

**Institute of Agronomy and Plant Breeding I
Justus Liebig University Giessen
Department of Plant Breeding**

Prof. Dr. Rod Snowdon

**Breeding for Climate Change: Genetics and Physiology of
Seed Vigor, Seedling Vigor and Early Drought Resistance in
Winter Oilseed Rape (*Brassica napus* L.)**

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Justus-Liebig-University Giessen

submitted by

Sarah Vanessa Hatzig, MSc
born in Siegen, North Rhine-Westphalia

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1 Introduction

1.1 Preface

Conventional plant breeding has led to a continuous improvement of crop yields and quality. However, important efforts are still needed to ensure future food security. On the one hand, a growing world population causes a constantly increasing demand for food, feed and feedstock. On the other hand, more frequent weather extremes resulting from climate change dramatically affect agricultural production. Furthermore, future breeding success might be further diminished by erosion of available diversity in breeding germplasm. These circumstances underline the need for breeding strategies which help to increase and stabilize crop yields in a changing environment. The improvement of secondary agronomic traits which contribute to proper plant development and therefore ensure high yield levels is therefore of overriding importance. Yield stability could be further enhanced by increasing the adaptive potential of crop plants to varying environmental conditions.

As a chain can only be as strong as its weakest link, plant breeding should not solely focus on generative plant growth, but already on the early stages in plant development. Plant yield formation can only rely on assimilates provided by the source organs. The formation of photosynthetically active plant organs requires adequate juvenile and seedling growth as well as proper seed germination. If one of these successional stages is limiting, this causes an incomplete exploitation of potential yield levels. In this context, the present work aimed to disclose and understand the genetic and physiological attributes which contribute to economic seed yield production in winter oilseed rape (*Brassica napus* L.), with a focus on seed vigor and seedling growth. In view of the forecasted variability in precipitation events due to climate change, an additional focus was placed on securing the stability of seedling growth under varying water conditions.

Seed vigor is determined by the three key factors seed germination, initial upward and downward growth. The investigations performed within the present work were therefore targeted on each of these developmental processes:

- 1) With a focus on germination, a genome-wide association analysis was carried out in order to disclose genomic regions underlying seed vigor in winter oilseed rape.

2) With respect to aboveground plant growth, seedling shoot responses to osmotic stress were investigated. This work aimed particularly at identification of physiological and metabolic markers related to improved seedling vigor under drought stress conditions in winter oilseed rape.

3) Seedling root architectural responses to osmotic stress were analysed in order to define root characteristics which contribute to seedling growth under water scarcity.

Within the course of the work several key genetic, physiological and anatomical factors could be identified that are associated with improved seed vigor and an enhanced early adaptive potential in winter oilseed rape. In ongoing studies these key traits can be further used as selection targets for improving seed vigor and seedling vigor stability in one of the most important oil crops of the world.

1.2 Oilseed rape: Current breeding practices and future challenges

1.2.1 Oilseed rape and its economic importance

Oilseed rape (*Brassica napus* L.) is one of the major oil crops worldwide. With respect to total oil production, oilseed rape (25 million tonnes [Mt]) currently ranks third behind oil palm (54 Mt) and soybean (43 Mt) and well ahead of sunflower in fourth place with just 12.5 Mt (<http://faostat3.fao.org>; world production data from 2013). Historical reports assume that agricultural production of oilseed rape goes back to the 16th century, when it was cultivated for the extraction of technical oils (Gómez-Campo and Prakash 1999). Original rapeseed oil contains high levels of erucic acid in the seeds. Besides its bitter taste, erucic acid has a negative impact on mammalian health causing pathological changes, such as an accumulation of triglycerides in the myocardium which lead to myocarditis (Beare-Rogers and Nera 1972). After the establishment of low-erucic acid cultivars in the early 1970s, oilseed rape became a major source for the production of highly nutritional vegetable oil, especially because of its high seed oil content of up to 50% and a beneficial fatty acid composition, with up to 60% monounsaturated oleic acid in the seed oil along with high levels of the polyunsaturated fatty acids linoleic and linolenic acid. Another milestone in the breeding history of rapeseed was the establishment of cultivars with reduced seed

glucosinolate content. As by-products from oil extraction, rapeseed press-cakes are a source for valuable protein-rich fodder. Since glucosinolates cause organ damages as well as thyroid dysfunctions in mammals and substantially lower food acceptance (Tripathi and Mishawa 2006), only the elimination of these antinutritive compounds allowed the use of rapeseed press-cake in animal production. The first double-low winter oilseed rape cultivar 'LIBRADOR' (low erucic acid, low glucosinolates) was registered in Germany in 1981.

The use of oilseed rape as feedstock for biofuel production has become increasingly important in Europe in recent decades. As the main feedstock for biodiesel production in the European Union (EU), which according to the European Biodiesel Board contributes one half of the world's total biodiesel production (<http://ebb-eu.org>), the economic importance of rapeseed oil is particularly high in Europe. The main producer of biodiesel in the EU is Germany, with 5.0 mio tons produced in 2012 (<http://ebb-eu.org>). According to Agrarmarkt Informations-Gesellschaft mbH (AMI, Bonn), 75% of the German rapeseed oil production in 2012 was fed into the production of biofuel and technical oils, while the remaining proportion of 25% was used for nutritional purposes.

Worldwide leading producers of oilseed rape are the EU, Canada and China with 21 Mt, 18 Mt and 14.5 Mt of seed, respectively, produced in 2013 (Figure 1; <http://faostat3.fao.org>). This amounts to nearly 75% of the worldwide rapeseed production. Depending on the differences in climatic and phenological conditions, different morphotypes are preferred for cultivation. In Europe, winter types are predominant, while lower-yielding spring types represent only a slight part of the total European rapeseed production. In contrast, spring-sown rapeseed is mainly cultivated in Canada, as continental conditions and strong winters curtail the cultivation period and prevent overwintering. Flowering initiation in winter oilseed rape requires vernalization, while there is no such demand in spring types. As an intermediate form of winter and spring type rapeseed, semi-winter types represent the main portion of Chinese oilseed rape production and have medium demands on photoperiod and adolescence. From Figure 1 it is obvious that highest yields per unit area were achieved in the EU, while there is a larger margin between production quantity and area harvested for regions in which spring and semi-winter types are grown. Differences between rapeseed morphotypes can be mainly ascribed to differences in flowering time control (Wang *et al.* 2011, Raman *et al.* 2013). Interestingly, several studies deal with a link between the control

of flowering time and germination in *Brassicaceae* (Deng *et al.* 2011, Chiang *et al.* 2009, Tyler *et al.* 2004). Thus it cannot be excluded that different morphotypes also differ in their regulation of seed germination. In this context, the scientific approaches in the present work focus on winter oilseed rape exclusively and general conclusions for other morphotypes should be drawn with caution.

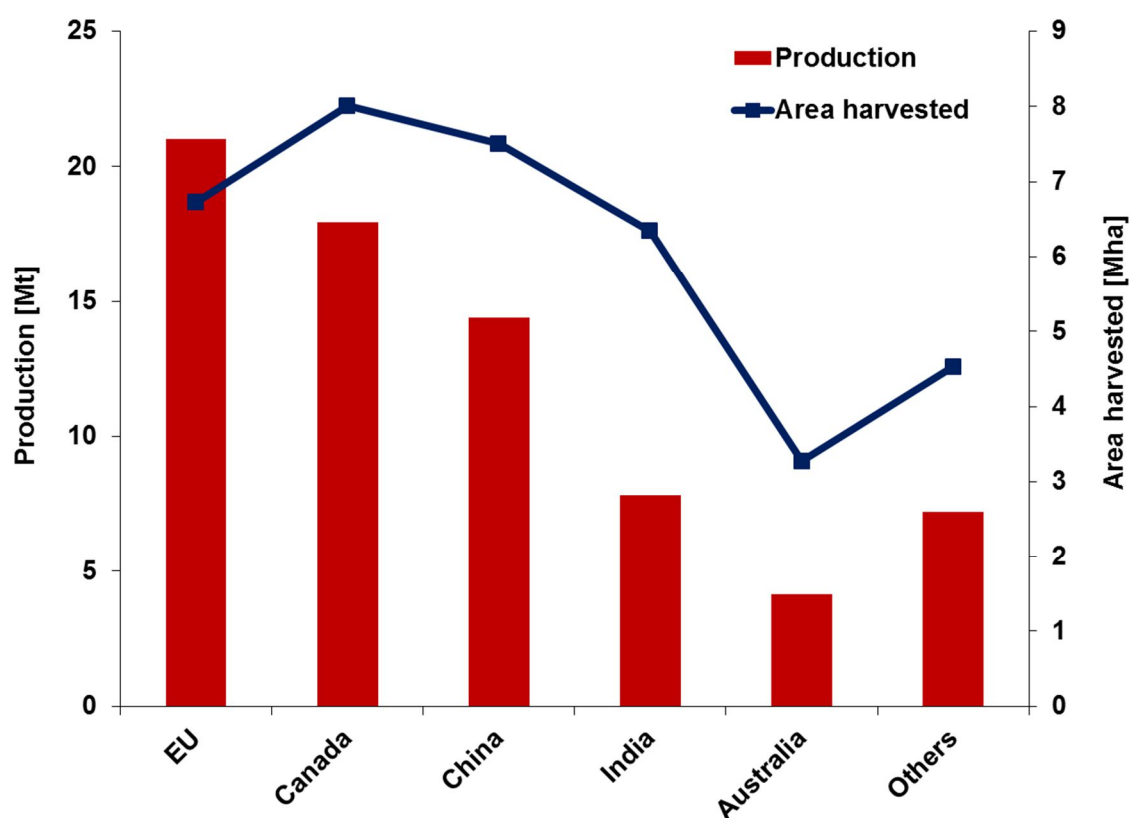


Figure 1: Rapeseed production quantity and area harvested, displayed for the main contributors to worldwide rapeseed production. Amounts produced are given in million tonnes [Mt] on the left ordinate. Areas harvested are represented in million hectares [Mha] on the right ordinate.

1.2.2 Current breeding aims of oilseed rape

Oilseed rape (*Brassica napus* ssp. *napus*, genome AACC, $2n=38$) belongs to the family of the *Brassicaceae*. The allotetraploid species *B. napus* L. resulted from a spontaneous

hybridization between turnip rape (*Brassica rapa* L., genome AA, 2n=20) and cabbage (*Brassica oleracea* L., genome CC, 2n=18) followed by a spontaneous chromosome doubling (U 1935). Oilseed rape is a facultatively cross-pollinating species. Since the establishment of the first male sterility systems (McVetty *et al.* 1989 and references therein, Frauen and Paulmann 1999), hybrid breeding became the most relevant breeding method for rapeseed. The first winter type hybrids were registered in 1995.

To ensure a high economic efficiency, breeding has to be aligned to the main utilization branches of oilseed rape. According to its use as an oil crop, a major aim is the improvement of oil yield and oil quality. Since oil content and composition are particularly dependent on environmental effects (Gao *et al.* 2010, Onemli 2014, Jensen 1996), it is especially important to take advantage of the genetically determined variation. Furthermore, high seed protein content, low glucosinolate levels and more recently reduced contents of crude fiber and specific antinutritive compounds, like tannins, phytates and sinapate esters, ensure the exploitation of rapeseed press cakes as a valuable fodder in livestock production. Oil, protein and glucosinolate contents are all quantitatively inherited and mainly determined by additive genetic effects (Grami and Steffanson 1977, Wittkop *et al.* 2009). Seed quality could therefore be further improved by the pyramiding of favorable alleles. However, a major constraint breeders have to deal with is the negative correlation between seed oil and seed protein content (Grami *et al.* 1977, Uppström 1995, Wittkop *et al.* 2012). For highest profitability the main attention in breeding tends to be paid to improvement of oil content, since at least at present no premium is paid to farmers for protein yields or quality.

Different use purposes demand different rapeseed oil quality. Whereas original rapeseed oil contains high levels of erucic acid with up to 50% of total fatty acid content, modern cultivars for edible oil production exhibit only marginal levels of this long-chained fatty acid. An erucic acid content of less than 0.2% of total fatty acids, as well as a glucosinolate concentration of less than 18 µmol/g are fixed criteria for the declaration of so-called “double-low” seed quality. While seed glucosinolate content is contingent on different environmental factors, such as plant water supply (Jensen *et al.* 1996) or fertilization (Asare and Scarisbrick 1995), the main contribution emanates from the genetic control of underlying biosynthetic pathways (Verkerk *et al.* 2009). For some industrial purposes erucic acid is an important commodity and provided by so-called HEAR cultivars (high erucic acid

rapeseed). These cultivars, which are only grown on small areas in specific production regions, have high levels of erucic acid but low glucosinolate concentrations in the press cakes. Beyond these niche varieties, high-oleic low-linolenic acid rapeseed cultivars (known as “HOLLI” types) supply a further special-quality oil, which is mainly used as a highly stable, nutritional frying oil in the fast-food industry. High levels of mono-unsaturated oleic acid (>75%), combined with low amounts of poly-unsaturated fats, prevent the formation of undesirable *trans*-fatty acids in HOLLI oils during heating.

In order to secure high seed yield levels and consequently a high oil yield by breeding, it is essential for breeders to also consider plant pest and disease resistances as well as a range of additional agronomic qualities. For winter oilseed rape the most important traits include tolerance to late planting, winter hardiness, plant height and lodging resistance, flowering and ripening time, nutrient efficiency, shattering resistance, herbicide tolerance and drought tolerance (Christen and Friedt 2007). Especially the latter – drought tolerance – is becoming a major new breeding aim in light of an elevated frequency of extreme weather events and a continuous increase in atmospheric temperature due to climate change.

1.2.3 Impact of climate change on European winter oilseed rape production

Contrary to popular perception, climate change is not just a recent occurrence. Indeed, many of the present natural realities reflect climatic changes in the distant past. One impressive example is that long-lasting drought periods at the end of the last ice age (15,000 to 13,500 years ago) caused a natural selection towards annual plants with dormant seeds or tubers – plant species to which most of today’s agronomically important crops might be traced back (Ceccarelli *et al.* 2010).

Today, extreme weather events in recent years, and their negative impact on agricultural production have renewed focus on climate change. Especially in the face of an increasing world population, climate change presents one of the main future challenges to global food security. According to Alexandratos and Bruinsma (2012), oil crop production must be increased by 30% until 2050 in order to secure food, feed and fuel demand of the growing world population.

Extreme drought and precipitation events during the last years enhance the importance of flexible cultivars with a broad adaptive potential under diverse meteorological conditions. As one example, the highest daily rainfall since more than 100 years was recorded in Germany in 2002, while already one year later, the hottest summer since at least 500 years was reported throughout Europe (Coumou and Rahmstorf 2012). In 2011, Western Europe experienced the hottest and driest spring since the beginning of weather recordings, while in the same year the wettest summer was recorded (Coumou and Rahmstorf 2012).

Compared to many other grain crops, oilseed rape is generally more sensitive to water deprivation. In consideration of increasing weather anomalies and the forecasted climate changes this could lower the competitive position of oilseed rape compared to the more drought compatible cereals, such as wheat and barley. Not only for Europe it is furthermore important to enhance the qualification of rapeseed in crop rotations, in order to retain its positive effects on soil fertility and nutrient enrichment. Independently of the time of occurrence, drought significantly impairs yield development in rapeseed, although the effects of water scarcity are most drastic during the sensitive phase of flowering (Richard and Thurling 1978). Significant effects on seed quality are also observed as a result of drought stress (Bouchereau *et al.* 1996).

In recent years rapeseed production suffered severe yield losses due to insufficient precipitation across Europe. In Germany in 2011, average yields of oilseed rape were decreased by more than 30% compared to previous years (<http://faostat3.fao.org>). These losses could be mainly traced back to the extreme low spring precipitation with only 90 L per m² (data from <http://www.dwd.de>). Field emergence and canopy formation during autumn of the same year was significantly impaired by marginal rainfalls in several countries across Europe. According to the German Weather Service (Deutscher Wetterdienst; <http://www.dwd.de>), Germany suffered its driest November in 2011 since the beginning of weather recordings. Similar impacts became perceptible in 2012, where insufficient soil moisture during spring affected agricultural production across western and central Europe. In fact, several modelling studies anticipate that drought events will appear more frequently (Good *et al.* 2004, Lehner *et al.* 2006, Bates *et al.* 2008). According to Lehner *et al.* (2006), a crucial increase is forecast in the frequency of 100-year precipitation extremes, and drought events are expected to become more frequent especially in southern and southeastern

Europe (FIGURE 2). Therefore, appropriate adaptation measures are needed in order to prevent yield losses under these future conditions. In this context, the establishment of cultivars with increased field emergence and early canopy formation is an important step to secure yield stability in oilseed rape production. Increased seed vigor and seedling growth accelerate plants' adaptive capacities and allow reduced exposure to external stresses, as sensitive stages of development are more rapidly completed.

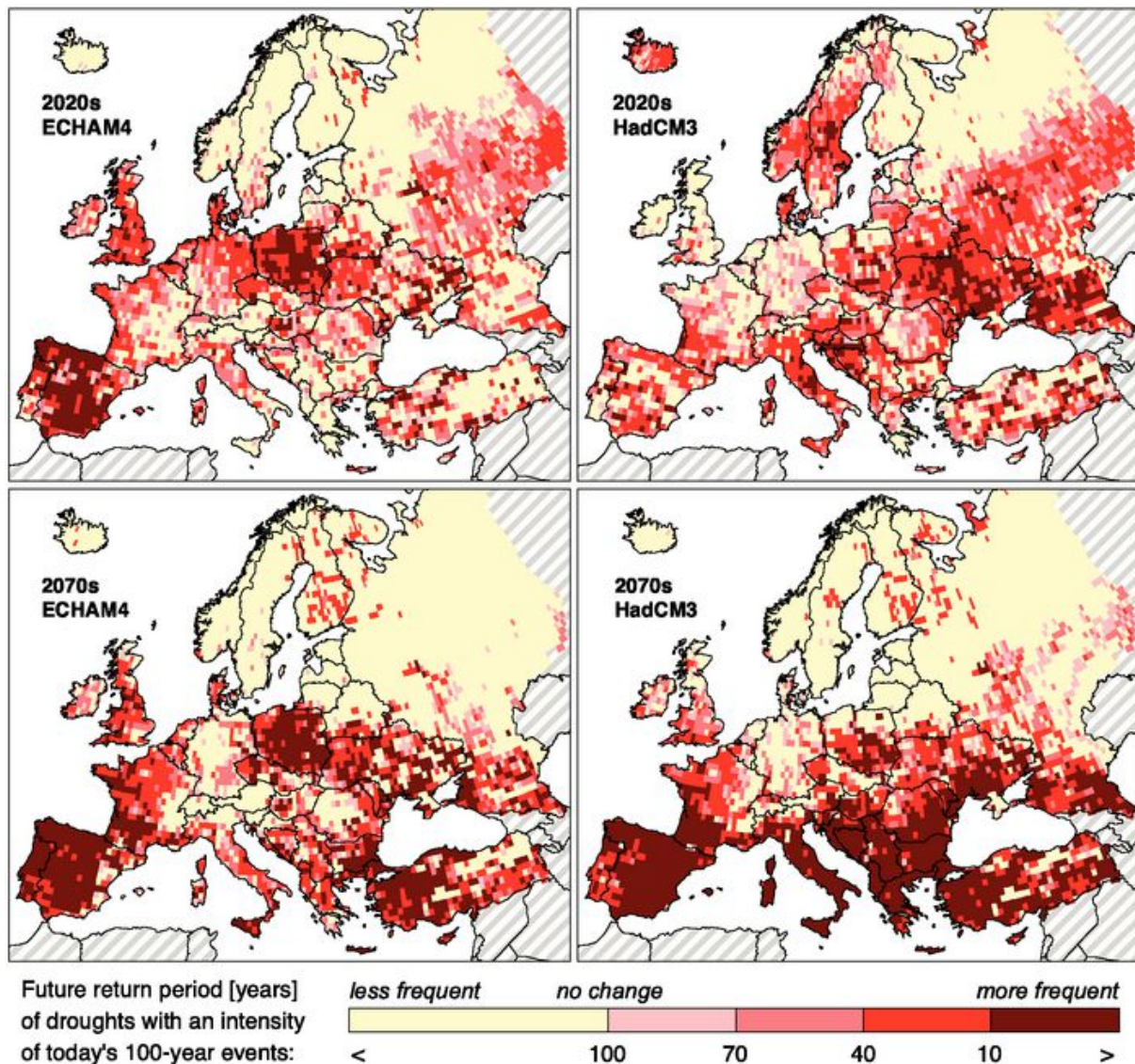


FIGURE 2: Change in the recurrence of 100-year droughts in Europe, based on comparisons between climate and water use in 1961 to 1990 and simulations for the 2020s and 2070s (based on the ECHAM4 and HadCM3 GCMs, the IS92a emissions scenario and a business-as-usual water-use scenario). Values calculated with the model WaterGAP 2.1 (Lehner et al. 2006; Use with permission from Springer Science + Business Media, Inc.).

1.2.4 The significance of seed vigor and plant establishment

Several studies emphasize the importance of germination performance and post-germination seedling growth on crop formation, vegetative growth and yield (Finch-Savage 1995, Ghassemi-Golezani *et al.* 2010, Mondo *et al.* 2013). However, seed vigor has a stronger effect on vegetative and early reproductive growth than on the later reproductive phase (TeKrony and Egli 1991). Field trials with diverse winter oilseed rape genotypes on different locations in France and Germany demonstrated that plant density and early leaf biomass development were strongly correlated with germination speed measured under lab conditions (S. Goertz, Norddeutsche Pflanzenzucht, Hohenlieth, unpublished data). Improved germination has different direct and indirect effects on crop yield. However, indirect effects are more distinct and lie in the fact that plant density, spatial arrangement and growth duration are positively influenced (Ellis 1992). Furthermore, uniformity of seedling emergence is a prerequisite for homogeneous maturity of a crop population and therefore also effects the exploitation of potential yield levels.

Vegetative plant growth and thus an adequate formation of photosynthetically active biomass is a major requirement for the realization of high seed yield levels. In accordance with Sun *et al.* (2009), biomass can be described as a function of available incident solar radiation across the season, the efficiency of light interception by the crop and the efficiency of conversion of absorbed energy into biomass. Therefore, one possibility to enhance biomass production and best exploit available solar radiation is an increased light interception by early canopy formation and delayed senescence. According to Diepenbrock (2000), growth rates of rapeseed are mainly limited by an inadequate light interception during spring, with potential growth rates being much higher than observed experimental values (FIGURE 3). A potential increase in light interception during this phase could be realized by increasing seedling vigor, canopy formation and leaf area index (LAI) in autumn before growth is interrupted during winter. Thus, plants would be able to access an adequate leaf apparatus for increased capture of solar radiation when growth continues in spring. Furthermore, Ghassemi-Golezani *et al.* (2010) showed that ground coverage in spring was significantly dependent on seed vigor in winter oilseed rape, showing that the latter is an important determinant for increasing early light interception and plant growth. Additionally, cultivars with improved seedling vigor seem to cope better with late sowing

dates, because they are able to compensate for a shorter pre-winter growing period through their enhanced seedling development.

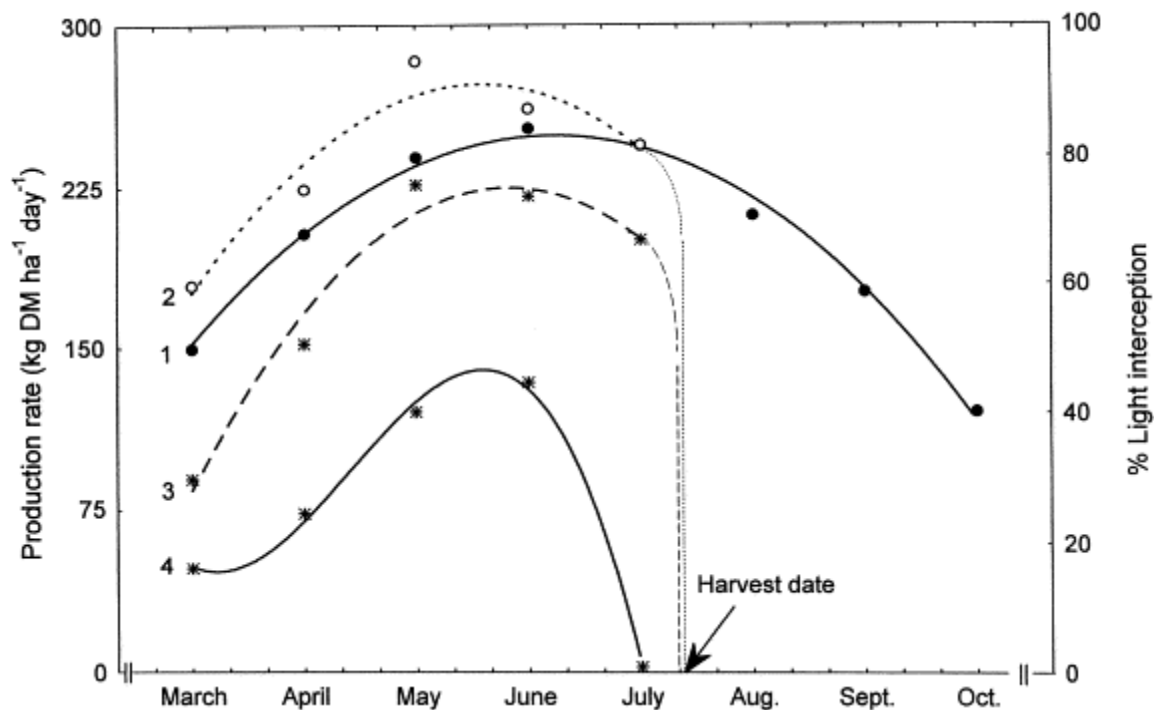


Figure 3: Potential production rate (line 1), percentage of light intercepted by the crop (line 2), calculated crop growth rate (line 3) and measured crop growth rate (line 4) of winter oilseed rape on experimental plots in the hercynian dry region of central Germany (Sibma 1977, Diepenbrock unpublished). (Diepenbrock 2000; Use with permission of Elsevier Science B.V.)

On the other hand, plant development during spring is not solely confined by the realized light interception. This is obvious from FIGURE 3, which shows a sigmoidal progression for the experimentally observed growth of rapeseed but a polygonal regression for potential growth rates at a given light intensity. This finding underlines the need for optimal early plant vigor in establishment of efficient rapeseed cultivars. High vigor increases the ability of a plant to withstand abiotic and biotic stresses during the sensitive stage of seedling establishment. Besides an increased viability and resistance of the seedling, there are also some indirect positive effects on stress compatibility. As one example, enhanced seedling vigor contributes to early canopy formation and can improve salinity or drought compatibility. Better ground covering reduces evaporation, which leads to an increased water-use efficiency, as available soil water could be more effectively used for growth

processes (Siddique *et al.* 1990, Richards 1996, Richards *et al.* 2002, Condon *et al.* 2004, Colmer *et al.* 2005).

1.3 The principles of genome-wide association mapping

1.3.1 Linkage analysis and trait heritability

For breeding of new cultivars with improved seed vigor it is crucial to understand the factors underlying germination performance and seedling growth. In general, these factors can be grouped into genetic and non-genetic factors, while the latter comprise any environmental effects, *inter alia* emanating from climate, soil or biotic actions. Especially complex traits underlying polygenic control, such as seed yield, seedling development (Basunanda *et al.* 2010) or even seed germination (Morrison *et al.* 2014) are strongly dependent on the environment. However, since external conditions are permanently changing, only that part of phenotypic variation which is genetically determined is able to be captured by selection. Therefore, improving crop performance requires disclosure of the genetic background influencing a trait of interest.

The genetic contribution to phenotype expression is defined as heritability (H^2) and can be calculated from the quotient of the genetic variance (V_G) and the phenotypic variance (V_P ; Wray and Visscher 2008):

$$H^2 = V_G / V_P$$

Within this definition of broad-sense heritability, overall genetic variance is taken into account, including additive as well as epistatic gene interactions. A special form of heritability is the so-called narrow-sense heritability, in which only the additive component of genetic variance (V_A) is included (Wray and Visscher 2008).

$$h^2 = V_A / V_P$$

Low H^2 and h^2 values indicate that trait performance is almost exclusively dependent on the environment and breeding success is restricted, whereas high values indicate a large influence of underlying genes. In the latter case, the trait of interest can potentially be

improved by breeding. When additive gene effects are predominant, so called pyramiding of favorable alleles might bring the greatest success to improve varieties.

In general, heritability is a good measure of how much advance might be achieved by breeding. Therefore, it is inevitable to ensure that genetically determined variation is sufficiently large to be effectively used in breeding programs. If so, genetic linkage analysis is a worthwhile tool for the disclosure of influential genes underlying a trait of interest.

1.3.2 Linkage analysis in the presence of population structure

Genome-wide association mapping is a powerful tool for the disclosure of genomic regions underpinning phenotypic trait variation. Originally developed in human medicine, association mapping has recently become increasingly relevant in plant genetics. The method is based on the same principle as classical quantitative trait locus (QTL) analysis, in which individuals are characterized phenotypically and genotypically and these joint datasets are used to calculate significant links between polymorphic DNA markers and phenotypic expression. In contrast to classical bi-parental linkage analysis, in genome-wide association mapping such associations are calculated for a broad set of unrelated individuals. One major advantage is that the time-intensive establishment of crossing populations is no longer necessary. Unrelated populations bear a larger genetic background due to the incorporation of numerous ancient meioses. For this reason association mapping provides a higher resolution power than classical QTL mapping (Oraguzie and Wilcox 2007). A higher resolution is mainly achieved due to a lower extent of linkage disequilibrium (LD), which is defined as the non-random association between alleles of different loci. Decisive factors affecting LD in any given population are recombination rate, mating system, natural and artificial selection, genetic isolation, mutation rate as well as population size, subdivision and admixture (Rafalski and Morgante 2004). Furthermore, genome-wide association mapping is not limited to the set of parental alleles which are contributed to a bi-parental mapping population by the selected parents. The effects of rare alleles might be better detected in bi-parental crosses, as equal allele frequencies are predominant. On the other hand, however, allelic effects are often overestimated since there is no capture of complete genomic variance in bi-parental populations.

