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Breeding *Brassica napus* for Shatter Resistance

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1. Introduction

Brassica napus (canola or oilseed rape) has emerged as an important cultivated oilseed crop species grown in temperate climates of both the northern and southern hemispheres. In 2009, canola was sown to approximately 23.8 million hectares worldwide and production was approximately 53.3 million tonnes (FAOSTAT, 2011). The name "canola" identifies the "double low" oil and meal quality (low erucic acid content in the oil and low glucosinolate content in the meal) of the crop. Innovations such as herbicide resistance have enhanced the value of canola in weed management and crop rotations and improved its profitability. Further oil quality improvements have resulted in specialty canola varieties producing high oleic and low linolenic acid oils suitable for frying applications. However, one requirement that has persisted through the relatively short history of domestication of *B. napus* is the need for substantial improvement in shatter resistance to prevent significant seed loss especially under adverse harvest conditions.

Dehiscence of siliqua due to external forces at or after maturity leads to siliqua shatter (Kadkol et al., 1986a). Siliqua shatter can occur both prior to harvest due to adverse weather conditions and at harvest due to impact from combine harvesters. Dehiscence of ripe, dry fruit is a natural process by which many plant species disperse their seed in order to survive and spread in the wild. Whilst this mechanism is advantageous in nature, siliqua dehiscence in agriculture results in significant yield loss. Moreover, the dehisced seed can persist in the soil up to 10 years in winter *B. napus*, giving rise to volunteer plants or weeds in subsequent crops (Pekrun et al., 1996; Gulden et al., 2003). Typically yield losses are in the range of 10%-25% (Price et al. 1996). Seed losses of as much as 50% of expected yield have been reported when adverse climatic conditions delayed harvesting (MacLeod, 1981; Child & Evans, 1989). Current cultural practices to reduce siliqua shatter and to achieve better uniformity of

ripening for harvest include windrowing (or swathing) and spraying desiccants. However, both these practices add to the cost of production and reduce flexibility in farm operations (Kadkol, 2009). Increased inherent shatter resistance could provide an option to delay harvesting to allow more even maturing of seeds and decrease the incidence of chlorophyll contamination from immature seeds in extracted oil (Morgan et al., 1998).

The fruits of *Brassicaceae* are botanically known as siliquae. Siliquae are derived from two carpels that form two locules separated by a thin, papery white replum. The fruit walls are valves that are attached to the replum forming a suture. The siliquae are attached to the raceme by a pedicel at the proximal end. At the distal end is the beak formed by the style (Fig 1). The suture is also known as dehiscence zone (DZ), where the valve margin is connected to the replum. Typically, a layer of thin parenchyma cells, that acts as a separation layer upon ripening, connects the valve margin to the replum. Dehiscence is usually initiated at the proximal end of the siliqua.

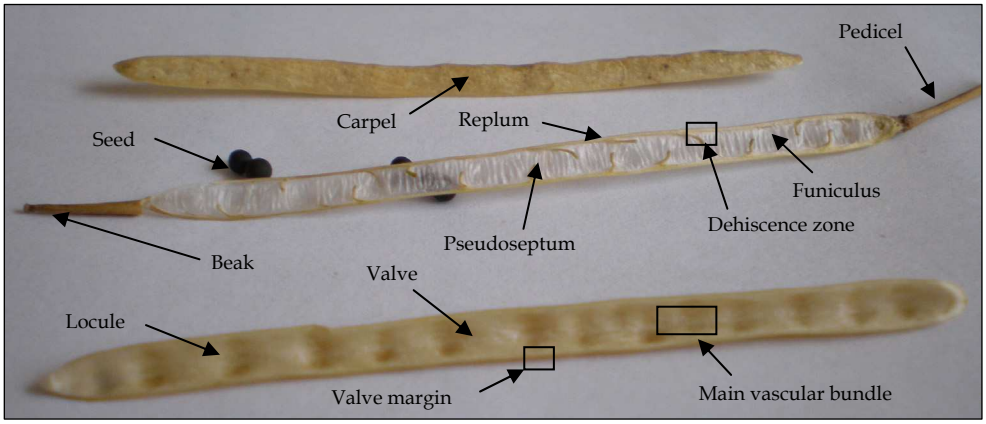


Fig. 1. The main structural features of a *Brassica napus* seed siliqua (from Kadkol, 2009)

Kadkol et al. (1986a) showed the presence of an abscission layer in the suture of siliquae of shatter susceptible *Brassica napus* and its absence in shatter-resistant *Brassica rapa* (Fig 2). They suggested that presence of an abscission layer is the basis of shatter susceptibility of *B. napus*. Differences in the vascular structure of siliquae and the width of the DZ has also been reported to be associated with variation for shatter resistance in a resynthesized *B. napus* line, 'DK142' in comparison with the shatter-susceptible winter *B. napus* line'Apex' (Child et al., 2003). The size of the main vascular bundle as it exited the valve and joined the vascular tissue of the replum was much larger in the resynthesized line.

Picart and Morgan (1984) investigated the physiological processes implicated in the control of siliqua dehiscence such as autolysis of the cells (degradation of pectic material in the middle lamella) of the DZ, senescence of the siliqua wall, water loss from thin walled cells, development of tensions resulting from different rates of drying of non-lignified and lignified cells of the valve and breakage of the vascular bundles at the base of the siliqua at the pedicel end. However, a study using polarizing microscopy by Kadkol et al. (1986a) suggested that development of tensions in the siliqua due to differential drying is unlikely.

