Genome-wide association analyses of loci for shatter resistance in *Brassicas*

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ABSTRACT

Dehiscence of siliquae (shattering) is a major issue for rapeseed (canola, *Brassica napus* L.) growers because it causes significant yield loss. To date, genetic variation for shatter resistance in cultivated *Brassicas* has not been comprehensively characterised. We evaluated 192 accessions of *Brassica napus*, *B. rapa*, *B. juncea* and *B. carinata*, representing contemporary cultivars and elite lines from Australian and international programs, for shatter resistance using a pendulum test. Accessions were grown in outdoor pot and field experiments in 2010 in two locations. At physiological maturity, five pods from five plants per genotype were collected in plastic vials. The strength of individual pods was tested using a pendulum machine that struck the pod with a known force and recorded the energy absorbed by the pod before breaking – the rupture energy (RE). This work revealed significant variation for RE that could provide resistance to field shattering (a higher level of RE is required to achieve improved shatter resistance). All accessions were genotyped with 1513 markers based upon DArT, SSRs and two candidate genes that are reported to be involved in shatter resistance in *Arabidopsis thaliana*, a distant relative of *Brassicas*. Bayesian and principal coordinate analysis revealed the significant population structure within the germplasm. Molecular marker data was used to identify associations using a mixed model approach that accounts for population structure as a fixed effect and genetic relatedness as a random effect, as implemented in the TASSEL software program. Our results suggest that an association mapping strategy can be applied to identify and validate loci/alleles associated with shatter resistance.

INTRODUCTION

Oilseed Rape (syn. rapeseed, *Brassica napus* L., spp. *oleifera*; genomes AACC, 2n=4×=38) originated through spontaneous hybridisation between turnip rape (*Brassica rapa* L.; genome AA, 2n=2×=20) and cabbage (*Brassica oleracea L.;* genome CC, 2n=2×=18), and was domesticated about 400-500 years ago (Go´ mez-Campo 1999). During its domestication and further genetic improvement, several traits have been targeted including increase in the seed number per pod, pod number per plant, plant architecture, adaptation of flowering time to local environments, oil content and quality, and resistance to insect-pest and diseases. However, dehiscence of siliqua (pod shattering) – a natural mechanism for survival and spreading of the species in the wild – has not been eliminated during the domestication of cultivated *Brassicas*. Shattering remains one of the significant problems for commercial production of rapeseed, as it could account for up to 50% yield loss (MacLeod 1981) in seasons conducive for pod shatter. Furthermore, shattered seeds produce 'volunteers' in following crops. For commercial success, canola genotypes require pods that will not shatter under normal conditions in the field but which can still be readily threshed in mechanical harvesters.

Mechanisms of pod shatter have been well studied in *Brassicas* and have been reviewed elsewhere (Kadkol 2009). In Arabidopsis, significant advances have been made in the molecular characterisation of several mutations which produce shatter resistance. Arabidopsis is a distant relative of rapeseed, estimated to have diverged approximately 43 million years ago. Dehiscence of Arabidopsis siliqua is very similar to the process in rapeseed and occurs via detachment of valves from the central replum (Ferrandiz *et al.* 1999). Several genes such as, polygalacturonase, β-1.4 glucanase, expansin, *SHATTERPROOF* (*shp1, shp2)*, *FRUITFULL (FUL), INDEHISCENT (IND), ALCATRAZ,* and *RUPLEMLESS,* have been identified that control

pod dehiscence in *Arabidopsis* (Liljegren *et al.* 2000; Ostergaard *et al.* 2006; Rajani and Sundaresan 2001; Sorefan *et al.* 2009).

Genetic variation for shatter resistance exists within *Brassica* species including *B rapa* , *B oleracea, B juncea* (genomes BBCC, 2n=2x=36), *B carinata* (genomes AABB, 2n=2x=34), *B. nigra (*genome BB, 2n=2×=16), and wild relatives of *Brassica* (Kadkol *et al.* 1985; Morgan *et al.* 1998; Prakash and Chopra 1988; Spence *et al.* 1996; Wang *et al.* 2007; Wei *et al.* 2010) and has been exploited to a limited extent in modern rapeseed breeding. Overseas research suggests that genetic variation for pod shatter resistance exists among *B napus* lines (Wen *et al.* 2008). Recently, Peng-Fei *et al.* (2011) evaluated 68 lines of *B. napus* for shatter resistance using a 'ripping' method (Tan *et al.* 2006) and showed that ripping force varies from 0.59N to 2.75N in different *B napus* accessions. This study further investigated the inheritance of shatter resistance and reported that two genes control shatter resistance, with heritability of 50%. This suggests that significant genetic gain can be made through conventional breeding in rapeseed. Genetic inheritance studies in *B rapa* revealed that shatter resistance is determined by 2-3 genes in the crosses from Brown Sarson/Torch and Yellow Sarson/Torch (Kadkol *et al.* 1986). Loci for shatter resistance have also been mapped in an intercross population derived from Torch (a shatter susceptible Canadian cultivar)/DS-17-D (a shatter resistant Indian line) (Mongkolporn *et al.* 2003). Recently, Banga *et al.* (2011) reported transfer of resistance to pod shattering from *B carinata* to *B napus*. Hybrid derivatives were characterised cytologically and further evaluated for shatter resistance using delayed harvesting.

