acid

proteins is very much decreased (Tabe et al. 2002 and references therein). Zhao et al. (1993) showed that sulphur fertilisation had no significant effect on the seed protein content in 00-quality oilseed rape. However, sulphur deficiency, in particular at high nitrogen fertiliser levels, reduces the seed protein content and its quality in terms of sulphur amino acid (Hawkesford and De Kok 2006, Zhao et al. 1993). Especially, the content of sulphur rich napins is reduced (Zhao et al. 1993 and references therein).

The aromatic and indol glucosinolate molecules contain two and the methionine derived aliphatic glucosinolate molecules contain three sulphur atoms. Sulphur makes up 15 to 20% of the molecular weight of the glucosinolates and the glucosinolate sulphur makes up 10 to 30% of the total seed sulphur content (Falk et al. 2007). There is a close relationship between seed sulphur and seed glucosinolate content. Hence, glucosinolate content of the seeds can be determined by analysis of its sulphur content (Schnug and Kallweit 1987). Finding this close correlation it has been anticipated that there is little variation in the amount of sulphur bound in the seed storage proteins. However, the results of the present study show that there is a quite close positive correlation between napin- and glucosinolate content.

This close correlation is also corroborating with the overlapping confidence intervals of the major QTL from glucosinolate (GSL-2) and napin (Nap-3) on linkage group N19 (Fig. 4), explaining vast amounts of the phenotypic variation of the traits (72% and 37%, respectively). Similarly, Radoev (2007) also reported of a hotspot area on linkage group N19 when examining this very same segregating biparental doubled haploid population, although tested at different locations and years. Radoev (2007) showed that for glucosinolates the major QTL was on N19 at similar position like in this study but only explaining 24% of the phenotypic variance. Furthermore a minor QTL for oil was likewise detected on N19, but the minor QTL for protein (Pro-2, see Tab. 4) was not reported by Radoev (2007). These differences might be owed to the altered environmental influence present at the conduction of the studies and due to dissimilar QTL software usage.

Though the increase of napin levels within the seeds and the resulting promotion of the nutritional value due to their providing of essential sulphur-containing amino acids for consumption purposes alone is tempting other functions of napins recently came into focus. Surplus the major role of napins as a nitrogen and sulphur supply source and their mobilisation during germination (Müntz 1998, Neumann et al. 1996a), glutamine-rich storage proteins of this type (napin-like) are associated with antimycotic and antibacterial properties (Neumann et al. 1996bc, Polya 2003) Furthermore, the expression of napins in *Brassica napus* L. is reliant on the promoter segment placed between -152 to -120, termed the B box which is vastly con-

served in all 2 S albumin promoters and shows many resemblances to ABA response elements (Rask et al. 1998). This assumes a possible interaction of napins in germination and dormancy processes due to their association with ABA, which is well known for its regulatory properties in the closely related species *Arabidopsis thaliana* L. (Finkelstein et al. 2002, Brocard-Clifford et al. 2003). But despite a loose positive correlation between ABA content and secondary seed dormancy rates could be detected in this doubled haploid population (0.14^{*}, see chapter 3), no correlation concerning ABA- and napin content was observed (data not shown). Nevertheless a negative correlation was perceived for napin content and secondary seed dormancy (-0.19^{*}), although this data was not supported on the QTL data level (cf. Tab. 4 with Tab. 4, chapter 3).

In spite of the various positive features of raising napin levels in the oilseed rape protein fraction the occurrence of napins or their degradation products are also of clinical interest according to a possible allergenic response (Monsalve et al. 1997, Müntz 1998, Teuber et al. 1998). These interactions might also result from the ability to act as trypsin and calmodulin inhibitor, whereas the trypsin inhibitory effect was alleviated when cut into its subunits (Gehrig and Biemann 1996, Neumann et al. 1996a). In contrast it seems that the calmodulin inhibition results from a structure similarity within the small napin subunit and calmodulin in form of a α-helix motif (Neumann et al. 1996a). Additionally Wu and Muir (2008) indicated because of the significant differences of the two major canola proteins in structural, thermal, and emulsifying properties the enhanced presence of napins in canola protein may detrimentally affect the excellent emulsifying properties of the cruciferins.