A main challenge in association mapping is that population structure has to be recognized and considered in the statistical analysis. Population structure is defined as the variation in genotype distribution across an admixed population. Generally, such kinds of stratification become noticeable in unevenly distributed allele frequencies, which can be mainly traced back to a non-random mating between subpopulations. Generally known factors causing changes in population structure are physical isolation, local selection, genetic drift or migration, for example. Population substructure therefore causes a reduction of heterozygosity within an entire population. This phenomenon, first described by Wahlund (1928) is accordingly known as the Wahlund effect. In genome-wide association studies, population structure is a confounding factor, as it bears a high risk for the detection of false-positive marker-trait associations (Korte and Farlow 2013). In simple words, LD between two loci could be observed solely due to their co-occurrence in sub-populations, and not due to their genomic location. Another concern within structured populations is the disregard of rare alleles and/or alleles associated with local adaptation (Nordborg and Weigel 2008). Therefore, correction for stratification is a prerequisite step prior to association testing. For the detection of systematic differences in ancestry within an admixed population Wright's F statistics can be applied (Wright 1949). The genetic differentiation between subpopulations is described by the fixation index or inbreeding coefficient F_{ST} , which measures the relative changes in heterozygosity within subpopulations (H_S) compared to the entire population (H_T).

$$F_{ST} = H_T - H_S / H_T$$

F_{ST} can theoretically reach values between 0 and 1. High values indicate a large contribution of substructure to total genetic diversity, while low values account for rather evenly distributed allele frequencies across an admixed population. Determination of F_{ST} is not only a powerful tool for the capture of genetic divergence between subpopulations. The calculation of locus-specific F_{ST} values is furthermore suitable for the identification of genomic regions subjected to selection (Akey et al. 2002, Wang *et al.* 2014), since such regions are a target of inbreeding and thus feature larger F_{ST} values.

An optimal case for association mapping would be the exclusive observation of causative relations between an observed trait and a genetic marker. However, in practice, this is not

commonly the case, as specific confounding variables such as population substructure or genetic relatedness are inherent in most test populations. For such populations, results of genome-wide association studies are only reliable when population stratification has been included in the test statistics. Otherwise there would be an inflation of false-positive associations. The extent of inflation can be quantified by the inflation factor λ , which describes the ratio between the median of the observed test statistics distribution and the expected median (Bacanu *et al.* 2000).

A simple method to correct inflation is dividing the calculated values from the test statistics by λ (Devlin and Roeder 1999). However, this method – commonly known as ‘genomic control’ – lacks power if high values of λ (> 1.1) are predominant (Aulchenko 2011). Thus a more powerful correction statistic is required in such cases. A suitable approach in case of substantial stratification is a mixed model approximation incorporating covariates from principal component analysis (Price *et al.* 2010). This calculation approach includes the genomic kinship between all single individuals in terms of their identity by descent and corrects well for common confounding like population and family structure as well as cryptic relatedness.

1.4 Drought and drought adaptation

1.4.1 The importance of adaptive plasticity for securing yield stability under drought

Plants have an enormous adaptive potential which allows them to grow and reproduce in several different environments. Long-term evolutionary adaptation due to natural selection within different habitats has led to the formation of so-called ecotypes with different genetically fixed demands on their growing sites. However, besides evolutionary adaptation, which is a steady constant during the growing period, plants are also able to react with spontaneous plasticity to varying conditions within their habitat. Both evolutionary adaptation and adaptive plasticity play major roles in the manifestation of drought compatibility.

Franks (2011) deduced that “(...) natural drought caused an evolutionary shift to earlier flowering in natural populations of *B. rapa* (...)”. Early flowering serves the purpose of

drought escape, as growth and reproduction take place before the stress occurs (Franks *et al.* 2007). On the other hand, this strategy might be disadvantageous when drought stress occurs during the early season. In such cases, water shortage can occur during flowering, the phase which is most sensitive to water deprivation. Another evolutionary concept for drought-prone environments would be the conservation of water due to a reduction of plant water use. However, as water use is highly linked with productivity, breeding for yield in water limited environments would result in cultivars with reduced plant size and leaf area (Blum 2005). In turn it is quite easy to conclude that such cultivars would be inferior under optimal conditions in which more vigorous varieties could achieve their full yield potential.

The two examples described in the previous paragraph demonstrate that crop yield in the face of environmental change would be more secure if adaptation is not a fixed constant, but would rather occur more dynamically. Thus the flexibility of a plant seems to be a key feature determining yield stability. Plants possessing a high adaptive plasticity cope better with a changing environment than “specialists” can. Therefore, it is essential to incorporate this feature into modern breeding varieties to enable them to deal with climate change and precipitation extremes. In this context it is crucially important to identify important key traits, such as morphological or metabolic markers, which could be used as reliable selection targets for improving drought stress adaptation.

As stated in the pioneer work of Levitt (1980), drought resistance is the “(...) capacity of a plant to withstand periods of dryness (...)”, while this capacity could be conditioned by drought avoidance and/or drought tolerance. As the latter aims for plant survival rather than plant growth, breeders should be mainly interested in advancing the potential for drought avoidance. Two main avoidance strategies are conceivable for the maintenance of plant water relations: 1) Plants diminish their water demand due to a retraction of transpiration activity and assimilative processes, which is mainly expressed in growth arrest. Plants which pursue this strategy are defined as ‘water savers’ (Levitt 1980). 2) Plants strive to maintain favorable tissue water content due to reinforcement of water uptake, while growth is sustained. This strategy is assigned to so-called ‘water spenders’, and often associated with osmotic adjustment and the formation of an extensive root system (Levitt 1980, Jones and Zur 1984).

1.4.2 Osmotic adjustment as a core attribute for drought adaptation

Osmotic adjustment is documented as an adaptation strategy to water stress over a wide range of important crop species, such as sorghum (Jones 1978), wheat (Kameli and Lösel 1995), maize (Chimenti *et al.* 2006), sunflower (Chimenti *et al.* 2002) or species from the *Brassicaceae* (Wright *et al.* 1997). If soil water potential decreases – which is commonly the case during soil desiccation – drought-adaptive genotypes react with a decrease in their cell osmotic potential, which is a driving force for water uptake into the cells and turgor maintenance. Species or cultivars that are able to respond with osmotic adjustment can therefore maintain their leaf water potential under water scarcity, hence meeting water demands necessary for sustained transpiration. The adaptation of cell osmotic potential mainly occurs due to the accumulation of cell compatible osmolytes, such as amino acids (Good and Zaplachiski 1994) or soluble sugars (Zhongchun and Stutte 1992). However, it has to be taken into account that drought-induced solute accumulation is not solely an adaptive response. Increased assimilate concentrations could also be a side-effect of inhibited assimilate expenditure, when growth is arrested during water deficiency (Kameli and Lösel 1995). Osmotic adjustment is often coupled with maintenance of transpiration, which allows growth to continue under water scarcity (Levitt 1980). Different studies demonstrated a significant relationship between transpiration intensity and leaf surface temperature (Hanson and Hitz 1982, Kumar *et al.* 1984, Singh *et al.* 1985, Munns *et al.* 2010). When stomata are open, gas exchanges have a cooling effect on the leaf surface. Thus if osmotic adjustment is observed, supporting measurements of leaf temperature might display whether or not they are growth-effective.

1.4.3 Phytohormones as key players in stress signalling

Several studies demonstrate a complex regulatory network behind abiotic stress adaptation (Zhu 2002, Xiong *et al.* 2002, Shinozaki and Yamaguchi-Shinozaki 2007), underlining the quantitative character of drought compatibility. It is discussed that phytohormones, such as auxins (Huang *et al.* 2008, Peleg and Blumwald 2011), cytokinins (Huang *et al.* 2008, Ha *et al.* 2012, Kudoyarova *et al.* 2013), gibberellins (Huang *et al.* 2008, Alonso-Ramírez *et al.* 2009) and abscisic acid (ABA; Huang *et al.* 2008, Kudoyarova *et al.* 2013) play a crucial role in stress

sensing and the initiation of adaptive pathways. Although much is known about several single strategies or pathways, the whole dimension of drought stress signaling has yet to be demonstrated. Indeed, some conflicting contiguities are observed. For instance, ABA is also involved in the activation of osmotic adjustment, a major prerequisite for maintained plant growth (as discussed above). However, the same phytohormone has also inhibiting effects on growth-related processes such as transpiration (Mittelheuser 1969) or lateral root formation (Xiong *et al.* 2006). Furthermore it has been shown that attenuated abscisic acid responsiveness is linked to increased drought sensitivity (Gosti *et al.* 1999, Arend *et al.* 2009), suggesting that not only the capacity to react to external changes, but even the ability to percept such alterations is a main attribute bringing success under drought stress. Therefore, further efforts are needed to gain a better understanding of drought resistance and the potential targets to improve this important trait in crop plants. Among these stress signaling transactions, a major role is taken over by the plant root system. The roots sense changes in water potential in the soil and transfer the attained information to the upper plant parts, which are ultimately responsible for reproductive growth and yield formation. When water scarcity is perceived, important stress-relevant signal molecules, such as ABA or cytokinins, are synthesized in the roots and allocated to the upper plant parts, triggering different adaptation responses (Schachtman and Goodger 2008). Therefore, in order to unravel the complexities of drought adaptation, a main focus has to be on the ‘hidden half’ of the plant, the roots.

1.4.4 The complexity of root responses to drought stress

The root system is responsible for a sufficient uptake of soil water, required for transpiration and assimilation processes. An occurrence often observed during soil desiccation is that the root/shoot ratio decreases, caused mainly by relatively stronger decreases in shoot growth than root growth (Blum 1996). In some cases this phenomenon might even be triggered by the promotion of root growth (Sharp and Davies 1989). A resulting increase in relative water uptake capacity per unit above-ground mass emphasizes the fact that maintenance of plant water status under drought is crucial for the sustainment of growth. Osmotic adjustment is a major prerequisite for maintaining root growth under water scarcity (Voetberg and Sharp 1991).

Water stress does not only affect absolute root growth, but also root complexity. Indeed, root morphology is highly variable depending on soil water availability and the manner on which water becomes available. For example, the exploitation of deep stored soil water reservoirs demands a root shape which is considerably different from that required for reduction of evaporative losses in the top-soil (Monneveux and Belhassen 1996). In this context, lateral root growth inhibition in favor of primary root growth seems to be advantageous in soils where water is available from the subsoil (Xiong *et al.* 2006). An elongated vertical root system can exploit deeper layers of the soil, while the corresponding energy and carbon costs are compensated by lateral root growth reduction. On the other hand, if sufficient moisture is available in the upper soil layers then enhanced root penetration in the top-soil is more cost-effective compared to the formation of longer vertical roots (Blum and Ritchie 1984). The formation of a limited root system with high hydraulic resistance is another interesting adaptive response to water scarcity, as such modifications aim to dose water extraction during early plant growth and conserve soil water for the remaining growing period (Passioura 1972). However, because of their manifoldness, the significance of root morphological and architectural changes cannot always be as clearly assessed as in the given examples. Especially in consideration of the resulting costs in energy and assimilates, it is difficult to generalize to what extent a decline, maintenance or enhancement of root growth under drought is economical (Passioura 1983) and therefore yield-relevant for a plant breeder. Especially for dicots like rapeseed, very little is known about the effects of water shortage on root shaping. In other words, further efforts to investigate beneficial root responses to drought stress are essential to gain an understanding of the best strategies for breeding.

2 From theory to practice: Current approaches towards the comprehension of improved seed vigor and seedling growth in rapeseed

This study aims to improve understanding of intrinsic factors determining seed vigor and seedling growth in winter oilseed rape (*B. napus*). As stated by Finch-Savage *et al.* (2010), seed vigor is determined by three key elements, namely seed germination, initial downward growth and initial upward growth. According to this partitioning three different approaches were pursued, focusing on (1) germination performance, (2) seedling shoot growth and (3) seedling root growth. However, as post-emergence seedling growth should be taken into consideration as well, a prolonged time-span was chosen for the investigation of shoot and root growth.

2.1 Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*

2.1.1 Publication outline

The publication in the following chapter describes a genome-wide association analysis carried out in order to identify genomic loci underlying the control of seed germination and early seedling growth in winter rapeseed. A large diversity panel, including winter-type forms of oilseed, fodder and exotic rape, was genotyped with the *Brassica* 60kSNP Illumina genotyping array. A large-scale automated phenotyping delivered germination-related parameters such as seed imbibition, germination rate and speed, as well as radicle growth. Narrow-sense heritability was calculated in order to estimate genetic variability for germination performance and to evaluate the breeding potential for improving seed vigor in rapeseed. Marker-trait associations were calculated using a mixed-model approximation including principal coordinate variates. Association mapping in two seed lots, produced in different years at different locations, enabled identification of loci which are universally involved in seed vigor performance. Besides the identification of marker alleles that are highly correlated with germination performance, this study aimed directly to disclose potential genes involved in this agronomic important trait complex.

Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*

Sarah V. Hatzig¹, Matthias Frisch², Frank Breuer³, Nathalie Nesi⁴, Sylvie Ducournau⁵, Marie-Helene Wagner⁵, Gunhild Leckband⁶, Amine Abbadi⁶ and Rod J. Snowdon^{1*}

¹ Department of Plant Breeding, Justus Liebig University, Giessen, Germany, ² Department of Biometry and Population Genetics, Justus Liebig University, Giessen, Germany, ³ KWS SAAT AG, Einbeck, Germany, ⁴ Institute for Genetics, Environment and Plant Protection, INRA, Le Rheu, France, ⁵ Groupe d'Etude et de contrôle des Variétés Et des Semences, Beaucouzé, France, ⁶ NPZ Innovation GmbH, Holtsee, Germany

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Edited by:

Rajeev K. Varshney,
International Crops Research Institute
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Reviewed by:

Dongying Gao,
University of Georgia, USA
Zhe Liang,
National University of Singapore,
Singapore

*Correspondence:

Rod J. Snowdon,
Department of Plant Breeding, Justus
Liebig University, IFZ Research Centre
for Biosystems, Land Use and
Nutrition, Heinrich-Buff-Ring 26-32,
35392 Giessen, Germany
rod.snowdon@agr.uni-giessen.de

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Rapid and uniform seed germination is a crucial prerequisite for crop establishment and high yield levels in crop production. A disclosure of genetic factors contributing to adequate seed vigor would help to further increase yield potential and stability. Here we carried out a genome-wide association study in order to define genomic regions influencing seed germination and early seedling growth in oilseed rape (*Brassica napus* L.). A population of 248 genetically diverse winter-type *B. napus* accessions was genotyped with the *Brassica* 60k SNP Illumina genotyping array. Automated high-throughput *in vitro* phenotyping provided extensive data for multiple traits related to germination and early vigor, such as germination speed, absolute germination rate and radicle elongation. The data obtained indicate that seed germination and radicle growth are strongly environmentally dependent, but could nevertheless be substantially improved by genomic-based breeding. Conditions during seed production and storage were shown to have a profound effect on seed vigor, and a variable manifestation of seed dormancy appears to contribute to differences in germination performance in *B. napus*. Several promising positional and functional candidate genes could be identified within the genomic regions associated with germination speed, absolute germination rate, radicle growth and thousand seed weight. These include *B. napus* orthologs of the *Arabidopsis thaliana* genes *SNOWY COTYLEDON 1* (*SCO1*), *ARABIDOPSIS TWO-COMPONENT RESPONSE REGULATOR* (*ARR4*), and *ARGINYL-t-RNA PROTEIN TRANSFERASE 1* (*ATE1*), which have been shown previously to play a role in seed germination and seedling growth in *A. thaliana*.

Keywords: seedling, emergence, high-throughput phenotyping, GWAS, rapeseed, canola

Introduction

Selection bottlenecks have a large impact on the diversity available for breeders to sustain selection gains for important traits, particularly in crop species like modern oilseed rape (*Brassica napus* L.) with relatively small gene pools. One approach to overcome this problem is introgression of

untapped germplasm in order to broaden the genetic basis of breeding materials (Friedt and Snowdon, 2010). A main focus in genetics and breeding of oilseed rape has been on analysis and improvement of general breeding targets, such as abiotic and biotic stress resistances (e.g., Obermeier et al., 2013; Hatzig et al., 2014) or flowering time optimization (Schiessl et al., 2014). Enhancement of such traits can contribute directly to an increase and stabilization of yield levels. On the other hand, less attention has been given to elucidation of the genetic control of seed germination and seedling vigor, although these are fundamental processes shaping field emergence (Matthews and Khajeh Hosseini, 2006; Wagner et al., 2011; Matthews et al., 2012) and yield performance (Larsen et al., 1998).

Germination is a major component of seed vigor, which is defined as the sum of those properties of the seed that determine the potential level of activity and performance of seed lots with acceptable germination under a wide range of environments (Perry, 1978). Although seed germination is influenced by both genetic as well as environmental factors, it is still an open question whether seeds performing well under optimal conditions also have advantages under stress conditions. However, different studies dealing with drought (Bettey et al., 2000), salt or cold stress (Foolad et al., 1999) strengthen such correlations.

Germination *per se* commences with the uptake of water by the quiescent dry seed, interruption of dormancy and the subsequent elongation of the embryonic axis (Bewley, 1997). Water uptake during germination is a triphasic process while per definition germination is completed upon entry into the third phase (Bewley, 1997). Initially, water uptake is driven by physical swelling of the seed when hydrophilic molecular groups form hydration shells. There is evidence that environmental influences already affect germination during this initial phase, in which water uptake takes place independently from metabolism. Factors like temperature or the presence of specific solutes are able to retard or accelerate seed swelling (Leopold, 1983). Subsequent to physical swelling, metabolic processes are initiated and germination becomes irreversible by water deprivation. This second phase is also described as the plateau phase (Bewley, 1997), as increases in fresh weight due to physical swelling are already completed, while active growth takes place due to cell differentiation and elongation after the initiation of underlying biochemical processes. Per definition germination stops upon entry into the

third phase, in which growth becomes visible and the primary root breaks through the testa (Bewley, 1997).

For breeding of vigorous plant cultivars with fast and uniform field emergence, it is crucial to understand the genetic factors that contribute to an adequate germination performance and seedling growth. As seed germination is a very complex trait controlled at transcriptional, translational and metabolic level (Rajjou et al., 2012), it is difficult to identify the contributing genetic factors by conventional genetic or physiological analyses. A preferential method for identification of genomic regions associated with complex quantitative traits is genome-wide association mapping, in which marker-trait associations are calculated across a broad set of diverse germplasm in order to define chromosome regions harboring promising genes.

The high resolution of genome-wide association studies, arising from the incorporation of numerous ancient meioses in a set of unrelated plant individuals, has become a popular method for disclosure of genomic polymorphisms affecting quantitative traits. However, a common drawback of GWAS is that population structure can shift allele frequencies within a diversity panel. This can lead to the detection of false-positive marker-trait associations (Korte and Farlow, 2013) or a non-detection of rare and/or region-specific alleles (Nordborg and Weigel, 2008). In such a case statistical methods have to be applied which correct for population stratification.

In this study we used genome-wide association studies for the investigation of quantitative trait loci (QTL) linked to germination performance and early vigor in winter oilseed rape (*Brassica napus* L.). An automated high throughput phenotyping platform (Demilly et al., 2014) was used to assay diverse traits, related to the three different phases of seed germination and post-germination radicle growth, in a large set of diverse winter rapeseed lines genotyped with genome-wide single-nucleotide polymorphism (SNP) markers. Genome-wide association analyses using these data sets enabled identification of highly promising candidate genes and markers for breeding towards improved germination in rapeseed. Phenotyping of seed lots produced in different environments helped to evaluate the effects of germination-independent factors, such as seed ripening and storage, on germination performance.

Materials and Methods

Seed Material and Phenotyping

A total of 248 genetically diverse, winter-type *B. napus* inbred lines were used in this study, including 216 winter oilseed rape (WOSR), 20 winter fodder (WF) and 10 exotic lines (Table S1). Seeds of all inbred lines were produced by controlled self-pollination in two distinct environments during the growing seasons of 2010/2011 (SL 2011) in Le Rheu, France and 2011/2012 (SL 2012) in Asendorf, Germany. The genotype panel comprises lines derived from modern oilseed rape cultivars with low seed erucic acid and glucosinolate content, old rapeseed varieties with high seed erucic acid and high glucosinolate content (++ quality), fodder rapes, kale vegetable forms, and resynthesized *B. napus* derived from interspecific hybridizations between its two diploid progenitors, *Brassica rapa* and *Brassica oleracea*.

Abbreviations: ABA, Absciscic acid; *ARR4*, *A. thaliana* gene two-component response regulator *ARR4* (AT1G10470.1); *ATE1*, *A. thaliana* gene Arginine-tRNA Protein Transferase 1 (AT5G05700.1); *Bna.ARR4*, *B. napus* ortholog of *A. thaliana* two-component response regulator *ARR4* (AT1G10470.1); *Bna.ATE1*, *B. napus* ortholog of *A. thaliana* gene *ATE1*, encoding for Arginine-tRNA Protein Transferase 1 (AT5G05700.1); *Bna.SCO1*, *B. napus* ortholog of *A. thaliana* gene *snowy cotyledon 1* (AT1G62750.1); DMSO, Dimethyl sulfoxide; ES, Elongation speed of the radicle (mm/h); FG, First germination (h); F_{ST} , Fixation index; GO, Gene ontology; GR36, Germination rate within 36 h (%); GR72, Germination rate within 72 h (%); GWAS, Genome-wide association study; IBD, Identity by descent; IS, Imbibition speed during first 4 h after initiation of imbibition (mm^3/h); LD, Linkage disequilibrium; MGT, Mean germination time (h); PC, Principal component; PCA, Principal component analysis; *SCO1*, *A. thaliana* gene *snowy cotyledon 1* (AT1G62750.1); SL2011, Seed lot produced in 2011 in LeRheu, France; SL2012, Seed lot produced in 2012 in Asendorf, Germany; SNP, Single nucleotide polymorphism; T50, Time necessary to reach 50% of germination (h); TSW, Thousand seed weight (g); VI, Volume increase within first 8 h (%).

Monitoring of seed imbibition, germination, and early radicle growth was conducted under *in vitro* conditions using the automated phenotyping platform of the variety control office of the French national seed testing agency (Station Nationale d'Essais de Semences, Groupe d'Etude et de contrôle des Variétés et des Semences—GEVES, Angers, France). The phenotyping platform is described in detail by Ducournau et al. (2004, 2005) and Wagner et al. (2011). Image acquisition, image analysis and data analysis methods are described in detail by Demilly et al. (2014). The following parameters were determined: Volume increase within first 8 h (VI; in %), imbibition speed during first 4 h after initiation of imbibition (IS; in mm³/h), total germination rate within 72 h after initiation of imbibition (GR72; in %), first germination time (FG; in h), mean germination time (MGT; in h), radicle elongation speed (ES; in mm/h), time to reach 50% of germination (T50; in h) and germination rate within 36 h after initiation of imbibition (GR36; in %). Additionally, thousand seed weight (TSW; in g) was measured before germination monitoring. Correlations were calculated applying the Pearson's product-moment correlation.

Pre-Processing of Marker Data

Genomic DNA was extracted from young leaf materials for all genotypes of the diversity panel ($n = 248$). Genotyping was performed with the *Brassica* 60k Illumina[®] Infinium consortium SNP array (Edwards et al., 2013), according to standard procedures of the manufacturer and using the same cluster file for SNP calling. For physical localization of SNP markers, flanking sequences were blasted onto the *Brassica napus* Darmor-bzh reference genome sequence assembly (version 4.1), recently published by Chalhoub et al. (2014). SNPs were blasted using the following criteria: minimum overlap of 50 bp length, minimum identity of 95%, no sequence gaps. SNPs which failed across the entire genotype collection, as well as all locus non-specific SNPs, for which more than 1 BLAST hit on the *B. napus* sequence was found, were excluded from further analyses. As heterozygous SNPs cannot be distinguished from multi-locus hemi-SNPs or false calls, heterozygous calls were treated as missing values. For visualization of population structure and calculation of genome-wide associations, SNP markers with more than 20% missing calls across the panel were excluded. Furthermore, all individuals which had more than 20% missing calls across the genotype data were excluded. In order to incorporate rare alleles with potential effects on germination performance, only markers with a minor allele frequency ≤ 0.025 were excluded from analysis. After pre-processing, 218 individuals and 22,169 SNP markers remained for further analyses.

Population Structure Analysis

Visualization of genetic relatedness was performed using the statistical software R studio version 0.98.501 and the integrated R package *SelectionTools* (<http://www.uni-giessen.de/population-genetics/downloads>). Genetic distances were calculated applying the euclidean modified Rodger's distance method according to Wright (1978). In order to visualize genetic relatedness among all genotypes, principal component analysis (PCA) was carried out regarding the first four

components. Correction for population stratification was performed by a mixed-model approach including principal component covariates. According to Price et al. (2010) this is the method of choice for correction of population structure, family structure and cryptic relatedness. Genotypes were assigned to specific clusters by k-means clustering (MacQueen, 1967).

Optimal number of clusters was estimated by calculating the within-cluster sum of squares (WSS), varying cluster number k from 1 to 15, and subsequently defined as 4. Linkage disequilibrium (LD) was calculated for each chromosome individually, quoting coefficients of determination (r^2) for all locus pairs localized on the same chromosome. Inter-chromosomal genome-wide LD decay was calculated for trait-associated markers that exhibited unexpected patterns of local LD. Locally paired scatterplot smoothing in R was employed for graphical representation of LD curves with a span of 0.1.

For characterization of genomic kinship, genome-wide allele identity by descent was computed for the whole diversity panel as well as separately for the four identified subpopulation clusters, using the R package GenABEL (Aulchenko et al., 2007). Additionally, to determine the proposition of genetic differentiation explained by differentiation among the subclusters, overall F_{ST} values were calculated using the software Genepop version 4.2.2 (Rousset, 2008).

Genome-Wide Association Analysis

Narrow-sense heritability for each trait was calculated separately for each seed lot, again using the R package GenABEL. Genome-wide associations were calculated with GenABEL. Adjustment for stratification was performed by mixed-model approximation combined with PC adjustment (Price et al., 2010; Svishcheva et al., 2012). For PC adjustment the first 4 components were taken into account.

False non-discovery rate was calculated with the R package *fdrtool* based on p-value statistics. Only associations exceeding the predetermined cutoff values were taken into account. For the integration of markers with weak associations to the phenotypic traits, all SNPs with a $-\log_{10}(p\text{-value}) > 3$ were considered as significant. Marker-trait associations were regarded as reliable when they could be confirmed in a second seed lot with $-\log_{10}(p\text{-value}) > 2.5$. To define regions of interest for selection of potential candidate genes, local LD decay was first calculated within the flanking regions up to 1000 kbp on either side of the associated markers. All genes anchored between the two markers next to an associated marker were taken into account for the disclosure of candidates. Furthermore, when flanking markers were in strong LD ($r^2 > 0.4$) with an associated marker, regions were defined as LD blocks and the whole LD block was taken into account for candidate gene disclosure. Genes within LD blocks containing trait-associated markers were characterized with the tool Blast2GO (Conesa et al., 2005) using default settings, and candidates were selected based on their gene ontology (GO) terms. For differentiation between coding and non-coding regions of selected candidate genes the Genscan web server (<http://genes.mit.edu/GENSCAN.html>) was used. The plant pathway database MetNet (<http://www.metnetonline.org>) was used to determine

common pathway memberships among the identified candidate genes.

Marker haplotypes were defined for each associated region and genotypes were bulked in regard to their associated-marker haplotypes. For T50 and ES boxplots were generated showing the phenotypic distribution of 4 and 6 classes, respectively, representing the cumulative phenotypes from haplotypes with favorable effects on the trait performance.

Analysis of Chlorophyll Content

For chlorophyll analyses, two groups of 10 extreme genotypes were selected with consistently high and low values, respectively, for ES (radicle elongation speed) in both seed lots. Seeds from SL2011 were used for chlorophyll content determination. Around 60 seeds of each genotype were germinated in Jacobsen vessels on circular filter papers soaked with H₂O dest. Continuous water supply was ensured by wicks connecting the filter papers with 100 mL H₂O dest at the bottom of each vessel. Germination was carried out in a climate chamber with the following conditions: 20°C, 16 h light, 8 h dark. Chlorophyll extraction was performed after 5 d. The cotyledons of eight seedlings per genotype were pooled and treated as one biological replicate. In total 3 biological

replicates were sampled per genotype. Fresh weights of all samples were measured. Subsequently all samples were homogenized (Ultra-Thurrax T25, IKA, Staufen / Germany) in 15 mL Falcon tubes filled with 5 mL dimethyl sulfoxide (DMSO, >99.8% GC). The samples were incubated at room temperature for 24 h in the dark. For chlorophyll quantification, 1 mL of the supernatant was collected and filled into a micro cuvette (1.6 mL) for spectrometric measurement. The absorbance of the sample solutions was measured after blank calibration (1 mL DMSO) with a Smart-Spec Plus spectrophotometer (Bio-Rad Inc., Hercules, CA, USA) at 665 nm and 649 nm wavelength. Chlorophyll a and chlorophyll b concentrations were calculated according to the equations of Wellburn (1994). Significant differences were calculated with Student's *t*-test.

Results

Phenotypic Distribution, Genetic Diversity and Linkage Disequilibrium

Phenotypic values for all traits showed a similar mean trait performance and phenotypic variability in the two seed lots SL2011

TABLE 1 | Phenotypic variation for different traits associated with seed germination performance in two seeds lots of a *Brassica napus* diversity panel (n=248) produced in Le Rheu, France in 2011 (SL2011) and Asendorf, Germany in 2012 (SL2012), respectively: Minimum (Min), maximum (Max), standard deviation (SD) and correlation between seed lots (r).