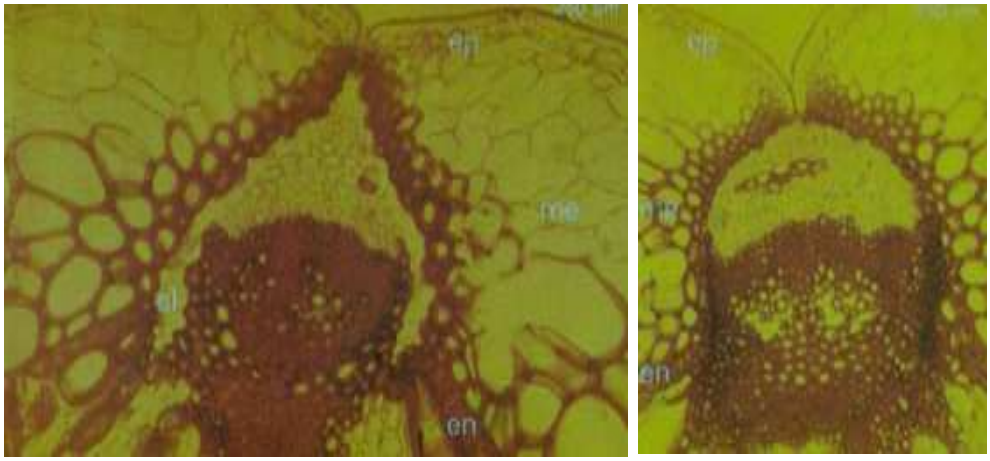
A number of possible factors involved in the expression of the siliqua shatter resistance include morphological, anatomical and biochemical aspects of siliqua development and physiology. It may even encompass biotic and abiotic stress factors (Kadkol et al., 1986a; Morgan et al., 1998; Morgan et al., 2003; Summers et al., 2003). A summary of siliqua and plant characters as well as other factors reported to be involved in siliqua shatter are presented in Table 1.

Source of trait	Trait	Trait type	Reference
Siliqua	Siliqua erectness	Morphological	Kadkol et al., 1984; Morgan et al., 2000
	Siliqua size, shape and weight	Morphological	Morgan et al., 2000; Squires et al., 2003; Dinneny and Yanofsky 2004
	Density of siliqua	Morphological	Kadkol et al., 1984
	Pedicel length	Morphological	Morgan et al., 1998; Kadkol et al., 1984
	Lignification of the suture/dehiscence zone	Anatomical	Kadkol et al., 1986a
	Lignification of the siliqua valves	Anatomical	Morgan et al., 1998
	Size of main vascular bundle	Anatomical	Child et al., 2003; Kadkol et al., 1989; Morgan et al., 1998
	Size of the dehiscence zone	Anatomical	Child et al., 2003
	Enzymatic activity	Biochemical	Morgan et al., 1998; Child et al., 2003
	Hormonal activity	Biochemical	Chauvaux et al., 1997; Child et al. 1998; Morgan et al., 1998
Canopy structure	Interaction between plants	Morphological	Bowman, 1984; Kadkol et al., 1989; Summers et al., 2003
Plant	Stem thickness	Morphological	Morgan et al., 1998
	Uniformity of flowering	Physiological	Chandler et al., 2005; Morgan et al., 1998
	Plant height	Morphological	Morgan et al., 1998; Morgan et al., 2000; Summers et al., 2003
	Raceme structure	Physiological	Child & Huttly, 1999; Summers et al., 2003
	Angle of the branches to the main stem	Morphological	Kadkol et al., 1984; Child & Huttly, 1999
	Number of primary branches	Morphological	Kadkol et al., 1984
Abiotic factors	Temperature	Environmental	Morgan et al., 2003; Summers et al., 2003
	Rain and drought	Environmental	Morgan et al., 2003; Summers et al., 2003
	Time of sowing	Environmental	Summers et al., 2003
Biotic factors	Pests e.g. siliqua midge, aphids	Environmental	Meakin & Roberts , 1991; Summers et al., 2003
	Pathogens e.g. alternaria	Environmental	Morgan et al., 2003

Table 1. Morphological, anatonomical, biochemical, physiological and environmental attributes implicated in siliqua shatter

2. Biochemical and molecular mechanisms underlying shatter resistance

Dehiscence of siliquae occurs as a result of highly coordinated and regulated events in growth and differentiation of the DZ and the degradation of the separation layer at ripening. This is due to triggering of enzymatic activity in DZ and cell separation predisposing siliqua to dehiscence from external forces. Several genes involved in growth and differentiation of the DZ have been identified and studied in *Arabidopsis* (e.g. Sorefan et al., 2009).