Even so Schwenke (1982) stated that the functionality of proteins is not singularly determined by its physicochemical properties of the basic raw material, but also by the processing steps during protein isolation as well as interaction with non-protein components. In summary breeding for genotypes with altered cruciferin to napin ratio looks rather challenging, although when conducted by transgenic approach (Kohno-Murase 1994) the negative association of napin content and secondary seed dormancy might prove beneficial under biosafety aspects (see also chapter 3) by possibly reducing the secondary seed dormancy potential. Despite the importance of the genetic interactions of the QTL (GSL-2, Nap-3 and Cru-2) indicated on linkage group N19 (see Fig. 4) the value for breeding purposes seem to be limited and making breeding for contents of low glucosinolates and high napin most complicated. For that reason the focus of ongoing research should also lie on linkage groups N2 and N16 and its status for explaining the phenotypic variance of the cruciferin to napin ratio within the seed. Malabat et al. (2003) already concluded that over the past decades breeding programs dedi-

cated to improve the oil fraction in oil seed rape did not only lead to a decrease in the protein content but also in an simultaneously increase of the cruciferin levels. We detected comparatively low cruciferin to napin ratios, but clearly resulting from the extraction method used. Moreover, the presented correlations and QTL interactions in this study further corroborates the theory of Malabat et al. (2003), although an increase of cruciferins likely is linked to the breeding of low glucosinolate cultivars combined with high oil levels. Furthermore advanced research must be done to explore the functional potential hidden in the molecular structure of storage proteins, and napins in particular.

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Chapter 5 - Final Discussion

Genetic variation for secondary seed dormancy in a set of current European winter oilseed rape cultivars

The work in this thesis was realised as part of the cooperation project "Confinement strategies for oilseed rape" and was implemented in the research program "Biosafety Research On Genetically Modified Plants" of the Federal Ministry of Education and Research (BMBF) in Germany. In this context we wanted to study the genetic variation and inheritance of secondary dormancy in winter oilseed rape (*Brassica napus* L.). Since volunteer rape seed is a common problem in following crops and also of relevance when changing oilseed crop qualities (e.g. from high erucic acid qualities to double low qualities or from double low to HOLL quality), it would be a simple and efficient way to reduce the soil seed bank inputs by selecting for low dormant potential genotypes. The objectives of these studies were to define the importance of the genotype and its environmental interactions on one hand and to estimate correlations between secondary seed dormancy and other seed traits on the other hand. For reasons of comparability to other conducted studies and practical relevance to breeders we examined 28 modern winter oilseed rape cultivars from six different locations.

Since dormancy is defined as a block to germination under favourable conditions and modern oilseed cultivars are selected with respect to high germination rates, the dormancy potential of those cultivars was expected to be low. As expected we observed a high germination rate mean of 98% but nevertheless also for secondary dormancy rates with a mean of 26% and a considerable range of 8 to 56%. A similar variation amongst genotypes of oilseed rape was previously reported in other publications (Pekrun et al. 1997, Momoh et al. 2002, Gulden et al. 2003, Gruber et al. 2004, Gulden et al. 2004). Nevertheless, in this study we observed a surprisingly high heritability (0.97) for the trait in modern oilseed rape cultivars indicating that selection for low dormancy should be very successful. A highly significant effect of the location was also observed, making it difficult to predict the real case potential of seed dormancy. However the genotype x location interaction was comparatively low and non-significant, indicating that the order of the genotypes across the locations did not differ largely.

In this study we did not detect any correlations between secondary dormancy and other seed traits, suggesting that seed dormancy similar to DOG1 in *Arabidopsis* (Bentsink et al.2006) might be also regulated by different independently genes. To further investigate secondary seed dormancy in *Brassica napus* on the genetic level we conducted QTL analyses for SD and corresponding traits in a segregating doubled haploid population (cf Chapter 5.2).