Trait	Unit	SL2011				SL2012				r
		Min	Max	Mean	SD	Min	Max	Mean	SD	
TSW	g	3.07	6.70	4.65	0.53	3.12	6.24	4.92	0.54	0.63***
VI	%	8.12	40.37	22.33	5.08	7.91	46.43	24.14	6.57	0.44***
IS	mm ³ /h	0.02	0.35	0.18	0.05	0.00	0.40	0.17	0.07	0.24***
FG	h	12.00	30.00	21.85	3.81	12.00	28.00	17.43	3.35	0.26***
MGT	h	27.62	48.75	36.29	3.41	27.17	46.16	35.88	3.22	0.56***
T50	h	26.14	62.00	35.14	3.88	27.08	45.75	35.20	3.22	0.57***
GR36	%	7.00	93.33	55.89	18.62	10.00	87.00	54.78	15.73	0.58***
GR72	%	44.00	100.00	96.96	5.53	75.00	100.00	94.68	4.00	0.44***
ES	mm/h	0.51	1.24	0.81	0.12	0.61	1.23	0.88	0.12	0.57***

Abbreviations of traits are explained in Materials and Methods.

Significances were calculated at a level of ****p* < 0.001.

TABLE 2 | Correlation coefficients (r) for different trait pairs associated with seed germination performance measured in two seed lots (SL2011 and SL2012) from an oilseed rape diversity panel (n = 248).

	TSW	VI	IS	FG	MGT	T50	GR36	GR72
VI	-0.04							
IS	0.21***	0.74***						
FG	-0.08	-0.30***	-0.10*					
MGT	0.10*	-0.41***	-0.19***	0.46***				
T50	0.15**	-0.38***	-0.18***	0.36***	0.94***			
GR36	-0.15***	0.39***	0.19***	-0.37***	-0.95***	-0.94***		
GR72	-0.08	-0.14**	-0.08	0.06	-0.15***	-0.36***	0.27***	
ES	0.17***	0.25***	0.17***	-0.36***	-0.29***	-0.18***	0.19***	-0.23***

Abbreviations of traits are explained in Materials and Methods.

Significances were calculated at levels of **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

and SL2012 (**Table 1**). However, only small to medium correlations ($0.24 < r < 0.63$) were calculated between the two seed lots. Strongest correlations were found between MGT, T50 and GR36, with $I r I > 0.9$, and between VI and IS ($I r I > 0.7$). Correlations with $0.3 \leq I r I \leq 0.5$ were observed between FG and

the traits VI, MGT, ES, T50 and GR36, as well as between VI and MGT, T50 and GR36 (**Table 2**).

With regard to genetic relatedness, the first four PCA components explained 20.98% of the total genetic variance, while the first two components contributed 7.1% (Comp 1) and 6.5% (Comp 2), respectively (**Figure 1**). Almost all fodder rape genotypes (13 out of 16) were assigned to the same cluster (herein referred to as cluster “green”). Taking k-means clusters into account, an overall F_{ST} value of 0.1448 was observed over the whole diversity set, indicating a medium genetic differentiation. Regarding the population pairs, lowest genetic differentiation was found between clusters “red” and “blue” ($F_{ST} = 0.10$), while strongest differentiation was calculated between clusters “green” and “blue” ($F_{ST} = 0.21$). For all genotype pairs within the diversity set, a mean identity by descent (IBD) of 0.69 was calculated, while values of 0.72, 0.73, 0.62, and 0.76 were found within clusters “black,” “red,” “green,” and “blue,” respectively (**Figure 2**). The largest cluster “blue” comprised 79 genotypes and showed the narrowest distribution, whereas the smallest cluster “green” comprised only 29 genotypes but showed the widest distribution of IBD values.

For all chromosomes except C1, C3, C4, and C8, LD decayed to $r^2 = 0.1$ at a distance between marker pairs ranging from 480 kbp (A1) to 1283 kbp (A9) (**Figure 3**). For chromosomes C3 and C8, r^2 did not drop to 0.1 until the distance between marker pairs reached 2014 kbp and 1939 kbp, respectively. For chromosome C8, strongly conserved LD was observed for marker

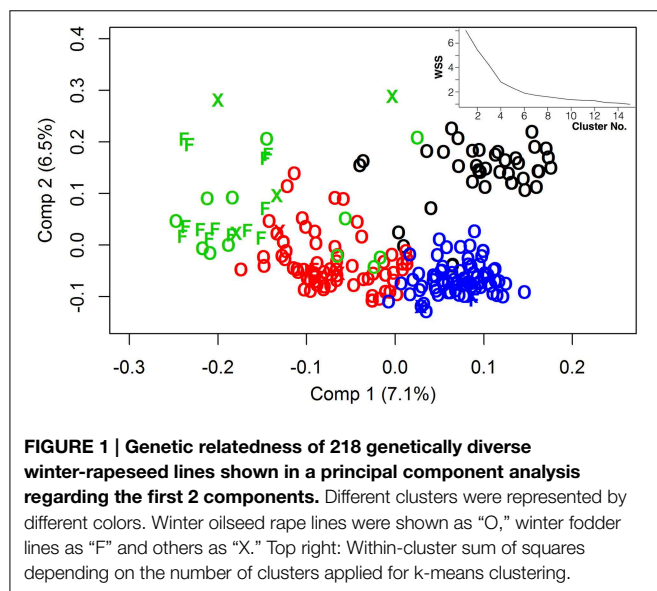


FIGURE 1 | Genetic relatedness of 218 genetically diverse winter-rapeseed lines shown in a principal component analysis regarding the first 2 components. Different clusters were represented by different colors. Winter oilseed rape lines were shown as “O,” winter fodder lines as “F” and others as “X.” Top right: Within-cluster sum of squares depending on the number of clusters applied for k-means clustering.

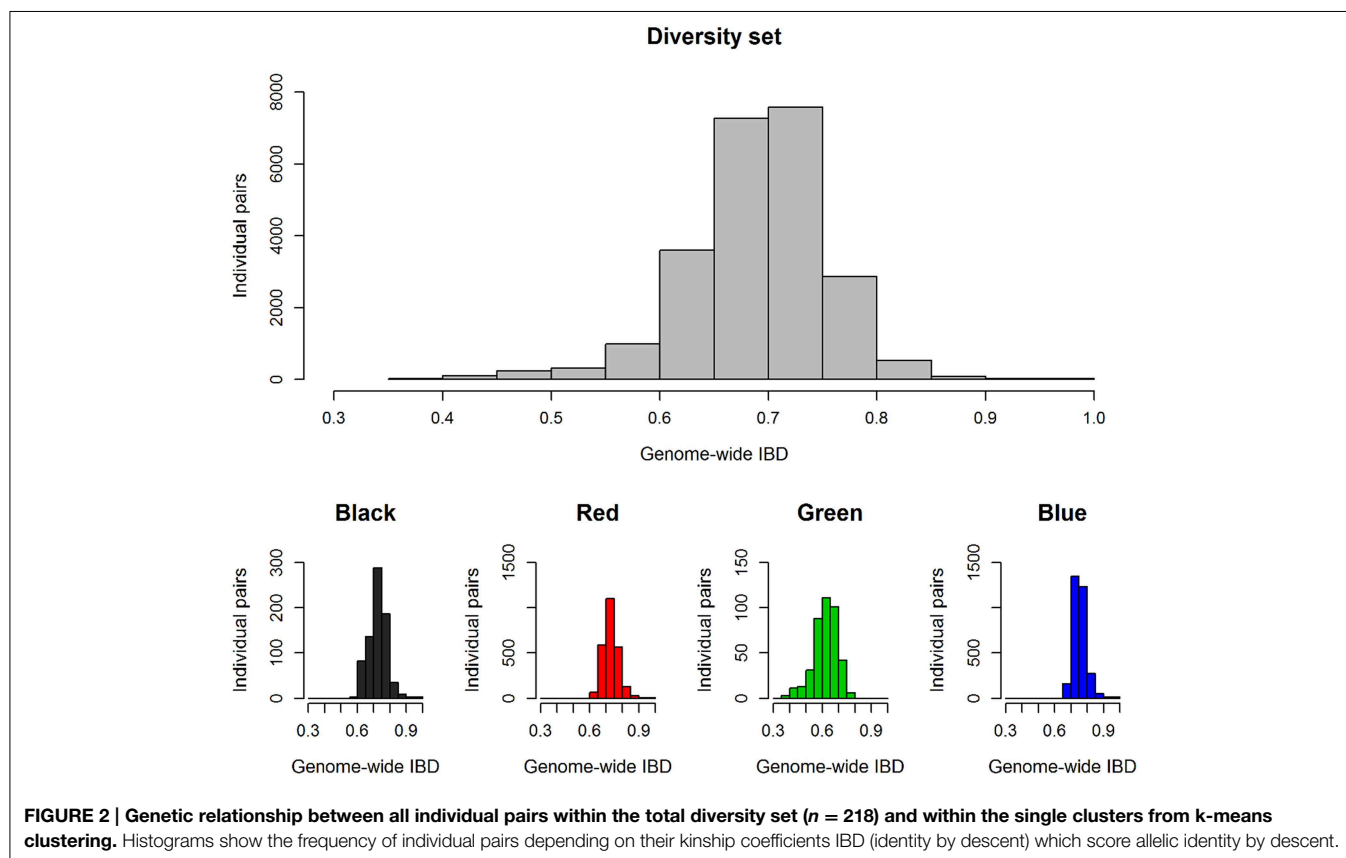
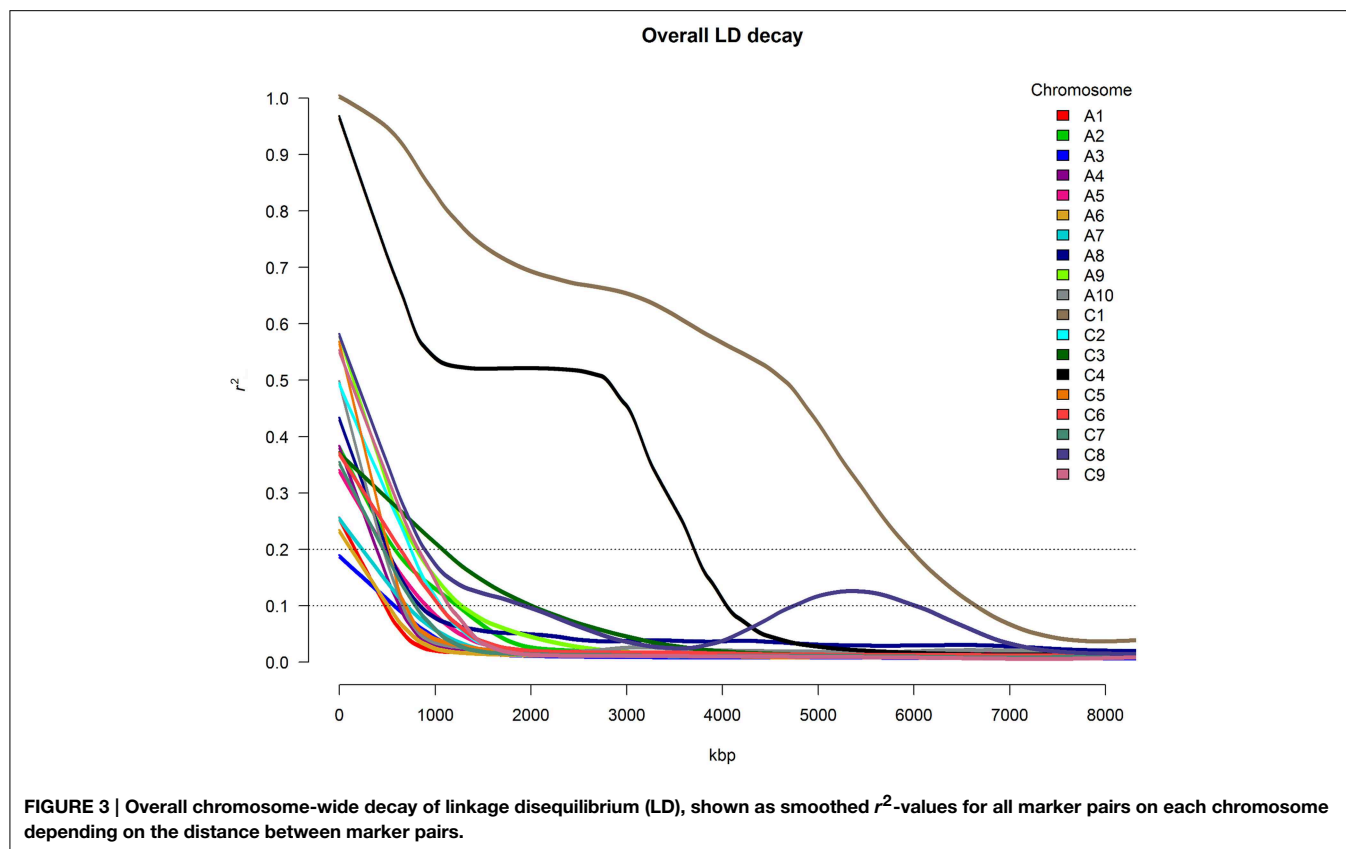


FIGURE 2 | Genetic relationship between all individual pairs within the total diversity set ($n = 218$) and within the single clusters from k-means clustering. Histograms show the frequency of individual pairs depending on their kinship coefficients IBD (identity by descent) which score allelic identity by descent.



distances between 4500 and 6000 kbp. Particularly strong patterns of LD were observed on chromosomes C1 and C4, with r^2 -values above 0.1 for up to 6651 kbp and 4048 kbp, respectively. The very slow average LD decay on chromosome C4 was mainly determined by the presence of a large conserved LD block localized between 15,429 and 20,449 kbp, whereas conserved LD between 17,787 and 26,635 kbp caused the extremely slow LD decay on chromosome C1.

Narrow-sense heritability differed considerably among traits and for some traits also between seed lots (Table 3). The strongest differences between the 2011 and 2012 seed lots were observed for the trait T50, with relatively low heritability observed within the 2011 seed lot and comparatively high heritability within the 2012 seed lot. The traits GR36, ES and TSW showed a medium to high heritability with $h^2 > 0.5$ in all cases. For VI and FG a low heritability was calculated with $h^2 > 0.3$. For GR72 the heritability approached zero for both seed lots. Marker-trait associations that could be confirmed in both seed lots were detected for T50, GR36, GR72, ES, VI, FG, and TSW (Table 3). The observed F_{ST} value revealed a low to medium genetic differentiation within the diversity set (Figure 2), with subdivision into clusters explaining 14.5% of the total genetic variation. Calculation of the inflation factor λ by genomic control (Devlin and Roeder, 1999; data not shown) confirmed that population structure was predominant. Hence, correction for stratification was performed by mixed-model approach including principal component covariates. Subdivision into clusters

caused an increase of IBD values within all clusters except for cluster “green,” which mainly comprises fodder rapes and exotic accessions.

Genome-Wide Association Analysis

The chromosomal regions delineated by haplotype blocks in strong LD ($r^2 > 0.4$) with trait-associated markers harbored a total of 681 genes (Table S2). Several genes ascribed to seed germination, seed dormancy and seed and embryo development were disclosed, associated with T50, GR72, ES, FG, and TSW (Table 4). Further investigation showed that none of the candidate genes listed in Table 4 was known to be involved in a mutual pathway. Identical chromosome regions were associated with T50 and GR72, hence the same candidate genes were assumed on chromosomes A9 and C6. The SNP marker Bn-A06-p23586019, associated with TSW, is localized within the non-coding region of the candidate gene *BnaA06g34100D*. Very strong local LD values ($r^2 > 0.8$) were found between markers Bn-A04-p4074166, Bn-A04-p4217227 and Bn-A04-p10196289, associated with GR72 on chromosome A4, whereas LD with the other surrounding markers was very low ($0 < r^2 < 0.06$). On the other hand, these markers showed strong extra-chromosomal LD to markers Bn-A03-p5072729 and Bn-A03-p16990947 on chromosome A03, both of which also showed associations with GR72. Although this might be due to co-selection of two epistatically interacting gene loci, this may also suggest a spurious allocation of markers Bn-A04-p4074166, Bn-A04-p4217227 and Bn-A04-p10196289 to

TABLE 3 | Intervals of linkage disequilibrium (LD) containing significant marker-trait associations for different traits related to germination performance in seeds of an oilseed rape diversity panel produced in Le Rheu, France in 2011 (SL2011) and Asendorf, Germany in 2012 (SL2012), respectively: Chromosome (Chr), Chromosomal position in base pairs [LD interval (bp)], narrow-sense heritability (h^2), additive allelic effect (Add. eff.), logarithmic scaled P -value ($-\log_{10}(p)$).

Trait	Chr	LD interval (bp)	SL2011			SL2012		
			h^2	Add. eff.	$-\log_{10}(p)$	h^2	Add. eff.	$-\log_{10}(p)$
TSW	A1	2,094,614 – 2,132,814	0.84	–0.23	3.28	0.76	–0.23	2.65
	A5	18,641,259 – 18,796,724		–0.28	2.53		–0.35	3.11
	A6	22,333,133 – 22,588,108		0.26	5.32		0.19	2.95
	C6	21,335,770 – 21,666,697		–0.13	2.66		–0.15	3.07
VI	C7	41,147,197 – 41,318,742	0.23	1.69	3.43	0.18	2.05	2.69
FG	C7	35,089,636 – 35,279,703	0.21	0.91	2.60	0.29	1.11	4.07
T50	A9	32,206,450 – 32,410,348	0.34	2.76	4.80	0.86	1.98	3.06
	C6	31,736,330 – 32,133,403		1.10	2.74		1.23	3.85
	C8	36,326,419 – 36,384,818		1.46	2.60		1.41	3.02
GR36	C8	36,326,419 – 3,638,4818	0.50	–8.36	3.47	0.77	–6.64	2.76
GR72	A3	4,478,594 – 4,515,865	0.07	–2.01	3.16	0.00	–1.78	4.28
		7,082,702 – 7,098,892		–1.96	2.64		–1.82	3.69
		16,074,818 – 16,173,009		–2.44	3.43		–2.01	4.22
	A4	4,102,377 – 4,144,135		–2.37	3.41		–2.01	4.04
		4,260,229 – 4,302,660		–2.21	3.13		–1.99	4.33
		11,328,102 – 11,529,657		–2.17	3.03		–1.79	3.59
	A9	32,206,450 – 3,241,0348		–3.00	3.00		–2.86	3.16
	C6	31,736,330 – 32,133,354		–1.59	3.10		–1.06	2.58
	C9	42,980,135 – 43,015,797		–1.89	2.70		–1.91	4.68
	A9	6,512,728 – 7,122,368	0.62	–0.04	2.75	0.72	–0.05	4.22
		11,631,861 – 11,677,068		–0.07	3.00		–0.08	3.14
		16,085,669 – 16,164,217		0.05	3.15		0.05	3.00
ES	C2	12,742,547 – 14,316,723		0.04	2.63		0.04	3.37
	C6	25,600,351 – 25,671,165		0.04	2.68		0.06	4.73

Abbreviations of traits are explained in Materials and Methods.

the wrong chromosome, hence they were excluded from further analysis.

Two of three particularly promising candidate genes were associated with blocks of strong LD containing QTL for different traits on chromosomes A9 (two independent QTL) and A10. The first of these three, *BnaA09g48160D*, is located on chromosome A9 only 80.789 kbp from marker Bn-A09-p35262679, which shows significant associations to T50 ($-\log_{10}(p) = 4.8$ and 3.1 and GR72 ($-\log_{10}(p) = 3.2$ and 3.0), but exhibits almost no LD to its nearest flanking markers (Tables 3, 4, Figure 4). *BnaA09g48160D* encodes for an ortholog of *A. thaliana* gene *ARABIDOPSIS TWO-COMPONENT RESPONSE REGULATOR (ARR4)*. In another independent association peak between 6.51 and 7.12 Mbp on chromosome A9 we localized the gene *BnaA09g12770D*, between two adjacent markers with very strong LD that both show significant associations to ES (Tables 3, 4, Figure 5). The SNP marker Bn-A09-p6021939 (associated with $-\log_{10}(p) = 4.1$ and 2.8 to ES) is located 141.051 kbp from *BnaA09g12770D*, which codes for an ortholog of the *A. thaliana* gene *SNOWY COTYLEDON 1 (SCO1)*. Another significant association peak for ES was localized on chromosome A10 (Tables 3, 4). This region harbors an LD

block containing four markers (Bn-A10-p15378551, Bn-A10-p15372875, Bn-A10-p15367240, and Bn-A10-p15361519) the latter of which lies directly adjacent (9.701 kbp) to the gene *BnaA10g24850D* (Figure 6). *BnaA10g24850D* is an ortholog of another *A. thaliana* gene implicated in germination and early development, *ARGINYL-t-RNA PROTEIN TRANSFERASE 1 (ATE1)*.

Pigment concentrations were measured in the cotyledons of 5d old seedlings from the two genotype bulks, featuring consistently low and high ES values, respectively. Mean concentrations of chlorophyll a and b were found to be significantly lower in genotypes with poor performance in ES (Figure 7).

Marker haplotypes for all disclosed regions associated with T50 and ES are displayed in Figure 8, which compares the most frequent locus-specific haplotypes with favorable and undesirable effects on trait performance. Because a threshold of $r^2 > 0.4$ was chosen for LD block definition, a smaller fraction of recombinant haplotypes also occurred. A list of all observed haplotypes and their frequencies is provided in Table S3. With increasing number of favorable alleles, performance is shown to be improved for both T50 and ES (Figure 9). For T50 an increase in the number of undesirable haplotypes was associated with a decrease in

TABLE 4 | List of candidate genes ascribed to seed germination, dormancy and seed development: Chromosome (Chr), Name of the candidate gene (Candidate gene) and absolute chromosomal position in base pairs [Position (bp)].

Trait	Chr	Candidate gene	Position (bp)
TSW	A6	BnaA06g34100D	22,551,497 – 22,558,918
		BnaA06g33970D	22,490,437 – 22,491,055
		BnaA06g33830D	22,421,795 – 22,424,461
		BnaA06g33880D	22,446,550 – 22,447,630
FG	C6	BnaC06g19160D	21,499,780 – 21,506,256
	C7	BnaC07g31020D	35,273,679 – 35,275,807
T50	A9	BnaA09g48160D	32,313,276 – 3,231,4748
	C6	BnaC06g31150D	31,787,778 – 31,792,013
GR72	A9	BnaA09g48160D	32,313,276 – 32,314,748
	C6	BnaC06g31150D	31,787,778 – 31,792,013
	C9	BnaC09g40630D	42,994,450 – 42,995,360
		BnaC09g40640D	42,997,059 – 42,998,540
ES	A9	BnaA09g12770D	6,802,082 – 6,804,959
		BnaA09g12820D	6,856,835 – 6,858,406
		BnaA09g12800D	6,834,031 – 6,835,814
		BnaA09g13040D	7,099,948 – 7,100,930
	A10	BnaA10g24850D	16,143,505 – 16,146,463
		BnaA10g24780D	16,125,049 – 16,127,616

Abbreviations of traits are explained in Materials and Methods.

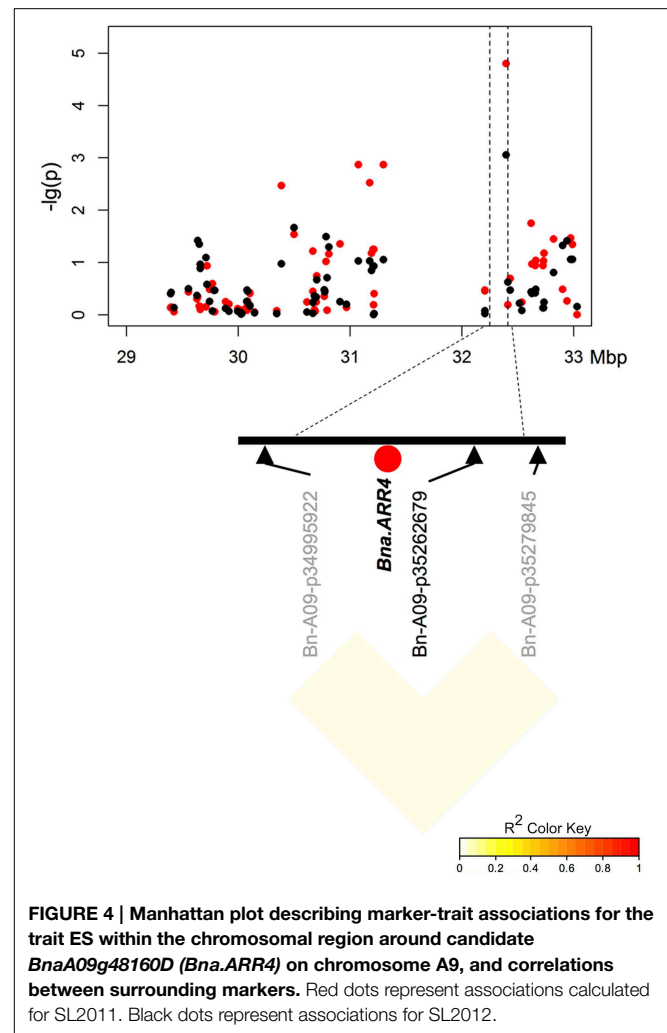
the number of individuals per bulk (Table S3). While the main fraction of lines ($n = 133$) exhibited the favorable haplotype at all three loci, a combination of all three unfavorable alleles was observed in only one line. For ES, neither a combination of all favorable, nor all undesirable haplotypes occurred, with most individuals ($n = 105$) exhibiting 2 favorable alleles (Table S3).

Discussion

The Key Elements of Seed Germination

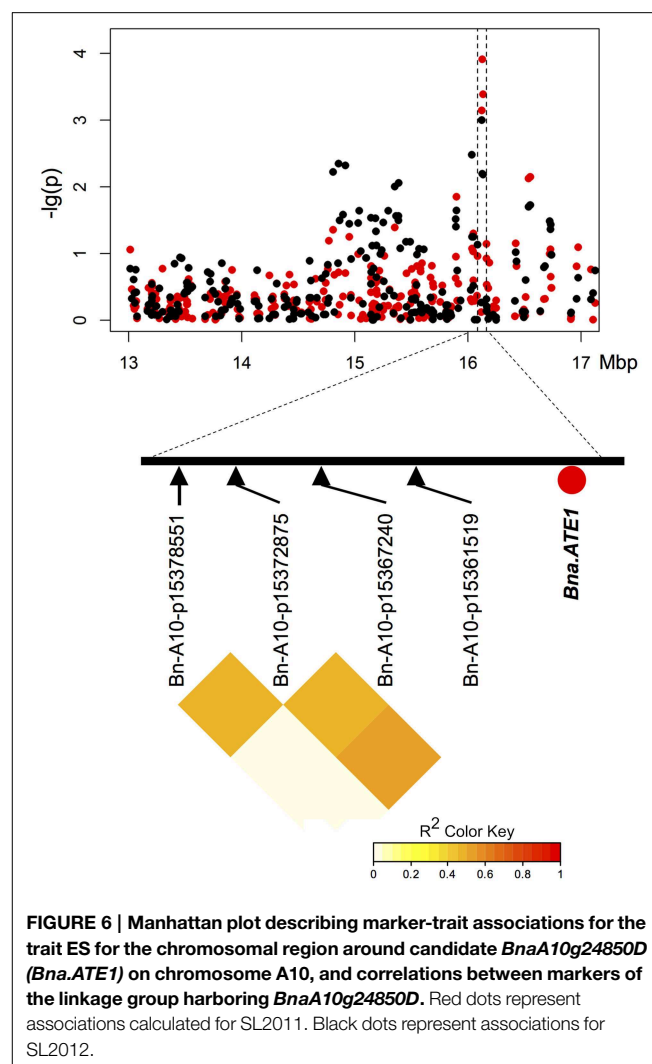
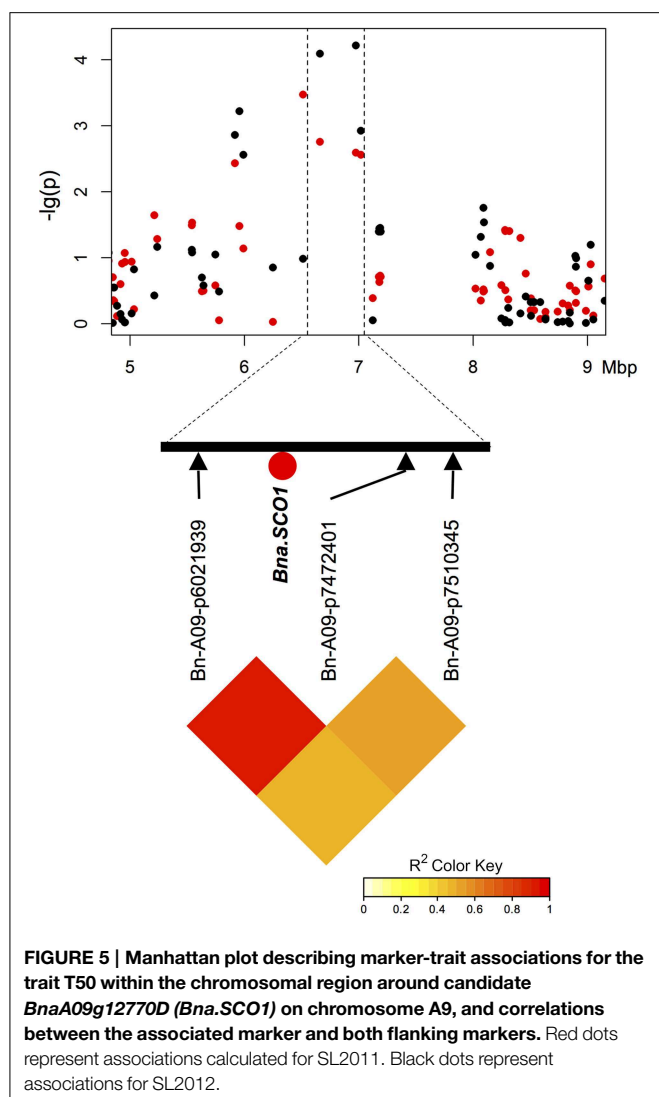
Seed germination has a decisive influence on homogeneous field emergence and successful seedling establishment and is therefore a basic target for the development of vigorous crop plants with stable yield performance. According to Finch-Savage et al. (2010), germination speed is a major key element of vigorous seeds, along with a rapid initial downward and upward seedling growth.