In recent years, association mapping (AM) based upon linkage disequilibrium has become an important tool in the identification and validation of genetic linkages between molecular markers and traits of interest. AM overcomes the major limitations of the QTL analysis, a technique that utilises bi-parental populations (such as doubled haploids and recombinant lines), and generally only samples two alleles at any given locus. AM enables researchers to (i) survey a large number of alleles at one locus, (ii) perform high resolution mapping exploiting historic recombination events which occurred during germplasm development, and (iii) save the resources and time required to construct purpose-designed 'mapping and validation' populations derived from structured crosses. This approach has extensively been exploited in various crops in recent years (Agrama and Eizenga 2008; Aranzana *et al.* 2005; Aranzana *et al.* 2006; Arunyawat *et al.* 2007; Bradbury *et al.* 2007; Buckler *et al.* 2009; Ehrenreich *et al.* 2009; Jestin *et al.* 2011; Myles *et al.* 2009; Raboin *et al.* 2008; Stich *et al.* 2008; Stracke *et al.* 2009; Tommasini *et al.* 2007; Yu *et al.* 2006).

In the present study, we assessed the extent of genetic variability for shatter resistance in *Brassica* germplasm and used association mapping approach to identify genomic regions that are associated with the variation. To our knowledge, no such study has been reported in rapeseed so far.

MATERIALS AND METHOD

Phenotyping

A diversity set of 192 accessions of *Brassica napus* L, one of *B. rapa*, three of *B. juncea* and two of *B. carinata*, representing contemporary cultivars and elite lines from Australian and international programs was assembled by Mr Neil Wratten, at the Wagga Wagga Agricultural Institute. The accessions were grown in outdoor pot and field experiments in 2010 at the Wagga Wagga Agricultural Institute (New South Wales, Australia). The birdcage pot trial was originally arranged in two replicates on two benches in a glasshouse in a 4 row by 94 column array. The 2 row by 94 column array of pots on each bench was composed of 24 trays, each containing 1 row by 8 columns of pots. The pots were transferred to an outdoor birdcage enclosure until the end of the trial. Among 178 genotypes raised to maturity, 159 genotypes were duplicated and 19 genotypes were un-replicated. Accessions in the field trial were arranged in two replicates in 15 rows by 26 ranges. Data were available for 184 genotypes from two replicates and 8 genotypes unreplicated. The genotype factor was treated as a random factor.

At physiological maturity, ten pods from five plants per genotype were collected from the middle portion of the main raceme in the plastic vials. The strength (rupture energy) of up to six individual pods from five random plants sampled from each genotype was tested for shatter resistance using a pendulum machine that struck the pod with a known force and recorded the energy used to split the pod open as described previously (Kadkol, 2009, Liu *et. al.* 1994). The

pod length (PodLen) and rupture energy (RE) were measured for each pod. RELSQ was calculated as a measure of RE adjusted for variation in pod length ($RE/(Podlen^2)*1000$).

Molecular analysis

All accessions were genotyped with 1513 markers based upon DArT, SSR and candidate genes. DArT analysis was carried out at DArT P/L (Yarralumla, Canberra, Australia). DArT markers that have more than 90% call rate and high reproducibility were rated as good markers and used for association analysis. Population structure was determined using the STRUCTURE program and cluster analysis, applying the admixture model, a burn-in of 10,000 iterations and 10,000 MCMC durations to test for a K value in the range of 1-10 as described previously (Raman *et al.* 2010).

Association mapping

Molecular marker data was used to identify associations using a general linear model and mixed model approach that accounts for population structure as a fixed effect and genetic relatedness as a random effect, as implemented in the TASSEL software program.