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

The main goal of the work was the evaluation of genetic variation and inheritance of secondary dormancy in a segregating doubled haploid (DH) population. DH populations are often used due to its 100% pure, homozygous properties which make them easily multipliable and reproducible. Since seeds from DH propagated selfings are identical material, the phenotypic and genotype data and subsequent QTL mapping results can be gathered across different laboratories (Young 1994). Nevertheless QTL can only be detected for traits that segregate between the parents (Flandez-Galvez et al. 2003), why previously conducted examination of the parents was necessary and revealed low dormancy potential for 'Express 617' and a high dormancy potential for the resynthesised 'R53'. In this study for each genotype seeds were gathered from main inflorescences of ten open pollinated plants per replication. Since paternal influences on seed dormancy have been discussed (Foley and Fennimore 1998, Flintham 2000) because of the diploid embryo’s maternal and paternal origin, its true contribution for dormancy is still to be discovered. Nevertheless, for oil- and protein content of summer oilseed rape seeds Grami and Stefansson (1977) observed no significant paternal impact. The heritabilities from both the modern oilseed cultivars and the DH-population in our studies were high (0.97 and 0.85 respectively), indicating that the resolution of the phenotypic data for secondary dormancy seemed suitably high, despite unknown paternal influence to the genotypes at different locations and/or years. Nevertheless these results reveal that secondary dormancy is a heritable trait and is predominantly determined by its genotype. From the additionally estimated traits the germination rate, glucosinolate- and ABA content showed significant correlations to secondary dormancy in the DH population. In contrast to the previous study (cf Chapter 5.1) we observed a large and highly significant negative correlation between secondary dormancy and germination rate. Figure 3 (Chapter 3) reveals an unexpected relation of SD and GR indicating that a selection for high germination rate does not effectively

affect the dormancy potential. The range of germination rates in the DH population was considerably higher (73-100%) than in the modern cultivars (94-100%) suggesting that the resynthesised parent 'R53' contributed some allels with ‘wild-type’ characteristics that might also affect secondary dormancy. The great variation for secondary dormancy in the DH population ranging from 5 to 96% further supports this assumption. We also detected a transgressive segregation for SD which was supported on the QTL level revealing that both parents’ alleles contribute additive effects at different loci. Both QTL found for GR overlapped with confidence intervals from QTL for SD with an inversed effect, with the 'Express 617' alleles increasing GR values by simultaneously decreasing SD rate values. Nevertheless three additional QTL were found hitherto unlinked to QTL relating to GR with two of them increasing the SD rate values by an 'Express 617' allele. These QTL might explain the unusual correlation between GR and SD and support the hypothesis that although SD and GR are associated the effect in modern cultivars is hidden due to the selection of high GRs. This also suggest that there are at least two different mechanisms for genetic regulation of SD, with one including GR and therefore obviously also primary dormancy rates and one independent from either of them. This difference in the plant material might also explain the requirement for additional viability tests in the DH population (cf Chapter 2 and 3) which have to be considered when testing for SD.

We also detected two QTL for ABA known for its involvement in seed development and germination processes in many species (Finkelstein 1994, Koornneef and Karssen 1994, Finch-Savage and Leubner-Metzger 2006, Finkelstein et al. 2008) but the correlation between SD and ABA was observed to be rather weak. Finkelstein et al. (1985) reported that endogenous ABA levels are associated with GR of excised embryos early during seed maturation, but ABA levels declined during desiccation. Consistently, Gulden et al. (2004) reported that endogenous ABA levels were at one level in mature seeds from a low and a high dormant genotype examined but increased differently during dormancy induction. Although the role of ABA in controlling the acquisition of SD after seed dissemination is unclear, *de novo* ABA synthesis in seeds is assumed to be essential for the maintenance of SD (Yoshioka et al. 1998, Grappin et al. 2000). Consistently, hence the samples came from mature seeds only, just a minor QTL for ABA was found in overlapping chromosome position with a major QTL for SD, with the 'Express 617' allele increasing both values. A loose correlation was also detected between glucosinolates content and SD but could not be explained on the QTL level, but an indirect effect eventually by influence of protein composition (see Chapter 5.3) might be assumed.