While traits like seed imbibition speed (IS) and seed volume increase (VI) can be used for characterization of initial water uptake within the first hours after soaking, mean germination time (MGT), time to reach 50% of germination (T50), germination rate within 36 h (GR36) and first germination (FG) provide insight into the speed of the total germination process until the radicle breaks through the testa. Elongation speed of the radicle (ES) is not part of the germination process *per se*, but is an important component of pre-emergence seedling growth after entry into the third phase of water uptake (Bewley, 1997). Although seed vigor is constituted by both germination speed and pre-emergence seedling growth, these two criteria are presumed to contribute independently to seed vigor performance (Dutt and



Geneve, 2007). This assumption is strengthened by the very low or absent phenotypic correlations we observed between ES and germination speed parameters (FG, MGT, T50, GR36) in the *B. napus* diversity panel. Interestingly, we found no evidence for a decisive influence of seed weight (TSW) on germination performance, absolute germination rate or radicle growth, with only extremely low correlations being observed to these parameters. This is in accordance to Bettey et al. (2000), who attributed no influence of seed weight to mean germination time or absolute germination in *Brassica oleracea*. On the other hand, Hanumiah and Andrews (1973) described a positive relationship between seed size and seedling growth for *B. rapa* and *B. oleracea*. According to our observations TSW should be regarded as a seed yield parameter in oilseed rape rather than a contributing factor for germination performance.

Weak correlations between germination speed (T50, MGT, GR36, and FG) and seed imbibition in terms of VI and IS indicate that the velocity of total germination is impacted only to a limited extent by initial water uptake. In turn, induction of the biochemical processes contributing to embryo expansion and leading to visible germination seem to be mainly independent



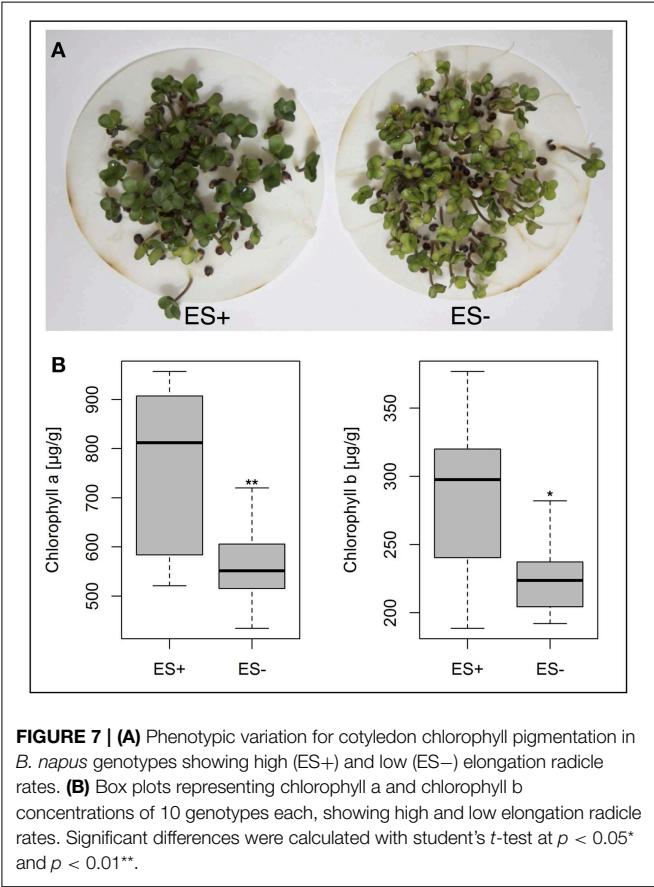
from imbibition speed. Apparently other factors induce the release of germination, and in this context it is worthwhile to consider seed dormancy. According to Finch-Savage and Leubner-Metzger (2006), seed dormancy is an innate seed property that defines the environmental conditions that must be met before the seed can germinate. With respect to the diverse regulation levels, dormancy can be divided into different dormancy types (Baskin and Baskin, 2004). For members of the *Brassicaceae* it is assumed that mainly physiological dormancy is predominant (Müller et al., 2006). Our results indicate that differences in germination speed in *B. napus* are not solely dependent on water availability or the presence of favorable conditions for the induction of biochemical actions contributing to seed germination. In fact, the observed variation in germination speed might be caused by differences in the manifestation of seed dormancy. However, because the *in vitro* germination assay we applied implements optimal and controlled germination conditions, two explanations are possible: On the one hand, the diversity panel we investigated appears to exhibit differences in genetically determined responsiveness to dormancy release factors. On the other hand,

the observed differences in germination performance also appear to be strongly determined by the conditions predominant during and after seed production. The latter hypothesis is strengthened by the observation of only moderate correlations between the two seed lots (Table 1). As both seed lots were tested for germination under constant laboratory conditions, the observed differences in performance between the two seed lots must reflect genotype by environment interactions during seed ripening in the field and/or during the subsequent seed storage. One possible mechanism could be epigenetic imprinting during seed development due to stress conditions in the maternal environment.

Exploring the Genetic Basis of Seed Germination and Seedling Growth

Against the background of a strong environmental dependence of seed germination and early seedling growth, breeders are interested in the fixed fraction of variables influencing seed vigor performance. Although the physiology of seed germination and dormancy is well studied and extensively described in literature (e.g., reviewed in Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008; Rajjou et al., 2012), there is still a lack of knowledge about

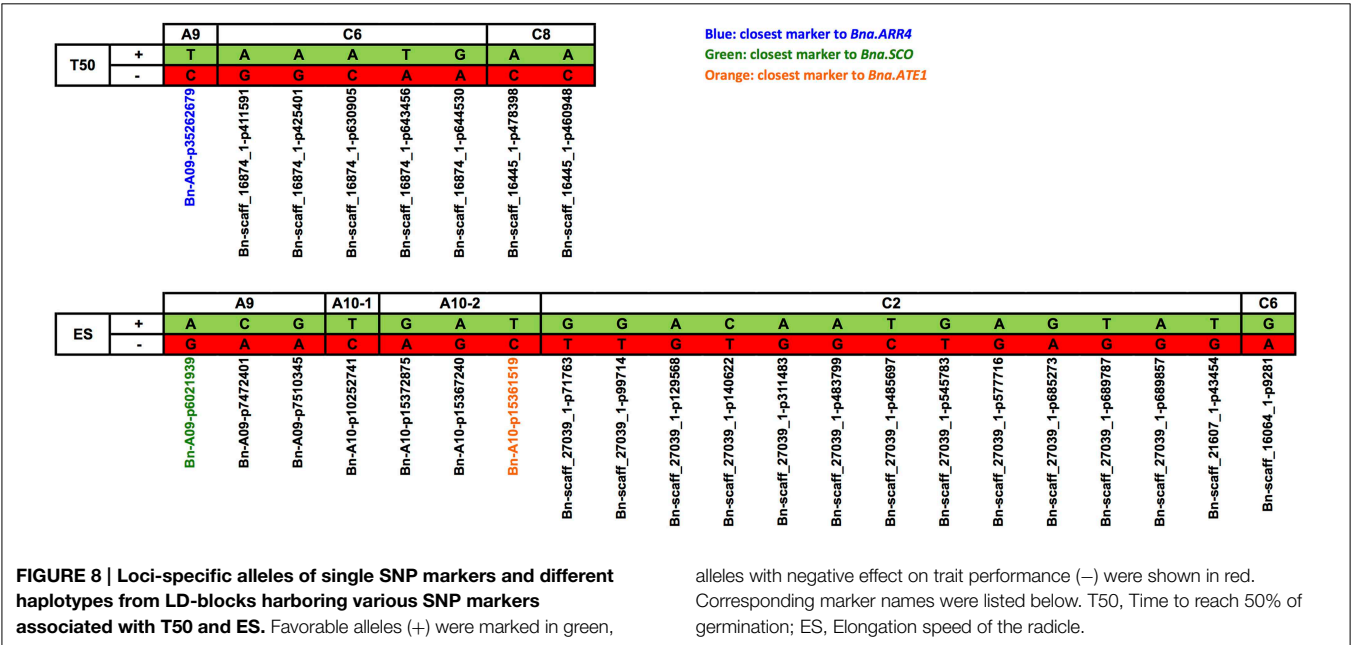
individual genes involved in these complex traits, particularly in crop species. Both germination and dormancy underlie polygenic control that can only be elucidated by quantitative genetics approaches.



To estimate how much genotypic variation contributes to the observed phenotypic variation we calculated narrow-sense heritability (h^2), which captures only that part of genetic variation caused by additive genetic values (Visscher et al., 2008). Narrow-sense heritability can only be calculated based on the detected associations, so that undetected associations may repress h^2 -values. The observed high h^2 -values for TSW agree with findings from earlier studies (Basunanda et al., 2010; Fan et al., 2010; Khan et al., 2013; Xing et al., 2014). In contrast, very low h^2 -values for GR72 suggested that the absolute germination rate is almost completely dependent on the environmental state and can hardly be influenced by selection. Nevertheless, identical associations for T50 and GR72 on chromosomes A9 and C6 indicate that both germination speed and absolute germination rate seem to be controlled by the same genetic factors (see Table 3), so that selection for T50 may also lead to improvement of GR72.

The candidate gene disclosure strategy we applied accounted for trait associations that were detectable in both of the investigated seed lots. The use of local LD to delineate regions of interest around significantly associated SNP markers proved a valuable method for defining potential candidate genes. The very strong LD on some C-sub-genome chromosomes corresponds to findings in other *B. napus* gene pools and reflects extreme selection for specific seed quality traits (Li et al., 2014; Qian et al., 2014). Strong LD conservation can complicate candidate gene identification from association peaks, hence it is important to consider local LD when defining QTL confidence intervals for candidate gene selection. The validity of the trait associations we detected appears to be confirmed by the identification of comparatively small regions of strong LD that harbor highly interesting genes involved in processes closely related to seed germination and seedling development.

Several genes ascribed to seed germination, dormancy, seed development and radicle emergence were identified within the associated regions (Table 4). For example, the *A. thaliana* gene



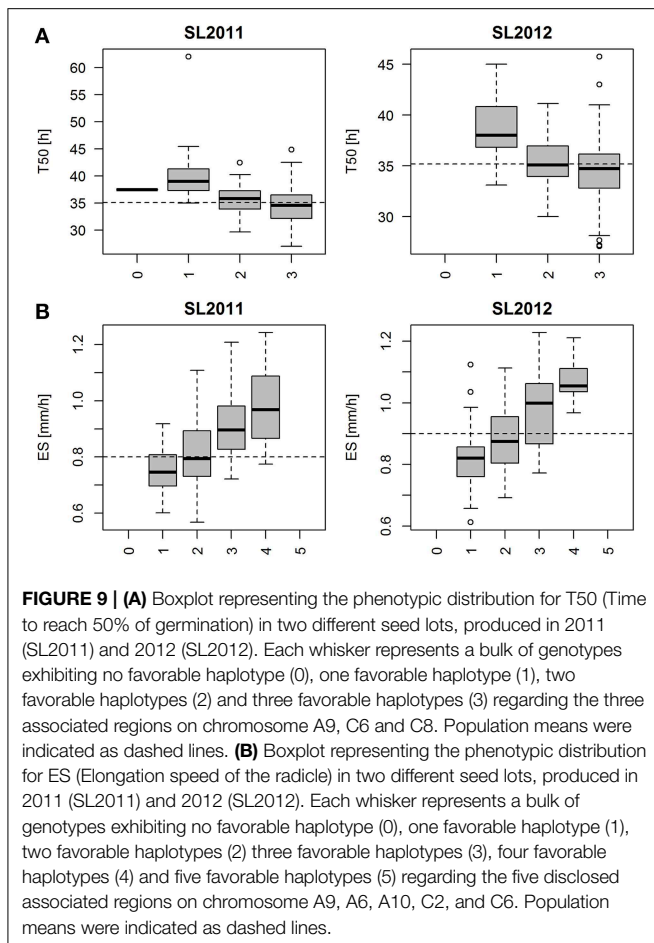


FIGURE 9 | (A) Boxplot representing the phenotypic distribution for T50 (Time to reach 50% of germination) in two different seed lots, produced in 2011 (SL2011) and 2012 (SL2012). Each whisker represents a bulk of genotypes exhibiting no favorable haplotype (0), one favorable haplotype (1), two favorable haplotypes (2) and three favorable haplotypes (3) regarding the three associated regions on chromosome A9, C6 and C8. Population means were indicated as dashed lines. **(B)** Boxplot representing the phenotypic distribution for ES (Elongation speed of the radicle) in two different seed lots, produced in 2011 (SL2011) and 2012 (SL2012). Each whisker represents a bulk of genotypes exhibiting no favorable haplotype (0), one favorable haplotype (1), two favorable haplotypes (2) three favorable haplotypes (3), four favorable haplotypes (4) and five favorable haplotypes (5) regarding the five disclosed associated regions on chromosome A9, A6, A10, C2, and C6. Population means were indicated as dashed lines.

ARR4 is a cytokinin-dependant antagonistic regulator of transcription activator *ABI5*, which is directly involved in the ABA mediated regulation of seed germination (Wang et al., 2011). The presence of a *Bn.ARR4* homolog in a region of LD with an association peak for T50 and GR72 corresponds to the expected role of ABA-mediated dormancy and germination release. In *A. thaliana* the gene *SCO1* was found to encode a plastidic translation elongation factor G, which is reported to be essential for eoplast to chloroplast transition during early germination (Ruppel and Hangarter, 2007). Furthermore, *A. thaliana* *sco1* mutations also cause a chlorophyll deficiency resulting in pale-green to white coloring of seedling cotyledons, and the seedling chlorophyll deficiency of *sco1* mutants is coupled with a delayed seed germination and development (Albrecht et al., 2006). Accordingly, *B. napus* genotypes with extreme segregation for ES also showed significant differences in seedling chlorophyll content. Associations of *Bna.SCO1* haplotypes to ES, together with the corresponding chlorophyll phenotypes, support a causal role for this gene in the speed of early radicle growth in *B. napus*. Mutations in *ATE* genes encoding for arginyl-t-rna protein transferases have also been shown to cause reductions in *A. thaliana* seedling root growth (Holman et al., 2009). Allelic differences in *Bn.ATE1* within LD haplotypes associated with ES are hence also strong positional and functional

candidates for control of radicle growth during and after germination.

Genotypic classification for T50 and ES revealed that both traits can potentially be improved by pyramiding of favorable alleles (Figures 9A,B). For both traits the absence among the diversity panel of individuals with three (T50) or five (ES) unfavorable haplotypes indicates that strong natural and artificial selection has occurred in cultivated forms of *B. napus* against individuals with very poor germination and vigor. Consequently, effects of loci associated with these traits might be underestimated because specific undesirable allele combinations are absent, thus restricting the extent of phenotypic variability within the diversity set. For ES we also observed a lack of favorable haplotype combinations, indicating that early seedling growth could be substantially improved beyond the phenotypic diversity in the present study.

Conclusions

Large-scale automated phenotyping revealed a broad phenotypic variability in germination performance across a *B. napus* diversity panel. As expected, narrow-sense heritability revealed that germination performance is influenced by both genetic as well as environmental factors. As the latter were constant during *in vitro* phenotyping, the observed differences in germination performance between two different seed lots could be attributed to the conditions predominant during seed production and storage. No relationship of germination traits to seed weight could be detected. Instead, the results indicate that genetic determinants for the manifestation of seed dormancy may be a decisive factor influencing inheritance of germination performance in *B. napus*. In a genome-wide association analysis several promising genes, including *Bna.SCO1*, *Bna.ARR4*, and *Bna.ATE1*, were found within regions showing LD with QTL for seed germination and vigor. Combination of positive alleles for the loci we identified should facilitate a decisive increase in germination performance and early seedling growth in oilseed rape, improving prospects for breeding of these complex, poorly selectable traits. As such the study provides a basis for the establishment of genomic selection tools for improved seed germination and seed vigor in rapeseed.

Author Contributions

SH and RS designed the study and interpreted the results. RS, NN, AA, and GL developed the diversity panel and generated seed lots for the phenotypic analysis. NN, AA, GL, and FB performed field phenotyping and analyzed the field phenotype data, SD and MW performed the automated germination phenotyping, and FB generated the genome-wide SNP data. SH and MF performed the GWAS analysis. SH and RS wrote the manuscript and all authors corrected and approved the final version.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00221/abstract>

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2.2. Early osmotic adjustment responses in drought-resistant and drought-sensitive oilseed-rape

2.2.1 Publication outline

The publication in the following chapter describes detailed physiological experiments in which rapeseed seedlings were investigated for their adaptive potential to osmotic stress in controlled climate chamber experiments. Metabolites assumed to be potential players involved in osmotic adjustment were quantified in drought-sensitive and drought-resistant winter oilseed rape lines and hybrids representing current breeding germplasm. Additional parameters, such as fresh and dry weight as well as leaf area, were monitored to capture the impact of osmotic stress on seedling growth and development. Furthermore, physiological traits such as shoot water content and leaf temperature were gathered as indicators for plant water status and transpirational activity. In order to get molecular insights into the control of drought stress recognition and signalling, patterns of important phytohormones as well as their precursors and catabolites were screened. For these purposes a novel hydroponic cultivation system was developed in which osmotic stress can be simulated by application of polyethylene glycol (PEG). The use of PEG assures a strict control of stress timing, duration and intensity. The study aimed to identify reliable physiological and metabolic markers suitable for selection of rapeseed germplasm with enhanced potential for drought adaptation during the seedling stage. Furthermore, a general link was made between drought adaptation during seedling stages and the resulting exploitation of yield potential.

Early osmotic adjustment responses in drought-resistant and drought-sensitive oilseed rape

Sarah Hatzig¹, L. Irina Zaharia², Suzanne Abrams³, Marie Hohmann¹, Laurie Legoahec⁴, Alain Bouchereau⁴, Nathalie Nesi⁴ and Rod J. Snowdon^{1*}

¹Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, 35392, Giessen, Germany, ²National Research Council of Canada, Aquatic and Crop Resource Development, Saskatoon, Saskatchewan S7N 0W9, Canada, ³Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C9, Canada, ⁴UMR 1349 INRA-AgroCampus Ouest-Université de Rennes 1, Joint Laboratory for Genetics, Environment and Plant Protection (IGEPP), Bât. 301, BP 35327 35653, Le Rheu Cedex, France. *Correspondence: rod.snowdon@agrar.uni-giessen.de

Abstract The impact of osmotic stress on growth, physiology, and metabolism of winter oilseed rape (*Brassica napus* L.) was investigated by detailed analysis of biomass traits, hormone metabolites and osmolytes in two genetically unrelated drought-tolerant genotypes and two unrelated drought-sensitive genotypes. Seedlings were grown *in vitro* under controlled conditions and osmotic stress was simulated by applying a gradual treatment with polyethylene glycol (PEG 6000), followed by hypo-osmotic treatment of variants used for metabolite determination. The results provide a basis for the identification of reliable selection criteria for drought resistance in oilseed rape. The *in vitro* cultivation system established during this study enabled effective discrimination of early osmotic stress responses between drought-resistant and -susceptible oilseed rape genotypes that also show large differences in relative seed yield under drought conditions in the field. Clear physiological and metabolic differences were

observed between the drought-resistant and drought-sensitive genotypes, suggesting that osmotic adjustment is a key component of drought response in oilseed rape. Unexpectedly, however, the drought-resistant genotypes did not show typical hormonal adjustment and osmolyte accumulation, suggesting that they possess alternative physiological mechanisms enabling avoidance of stress symptoms.

Keywords: Absciscic acid; *Brassica napus*; drought; hormones; metabolites

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INTRODUCTION

Drought stress is one of the main limiting factors for plant productivity worldwide. Recent extreme climatic events, reflecting climatic changes documented in the last decades, have shown that water shortage is not only a problem for arid zones but is also becoming more and more important for the temperate European zone (Ciais et al. 2005; Collins et al. 2009). The variable European climate demands breeding of cultivars adapted to a broad environment in which rainy periods are frequently followed by periods of water shortage. To avoid limitations of agricultural productivity during water deprivation, breeding of drought compatibility is of overriding importance.

The physiology of a plant strongly depends on the given environmental conditions. Changes in water availability always require a rapid adaptation of plant metabolism in order to regain a balanced relationship between the plant and the environment. Physiological and anatomical changes contributing to the maintenance of water provision under drought conditions are the result, as higher plants generally maintain a steady-state water potential, which is greater than that of their environment (Walter and Stadelmann 1968). Plants have different possibilities to adjust to water scarcity and thus to

protect their tissue from water losses. According to Levitt (1980), higher plants can be classified into two major categories, for which he coined the terms “water savers” and “water spenders.” A water saving species maintains its water relations by minimizing water losses via a strong control of stomatal conductance along with beneficial morphological properties. Water spenders pursue another strategy, maintaining their water balance by an increase in water uptake while transpiration remains unaffected. Osmotic adjustment has been discussed as a water-spending characteristic (Levitt 1980), as it forms the basis for maintenance of turgor pressure and therefore maintenance of plant growth under drought conditions. More recently, it was proposed that the main mechanisms of dehydration avoidance are accumulation of solutes and cell wall hardening (Verslues et al. 2006). The accumulation of so-called “compatible solutes”—which do not disrupt the equilibrium of cellular metabolic processes and are nontoxic even under high cytosolic concentrations—causes a decrease of cell osmotic potential. This allows water uptake from the growth medium even under lower water potentials.

In this study, we present a detailed investigation of the impact of osmotic stress on the growth, physiology, and metabolism of winter oilseed rape (*Brassica napus* L.)

seedlings. The aim of the study was to clarify (i) if osmotic stress triggers specific adjustment processes, like osmolyte accumulation and hormone responses, in seedlings of this important oilseed crop; and (ii) whether drought resistance in oilseed rape can be attributed to differences in these adjustment processes, or if additional response mechanisms are likely to be involved.

RESULTS

Vegetative plant growth and leaf area

After 4 d of *in vitro* osmotic stress, no effects on fresh weight could be observed in any of the four genotypes we investigated (data not shown). Fresh and dry weights (DW) of shoots and roots were, however, affected after 10 d of *in vitro* osmotic stress application (Figure 1).

During *in vitro* osmotic stress conditions, the two sensitive genotypes “M17473-06” and “NPZ208-03” (hereinafter referred to as S1 and S2) reacted with significant decreases in shoot fresh weight and DW. In contrast, neither parameter showed significant changes in the two resistant genotypes “Ferdie” and “P09H60569,” hereinafter referred to as R1 and R2 (Figure 2A). Decreases of 46% and 60% in the shoot fresh weight were observed for S1 and S2, respectively (Figure 2A). Similar observations were recorded for the shoot DWs, whereby S1 responded with a decrease of around 26% and S2 with a decrease of 52% (Figure 2B). Average leaf area was reduced under osmotic stress conditions for all genotypes; however, only the decreases for S1 and S2 could be statistically confirmed (Figure 3). S1 showed a reduction in leaf area of around 47% in the stress treatment relative to the control, whereas the stress treatment in S2 caused a reduction in leaf area of around 61% relative to the control (Figure 3). Besides plant growth, tissue water content can be regarded as a relevant parameter for the characterization of short-term osmotic stress reactions. All genotypes showed significant decreases of 28% (S1), 20% (S2), 19% (R1), and 11% (R2), respectively, in shoot water content under stress conditions (Figure 4).

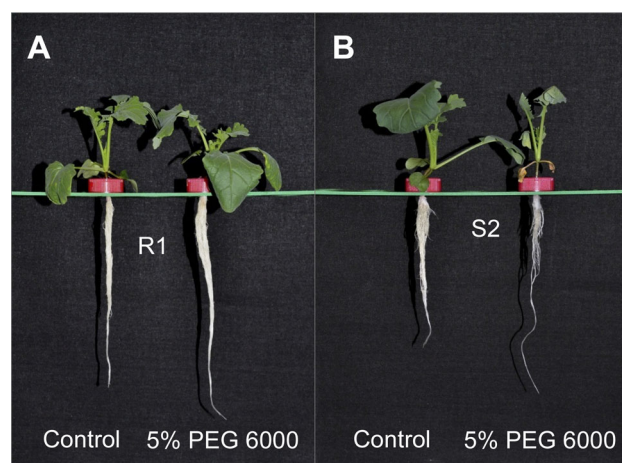


Figure 1. Visible effect of *in vitro* osmotic stress on morphology of (A) the drought-resistant oilseed rape genotype R1, and (B) the drought-sensitive genotype S2, in plants grown under control conditions or treated with 5% PEG 6000 for 8 d, respectively

Whereas R1 shows no visible symptoms of the stress treatment, in S2 a strong reduction of root biomass is visible under the osmotic stress conditions, along with typical wilting and dehydration symptoms.

Leaf temperature

Leaf temperature has been shown to be negatively related to transpiration intensity (Hanson and Hitz 1982; Kumar et al. 1984; Singh et al. 1985; Munns et al. 2010). Therefore, leaf temperature measurement is a practical method for an indirect characterization of transpirational changes.

Leaf temperature showed no significant differences between the four genotypes under control conditions, ranging from 17 to 18°C (data not shown). After 24 h on 2.5% polyethylene glycol (PEG), all genotypes were able to retain

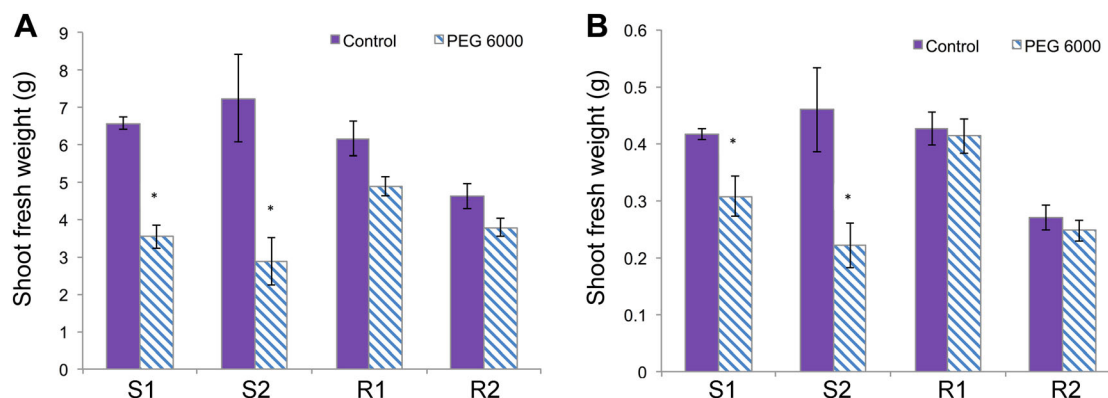


Figure 2. Effect of *in vitro* osmotic stress on (A) shoot fresh weight and (B) shoot dry weight of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured after 8 d on 5% PEG 6000
Bars are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

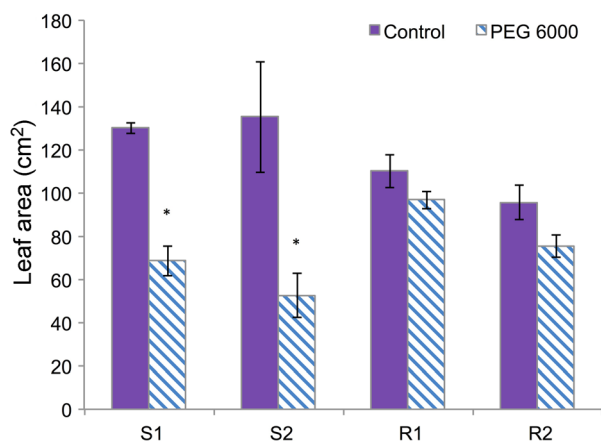


Figure 3. Effect of *in vitro* osmotic stress on total leaf area of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured after 8 d on 5% PEG 6000

Bars are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

the same leaf temperature as the control plants (Figure 5). After 2 d on 2.5% PEG, however, S1 and S2 showed a significant increase in leaf temperature to 118% and 125%, respectively, while R1 and R2 remained unaffected by the stress treatment. Similar values were measured on Day 3 (5% PEG), when the leaf temperature in S1 and S2 increased further in the stress treatment to 121% and 127%, respectively, while R1 and R2 reacted with slight but non-significant increases in their average temperature levels. On Day 4, the leaf temperature was still significantly increased for S1 and S2, but with declining values of 114% and 116%. After removing the osmotic stress

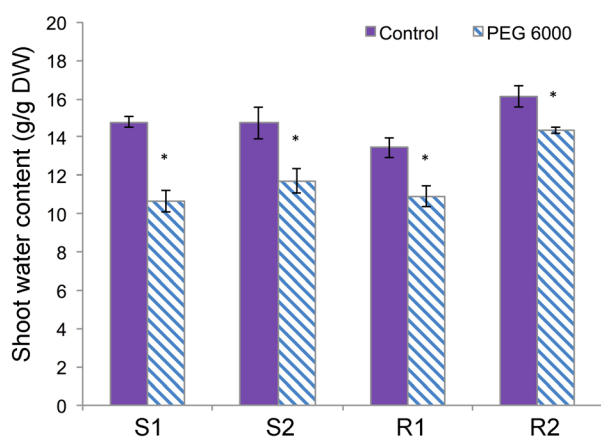


Figure 4. Effect of *in vitro* osmotic stress on shoot water content of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured after 8 d on 5% PEG 6000

Bars are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

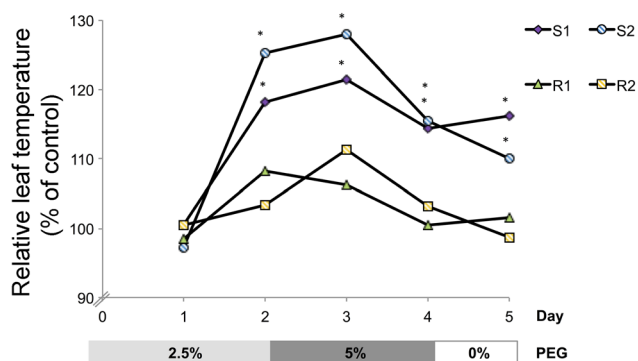


Figure 5. Effect of *in vitro* osmotic stress on leaf temperature of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes on every day of the stress treatment (Days 1–4) and 24 h after removing the osmotic stress (Day 5)

Points represent the mean percentage of leaf temperature reached under *in vitro* osmotic stress conditions relative to control. *Significances refer to differences between the absolute temperature values at $P < 0.1$.