(a) (b)

Fig. 2. Transverse sections (x120) of fresh siliquae of *B. napus* (2a) and *B. rapa* cv. DS17D (2b) through dehiscence zones, stained with phloroglucine (al = abscission layer, en = endocarp, me = mesocarp, ep = epicarp) (from Kadkol et al., 1986a)

Many growth regulators such as abscicic acid (ABA), ethylene and auxin are well known for their role in abscission (Nemhauser et al., 2000; Ferrándiz, 2002; Sohlberg et al., 2006; Child et al., 1998; Meakin & Roberts, 1990*b*; Roberts et al., 2002). In *Brassica*, the role of an abscission cell layer in the siliquae dehiscence was first investigated by Kadkol et al. (1986a). Dehiscence is caused by the loss of cellular cohesion in the abscission layer, primarily attributable to the degradation of the middle lamella which appeared to result from an increased activity of hydrolytic enzyme cellulase leading to the cell separation process (Meakin & Roberts, 1990*a*; Meakin & Roberts, 1990*b*).

Johnson-Flanagan and Spencer (1994) found a climacteric of seed-produced ethylene preceding the pre-desiccation phase of *B. napus*. The evidence for ethylene acting as a regulator of dehiscence is unclear but it could still be a trigger for cellulase activity in DZ. Child et al. (1998) observed a correlation between delayed shattering and reduced ethylene production. The suppression of ethylene production by the treatment of siliquae with amino-ethoxyvinylglycine (AVG) delayed siliqua shatter. However, Roberts et al. (2002) reported *Arabidopsis* mutants that have nonfunctional ethylene receptors still exhibit a normal time-course of siliqua dehiscence and that the elevation in the cellulase β-1,4-glucanase in *B. napus* DZ occurs when the ethylene level in the siliqua is falling.

The activity of hydrolytic enzymes including β-1,4-glucanase and polygalacturonase involved in cell separation in the DZ appears to be regulated by auxin (Coupe et al., 1993). Chauvaux et al. (1997) observed that a decrease in auxin content in the DZ just prior to moisture loss in siliquae was correlated with a tissue specific increase in β-1,4-glucanase activity and hence with siliqua dehiscence. Auxin appears to have the opposite effect to ethylene and negatively regulates β-1,4-glucanase. Sorefan et al. (2009) demonstrated that formation of a local auxin minimum is required for specification of the valve margin abscission layer in *Arabidopsis* where dehiscence takes place. Thus, a low level of auxin

seems to be a prerequisite for siliqua dehiscence and may allow for the induction of the activity of cell wall degrading enzymes.

In addition to cellulase activity, dissolution of the middle lamella in the DZ is another important process leading to cell separation. Jenkins et al. (1996) and Petersen et al. (1996) cloned and characterised two DNA fragments, SAC66 and RDPG1 associated with an endo-polygalacturonase (endo-PG). Both the DNA fragments related to a single *Arabidopsis* ortholog (called SAC70). Both transcriptional and post-translational control of PG activity has also been proposed (Roberts et al., 2002; Sander et al., 2001). However, in contrast to the activity of the cell wall degrading enzyme β-1,4-glucanase, polygalacturonase exhibits no correlation either temporally or spatially with siliqua dehiscence (Meakin & Roberts, 1990). This lack of siliqua DZ specificity of the endo-PG promoter has prevented the engineering of shatter resistance by silencing the endo-PG (Ostergaard et al., 2007).

3. Methods for screening germplasm for shatter resistance

Many of the early assessments used to evaluate siliqua shatter resistance have been based on imprecise, visual field observations (e.g., harvest yield and visual assessments) or manual tests (Table 2). These tests are somewhat subjective and are often not necessarily comparable due to the difference in maturity and moisture status of siliquae or differences in environmental conditions (Morgan et al., 1998).

Approach	Type	Measure	Reference
Field observations	Visual scoring	Index	Josefsson, 1968
	Direct harvesting vs. windrowing	Yield	Josefsson, 1968
	Number of volunteer plants after harvest	Plants/area	Josefsson, 1968
	Seed counting after harvest	% seed loss	Josefsson, 1968
	Count shattered siliquae	% shattered siliquae	Tomaszewski & Koczowska, 1971
Mechanical test	Compress plants between plates	% shattered siliquae	Jakubiec & Growchowski, 1963
	Vibrate whole plants	% shattered siliquae	Voskerusa, 1971
	Squeeze siliquae between fingers	Index	Tomaszewski & Koczowska 1971
Anatomical test	Size of sclerenchymatic bridges between valves and replum	Thickness of sclerenchymatic bridge	Loof & Jonsson 1970

Table 2. Early tests used to identify shatter resistance in *Brassica* species

Kadkol et al. (1984) suggested that the methodology used to test siliqua shatter resistance should simulate shattering as it occurs in the field and during harvesting. They further

suggested that it would be most appropriate to test the siliqua as a cantilever because most external forces acting on the siliqua would load it at the distal end whilst it is attached to the plant at the proximal end. However, many of the mechanical tests (Table 3) including the random impact test do not achieve this requirement. Another requirement of testing procedures is that they should be low cost, fast and efficient. This criterion is not met by tests of the DZ that involve considerable preparation of the sample and subsequent technical demanding analysis.

To date, several mechanical testing procedures have been employed to investigate shatter resistance (Table 3) which allowed for greater comparability, accuracy and repeatability across different lines and cultivars. Liu et al. (1994) developed a pendulum-based test (Fig 3) that was a further development from the quasi-static cantilever test developed by Kadkol et al. (1984). The use of a pendulum provided a dynamic cantilever test of the siliquae that simulates the natural process in the field and achieves rates of loading comparable to those in the field. Recently, Kadkol (2009) reported further refinements of computer software and the apparatus for the pendulum test (Fig 4) which have improved the efficiency of the process as a screening method for use in breeding.