Gruber (2004) reported a reduction of dormancy potential after a certain storage period. Due to the vast amount of samples examined in this study we had to divide the seed samples into different lots. This might have resulted in an impact on the calculated environmental effects since each replicate was analysed as a block. The blocks from one location were analysed first in both years and therefore the storage time of seeds did differ up to 3 weeks. The same was true for the years since we started analysis of seeds 3 weeks and 6 weeks after harvest respectively.

Since in *Arabidopsis* the DOG1-gene has already been cloned (Bentsink et al.2006), syntheny approaches by aligning known marker sequences from this study with the DOG1 sequence might give further information about the regulation mechanism of SD. This information might be even more useful when the total *Brassica napus* genome sequencing is completed.

Mapping of QTL for the seed storage proteins cruciferin and napin in a winter oilseed rape doubled haploid population and their inheritance in relation to other seed traits

Enhancing the seed oil content and improving the oil quality as well as reducing the concentration of undesirable compounds in the meal like e.g. sinapic acid esters (Zum Felde et al. 2006, Hüsken et al. 2005), phytic acid (Lickfett et al. 1999) and fibre content (Wittkop et al. 2009, Dimov et al. 2012) have been the objective of previous studies. In this study we analysed the genetic variation and the inheritance of napin and cruciferin content of the oilseed rape seed protein in a winter oilseed rape doubled haploid population. Since the consequences of the changed protein composition of the seeds on traits like germination, primary and secondary dormancy, seed longevity in the soil (Nagel and Börner 2011) and fungal disease resistance (Terras et al. 1992, Barciszewski et al. 2000) has not been hitherto investigated, we also determined correlations to other seed traits previously valued in this population. With 229 genotypes chosen for evaluation the vast sample numbers required a fast and easy routine protocol. Due this fact, we chose a simple water extraction method for all samples, despite the solubility of napins and cruciferins are quite different (Kroll et al. 2007). This resulted in non-absolute but between genotypes comparable amounts of both proteins but might be the major explanation for the from literature dissenting cruciferin to napin ratios (Raab et al. 1992, Malabat et al. 2003). Nevertheless we determined highly significant effects of the year and the genotype on napin and cruciferin content as well as on the cru/nap ratio. Whereas the heritability for either of the proteins was moderate (0.79 and 0.77 respectively) the heritability for

the cru/nap ratio was surprisingly high (0.92). We also detected a large variation for either protein. Furthermore we discovered several correlations between cruciferin and napin itself (-0.36*) and other seed traits. Napin was significantly associated with sulphur and glucosinolates but interestingly also with TKW and secondary dormancy. For the relation to sulphur and glucosinolates we retrieved supporting data by the QTL analysis in identification of a hotspot of QTL for several traits on N19. This chromosome region was assigned to QTL of oil-, protein-, glucosinolate-, sulphur-, napin- and cruciferin content, mostly represented by major QTL, except for oil and protein. An 'Express 617' allele at this locus therefore greatly reduces glucosinolates and napin contents by simultaneously increasing the amount of cruciferin. The associations between napin and TKW and ABA respectively did not corroborate with the results from the QTL analysis. An indirect effect is therefore assumed as napin has been associated with several non-storage functions. Nevertheless the QTL detected for cruciferin and napin should prove valuable tools for changing the storage protein composition in *Brassica napus.* A breeding for low secondary dormancy genotypes (cf chapter 2 and 3) could also indirectly be associated with a breeding for higher total protein and napin contents with respect to QTL that are unlinked from those of GSL.

