University of Alberta

Genetic study on *Brassica rapa* and *Brassica napus* for seed color and identification of molecular markers

by

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Abstract

Yellow seed in *Brassica* oilseed crops is favoured for its association with lower fibre and higher oil contents. Yellow-seeded *Brassica napus* lines have been developed through introgression of this trait from its allied species; however, the trait in *B. napus* is often influenced by environmental conditions. The yellow seed color of 'yellow sarson' (*B. rapa*) is stable under wide environments, and can be used in the breeding of yellow-seeded *B. rapa* and *B. napus* cultivars. In this study, the major seed color locus of *B. rapa* has been mapped, and several SSR markers linked to the yellow seed color allele of 'yellow sarson' were identified for use in marker assisted breeding. Additionally, genetic relationship between two yellow-seeded *B. napus* lines was investigated by use of doubled haploid and pedigree populations. Transgressive segregation for seed color in these populations suggests different genetic control of seed color in these two lines.

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List of Symbols and Abbreviations

⊗	Self pollination
±	Plus/minus
°C	Degrees Celsius
µl	Microliter
µm	Micrometer
µmol/g	Micromoles per gram
µmoles	Micromoles
χ^2	Chi-square test statistics
2n	Diploid number of chromosomes
AAFC	Agriculture and Agri-Food Canada
ADF	Acid detergent fibre
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
B_1	Plants recovered after first backcross
BC_1	First backcross generation
BC_2	Second backcross generation
BC_2F_2	Second filial generation after second backcross
BC_3F_1	First filial generation after third backcross
BC_3F_2	Second filial generation after third backcross
B_1S_1	First self-pollinated generation after first backcross
B_1S_2	Second self-pollinated generation after first backcross
BC_6F_2	Second filial generation after sixth backcross
bp	Base pair
CAPS	Cleaved Amplified Polymorphic Sequences
cm	Centimeter
cM	Centimorgan
cv.	Cultivar
DH	Doubled haploid

DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
F_1	First filial generation
F_2	Second filial generation
F_3	Third filial generation
F_{10}	Tenth filial generation
Fam	Family
Fig.	Figure
FISH	Fluorescent in situ hybridization
g	Gram
GDP	Gross domestic product
HEAR	High Erucic Acid Rapeseed
HOLL	High-oleic and low-linolenic acid
kPa	Kilo pascal
malonyl-CoA	Coenzyme A of malonic acid
mg	Milligram
M ha	Million hectare
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimole
n	Haploid number of chromosomes
N	No. of individuals in population under study
NDF	Neutral detergent fibre
NDSP	Neutral-detergent-soluble polysaccharides
NIRS	Near infra-red spectroscopy
nm	nanometer
No.	Number
NPZ Lembke	Norddeutsche Pflanzenzucht Lembke
OP	Open-pollination
p	Probability

PAs	Proanthocyanidins
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rp gene	Repressor gene
rpm	Revolutions per minute
sec	Second
SCAR	Sequence characterized amplified region
S.E.	Standard Error
SP	Self-pollination
SSR	Simple sequence repeat
SRAP	Sequence-related amplified polymorphism
SNP	Single nucleotide polymorphism
Taq (polymerase)	Polymerase from the bacterial species *Thermus aquaticus*
Var.	Variety

Chapter 1

Literature Review

1.1 Introduction

Brassica oil crops, *Brassica rapa* (AA, 2n=20), *B. napus* (AACC, 2n=38) and *B. juncea* (AABB, 2n=36), are an important source of edible oil in many countries in the world. Of these three species, *B. rapa* and *B. napus* are collectively called as rapeseed, while *B. juncea* is called Indian mustard. Spring type rapeseed is primarily grown in Canada, Australia and Northern Europe, semi-winter type grown in China, and the winter type mainly grown in Central Europe. In Canada, northern United States and in Europe, spring type is planted in April-May and harvested in September-October; while in Australia the same spring type is grown as winter crop. Winter type rapeseed is planted in August-September and is harvested in June-July of the following year. Winter type typically yields 20% to 30% more than spring type.

Currently, spring type *B. napus* is the second most important crop in Canada after wheat. In 2008, this crop was cultivated in 6.55 million hectares, of which 99.98% was canola quality type (Statistics Canada 2008). The 'canola' quality cultivars are characterized by having less than <2% erucic acid in the fatty acid profile of the seed storage lipid and less than 30 μmoles glucosinolates per gram seed meal at 8.5% moisture basis (Canola Council of Canada, http://www.canolacouncil.org/canola_the_official_definition.aspx). Successful development of canola quality *Brassica* oilseed crops has led to an increased

acreage and production of these crops worldwide, and they are currently the world's second largest oilseed crop after soybean.

Canola (*B. napus* and *B. rapa*) introduced in Canada in 1940's and since then its acreage has increased continuously, reaching approximately 6.87 million ha in 2010 (Fig. 1.1) and producing about 12 million tonne seeds (Statistics Canada, 2010) (Fig. 1.2). Recently, Canola Council of Canada set a target of producing 15 million tonnes canola by 2015 (Canola Council Annual Report 2009). To achieve this goal, productivity of this crop needs to be increased, for which plant breeding and improved agronomic practices will play an important role.

Canola contributes an estimated $13.8 billion economic activity to the Canadian economy, accounting for 1.18% of the nation's Gross Domestic Product (GDP). Its production and processing sectors have generated approximately 216,000 jobs in Canada (Fig. 1.3). Processed oil and meal exports further added value to this crop. Canadian exports of oil increased from $341 million in 2002 to $602 million in 2006. During the same period, meal sales to the United States went from $154 million to $203 million, and thus combined meal and oil benefits raised by 88% in years 2002 through 2006 (Mark Goodwin consulting Ltd. 2008).

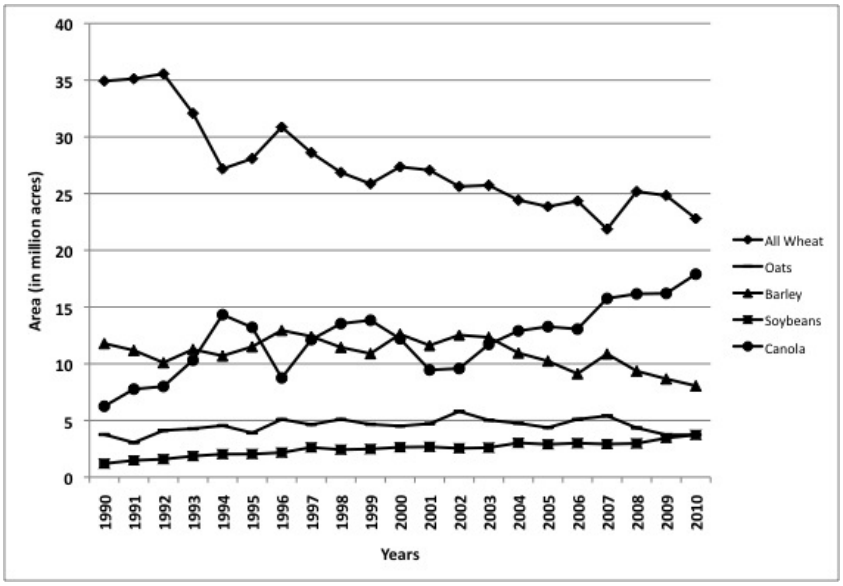

Fig. 1.1: Agricultural land usage statistics for five important crops in Canada (Statistics Canada 2010, data retrieved on March 2011)

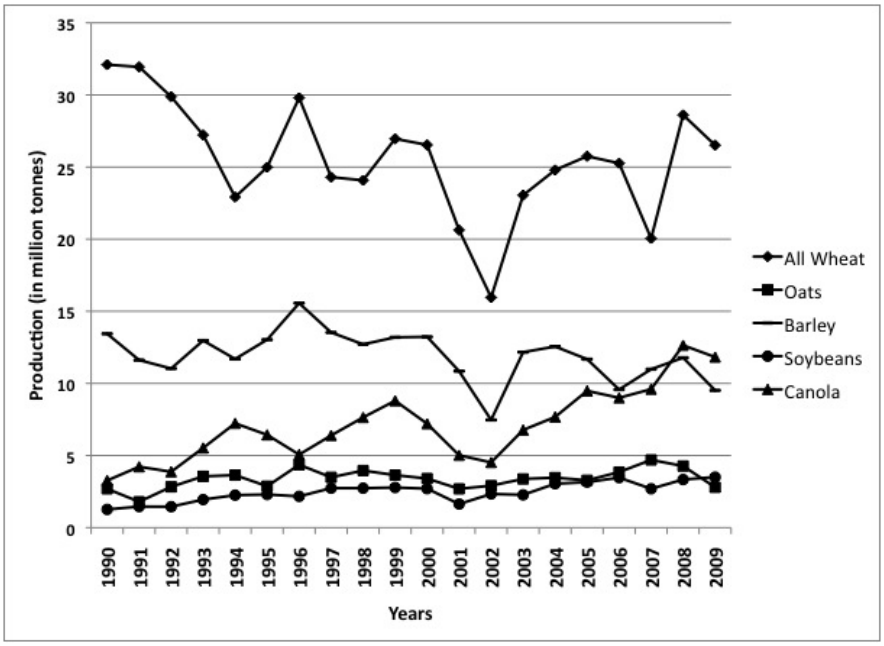

Fig. 1.2: Production of major crops in Canada over the past decade (Statistics Canada 2010, data retrieved March 2011)

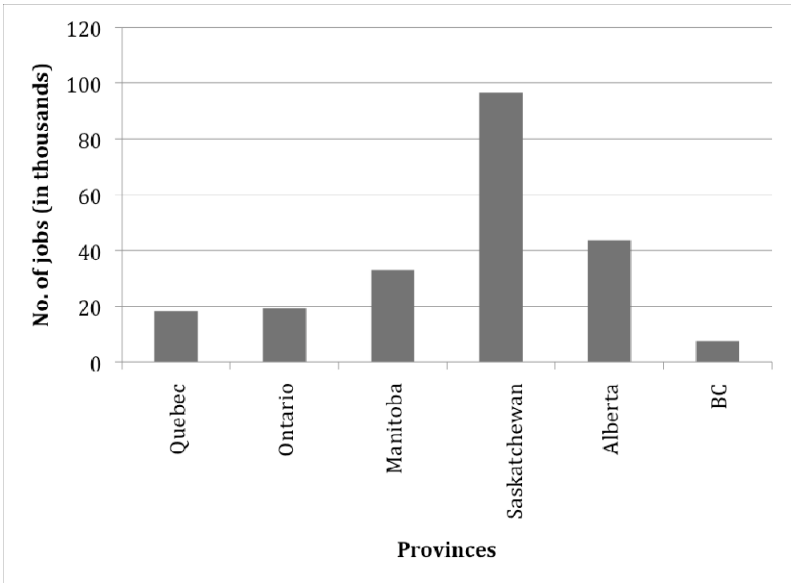

Fig.1.3: Number of jobs created based on canola industry in Canada (Goodwin 2008)

1.2 *Brassica* species and genome relationships

Cytological analysis of chromosomes is the traditional method used for understanding the relationships between different species within a genus. In case of *Brassica*, chromosomes are small and not easy to differentiate based on karyotype analysis, and this is the major constraint for establishing relationship between different species based on chromosome morphology (Olin-Fatih 1994). U (1935) described the relationship between the six *Brassica* species in the form of a triangle (Fig 1.4), based on their crossability and chromosome pairing/fertility in the interspecific hybrids. The three diploid genomes are designated as A for *B. rapa* (n=10), B for *B. nigra* (n=8) and C for *B. oleracea* (n=9). The three

amphidiploid species *B. napus* (AACC, 2n=38), *B. juncea* (AABB, 2n=36) and *B. carinata* (BBCC, 2n=34) are evolved from the three diploid progenitors.

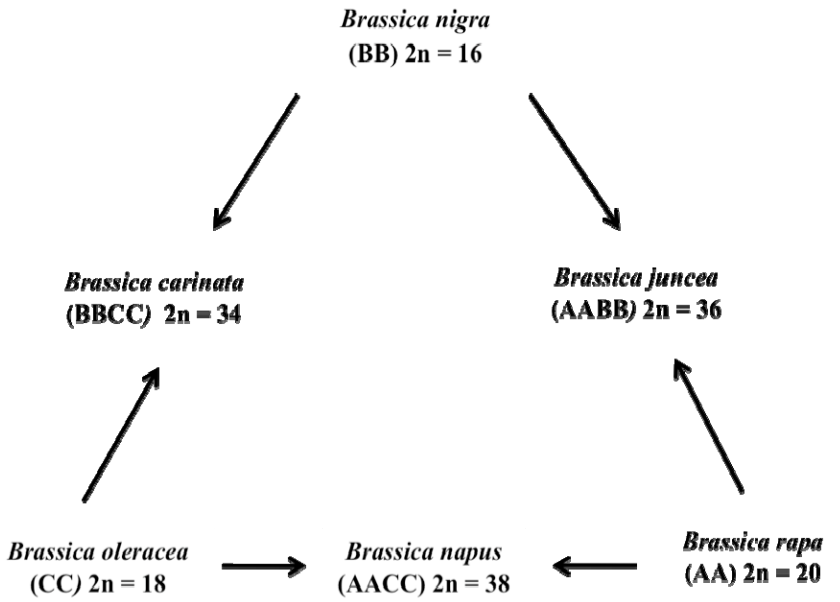

Fig. 1.4: Genome relationships between the diploid and amphidiploid *Brassica* species as described by U (1935)

Over the past 90 years, extensive research has been conducted to study the evolution and phylogenic relationship between different species of the family Brassicaceae. Studies underwent change over time from morphological characterization to cytogenetic analysis, cytoplasmic characterization, biochemical analysis, and more recently to molecular and genomic approaches. Truco et al. (1996) compared linkage maps of *B. rapa*, *B. nigra* and *B. oleracea* and found intergenomic conserved regions as well as extensive duplication and reordering of the regions among the three *Brassica* genomes, demonstrating that these three genomes evolved from a small ancestral genome closely related to *Arabidopsis*. The split of *Brassica* and *Arabidopsis* genomes occurred about 20 million years ago (Yang et al. 1999). According to Lysak et al. (2005),

polyploidization in the *Brassica* genome has occurred after the split of *Arabidopsis* and *Brassica* genomes, between 7.9 and 14.6 million years ago. Comparative mapping of *Arabidopsis thaliana* and *B. napus* genome showed that, in most cases, for each genomic region of *Arabidopsis* three genomic segments can be found in *Brassica* (Parkin et al. 2005). This suggests that the diploid *Brassica* species have evolved from a hexaploid ancestor.

Song et al. (1988) studied the evolution of cultivated *Brassica* species by the use of restriction fragment length polymorphism (RFLP) of nuclear DNA. Based on RFLP data, they suggested that *B. nigra* evolved through a distinct evolutionary pathway with *Sinapis arvensis* or a closely related species as progenitor; whereas *B. rapa* and *B. oleracea* evolved through a separate pathway from a wild *B. oleracea* or a related chromosome species as ancestor. Based on chloroplast genome analysis, Warwick and Black (1991) also suggested two lineages, the 'nigra' and 'rapa/oleracea', for the evolution of the three diploid *Brassica* species. In the 'nigra' lineage, *B. nigra* was found to be closely related to *Sinapis alba* and *S. arvensis*; while in the 'rapa/oleracea' lineage, *B. rapa* and *B. oleracea* were found to be related to the n=7 species *Diplotaxis erucoides* and *B. deflexa*. According to Cheung et al. (2009), the *B. rapa* and *B. oleracea* lineages diverged between 2.57 and 4.23 million years ago.

Palmer et al. (1983) studied chloroplast DNA variation and found that the cytoplasm of *B. rapa* and *B. oleracea* are more closely related to each other than *B. nigra*. According to these authors, *B. nigra* functioned as the maternal parent during the evolution of *B. carinata,* while *B. rapa* was the maternal parent for *B.*

juncea. Similar results have also been reported by Erickson et al. (1983) based on chloroplast DNA analysis of cytoplasms. Mitochondrial DNA analysis by Palmer (1987) provided further evidence that the cytoplasms of *B. nigra* and *B. rapa* are distinct, and these species were the maternal parents respectively for *B. carinata* and *B. juncea*. Song and Osborn (1992) used restriction fragment length polymorphisms (RFLPs) of chloroplast and mitochondrial DNA to analyse the relationship between *B. rapa*, *B. oleracea* and *B. napus*. They classified the cytoplasmic genomes of the two diploid species into four major types: *B. rapa*, Broccoletto (a specialized from of *B. rapa*), *B. oleracea* and *B. montana*; and suggested that *B. montana* might be the closely related prototype that gave rise to cytoplasm of *B. oleracea* and *B. rapa*, and consequently of *B. napus*. However, they also found all four types of cytoplasm in *B. napus*, suggesting multiple origins of this species.

Attia and Röbbelen (1986) investigated chromosome pairing in three amphihaploids, AC, AB and BC, derived from the three diploid *Brassica* species. They observed frequent pairing of chromosomes in AC, compared to AB and BC amphihaploids. Lowest chromosome pairing was observed in BC amphihaploids. Based on fluorescent in situ hybridization (FISH), Mason et al. (2010) studied the allosyndetic pairing of chromosomes between the three *Brassica* genomes. They observed an average pairing association per cell of 4.0 (range 2.3 to 6.0) for the A-C chromosomes, 0.8 (range 0.5 to 1.4) for A-B chromosomes, and 0.3 (range 0.03 to 0.6) for B-C chromosomes. These cytogenetic studies provided further evidence that the three *Brassica* genomes evolved following two lineages; and

among the three genomes, the A and C genomes are close to each other than the B genome.