(Day 5), a further decline in relative leaf temperature was measured for S1; however, both drought-sensitive genotypes still showed significantly higher temperatures compared with the control treatment. In the resistant genotypes R1 and R2, a slight increase in temperature was observed; however, the differences between the treatments were non-significant.

Hormone analysis

The quantification of auxins, gibberellins, and cytokinins did not reveal clear response patterns to *in vitro* osmotic stress (data not shown). On the other hand, an effect of osmotic stress on shoot abscisic acid (ABA) levels was observed. Under osmotic stress conditions, the average concentrations of ABA and ABA catabolites, measured on Day 4 of the stress treatment, were significantly increased in the shoot of S1 (200% relative to control) (Figure 6). A corresponding 30% increase in average ABA levels in S2 could not be confirmed as significant. On the other hand, concentrations of ABA and ABA catabolites remained unaffected by osmotic stress in R1 and R2. Concentrations of biologically active ABA showed a similar distribution: in S2, a significant increase was measured but all other genotypes were not significantly affected by the *in vitro* osmotic stress. Comparisons of ABA concentrations with ABA catabolites showed equivalent distributions, with concentrations being significantly increased in S1. S2 reacted with a tendential but non-significant increase, while ABA levels in R1 and R2 were unaffected by the stress treatment (data not shown).

Proline

Under *in vitro* osmotic stress conditions, shoot proline concentrations were increased in all genotypes (Figure 7). The sensitive genotypes S1 and S2 reacted with stronger increases, however, peaking with maximum concentrations on Day 4 of the stress treatment. On the same day, the highest differences were found between the treatments for S1 and S2,

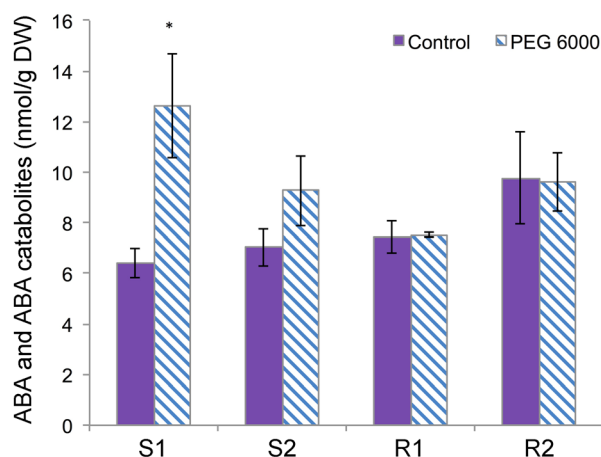


Figure 6. Effect of *in vitro* osmotic stress on concentrations of abscisic acid (ABA) and ABA catabolites of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured on Day 4 of the stress treatment

Bars are means of three replicates with standard errors.

*Significant differences at $P < 0.1$.

with threefold higher proline concentrations in the stress treatment than in the control. For the resistant genotypes R1 and R2, the highest differences were already observed on Day 3, when twofold higher proline concentrations were

measured in the stress treatment. After hypo-osmotic treatment on Day 5, the sensitive genotypes S1 and S2 showed a reduction in proline concentration again.

Ornithine

Average ornithine concentrations in the shoot were significantly decreased on almost all days of the stress treatment for S1 and S2 (Figure 8). On the other hand, R1 showed no differences between the treatments during the stress period but reacted with a strong increase in ornithine concentration after removal of the osmotic stress. For R2 significant differences could only be found on Day 4, when the ornithine concentration increased in the osmotic stress treatment.

Sugars and sugar alcohols

Significant differences in fructose, glucose, sucrose, and myo-inositol concentration were first measured after 4 days of *in vitro* osmotic stress (Figure 9). The two sensitive genotypes S1 and S2 reacted with significant increases in myo-inositol, sucrose, and fructose concentrations. The sensitive genotype S1 revealed a significantly higher glucose concentration in the stress treatment, while an increase in average glucose level was also observed for S2 but could not be statistically confirmed. The resistant genotype R1 showed no differences in sugar or myo-inositol concentrations between the treatments during the whole stress period. For R2, a significant decrease in the concentrations of glucose, fructose, and sucrose was measured under stress conditions, whereas the myo-inositol concentration remained unaffected. After removing the osmotic stress, sugar and myo-inositol concentrations in the

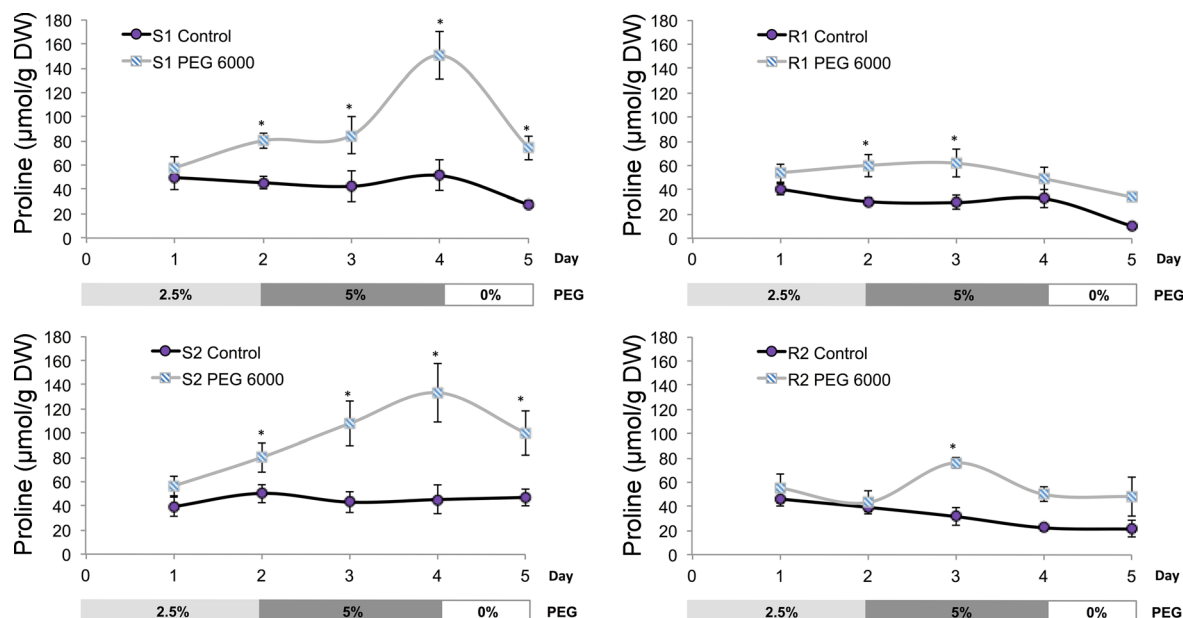


Figure 7. Effect of *in vitro* osmotic stress on shoot proline concentrations of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured on every day of the stress treatment (Days 1–4) and 24 h after removing the osmotic stress (Day 5)

Points are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

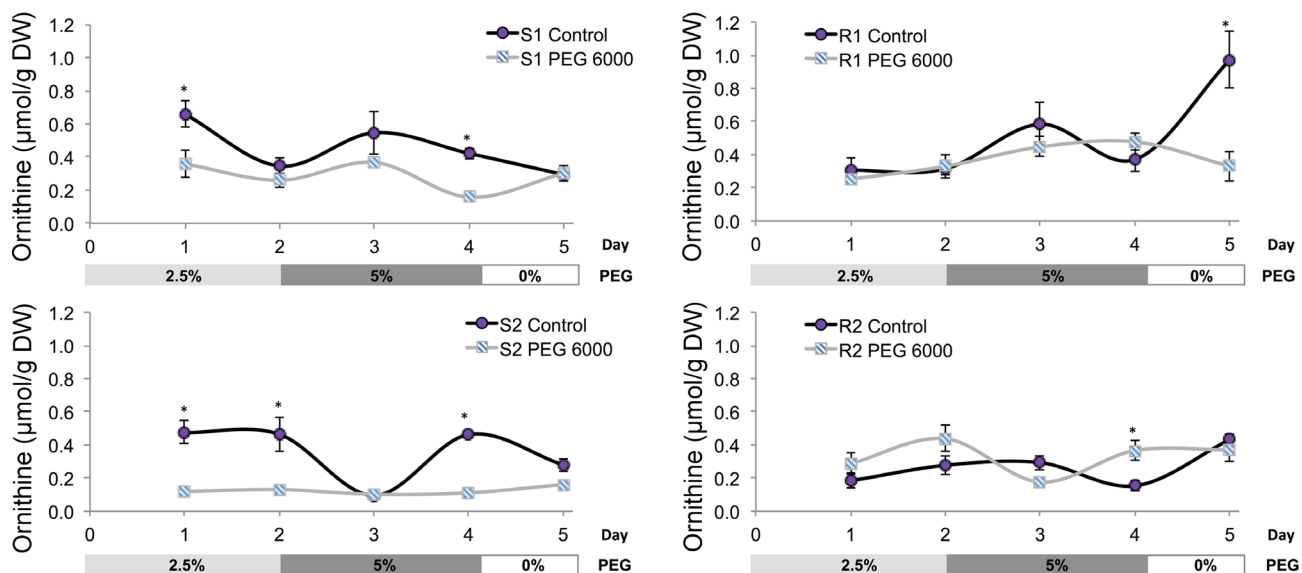


Figure 8. Effect of *in vitro* osmotic stress on shoot ornithine concentrations of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured on every day of the stress treatment (Days 1–4) and 24 h after removing the osmotic stress (Day 5)

Points are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

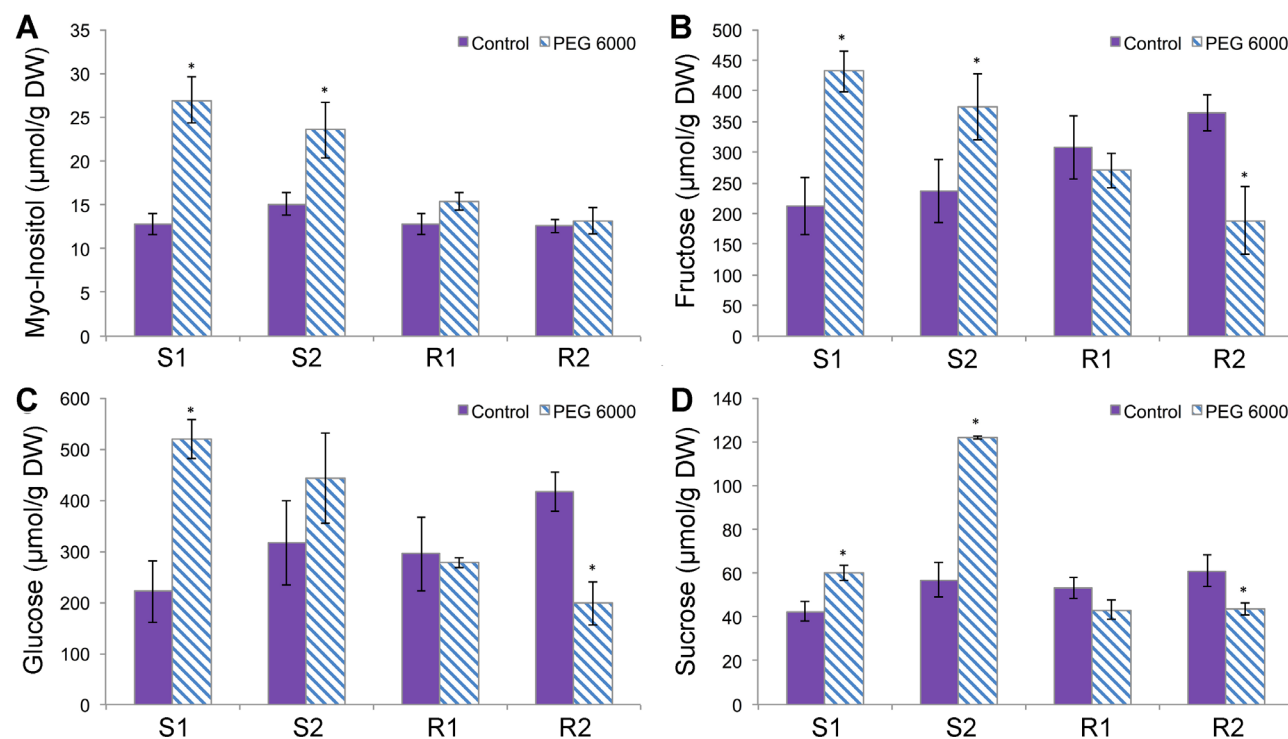


Figure 9. Effect of *in vitro* osmotic stress on (A) myo-Inositol, (B) fructose, (C) glucose, and (D) sucrose concentrations in the shoots of selected drought-sensitive (S1 and S2) and drought-resistant (R1 and R2) winter oilseed rape genotypes measured on Day 4 of the stress treatment

Points are means of four replicates with standard errors. *Significant differences at $P < 0.1$.

stress treatment declined to the same level measured in the control treatment (data not shown).

DISCUSSION

Early osmotic stress affects plant growth and vigor

Drought stress has wide-reaching effects on the physiology of a plant. Besides cellular dehydration, damage to cellular structures by arising reactive oxygen species (ROS) necessitates diverse adaptive metabolic responses. According to [Zhu \(2002\)](#), these responses can be grouped into three categories: osmotic homeostasis, damage control, and growth control in terms of regulation of cell division and expansion. In our profiling of metabolic responses in oilseed rape to osmotic stress, we observed diverse changes in growth properties, hormone signaling, and metabolite patterns that can be assigned to each of these different response categories.

Drought stress limits plant growth and productivity. First reductions in plant growth can be ascribed to an inhibition of cell elongation growth when turgor pressure is insufficient. According to [Cossgrave \(1986\)](#), a decrease in cell elongation rate can result from both a decrease in cell wall extensibility and a decrease in growth-effective turgor. It is known that apoplast acidification, a process necessary to afford loosening of cell wall structure ([Hager et al. 1971](#); [Rayle and Cleland 1992](#)), is decreased during osmotic stress in maize ([Van Volkenburgh and Boyer 1985](#); [Pitann et al. 2009](#)) and might be a main limiting factor during drought stress. On the other hand, it is assumed that additional factors, such as a change in cell wall elasticity and cell wall composition, can also influence cell wall extensibility under osmotic stress conditions ([Martínez et al. 2007](#); [Hatzig et al. 2010](#)). Furthermore, if no adequate decrease in cell water potential is affected, the turgor pressure will not be sufficient to trigger cell growth and consequent tissue expansion under drought conditions. Osmotic adjustment is therefore a fundamental mechanism for the maintenance of a growth-effective turgor under drought conditions. In our profiling, the accumulation of osmotically active solutes such as proline or soluble carbohydrates under *in vitro* osmotic stress indicates that a lowering of the cell osmotic potential is also a key drought-stress response in oilseed rape.

A second cause for limited plant growth under drought conditions is that a decrease of transpiration intensity, and a subsequently lowered photosynthesis and assimilation, lead to reductions in productivity and plant growth. Our observations with regard to leaf temperature support this assumption. Leaf temperature is negatively correlated with transpiration intensity ([Hanson and Hitz 1982](#); [Kumar et al. 1984](#); [Singh et al. 1985](#); [Munns et al. 2010](#)) and the drought-sensitive genotypes S1 and S2 reacted with significant increases in leaf temperature ([Figure 5](#)). Interestingly, these two genotypes were able to lower their temperature level again. This may indicate that some adjustment processes contributing to osmotic homeostasis take place in order to maintain cell water relations and, thus, transpiration.

The patterns we observed for plant growth parameters revealed distinct genotypic differences in the osmotic stress responses of the drought-resistant and drought-sensitive genotypes. Whereas the two resistant genotypes R1 and R2 were able to maintain biomass levels and leaf area, the

sensitive genotypes S1 and S2 revealed reduced biomass levels ([Figures 2, 3](#)). After 10 days of *in vitro* osmotic stress, however, the water content was shown to be significantly decreased in all genotypes ([Figure 4](#)). Thus the two drought-resistant genotypes R1 and R2 seem to continue growing even though tissue water content is reduced, while the growth of the sensitive genotypes S1 and S2 was already suppressed. One possible reason for restricted plant growth of these genotypes might be that the expenditure of energy and carbon for the synthesis of osmotically active compounds, such as proline and sugars, occurs at the expense of energy and assimilates which consequently become unavailable for growth processes.

Absciscic acid enhances proline accumulation under osmotic stress

In higher plants, ABA is well known as an activator of different resistance mechanisms during diverse abiotic stresses such as osmotic stress. Previous studies showed that drought resistance could be decreased both by a knock-out of ABA-biosynthetic enzymes or by enhancement of ABA catabolism (reviewed in [Zhu 2002](#); [Seki et al. 2007](#)). Absciscic acid generally regulates diverse processes and pathways that contribute to maintenance of cell water balance. On a physiological level, an ABA-triggered stomatal closure leads to minimized water losses during drought conditions, while ABA-induced osmotic adjustment processes help to maintain water uptake from the growth medium and to resist cellular dehydration. On a molecular level, ABA regulates the expression of different stress responsive genes that contribute to a better drought compatibility (reviewed by [Zhu 2002](#); [Huang et al. 2008](#); [Roychoudhury et al. 2013](#)).

As observed by [Huang et al. \(2008\)](#), consideration of the combined pools of ABA and its principal catabolites (phaseic acid, dihydrophaseic acid, and ABA glucose ester) provides a more detailed view of how much ABA that has been biosynthesized and further metabolized in the course of a stress treatment. In the two drought-sensitive genotypes S1 and S2, it was evident that ABA metabolism was enhanced during the stress conditions, although the mean increases could not be confirmed statistically for S2 ([Figure 6](#)). The relationship between increased ABA levels and inhibited plant growth in the sensitive genotypes can be explained by the fact that ABA induces stomatal closure and thus inhibits photosynthesis and growth. The increased leaf temperatures under osmotic stress in S1 and S2 underline this supposition.

In accordance with [Larosa et al. \(1987\)](#), it is evident from our results that osmotic adjustment was stimulated by an ABA-triggered accumulation of osmotically active solutes, such as proline as well as reducing and non-reducing sugars. [Verslues and Bray \(2006\)](#), and more recently [Larher et al. \(2013\)](#), hypothesized that increased ABA levels and changes in ABA sensitivity under osmotic stress conditions can trigger the accumulation of proline. An increase in ABA concentration is not a prerequisite for osmotic adjustment, however. Our results corroborate the assumption that proline accumulation is enhanced by increased ABA concentrations in the tissue: Concentrations of proline showed a stronger increase under osmotic stress conditions in the sensitive genotypes S1 and S2, which also reacted with increases in their average ABA concentrations under osmotic stress ([Figures 6, 7](#)). However, ABA accumulation does not seem to be a prerequisite initiator

for proline accumulation, as the resistant genotypes R1 and R2 reacted with an accumulation of proline but did not show increases in ABA concentration under osmotic stress conditions. One possible explanation for a stronger ABA accumulation in the sensitive genotypes is that the resistant genotypes did not sense the stress to the same extent and therefore did not respond by increasing biosynthesis of ABA.

Osmotic stress triggers proline accumulation but decreases ornithine formation

Proline is known to play a crucial role in osmoregulation and the adaption to osmotic stress conditions (Handa et al. 1986; Delauney and Verma 1993). Besides its function as an osmolyte, proline is ascribed to play roles as an osmoprotectant and as a stabilizer of subcellular structures and macromolecules (Schobert and Tschesche 1978; Kavi Kishor 1995). Proline also acts as a radical scavenger (Smirnoff and Cumbes 1989) in the prevention of photoinhibitory damage.

All four genotypes accumulated proline in their shoots. A concentration effect due to inhibited plant growth can be excluded as an explanation for this, because plant growth was not significantly affected after 4 d of stress treatment (data not shown). Furthermore, it seems that proline accumulation under osmotic stress is an active process, as concentrations of other amino acids such as ornithine (Figure 8) and glutamate (data not shown) were shown to be decreased and unaffected, respectively. If a concentration effect is predominant, this would cause an increase of these amino acids too. Additionally, it can be assumed that proline accumulation is strongly osmoregulated, as the osmotic stress treatment causes increased proline concentrations. In the hypo-osmotic treatment, the proline content decreased correspondingly.

It appeared that increases in proline accumulation contributed to the maintenance of cell water potential in all four tested genotypes. This might also explain the drop in relative leaf temperature after 2–3 d of *in vitro* osmotic stress,

as osmotic adjustment is sufficient to strengthen transpiration intensity again (Figure 5). In the resistant genotypes R1 and R2, however, it is likely that additional mechanisms or qualities contribute further to the maintenance of transpiration intensity, as these genotypes were able to constrain increases in leaf temperature while showing only slight increases in proline concentration compared to the sensitive genotypes. Furthermore, a lower proline accumulation during the short-term stress phase did not cause stronger decreases in water content in the resistant genotypes, as it did for the sensitive genotypes after 10 d of stress (Figure 4).

Besides, its function as osmolyte, proline is also involved in the prevention of oxidative stress when stomatal conductance, and consequently photosynthesis, are inhibited during stress (Matysik et al. 2002). A decrease in photosynthetic electron transport under unchanged light absorption leads to an overload of energy and the formation of ROS (Lawlor and Tezara 2009). This necessitates an adequate quenching of ROS in order to prevent oxidative damage. One additional reason for a stronger increase in proline content in the sensitive genotypes S1 and S2 might be its antioxidative qualities; these may help to prevent oxidative damage resulting from the inhibition of transpiration and photosynthesis during the *in vitro* osmotic stress. Proline metabolism also drives the consumption of reducing power, which is essential when CO₂ becomes limiting, as is the case in S1 and S2 where stomata are likely to be closed as a result of the osmotic stress.

Two alternative processes are responsible for proline biosynthesis in plants: The glutamate pathway and the ornithine pathway (Delauney and Verma 1993; Figure 10). Under osmotic stress conditions, however, expression of genes encoding specific enzymes involved in the glutamate pathway (Δ -Pyrroline 5-carboxylate-synthetase and Δ -Pyrroline 5-carboxylate-reductase) is upregulated, whereas the key ornithine pathway gene ornithine- δ -aminotransferase is repressed (Hu et al. 1992; Delauney et al. 1993). In addition, Kavi Kishor et al.

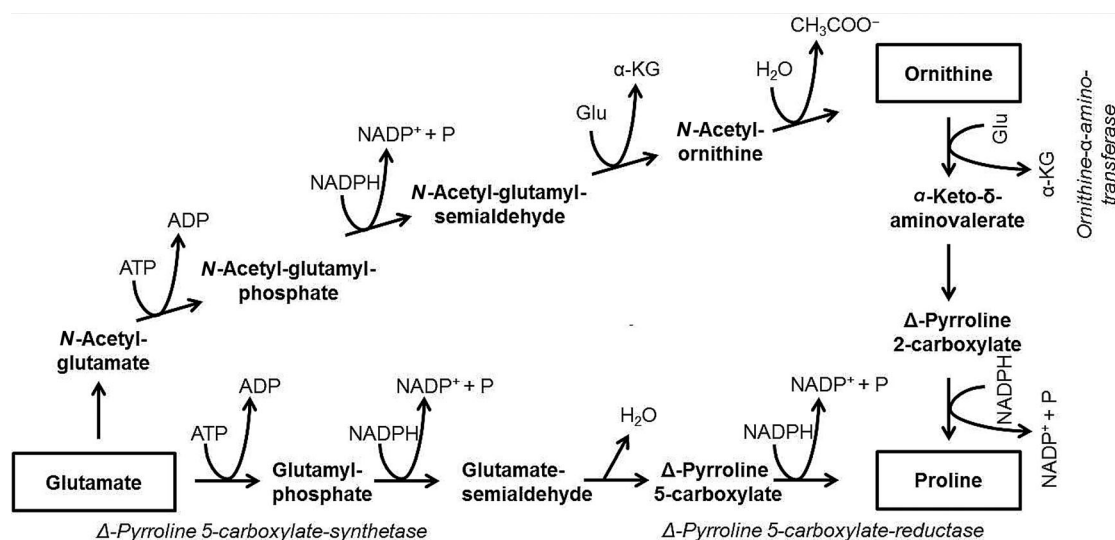


Figure 10. Proline biosynthesis by glutamate and ornithine pathway in plants, adapted from Delauney and Verma (1993) and Shargool et al. (1988)

(1995) showed that proline biosynthesis in transgenic tobacco plants is initially limited by Δ -Pyrroline 5-carboxylate-reductase activity under water stress conditions. This finding led to a conclusion that proline synthesis from glutamate is predominant under stress conditions (Delauney and Verma 1993; Yang and Kao 1999). Our findings also support this assumption for osmotic stress in drought-sensitive oilseed rape genotypes, whereby not only the ornithine pathway itself but also previous steps in ornithine biosynthesis seem to be repressed during osmotic stress. In particular, glutamate, which is also a requisite intermediate in ornithine synthesis, is prevalently used for the accumulation of proline in the glutamate pathway. This may occur at the expense of a decrease in ornithine formation and thus a decrease in the ornithine pool under osmotic stress conditions. This also seems to apply in the present case: Proline concentrations in the shoots of S1 and S2 showed a strong increase, ornithine concentrations decreased and glutamate concentrations were not affected significantly by the *in vitro* osmotic stress (data not shown). One possible reason for the preference for the glutamate pathway under stress conditions might be the regeneration of reducing equivalents in order to prevent photoinhibitory damage when light absorption exceeds carbon assimilation (Hare and Cress 1997). It nevertheless remains unclear why R1 showed a significantly lower ornithine concentration in stressed plants after hypo-osmotic treatment. Because R1 and R2 come from different backgrounds it might be speculated that the two genotypes have an overlapping but not identical suite of responses to osmotic stress. Further studies are necessary to elucidate the reasons for its difference.

Drought-sensitivity coincides with sugar accumulation

Analogous to proline, sugar and myo-inositol concentrations were shown to be increased in the shoots of stressed plants from S1 and S2; however, significant delayed differences occurred after 4 d of stress. It can be assumed that an accumulation of these compatible solutes addresses osmotic homeostasis, due to a reduction of cell osmotic potential in order to maintain water uptake. However, it cannot be ruled out completely that an accumulation of carbohydrates might be due to a lowered carbon usage, as a consequence of growth impairment under stress conditions. Several studies have presented evidence that osmotic stress resistance correlates with fructan accumulation (Munns and Weir 1981; Pilon-Smits et al. 1995, 1999; Sánchez et al. 1998; Kerepesi and Galiba 2000). Many other motives have also been discussed in the context of sugar accumulation under osmotic stress conditions. It is known that sugars are involved in the protection and stabilization of macromolecules and cell structures, and that they act as radical scavengers to prevent oxidative damage (reviewed in Hoekstra et al. 2001).

Interestingly, we were able to show that an accumulation of glucose, fructose, sucrose, and myo-inositol only occurred in the sensitive oilseed rape genotypes, while R2 even reacted with decreased concentrations (except for myo-inositol). Transpiration did not seem to be affected by the stress treatment, so that photosynthesis was clearly not limiting. Hence, it can be assumed that these decreases are due to the conversion of hexoses into other metabolites or macromolecules, and this conversion process is stimulated by osmotic stress in R2.

Early osmotic adjustment responses as indicators for drought resistance in oilseed rape

In this study, we developed an effective *in vitro* growth system for metabolic and physiological characterization of responses to osmotic stress simulated by PEG application to seedling roots. Application of the system enabled us to demonstrate clear differences between the physiological responses between selected drought-stress sensitive and drought-stress tolerant winter oilseed rape materials. The results showed that key differences in drought resistance were already manifested during early growth stages. Genotypes that were able to realize a high proportion of their yield potential under drought conditions in the field also showed correspondingly lower seedling biomass reductions during short-term *in vitro* osmotic stress experiments. Additional traits including leaf temperature, along with characteristic changes in metabolite patterns, could also be used to predict early osmotic stress response in oilseed rape genotypes.

Metabolite and hormone profiling provided useful information about the physiological fundament leading to differences in osmotic stress resistance. The results of the ABA and ABA catabolite analysis suggest that stress sensing is different between the resistant and sensitive genotypes we studied. Whereas the sensitive genotypes showed an increase in average ABA concentration, and already reacted during the short-term stress phase with increased concentrations of compatible solutes, these adjustment processes did not occur to the same degree in the resistant genotypes. In contrast, these drought-resistant genotypes seem to overcome the preliminary phase of *in vitro* osmotic stress by other strategies. It appears that they are not reliant on stronger adjustment processes during the short-term stress phase, as plant growth and leaf temperature were not significantly affected by the osmotic stress.