Outlook

Although the here presented data looks promising, the results of the secondary dormancy test still remain a limited diagnostic for sufficient prediction of the actual SD values for an upcoming season, since large environmental interactions are undeniable and unpredictable. Nevertheless the results from this study further support the predominant influence of the trait SD by the genotypes and its heritability for SD potential. A selection for genotypes with a low SD potential should consequently be possible. The here presented QTL and marker data could therefore represent a valuable tool for future breeding programs to limit the risks of unwanted propagation of voluntary oilseed rape by decreasing the seed soil bank inputs during and after harvest.

Limiting factors for SD analysis hitherto were the number of genotypes and/or environments analysed. By increasing these limiting factors e.g. by use of the improved laboratory test by Weber et al. (2010) the quality of phenotypic data and therefore also possible QTL analysis resolution could be further enhanced. This could also be achieved by analyzing a different population for additional QTL not present in the parents 'Express 617' and 'R53'". Future research should also target the influence of the origin of seed material analysed by laboratory

tests, since DH material seem to vary from modern cultivars. This could be also including the relationship between primary and secondary dormancy as previously stated.

Since seed coat derived SD is exclusively dependent on maternal tissue in mature seeds of *Brassica napus*, we currently survey the impact of seed hull proportion and seed fibre contents (Suprianto et al. 2011). Additionally the paternal influence on SD values might be estimated via subset studies of the here presented genotypes from either open pollinated or selfed plants.

Another possible problem represents pre-harvest sprouting in *Brassica napus*. Therefore shifting gene pools of *Brassica* in direction of low seed dormancy potential of genotypes might further increase this problem. However pre-harvest sprouting is mainly associated with primary dormancy and the results from our study suggest that primary and secondary dormancy might be regulated at least partly by different genes. Feng et al. (2009) detected 5 QTL for pre-harvest sprouting on chromosome N3 and N11. These linkage groups were not associated with QTL for GR or SD. An independent selection for low SD potential should therefore be possible.

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SUMMARY

Secondary seed dormancy in oilseed rape is a phenomenon that allows seeds to survive in the soil for many years without germination. Following soil cultivation, dormant seeds may germinate in subsequent years and are the reason for the occurrence of volunteer oilseed rape plants in successive crops. These volunteer plants pose a common threat, especially under the assumption of previously cultivated transgenic plant material. Genetic reduction of secondary dormancy in oilseed rape could therefore provide a mean to reduce the frequency of volunteer plants and its subsequent pollen dispersal. However, little is known about the genetic variation for and the environmental influence on the secondary seed dormancy of winter oilseed rape cultivars. Therefore one aim of the present study was to analyse secondary seed dormancy in a set of 28 current winter oilseed rape cultivars tested in 2008/2009 in field experiments at six different locations in Germany.

To further investigate the inheritance of this trait a double haploid (DH) winter oilseed rape population consisting 229 genotypes from a cross 'Express 617' x 'R53' was tested for correlations to other seed traits. Field experiments were performed in two consecutive years from 2008/2009 to 2009/2010 at two locations in Germany with two replicates. Seeds harvested from open pollinated plants were used for all analyses, including a laboratory test for secondary dormancy. A previously developed molecular marker map of the DH population was used to map QTL of the relevant traits.

In the ANOVA highly significant effects were found for the influence of the locations and for the variation among the genotypes of the 28 winter oilseed rape cultivars. Among these genotypes, secondary seed dormancy ranged from 8% to 56% and the heritability was high (0.97). The means for the six locations ranged from 12% to 38%.

The results of the DH-population showed significant effects of the location, the genotypes and their interactions on secondary seed dormancy. Heritability was high (0.85) and secondary dormancy ranged from 5% to 96%. Two major (above 10% of explained phenotypic variance) and three minor (under 10% of explained phenotypic variance) QTL for secondary dormancy were detected which together explained 42% of the phenotypic variance. Secondary dormancy values showed significant negative correlations with the germination rate (-0.71), glucosinolate content (-0.14) and the content of the storage protein Napin (-0.19). Additionally a significant positive correlation between secondary dormancy and the abscisic acid content was revealed (0.14).