Parkin et al. (1995) constructed an RFLP linkage map, based on a doubled haploid (DH) mapping population derived from a cross between a resynthesized *B. napus* line, generated from *B. rapa* and *B. oleracea*, and a natural *B. napus* line. Based on segregation of RFLP alleles, they demonstrated that in microspore formation in F_1 plants of resynthesized x natural *B. napus*, the *B. rapa* chromosomes were pairing exclusively with the A-genome homologues of natural *B. napus* while the *B. oleracea* chromosomes were likewise pairing exclusively with the C-genome homologues of natural *B. napus*. This behaviour of A and C-genome chromosomes suggest that the nuclear genomes of *B. rapa*, *B. oleracea* and *B. napus* remain unaltered since the evolution of *B. napus*. Axelsson et al. (2000) constructed two RFLP linkage maps to study the genome evolution of the amphidiploid species *B. juncea*. One linkage map was constructed based on a *B. juncea* line- resynthesized from *B. rapa* x *B. nigra*- and crossed with a natural *B. juncea;* while the other map was constructed from a cross between two natural *B. juncea* cultivars. They found that the *B. rapa* chromosomes pair exclusively with the A-genome homologues of natural *B. juncea* and *B. nigra* chromosomes pair with the B-genome homologues. The two maps were perfectly collinear suggesting that the A and B genomes of *B. juncea* remained unchanged since the evolution of this amphidiploid from the two diploid progenitors *B. rapa* and *B. nigra*.

1.3 Rapeseed/ canola oil

Brassica napus seed contains about 45% oil. Increasing the oil content in *Brassica* oilseed crops is one of the important breeding targets. Plant breeding acted as an important tool in improving seed yield and oil content in *B. napus* cultivars. Moderate heritability for seed oil content combined with effective selection methods has increased the oil content in this crop to some extent in the last few decades (Weselake et al. 2009). With the increasing interest for the use of canola oil as biofuel, the demand for further increase in oil content in this crop will increase in the coming years.

The fatty acid composition of traditional rapeseed oil is 5% palmitic acid (C16:0), 1% stearic acid (C18:0), 15% oleic acid (C18:1), 14% linoleic acid (C18:2), 9% linolenic acid (C18:3) and 45% erucic acid (C22:1) (Ackman 1990). High content of erucic acid in oil is nutritionally undesirable for human consumption. The discovery of a *B. napus* plant in which seed oil is essentially free from erucic acid opened a new era for quality improvement in this oil crop (Steffansson and Hougen 1964). This genetic variation has been used extensively in breeding for the development of erucic acid free *B. napus* cultivars (Stefansson and Downey 1995). Complete conversion of Canadian rapeseed acreage to zero erucic acid type (single low) occurred by 1980s. Fatty acid profiles of zero-erucic acid rapeseed oil is 4% palmitic acid (C16:0), 1.8% stearic acid (C18:0), 63% oleic acid (C18:1), 20% linoleic acid (C18:2), 9% linolenic acid (C18:3) and 0% erucic acid (C22:1). This zero erucic acid oil is favored in various food applications e.g. margarine, salad oil, frying oil, confectionary items, etc.

However, high content of erucic acid in oil has many industrial applications, e.g. in polymers, cosmetics, lubricants, detergents, surfactant, pharmaceuticals, etc. (Topfer et al. 1995). Therefore, it is desirable to increase the content of erucic acid in High Erucic Acid Rapeseed (HEAR) cultivars.

Breeding cultivars with reduced level of linolenic acid and increased level of oleic acid further increased the value of canola oil. High-oleic and low-linolenic acid (HOLL) canola oil is considered as specialty oil; and demand for this type of oil is increasing owing to its greater stability, functionality and excellent food flavors. Fatty acid composition of HOLL canola is: palmitic acid 3.6%, stearic acid 2%, oleic acid 75%, linoleic acid 15% and linolenic acid <3%. Oleic acid is cholesterol neutral fatty acid, and therefore HOLL oil is also nutritionally desirable (Orthoefer 2005).

1.4 Rapeseed/ canola meal

Canola is mainly grown as an oil crop where oil is considered as the main product. The seed meal left over after oil extraction is rich in protein and used as animal feed. Meal from traditional *Brassica* oilseeds contain high amount (>60μmol/g seed) of glucosinolates, which is nutritionally undesirable in animal feed (Bell 1993, Clandinin and Robblee 1981). Plant breeders have always been keen to lower the glucosinolate content in the rapeseed meal to enhance its feeding value. The Polish researcher Dr. Kzrymanski, during working at Agriculture and Agri-Food Canada, Saskatoon Research Station discovered that the Polish cultivar Bronowski contain low glucosinolate content in seed. This

genetic variation been used extensively by the Canadian breeders for the development of low glucosinolate cultivars. The first low glucosinolate *B. napus* cultivar Tower was released by the University of Manitoba in 1974, and the *B. rapa* cultivar Candle was released in 1978 by Agriculture and Agri-Food Canada, Saskatoon. These new cultivars meant that Canada could now produce oil and meal superior to those produced from traditional rapeseed in other parts of the World. By the end of year 1980, Canadian rapeseed acreage was totally replaced by canola quality type (Stefansson and Downey 1995).

Canola meal contains about 35% protein (Schroeder 1999). The amino acid composition of canola meal protein is excellent; and rich in some of the essential amino acids, e.g. lysine (6.0%), methionine (1.8%) and cystine (1.2%), which are generally low in most other oilseeds. The protein value of canola meal is higher than many other vegetable proteins containing lysine and sulphur amino acids. Similarly, the amounts of minerals Calcium and Phosphorus of the vitamin niacin and of choline are higher in canola meal compared to soybean and sunflower meal (Rutowski 1971). However, the traditional rapeseed meal contain about 12% crude fibre, which is almost double compared to soy meal; and this is one of the major constraints of using this meal for monogastric animals (Bell and Shires 1982). Improvement of canola meal for low fibre content is therefore needed for better utilization of this protein source.

1.5 Yellow-seeded *Brassica*

1.5.1 Occurrence of yellow seed

Not all *Brassica* species have yellow-seeded forms naturally available. Amongst the diploid species, yellow seed naturally exists in *B. rapa* (AA, 2n=20) but not in *B. nigra* (BB, 2n=16) and *B. oleracea* (CC, 2n=18). The yellow seed is naturally available in amphidiploids *B. juncea* (AABB, 2n=36) and *B. carinata* (BBCC, 2n=34), but not in *B. napus* (AACC, 2n= 38). Availability of yellow-seeded form in *B. carinata,* but not in its parental species, indicates that this trait might have evolved in this amphidiploid species after its origin.

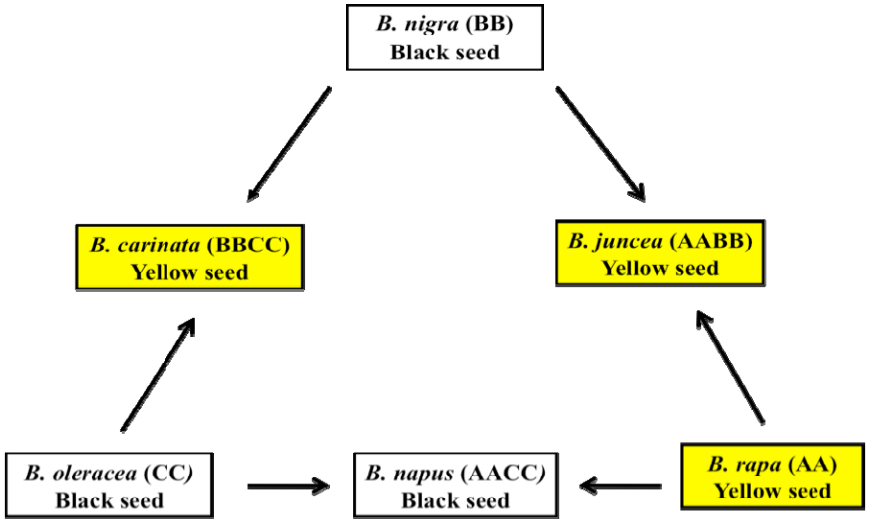

Fig 1.5: Natural occurrence of yellow seed in nature in *Brassica*

1.5.2 Yellow seed and meal quality

Yellow seeds contain higher oil and protein and lower fibre content as compared to brown seeds (Stringam et al. 1974, Shirzadegan and Röbbelen 1985, Rahman et al. 2001). Most of the seed oil and protein are present in embryo and

small amounts in seed coat. Seed coat of yellow seed is thinner than that of brown/black seeds; thus embryo comprises greater proportion of the whole seed in yellow seeds and resulting higher amount of oil and protein in this type. The proportion of seed coat in black/brown seeded canola meal is about 30%; and brown seed coat contains more fibre and lignin as compared to the coat from yellow seed (Bell and Shires 1982). The lower fibre in yellow seed meal is also due to the lower proportion of seed coat to the whole seed in this type as compared to black/brown seeds (Stringam et al. 1974).

Estimation of total dietary fibre generally includes neutral detergent fibre (NDF) (cell wall including cellulose, hemicellulose and lignin) and neutral-detergent-soluble polysaccharides (NDSP) (Slominski et al. 1994). Fibre of plant material is generally less digestible (Kritchevsky 1988), and fibre digestibility is best determined by NDF values. According to Slominski et al. (1994), the NDF value for meal from yellow-seeded canola is significantly lower (19%) as compared to meal from brown-seeded canola (26%). Yellow seed also contain 3-4% higher sucrose and less lignin and polyphenols. Simbaya et al. (1995) compared quality characteristics of seed meal of 26 yellow-seeded genotypes of *B. napus*, *B. rapa*, *B. juncea* and *B. carinata* and seven brown-seeded genotypes of conventional canola. Overall, meal from yellow seed was found to be superior to black seed meal in respect to higher protein (44.5% vs. 42.7%), lower dietary fibre (28% vs. 33%) and higher sucrose (8.7% vs. 7.5%) content. Mitaru et al. (1982) analysed seed hull tannins from different colored *B. napus* seeds and found

higher content of acid detergent lignin in dark colored seed compared to yellow colored seeds.

1.5.3 Seed meal nutritive value

The canola meal, left after extraction of oil of seed, contains about 40% protein. This is an excellent source of protein for ruminant and non-ruminant animals. However, high content of glucosinolates (>60μmol/g seed) in traditional rapeseed meal has been a limiting factor for use of this meal in animal feed. Plant breeding efforts has lowered the content of seed glucosinolates in canola (≈12μmol/g seed), and thus made this seed meal acceptable for feeding animals. However, high content of fibre in canola meal, compared to soybean meal, is one of the limiting factors for maximum utilization of this protein source. Other antinutritional compounds in canola seed meal are sinapine, tannin and phytic acid (Bell 1993).

Bell and Shires (1982) studied the effect of hull from yellow and brown seed in pig diet and found that increasing levels of hulls in diet depresses the digestibility of dry matter, energy, crude fibre and ether extract. The energy components of yellow seed hull were more digestible than of brown seed hull. Protein of brown hull was indigestible, while yellow seed hull protein was about 20% digestible. Bell (1993) reviewed the nutritional value of canola meal, and suggested that development of yellow-seeded canola cultivars with reduced hull content and increased level of protein can increase the gross energy of this seed meal. Thus, development of triple-low (double low + yellow seed/low fibre)

canola cultivars would allow greater freedom of inclusion of this seed meal in feed for poultry and other monogastric animals. Protein quality of canola meal, in respect to the availability of amino acids, can be affected by processing temperatures. Parsons et al. (1992) showed that autoclaving of meal at 121ºC and 105 kPa results in reduction in lysine content. Since lysine is one of the limiting amino acids in animal feed, its reduction would not be desired in canola meal.

1.6 Biology of seed coat

The seed coat of yellow seed is translucent (transparent), which makes its yellow-embryo visible, thus making the seed phenotypically yellow. Brown and black seeds contain dark pigmentation in seed coat, thus hiding the internal components of seed. The seed coat of yellow seed is thinner than black or brown seed (Stringam et al. 1974).

According to Van Caeseele et al. (1982), seed coat of *B. napus* is made up of outer and inner integuments and composed of a palisade layer, several layers of crushed parenchyma cells and single aleurone cell layer. Mature and unfertilized ovule has two integuments formed of undifferentiated parenchyma cells. The differentiation of the outer integument into various layers starts after pollination and is completed in 30 days. The innermost layer of outer integument forms the palisade layer of the seed coat. Deposition of seed coat coloring pigment flavonoids occurs in the palisade layer i.e. innermost layer of outer integument of the seed (Vaughan 1970, Stringam et al. 1974). Palisade layer is the cell layer that contains higher fibre, which is thinner in yellow seeds than in black/brown seeds.

According to Wan et al. (2002) the maternal plant uses the outer and inner ovule integuments for physical and physiological contact with developing embryo, giving rise to seed coat. At the time of flowering, the outer integument contains four cell layers and the inner integument contains six to eight cell layers. However, after fertilization, the number of cell layers reduced in the inner integument, whereas the outer integument remains as a four-cell layer. Vaughan and Whitehouse (1971) described the seed coat developmental process in *Arabidopsis*, which is comparative to *Brassica*. According to the authors, the seed coat is making up of four cell-layers, three layers derived from the outer integument and one layer from the inner integument. The outermost layer of the outer integument may or may not contain mucilage; and the second layer is subepidermal parenchymatous layer, which is found to be collenchymatous and sclerotic in *B. nigra* and *Sinapis alba* (Bouman 1975). The innermost layer of the outer integument is palisade layer. This is a thick-walled cell layer where most of the seed coat pigments are deposited (Vaughan and Whitehouse 1971, Bouman 1975: cited by Moïse et al. 2005). The fourth layer is derived from the inner integument, composed of parenchyma cells and is called pigment layer (endothelium).

Seed coat pigmentation occurs mainly due to the deposition of condensed tannins, which are oxidized proanthocyanidins (PAs) derived from phenylpropanoids and malonyl-CoA (Leung et al. 1979, Akhov et al. 2009). Proanthocyanidins (PAs) are the main seed coat flavonoids that impair the digestibility of canola seed meal (Auger et al. 2010). Akhov et al. (2009) studied

biosynthesis of PAs in the seed coat of *B. napus*. Comparative study between yellow- and black-seeded genotypes showed that robust accumulation of PAs occur in black-seeded genotypes between 10 and 20 days post-anthesis, whereas PAs accumulation was barely detectable in yellow-seeded line YN01-429. The expression of dihydroflavonol 4-reductase (an enzyme involved in the synthesis of PAs from phenolics) was found to be lower in yellow seed compared to black seed. Marles and Gruber (2004) performed in situ histochemical characterization of the seed coat pigments, and found that the unextractable seed coat proanthocyanidins form the black seed color in *B. napus*. They also found lack of anthocyanins in seed coat tissues. According to these authors, the seed coat of yellow-seeded type contains lower content of PAs and lignin.

1.7 Genetics of seed color and molecular mapping of the genes

Brassica rapa

Seed coat pigment is deposited in the palisade and parenchyma layers of testa; and testa originates from the integuments that is maternal tissue. Thus, genotype of the maternal parent plays an important role in seed color determination. However, Schwetka (1982) found that seed color in *B. rapa* is not completely determined by the mother plant genotype, but genotype of the developing embryo also played role in color determination in the seed coat. This direct influence of pollen on the developing seeds is called 'Xenia' effect. The Xenia effect has also been reported in *B. rapa* by Chen and Heneen (1992) and

Rahman et al. (2007), and in maize for endosperm and aleurone layers by Focke (1881) and Kornicke (1872), cited by Sandler and Sandler (1986).

Several studies have been conducted to understand the genetic control of seed color in *B. rapa*. Mohammad et al. (1942) reported three independent genes controlling seed color in this species. Stringam (1980) studied genetic control of seed color in *B. rapa* based on a population derived from Canadian/Swedish brown-seeded type crossed with *B. rapa* var. 'yellow sarson', and reported a digenic inheritance of this trait. According to this model, presence of dominant alleles at both loci (Br_1 and Br_3) or presence of dominant alleles only at the first locus (Br_1) produce brown seed, while presence of dominant allele at the second locus (Br_3) and homozygous recessive alleles at the first locus (br_1br_1) produce yellow-brown seeds. Yellow seeds are produced when all loci are in homozygous recessive condition (br_1br_1, br_3br_3). Similar results have also been reported by Zaman (1989) and Rahman et al. (2007). Schwetka (1982) studied seed color genetics by use of different yellow-seeded genotypes including 'yellow sarson' and reported more complex genetic control of this trait. According to this author, two genes with epistatic effect (Br_1br_1; Br_6br_6) and four genes with hypostatic effect (Br_3br_3; Br_4br_4; Br_5br_5; Br_7br_7) are involved in the control of seed color in *B. rapa*. Schwetka also identified two alleles for yellow seed color (br_1^1 and br_1^2) in the locus Br_1br_1, but only one allele in the locus Br_6br_6 for yellow seed color (br_6^1). Rahman (2001a) studied the inheritance of seed color and flower (petal) color in F_2 and backcross population of Canadian *B. rapa* x 'yellow sarson' crosses. Petal color was found to be controlled by a single gene where the yellow

color is dominant over creamy white color; while seed color was found to be under digenic control where yellow seed color genes of 'yellow sarson' are recessive to brown/partially yellow seed color genes of Canadian *B. rapa*. Joint segregation of these two traits in F_2 population revealed that the genes controlling petal color and seed color are inherited independently. Ahmed and Zuberi (1971) found monogenic inheritance for seed color in a population derived from brown-seeded Toria (*B. rapa*) crossed with a yellow-seeded type. Monogenic inheritance can be observed if the parents are differing for one locus only. Single dominant gene control of seed color control has also been reported by Hawk (1982) and Chen and Heneen (1992). Choudhary (2008) studied inheritance of seed color in *B. tournefortii* (TT, 2n=20) and reported a monogenic control of this trait where brown seed color was found to be dominant over yellow seed color. Rahman et al. (2007) identified a sequence-related amplified polymorphism (SRAP) marker tightly linked to the major seed coat color gene Br_1/br_1. This dominant marker was converted into single nucleotide polymorphism (SNP) and sequence characterized amplified region (SCAR) markers through chromosome walking techniques.