Furthermore, it is evident that a stronger stress sensing and accumulation of osmotically active compounds in the sensitive genotypes did not improve the relative tissue water concentrations under stress, as it did in the resistant genotypes. In fact the smallest decreases in average shoot water content were calculated for the resistant genotypes R2 and R1. Another important finding is that osmotic adjustment by proline and sugar accumulation appears insufficient for drought-sensitive genotypes to regain their original transpiration intensity. Collectively, our results imply that drought resistance in oilseed rape appears to be not only imparted by stress sensing and the accumulation of osmolytes, so that other resistance strategies or differences in constitutive properties are likely to play an additional role. The stronger stress sensing and enhanced osmolyte accumulation observed in the drought-sensitive genotypes suggest that normal mechanisms for adjustment to osmotic stress are intrinsically active in *B. napus*. On the other hand, however, osmotic adjustment seemed to be unexpectedly dispensable for the two drought resistant under the osmotic stress conditions we applied. It is still possible that delayed osmotic adjustment may occur when other resistance attributes are overcome by more intense or prolonged stress.

Indeed, the question remains whether osmotic stress resistance, in terms of reduced stress sensing and responsiveness, can be maintained under more severe osmotic stress. On the one hand, the accumulation of compatible solutes is

associated with an energy cost at the expense of growth processes (reviewed in Munns and Tester 2008). A lower investment in the accumulation of proline and sugars, coupled with the maintenance of transpiration, seems to be advantageous under the chosen experimental stress conditions, as less energy must be spent on these adjustment processes and plant growth is maintained. On the other hand, if stress intensity becomes higher and the stress duration is extended, a stronger active responsiveness may become beneficial when these adaptive processes are aimed at plant survival and recovery rather than plant growth and productivity. It cannot be excluded that drought-resistant genotypes react in the same manner as the sensitive genotypes when exposed to long-term stress; however, it is difficult to prove this supposition using *in vitro* experiments.

Our study discloses striking physiological and metabolic differences between selected drought-resistant and drought-sensitive oilseed rape materials. As such we provide a potential basis for the identification of selection criteria for drought resistance in this important crop. Although the *in vitro* screening system needs to be validated by larger scale testing in diverse populations, an application of our methods and the metabolic and physiological parameters we identified in larger populations might provide a unique opportunity for early screening and pre-selection for drought resistance in oilseed rape.

Heterosis as a factor in drought tolerance

Investigations into the genetic mechanisms underlying the phenotypic differences between the four test genotypes were beyond the scope of this study. Nevertheless, it is interesting that the two genotypes with improved drought resistance, “Ferdie” (R1) and “P09H60569” (R2), are both F1 hybrids, whereas the two genotypes “M17473-06” (S1) and “NPZ208-03” (S2) with sensitive reactions to drought are both homozygous inbred lines. Heterozygous F₁-hybrids of winter oilseed rape often show improved vigor phenotypes and a general resilience against abiotic stress constraints in comparison with homozygous inbred lines (Basunanda et al. 2010). The genetic and molecular basis of this phenomenon is still unclear; however, the present results support the notion that heterosis may impart an improved general stress tolerance. More detailed genetic mapping and transcriptomic studies, to elucidate the genetic mechanisms of hybrid vigor, in relationship to the metabolic and hormonal control of stress responses, would be of considerable interest in this context.

MATERIALS AND METHODS

Field evaluation and selection of plant materials

In preliminary field experiments, 20 elite winter oilseed rape cultivars and breeding lines were tested for their yield potential under natural drought conditions at six different locations in Germany during the growing season of 2010/2011. This growing season was characterized by an extremely dry spring, which led to strong yield reductions in autumn-sown oilseed rape (M Hohmann, Justus Liebig University, Giessen, Germany, unpubl. data). The materials were grown in 10.0–13.5 m² plots (depending on the location), with two replications per location and treatment. Each location included a non-irrigated control and an irrigated variant. Depending on the location, drip or

spray irrigation was applied to the irrigated variant to maintain the field water capacity during the flowering and seed setting phases in spring and summer. Three locations (Gross Gerau, Leutewitz, Seligenstedt) showed consistent and significant reductions across the test panel for yield performance in the non-irrigated drought treatment compared to the irrigated control. These three locations were therefore chosen to select materials showing strong differential drought responses. At each location, all materials were sown together in August 2010 and harvested in July 2011.

For further analysis, four unrelated winter oilseed rape genotypes were selected. Two showed consistently low relative seed yield reductions under drought stress over the three locations, while two showed consistently high yield reductions, respectively. According to their reaction during drought stress, the materials were classified as “drought resistant” (average yield reduction under drought around 20%, referred to here as R1 and R2) and two as “drought sensitive” (average yield reduction under drought around 40%, referred to in the following as S1 and S2). S1 is the inbred line “M17473-06,” while R2 is the F1 hybrid “P09H60569” (both sourced from Deutsche Saatveredelung AG, Lippstadt, Germany). S2 is the breeding line “NPZ208-03” (Norddeutsche Pflanzenzucht H.G. Lembke, Hohenlieth, Germany) and R1 is the F1 hybrid “Ferdie” (Monsanto Saaten, Nienstädt, Germany). Seeds from the materials may be obtained from the respective owners for research purposes under a material transfer agreement. Based on their consistently poor (S1, S2) or good (R1, R2) reactions to field drought stress, these four genotypes were used to develop and test an *in vitro* cultivation system for screening of simulated osmotic stress in oilseed rape seedlings under highly controlled conditions.

Plant cultivation and growth monitoring

Seeds were surface-sterilized in 3% NaOCl solution, containing detergent to reduce the surface tension, for 10 min on a rotary shaker. Afterwards the seeds were washed repeatedly five times in H₂O dest. and prepared for germination. Plastic tubes with 0.2 mL volume were filled with agar gel (1.5% (w/v)) and one sterile seed per vessel was inserted at 1 mm depth in the gel matrix. The lower ends of the tubes were cut off so that the roots could grow into the nutrient solution in the later stage of the experiment. The tubes were placed into a rack, wrapped in aluminum foil and stored at room temperature. After 3 d, the seedlings were transferred into 50 mL plastic tubes containing MS medium (Murashige and Skoog 1962) with the following nutrient concentrations: NH₄NO₃ (10.54 mmol/L), KNO₃ (9.61 mmol/L), CaCl₂ (1.53 mmol/L), MgSO₄ (0.77 mmol/L), KH₂PO₄ (0.64), H₃BO₃ (51.28 µmol/L), MnSO₄ (51.14 µmol/L), FeNa EDTA (51.14 µmol/L), ZnSO₄ (15.30 µmol/L), KI, Na₂MoO₄ (0.53 µmol/L), CoCl₂ (0.06 µmol/L), and CuSO₄ (0.05 µmol/L). For further plant development, the seedlings were transferred, inside the lids of the tubes, to open tubes inserted into 15 cm deep 10 L aquaria (Figure 11A, B) in a climate chamber. For all analyses except the measurement of leaf temperature, the following conditions were used: Day period: 16 h, 16 °C, 65% RH, night period: 8 h, 12 °C, 75% RH. For the leaf temperature measurements, a separate chamber was used with day/night temperatures of 20 °C/15 °C. After 7, 14, and 21 d, the nutrient solution was changed and the nutrient concentration was doubled from Day 14.

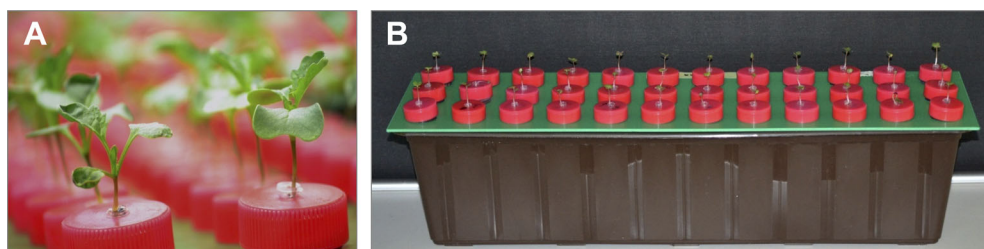


Figure 11. Hydroponic system for the cultivation of oilseed rape

(A) Seedlings were germinated in 0.2 mL Eppendorf tubes which were fixed in lids of 50 mL Falcon tubes. (B) Open Falcon tubes with 5-d-old seedlings were placed in aerated 15 cm deep aquaria containing 10 L nutrient solution.

The stress treatment was started 26 d after sowing, when all plants had reached growth stage Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) 13–14 (see Weber and Bleiholder 1990; Figure 12). Each genotype was cultivated both under control conditions and under simulated osmotic stress conditions. The plants were transferred into 10 L plastic aquaria containing nutrient solution. In the stress treatment, polyethylene glycol (PEG 6000) was added, initially at a PEG concentration (w/w) of 2.5% and increasing to 5% after 48 h. After 4 d of stress, one set of the plants in both the stress and control treatments were harvested for fresh weight determination. In a second set of the plants, the stress treatment was removed after a further 48 h by applying nutrient solution without PEG 6000. A third set of plants in the stress treatment was kept on 5% PEG and harvested on Day 35 (after 10 d on PEG with 8 d on 5% PEG) for fresh weight determination. The shoots were dissected from the lower end of the hypocotyl, and at the same time point total leaf area was scanned and measured as pixel area, using the software ImageJ (NIH), as an indicator for seedling vigor under osmotic stress conditions. For determination of DW, the plant material was dried at 85 °C for 24 h.

The corresponding osmotic potential of the different PEG solutions can be calculated as follows using the formula of Michel and Kaufmann (1973): (−0.022) MPa at 2.5% PEG 6000 and (−0.058) MPa at 5% PEG 6000. To ensure oxygen supply, the solutions were aerated using a pressure air pump.

Leaf temperature was monitored on the second fully developed true leaf, at midday every 24 h after the onset of stress treatment. Leaf temperature, which is negatively correlated with a decrease in transpiration (Hanson and Hitz 1982; Kumar et al. 1984; Singh et al. 1985; Munns et al. 2010), was measured under windless conditions using an infrared thermometer (Votcraft IR650-12D, Hirschau, Germany).

Hormone profiling

For hormone profiling, the shoot material was sampled 8 h into the light period on Day 29 (after 4 d on PEG with 2 d on 5% PEG). For shoot hormone analysis, only leaf material from the true leaves was used. All samples were shock frozen in liquid nitrogen immediately after harvest and freeze-dried. The procedure for quantification of ABA, cytokinin, auxin, and gibberellins in *Brassica* shoot tissue was performed as described in detail in Lulsdorf et al. (2013).

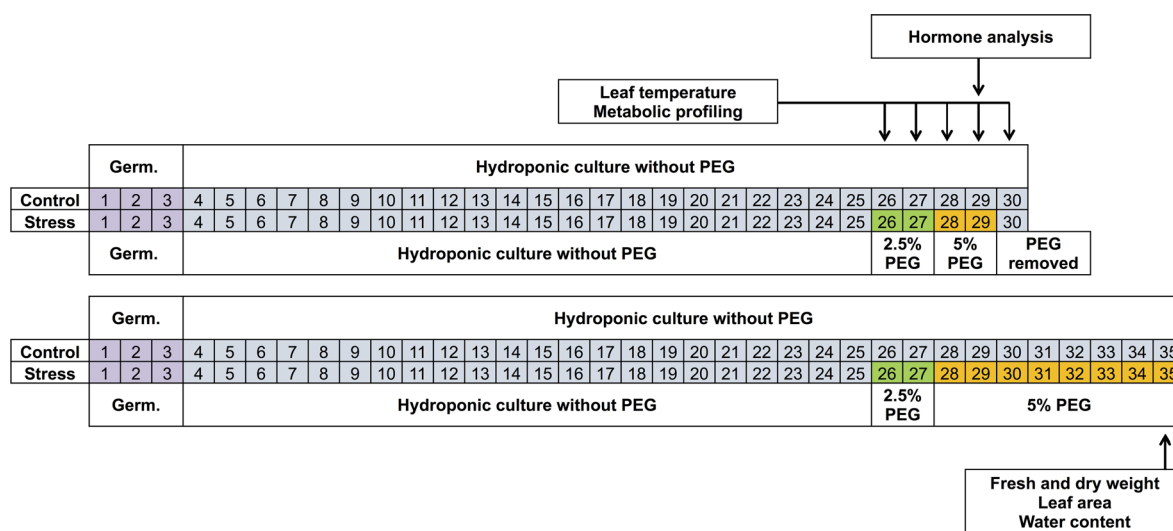


Figure 12. Course of *in vitro* plant cultivation and sampling time-points

Values represent the number of days after sowing the seeds.

Metabolic profiling

For shoot amino acid and sugar analysis, only leaf material from the true leaves was used. Leaf material was sampled at the following times: 8 h after the light period started; 24 and 48 h after starting the stress treatment with 2.5% PEG; 24 and 48 h after increasing the PEG concentration to 5%; 24 h after restoring the osmotic stress. Samples were shock frozen in liquid nitrogen immediately after harvest and freeze-dried.

The freeze-dried plant material was ground using the TissueLyserII (Qiagen, Germany) and 400 μ L methanol (100%) containing β -amino-butyric acid (BABA, 100 μ mol/L) and adonitol (100 μ mol/L) were added to 10–15 mg of the ground powder. β -amino-butyric acid and adonitol were used as internal standards for amino acid and carbohydrate quantification, respectively. The mixture was shaken for 15 min at room temperature and 200 μ L chloroform (100%) were added before shaking again for 10 min at room temperature. Subsequently, 400 μ L of distilled water was added, the mixture was vortexed and then centrifuged for 5 min (12,000 g, 15 °C). Afterwards aliquots of 50 μ L supernatant were used for amino acid and sugar analysis. The solutions were completely evaporated and stored at –20 °C before analysis.

Analysis was performed on a Waters Acquity ultra-performance liquid chromatography with diode array detection using methods and software described in the Waters Corporation user manual and developed for oilseed rape tissue by Albert et al. (2012) and Deleu et al. (2013). The AccQtag Method was used to quantify aminoacids and integration software Empower (Waters Corporation, Milford, USA) was used for analysis. Samples were resuspended in 100 μ L distilled water. Subsequently, 5 μ L were derivatized using AccQtag Ultra Derivatization Kit, according to the manufacturer's recommendations. An external standard of 100 μ mol/L each of proline, ornithine, and glutamate was run every 10 samples.

Quantification of sugars was performed using a gas chromatography-flame ionization detector (GC-FID) System from Agilent Technologies (Santa Clara, CA, USA) according to Lugan et al. (2009). For data analysis, the integrated Agilent software ChemStation Rev.B.04.02 was used. Samples were resuspended in 50 μ L pyridine (100%) with methoxamine hydrochloride (240 mmol/L), then derivatized with 50 μ L MSTFA (N-methyl-N-(trimethylsilyl)trifluoro acetamide) (100%). An external standard containing 400 μ mol/L each of fructose, glucose, sucrose, and myo-inositol was run every 10 samples.

Statistical analysis

The experiment was laid out as a completely randomized block design. For fresh weight determination and metabolic profiling, four biological replicates per genotype and treatment were sampled. Hormone analysis was performed using three biological repeats per genotype and treatment. Measurements of leaf temperature were carried out with four biological repetitions and three additional technical repetitions per leaf.

Data analysis was carried out using the free software R (version 2.13.1, 2011). Outliers were identified applying Cook's distance (D_i) test. Because all values are expected to lie within a tight range, values with $D_i > 0.4$ were excluded from further analyses.

Compliance with normal distribution and homogeneity of variances were evaluated using a Bartlett test. If variances were homogeneous, significances were calculated using a pairwise t-test with `var.equal=TRUE`. For datasets with inhomogeneous variances, a pairwise t-test with `var.equal=FALSE` was implemented. In terms of consistency testing, no adjustment was chosen for the statistical analysis.

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2.3 Characterising root response phenotypes by neural network analysis

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2.3.1 Publication outline

The following paper outlines the influence of osmotic stress on root architecture in seedlings of winter oilseed rape genotypes with differences in their drought resistance. The aim was to identify root characteristics involved in the manifestation of drought compatibility during early plant growth of rapeseed and might be potential key targets for the selection of drought resistant rapeseed germplasm. The genotypes were cultivated under controlled conditions in the climate chamber using the hydroponic cultivation system described in the previous chapter (see Paper 2), in which osmotic stress was accordingly simulated with polyethylene glycol. Furthermore, a novel root phenotyping tool was developed, based on a common method for neural network analysis in neuroscience, called 'Sholl analysis'. A comparison with manual root measurements showed that Sholl analysis provides relevant supporting information about the interactive changes in seedling root architecture and spatial distribution under osmotic stress, making it well suited for the exploration of plant root system development. The data showed that interactive root architectural changes captured by Sholl analysis cannot be disclosed by the measurement of single root properties. Sholl analysis therefore helps to overcome a limitation of conventional root phenotyping tools that assess only root length measurements, numbers of lateral roots, or root zone-specific responses, respectively.



RESEARCH PAPER

Characterizing root response phenotypes by neural network analysis

Sarah V. Hatzig*, Sarah Schiessl, Andreas Stahl, and Rod J. Snowdon

Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26–32, 35392 Giessen, Germany

* To whom correspondence should be addressed. E-mail: sarah.hatzig@agrar.uni-giessen.de

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Abstract

Roots play an immediate role as the interface for water acquisition. To improve sustainability in low-water environments, breeders of major crops must therefore pay closer attention to advantageous root phenotypes; however, the complexity of root architecture in response to stress can be difficult to quantify. Here, the Sholl method, an established technique from neurobiology used for the characterization of neural network anatomy, was adapted to more adequately describe root responses to osmotic stress. This method was used to investigate the influence of *in vitro* osmotic stress on early root architecture and distribution in drought-resistant and -susceptible genotypes of winter oilseed rape. Interactive changes in root architecture can be easily captured by individual intersection profiles generated by Sholl analysis. Validation using manual measurements confirmed that the number of lateral roots decreased, while mean lateral root length was enhanced, under osmotic stress conditions. Both genotypes reacted to osmotic stress with a shift in their intersection patterns measured with Sholl analysis. Changes in interactive root architecture and distribution under stress were more pronounced in the drought-resistant genotype, indicating that these changes may contribute to drought resistance under mild osmotic stress conditions. The Sholl methodology is presented as a promising tool for selection of cultivars with advantageous root phenotypes under osmotic stress conditions.

Key words: *Brassica napus*, drought resistance, lateral roots, phenotyping, root architecture, Sholl method.

Introduction

This paper presents a novel and practicable method for the characterization of plant root architectural properties. The method was adapted from the analysis technique of Sholl (1953), an established neurobiological approach still applied today for morphological characterization of neural networks (Binley *et al.*, 2014; Ferreira *et al.*, 2014; Garcia-Segura and Perez-Marquez, 2014). Known as the Sholl method, this approach uses a coordinate system consisting of a series of concentric circles centred at the soma of a neuron. Local variables are extracted by counting the number of intersections

on regular concentric circles surrounding cell dendrites, giving a metric representation of the architectural characteristics of the network and how they change with distance and time. Because roots have similarly dynamic spatial and developmental characteristics to those of a neural network, the Sholl method was tested for its ability to describe topological differentiation in root distribution responses to abiotic stress.

Several climate reports and modelling studies predict a future increase of flooding events in northern Europe, while an increase of drought events is forecast for southern and

Abbreviations: DR, drought resistant; DS, drought sensitive; FW, fresh weight; LRL, lateral root length; NaOCl, sodium hypochlorite; MLRL, mean lateral root length; MS powder, Murashige and Skoog nutrient mixture; NLR, number of lateral roots; PEG, polyethylene glycol; r, radius; PC, principal component; PCA, principal component analysis; PRL, primary root length; RL, total root length; dpi, dots per inch.

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southeast European countries (Lehner et al., 2006; Alcamo et al., 2007; Bates et al., 2008). While precipitation during winter months is expected to increase slightly, the risk for drought events during the summer cropping season is expected to rise in many areas of Europe (Alderlieste and van Lannen, 2013). In major agricultural areas of North and South America, Asia, and Australia, crop production is already increasingly threatened by drought. Breeding of cultivars adapted to a broad range of environmental conditions and distinctive annual changes in precipitation are therefore of overriding importance and drought resistance is essential for future crop breeding. *Brassica napus* (oilseed rape) is a major international crop produced in diverse eco-geographical regions and a model polyploid for study of crop evolution and adaptation (Chalhoub et al., 2014).

In a previous study, different winter oilseed rape genotypes were tested in a climate chamber for their *in vitro* osmotic stress responses during a short-term stress period (Hatzig et al., 2014). Drought-resistant genotypes were able to maintain shoot growth under stress conditions, whereas drought-sensitive genotypes reacted with a strong decrease in shoot fresh and dry weight. During different replications of the *in vitro* experiment, one drought-resistant genotype appeared to respond to drought stress by additionally modifying its root architecture; however, differences in fresh or dry weight could not be significantly confirmed. Thus, a need arose for a more sophisticated root phenotyping tool that is able to illuminate potentially beneficial changes in root architecture associated with (*in vitro*) drought stress. Several root evaluation techniques exist for the investigation of root growth characteristics. Many of these also allow a manual or semi-automated analysis of root zone-specific responses [for example, RootReader2D (Clark et al., 2013), RootTrace (French et al., 2009), or WinRhizo (Arsenault et al., 1995)]. However, these methods do not completely capture the interactive architectural properties of the root system as a whole. Here, an easily manageable root phenotyping method, based on the principles of Sholl analysis, was applied for the investigation of root spatial distribution and architecture and their responses to abiotic stress *in vitro*. As far as could be ascertained, this is the first time that this established neurobiological method has been adopted for characterization of plant roots.

Materials and methods

Plant materials and cultivation

Two winter oilseed rape (*B. napus* L.) genotypes with different physiological and morphological responses to drought stress were tested for their root responses to osmotic stress during the seedling stage, applying the hydroponic cultivation system as described by Hatzig et al. (2014). The two genotypes 'Ferdie' (Monsanto Saaten, Nienstädt, Germany) and 'NPZ 208/03' (Norddeutsche Pflanzenzucht H.G. Lembke, Hohenlieth, Germany) were classified as drought resistant (DR) and drought sensitive (DS), respectively, corresponding to their drought compatibility (Hatzig et al., 2014). Seedlings were cultivated hydroponically for 22 days in a climate chamber as follows: seeds were sterilised in 3% NaOCl solution for 15 min on a shaker. To reduce the surface tension a drop of dishwasher detergent was added. Subsequently the seeds were washed

five times in distilled H₂O and prepared for germination in 0.2 mL plastic vessels filled with agar gel [1.5% (w/v)]. One sterile seed per vessel was placed at 1 mm depth in the gel matrix. The lower ends of the vessels were cut off so that the roots could reach the nutrient solution in a later stage of the experiment. The vessels were put into a bracket and wrapped in aluminium foil so that germination took place in the dark. After 3 days the seedlings were transferred into 50 mL falcon tubes containing MS medium [1.1 g L⁻¹ (Murashige and Skoog, 1962)]. Further plant development proceeded in a fully controlled growth chamber under the following conditions: Day period: 16 h, 20°C, 65% relative humidity; night period: 8 h, 15°C, 65% relative humidity. After 7 days the nutrient solution was changed and nutrient concentration was increased up to 2.2 g MS powder per litre.

Each genotype was cultivated under control conditions and under simulated drought stress conditions, with stress treatment commencing on day 17. The plants were transferred into 10 L plastic bins (52 cm × 12 cm × 13 cm) containing fully concentrated nutrient solution (4.3 g MS powder per L). For the stress treatment 2.5% PEG 6000 was added to the hydroponic medium on day 17, with the PEG concentration (w/w) being increased to 5% after a further 48 h. The corresponding osmotic potentials can be calculated as follows using the formula of Michel and Kaufmann (1973): −0.022 MPa at 2.5% PEG 6000 and −0.058 MPa at 5% PEG 6000. To ensure oxygen supply the solutions were constantly aerated using an air pump. After 3 days at 5% PEG the roots of control and stressed plants were harvested for image analysis.

Root evaluations and imaging

Roots were placed for 5 min on an absorbent paper and fresh weights (FW) were subsequently determined using a fine scale. The roots were placed on a glass scanner plate and disentangled by pipetting a thin layer of water onto the root body. Roots were scanned with a common office scanner at a resolution of 200 dpi. For each genotype and treatment five biological replicates were imaged. Significant differences were calculated with pairwise t-test ($P < 0.1$) in the R statistical software package (R Development Core Team, 2008), version 3.0.2. Compliance with normal distribution and homogeneity of variances were evaluated using a Bartlett test. If variances were homogeneous, significances were calculated using a pairwise t-test with var.equal = TRUE. For datasets with inhomogeneous variances, a pairwise t-test with var.equal = FALSE was implemented.

Total root length (RL), primary root length (PRL), and lateral root length (LRL) were measured manually by tracing the roots with the freehand line tool of the software ImageJ (rsb.info.nih.gov/ij/). Mean length of lateral roots (MLRL) was calculated. The number of lateral roots (NLR) was determined by manual counting. Concentric circles were drawn with a common compass around the root origin (Fig. 1) at intervals of 0.5 cm. The first circle was drawn at a distance of 0.5 cm from root origin and the outer circle was drawn beyond the outermost root tip.

Sholl analysis

Sholl analysis was performed with the software ImageJ using the plugin Sholl analysis (version 3.0). Before starting the image analysis, the root pictures were converted into a 16 bit threshold greyscale image and the pixel distance was converted into a known distance with the 'Set scale' function accessible in the 'Analyze' menu. After brightness adjustment and manual setting of the root origin, Sholl analysis was started. The linear method was selected, in which the starting radius, radius step size, and ending radius are chosen by the user. Concentric circles were then drawn automatically at regular intervals around the root (Fig. 1). According to the growth characteristics of the *B. napus* seedling roots used as a model, a circle interval of 0.5 cm was chosen, with a starting radius of 0.5 cm for the innermost circle and an outer radius beyond the outermost root tip. A further decrease in circle intervals did not lead to an additional

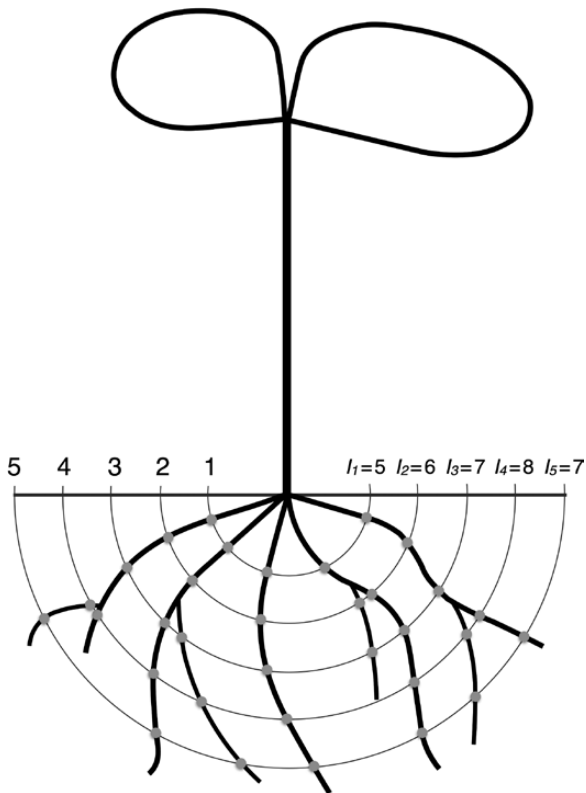


Fig. 1. Principle of root phenotyping by Sholl analysis, in which concentric circles are drawn at regular intervals around the root origin and the number of root-circle intersections was counted for each circle (this figure is available in colour at JXB online).

gain of information. Sholl analysis determines the architectural characteristics of a network by documenting the number of root-circle intersections in relation to their distance from the centre of origin. After parameter setting, the counting of intersections per radius occurs automatically. Depending on the chosen radius step size, the analysis takes up to a few seconds. For specification of root characteristics, two parameters were evaluated. The first was the absolute number of root-circle intersections, defined as the sum of root intersections of all concentric circles (I_T). This number can be represented by the following formula:

$$I_T = \sum_{k=1}^n I_k$$

where I_k is the count of intersections of circle k .

Furthermore, the circle-specific numbers of intersections were plotted against the corresponding circle radii, in order to gain information about the number of roots located at a specific distance to the root origin. To evaluate the effect of the root spreading on the positional data delivered by the Sholl analysis, the winter oilseed rape line 'Adriana' (Limagrain, Germany) was cultivated in pots (11 × 11 × 12 cm) filled with sand for 22 days in the greenhouse under semi-controlled conditions. The washed root system from a single plant was scanned 10 times independently, with repeated rinsing and re-spreading of the roots each time.

Data validation via manual counts

The values from manual counting of circle-specific intersections and the automated counting by Sholl analysis were correlated separately for each biological replication. Pearson's product moment correlation was then calculated after Fisher's z-transformation. Principal component

analysis (PCA) was carried out with results from Sholl analysis (circle-specific intersection patterns) and first components were then correlated with the manual measurements of root traits (RL, NLR, PRL, LRL, MLRL) using Pearson's product moment correlation.

Results

For both genotypes no significant differences in root FWs could be observed between control and PEG 6000 treatment (Fig. 2A). For genotype DR the mean root FW was 51.2 mg in the control treatment and 60.2 mg in the PEG 6000 treatment. For genotype DS a mean root FW of 35.1 mg was measured in the control treatment, with 31.5 in the PEG 6000 treatment. Under osmotic stress conditions, NLR decreased significantly in both genotypes (Fig. 2B). In genotype DR, NLR decreased to almost 50% compared to the control, while genotype DS showed a reduction of 40% under stress. In contrast, osmotic stress caused an increase in MLRL in both genotypes. However, significant differences between the treatments could be confirmed for genotype DR only, which showed an increase from 1.48 cm (control) to 6.19 cm (PEG 6000) (Fig. 2C). Mean values for RL and LRL in both genotypes were increased under stress but not significantly different from the measurements in the control treatment (Fig. 2D, E). PRL was significantly reduced under osmotic stress in genotype DR, while no differences could be observed for DS (Fig. 2F).