Name of the test	Purpose	Methodology	Reference
Manual bending test	Evaluate shatter resistance	Collected siliqua placed on flat surface with angles marked and with pedicel held firm. The siliqua is bent anticlockwise causing bending stress at which the angle is noted (this bending stress is similar to wind stress in field).	Roy, 1982
Cantilever test	To measure the bending moment and energy required to cause siliqua fracture	Siliqua is clamped at the pedicel end in a Universal Testing Machine. A steel wedge fixed to the load cell was used to load the siliqua as a cantilever, the applied force is recorded on the chart. Shatter resistance was defined as the bending moment at the peak of the force displacement graph. Another measure of shatter resistance was energy measured as the area under the curve up to the peak.	Kadkol et al., 1984
Microfracture test (MFT)	To establish the contribution of the main vascular bundle of the valve to the amount of energy needed to separate the valve from the replum.	Siliqua wall tissue is excised at the pedicel end of the valve or from the middle of the siliqua half-way between pedicel and the beak in order to isolate areas for testing that were ~1mm in length containing the septum and valve between which the DZ was intact. An L-shaped steel device is raised by a Universal Test Machine until fracture occurred.	Child et al., 2003

Siliqua twisting (applying torque)	To determine the strength of the DZ by applying twisting force to the siliqua. Angle at which seed siliqua rupture occurs and the maximum torque required for siliqua rupture	Torque applied under twist of 180° in a holder using an INSTRON device.	Tys et al., 2007
'Ripping' method	To quantitatively determine siliqua dehiscence strength at 2.5 cm from pedicel	6 siliqua per variety kept at 25°C and 50% RH for 2 weeks. A metallic thread laced around the siliqua 2.5 cm from pedicel and laced to the pedicel, siliqua glued to plate. An L-shaped probe of the texture analyser lifted thread and opened siliqua; probe recorded opening strength.	Tan et al., 2007
Pendulum test	To measure energy absorbed by the pendulum in siliqua rupturing process	Siliqua is clamped vertically by its stalk at the bottom dead centre of the pendulum swing. An optical encoder is used to measures the loss of pendulum movement upon striking and shattering the siliqua which provides an estimate of the energy absorbed by the siliqua.	Kadkol et al., 1991; Liu et al., 1994
Random Impact Test (RIT)	Measure breaking response of siliqua by mimicking conditions in the crop canopy caused by agitation during harvest or caused by poor weather conditions, fit a model and estimate half life of sample	Equilibrate siliqua in atmosphere of constant relative humidity (50%) and temperature (105°C) to achieve constant weight; 20 siliqua per sample (2 replications), Controlled agitation of sample in a receptacle (cylindrical of 20cm diameter, axis vertical) containing 6 steel balls (12.5mm diameter) and shaken in the horizontal plane, 17 seconds ; remove siliqua and classify them as shattered or intact.	Bruce *et al*. (2002); Morgan *et al*. (1998; 2003); Squires *et al*. (2003)

Table 3. Recent attempts to evaluate siliqua shatter resistance.

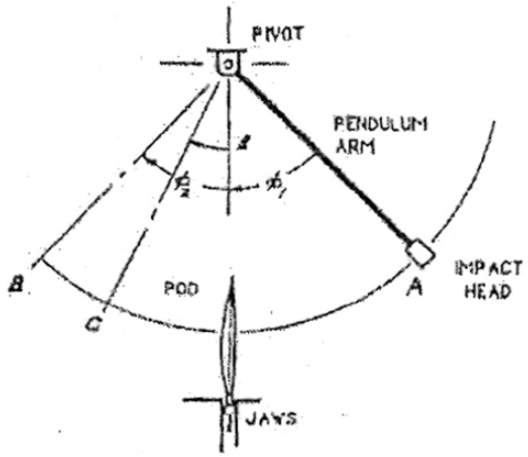


Fig. 3. Arrangement and analysis of pendulum (from Liu *et al.,* 1994).

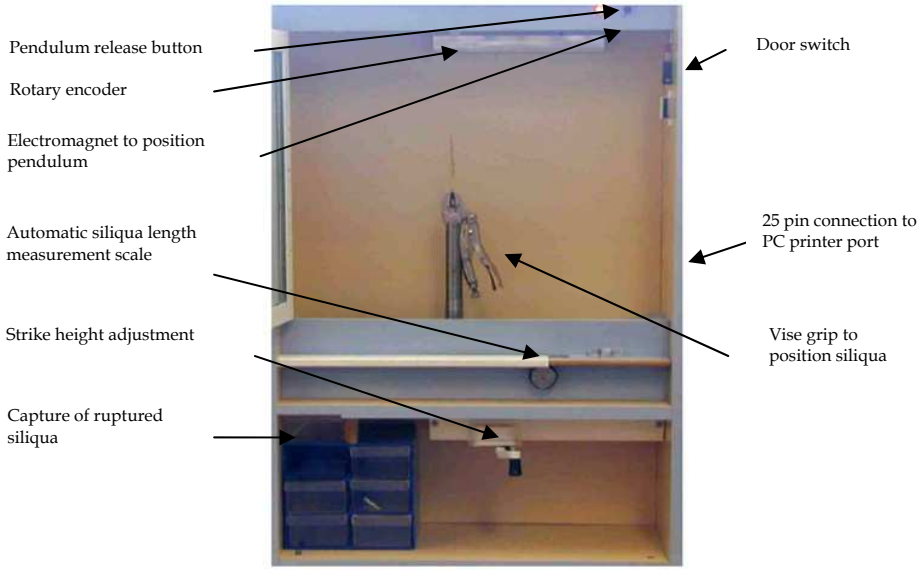


Fig. 4. The new pendulum machine for testing *Brassica* siliquae.