Thus, it is apparent that extensive studies have been conducted on the genetics of seed color in *B. rapa*, and SNP/SCAR molecular markers for the major seed color locus is available in this species. However, identification of marker(s) of the most widely used marker system, i.e. simple sequence repeat (SSR, microsatellite), would be desired for use in marker assisted selection by wider breeding community.

Brassica juncea

Seed color in *B. juncea* is controlled by the genotype of the maternal plant (Xu et al. 2010). Two duplicate pairs of genes are involved in the control of seed color in *B. juncea*. Brown seed color is produced when a single dominant allele is present in either of the loci, and yellow seeds are produced when alleles at both loci are homozygous recessive (Vera et al. 1979, Rawat 1989, Lionneton et al. 2004, Yan et al. 2009). However, monogenic inheritance of this trait has also been reported in this species (Xu et al. 2010) as in *B. rapa*, apparently due to the involvement of brown and yellow-seeded parents differing for one seed color gene locus.

Different types of markers have been developed for seed color genes by different research groups. Negi et al. (2000) identified an amplified fragment length polymorphism (AFLP) marker linked to a brown seed color gene and converted this marker to a co-dominant SCAR marker. Based on RFLP markers, Mahmood et al. (2005) mapped the two seed color genes on the linkage groups A6 and B4. Yan et al. (2009) used a BC_6F_2 population, segregating for a single seed color gene locus, and identified two sequence-related amplified polymorphism (SRAP) markers linked at a distance of 2.35 cM and 7.06 cM from the brown seed color gene. By use of an F_2 population, Xu et al. (2010) identified 15 AFLP markers linked to one of the seed color gene, where two markers were only 0.3 cM apart from the gene. They converted four AFLP markers into sequence characterized amplified region (SCAR) markers for use in marker assisted breeding.

Brassica carinata

In *B. carinata*, seed color is determined by the genotype of the maternal plant (Getinet and Rakow 1997), and xenia effect is apparently absent in this species (Rahman and Tahir 2010). Getinet and Rakow (1997) found that F_1 plants produced from reciprocal crosses between brown and yellow-seeded parent produce almost yellow seed (slightly darker, but close to yellow parent) indicating incomplete dominance of yellow seed color over brown. In F_2, a 1:3 segregation for brown and light yellow-brown to yellow suggested that a single gene locus is involved in the control of this trait in *B. carinata*. The yellow seed phenotype is due to a dominant repressor (*Rp*) gene in homozygous condition. The repressor gene inhibits the synthesis of seed coat pigment; the brown-seeded plants lack this gene. Rahman and Tahir (2010) studied the inheritance of seed color in this species by using F_1, F_2, F_3 and backcross populations derived from cross between brown- and yellow-seeded genotypes and confirmed the findings of Getinet and Rakow (1997). Thus, it is apparent that the genetic control of yellow seed color in *B. carinata* is strikingly different from *B. rapa* and *B. juncea*, where in *B. carinata* the yellow seed color is due to a dominant repressor gene; while in *B. rapa* and *B. juncea*, this trait is due to homozygous recessive condition in all seed color loci. Molecular mapping of this seed color gene of *B. carinata* has not yet been done, and no marker is available for this repressor gene.

Brassica oleracea

Yellow-seeded genotypes are not naturally available in *B. oleracea*. Cheng et al. (1994) produced several *B. rapa-alboglabra* monosomic addition

lines through recurrent backcrossing a *B. napus* line, resynthesized from yellow-seeded *B. rapa* and black-seeded *B. alboglabra* (a form of *B. oleracea*), to *B. rapa*. One of these lines carried a *B. alboglabra* chromosome with gene for black seed color. This research group reported that three *B. alboglabra* chromsomes, the chromsome 1, 4 and an unidentified chromosome, carry seed color genes. *Brassica rapa* plants carrying the *B. alboglabra* chromosome 1 showed maternal control of seed color and produce only brown seeds; while the plants carrying the chromosome 4 or the unidentified chromosome showed embryonic control of seed color and produce a mixture of yellow and brown seeds (Heneen and Brismar 2001). Chen et al. (1997) identified 19 randomly amplified polymorphic DNA (RAPD) markers specific to the chromosome 1, where one of these markers showed linkage with the seed color gene.

1.8 Development of yellow-seeded *B. napus*, genetic study and molecular mapping

In contrast to *B. rapa*, *B. carinata* and *B. juncea*, no yellow-seeded form is available in natural *B. napus* germplasm. Therefore, development of yellow-seeded *B. napus* has been one of the prime objectives to the researchers and breeders.

Chen et al. (1988) resynthesized several *B. napus* lines through interspecific hybridization between two black- and light brown-seeded *B. alboglabra* and one brown and ten yellow-seeded *B. rapa* from different sources for developing yellow-seeded *B. napus*. The light brown-seeded *B. alboglabra*

was developed from a cross between a yellow-seeded *B. carinata* and black-seeded *B. alboglabra* No. 4003 followed by backcrossing the interspecific hybrid to No. 4003, and selection for yellow-seeded *B. alboglabra* in self-pollinated generations of the backcross hybrids. Seed color of the resynthesized *B. napus* lines was either black or light-brown depending upon the seed color of the parents. Crosses between black x brown and black x yellow-seeded lines produced black-seeded *B. napus*. However, crosses between light-brown seeded *B. alboglabra* and yellow-seeded *B. rapa* resulted black- and light brown-seeded progeny. Liu et al. (2005a) developed several DH lines from the resynthesized *B. napus* developed by Chen et al. (1988); where the DH line 2127-17 was found to possess the yellow seed property. Yellow seed trait in this DH line was found to be partially dominant over black seed and is controlled by a major gene (Liu et al. 2005a, Liu et al. 2005b, Xiao et al. 2007). Zhang et al. (2010) used this yellow-seeded line and developed two mapping populations for QTL analysis, and detected seed color QTL on N18, N5 and N3 in one population where the N18 QTL explained more than 50% phenotypic variation. In case of the second population, they detected three QTL on linkage groups N9, N18 and N8 where the QTL on N9 explained more than half of the total phenotypic variation.

Rashid et al. (1994) designed an interspecific crossing programme to develop yellow-seeded *B. napus* through introgression of yellow seed color genes from the A genome of *B. juncea* and C genome of *B. carinata* into the A and C genome of *B. napus*. For this, they backcrossed the interspecific *B. napus* x *B. juncea* and *B. napus* x *B. carinata* hybrids plants to *B. napus*; and subsequently

crossed the backcross F_2 plants of (*B. napus* x *B. juncea*) x *B. napus* with the backcross F_2 plants of (*B. napus* x *B. carinata*) x *B. napus*. They obtained 91 yellow-seeded plants among 4,858 plants in F_2 generation of this backcross intercross. Rakow et al. (2011) reported the yellow-seeded *B. napus* line YN01-429, developed from interspecific crosses between *B. rapa* 'yellow sarson' and yellow-seeded *B. carinata* and *B. juncea*, and backcrossing of the interspecific hybrids to *B. napus* followed by selection for yellow-seeded *B. napus*; and intercrossing of the yellow-seeded plants and selection for stable yellow seed phenotype and *B. napus* plants. The initial true breeding yellow-seeded *B. napus* line had poor agronomic traits and seed yield. However, the agronomic and seed quality of this yellow-seeded *B. napus* has been improved through repeated cycles of breeding and seed yield has been improved to about 109% of the official check cultivar 46A65/Q2. The yellow seed color in this material is due to a partial dominant gene (Rakow, personal communication).

Rahman (2001b) developed yellow-seeded *B. napus* from interspecific crosses between yellow-seeded *B. rapa* var. 'yellow-sarson' (AA), black-seeded *B. alboglabra* (CC), yellow-seeded *B. carinata* (BBCC) and black-seeded *B. napus* (AACC) through introgression of yellow seed color gene(s) of 'yellow sarson' into the C-genome of *B. napus*. This yellow-seeded *B. napus* carry the yellow seed color genes of 'yellow sarson' in both A and C genomes. Working with this yellow-seeded *B. napus* line, Rahman et al. (2010) at the University of Manitoba confirmed introgression of the yellow seed color allele (br_1) from the A genome of 'yellow sarson' into C3 (N13) of the C genome.

The Southwest Agricultural University in China, identified a dominant gene controlling yellow-seeded trait in *B. napus* line GH06 from black-seeded *B. napus* x yellow-seeded *B. juncea*) x yellow-seeded *B. napus* cross followed by self-pollination for 10 generations. QTL mapping for seed color based on F_2 derived from crossing of this line with black-seeded *B. napus* disclosed two loci on LG5 and LG19 which explain 46% and 30.9% of the total phenotypic variation for these two loci respectively (Liu et al. 2006). Based on this yellow-seeded line, the yellow-seeded *B. napus* cultivar 'Yu-Yellow No. 1' has been developed and marketed in South West of China.

Burbulis and Kott (2005) identified a yellow-seeded *B. napus* line in a doubled haploid (DH) population derived from a cross between two black-seeded *B. napus*. Out of 26 DHs produced from this cross, six were yellow-seeded; however no further report on this material is available. Badani et al. (2006) performed QTL analysis based on three populations derived from two different yellow-seeded *B. napus* lines, 25629-3 and 1012/98. The yellow-seed trait in 25629-3 was introgressed from a Chinese *B. rapa* ssp. *oleifera* accession; while the line 1012/98 was developed from a resynthesized *B. napus,* originally created at the University of Göttingen, Germany. Based on AFLP and SSR markers, a major QTL for yellow-seed color was identified on the linkage group N18 in all three populations and this QTL was found to be colocalized with a major locus for ADF (acid detergent fibre) content. It was found that this major yellow seed color gene of N18 is partially dominant over black seed color gene, and contributes to a reduction in fibre content.

Van Deynze and Pauls (1994) studied the inheritance of seed color in *B. napus* by using DH and F_2 populations derived from cross between yellow and black-seeded types, and reported a trigenic inheritance of this trait, where black seed color is being dominant over yellow seed color. According to these authors, the maternal parent primarily determined the seed color in the material; however, the pollen parent also plays a minor role in seed color determination. According to their proposed genetic model, black color seed is formed only when the first locus is homozygous dominant and at least one dominant allele is present at the second locus; brown seed is formed when one or more recessive allele is present at the first locus and one or more dominant alleles at any of the three loci; while yellow-seed is due to homozygous recessive condition at all three loci. Van Deynze et al. (1995) identified RFLP markers for two of the three seed color genes for use in marker-assisted breeding. However, no yellow-seeded cultivar has thus far been developed based on this yellow-seeded source.

1.9 Environmental influence on seed color

Seed color of yellow-seeded *B. napus* is often influenced by temperature under which the plants are being grown and set seed. Van Deynze et al. (1993) evaluated two black-seeded and four yellow-seeded *B. napus* genotypes under 16ºC /12ºC to 24ºC /20ºC day/night with constant photoperiod. They found that the yellow-seeded genotypes become increasingly yellow (or lighter in color) under higher temperature; however, the dark-seeded genotypes are not affected by temperature changes. Burbulis and Kott (2005) studied the effects of temperature

on the seed color by growing yellow-seeded genotypes under 20ºC, 28ºC and over 30ºC, and found that seed color become increasingly yellow at hot temperatures, while cooler temperatures tend to produce dark color seeds.

1.10 Research objectives

To date, several researchers have developed yellow or partially yellow-seeded *B. napus* through introgression of yellow-seed color genes from *B. rapa*, *B. carinata* and *B. juncea* into *B. napus*. However, uniformity of yellowness and stability of this trait under different environmental conditions is still an issue for the development of bright yellow-seeded (like 'yellow sarson') canola cultivars with maximum reduction in fibre content. In these yellow-seeded *B. napus*, the yellow seed color genes are originated from natural *B. rapa* and/or *B. juncea* and/or *B. carinata*, depending on the interspecific cross designed by the researchers; and the genetic control of yellow seed color is quite different in these three species. As reviewed in section 1.8, the yellow-seeded *B. napus* developed by AAFC, Saskatoon and Rahman (2001b) has been developed through different approaches of interspecific hybridization. Thus, it is hypothesized that the genetic control of yellow seed color in these two sources are different, and cross between these two yellow-seeded *B. napus* types will generate transgressive segregants for brighter yellow seed color through re-shuffling of the yellow seed color genes.

The yellow seed color trait of *B. rapa* 'yellow sarson' has been of interest to the researchers/breeders for introgression into *B. napus* due to its bright yellow seed color and greatest stability of this trait under diverse environmental

conditions. Use of molecular markers, linked to the yellow seed color allele of 'yellow sarson' would greatly facilitate rapid introgression of this allele in a breeding programme.

Thus, the objectives of this research are:

- To map the seed color gene(s) of *B. rapa* and identify SSR makers linked to the yellow seed color allele of 'yellow sarson' for use in marker-assisted breeding.
- To study the genetic relationship between the yellow-seeded *B. napus* lines YN01-429 and CH5034/06 obtained from AAFC, Saskatoon and NPZ, Lembke, Germany. The yellow seed trait of CH5034/06 was originally developed by Rahman (2001b) and agronomic property of this line has been improved through breeding by NPZ Lembke, Germany.

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Chapter 2

Mapping of the major seed color locus in *Brassica rapa*

2.1 Introduction

Rapeseed (*Brassica rapa* AA, 2n= 20 and *B. napus* AACC, 2n=38) is cultivated as an important oil crop in many parts of the World. Oil and protein components are the major products of this crop. Seeds of naturally occurring *B. napus* are black in color; while in other *Brassica* oilseed crops, e.g. *B. rapa*, *B. carinata* and *B. juncea*, black/brown and yellow seed color type exists. Stringam et al. (1974) studied the brown and yellow-seeded *B. rapa* and reported that yellow seed contain more oil and protein and less fibre as compared to brown seed. Yellow seed of *B. napus* can have 3-4% lower fibre content as compared to brown/black seed (Shirzadegan and Röbbelen 1985, Rahman et al. 2001). Therefore, developing yellow-seeded cultivars has been of interest to the breeders and researchers primarily to reduce the content of fibre in seed meal, as this seed component decreases protein digestibility (Lee et al. 1984) and consequently lowers the nutritional value of this protein-rich meal for use as animal feed.

Genetic study of seed color in *B. rapa* was first reported in 1942 by Mohammad et al. They reported that seed color in this species is controlled by three independent gene loci. However, Stringam (1980) found that this trait in *B. rapa* is controlled by two gene loci, and suggested a genetic model of inheritance of this trait. According to this model, presence of dominant alleles at both loci (Br_1 and Br_3) or only at the first locus (Br_1) produce brown seed, while presence

of dominant alleles at the second locus (Br_3) and homozygous recessive condition at the first locus (br_1br_1) produce yellow-brown seeds. Yellow seeds are produced when both loci are in homozygous recessive condition (br_1br_1, br_3br_3). Similar digenic inheritance of seed color has also been reported by Zaman (1989) and Rahman et al. (2007). Schwetka (1982) distinguished two genes with epistatic effect *(Br_1br_1; Br_6br_6)*, and four genes with hypostatic effect *(Br_3br_3; Br_4br_4; Br_5br_5; Br_7br_7)* for seed color; and identified two alleles for yellow seed color in Br_1br_1 locus, while only single allele for yellow seed color in Br_6br_6 locus. Rahman (2001) studied joint segregation of seed color and flower (petal) color in *B. rapa* based on cross between *B. rapa* var. 'yellow sarson' and brown- or partially yellow-seeded Canadian *B. rapa,* and reported a digenic inheritance of seed color, where the yellow seed color genes of 'yellow sarson' are recessive to brown or partially-yellow seed color genes of Canadian *B. rapa*. Petal color was found to be controlled by single gene locus, and the genes controlling petal color and seed color inherit independently.

DNA marker techniques have led to enormous progress in plant genetic research and breeding. These techniques emerged as big hand in assisting breeders towards developing cultivars in short span of time by using marker-assisted selection. Genome sequencing in crops, such as *Arabidopsis*, Rice and Tomato, has opened a new era of molecular technologies to be used in breeders' manual. The genome sequencing of *Arabidopsis* (The Arabidopsis Genome Initiative, AGI, 2000), to which *Brassica* is a close relative, as well as *B. rapa* (The *Brassica rapa* Genome Sequencing Project Consortium, 2011), has opened a new era for

studying the *Brassica* genomes including study of simple as well as complex traits. In *Brassica*, molecular mapping of genes has started in late 1980's with the development of restriction fragment length polymorphism (RFLP) linkage maps in *B. oleracea* (Slocum et al. 1990), *B. rapa* (Song et al. 1991) and *B. napus* (Landry et al. 1991). With the discovery of polymerase chain reaction (PCR), more different types of markers were developed, e.g. randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNP) etc. The number of SSRs available (~ 2825 SSRs available now, source: http://www.brassica.info/resource/markers.php) in *Brassica* has been increasing as a result of publicly funded international initiative (www.brassica.info). Another set of 627 SSRs developed by Cheng et al. (2009) based on public funding are also available to the scientific community. The sequence-related amplified polymorphism (SRAP) markers developed by Li and Quiros (2001) also provided enormous number of markers for mapping and gene tagging in *Brassica*. Single nucleotide polymorphism markers are not publicly available yet; however, these are expected to be released soon. This marker type expected to have wide application in plant breeding.