The large genetic variation and the high heritability indicate that secondary dormancy is a heritable trait and that selection for low secondary dormancy is possible and effective breeding for reduced secondary seed dormancy could be performed.

ZUSAMMENFASSUNG

Das Auftreten von sekundärer Dormanz bei Raps bedeutet, dass nach der Ernte auf dem Feld verbleibende und in den Boden eingearbeitete Samen für viele Jahre lebensfähig bleiben können ohne zu keimen. Diese dormanten Samen führen bei einem späteren Rapsanbau auf gleicher Fläche zu ungewolltem Durchwuchs und werden als besonders problematisch bei wechselnden Rapsqualitäten und dem Anbau von transgenen Sorten erachtet. Demnach könnten Genotypen ohne starke Ausprägung des Merkmals sekundärer Dormanz im Samen auf einfachem und effizientem Wege zur Verringerung von Rapsdurchwuchs und eines anschließenden Pollenaustrages beitragen. Bisher ist jedoch wenig über die genetische Variation von, und den Umwelteinflüssen auf, das Merkmal sekundärer Dormanz bei Winterrraps Kultursorten bekannt. Deshalb war eines der Ziele dieser Studie die sekundäre Dormanz in einer Zusammenstellung von 28 aktuellen Winterrapssorten, die in der Saison 2008/2009 in Feldversuchen an sechs unterschiedlichen Standorten in Deutschland angebaut worden sind, zu analysieren.

Um die Erblichkeit des Merkmals näher zu untersuchen wurde die sekundäre Dormanz ebenfalls in einer doppelt haploiden (DH) Winterraps Population, bestehend aus 229 Genotypen aus der Kreuzung 'Express 617' x 'R53', bestimmt und Korrelationen zu anderen Samenmerkmalen berechnet. Die Feldversuche wurden in zwei aufeinanderfolgenden Jahren von 2008/2009 bis 2009/2010 an zwei Orten, mit jeweils zwei Wiederholungen, in Deutschland durchgeführt. Dabei wurden Samen von offen abgeblühten Pflanzen geerntet und für alle Analysen, inklusive eines Labortestes für sekundäre Dormanz, verwendet. Eine bereits entwickelte molekulare Markerkarte der DH Population wurde verwendet, um die QTL für relevante Merkmale zu bestimmen.

Bei der ANOVA zeigten sich signifikante Effekte zum Einfluss der Orte und der Varianz unter den Genotypen der 28 untersuchten Winterraps Sorten. Bei den Genotypen zeigten die Werte für sekundäre Dormanz eine Spannbreite von 8% bis 56% und eine hohe Heritabilität (0.97). Die jeweiligen Mittelwerte der sechs Orte erstreckten sich von 12% bis 38% sekundärer Dormanz.

Die Ergebnisse der an den Werten der DH Population durchgeführten ANOVA zeigten signifikante Effekte der Orte, der Genotypen und deren Interaktion auf sekundäre Dormanz. Die Heritabilität war hoch (0.85) und die Spannbreite für sekundäre Dormanz betrug 5% bis 96%. Außerdem konnten drei Haupt-QTL (mehr als 10% der phänotypischen Varianz werden erklärt) und 3 Neben-QTL (weniger als 10% der phänotypischen Varianz werden erklärt) für sekundäre Dormanz identifiziert werden, die zusammen 42% der phänotypischen Varianz erklären. Die Werte für sekundäre Dormanz waren signifikant negativ korreliert mit den Werten der Keimrate (-0.71), des Glucosinolatgehaltes (-0.14) und des Gehaltes des Speicherproteins Napin (-0.19). Außerdem zeigte sich eine signifikant positive Korrelation zwischen den Werten von sekundärer Dormanz und dem Abscisinsäuregehalt (0.14).

Die große genetische Variation und die hohe Heritabilität zeigen, dass es sich bei der sekundären Dormanz um ein erbliches Merkmal handelt und dass eine Selektion und effektive Züchtung von Genotypen mit niedrigem sekundärem Dormanzpotential möglich ist.

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