Morgan et al. (2003) cited the random impact test (RIT) as a good overall measure to compare the relative susceptibility of lines. The RIT involves agitation of 20 siliquae with ball bearings for 20 s and counting the number of intact siliquae. This test does not simulate the process of shatter as it happens in the field. These authors also quoted the tensile strength test as a useful test which correlates well to the RIT and field scores of shatter. However, the test appears to involve considerable sample preparation and hence is unsuitable for application in breeding programs. Wang et al. (2007) compared the degree of

correlation between field data and results from pendulum test and RIT. Although it is difficult to accurately quantify harvest losses due to shatter in the field, in the study of Wang et al. (2007), RIT showed a lower level of association with field shatter than the pendulum test.

Morphological characters associated with shatter are more difficult to quantify. Delayed harvesting restricts the accuracy and effectiveness in discriminating between small differences in shattering affected by climatic and other environmental factors such as bird damage.

4. Genetic variation for shatter resistance

Genetic variation for shatter resistance exists both within *Brassica* species including *B. rapa* L., *B. juncea* L., *B. hirta* L. and within wild relatives of *Brassica* (Kadkol et al., 1985; Wang et al., 2007). Although there is some variation in *B. napus*, the level of resistance available is generally considered inadequate to avoid windrowing of crops on a routine basis (Raman et al., 2011). There have been a small number of reports characterizing genetic variation for shatter resistance in *Brassica* in germplasm collections. Wen et al. (2008) investigated the siliqua shattering resistance index of 229 accessions (mostly of Chinese origin) of *B. napus* using RIT. Most of the accessions (59.4%) were very susceptible to siliqua shatter. However, there were two lines considered to be shatter resistant which could potentially be used as parents to develop new varieties for improved this trait. Peng-Fei et al. (2011) evaluated 220 lines of *B. napus* for shatter resistance using 'ripping' method (Tan et al. 2007) and showed that ripping force ranged from 1.46N to 4.23N. The levels of pod strength reported in this study appear to be in general agreement with studies in Australia (Kadkol et al., 1984; Raman et al., 2011) indicating limited genetic variation in *B. napus*. Raman et al. (2011) evaluated 181 accessions of *Brassica napus*, one *B. rapa*, three *B. juncea* and two accessions of *B. carinata*, using a pendulum test (Kadkol, 2009) in two separate experiments. These accessions were collected from different parts of the world, representing contemporary cultivars and elite lines from Australian and international programs for shatter resistance. There was a moderate degree of correlation between the two sets of data. Siliqua strength (rupture energy - RE) values varied from 2.09 to 5.28 mJ and 2.34 to 5.58 mJ respectively, in the two experiments, indicating good correspondence between the two trials. These levels of RE are associated with intermediate shatter resistance which could prevent pod shatter in standing crops but insufficient to prevent harvest shatter (Kadkol, 2009). Genetic variation for higher levels of siliqua strength necessary for resistance to harvest shatter is present in *B. rapa vars Yellow Sarson* and *Brown Sarson* (Kadkol et al., 1984; Liu et al., 1994; Mongkolporn et al., 2003; Kadkol, 2009). Shatter resistance could be improved by introgressing the trait from these types and *B. juncea* (Kadkol 2009; Raman et al., 2011).

5. Inheritance of shatter resistance

Kadkol et al. (1986b) considered the genetic variation for shatter resistance within *B. napus* to be limited and studied inheritance of shatter resistance (measured as siliqua strength) in *B. rapa* in crosses between *Brown Sarson* (shatter resistant) and Torch (shatter susceptible) and *Yellow Sarson* (resistant) and Torch (susceptible). Segregation in the F_2 generation indicated the presence of 2 to 3 recessive genes which showed dominant epistatic interaction controlling shatter resistance. Further genetic analysis in one cross (Torch x DS-17-D)

showed the presence of significant non-additive and additive genetic variances and a high broad sense heritability of shatter resistance (Kadkol et al., 1986c). The degree of dominance for shatter resistance was close to one supporting results from Mendelian analysis. In a subsequent study, Mongkolporn et al. (2003) confirmed a phenotypic segregation ratio of 12:3:1 (susceptible: intermediate: resistant) in an F_2 population derived from the Torch x DS-17-D, which indicated two recessive major genes (*sh1* and *sh2*) with dominant epistasis conferring the resistance. This supports the earlier findings of Kadkol et al. (1986b).