To date, several traditional genetic studies have been conducted by different researchers to understand the genetic control of seed color in *B. rapa*; however, only two reports are available on mapping and identification of molecular marker for this trait in this species. Based on a backcross populations of *B. rapa*, derived from a cross between brown-seeded cv. 'Span' and 'yellow

sarson' cv. 'BARI-6', Rahman et al. (2007) identified a SRAP marker linked to the major seed color gene Br_1/br_1; and based on this marker they developed several SNP and SCAR markers for use in marker assisted breeding. Recently, Xiao et al. (2011) identified several AFLP markers linked to the major seed color gene of *B. rapa*; and by use of SSR markers from a reference map, they mapped this gene on A9.

In contrast to *B. rapa*, several researchers mapped the seed color genes of *B. napus* and developed molecular markers for use in marker assisted breeding. Van Deynze et al. (1995) identified RFLP markers for two of the three seed color genes in *B. napus*. Somers et al. (2001) identified eight RAPD markers co-segregating with a major seed color gene in *B. napus*. They also identified two additional loci for seed color, which seems to interact with the major seed color gene locus. Rahman et al. (2010) used SRAP markers to tag the yellow seed color genes in a *B. napus* population. A SRAP marker was identified on A9 (≈N9) of the A-genome and another marker on the C3 (≈N13) of C-genome of *B. napus*. Snowdon et al. (2010) identified a major QTL for seed color, fibre content and phenolic compounds on the chromosome A9 of *B. napus*, and this QTL region shows synteny to a sequence contig to chromosome A9 of *B. rapa*. Liu et al. (2005) identified several RAPD and AFLP markers linked to yellow seed color gene in *B. napus*. This yellow seed color gene is partially dominant over black seed color. Using a doubled haploid population derived from the F_1 of No. 2127-17 (yellow) x 'ZY821' (black) cross, Xiao et al. (2007) identified two AFLP markers flanking the partially dominant yellow seed color gene only at 0.5 cM

away. They converted the AFLP markers into SCAR markers, and the markers mapped to the linkage group A9 (≈N9). They also identified two SSR markers (Na14-E08 and Na10-B07) linked to the seed color locus.

Thus, it is apparent that several studies have been conducted on molecular mapping of seed color genes in *B. napus*, and chromosomal location of some of the seed color genes has been identified. *Brassica napus* share the A-genome of *B. rapa*. Therefore, based on the studies on *B. napus* the genomic location and molecular markers for seed color in *B. rapa* can be inferred. It is well established today that the amphidiploid species *B. napus* evolved in nature from the A- and C-genome species *B. rapa* and *B. oleracea*; and these two *Brassica* genomes evolved from a much smaller genome through polyploidization including chromosome fission, fusion and rearrangements (Lysak et al. 2005). Therefore, multiple copies of a gene can be found in the diploid genome (Parkin et al. 2003) and allopolyploidy can stimulate homeologous chromosome exchange and thus alter gene expression/function through the loss and doubling of homeologous genes within the rearrangements (Gaeta 2009). Therefore, it is important to understand a trait in the diploid species; and this knowledge can be used to understand the trait in the amphidiploid species. Mapping of the seed color gene in the diploid species would also facilitate map-based cloning of the gene due to its simpler genome. Thus, the objective of this study was to map the seed color genes of *B. rapa* as well as to identify SSR markers linked to the yellow seed color gene for use in marker assisted breeding.

2.2 Materials and methods

2.2.1 Plant material and mapping population development

Brassica rapa mapping population was developed by crossing the 'yellow sarson' cultivar Sampad with the breeding line 3-0026.027. The characteristics of the two parental lines are presented in Table 2.1. Sampad is a registered cultivar in Bangladesh, while 3-0026.027 is a breeding line derived from cross between *B. rapa* var. 'yellow sarson' line M-91 and Canadian *B. rapa* cultivar Tobin (Rahman et al. 1996, Rahman 2001) (Fig. 2.1).

Table 2.1: Agronomic and seed quality characteristics of the *Brassica rapa* var. 'yellow sarson' cultivar Sampad and breeding line 3-0026.027

Character	Sampad	3-0026.027
Flower color	Yellow	Creamy white
Seed color	Yellow	Yellowish brown
Erucic acid in oil	High	Zero
Glucosinolates in seed	High	Low
Silique size	Normal	Short
Pollination type	Self-compatible	Self-compatible

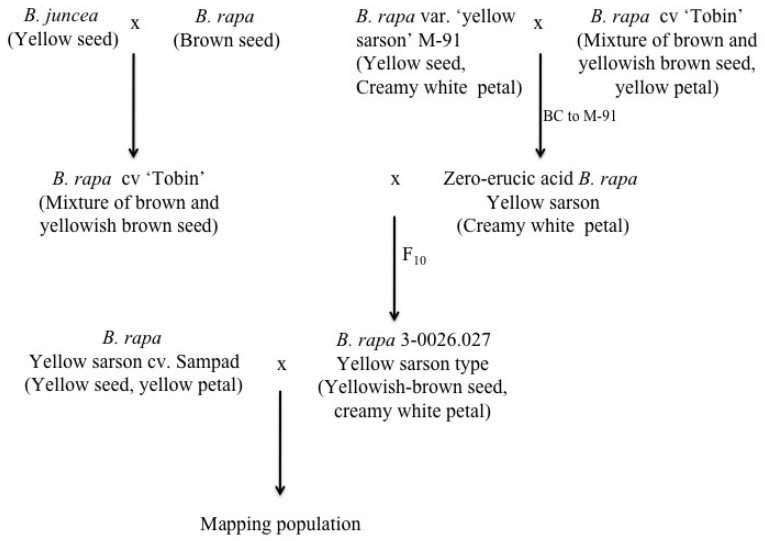


Fig 2.1: Pedigree of the self-compatible *Brassica rapa* line 3-0026.027 (Rahman 2001, Rahman et al. 1996)

Reciprocal crosses were made between Sampad and 3-0026.027 and F_1 seeds were produced. The F_1 plants of Sampad x 3-0027.027 and 3-0026.027 x Sampad were self-pollinated to produce F_2 seeds, as well as backcrossed to Sampad to produce backcross (B_1) seeds. The parents, F_1, F_2, and backcross populations were used by Zimmerli (2006) to study the inheritance of seed color and other agronomic traits. Self-pollinated seeds of the backcross plants, i.e. B_1S_1 seeds, were used in the present research project.

Ten plants of each of 11 B_1S_1 families of (Sampad x 3-0026.027) x Sampad and eight families of (3-0026.027 x Sampad) x Sampad were grown in a growth chamber to study segregation of seed color for development of the mapping population. Of the 19 (11+8) families, four families showed a 3:1 segregation for brown : yellow seed color. Two of the four B_1S_1 families were also grown in field in 2007 to study segregation of seed color. Based on seed color segregation in growth chamber and in field, one B_1S_1 family of (Sampad x 3-

0026.027) x Sampad cross was selected and population size of this family was increased to 120.

A total of 15 B_1S_2 families, derived from the above mentioned selected B_1S_1 family, were grown to study seed color segregation as well as for validation of the identified marker(s) linked to the seed color gene. For this, 10 plants from each family were grown and evaluated for seed color and molecular marker.

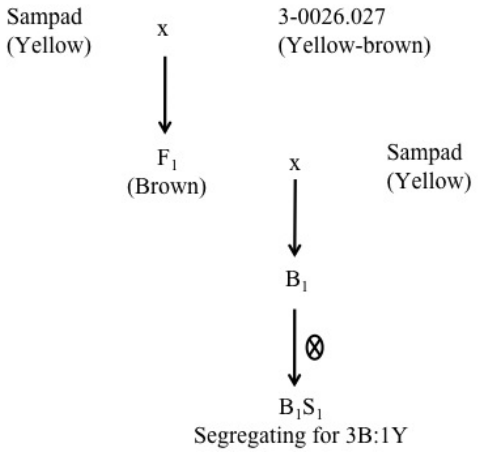


Fig 2.2: Schematic diagram showing the development of mapping population from
(Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

2.2.2 Seed color analysis

Self-pollinated seeds produced by bag isolation of individual plants were assessed visually for seed color following the scale developed by Zimmerli (2006) (Fig. 2.3). The parent Sampad had uniform bright yellow color seed and grouped as A. The group B and C were intermediate between the two parents. Group B had approximately 75% yellow seeds and 25% yellowish-brown and/or partly yellow seeds, while the group C had approximately 50% brown and/or yellowish-brown

seeds and 50% yellow or partly yellow seeds. Group D is yellowish-brown color, like the parent 3-0026.027, and is different from the group C by having more than 50% brown seeds. The group E is uniform red in color which marks the edge of yellowish-brown (group D) and group F, which is brown in color. The group G consisted of black/brown seeds and group H is 90% black seeds (Fig. 2.3).

Fig 2.3: Seed color classification of the segregating population of Sampad x 3-0026.027 cross of *Brassica rapa*. Parental and F_1 seed color is also presented (source: Zimmerli 2006).

A total of 170 seed samples from the same cross segregating for seed color were visually scored for seed color (1 to 8 ≅ A to H), as well as analysed by NIRS (near infra-red spectroscopy) for this trait based on whiteness index. NIRS analysis was done in collaboration with Dr. Gerhard Rakow, Agriculture and Agri-Food Canada (AAFC), Saskatoon. Correlation between visual score and NIRS assessment for seed color was 0.94, suggesting that visual assessment of seed color can be reliably used.

2.2.3 Molecular marker analysis

DNA extraction

Leaf samples of 3-5 weeks old plants of the parents, F_1, B_1S_1 and B_1S_2 populations grown in greenhouse were collected in 2 ml Eppendorf tubes and kept at minus 80°C until use. DNA of the samples was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co, St. Louis, MO, USA) following the instructions of the manufacturer. In brief, 100 mg leaf tissue was ground in liquid nitrogen and 350 µl lysis buffer part A and 50 µl lysis buffer part B were added to the samples. Both lysis buffer A and B rupture the cell walls. The lysed plant tissue samples were incubated at 65°C for 30 min and 130 µl precipitation solution was added. After mixing properly, the samples were incubated on ice for 5 min and this followed spinning at 10,000 rpm for 5 min. The supernatant was transferred to the GenElute Filtration column. Filtration column was centrifuged for 1 min at 10,000 rpm, and 700 µl binding solution was added to the filtrate. Binding solution binds with the nucleic acids in the supernatant. The GenElute Nucleic Acid Binding Column was prepared by adding 500 µl of column preparation solution followed by centrifugation at 10,000 rpm for 1 min and discarding the flow through. The GenElute Nucleic Acid Binding Column was aliquot with 700 µl mixture of the filtrate and binding solution; centrifuged for 1 min and the flow through was discarded. The rest of the mixture of the filtrate and binding mixture was then loaded into the same GenElute Nucleic Acid Binding Column, and the process was repeated. The GenElute Nucleic Acid Binding Column was transferred into a collection tube. The binding

column with the bound DNA was washed with 500 µl of Wash Solution followed by centrifugation at 10,000 rpm for 1 min. The binding column was transferred to a new collection tube and the binding column was washed for the second time by adding 500 µl of Wash Solution and followed by centrifugation for 3 min at 10,000 rpm. The binding column was transferred to a new collection tube and 100 µl of pre-heated Elution Solution (pre-heated to 65°C) was added. The binding column was then centrifuged at 10,000 rpm for 1 min. The eluting process was repeated with another 100 µl Elution Solution. The quality and quantity of the DNA was evaluated by spectrometry using 260/280 nm absorbance ratio method, and the DNA concentration was estimated at 260 nm with an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA).

Simple sequence repeat (SSR) primers

Simple sequence repeat (SSR) markers were used for mapping the seed color gene. Primer sequences were obtained from various sources: Biotechnology and Biological Science Research Council (BBSRC) (http://brassica.agr.gc.ca/index_e.shtml), Agriculture and Agri-Food Canada (AAFC), Saskatoon, National Institute of Vegetable and Tea Science, (http://vegmarks.nivot.affrc.go.jp/VegMarks/jsp/index.jsp) (Suwabe et al. 2002), and Celera AgGen Brassica Consortium (Piquemal et al. 2005). Additional 126 SSR primer pairs were designed from genomic DNA sequence of *B. rapa* var. Chiifu available at http://www.brassica.bbsrc.ac.uk/BrassicaDB.

Polymerase chain reaction (PCR)

Polymerase chain reaction was performed in a total volume of 10 µl containing 10 ng of DNA, 1x PCR Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs (Promega, Madison, WI, USA), 0.25 µM of each of forward and reverse primer, 1 pmol of labelling dye, FAM, VIC, NED, and PET, (Applied Biosystems, Foster City, CA) and 1 unit of *Taq polymerase* enzyme (Applied Biosystems, Foster City, CA). Temperature profile for PCR was 94°C for 5 min, 30 cycles at 94°C for 30 sec, 56°C for 45 sec, 72°C for 45 sec, and 8 cycles at 94°C for 30 sec, 53°C for 45 sec, 72°C for 45 sec and 10 min of final extension at 72°C. The final products were resolved on a capillary ABI sequencer No. 3730 (Applied Biosystems, Foster City, CA), and software programme 'Genemapper version 3.7' was used for analysing the PCR products.

Bulk segregant analysis (BSA)

Bulked segregant analysis (Michelmore et al. 1991) was used for identification of polymorphic markers, and eventually to identify the markers linked to the seed color gene(s). For this, equal quantities of DNA of 10 yellow and 10 dark-brown seeded plants of the B_1S_1 populations were pooled to make the yellow and brown seed bulks. In addition to these two bulks, the two parental lines were also included for identification of polymorphic markers. For this, bulks of the two parental lines were prepared by pooling DNA from 4-5 plants of Sampad and 3-0026.027 and included in the study.

2.2.4 Genotyping

Polymorphic markers were used to genotype the B_1S_1 mapping population. Markers closely linked to the seed color gene in B_1S_1 mapping population were tested in B_1S_2.

2.2.5 Data analysis and gene mapping

Genotypic and phenotypic data obtained from B_1S_1 mapping population were used for mapping the seed color gene. The segregation of marker genotypes were tested for goodness of fit using χ^2-test for 1:2:1 (homozygous for allele A: heterozygous: homozygous for allele B) ratio. Linkage analysis was done using software package Mapmaker (Lander et al. 1987). Recombination frequencies were designated as cM using Kosambi's map function (Kosambi, 1944). To determine the correct marker linked to seed color, 'compare' and 'try' commands were used with three-point analysis. The order of markers in the linkage groups was further verified by 'ripple' command.

2.3 Results

2.3.1 Seed color segregation in mapping populations

Segregation in B_1S_1 population

A total of 19 B_1S_1 families of (Sampad x 3-0026.027) x Sampad and (3-0026.027 x Sampad) x Sampad crosses were evaluated of which 15 families were segregating for seed color while four families were true breeding for yellow seed color (Table 2.2). This 15:4 distribution follow a 3:1 segregation ($\chi^2 = 0.16$, p =

0.7-0.9) as could be expected among B_1S_1 families for a trait controlled by two major gene loci.

Table 2.2: Type of the B_1S_1 families of (Sampad x 3-0026.027) x Sampad and (3-0026.027 x Sampad) x Sampad crosses of *Brassica rapa* in respect to segregation for seed color

Cross	Total families	No. of families segregating for seed color				
		Y:YB:B	Y:YB[a]	YB:B	Y:B	Yellow[b]
(Sampad x 3-0026.027) x Sampad	11	1	4	3	1	2
(3-0026.027 x Sampad) x Sampad	8	1	1	1	3	2
Total	**19**	**2**	**5**	**4**	**4**	**4**

[a] B = brown, YB = mixture of yellow, yellowish brown and brown seeds, Y= yellow
[b]non-segregating

Of the 15 segregating B_1S_1 families, four families were segregating only for brown and yellow seed color in 3:1 ratio under both growth chamber and field conditions (Table 2.3). Based on this data, the B_1S_1 Family #61 of (Sampad x 3-0026.027) x Sampad cross was selected for mapping the seed color gene.

Table 2.3: Segregation for seed color in four B_1S_1 families of (Sampad x 3-0026.027) x Sampad and (3-0026.027 x Sampad) x Sampad crosses of *Brassica rapa*

Cross	B_1S_1 family	Growth conditions	Total plants	Seed color segregation, 3:1			
				Brown	Yellow	χ^2	p-value
(Sampad x 3-0026.027) x Sampad	Fam #61	Growth chamber	107	78	29	0.25	0.5-0.7
		Field	23	17	6	0.31	0.5 - 0.7
(3-0026.027 x Sampad) x Sampad	Fam #72	Growth chamber	10	8	2	0.13	0.7-0.9
		Field	25	21	4	1.08	0.2-0.3
(3-0026.027 x Sampad) x Sampad	Fam #82	Growth chamber	10	8	2	0.13	0.7-0.9
(3-0026.027 x Sampad) x Sampad	Fam #85	Growth chamber	10	9	1	1.2	0.2-0.3

Segregation in B_1S_2 families

A total of 15 B_1S_2 families were grown – three from yellow-seeded and 12 from brown-seeded B_1S_1 plants. All 30 plants of the three yellow-seeded families were true breeding for yellow seed color (Table 2.4). On the other hand, of the 12 brown seed families, four were true breeding for brown seed color, while eight families were segregating for brown and yellow seed color. This 4:8 distribution for non-segregating to segregating families for seed color is in accordance to 1:2 ratio ($\chi^2 = 0$, $p = 1.0$) as could be expected for a trait controlled by a single gene locus.