RESULTS AND DISCUSSION

Statistical analysis revealed that there was a mean variance relationship in rupture energy measurements. Square root transformation of the rupture energy reduced this dependence. The component of variance analysis revealed a significant variation for shatter resistance due to genotype. Pod length was a significant covariate in both trials. Pod strength (rupture energy) measured using the pendulum test varied from 2.09 to 5.28, and 2.34 to 5.58 mJ under birdcage and field experiments, respectively. These levels of RE are associated with intermediate shatter resistance which could prevent shatter in standing crops but would be insufficient to prevent harvest shattering (Kadkol, 2009). Whilst we have yet to fully characterise the high RE accessions, such variation would most likely be due to subtle differences in the physiology and development of the dehiscence zone (Kadkol *et. al.* 1986). Pod strength measurements from both birdcage and field experiments (Fig 1.) showed positive correlation (Pearson correlation coefficient=0.56). This suggests that pod strength can be used as a reliable measure, for predicting shatter resistance in rapeseed. However, due to wet seasonal conditions, it was not possible to collect the data to correlate pod strength and shatter resistance in the field in 2010. This work will now be conducted in the 2011 growing season.

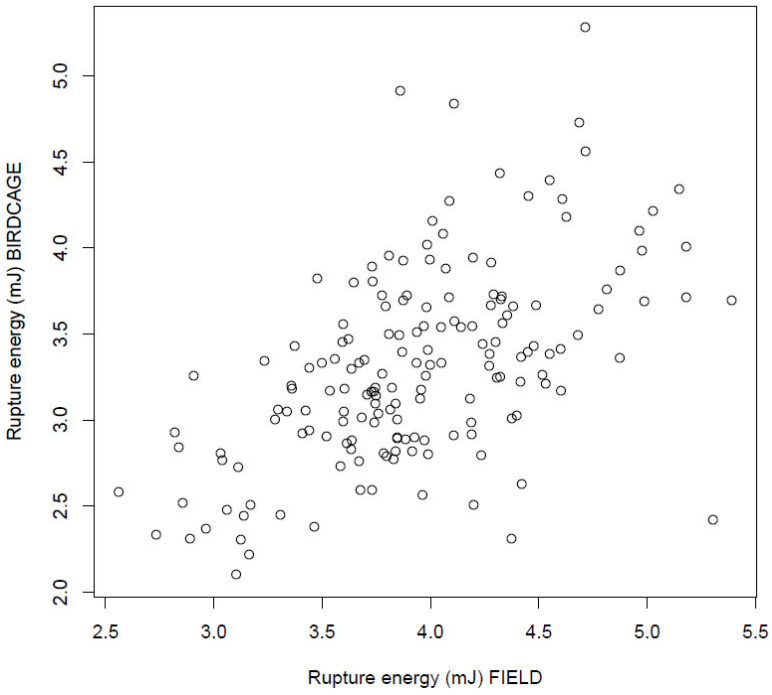

Fig 1: Relationships among different accessions for pod strength (mJ: millijoules) measured as rupture energy using the pendulum machine in two experiments (X) field and (Y) birdcage.

Bayesian and principal coordinate analysis revealed the significant population structure within the germplasm. This was due to use of contemporary Australian cultivars and elite breeding lines that had familial lineages. In order to reduce spurious associations between markers and shatter resistance due to genetic relatedness among accessions, we employed the STRUCTURE program (Pritchard *et al.* 2000). AM revealed that 150 markers were significantly associated ($P<0.05$) by the MLM model whereas the GLM detected a total of 266 alleles showing significant associations with rupture energy. Significantly associated markers were located on chromosomes A1, A2, A4, A6, A7, A8, A10, C2, C3, C5, C8 and C9. These map locations were based upon the linkage map of the DH population from Skipton/Ag-Spectrum. There are several DArT markers that showed significant associations with shatter resistance, but have not been mapped yet. Therefore, we were unable to assign them on the designated chromosomes/linkage groups and determine the linkage disequilibrium across different chromosomes. Attempts are being made to develop a consensus map based upon DArT and other marker systems employed in a range of various rapeseed populations.

We employed both GLM and MLM approaches for association mapping. In general, MLM proved to be a more 'stringent', as it is based upon Structure (Q) and kinship (K) matrices, and it identified a reduced number of significant marker-shatter resistance associations compared to GLM. Some of the loci are consistent with the map location of candidate genes involved in shatter resistance in Arabidopsis and *Brassicas*. New loci now need to be validated using a standard QTL mapping approach.

The large number of loci involved in determining shatter resistance in this study is in contrast with the small number of genes reported by Kadkol *et al.* (1986) and Mongkolporn *et al.* (2003), and is most likely due to the type of shatter resistance involved in this study. Both the previous studies involved shatter resistant *B rapa* with significant anatomical differences in the dehiscence zone. It is likely that the moderately high RE lines involved in this study only possess subtle physiological differences from the low RE lines but the anatomical features would most likely be similar. Further studies are needed to characterise this variation anatomically and genetically.

In summary, our results suggest that (i) an association mapping strategy can be applied to identify and validate loci/alleles associated with pod strength (shatter resistance), (ii) useful genetic variation for shatter resistance exists within cultivated Brassicas, and (iii) multiple loci control shatter resistance.

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