Morgan et al. (2000) reported that shatter resistance in *B. napus* was recessive and mostly determined by additive genes. In their study, correlation of shatter resistance with important agronomic traits was low, suggesting that it would be feasible to introgress the shatter resistance trait into commercial breeding lines. They also noted the absence of genetic linkage of siliqua strength with other siliqua characters such as short/long siliqua or erect/horizontal siliqua. This suggested that it should also be possible to enhance shatter resistance by combining it these characters. Peng-Fei et al. (2011) investigated inheritance of shatter resistance in *B. napus* by mixed model analysis of parental lines, F_1, BC_1, RBC_1 and F_2 generations. They showed that two genes with additive–dominance–epistatic effects plus polygenes with additive–dominance-epistatic effects control shatter resistance. The heritability of two major genes in the F_2 and backcross generation ranged from 49.4% to 50%, suggesting that significant genetic gain can be made through conventional breeding.

Molecular studies of dehiscence zone specific mRNAs have led to isolation of genes which have been considered to be involved in production and regulation of enzymes involved in degeneration of the separation layer upon siliqua ripening (Coupe et al., 1993, 1994; Petersen et al., 1996; Whitelaw et al., 1999). In *Arabidopsis*, seed shattering is controlled by the several MADS-box and homeodomain genes. Screening of *Arabidopsis* enhancer or gene trap lines (Ferrándiz, 2002) identified genes involved in DZ differentiation. SHATTERPROOF (*SHP1*) and SHATTERPROOF (*SHP2*), previously called *AGL1* and *AGL5* respectively, are closely related MADS-box genes and are members of a monophyletic clade that also includes A*GAMOUS* and *AGL11* control and promote DZ differentiation at the valve-replum boundary in *Arabidopsis* (Liljegren et al., 2000). The expression of *shp1* and *shp2* is regulated by *AGAMOUS* (Savidge *et al.,* 1995), *FRUITFULL* (Ferrandiz et al., 2000) and *REPLUMLESS* genes (Roeder et al., 2003). Recently, *SHP1* and *SHP2* have been shown to play an important role in promoting stigma, style and medial tissue development (Colombo et al., 2010). Another indehiscent mutant gene, *ALCATRAZ* (ALC), corresponding to the bHLH transcription factor, has been isolated which is involved in the development of the abscission layer in the DZ and direct cell differentiation (Rajani & Sundaresan, 2001). Girin et al. (2010) reported that the *REPLUMPNESS* (*RPL*) gene which acts by limiting the expression of the valve margin identity genes; *Shp-1* and *Shp-2*, *INDEHISCENT* and *ALC* to the narrow strips where wall margins will form. In the valves, the *FRUITFULL* gene is required for post-fertilization development and elongation of the fruit and it acts similarly to the *RPL* by repressing *Shp1/Shp2* and *IND* gene activity.

6. Breeding *B. napus* for shatter resistance

Previous research on evaluation of *B. napus* germplasm have revealed that there is limited variation in siliqua shatter resistance among current cultivars (Bowman, 1984; Kadkol et al., 1985; Downey & Röbbelen, 1989; Roberts et al., 2002). Ostergaard et al. (2007) ascribed this to

the narrow genetic base as a result of breeding focus on 'double-low' cultivars originated from two cultivars, Bronowski and Liho. Also, the recent studies of variation for shatter resistance in germplasm collections (Raman et al., 2011; Wen et al., 2008) support previous reports of a general lack of variation for high levels of shatter resistance in *B. napus*.

Tolerance to field shattering has been developed in some Australian breeding programs by direct heading of breeding trials and plots as an indirect selection method for shatter resistance. Although the varieties from Australian programs have not been properly characterized for shatter resistance, there appears to be significant improvement in field shatter tolerance in new lines relative to older varieties (Kadkol, 2009; Hossain et al., 2011a). However, further improvement in shatter resistance is required to allow direct heading of commercial crops. The conventional approach to breed *B. napus* for higher levels of shatter resistance has been based on interspecific hybridisation or resynthesis of *B. napus* using shatter-resistant species from the triangle of U. This approach requires several cycles of breeding and selection to overcome chromosomal imbalances and consequent impairment of meiosis and improve fertility of the shatter-resistant segregants. Often, malformation of the siliqua on partly sterile plants results in high siliqua strength.

Prakash and Chopra (1990) carried out interspecific hybridisation between *B. juncea* and *B. napus* and were able to isolate a reconstituted *B. napus* plant with complete nondehiscent fruits. This plant had normal meiosis and formed 19 bivalents. However, the seed fertility was very poor (23%) although pollen fertility appeared acceptable (84%) and this indicated significant chromosomal imbalances which might not have been apparent in meiosis studies. Agnihotri et al. (1990) attempted to transfer shatter resistance from *Raphanus* into *B. napus* using *Raphanobrassica* as the bridging material. This resulted in genetic material with variable fertility. In a Canadian study, lines derived from complex crosses made for development of yellow seeded *B. napus* showed better shatter resistance than standard Canadian *B. napus* varieties (Wang et al., 2007). Summers et al. (2003) resynthesised *B. napus* from crosses between *B. oleracea var. alboglabra* and *B. rapa var. chinensis* and developed DK142 that showed superior shatter resistance based on RIT assessments. However, the line turned out to have significantly lower levels of seed set relative to Apex, a commercial check variety. Recently, Banga et al. (2011) transferred shatter resistance from *B. carinata* to *B. napus*. Hybrid derivatives were characterized cytologically and further evaluated for shatter resistance using delayed harvesting. Anatomical analysis of shatter susceptible lines indicated the presence of DZ comprising thin-walled parenchymatous cell and showed dissolution in 40 days. Whilst shatter resistant genotypes displayed well defined DZ but remained intact and no sign of dissolution of cells or change that could lead to separation of siliquae valve margins from replum. The degree of improvement achieved in siliqua strength in this work is unclear.