Table 2.4: Segregation for seed color in B_1S_2 families of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

B_1S_1 seed color	No. B_1S_2 families	Total plants	Brown seed	Yellow seed	segregation	χ^2	p-value
Yellow	3	30	0	30	0:1	0	
Brown	4	40	40	0	1:0	0	
Brown	8	80	63	17	3:1	0.6	0.3-0.5

Seed color segregation in the eight segregating families followed similar pattern (homogeneity $\chi^2 = 4.73$, $p = 0.05 - 0.1$). A total of 80 B_1S_2 plants from these eight families were evaluated, where 63 were brown-seeded and 17 yellow-seeded. This 63:17 distribution clearly followed a 3:1 segregation (Table 2.4).

2.3.2 Polymorphism of the SSR markers

A total of 513 SSR markers were tested for polymorphism where 117 (35%) were found to be polymorphic between the parents, Sampad and 3-0026.027, and similar number were found to be polymorphic between the brown and yellow bulks. On the other hand, of the 126 newly designed markers (designed based on genomic DNA sequence of *B. rapa* var. Chiifu), 52 (41%) were polymorphic amongst the parents; but only 6 (8.2%) were polymorphic between the yellow and brown bulks. Based on parental polymorphism and BSA, a total of 11 SSRs from the linkage group A9, including four newly designed SSRs developed from the genomic DNA sequence of *B. rapa* var. Chiifu, were used for genotyping the mapping population.

Table 2.5: Analysis of simple sequence repeat (SSR) markers for polymorphism between the parents Sampad and 3-0026.027, and between bulks of brown and yellow-seeded B_1S_1 plants of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

Total SSR	No. amplified	Polymorphism between parents			Polymorphism between bulks		
		No. monomorphic	No. polymorphic	% polymorphism	No. monomorphic	No. polymorphic	% polymorphism
513	333	216	117	35	216	117	35
126[a]	73	21	52	42	67	6	8.2

[a]SSR markers designed based on genomic DNA sequence of *B. rapa* var. Chiifu

2.3.3 Segregation of the SSR marker alleles in B_1S_1 and B_1S_2 populations

Segregation of the 11 SSR marker alleles in B_1S_1 Fam #61 of (Sampad x 3-0026.027) x Sampad cross is presented in Table 2.6. Of the 11 markers, five markers showed significant deviation from expected 1:2:1 segregation for the marker alleles.

Segregation of the SSR marker alleles in B_1S_2 population, segregating for seed color, is presented in Table 2.7. Of the five markers tested, three showed significant deviation from expected 1:2:1 segregation. These three markers however did not show segregation distortion in B_1S_1 population.

Table 2.6: Segregation of SSR markers alleles in Fam #61 of B_1S_1 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

Marker	Total plants	Observed marker genotype			Segregation, 1:2:1	
		No. homozygotes (Sampad type)	No. heterozygotes	No. homozygotes (3-0026.027 type)	χ^2	p-value
BRAS036	117	32	77	8	21.5	<0.05
CB10103A	119	36	62	21	4.0	0.14
CB10298	44	15	21	8	2.3	0.31
CB10022A	113	28	68	17	6.8	<0.05
BRAS002A	15	0	11	4	5.4	0.07
CB10255	101	31	45	25	1.9	0.38
nrc 773	93	29	48	16	3.7	0.15
nrc 781	93	26	49	18	1.6	0.44
nrc 782	92	30	43	19	3.0	0.22
nrc 795	81	27	48	6	13.7	<0.05
CB10311	73	5	11	57	109.7	<0.05

Table 2.7: Segregation of SSR markers alleles in B_1S_2 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

Marker	Total	Observed Marker genotype			Segregation, 1:2:1	
		No. homozygotes (Sampad type)	No. heterozygotes	No. homozygotes (3-0026.027 type)	χ^2	p-value
BRAS036	61	15	28	18	0.70	0.70
CB10103A	69	29	22	18	12.57	<0.05
CB10298	68	31	24	13	15.41	<0.05
CB10022A	73	16	38	19	0.37	0.83
CB10255	62	17	17	28	16.55	<0.05

2.3.4 Linkage association of the SSR markers with seed color

Of the 11 SSR markers from linkage group A9 used for genotyping the B_1S_1 mapping population, six marker alleles showed different level of co-segregation with the seed color alleles. The 232 bp (base pair) allele of the SSR marker BRAS036, present in the yellow-seeded parent Sampad, was present in 93.5% yellow-seeded B_1S_1 plants; while 6.5% plants in addition to carrying the 232 bp allele of Sampad also carried the 249 bp allele of 3-0026.027 which resulted from recombination between the marker and seed color alleles (Table 2.7). The 249 bp marker allele of BRAS036 of the parent 3-0026.027 was detected in more than 96% of the brown-seeded B_1S_1 (Table 2.8) and B_1S_2 (Table 2.10) plants. However, this marker allele showed distorted segregation in brown-seeded plants of B_1S_1 population (Table 2.6, 2.7) – but not in B_1S_2 population. For example, of the 86 brown-seeded B_1S_1 plants, only 9.3% plants were

homozygous for 3-0026.027 allele (249 bp) while 87.2% plants were heterozygous for this marker alleles (Table 2.8). This observed distribution of homozygous and heterozygous marker genotypes deviated significantly from expected genotype distribution of 33.3% homozygous and 66.6% heterozygous for the marker alleles ($\chi^2 = 20.5$, $p < 0.05$). About 3.5% brown-seeded B_1S_1 plants carried only the 232 bp allele of Sampad, i.e. were recombinants.

The marker alleles of CB10022A, CB10298 and CB10255 also showed co-segregation with seed color alleles in B_1S_1 and B_1S_2 plants, however, with higher recombination than the marker BRAS036. These markers also showed variable recombination among the yellow-seeded plants of different population. For example, the marker allele of Sampad of CB1022A was present in 83.3% of the yellow-seeded B_1S_1 plants in homozygous condition; while this was found in 94.1% yellow-seeded plants in B_1S_2 population segregating for seed color, and 96.7% plants of the true breeding yellow seed color B_1S_2 families. The markers BRAS036 and CB10022A map on the linkage group A9, and exactly at the same position on the chromosome (Dr. Berisso Kebede, unpublished map, personal communication).

Table 2.8: Linkage association of SSR marker alleles with seed color in B_1S_1 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa* segregating for yellow and brown seed color

SSR marker	Yellow seed					Brown seed				
	No. of plants	% plants for marker allele(s)				No. of plants	% plants for marker allele(s)			
		Sampad allele (a)	3-0026.027 allele (b)	Both alleles (c)	a+c[1]		Sampad allele (d)	3-0026.027 allele (e)	Both alleles (f)	e+f[2]
BRAS036	31	93.5	0	6.5	100	86	3.5	9.3	87.2	96.5
CB10103A	32	65.6	3.1	31.3	96.9	87	17.2	23	59.8	82.8
CB10298	11	90.9	9.1	0	90.9	33	15.2	21.2	63.6	84.8
CB10022A	30	83.3	3.3	13.3	96.7	83	3.6	19.3	77.1	96.4
CB10255	30	86.7	3.3	10	96.7	71	7	33.8	59.2	93

[1]percent yellow-seeded plants carrying Sampad allele
[2]percent brown-seeded plants carrying 3-0026.027 allele

Table 2.9: Linkage association of SSR markers with yellow seed color in true breeding yellow-seeded B_1S_2 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

SSR marker	No. of plants	Percent plants with marker allele(s)			
		Sampad allele (a)	3-0026.027 allele (b)	Both alleles (c)	a+c[1]
BRAS036	26	100	0	0	100.0
CB10298	30	96.7	0	3.3	100.0
CB10022A	30	96.7	0	3.3	100.0
CB10255	22	90.9	9.1	0	90.9

[1]percent yellow-seeded plants carrying Sampad allele

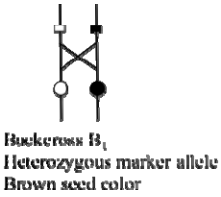
Table 2.10: Linkage association of SSR marker alleles with seed color in B_1S_2 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa* segregating for yellow and brown seed color

SSR Marker	Yellow Seed					Brown Seed				
	No. of plants	Percent plants with marker allele(s)				No. of plants	Percent plants with marker allele(s)			
		Sampad allele (a)	3-0026.027 allele (b)	Both alleles (c)	a+c[1]		Sampad allele (d)	3-0026.027 allele (e)	Both alleles (f)	e+f[2]
BRAS036	15	100.0	0	0	100.0	46	0	39.1	60.9	100
CB10298	16	100.0	0	0	100.0	52	28.8	25.0	46.2	71.2
CB10022A	17	94.1	0	5.9	100.0	56	0	33.9	66.1	100
CB10255	16	75.0	12.5	12.5	87.5	45	11.1	55.6	33.3	88.9

[1]percent yellow-seeded plants carrying Sampad allele, [2]percent brown-seeded plants carrying 3-0026.027 allele

To further test linkage association between the markers and seed color alleles, χ^2-test was done on B_1S_1 population. Assuming the B_1 plants were heterozygous for markers and seed color alleles, and independent assortment ($\geq$ 50% recombination ratio) of the marker and seed color alleles occurred (Fig. 2.5), following six types of plants are possible in B_1S_1 population.

B_1 plant:



↓

B_1S_1 population:

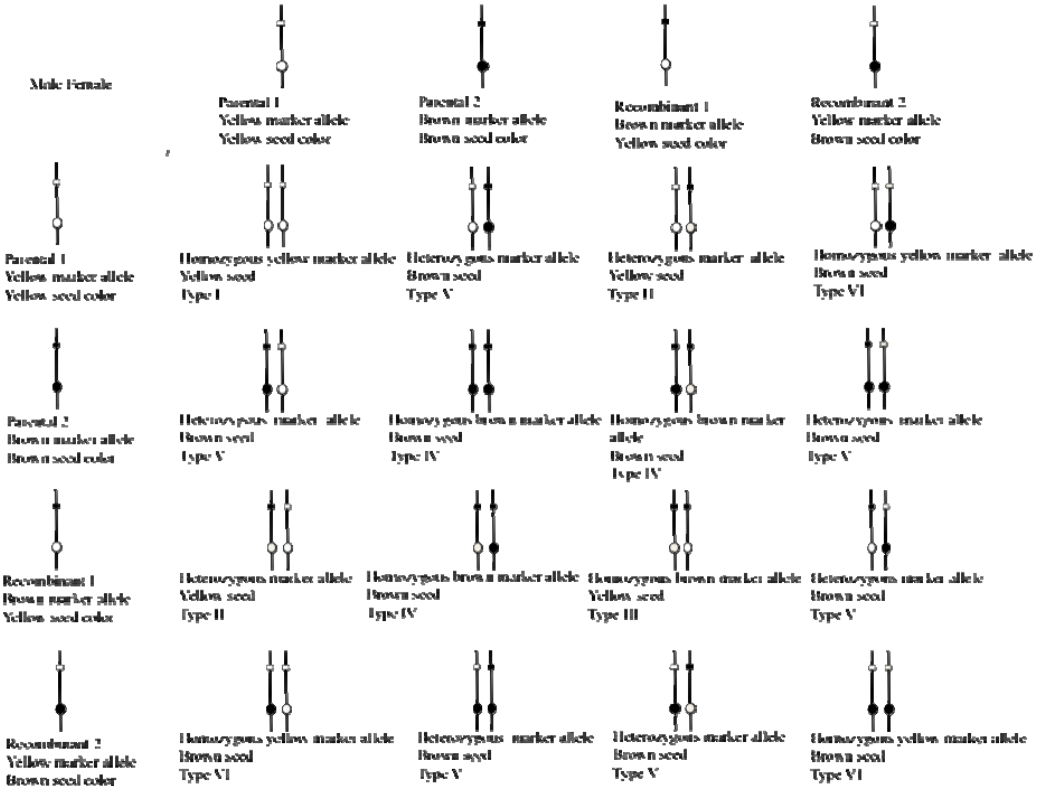


Fig 2.4: Possible marker genotypes and seed color phenotypes of B_1S_1 plants based on independent assortment of seed color and molecular marker alleles in (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

Table 2.11: Possible marker genotypes and seed color phenotypes of B_1S_1 plants based on independent assortment of seed color and molecular marker alleles in (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa*

	Seed color/marker genotype	Expected No.	Expected %
Type I	yellow seed, homozygous for yellow marker allele	1	6.25
Type II	yellow seed, heterozygous for marker allele	2	12.50
Type III	yellow seed, homozygous for brown marker allele	1	6.25
Type IV	brown seed, homozygous for brown marker allele	3	18.75
Type V	brown seed, heterozygous for marker allele	6	37.50
Type VI	brown seed, homozygous for yellow marker allele	3	18.75
	Total	16	100

Type I, II and III are all yellow seed color but differ for molecular marker alleles. Type I is homozygous for yellow marker allele, Type II is heterozygous markers alleles and Type III is homozygous brown marker alleles. Type IV, V and VI are brown seed color phenotypes, where Type IV is homozygous for brown marker allele. Type V is heterozygous for marker alleles, and Type VI is homozygous for yellow marker allele. Proportion of these six types in B_1S_1 population will vary depending on linkage association between seed color and molecular marker alleles. Chi-square test was done in B_1S_1 and B_1S_2 populations genotyped with SSR markers to estimate the assortment of seed color and marker alleles. In this case, χ^2-value with $p<0.05$ would indicate non-random assortment of marker and seed color alleles. Chi-square values were

non-significant ($p<0.05$) for all tested markers in both B_1S_1 and B_1S_2 populations suggesting non-random assortment of markers and seed color alleles. Greatest χ^2-value was obtained for the marker BRAS036 in both B_1S_1 and B_1S_2 populations suggesting its strongest association with the seed color allele.

Table 2.12: Chi-square test for joint segregation of seed color and molecular marker alleles in B_1S_1 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa* based on the assumption of their independent assortment

Marker	Total plants	No. plants for seed color and marker alleles						Segregation, 1 : 2 : 1 : 3 : 6 : 3	
		Type I	Type II	Type III	Type IV	Type V	Type VI	χ^2	p-value
BRAS036	117	29	2	0	8	75	3	129.93	<0.05
CB10103A	119	21	10	1	20	52	15	38.55	<0.05
CB10298	44	10	0	1	7	21	5	29.94	<0.05
CB10022A	113	25	4	1	16	64	3	85.77	<0.05
CB10255	101	26	3	1	24	42	5	85.69	<0.05
CB10311	73	4	3	10	47	8	1	116.92	<0.05

Table 2.13: Chi-square test for joint segregation of seed color and molecular marker alleles in B_1S_2 population of (Sampad x 3-0026.027) x Sampad cross of *Brassica rapa* based on the assumption of their independent assortment

Marker	Total plants	No. plants for seed color and marker alleles						Segregation, 1 : 2 : 1 : 3 : 6 : 3	
		Type I	Type II	Type III	Type IV	Type V	Type VI	χ^2	p-value
BRAS036	61	15	0	0	18	28	0	60.62	<0.05
CB10298	68	16	0	0	13	24	15	45.72	<0.05
CB10022A	73	16	1	0	19	37	0	59.60	<0.05
CB10255	61	12	2	3	25	15	5	44.32	<0.05

2.4 Discussion

The B_1S_1 mapping population developed in this study from (Sampad x 3-0026.027) x Sampad cross of *B. rapa* clearly showed simple Mendelian segregation for brown and yellow seed color. This was validated through progeny test of the yellow- and brown-seeded B_1S_1 plants, i.e. seed color in B_1S_2 families. Genetic analysis of seed color (Stringam 1980, Zaman 1989, Rahman 2001, Rahman et al. 2007) demonstrated that more than one locus is involved in the control of this trait in *B. rapa*. However, monogenic inheritance for brown and yellow seed color is still possible if the parents in the cross differ only for one locus, while the other locus is in homozygous recessive condition. Monogenic inheritance of seed color has been reported by Ahmed and Zuberi (1971) and Chen and Heneen (1992) based on cross between brown- and yellow-seeded *B. rapa* parents. Therefore, in this study, it was also possible to develop a B_1S_1 family, segregating for one of the seed color loci. By use of this mapping population, SSR markers linked to the major seed color locus was identified through application of bulk segregant analysis (BSA) followed by genotyping of the whole population. BSA followed by genotyping of the population has been used by several researchers for mapping of genes and identification of molecular markers. For example, Du et al. (2011) identified RAPD markers linked to gene controlling bolting in *Brassica rapa*, where the distance between RAPD marker $S295_{750}$ and the gene locus was found to be 3.14 cM. Similarly, Cheema et al. (2008) applied BSA for identification of bacterial blight resistance gene in *Oryza*.

By use of F_1, BC_2F_2, BC_3F_1 and BC_3F_2 population, bacterial blight resistance gene was localized in the putative region of ~35 cM, on chromosome 4L, and fine mapping of this region lead to confine the region of resistance gene between ~38.4 kb. El-Kadi et al. (2006) identified salt tolerance in Egyptian cotton using RAPD markers.