Sholl analysis reveals differentiation of root branching characteristics

No difference in absolute number of root-circle intersections was observed between the control treatments of the two investigated *B. napus* genotypes (Fig. 3). Under osmotic stress conditions, however, genotype DR reacted with a significant increase in intersection number, while for genotype DS no difference in intersection number could be observed between treatments. In genotype DR the absolute number of intersections increased from 173 (control) to 258 (PEG 6000), while DS showed 165 intersections under control conditions and 163 after PEG 6000 treatment. Under control conditions the last root-circle intersection in genotype DR occurred at a radius of 26 cm, whereas for genotype DS the farthest circle intersection was measured at 21 cm from the root origin (Fig. 4). Outermost root intersection under PEG 6000 treatment was measured at radii of 22 cm and 18 cm for DR and DS, respectively. Under control conditions, both genotypes showed a similar number of root-circle intersections between 0.5 and 4 cm distance to the root origin. The number of root intersections was significantly lower at 6.5 cm for genotype DR than for DS, whereas it was significantly higher at most circles between 9.5 and 26 cm from the origin. Both genotypes showed a significant circle-specific increase in intersection number in the PEG 6000 treatment. In DR the intersection number increased significantly between 3 and 10 cm, whereas DS showed significantly higher intersection numbers from 2.5 to 3 cm from the origin. Intersection number in DS dropped significantly below that of the control treatment between 7.5 and 9 cm, while no differences between treatments could be

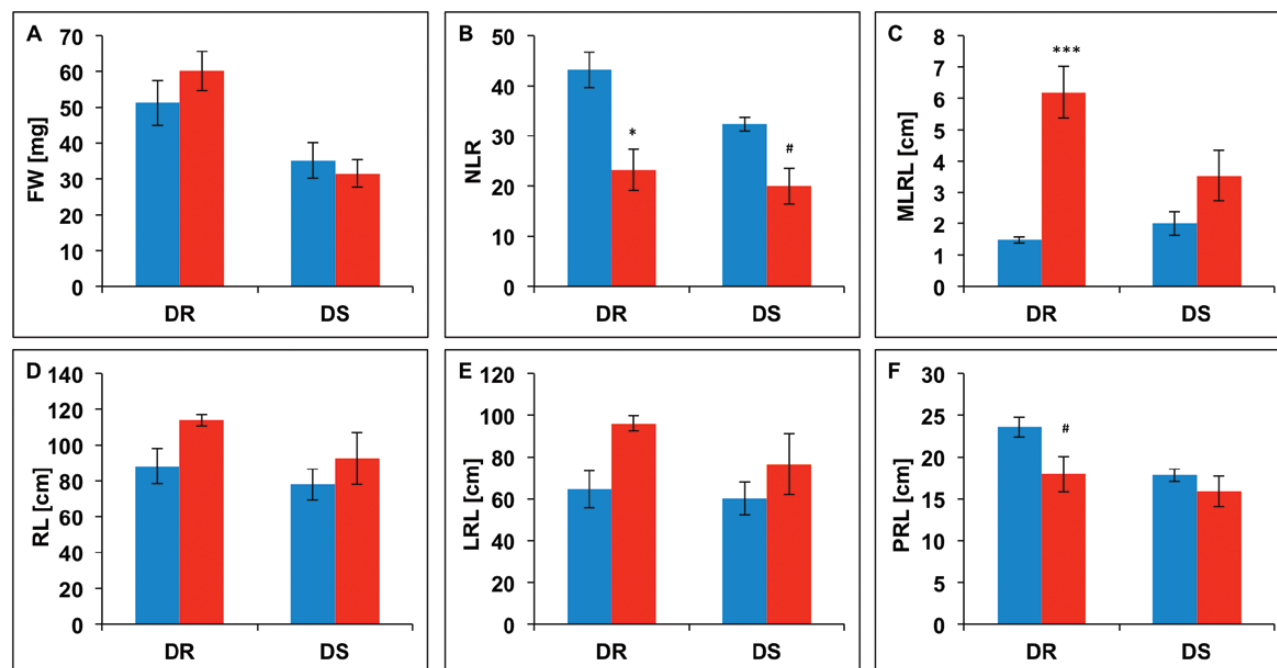


Fig. 2. Effect of *in vitro* osmotic stress on (A) root fresh weights (FW), (B) the absolute number of lateral roots (NLR), (C) the mean length of lateral roots (MLRL), (D) the total root length (RL), (E) the total length of lateral roots (LRL), and (F) the length of the primary root (PRL) of a DR and a DS winter oilseed rape genotype, measured under control conditions (left bars) and after 5 days of PEG 6000 treatment (right bars). Bars show mean measurements of five replicate plants with standard errors indicated by whiskers. Significant differences at * $P < 0.1$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (this figure is available in colour at JXB online).

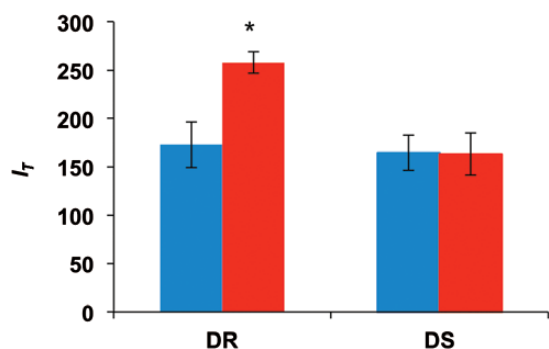


Fig. 3. Effect of *in vitro* osmotic stress on the absolute number of intersections measured with Sholl analysis of a DR and a DS winter oilseed rape genotype, measured under control conditions (left bars) and after 5 days of PEG 6000 treatment (right bars). Significant differences at * $P < 0.05$ (this figure is available in colour at JXB online).

observed between 9.5 and 18 cm. For DR the intersection number between 13 and 21 cm was almost always significantly lower in the PEG 6000 treatment than that of control treatment. A very strong correlation ($r > 0.967$) was observed between the manual counting of circle-specific root intersections and the automated counting by Sholl analysis (Fig. 5).

Correlations among root traits

PRL was significantly correlated with FW and NLR. LRL showed a very strong correlation with RL ($r > 0.9$) (Table 1). For MLRL significant correlations with NLR, RL, PRL, and LRL were calculated. The absolute number of intersections measured with Sholl analysis showed significant correlations of $r > 0.5$ with

the traits FW, RL, LRL, and MLRL. All manually measured root traits (NLR, MLRL, RL, PRL, and LRL) showed significant correlations with the first two components of the PCA, including circle-specific intersection patterns of Sholl analysis (Table 2).

Discussion

Interactive root responses to osmotic stress

Water deprivation has a manifold nature, with its impact *inter alia* being strongly dependent on the drought pattern (Fischer and Turner, 1978) and seasonal timing (Richards, 1996). On the other hand, plant reactions in terms of architecture and physiology are also multifarious. Generally, changes in root topology are connected with maintenance of the hydraulic status under drought conditions (Comas et al., 2013). A frequently observed phenomenon is that plants adopt physiological strategies to maintain root growth during times of water scarcity. Osmotic adjustment is shown to be a suitable tool for the maintenance of growth effective turgor pressure in the root elongation zones (Voetberg and Sharp, 1991). However, the nature of root architectural responses to drought is strongly dependent on the species, stress shaping, and soil conditions (Bengough et al., 2006; Ito et al., 2006). Any particular part of the root system has a highly specific physiological age that can differ strongly from other roots on the same plant. Hence the phenotypic variation along the length and breadth of the root system reflects not only differences in root physiology and responsiveness, but also the range of genotype-by-environment interactions to which the plant was exposed following germination (Bengough et al., 2006; Forde, 2009). The results demonstrated

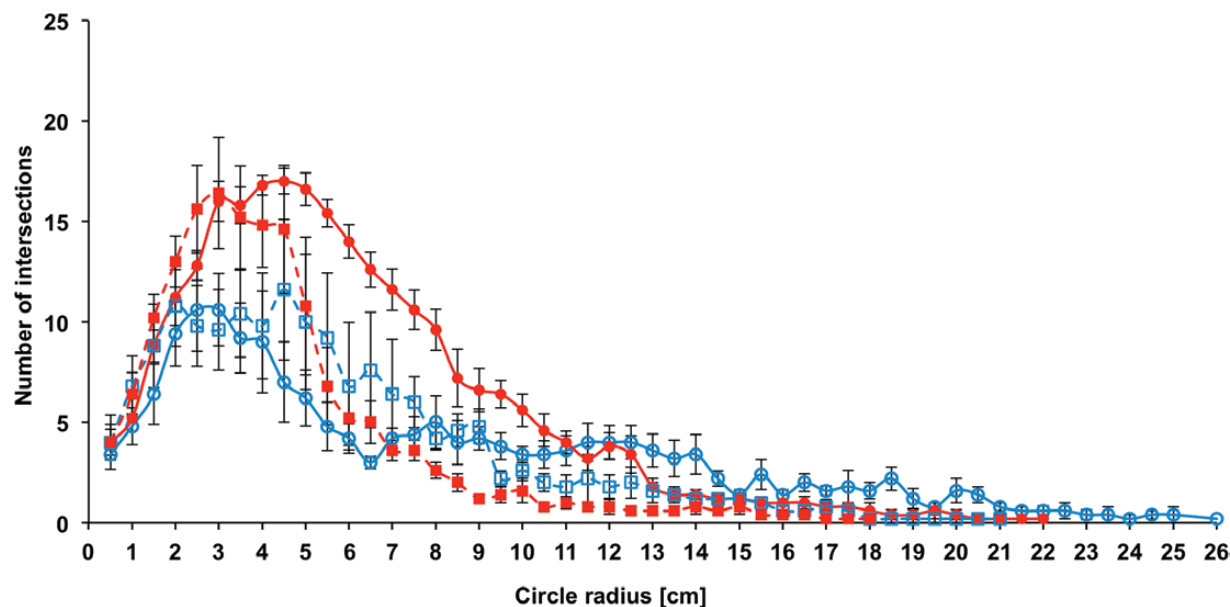


Fig. 4. Number of root intersections depending on the distance from root origin of a DR (dotted lines) and a DS (squared lines) winter oilseed rape genotype, measured under control conditions (open symbols) and after 5 days of PEG 6000 treatment (closed symbols). Points are means of five replicates with standard errors (this figure is available in colour at JXB online).

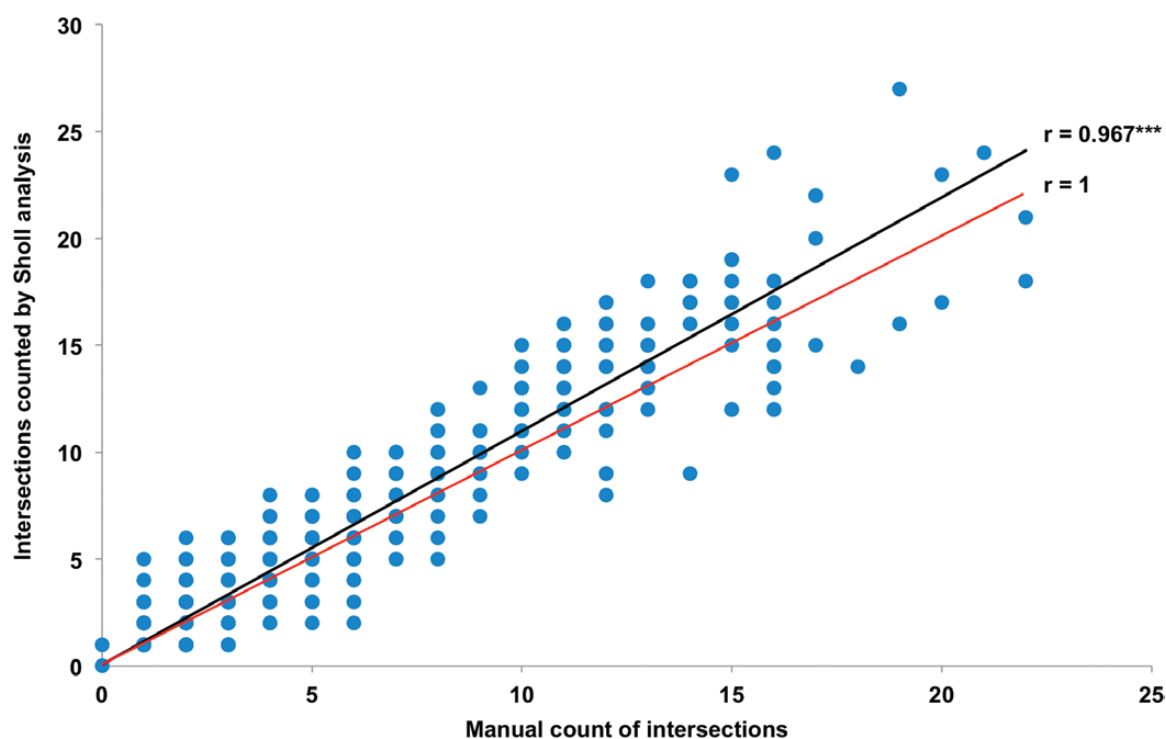


Fig. 5. Correlations between the manually counted number of intersections and the number of intersections measured with Sholl analysis. Measurements were done on roots of two different winter oilseed rape genotypes cultivated under control and osmotic stress conditions. Regression lines were indicated for the calculated correlation ($r = 0.967$) and the maximum possible correlation ($r = 1$) between manually counted intersections and intersections counted by Sholl analysis. Total sample size: $n = 20$. Significance at $***P < 0.001$ (this figure is available in colour at JXB online).

in this study suggest that osmotic stress in winter oilseed rape invokes an increase in the mean length of lateral roots as an early adaptive response. The 4-fold higher MLRL observed in a genotype resistant to drought under osmotic stress (Fig. 2C) was not observed in the drought-sensitive genotype and was only seen as a trend under stress conditions. However, this apparently drastic response contributed neither to a significant

increase of RL nor of total LRL, as it occurs at the expense of the absolute NLR in both genotypes. A reduction in the NLR was shown to be correlated with a decrease in the PRL, a phenomenon that was observed in the stress treatment as well (Table 1).

A stimulating effect of drought on lateral root length has previously been observed (Jupp and Newman, 1987; van

Table 1. Correlations between root parameters and total number of root-circle intersections measured with Sholl analysis

	FW	NLR	RL	PRL	LRL	MLRL
NLR	0.10					
RL	0.31	−0.16				
PRL	0.41 [#]	0.84***	0.11			
LRL	0.24	−0.31	0.98***	−0.07		
MLRL	0.27	−0.71***	0.69***	−0.48*	0.77***	
IT	0.56**	0.00	0.67**	0.23	0.63**	0.54*

FW, fresh weight; LRL, lateral root length; MLRL, mean length of lateral roots; NLR, absolute number of lateral roots; PRL, primary root length; RL, total root length. Total sample size: n = 20. Significances at [#]0.1, *P < 0.05, **P < 0.01, and ***P < 0.001.

Table 2. Correlations between the first five components of a PCA including values from Sholl analysis and manually measured root traits

	PC1Sholl (37.4%)	PC2Sholl (20.6%)	PC3Sholl (11.1%)	PC4Sholl (7.7%)	PC5Sholl (5.7%)
NLR	0.69***	0.42 [#]	0.18	−0.15	0.09
MLRL	−0.14	−0.77***	−0.25	0.25	0.04
RL	0.32	−0.56**	0.13	0.05	0.13
PRL	0.79***	0.16	0.09	−0.33	−0.06
LRL	0.18	−0.59**	0.12	0.11	0.14

LRL, lateral root length; MLRL, mean length of lateral roots; NLR, absolute number of lateral roots; PCSholl, PCA including values from Sholl analysis; PRL, primary root length; RL, total root length. Total sample size: n = 20. Significances at [#]0.1, *P < 0.05, **P < 0.01, and ***P < 0.001.

der Weele et al., 2000; Yang et al., 2004). However, different effects of drought were observed for the number of lateral roots. On the one hand, a reduction in the number of lateral roots, as found in the present study, was confirmed by other studies (van der Weele et al., 2000; Placido et al., 2013). On the other hand, drought can also cause an increase in the number of lateral roots (Jupp and Newman, 1987; Yang et al., 2004). The coherence between drought compatibility and root plasticity is a controversial topic. Xiong et al. (2006) provided evidence for an inhibitory effect of drought stress on lateral root formation in *Arabidopsis thaliana* and concluded that the formation of longer vertical roots, reaching deeper soil layers, is more advantageous for water uptake. While this assumption probably holds true for certain climatic scenarios and soil conditions, it cannot be regarded as a general rule. In contrast, Ehdaie et al. (2012) showed that an increase in lateral root formation in *Triticum aestivum* (bread wheat) was associated with increased yield stability under certain forms of drought. Although a decrease in the number and an increase in the mean length of lateral roots under osmotic stress is not a commonly observed phenomenon, the present results show that this seems to be an adaptive response of young rapeseed plants to mild osmotic stress. Furthermore, osmotic stress causes a shift in circle-specific intersection patterns of both genotypes, indicating changes in root spatial distribution (Fig. 4). The stronger reaction in both the

absolute number of root-circle intersections (Fig. 3) and the number of circle-specific intersections under osmotic stress in the DR genotype compared to the DS genotype (Fig. 4), suggest that root architectural changes and changes in root distribution are more distinct in DR and consequently lead to better drought compatibility, while architectural changes in DS are not sufficient to impart drought resistance.

Sholl analysis quantifies root architectural plasticity

As demonstrated in the examples presented here, Sholl analysis helps to capture the interactive architectural properties of an entire root system and its response to the environment (Fig. 6). The Sholl parameters assessed revealed different root architectural and distributional responses to osmotic stress in two contrasting genotypes that seem to be involved in the manifestation of drought compatibility in *B. napus*. The results of the Sholl analysis suggest that root architectural characteristics are significantly influenced by osmotic stress even when such changes are not necessarily obvious from root biomass or RL (Fig. 2A,D). The absolute number of intersections captured depends particularly on the RL and on LRL and MLRL (Table 1). Sholl analysis revealed a significant increase in the absolute number of root-circle intersections in the drought-resistant genotype under osmotic stress, whereas the drought-sensitive genotype did not show a corresponding response (Fig. 3). This indicates that genotype DR has a greater reaction in the above-mentioned root properties than DS, likely conferring effective adaptation and growth maintenance under the given stress conditions.

A very interesting insight into the changes of root architecture and distribution was given by the spatial values provided by Sholl measurements. Different distance-specific intersection patterns were generated depending on the genotype and the treatment (Fig. 4). These patterns were converted into principal components that showed marked correlations to all manually measured root traits (Table 2). It is thereby evident that Sholl analysis is a phenotyping tool suitable for an interactive discovery of root architectural properties such as the root length, the number of roots, and their spatial arrangement. Especially the latter could also be described by the absolute values of the distance-specific intersection patterns. In contrast to root density, which captures the cumulative number of roots per volume or area, Sholl analysis captures the absolute value of roots, located at a specific distance to the root origin. From the patterns shown in Fig. 4, it can be concluded that osmotic stress in oilseed rape enhances the number of roots present in the upper half of the root, while it causes a reduction in the number of roots towards the root tip.

Advances and limitations of Sholl analysis for root characterization

Sholl analysis is a very simple and non-destructive method for characterization of root architecture and distribution in plants grown under hydroponic cultures. It is easy to apply Sholl analysis using the free, open-source software ImageJ.

As the Sholl method relies on punctual measurements, it does not require the extremely high image quality and resolution necessary for measurements of root longitude and growth dynamics. The method could be validated by the manual counting of roots intersecting hand-drawn circles (Fig. 5). The slight overestimation of intersection number in comparison to the manual counts might be caused by sampling errors caused by pixel background noise in the images. These artefacts might be eliminated by an increase in image quality. To evaluate positional effects, the complex root system of a 22-d-old, soil-grown rapeseed plant was placed 10 times individually on the scanner plate. Calculations of standard deviations accounted for slight positional effects, while intersection patterns remain highly similar for all root images (Fig. 7). Positional effects might be excluded by using roots that are fixed in gel-based rhizotrons, for example.

One potential limitation of the Sholl method is the restriction to 2D root properties. Furthermore, Sholl analysis does not differentiate whether changes in intersection number are caused by alterations in root length or by the number of roots.

Results could be biased by curved root segments that cross the same circles more than once. Moreover, different root types cannot be distinguished. Therefore, if specific information about these root characteristics is required, the method has to be complemented by other specific software for measuring global root characteristics. A number of such packages provide information on type-specific root number and length or growth dynamics, for example RootReader2D (Clark *et al.*, 2013), RootTrace (French *et al.*, 2009), or WinRhizo (Arsenault *et al.*, 1995). In fact Sholl analysis represents a potentially useful additional tool for commercial root analysis software, because it captures interactive and responsive properties of a root system in a single step. The presented results showed that a significant effect of osmotic stress could be observed by intersection number and pattern, but not in terms of FW or root length characteristics such as RL, LRL, or PRL (except for PRL in genotype DR). This indicates that the Sholl method is more sensitive to mild osmotic stress applications than single biomass or length measurements. These results show that differences in interactive root

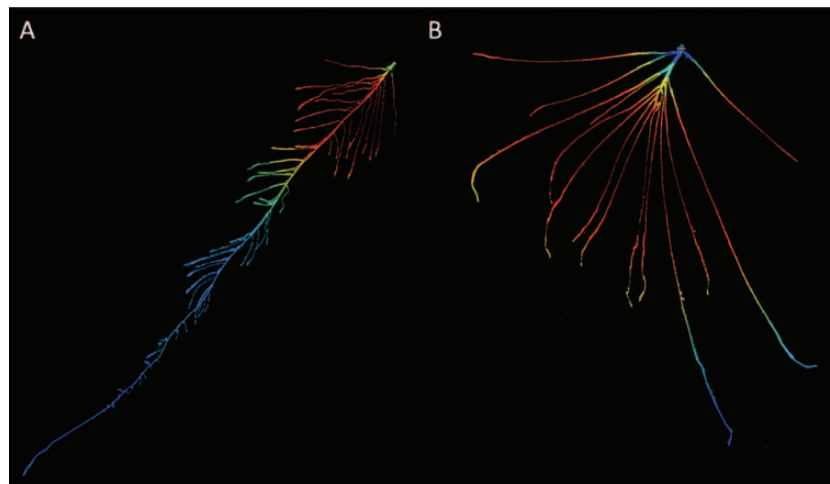


Fig. 6. Examples for Sholl's intersection masks of two contrasting extreme winter oilseed rape root phenotypes A (DR, control) and B (DR, PEG 6000).

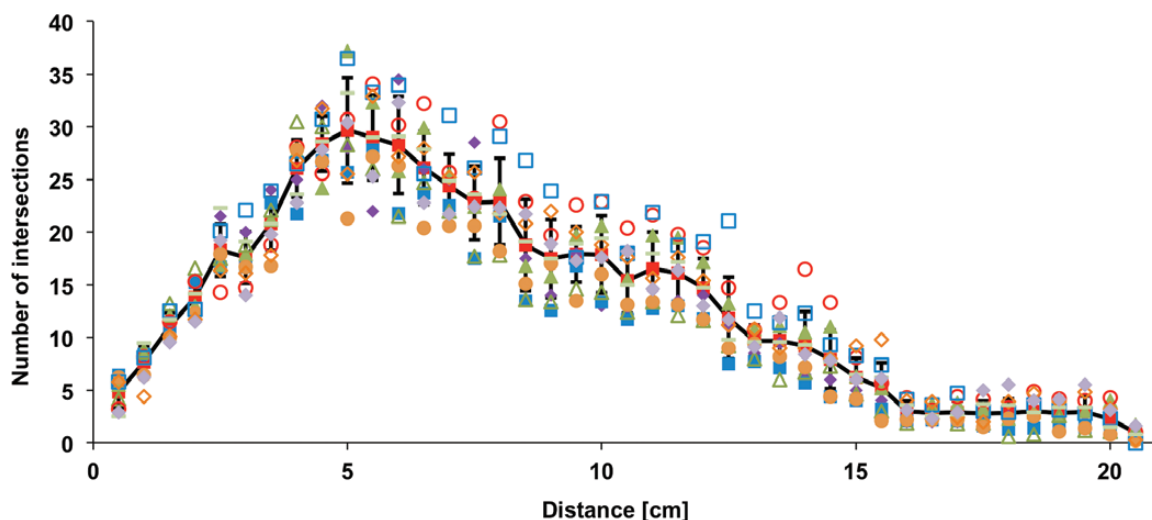


Fig. 7. Number of root intersections depending on the distance from root origin of a single soil grown winter oilseed rape root, repeated in 10 technical replications by re-spreading the root 10 times on the scanner plate. Sholl analysis was repeated on each scan. The single replications are represented by different symbols. Mean values are represented by a solid line. Error bars represent standard deviations (this figure is available in colour at JXB online).

architecture and spatial arrangement can be reliably investigated in a single time-saving step by applying the principles of Sholl analysis.

Conclusions

Sholl analysis, an established method for neural network analysis, was shown to be a powerful tool for the disclosure of early interactive root properties in seedlings of winter oilseed rape. This method unravels complex architectural changes, including root length properties and number of roots along with their spatial arrangement, that may be overlooked by singular-dimensional measurements. The method thus provides a novel opportunity for the selection of plant cultivars with beneficial root phenotypes and enhanced drought compatibility. An acceleration of data extraction by semi-automatic root image analysis might form a basis for inclusion of Sholl analysis in high throughput phenotyping platforms. Use of Sholl analysis can potentially provide added power to established root phenotyping software. The usefulness of Sholl analysis for studies investigating the basis of drought resistance in crop plants has been demonstrated here.

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3 Discussion

3.1 New insights for improving seed vigor in winter oilseed rape

High-throughput genomics technologies have emerged during the past decade as a powerful and useful toolbox for the dissection of molecular factors influencing complex, agronomically important traits. In genome-wide association studies, complex genotype and phenotype data sets are processed in order to delineate genomic regions associated with a trait of interest. In the present study this method was implemented for the disclosure of relevant genes involved in seed germination and seedling growth in rapeseed. Different quantitative trait loci (QTL) could be identified harboring promising candidate genes for improved seed vigor performance. The identification of several SNP markers showing linkage to QTL and potential candidate genes facilitates genomics based selection for improved seed vigor. Furthermore, a large potential was revealed for enhancement of seed germination and early radicle growth by pyramiding of desirable QTL alleles. The results provide new genetic insights into the complex regulation of seed germination and early seedling growth. However, little is known about the physiological function of the gene products encoded from the associated loci. A selection of promising genes was disclosed by gene ontology analysis. The following section provides an overview of the most interesting candidate genes and their possible involvement in the processes of seed germination and seed vigor in winter oilseed rape.

3.1.1 The hypothetical role of *Bna.SCO1* in chloroplast biogenesis during seed germination

In *Arabidopsis thaliana*, different gene mutations have been identified causing a disturbance of chloroplast development in the cotyledons, which consequently leads to impaired pigmentation (Albrecht *et al.* 2006, 2008, 2010). According to this phenotypic occurrence, the corresponding mutants were named *snowy cotyledon* (*sco*). While mutations in the *SCO1* gene impair the translation of plastid-specific mRNA (Albrecht *et al.* 2006), *SCO2* is assumed to be involved in chloroplast protein folding after translation (Albrecht *et al.* 2008). The gene *SCO3* is suggested to be a microtubule-associated peroxisomal protein (Albrecht *et al.* 2010) which is also required for chloroplast biogenesis.

A *B. napus* ortholog of *SCO1* (*Bna.SCO1*) was found to be localized within a QTL region on chromosome A9 associated with elongation speed of the radicle. In *A. thaliana*, mutations in *SCO1* were also associated with a delay in seed germination and later developmental processes, such as bolting or flowering (Albrecht *et al.* 2006). According to Ruppel and Hangarter (2007) and Albrecht *et al.* (2006), the *SCO1* gene encodes for chloroplast elongation factor G (EF-G) which catalyzes the translocation of the peptidyl-tRNA complex from the A to the P site of the ribosome. It is not yet clarified why mutations in *SCO1* cause chlorophyll deficiency in the cotyledons, while normal pigmentation occurs in the true leaves. One explanation might be that other EF-G proteins can compensate for disturbed *SCO1* function during later developmental stages (Ruppel and Hangarter 2007).

Under the assumption that seed vigor in *B. napus* is impaired by a corresponding *Bn.sco1* mutation, it might be expected that this mutation would also lead to a disrupted translation of chloroplast mRNA. To validate this expectation, chlorophyll a and b concentrations were determined in the cotyledons of rapeseed seedling with extreme vigor performance. The results showed consistently lower chlorophyll concentrations in the poor performing lines, accounting for impaired chloroplast biogenesis. However, the observed differences were only gradual. One reason for this could be that other gene copies partially compensate a loss of function of a putative *Bn.sco1* mutation on chromosome A9. In total 10 copies of *Bn.SCO1* were localized across the entire *B. napus* genome. Another reason might be that allelic differences in *Bna.SCO1* do not cause a complete loss of protein function. Ruppel and Hangarter (2007) confirmed that a knock-out of *SCO1* causes embryo lethality in *A. thaliana* and concluded that *SCO1* encoded EF-G is essential for embryo survival. Furthermore, allelic differences in *SCO1* were shown to have a significant effect on the binding affinity of EF-G to the ribosomal complex, for example by single amino acid exchange in the protein binding site (Albrecht *et al.* 2006), whereas a reduced level of chloroplast protein synthesis could be sustained. In conclusion, impaired eoplast to chloroplast transition is considered to be an obvious reason for a delay in seed germination and radicle growth in *B. napus*. The present study provides a basis for the elimination of undesirable *Bna.SCO1* alleles impeding chloroplast translation in future breeding materials with the help of marker assisted selection.