Interspecific hybridisation of *B. napus* with *B. rapa var. Brown Sarson* and *var. Yellow Sarson* (Kadkol et al., 1991; Hossain et al., 2011b) has provided promising initial results. Stable segregants with high levels of siliqua strength have been produced with potential to provide harvest shatter resistance. However, further work is required to fully characterise and assess the shatter-resistant selections for meiotic stability, seed set and agronomic traits.

There have been a few reports of genetic transformation for improving shatter resistance in *Brassicas*. Chandler et al. (2005) over-expressed *Sinapis alba MADSB* gene, a close homologue

to *FRUITFULL* in *Arabidopsis,* using a transgenic approach in winter and summer oilseed rape plants. The expression of the *MADSB* transgene modified the dehiscence zone differentiation and produced indehiscent plants. Ostergaard et al. (2006) showed that ectopic expression of the *Arabidopsis FRUITFULL* gene in *B. juncea* is sufficient to produce shatter-resistant *Brassica* fruit and that the genetic pathway leading to valve margin specification is conserved between *Arabidopsis* and *Brassica*. Studies have shown that transgenic fruit produced this way were completely shatter-resistant and were too tough for a combine harvester to thrash (Ferrandiz et al., 2000; Vancanneyt et al., 2003; Ostergaard et al., 2006). This is possibly because of the loss of the basic siliqua structure with valves and sutures that facilitates siliqua rupture. Authors suggested that the use of mutated forms of *FUL* or RNAi techniques to inactivate valve margin identity genes will probably prove useful in the fine–tuning of the degree of shatter resistance. Although these studies have been unsuccessful in producing the correct anatomical phenotype, they demonstrate a genetic strategy that can be used for improving shatter resistance.

6.1 Targeting Induced Local Lesions IN Genomes (TILLING)

The TILLING approach has been utilized for a large number of plants such as in *Arabidopsis*, wheat, barley, maize, lotus and *B. napus* (Comai et al., 2004; Slade et al., 2005; Slade & Knauf, 2005; Dreyer et al., 2007). The major advantage of this approach is the identification of mutants in target genes without genetic transformation. It allows the identification of single base-pair allelic variation in a target gene in a high throughput manner and may offer an alternative approach to identifying variation in shatter resistance among *B. napus* cultivars. Using this approach, Laga et al. (2011) achieved down-regulation of *IND* (indehiscent) gene which led to an indehiscence in *B. napus*, however, siliquae had a tube –like phenotype and did not rupture during mechanical harvesting obviously due to the loss of the valve and DZ structure similar to the transgenic canola discussed above. Use of a reverse genetics approach has produced an agronomically desirable phenotype that has optimal levels of seed shatter reduction. This study isolated and combined a set of mutant (null, weak and dominant negative) *IND* allele combinations that generated a range of seed shattering levels from natural shattering to pods that were shatter-resistant. Mutant plants displayed a range of reduction in shattering (5 to 15%) depending upon the combination of mutations used. This variation is being utilized for variety development. However, the method of screening for shatter resistance is unclear.

6.2 Molecular marker assisted breeding for shatter resistance

Identification of markers for shatter resistance in *B. napus* has not been reported extensively in the published literature due to lack of 'useful' variation for this trait in *B. napus* germplasm. Mongkolporn et al. (2003) utilized bulk segregant analysis (BSA) and identified three RAPD markers in an F_2 population derived from Torch X DS17D. Two of these markers (RAC-3_{900} and RX-7_{1000}) were linked to the *sh1* and *sh2* major genes for shatter resistance. RAPD marker SAC-20_{1300} showed a complete linkage with dominant alleles *SH1* and *SH2* for shatter susceptibility. The authors suggested it is likely that the recessive alleles, *sh1* and *sh2*, could have originated from independent mutations at two duplicate loci during the evolution of *B. rapa*. The marker linked to the dominant alleles can be used for marker-assisted selection (MAS), once validated in genetically diverse backgrounds. Mongkolporn

et al. (2003) did not determine the chromosomal location of the loci associated with shatter resistance and this will require further research.

Association mapping (AM) is a promising new strategy for identification of markers for shatter resistance. AM approach is based upon the principle that linkage disequilibrium is maintained between loci over many generations in a given gene pool. Association mapping has been used for discovery and validation of trait-marker associations identified in the classical quantitative trait loci (QTL) mapping for loci associated with blackleg resistance, flowering time, leaf traits, seed phytate content in rapeseed (Jestin et al., 2010; Raman et al., 2010; Raman et al., 2011; Zhao et al., 2007). AM overcomes the major limitations of the QTL analysis that utilize bi-parental populations such as doubled haploids and recombinant inbred lines, as it surveys a large number of alleles at one locus and saves resources and time required to construct purpose designed 'mapping and validation' populations derived from structured biparental crosses.