Previously, Rahman et al. (2007) at the University of Manitoba, identified a SRAP marker linked to the major seed color gene in *B. rapa* and converted this to a SCAR marker for use in marker assisted breeding. However, genomic position of this seed color locus in *B. rapa* was not reported. In another study, Rahman et al. (2010) found a SRAP marker (SA12BG18388) in F_2 and backcross populations of *B. napus* linked to the black/brown seed color gene. This SRAP marker was anchored to linkage group A9 (≈N9) of A-genome in *B. napus*. Recently, Xiao et al. (2011) mapped a seed color gene on A9 of *B. rapa*, and identified several AFLP markers linked to this seed color gene. Among the molecular markers, SSRs have become markers of choice, owing to their user-friendly nature. They are co-dominant, highly polymorphic, abundant and distributed evenly across the genome. This is a PCR based marker technique; therefore require small amount of DNA as compared to non-PCR based techniques, e.g. RFLP. This type of markers has been shown to be applicable within and between different *Brassica* species (Lowe et al. 2004, Plieske and Struss 2001, Saal et al. 2001, Suwabe et al. 2002). These types of markers are highly preferred by breeders for use in marker-assisted breeding. Therefore, identification of SSR markers linked to the seed color gene in *B. rapa* was one of

the objectives of this research. By use of 639 SSR markers, we identified several markers from the linkage group A9 linked to the yellow seed color allele of 'yellow sarson'.

Among the SSR markers tested, the marker BRAS036, which is mapped at the linkage group A9, showed the best co-segregation with the yellow seed color allele of 'yellow sarson' cv. Sampad. This was verified through progeny testing of B_1S_1 population, i.e. in B_1S_2 population. Although, almost all yellow-seeded plants carried the marker allele of Sampad, a small proportion of yellow-seeded plants also carried the marker allele of the brown-seeded parent, either in homozygous or in heterozygous condition, due to recombination between seed color and marker alleles. In conclusion, the SSR BRAS036 appeared to be the best marker for use in marker assisted breeding for the development of yellow-seeded *B. rapa* cultivar, as well as for introgression of the yellow seed color allele of 'yellow sarson' into *B. napus* for the development of yellow-seeded *B. napus* cultivars.

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Chapter 3

Genetic analysis of seed color and other quality traits in *Brassica napus*

3.1 Introduction

Increasing demand for vegetable oil in the world has brought *Brassica* oilseed crops second in line after wheat in Canada. Cultivation of this oilseed crop started in Europe and North America in 18th century since development of steam engines where this vegetable oil is found to be suitable for use as lubricant oil. In Canada, *Brassica* oilseeds primarily include *Brassica rapa* and *B. napus*, which collectively called rapeseed. Recently, the species *B. juncea* is also grown in limited acreage in Canada. This species is primarily grown in Indian sub-continent. *Brassica napus* is also known as 'Argentine rape', as it was originally introduced from Argentina, while '*B. rapa* is called as 'Polish rape' as it was introduced from Poland (Downey et al. 1975). During the early stage of establishment of *Brassica* oilseed crop in Canada, *B. rapa* equally shared the acreage with *B. napus*; but with the development of early maturing *B. napus*, the *B. rapa* acreage is replaced by *B. napus*. Currently *B. napus* is grown over >98% of the total *Brassica* oilseed acreage in Canada.

Development of *B. napus* and *B. rapa* cultivars with zero erucic acid content in oil and low glucosinolate content in seed meal by Canadian researchers/breeders has been the major breakthrough in the breeding history of these *Brassica* oilseed crops. These improved cultivars are designated as 'Canola'

(Stefansson and Downey 1995). Improvement of *Brassica* oilseed meal for low fibre content has also been one of the major interests to the breeders in the last few decades. Yellow seed in *B. rapa* is superior over brown/black-seed due to its higher oil and protein content, and lower fibre content (Stringam et al. 1974). Fibre content in meals from yellow seed of *B. napus*, *B. rapa*, *B. juncea* and *B. carinata* averaged at 28% and for brown seed averaged at 33% (Simbaya et al. 1995). The lower fibre content in yellow seed also reflected to lower content of lignin in meal.

Natural variants of yellow-seeded type are available in *B. rapa*, *B. carinata* and *B. juncea*, but not in *B. napus*. Seed color in *B. rapa* is controlled by two to three gene loci (Rahman 2001a, Mohammad et al. 1942, Stringam 1980, Schwetka 1982, Zaman 1989, Rahman et al. 2007), while in *B. juncea*, two gene loci with duplicate gene effect control this trait (Vera et al. 1979, Rawat 1989, Lionneton et al. 2004, Yan et al. 2009). In case of *B. carinata*, a single gene locus is involved in the control of this trait (Getinet and Rakow 1997, Rahman and Tahir 2010). Thus, yellow seed color in these three species is under different genetic control. Detailed review of this can be found in Chapter 1.

As yellow-seeded form is not naturally available in *B. napus*, extensive interspecific hybridization has been done during the past few decades to introgress the yellow seed color trait in this species from *B. rapa*, *B. juncea* and *B. carinata*. The first yellow-seeded *B. napus* cultivar, 'Hua-yellow No. 1' was registered in China in 1990 (Liu 1992), hence opening a realm for developing yellow-seeded *B. napus*. But, frequent occurrence of black spots in this yellow-seeded *B. napus*;

and also, change in seed color over generations and under different environmental condition was the major constraint for use of this material in breeding. Since then, several researchers (Rakow et al. 1999, Badani et al. 2006, Rahman 2001b, Liu et al. 2006) developed yellow-seeded *B. napus* through different approaches of interspecific hybridization, (for detail review, see section 1.8). However, a yellow-seeded *B. napus* with seed color like 'yellow sarson' is not available yet.

As mentioned above, the yellow-seeded *B. napus* lines reported by different researchers are developed through utilizing the yellow seed color genes of *B. rapa*, *B. juncea* and *B. carinata*, therefore, it can be hypothesize that genetic control of seed color in these yellow-seeded *B. napus* would be different and crossing of these types would generate even brighter and uniform yellow-seeded *B. napus*. The present study was conducted to investigate the genetic relationship between two yellow-seeded *B. napus* lines developed by two research groups through the use of different yellow-seed sources and different approaches of interspecific hybridization.

3.2 Materials and Methods

3.2.1 **Plant material and cross:** Two yellow-seeded *B. napus* lines were used in this study: YN01-429 and CH5034/06. The line YN01-429 was developed by Agriculture and Agri-Food Canada (AAFC, Saskatoon) through interspecific crosses involving *B. rapa* var. 'yellow sarson', yellow-seeded *B. carinata*, yellow-seeded *B. juncea* and black-seeded *B. napus* (Rakow et al. 2011). The line CH5034/06 is owned by Norddeutsche Pflanzenzucht (NPZ), Germany, where the

yellow seed color genes are derived from *B. rapa* var. 'yellow sarson'. This line was developed based on the yellow-seeded *B. napus* line 13-217 developed by Rahman (2001b) through introgression of the yellow seed color genes of *B. rapa* var. 'yellow sarson' into the A and C genome of *B. napus*.

Crosses were made between the two yellow-seeded lines (YN01-429 and CH5034/06) and F_1 seeds were harvested. The F_1 plants were subjected to microspore culture for production of DH lines; and self-pollinated for F_2 seeds, and backcrossed to both parents for backcross seeds.

3.2.2 Doubled haploid (DH) production: Microspore culture protocol, as described by Coventry et al. (1988), was applied for production of DH lines. Approximately 20-25 buds of 3-4 mm size were used for isolation of microspores. Buds of this size generally contain microspore at uninucleate stage. Buds were surface sterilized in calcium hypochlorite solution for 15 min and washed 3-4 times with distilled water to remove the traces of calcium hypochlorite. Buds were crushed in 1 ml liquid B5 medium (Gamborg et al. 1968) containing 13% sucrose, poured through two layers of nested sterile filters (Nytex 63cμM top and 44 μM bottom), making final volume of 2 ml of microspore suspension with additional B5 medium. The suspension was centrifuged, the supernatant was decanted and the pellet was re-suspended in B5 medium containing 13% sucrose. The centrifugation was repeated 3-4 times to remove chlorophyll from the suspension. Microspore suspension was cultured in 25 ml liquid NLN-13 medium (Lichter 1982) containing colchicine (50 mg per liter) in the medium, and placed in an

incubator at 30ºC for 24 hours. Colchicine disrupts the spindle apparatus in mitosis, and thus doubles chromosome number of the haploid cells. After 24 hours, suspension was transferred to a falcon tube and centrifuged at 2000 rpm for 5 min. Supernatant was decanted and 25 ml of NLN-13 medium (without colchicine) was added to the microspores in petridish, and the culture was kept at 30ºC for 14 days.

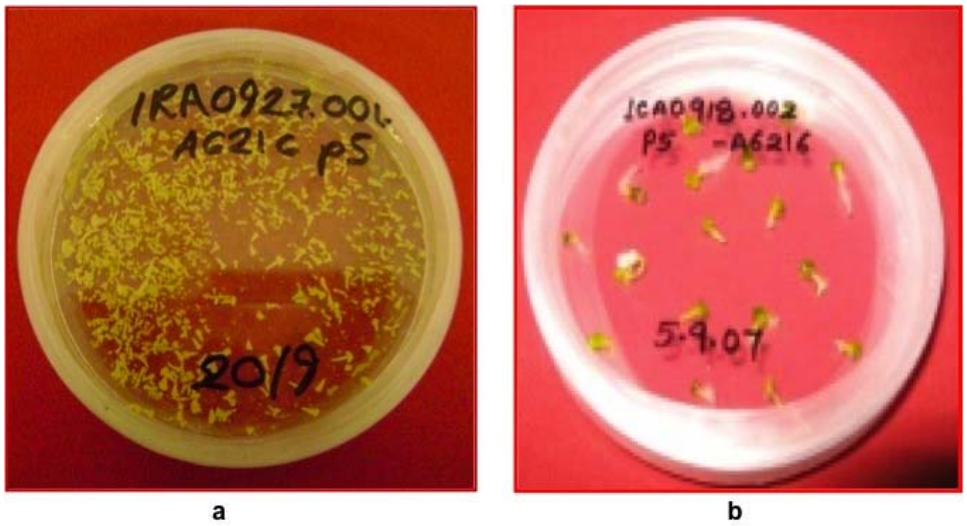


Fig 3.1: (a) Microspore culture in liquid medium resulted embryos, (b) embryos plated on solid B5 medium for seedling development of F_1 of YN01-429 x CH5034/06 cross of *Brassica napus*

After 14 days, the plates were transferred on a shaker to provide enough air to the developing of embryos. The torpedo-shaped embryos were transferred to solid B5 medium (liquid B5 with 20% sucrose and 2 g agar) at the rate of 20 embryos per plate (Fig 3.1b). The plates were kept at 4ºC for 10 days, and then were transferred to room temperature under light for embryos germination and plantlet development (Fig 3.2). Seedlings with well-developed roots were transferred to soil free growth media in a greenhouse and covered with transparent

cups for about a week to maintain high humidity, and then allowed to grow into mature plants (Fig 3.3).

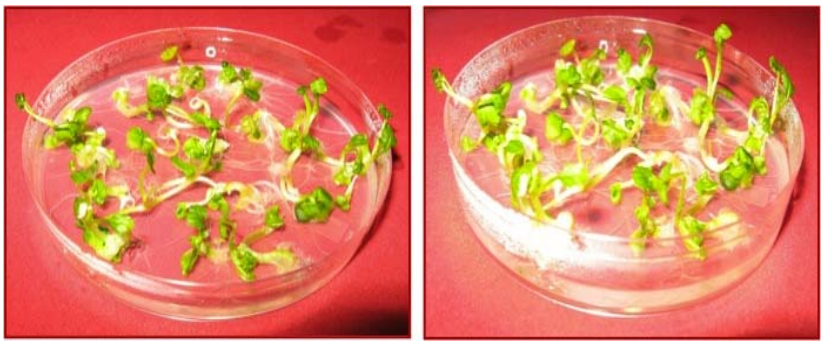


Fig 3.2: Microspore derived seedlings of *Brassica napus* growing on solid NN-13 medium and developing root system

Fig 3.3: Microspore derived seedlings of *Brassica napus* transferred to soil medium in greenhouse

At flowering, fertility of the plants was checked regularly. The pollen-producing plants were considered to be doubled haploids, and the sterile plants with smaller size flower/petal and lacking pollen were considered as haploids. The haploid plants without pollen were treated with 0.35% aqueous colchicine solution for doubling of chromosomes (Fig 3.4). For this, roots were washed properly and placed in colchicine solution for two hours. The roots of the treated

plants were washed thoroughly, shoots were cut off leaving few auxiliary buds (also called as lateral buds) and plants were transplanted to soil free growth medium in pots (Fig. 3.5). Fertile plants with pollen grains were self-pollinated under bag isolation, and harvested as doubled haploids.

Fig 3.4: (a) A microspore derived doubled haploid plant of YN01-429 x CH5034/06 cross of *Brassica napus* with pollen grains and (b) a sterile haploid plant without any pollen grain

Fig 3.5: (a) A microspore-derived plant of YN01-429 x CH5034/06 cross of *Brassica napus* after colchicine treatment, and (b) a plant with developing shoot after colchicine treatment

3.2.3 Production of F_2 and backcross seeds: F_1 plants were self-pollinated by bag isolation for F_2 seeds. The F_1 plants were also backcrossed to the parents for backcross seeds.

3.2.4 Field trials: The DH lines together with their parents and F_1 were grown in two replication field trials in 2008 and 2010 at Edmonton Research station, University of Alberta, Edmonton, Canada. Plot size was two-meter long and single row. The two parents YN01-429 and CH5034/06 were seeded every 20 rows and used as control. Two to three plants of each DH line and their parents and F_1's were self-pollinated by bag isolation. Plants were harvested at maturity, and seed color was recorded on both self- and open-pollinated seeds. The F_2 and backcross populations were grown in field plots in 2010, and the plants were self-pollinated. The self-pollinated seeds were harvested at maturity and seed color was recorded. Crop management, e.g. fertilizer application, pest control etc., were done as recommended for the location.

3.2.5 Seed color score: A panel of two people performed seed color grouping, and eleven seed color classes/scales were established (Table 3.1, Fig 3.6). The 'Class 1' composed of seeds with brighter yellow color than the two parents.

Table 3.1: Classification of *Brassica napus* plants for seed color in respect of proportion of different color seeds

Seed color class/scale	Percent seeds for color				
	Yellow	Yellow-brown	Brown	Red	Black
1	≅100	-	-	-	-
2	80-90	10-20	-	-	-
3	70-80	20-30	-	-	-
4	60-70	30-40	-	-	-
5	0-5	60-70	20-25	-	-
6	>1	40-50	50-60	-	-
7	-	5-10	60-70	5-10	5-10
8	-	-	10-20	80-90	-
9	-	-	40-50	20-30	30-40
10	-	-	-	40-50	40-50
11	-	-	-	-	100

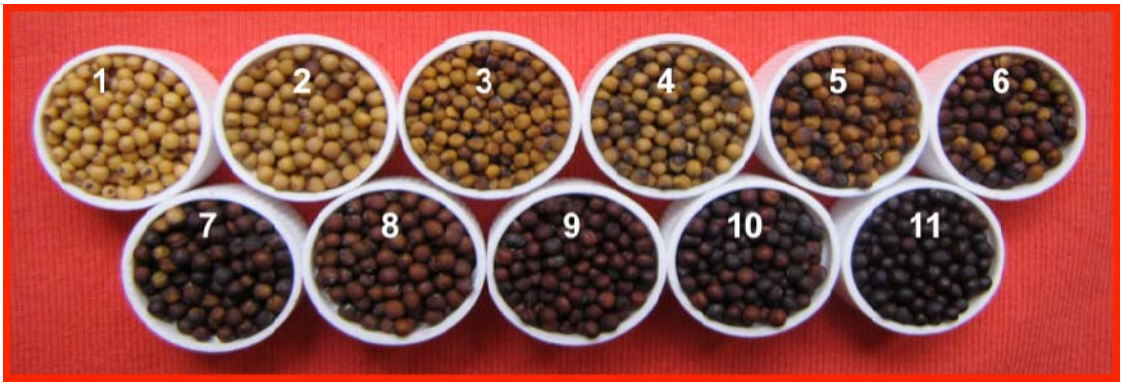


Fig 3.6: Seed color classes/scales of the doubled haploid lines and pedigree populations derived from YN01-429 x CH5034/06 cross of *Brassica napus*

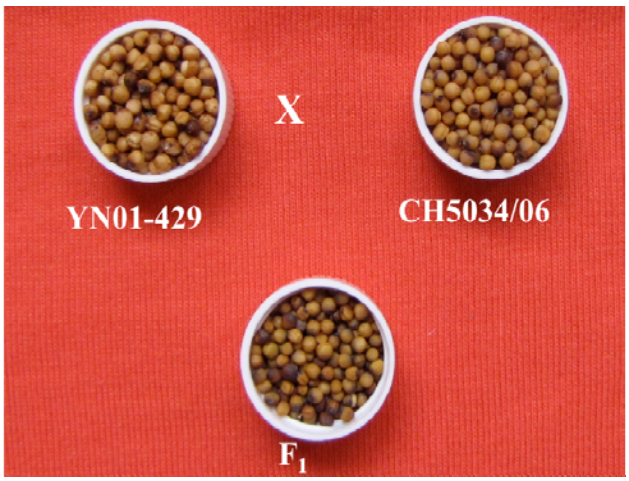


Fig 3.7: Seed color of the two yellow-seeded parents, and seed harvested from F_1 plants of YN01-429 x CH5034/06 cross of *Brassica napus*

3.2.6 Near infra-red spectroscopy (NIRS) analysis: Seeds harvested from 2008 and 2010 field trial were analysed for oil, protein and glucosinolate contents using near-infrared spectroscopy (NIRS) (Model 6500, Foss North America, Eden Prairie, MN) following the protocol approved by the Canadian Grain Commission (Daun, 1994). Oil and protein content were expressed as percent of whole seed dry weight basis, and glucosinolate content on whole-seed basis and expressed as µmol/g seed. Open-pollinated and self-pollinated seeds were analysed separately.