3.1.2 Hormone sensitivity and the initiation of seed germination

The intrinsic regulation of germination induction underlies a complex hormonal control. A major role is thereby taken over by the two phytohormones abscisic acid (ABA) and gibberellic acid (GA), which seem to have antagonistic effects within the mature seed. While ABA is responsible for the sustainment of dormancy and a suppression of seed germination, GA plays a key role in the release of dormancy and the initiation of seed germination (Hilhorst and Karssen 1992). Besides absolute hormone concentration and hormone ratio, it is known that hormone sensitivity status is a major factor of influence for dormancy release (Chiwocha *et al.* 2005, Finch-Savage and Leubner-Metzger 2006, Riefler *et al.* 2006). The impact of variable hormone sensitivity within the mature seed is illustrated by the prominent example of *A. thaliana* gene *abscisic acid insensitive 5* (*ABI5*). The gene *ABI5* encodes for a transcription factor belonging to the basic leucine zipper (bZIP) family involved in the regulation of ABA-responsive gene expression (Finkelstein and Lynch 2000). Functional mutations in *ABI5* are shown to cause a substantial decrease in ABA sensitivity which is associated with a reduced inhibition of germination (Finkelstein 1994). In contrast, overexpression of *ABI5* results in hypersensitivity to ABA and consequently in a stronger inhibition of seed germination and early seedling growth (Lopez-Molina *et al.* 2001).

While ABA and GA seem to be the key actors, several other signal molecules are discussed to be involved in the complex regulation of seed germination as well, for example auxin (Liu *et al.* 2007) or ethylene (Chiwocha *et al.* 2005). Furthermore, brassinosteroids are suggested to antagonize ABA signaling in *Arabidopsis* (Steber and McCourt 2001). The involvement of cytokinins is controversially discussed. Riefler *et al.* (2006) showed that germination speed was significantly enhanced in cytokinin receptor loss-of-function mutants, suggesting that cytokinins are negative regulators of germination. In contrast, cytokinin was confirmed to counteract ABA signaling and might therefore have a stimulating effect on germination (Wang *et al.* 2011). In *A. thaliana*, cytokinin signaling is mediated by different cytokinin-dependent two-component response regulators (*ARRs*) which act either as transcription activators (*ARR1*, *ARR2* and *ARR10*) or transcription repressors (*ARR4*, *ARR5*, *ARR6* and *ARR7*). *ARRs* are involved in the control of several developmental processes, such as meristem proliferation, cell division, leaf formation and senescence (Hwang and Sheen 2001) or even germination (To *et al.* 2004, Wang *et al.* 2011). One of these response regulators,

ARR4, represses the expression of the ABA-dependent transcription factor *ABI5*, which consequently inhibits the transcription of ABA-responsive genes and thus superposes ABA-dependent suppression of seed germination (Wang *et al.* 2011). In the present study, linkage analysis in rapeseed revealed that a genomic region on chromosome A9, harboring an ortholog of *A. thaliana* type-A *ARR4* (*Bna.ARR4*), was associated with germination speed and absolute rate of germination. This suggests a possible similar involvement of *Bna.ARR4* in the cytokinin-dependent regulation of seed germination in rapeseed, as in *A. thaliana*. Another interesting aspect is that *ARR4* is shown to be involved in the regulation of the circadian rhythm (Salomé *et al.* 2006). Since *Bna.ARR4* is also localized within a confidence region associated with flowering time in winter oilseed rape (Sarah Schießl, personal communication), this suggests a possible link between the regulation of germination and flowering time in rapeseed.

The importance of hormone sensitivity during germination is also illustrated by the following example: Holman *et al.* (2009) found that mutations in the *ATE* genes encoding for arginyl-tRNA protein arginyl-transferases in *A. thaliana* have a considerable influence on ABA sensitivity during seed dormancy, which is expressed in impaired seedling growth. As reviewed by Varshavsky (1997), *ATE1* is an important player in the N-end rule pathway and responsible for degradation of proteins labelled by specific destabilizing residues on their N-termini. Within this pathway, *ATE1* participates in the modification of secondary destabilizing residues into primary residues that are then further degraded into short peptides via the proteasome. It is suggested that mutations in *ATE* genes in *A. thaliana* cause ABA-hypersensitivity, due to impaired degradation of key factors involved in ABA signaling, although the nature of these components is still unknown (Holman *et al.* 2009). Similar effects might be expected for mutations in *B. napus* orthologs of *ATE1* (*Bna.ATE1*). In the present study *Bna.ATE1* was found to co-localize with SNP markers associated to a QTL for early radicle growth on chromosome A10. It might therefore be assumed that allelic differences in *Bn.ATE1* are expressed in a different determination of protein degradation due to the N-end rule pathway. These alterations seem to be coupled with modified ABA sensitivity, which might be again associated with the observed differences in onset of germination in rapeseed.

In summary, functional studies of the *A. thaliana* genes *ARR4* and *ATE1* demonstrate that variation in germination performance and early seedling growth is strongly associated with differences in hormone sensitivity within the mature seed. Results from the present genome-wide association study account for a possible involvement of *Bna.ARR4* and *Bna.ATE1* in the regulation of seed germination in *B. napus* as well. This suggests that hormone sensitivity status and hormone responsiveness are likely to significantly influence seed vigor performance in *B. napus*.

3.1.3 The genetic control of embryo and seed development

Embryo-defective (*emb*) mutations represent one of the main fractions of classical markers in the *A. thaliana* genome (Franzmann *et al.* 1995, Meinke *et al.* 2009). Mainly identified via t-DNA insertion, x-irradiation or by use of ethyl methanesulfonate, *emb* mutations cause defective seed development and embryo abortion (Meinke 1985, Erampalli *et al.* 1991, Franzmann *et al.* 1995). A broad set of these mutations could be assigned to different classes, appointing the embryonic stage in which development was arrested (<http://www.seedgenes.org>, Meinke *et al.* 2008). While several genes disrupted by *emb* mutations could already be functionally characterized, a large fraction of *EMB* genes may still be unidentified.

Because of their large incidence in the *A. thaliana* genome, it is not surprising that different *EMB* genes were found to be co-localized with QTL for seed quality and seed vigor related traits in the close relative *B. napus* within the present work. Orthologs of the two genes *EMB93* (*Bna.EMB93*; *BnaA06g33880D*) and *EMB2753* (*Bna.EMB2753*; *BnaC06g19160D*) were localized within genomic regions associated with thousand seed weight (TSW). An ortholog of *EMB25* (*Bna.EMB25*; *BnaC06g31150D*) is found to be associated with germination speed (T50) and absolute rate of germination (GR72). Additionally, the gene *Bna.EMB2474* (*BnaA09g13040D*) was co-localized with a genomic region associated with radicle elongation growth (ES).

A very interesting finding is that known lethal mutations in *A. thaliana* genes *EMB93*, *EMB2753* and *EMB2474* (*emb93*, *emb2753* and *emb2474*) cause developmental arrest during very early stages of the embryo (pre-globular, globular and transition stage), whereas

growth arrest in *emb25* occurs during the stage of cotyledon formation (Franzmann *et al.* 1989, Erampalli *et al.* 1991, Tzafrir *et al.* 2004, Johnston *et al.* 2007). Given that *Bna.EMB93* and *Bna.EMB2753* were identified as candidate genes for TSW, it might be assumed that early growth impairment partially has a decisive effect on embryo weight, which could therefore be measured in terms of TSW. A disruption of embryo development during later stages, as is the case for *emb25*, might not be reflected in reduced seed weight, but seems to affect subsequent germination performance. However, although embryogenesis was disrupted during the very early pre-globular stage in *emb2474*, no phenotypic alterations in TSW were found to be associated with the corresponding gene. In regard to the present results from genome wide association analysis it is suggested that allelic differences in *Bna.EMB93*, *Bna.EMB2753* and *Bna.EMB2474* cause gradual but non-lethal alterations in embryo and seed development in *B. napus*, as none of these genes are associated with GR72. In contrast, allelic variations in *EMB25* seem to be related to embryo survival rate, as an association to GR72 (absolute rate of germination) was observed in this case.

According to their orthologs in *A. thaliana*, *Bna.EMB93*, *Bna.EMB2753* and *Bna.EMB2474* are predicted to fulfill essential roles in the early embryogenesis, while *Bna.EMB25* is expected to be involved in later embryogenesis. However, their specific functions during embryogenesis are not completely deciphered yet. As stated by Meskauskienė *et al.* (2009), *EMB93* encodes for a plastid mTERF-related protein. A non-lethal mutation (*soldat10*) in *EMB93* led to impaired plastid homeostasis, associated with a disruption in plastid-to-nucleus signaling along with an altered expression of stress-related genes (Meskauskienė *et al.* 2009). It is not yet known if this is the cause for affected embryo growth. *EMB2753* is predicted to encode for an N-terminal acetyl-transferase involved in protein modification (<http://www.seedgenes.org>, Johnston *et al.* 2007). No information is available about its specific role during embryo and seed development. As stated by Deng *et al.* (2014), a lethal mutation in *EMB2474*, encoding for the mitochondrial membrane transporter protein TRANSLOCASE OF THE INNER MEMBRANE 9, causes a breakdown of mitochondrial structure and activity during embryogenesis due to crucial changes in mitochondrial membrane permeability. According to Kobayashi *et al.* (2007), *EMB25* encodes a chloroplast-localized DEVH-type RNA helicase. A mutation of type *emb25* is associated with an increase in plasmodesmatal size exclusion limit during the torpedo stage of embryogenesis (Kim *et al.* 2002), a quality which controls macromolecule movement between the cells. In this context

it is suggested that *EMB25* is involved in organelle-nucleus-plasmodesmata signaling, which is strongly associated with the expression of nuclear genes encoding products with plastid functions (Burch-Smith *et al.* 2011).

3.2 Integration of drought resistance traits in rapeseed breeding

3.2.1 Key features enhancing drought compatibility in winter oilseed rape

The impact of drought on a plant strongly depends on stress intensity, duration and timing. Small changes in these variables even during the early course of plant development can have a very large effect on plant growth and physiology. According to Blum *et al.* (1980), considerable differences in seedling survival rate can be noticed when stress application is shifted by only a few hours. The various forms of drought stress thus make it difficult to define plant key attributes which contribute to better growth and development under drought conditions. Indeed, plant adaptation responses are as manifold as the diverse shapes of drought stress. As reviewed by Passioura (1996), a plant pursues several adaptation strategies depending on the duration of drought stress: Osmotic adjustment already occurs after a few hours of water shortage, followed by subsequent architectural alterations, for example changes in root structure. During ongoing water stress plants then react with changes in their phenological behavior, such as an adaptation of flowering time or maturity (Passioura 1996). A different responsiveness dependent on the stress duration was also shown within the present study in winter oilseed rape. First, seedlings reacted with a decrease in stomatal conductance followed by abscisic acid (ABA) enhanced osmotic adjustment and a partial recovery of transpiration. Furthermore, changes in root and shoot fresh weight, as well as in root architecture, became obvious after a few days of ongoing stress.

Considering the large variability in the shaping of stress as well as in the adaptive responsiveness of an affected plant, it is even more interesting to reveal factors or attributes that generally contribute to drought compatibility, independently of stress timing, duration or intensity. Results of the *in vitro* experiments described here indicate that relative yield formation under drought in the field is already correlated with drought compatibility during seedling stage. These findings suggest the existence of resistance factors with a universal

character. One key trait involved in drought adaptation in rapeseed might be osmotic adjustment. This strategy has already been observed as an adaptive response to water stress in different members of the *Brassicaceae* family (Ashraf and Mehmood 1990, Wright *et al.* 1997, Ma *et al.* 2004, Norouzi *et al.* 2008). Findings from the present work revealed evidence of this strategy for rapeseed seedlings as well, but a broad difference was found between the observed cultivars. Osmotic adjustment occurred to a larger degree in the drought sensitive genotypes, but was not sufficient to completely restore transpiration. In contrast, osmotic adjustment occurred to a much lower extent in the drought resistant genotypes, while the latter could sustain their transpiration intensity. Additionally, results from the root phenotyping experiment suggest that effective root responses were more distinct in the resistant genotype than in the sensitive genotype which showed stronger osmolyte accumulation. Indeed, it seems likely that the drought resistant cultivars did not sense the stress to the same extent as the sensitive cultivars, since ABA concentrations increased only in the latter. Osmotic adjustment may not be the only factor triggering drought compatibility in the resistant cultivars, and the drought resistance seemed to be further constituted by another unknown quality. While both sensitive cultivars represent homozygous inbred lines, the two resistant cultivars are F1 hybrids. Thus it cannot be excluded that better drought compatibility is contributed in some way by positive effects from heterosis. Heterosis is defined as the superior performance of a hybrid relative to its homozygous parents. The impact of heterosis on stress compatibility is already well-documented. For example, Cheema and Sadaqat (2003) showed that rapeseed F1 hybrids generally performed better in seedling growth under drought conditions than their parents, in a manner independent from parental drought compatibility. Einfeldt *et al.* (2005) documented a considerable effect of heterosis on yield development and its components under drought conditions in barley. Furthermore, within the same work it was shown that the positive impact of heterosis increases with stress intensity. An increase in heterosis effect for grain yield relative to drought stress intensity was also described by Betrán *et al.* (2003). Araus *et al.* (2010) presented further evidence that hybrids are more drought resistant than inbred lines under very different water treatments, and hypothesized that “(...) heterosis may be considered an example of nonexistence of significant interactions between the genotype and the environment.” Consequently, heterosis must be discussed as a general contributor triggering drought compatibility.

It is generally accepted that heterosis is controlled by three different genetic interactions: dominance, overdominance and epistasis (Blum 2013). Another suggestion is that differences in gene expression in the F1 relative to its parents might play an important role in heterosis (Sun *et al.* 2004). For example, Stupar *et al.* (2008) hypothesized that additive complementation of parental transcriptional variation is more likely to cause heterosis in the F1, and Zhou *et al.* (2012) found that complementary gene expression patterns corresponding to insertion-deletion polymorphisms in different hybrid pools contribute to heterosis in rice. Although the causes for heterosis could be theoretically delineated, there is still a lack in knowledge about the molecular and physiological factors determining this phenomenon, especially in regard to complex traits such as drought resistance. One reason for enhanced drought compatibility might be a more effective water use in hybrids relative to inbred lines (Araus *et al.* 2010). According to Blum (2013), the advantage of heterosis under suboptimal growing conditions is maintenance of photosynthetic activity and formation of adequate source strength. He pointed out that these traits are already dependent on embryo growth and seedling vigor, stages at which hybrids seem to have decisive advantages due to an increased rate of cell division. A resulting rapid formation of initial leaf area is a main factor enhancing early light perception, source strength and seedling resisting power (Blum 2013). Correspondingly, *in vitro* experiments with rapeseed lines differing in their germination performance showed that increased seed vigor is not only correlated with seedling biomass but also with relative biomass formation under osmotic stress (data not shown). This again emphasizes the importance of seed and seedling vigor on general plant performance under abiotic stress.

3.2.2 Ideotype based breeding for drought prone environments

A frequently discussed current breeding concept is that of ideotype breeding, originally described by Donald (1968). He stated that traditional breeding programs mainly focus on either 'defect elimination' or 'selection for yield' and suggested a third kind of breeding model to which he referred as the 'ideotype concept'. Donald defined an ideotype as "(...) a plant model which is expected to yield a greater quantity or quality of grain, oil or other useful product, when developed as a cultivar (...)". The contrast to traditional concepts is that ideotype breeding requires a strict definition of a model plant to be developed for a

target environment, while selection is not focused on single individual traits in terms of 'defect elimination' or on yield *per se*. Thus a central point in ideotype breeding is the understanding of how single characters contribute to yield performance and might be auspiciously combined in one cultivar. Today, ideotype breeding has been widely accepted for the definition of model forms of several important crops, such as rice (Peng *et al.* 2008), maize (Lynch 2013) or oilseed *Brassicaceae* (Thurling 1991). As every environment makes different demands on plant architecture, physiology or phenology (see examples from chapter 1.4.1 and 1.4.4), a first step in ideotype breeding is the definition of the target environment. Afterwards, a plant model can be developed by incorporating architectural, physiological and phenological characteristics that are beneficial for plant and yield performance within the target environment.

The application of the ideotype concept is very often constrained by different facts and factors. Drought resistance indices seem to be very dependent on the given stress intensity (Sio-Se Mardeh *et al.* 2006). Thus, a clear definition of beneficial target traits is impeded. Blum (1996) reasoned that water stress is too manifold for defining a superior drought-compatible ideotype. Indeed, ideotype definition seems to be restricted if the target environment cannot be clearly defined. This can be illustrated by the following scenarios: Assuming that environment 'A' is characterized by longer-lasting moderate water scarcity, while environment 'B' is marked by severe short-term drought events, water acquisition due to a cost-intensive extension of the root system might result in higher yields in environment 'A', whereas a distinct reduction in water demand due to transient growth arrest might form a better exploitation of yield potential in environment 'B'. In other words, a morphological or physiological feature which is advantageous in environment 'A' might be associated with a fitness drawback in environment 'B'. In conclusion, the success of ideotype breeding depends on both a clear specification of the target environment and a clear definition of target traits that are beneficial in that environment. If both are available, a high yielding model plant can theoretically be effectively designed.

3.2.3 Novel breeding opportunities for the development of modern cultivars for European winter oilseed rape production

In the face of climate change, future European rapeseed production has to cope with more frequent drought extremes. However, cultivation of rapeseed is commonly realized on deep well-watered soils with proper nutrient and adequate water holding capacity. Therefore, breeding for European rapeseed ideotypes must focus on an environment characterized by an efficient water supply, but carrying a potential risk for temporary drought events during the growing period. Modern rapeseed cultivars should therefore combine a high yield potential with a broad adaptive potential to transient water scarcity. A high economic efficiency should furthermore be ensured by growth sustainment during periods of water scarcity, in order to maintain yield-promoting assimilation processes. The results of the present study suggest that different phenological, physiological and architectural attributes contribute to better growth and yield performance in winter oilseed rape under the above-described conditions. In the following paragraphs these aspects are discussed in terms of their potential consideration in determining and breeding a rapeseed ideotype in the context of climate change.

Phenology

Stress resistance is already formed during very early stages of plant development. As stated in Chapter 1.2.4, fast germination and rapid canopy development contribute to increased seedling resisting power and should be regarded as key characteristics for ideotype definition. Additionally, rapid ground covering minimizes evaporation and thus increases water use efficiency. A broad potential for the improvement of seed vigor was observed within this study, providing a novel breeding opportunity to enhance seedling development and robustness.

Physiology

The results of the present study justify the consideration of osmotic adjustment in the definition of a drought compatible rapeseed ideotype. Investigations showed that transpiration intensity, which is associated with photosynthetic activity, was partially restored by the accumulation of osmotically active compounds like proline or sugars. Although osmotic adjustment was more distinct in the genotypes which reacted with stronger growth impairment under osmotic stress, it should not be regarded as an index for

drought sensitivity. Rather, osmotic adjustment appears to be an adaptive response to drought that aims for the maintenance of plant water balance and a sustainment of assimilation and growth. Nonetheless, the experimentally observed dimension of osmotic adjustment in the sensitive genotypes was not sufficient to restore transpirational activity under the chosen stress conditions. However, it seems likely that better plant growth of the resistant genotypes is due to a combination of osmotic adjustment and other unknown qualities – one of which might be heterosis.

Architecture

Results from 'Sholl analysis' indicate that sustainment of root growth is another contributing characteristic under the experimentally applied stress conditions. A strong enhancement of lateral root elongation was observed in a drought-resistant cultivar. Under field conditions, this might be associated with better water acquisition due to a more extensive exploitation of the soil volume. Furthermore, the same cultivar reacted with a distinctive shift in its root distribution pattern, with an increase of root intersections towards the root tip that might be related to better water acquisition in deeper soil layers. On the other hand, the present results do not fully elucidate the extent to which root growth was promoted by osmotic adjustment, hence additional data is required to resolve this question.

To some extent, drought adaptation could not be defined as a measurable trait within this study. As discussed above, heterosis seems to play a significant role in the manifestation of drought resistance in rapeseed. From the present results it is unclear whether drought compatibility associated with heterosis has a constitutive or adaptive character. Furthermore it is unsure to which extent heterosis is expressed in the formation of the key traits discussed above, such as seed vigor, osmotic adjustment or enhanced root growth. In terms of ideotype breeding, the dimension of heterosis needs to be dissected into single components that can potentially be assayed as simple measurable traits.

In conclusion, ideotype-based breeding seems to be a good opportunity for breeding of drought compatible winter oilseed rape ideotypes. However, the concept reaches its limit if yield-determining factors and their complex interactions are not completely enlightened, as is the case for heterosis. Further investigation of this complex phenomenon might give some more interesting insights about the core issues underlying drought resistance.

3.3 Technical innovations in plant phenotyping

The true value of a cultivar can only be assessed in field experiments on several locations and in multiple years. However, drought stress experiments in the field are complicated by a lack of consistently drought-prone environments in most European countries. Additionally, a controlled application of drought stress is extremely difficult in field trials. The effects of other biotic and abiotic factors predominant under field conditions might furthermore bias observed relationships between drought stress and plant responses, thus making it difficult to clearly identify the key factors involved in drought resistance. Greenhouse or climate chamber experiments are therefore a good complement to field assessments, particularly for assessment of drought responses at seedling stages.

The efficiency of selection as well as the development of reliable genetic screening tools are both strongly dependent on the accuracy of phenotyping and the scope of individuals being considered in the phenotyping process (Araus and Cairns 2014). Within the last years conventional field phenotyping is therefore increasingly being replaced by high throughput tools which allow labor- and time-saving evaluation of large genotype panels (Munns *et al.* 2010, Clark *et al.* 2013, Yang *et al.* 2014). In order to increase the breeding success for drought resistance, there is still a need for tools which allow large scale phenotyping of drought responsive traits, for example transpiration intensity, metabolite patterns, or root architecture.

Within the present work an *in vitro* cultivation system was developed for controlled climate chamber experiments with young rapeseed plants. Osmotic stress conditions were simulated by the addition of polyethylene glycol to the nutrient solution. This long-chained polymer is commonly used for the investigation of drought stress responses in higher plants (Rhodes *et al.* 1986, Blum 1989, Ehlert *et al.* 2011, Ilhan *et al.* 2015), as it lowers the osmotic potential of an aqueous solution. The applicability of the described hydroponic cultivation system was demonstrated in two independent experiments (Chapters 2.2 and 2.3) focusing on the investigation of shoot and root responses of rapeseed seedlings to osmotic stress. A major advantage of the established *in vitro* cultivation system is that it avoids laborious excavation of roots, as is the case in soil experiments. Based on this hydroponic cultivation system, a new low-cost phenotyping tool for root analysis was developed inspired by a longstanding neuroscientific method called 'Sholl analysis'. The method was originally developed for the

investigation of neural network architecture. Applied to root systems, the Sholl method captures features of root architectural variation and plasticity that cannot be captured by conventional phenotyping software. Thus, selection for desirable root characteristics could be further improved when integrating the principles of Sholl analysis into conventional phenotyping procedures. The method has considerable promise for implementation into large-scale, automated root imaging and analysis systems, as described for example by Nagel *et al.* (2012).

4 Summary

Within the last decades enormous progress has been made in increasing crop yields by conventional breeding approaches. For rapeseed, a mean yield increase of over 1.5% per year has been realized in Europe since 1961 (<http://faostat3.fao.org>). However, further progress threatens to be diminished by loss of genetic diversity in current breeding pools. In consideration of a growing world population this could lead to food and feedstock shortfalls, and the situation is further aggravated by the effects of climate change. As long as gene technology is not generally accepted by European citizens, other strategies have to be developed for securing food, feed and fuel production.

One possibility is the improvement of secondary traits which have an indirect effect on yield potential and yield stability, such as biotic and abiotic stress resistances or enhanced vigor. Particularly in the face of climate change, it seems crucial to increase crop resistance in terms of improved vigor and stress adaptability. To achieve this aim, new screening tools must be developed which allow a reliable selection for beneficial trait or gene combinations contributing to enhanced seed vigor and stable seedling development. A major aim of the present work was the identification of physiological, anatomic and genetic markers associated with improved seed germination and stable seedling development under optimal and suboptimal water conditions in rapeseed.

A considerable potential for the improvement of seed vigor could be demonstrated within a genomics screening approach, in which several quantitative trait loci (QTL) could be identified throughout the rapeseed genome underlying seed germination and early seedling growth. Estimations of heritability indicate that seed vigor performance is strongly

dependent on both the environmental state and the genetic background. Promising candidate genes for seed germination and seedling growth were found in co-localization with the identified QTL. The most interesting candidates were the genes *Bna.SCO1*, *Bna.ATE1* and *Bna.ARR4*, which are suggested to be involved in chloroplast biogenesis or hormone signaling during dormancy release. Haplotype analysis revealed that seed vigor could be substantially improved by a pyramiding of desirable alleles. Genomic SNP markers were identified which are anchored in the genomic regions associated with seed vigor. These markers are now available for genomics-based selection for improved seed vigor.

Within two independent physiological experiments, osmotic stress responses of rapeseed genotypes differing in their drought resistance were investigated with regard to the seedling shoot and root. Genotypes were selected with regard to their relative yield development under drought conditions in the field. For a controlled application of osmotic stress, a novel hydroponic cultivation system was developed. In response to the osmotic stress treatment genotypes reacted with a shift in their shoot metabolite and hormone patterns. An accumulation of osmotically active compounds, such as proline or sugars, suggest that osmotic adjustment is an important factor in the adaptation of rapeseed to drought. However, osmotic adjustment was more distinct in homozygous lines than in hybrids, while the latter showed better growth performance under osmotic stress. It is therefore assumed that enhanced drought compatibility can be at least partially, and possibly mainly attributed to heterosis. For the characterization of root responses to osmotic stress, a new phenotyping tool was established, basing on the principles of 'Sholl analysis', a neuroscientific method applied for neural network analysis. The results showed that Sholl analysis captures interactive root properties which are normally not captured by conventional root phenotyping software. Under osmotic stress, rapeseed seedlings reacted with altered root architecture, due to enhanced lateral root growth at the expense of the number of lateral roots. A stronger reaction was observed in the resistant genotype. This suggests that the observed changes in root architecture contribute to a better water acquisition during soil desiccation in the field.

The innovative value of this work relies on the identification of several genomic, physiological and anatomic key factors which contribute to improved seed germination and seedling growth under optimal and suboptimal water supply. Furthermore, technical

innovations were achieved, amongst them a hydroponic cultivation system and a novel root phenotyping method that simplify selection for improved drought adaptation in rapeseed. An introgression of the genomic markers and key traits disclosed within this work could enhance modern breeding programs focusing on the development of stable and high yielding cultivars of winter oilseed rape.

5 References

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6 Appendix

6.1 List of figures

Figure 1: Rapeseed production quantity and area harvested, displayed for the main contributors to worldwide rapeseed production. Amounts produced are given in million tons [mio t] on the left ordinate. Areas harvested are represented in million hectares [mio ha] on the right ordinate. Worldwide oilseed rape production and total area harvested are equal to the sums of represented values. (Source of data: <http://faostat3.fao.org>)

FIGURE 2: Change in recurrence of 100-year droughts, based on comparisons between climate and water use in 1961 to 1990 and simulations for the 2020s and 2070s (based on the ECHAM4 and HadCM3 GCMs, the IS92a emissions scenario and a business-as-usual water-use scenario). Values calculated with the model WaterGAP 2.1 (Lehner *et al.*, 2006).

Figure 3: Potential production rate, percentage of light intercepted by the crop, calculated crop growth rate and measured crop growth rate of winter oilseed rape on experimental plots in the hercynian dry region of central Germany (Sibma, 1977; Diepenbrock, unpublished). (Diepenbrock, 2000)

6.2 List of abbreviations

AA	<i>Brassica rapa</i> genome
ABI5	<i>Arabidopsis thaliana</i> gene <i>Abscisic acid-insensitive 5</i>
ARR	<i>Arabidopsis response regulator</i>
AACC	<i>Brassica napus</i> genome
ABA	Abscisic acid
ATE	<i>Arginyl-tRNA protein arginyl-transferase</i>
Bna.ARR4	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>Arabidopsis response regulator 4</i>
Bna.ATE1	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>arginyl-tRNA protein arginyl-transferase 1</i>
Bna.SCO1	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>Snowy cotyledon 1</i>

<i>Bna.EMB25</i>	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>Embryo defective 25</i>
<i>Bna.EMB93</i>	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>Embryo defective 93</i>
<i>Bna.EMB2474</i>	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> gene <i>Embryo defective 2474</i>
<i>Bna.EMB2753</i>	<i>Brassica napus</i> ortholog of <i>Arabidopsis thaliana</i> <i>Embryo defective 2753</i>
CC	<i>Brassica oleracea</i> genome
DNA	Deoxyribonucleic acid
EF-G	Translation elongation factor G
<i>EMB, emb</i>	<i>Embryo defective</i>
ES	Elongation Speed (mm/h)
F1	First filial generation
F _{ST}	Fixation index
GA	Gibberellic acid
GR72	Germination rate within 72 h (%)
HEAR	<i>High erucic acid rape</i>
HOLLI	<i>High oleic low linoleic</i>
H _S	Heterozygosity of the subpopulation
H _T	Heterozygosity of the entire population
H ²	Broad-sense heritability
h ²	Narrow-sense heritability
LD	Linkage disequilibrium
mRNA	Messenger ribonucleic acid
mTERF	Mitochondrial transcription termination factor
PEG	Polyethylene glycol
QTL	Quantitative trait loci

<i>SCO</i>	<i>Snowy cotyledon</i>
SNP	Single nucleotide polymorphism
T50	Time necessary to reach 50% of germination (h)
tRNA	Transfer ribonucleic acid
TSW	Thousand seed weight
V_A	Additive component of genetic variance (V_A)
V_G	Genetic variance
V_P	Phenotypic variance

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Affidavit

I declare: this dissertation submitted is a work of my own, written without any illegitimate help by any third party and only with materials indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At any time during the investigations carried out by me and described in the dissertation, I followed the principles of good scientific practice as defined in the “Statutes of the Justus Liebig University Giessen for the Safeguarding of Good Scientific Practice”.

Giessen, May 2015