Association mapping was used by Raman et al. (2011) to identify loci for shatter resistance in 188 genotypes of *Brassica napus, B. rapa, B. juncea* and *B. carinata*. These lines were phenotyped for siliqua strength using the pendulum method (Kadkol 2009). All accessions were genotyped with 1513 markers based upon Diversity Array Technology (DArT), Simple Sequence Repeats (SSR) and candidate genes that are reported to be involved in shatter resistance. Association analysis revealed that 150 markers were significantly associated ($P<0.05$) by the mixed linear model whereas the generalized linear model detected a total of 266 markers 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 results are consistent with the findings of a comprehensive transcriptome analysis of silique development and dehiscence in *Arabidopsis* and *Brassica* (Jaradat et al 2010). This study identified 131 cell wall related genes and 112 transcription factors that may be involved in silique dehiscence. Raman et al (2011) utilized markers that were largely based upon Diversity Array Technology. The majority of these markers have not been genetically mapped yet on the linkage maps of *Brassica napus*. It is possible that many DArT markers may be cosegregating and therefore map on the same loci. Previous studies have shown that *B. napus* genome has several chromosome rearrangements and therefore some of the DArT markers may represent to multiple copies of the same gene. Validation and fine mapping of these genomic regions, utilizing structured (doubled haploid or intercross) populations, will allow identification of candidate genes and/or their pathways associated with shatter resistance. The genes/QTLs identified in this work would mainly include loci that influence biochemical processes leading to formation of a separation layer in the DZ and its degradation at ripening.

7. Conclusions

Resistance to shatter is an important trait for *B. napus* improvement. It is a difficult trait to measure and breed into adapted germplasm and requires multiple years of selection and screening. To date, various breeding approaches have been attempted for improving shatter resistance of *B. napus,* mainly through interspecific hybridization or resynthesis of *B. napus* using shatter-resistant species from the triangle of U. In recent years, the power of high-density genetic maps and candidate gene studies in *Brassica* crops have demonstrated that an understanding of the number of genes underpinning the trait and their mode(s) of inheritance

is important for further progress. In addition, an understanding of the potential of environment to impact on genetics is also required for the successful introduction of this trait into commercial oilseed rape. This information will greatly enhance breeding efficiency by identifying associated QTL or development and use of molecular markers for marker assisted selection.

Field screening using delayed harvest and visual assessments has been widely used to evaluate pod shatter resistance. It still remains the simplest way to get an approximate understanding of the shatter susceptibility of a large subset of lines. However, such assessments may be somewhat unreliable due to large environmental influences and subjective due to the vague boundaries of the assessment criteria. The development of pendulum method and the associated software facilitating rapid tests of shatter resistance have made it possible to characterize large germplasm collections in an objective way.

8. Future approaches in incorporating shatter resistance

To date, various approaches have been attempted for improving shatter resistance of *B. napus,* including indirect selection in breeding programs by direct heading, interspecific hybridization and also transformation with genes from other species. Breeding and selection within the species has limited potential due to the low genetic variation for the trait but could still result in development of varieties that are tolerant to field shattering.

To achieve higher levels of shatter resistance, it would be necessary to obtain siliqua strength levels that are available only in other species such as *B. rapa* and *B. juncea*. Although the reported attempts have generally not demonstrated complete success, interspecific hybridization could still achieve transfer of shatter resistance into *B. napus* combined with genetic stability, normal meiosis, and complete fertility together with absence of association with yield negative traits. The power of interspecific hybridization as a means of incorporating useful traits has been demonstrated in *B. napus* notably for blackleg resistance (Crouch et al., 1994) and yellow seed colour (Relf-Eckstein et al., 2007) but such work often needs a consistent, targeted breeding program over several generations after the initial isolation of the segregates to improve genetic stability and fertility. Molecular marker technology, such as marker assisted backcrossing would be very important for efficient development of shatter-resistant commercial cultivars upon achievement of successful incorporation of shatter resistance into *B. napus.*

Genetic engineering offers a promising a alternative approach for developing shatter-resistant *B. napus* in view of the advances in research on biology of shattering in *Arabidopsis*. However, shatter-resistant transgenics developed to date appear to have radically altered siliqua anatomy such that valve differentiation, DZ structure and consequently threshability are lost. Further research could develop mutations that retain valve differentiation and siliqua DZ structure whilst eliminating the separation layer similar to the anatomical phenotype of the *Brown* and *Yellow Sarson* varieties.

9. References

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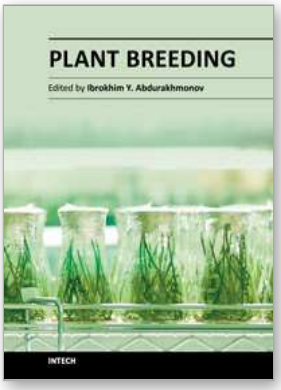

Plant Breeding

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Modern plant breeding is considered a discipline originating from the science of genetics. It is a complex subject, involving the use of many interdisciplinary modern sciences and technologies that became art, science and business. Revolutionary developments in plant genetics and genomics and coupling plant "omics" achievements with advances on computer science and informatics, as well as laboratory robotics further resulted in unprecedented developments in modern plant breeding, enriching the traditional breeding practices with precise, fast, efficient and cost-effective breeding tools and approaches. The objective of this Plant Breeding book is to present some of the recent advances of 21st century plant breeding, exemplifying novel views, approaches, research efforts, achievements, challenges and perspectives in breeding of some crop species. The book chapters have presented the latest advances and comprehensive information on selected topics that will enhance the reader's knowledge of contemporary plant breeding.

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