Some of the DH lines tested in 2008 field trial were analysed for oil, meal protein and fibre content at the University of Manitoba, in a collaborative research with Dr. Bogdan Slominski, following the method described by Slominski et al. (1994).

3.2.7 Statistical analysis: Statistical software SAS 9.2 (English) version was used for various statistical analyses. Analysis of variance (ANOVA) were performed to study the effect of replication and year on seed color. Genotypes x environmental

interactions were analysed using replication and year as fixed variables. Frequency distribution graphs were generated based on Least square Means using PROC GLM (SAS Institute, 2003).

Analysis of variance: ANOVA was calculated using PROC GLM procedure of SAS (SAS Institute, 2003) with the following models:

Simple linear model: This model is used for analysis of data, where two replications were analysed in two years.

$$Yijk = \mu + gi + ek + rj + geik + \varepsilon ijk$$

Where,

Y*ijk* = observation of genotype *i* in year *k* in replication *j*

μ = the general mean

g*i* = the effect of genotype *i*

ek = the effect of year (environment) *k*

rj = the effect of replication *j*

ge*ik* = the genotype × year interaction of genotype *i* in environment *k*

ε*ikj* = the residual error of genotype *i* in environment *k* in replication *j*

Levene's test for homogeneity was done to check if variances are equal. The null hypothesis was variances over the years, 2008 and 2010, are equal; while alternate hypothesis was variances are not equal across two years.

H_0: $\sigma^2_{2008} = \sigma^2_{2010}$

H_1: $\sigma^2_{2008} \neq \sigma^2_{2010}$

Spearman correlation coefficients were calculated using least-squares means by PROC CORR procedure of SAS (SAS Institute, 2003) to estimate relation between seed color, seed oil, protein and fibre content.

3.3 Results

3.3.1 Doubled haploid (DH) production

Microspore culture technique was applied on F_1 plants of YN01-429 x CH5034/06 cross for production of DH lines. About 550 embryos were transferred to solid media, of which 337 germinated and produced roots. These 337 seedlings were transferred into pots of which 148 produced viable pollen and deemed to be doubled haploids. The remaining 189 plants were treated with colchicine from where 61 DH were obtained. Thus, a total of 209 DH lines were harvested from this cross (Table 3.2).

Table 3.2: Number doubled haploids produced from F_1 of YN01-429 x CH5034/06 of *Brassica napus* cross

No. of embryos		No. of spontaneous DHs	% spontaneous	No. of plants treated with colchicine	No. induced DH	% induced	Total no. DH harvested
Solid media	No. of seedling to soil						
565	337	148	43.9	189	61	32.3	209

3.3.2 Seed color of the parents and F_1's

Seed color of the parent YN01-429 ranged from 3.0-4.0 and CH5034/06 ranged from 2.0-3.0 under greenhouse and field conditions. F_1 seed color ranged from 2.0-3.0, i.e. were similar or intermediate to the parents (Fig. 3.7, Table 3.3).

Table 3.3: Seed color of the parents and F_1 of *Brassica napus* in field trials in 2008 and 2010, and in greenhouse (GH) in 2009. For detailed description of seed color class/scale, see Table 3.1 and Fig. 3.6.

Year/pollination control	Parents and F_1	N	Range	Mean ± S.E.	Confidence limit
2008 Open-pollinated	YN01-429	10	3.0-4.0	3.5 ± 0.17	3.16-3.84
	CH5034	10	2.0-3.0	2.4 ± 0.16	1.91-2.89
	F_1	10	2.0-4.0	2.8 ± 0.25	2.16-3.44
2008 Self-pollinated	YN01-429	10	3.0-4.0	3.7 ± 0.15	3.40-4.00
	CH5034	10	2.0-4.0	2.6 ± 0.22	1.99-3.21
	F_1	10	2.0-3.0	2.5 ± 0.17	2.02-2.98
2009 GH Self-pollinated	YN01-429	5	3.0-4.0	3.2 ± 0.20	2.81-3.59
	CH5034	5	2.0-3.0	2.4 ± 0.24	1.77-3.03
	F_1	5	2.0-3.0	2.6 ± 0.24	2.02-3.18
2010 Open-pollinated	YN01-429	10	3.0-4.0	3.5 ± 0.17	3.16-3.84
	CH5034	10	2.0-3.0	2.6 ± 0.16	2.15-3.15
	F_1	10	2.0-4.0	2.9 ± 0.23	2.32-3.48
2010 Self-pollinated	YN01-429	10	3.0-4.0	3.4 ± 0.16	3.06-3.74
	CH5034	10	2.0-3.0	2.5 ± 0.17	2.02-2.98
	F_1	10	2.0-3.0	2.6 ± 0.16	2.15-3.05

3.3.3 Self-pollinated seed color of the doubled haploid lines

2008 and 2010 field trial

A total of 175 and 209 DH lines were tested in field trials, of which data from 98 and 140 lines respectively of 2008 and 2010 trial were included in statistical and frequency distribution analysis. However, 92 DH lines were common in these two years trials, and data of these lines were used for homogeneity analysis and analysis of variance. Levene's test for homogeneity showed that variance for seed color is equal over the two years, 2008 and 2010. Welch's ANOVA showed p-value of 0.69 for years 2008 and 2010, thus not rejecting the null hypothesis and variance analysis was done using two years combined data.

ANOVA for seed color showed that significant difference existed among the DH lines (Table 3.4). The interaction between genotypes (DH) and year is found to be significant, suggesting that environments significantly affected seed color of the DH lines. Replication showed non-significant effect on DH lines for seed color and year has significant effect on this trait. Genotype x environment effect is found to be significant in this data (Table 3.4).

Table 3.4: ANOVA for self-pollinated seeds of the doubled haploid lines of YN01-429 x CH5034/06 cross of *Brassica napus* tested in 2008 and 2010 field trials

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DH	91	1292.889842	14.207581	43.91	<0.0001
Year	1	1.331654	1.331654	4.12	0.0458
Rep	1	0.999024	0.999024	3.09	0.0827
DH*year	91	50.011115	0.549573	1.70	0.0079

Frequency distribution of the DH lines for seed color is presented in Fig. 3.8. Of the 98 DH lines tested in 2008, two lines (2.0%) produced brighter yellow seeds than the parents (seed color 'Class 1'); while, seed color in 96 lines ranged from parental type to black (Fig 3.8a). In 2010, out of 141 DH lines, four (2.8%) produced brighter yellow seeds than the parents. Seed color in the remaining 137 lines ranged from parental type to dark brown/black (Fig 3.8b). In both years, a continuous distribution for seed color was found. In 2008, highest number of DH lines was included in 'Class 6', which is intermediate color between yellow-brown and brown seed color class. In 2010, the distribution skewed towards the black/brown seed color with increased number of DH lines falling into brown seed color class. However, none of the DH lines had black seed color, i.e, seed color 'Class 11'. Frequency distribution of 2008 and 2010 combined data followed normal distribution; with highest number of lines falling into seed color 'Class 5'. Combined data analysis of the DH lines tested in both 2008 and 2010

showed one (1.08%) DH line having brighter yellow seed color than the parents (Fig. 3.8).

2009 greenhouse trial

Ninety-two DH lines were grown in greenhouse in 2009, to study seed color under controlled environmental conditions. Frequency distribution of the DH lines for seed color is presented in Fig. 3.8d. Almost a trimodal distribution is evident in this case, which is opposed to data from field trials. None of the DH lines produced black seed color, i.e., seed color 'Class 10' and 'Class 11'. Thus, increased number of DH lines in seed color 'Class 1' compared to field trial and absence of any line with seed color 'Class 10' and 'Class 11' indicate that environmental condition can significantly alter seed color in *B. napus*.

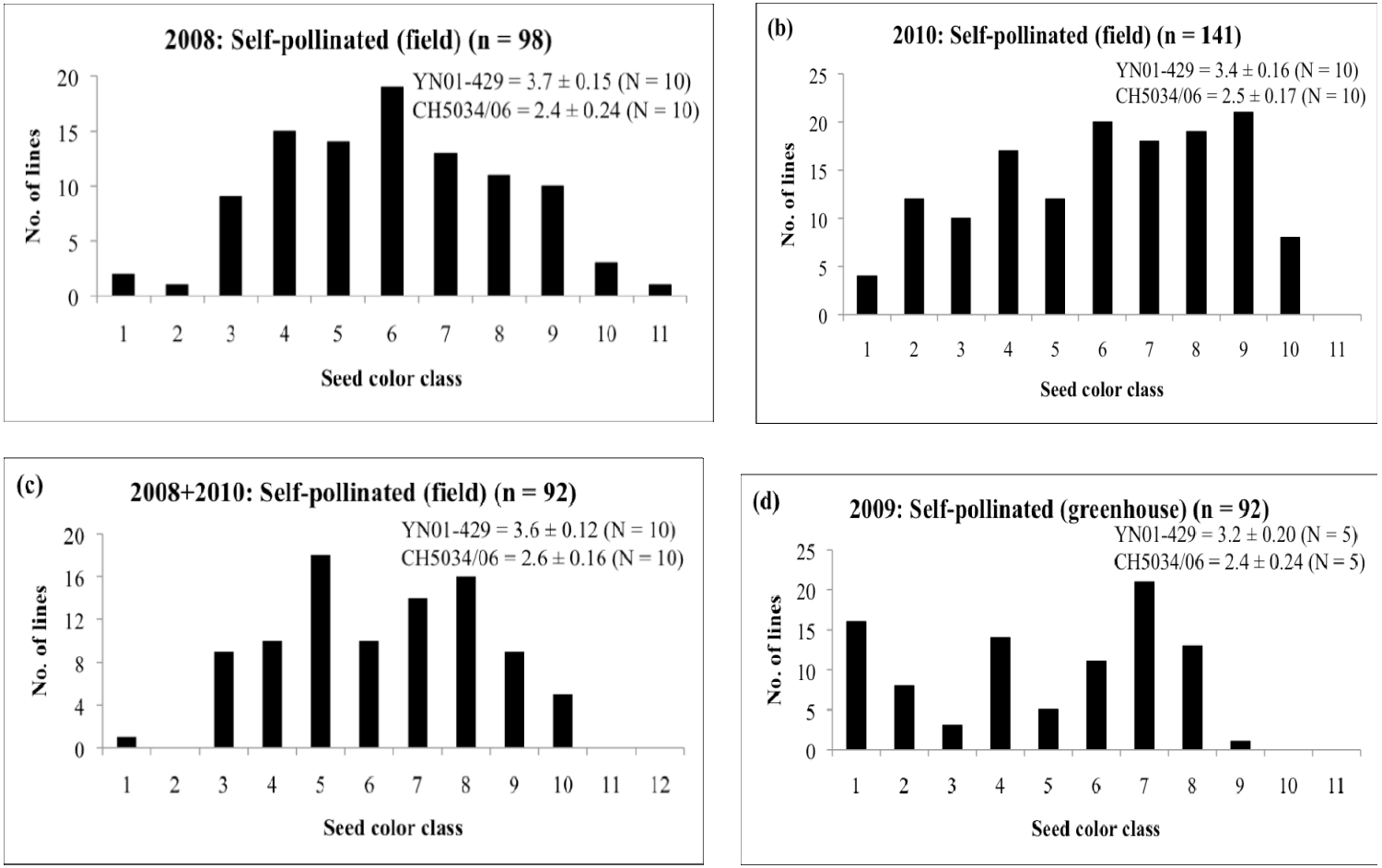


Fig 3.8: Frequency distribution of the doubled haploid lines of YN01-429 x CH5034/06 cross of *Brassica napus* for self- and open-pollinated seed color in 2008 and 2010 field trials and 2009 greenhouse trial . Parental values are also given in the Figures.

3.3.4 Open-pollinated seed color of the doubled haploid lines

2008 and 2010 field trials

A total of 175 and 209 DH lines were tested in field trials, of which data from 98 and 140 lines respectively of 2008 and 2010 trial were included in statistical and frequency distribution analysis. However, 101 DH lines were common in these two years trials, and data of these lines were used for homogeneity analysis and analysis of variance. Levene's test for homogeneity showed that variance for seed color is equal over the two years, 2008 and 2010. Welch's ANOVA showed p-value of 0.67 for years 2008 and 2010, thus not rejecting the null hypothesis and variance analysis was done using two years combined data.

ANOVA for seed color showed that significant difference existed among the DH lines (Table 3.5). The interaction between genotypes (DH) and years is found to be significant, suggesting that environments significantly affected seed color of the DH lines. Replication showed non-significant effect on DH lines for seed color and year has significant effect on this trait. Genotype x environment effect is found to be significant in this data (Table 3.5).

Table 3.5: ANOVA for open-pollinated seeds of the doubled haploid lines of YN01-429 x CH5034/06 cross of *Brassica napus* tested in 2008 and 2010 field trials

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DH	100	2167.575499	21.675755	15.98	<0.0001
year	1	5.296359	5.296359	3.90	0.0489
rep	1	3.374270	3.374270	2.49	0.1156
DH*year	95	206.513925	2.173831	1.60	0.0011

Frequency distribution of the DH lines for open-pollinated seed color is presented in Fig 3.9. Distribution was nearly normal in 2008; while in 2010, a bimodal distribution is more evident. Of the 117 lines scored in 2008, three lines (2.6%) had brighter yellow color seeds than the parents; while in 2010, of the 142 lines, two (1.4%) were brighter than the parents. Frequency distribution of combined data from 2008 and 2010 trials however showed almost normal distribution. Of the 101 DH lines tested in both 2008 and 2010, none of the lines produced brighter yellow seeds than the parents. This is due to the reason that the DH line showed brighter yellow seed color in one year was not holding true in the other year – probably due to 'xenia effect' of pollen.

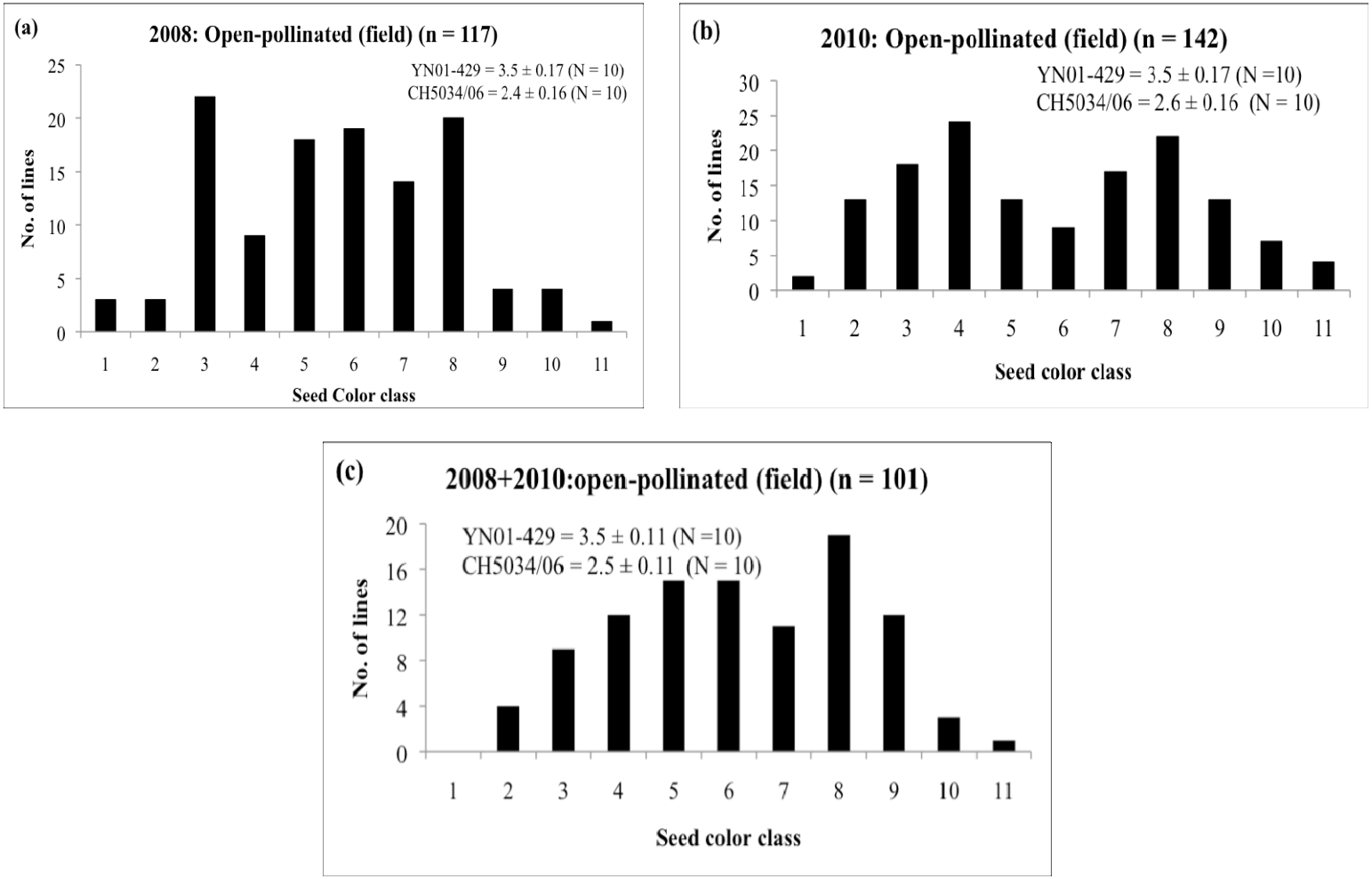
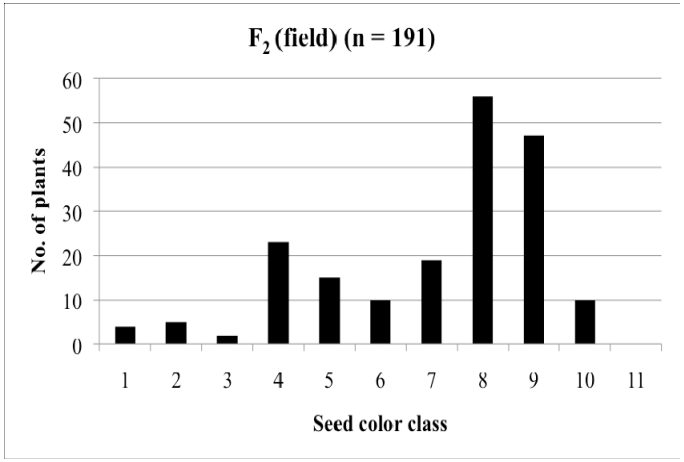
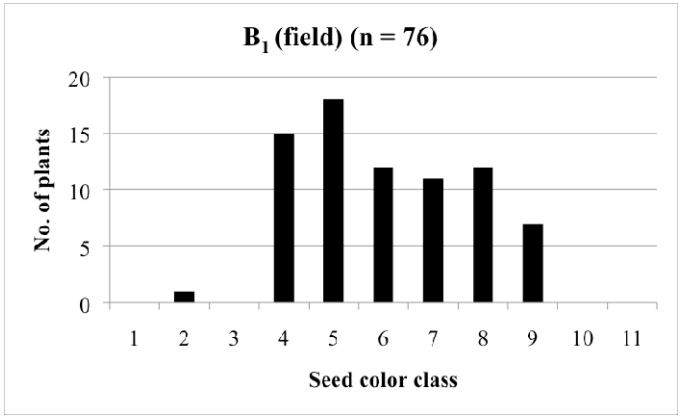


Fig 3.9: Frequency distribution of the doubled haploid lines YN01-429 x CH5034/06 cross of *Brassica napus* for open-pollinated seed color in 2008 and 2010 field trials. Parental values are also given in the Figures.

3.3.5 Seed color of the pedigree populations

Frequency distribution of F_2, B_1 and B_2 populations for seed color is presented in Fig. 3.10. Of the 191 F_2 plants evaluated for seed color, 4 (2%) plants had brighter yellow seed color than the two parents, i.e. transgressive segregants. Similarly, plants with brighter seed color than the parents were also found in B_2 population, i.e. F_1 x CH5034/06, at a frequency of 2.1% (2 out of total 94); however, this was not observed in case of B_1 population, i.e. F_1 x YN01-429.





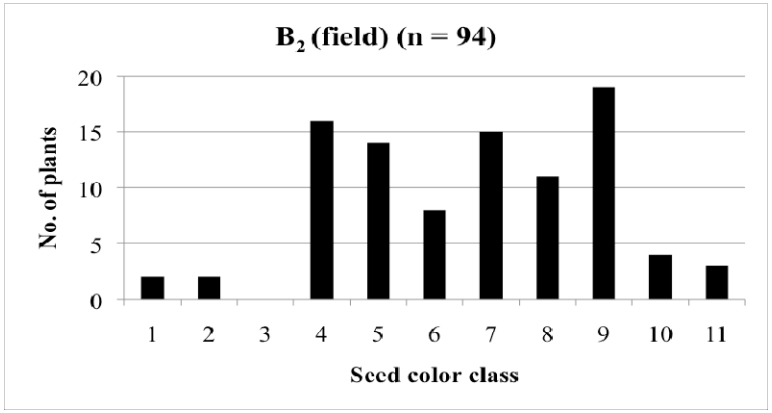


Fig 3.10: Frequency distribution of F_2 (YN01-429 x CH5034/06), B_1 [(YN01-429 x CH5034/06) x YN01-429] and B_2 [(YN01-429 x CH5034) x CH5034/06] plants of *Brassica napus* crosses from field in 2010.
Seed color of the parents: YN01-429 = 3.6 ± 0.12 (N = 10); CH5034/06 = 2.6 ± 0.16 (N = 10)

3.3.6 Genetic study of seed color

Seed color segregation in DH population

Seed color data of both self- and open-pollinated seeds of the DH lines from 2008 and 2010 field trials and 2009 greenhouse trial was subjected to χ^2 test for fit to 1:1, 1:3, 1:7, 1:15, 1:31 and 1:63 ratio (Table 3.6). For this, DH lines with seed color brighter than the parents (Class 1) was considered as one group, and the DH lines of all other seed color classes (Class 2-11) were included in the second group. Seed color data for 2008 field trial showed that segregation of the DH lines fitted to 1:15, 1:31 and 1:63 ratio, for both self- and open-pollinated seeds, suggesting involvement of four to six gene loci in the control of this trait. Similar results were also found in case of 2010 field trial data; though, in both years, better fit of data was found for five to six gene loci model. However, 2009 greenhouse data showed good fit to 1:3 and 1:7 ratio indicating only two to three gene loci determined seed color variation under greenhouse conditions.

Seed color segregation in pedigree populations

Self-pollinated seeds of 191 F_2 plants were evaluated for seed color, where four produced brighter yellow seeds (Class 1) than the parents. This 4:187 distribution fit to 1:63 ratio (χ^2 = 0.4, p = 0.5-0.7), suggesting involvement of three gene loci in the control of seed color. In B_2 population, i.e. (YN01-429 x CH5034/06) x CH5034/06, observed segregation fit to 1:15 and 1:31 ratios (Table 3.7).

Table 3.6: Segregation in a doubled haploid (DH) population of YN01-429 x CH5034/06 cross of *Brassica napus* for seed color (yellow vs. all other color) under field and green house conditions in 2008, 2009 and 2010

Growth condition	Pollination[a]	No. of DH lines	Yellow DH[b]	Other seed colors DH[c]	Segregation[d]	χ^2	p-value
Field 2008	SP	98	2	96	1:1	90.16	< 0.05
					1:3	27.55	< 0.05
					1:7	9.80	< 0.05
					1:15	2.96	0.05-0.1
					1:31	0.38	0.1-0.5
					1:63	0.15	0.7-0.9
Field 2008	OP	117	3	114	1:1	105.31	< 0.05
					1:3	31.41	< 0.05
					1:7	10.56	< 0.05
					1:15	2.72	0.1-0.5
					1:31	0.14	0.7-0.9
					1:63	0.76	0.7-0.9
GH 2009	SP	92	16	76	1:1	39.13	< 0.05
					1:3	2.84	0.05-0.1
					1:7	2.01	0.1-0.5
					1:15	19.49	< 0.05
					1:31	61.70	< 0.05
					1:63	142.49	< 0.05

Continued..

Growth condition	Pollination[a]	No. of DH lines	Yellow DH[b]	Other seed colors DH[c]	Segregation[d]	χ^2	p-value
Field 2010	SP	141	4	137	1:1	125.45	< 0.05
					1:3	36.94	< 0.05
					1:7	12.04	< 0.05
					1:15	2.81	0.05-0.1
					1:31	0.04	0.99
					1:63	1.50	0.1-0.5
Field 2010	OP	142	2	140	1:1	134.11	< 0.05
					1:3	42.15	< 0.05
					1:7	15.97	< 0.05
					1:15	5.69	< 0.05
					1:31	1.43	0.1-0.5
					1:63	0.02	0.99

[a]SP = self-pollinated seeds; OP = open-pollinated seeds
[b]DH lines with seed color brighter than the parents included in this group
[c]all other DH lines, i.e. parental color to black, included in this group
[d]segregation for one to six gene loci

Table 3.7: Segregation in F_2 (YN01-429 x CH5034/06) and B_2 [(YN01-429 x CH5034) x CH5034/06] populations of *Brassica napus* crosses under field condition in 2010

Growth condition	Pollination[a]	No. of DH lines	Yellow DH[b]	Other seed colors DH[c]	Segregation[d]	χ^2	p-value
Field 2010	SP	191	4	187	1:3	53.94	< 0.05
					1:15	5.60	< 0.05
					1:63	0.42	0.5-0.7
					1:255	14.42	< 0.05
					1:1023	81.14	< 0.05
Field 2010	SP	94	2	92	1:1	86.17	< 0.05
					1:3	26.23	< 0.05
					1:7	9.25	< 0.05
					1:15	2.65	0.1-0.5
					1:31	0.30	0.5-0.7

[a]SP = self-pollinated seeds
[b]DH lines with seed color brighter than the parents included in this group
[c]all other DH lines, i.e. parental color to black, included in this group
[d]segregation for one to five gene loci

Thus, seed color segregation in DH and pedigree populations suggests that three to six gene loci might be involved in determining seed color in this cross. Environment also played a role in the expression of seed color genes, as evident from the difference in seed color segregation under field and greenhouse condition.

3.3.7 Chemical analysis of seed components

Open-pollinated and self-pollinated seeds harvested from field trials were subjected to near infra-red spectroscopy (NIRS) analysis for oil, protein, glucosinolate and fibre contents.

Oil content

Oil content of the two yellow-seeded parents, YN01-429 and CH5034/06, presented in Table 3.8. Compared to CH5034/06, oil content in YN01-429 was higher in both 2008 and 2010 trials, except self-pollinated seeds harvested in 2010. For example, in 2008, self- and open-pollinated seeds of YN01-429 had 48.2% and 49.2% oil, respectively; while in the same year, self- and open-pollinated seeds of CH5034/06 had 47.8% and 48.8% oil, respectively. On the average of two years, oil content in self- and open-pollinated seeds of YN01-429 was 46.3% and 48.9%, respectively; while for CH5034/06 it was 46.9% and 48.3%, respectively. Oil content was generally higher under open-pollination condition − apparently due to influence of environmental conditions under which seeds were developed and matured.

Frequency distribution of the DH population for oil content is presented in Fig. 3.11. The distribution of the DH lines for oil content in self- and open-pollinated seeds was continuous. Confidence limit of the high parent YN01-429 for oil content was: 2008 self-pollinated seed = 48.1-48.2%; 2008 open-pollinated seed = 49.0-49.4%; 2010 self-pollinated seed = 44.0-44.6%; 2010 open-pollinated seed = 48.4-48.7%. In 2008, self-pollinated seeds of 21/97 (21.6%) DH lines and

open-pollinated seeds of 43/97 (44.3%) DH lines had higher oil content than the high parent. In 2010, self-pollinated seed of 23/136 (16.9%) DH lines and open-pollinated seeds of 84/136 (61.8%) DH lines had higher oil content than YN01-429. Thus, a transgressive segregation for higher oil content is evident in this DH population.

Table 3.8: Range, mean and confidence limit of the yellow-seeded *Brassica napus* parents YN01-429 and CH5034/06 for oil content

Year	Pollination[a]	Parent	N	Range	Mean ± S.E.	Confidence limit
2008	OP	YN01-429	3	46.7-50.5	49.18 ± 1.24	49.0-49.4
		CH5034/06	2	48.7-48.9	48.78 ± 0.08	48.7-48.8
2008	SP	YN01-429	3	48.0-48.9	48.19 ± 0.13	48.1-48.2
		CH5034/06	2	47.7-47.9	47.77 ± 0.09	47.7-47.8
2010	OP	YN01-429	5	45.3-51.7	48.54 ± 1.07	48.4-48.7
		CH5034/06	3	45.5-49.5	47.87 ± 1.22	47.7-48.1
2010	SP	YN01-429	5	37.8-49.2	44.32 ± 2.01	44.0-44.6
		CH5034/06	3	43.3-49.2	45.98 ± 1.74	45.7-46.3

[a]SP = self-pollinated seeds; OP = open-pollinated seeds

Protein content

Protein content data of the yellow-seeded parents, YN01-429 and CH5034/06, is presented in Table 3.9. It was found that protein content of

CH5034/06 was higher in both 2008 and 2010 trials than YN01-429. For example, in 2008, self- and open-pollinated seeds of CH5034/06 had 25.7% and 25.8% protein, respectively; while in the same year, self- and open-pollinated seeds of YN01-429 had 25.1% and 24.1% protein, respectively. On the average of two years, protein content in self- and open-pollinated seeds of CH5034/06 was 26.4% and 25.5%, respectively; while for YN01-429, it was 26.1% and 23.9%, respectively.

Frequency distribution of the DH lines for protein conent is presented in Fig. 3.11. The distribution of the DH lines for protein content in self- and open-pollinated seeds was continuous. Confidence limit of the high parent CH5034/06 for protein content was: 2008 self-pollinated seed = 25.6-25.9%; 2008 open-pollinated seed = 25.6-25.9%; 2010 self-pollinated seed = 26.6-27.6%; 2010 open-pollinated seed 24.8-25.7%. In 2008, self-pollinated seeds of 74/97 (76.2%) DH lines and open-pollinated seeds of 53/97 (54.6%) DH lines had higher protein content than the high parent CH5034/06. In 2010, self-pollinated seed of 108/136 (79.4%) DH line and open-pollinated seeds of 44/136 (32.3%) DH lines had higher protein content than the high parent. Thus, a transgressive segregation for protein content is evident in this DH population.

Table 3.9: Range, mean and confidence limit of the yellow-seeded *Brassica napus* parents YN01-429 and CH5034/06 for protein content

Year	Pollination[a]	Parent	N	Range	Mean ± S.E.	Confidence limit
2008	OP	YN01-429	3	23.0-25.3	24.13 ± 1.01	23.8-24.4
		CH5034/06	2	25.5-26.0	25.77 ± 0.20	25.6-25.9
2008	SP	YN01-429	3	24.8-25.3	25.11 ± 0.14	25.1-25.2
		CH5034/06	2	25.5-26.0	25.74 ± 0.25	25.6-25.9
2010	OP	YN01-429	5	19.7-28.7	23.68 ± 1.48	23.3-24.1
		CH5034/06	3	23.6-28.1	25.25 ± 1.42	24.8-25.7
2010	SP	YN01-429	5	22.9-31.5	27.18 ± 1.73	26.8-27.6
		CH5034/06	3	23.9-29.3	27.10 ± 1.65	26.6-27.6

[a]SP = self-pollinated seeds; OP = open-pollinated seeds

Glucosinolate content

Glucosinolate content (μmol/g) data of the yellow-seeded parents, YN01-429 and CH5034/06, is presented in Table 3.10. It appears that glucosinolate content of YN01-429 was significantly lower in both 2008 and 2010 trials than CH5034/06. For example, in 2008, self- and open-pollinated seeds of YN01-429 had glucosinolate content of 15.5 and 15.7 μmol/g seed, respectively; while in the same year, self- and open-pollinated seeds of CH5034/06 had 26.6 and 31.6 μmol/g seed, respectively. On average of two years glucosinolate content in self-

and open-pollinated seeds of YN01-429 was 15.4 and 15.1μmol/g, respectively; while for CH5034/06 it was 29.7 and 26.6 μmol/g, respectively.

Frequency distribution of the DH population for glucosinolate content is presented in Fig. 3.11. Confidence limit of the low parent YN01-429 for glucosinolate content was: 2008 self-pollinated seed = 15.4-16.0; 2008 open-pollinated seed = 14.8-16.2; 2010 self-pollinated seed = 14.5-15.5; 2010 open-pollinated seed = 14.2-15.1. In 2008, self-pollinated seeds of 4/97 (4.1%) DH lines and open-pollinated seeds of 1/97 (1.0%) DH lines had lower glucosinolate content than the low parent YN01-429. In 2010, none of the DH lines showed lower glucosinolate content than the low parent.

Table 3.10: Range, mean and confidence limit of the yellow-seeded *Brassica napus* parents YN01-429 and CH5034/06 for glucosinolate content (μmol/g)

Year	Pollination[a]	Parent	N	Range	Mean ± S.E.	Confidence limit
2008	OP	YN01-429	3	13.0-18.3	15.51 ± 1.52	14.8-16.2
		CH5034/06	2	25.9-27.2	26.60 ± 0.63	26.2-27.0
2008	SP	YN01-429	3	14.6-16.9	15.70 ± 0.66	15.4-16.0
		CH5034/06	2	30.6-32.5	31.57 ± 0.98	31.0-32.1
2010	OP	YN01-429	5	11.5-17.8	14.62 ± 1.02	14.2-15.1
		CH5034/06	3	24.6-28.5	26.63 ± 1.12	26.3-26.9
2010	SP	YN01-429	5	11.5-19.6	15.00 ±1.32	14.5-15.5
		CH5034/06	3	24.2-32.4	27.79 ± 2.41	27.1-28.4

[a]SP = self-pollinated seeds; OP = open-pollinated seeds

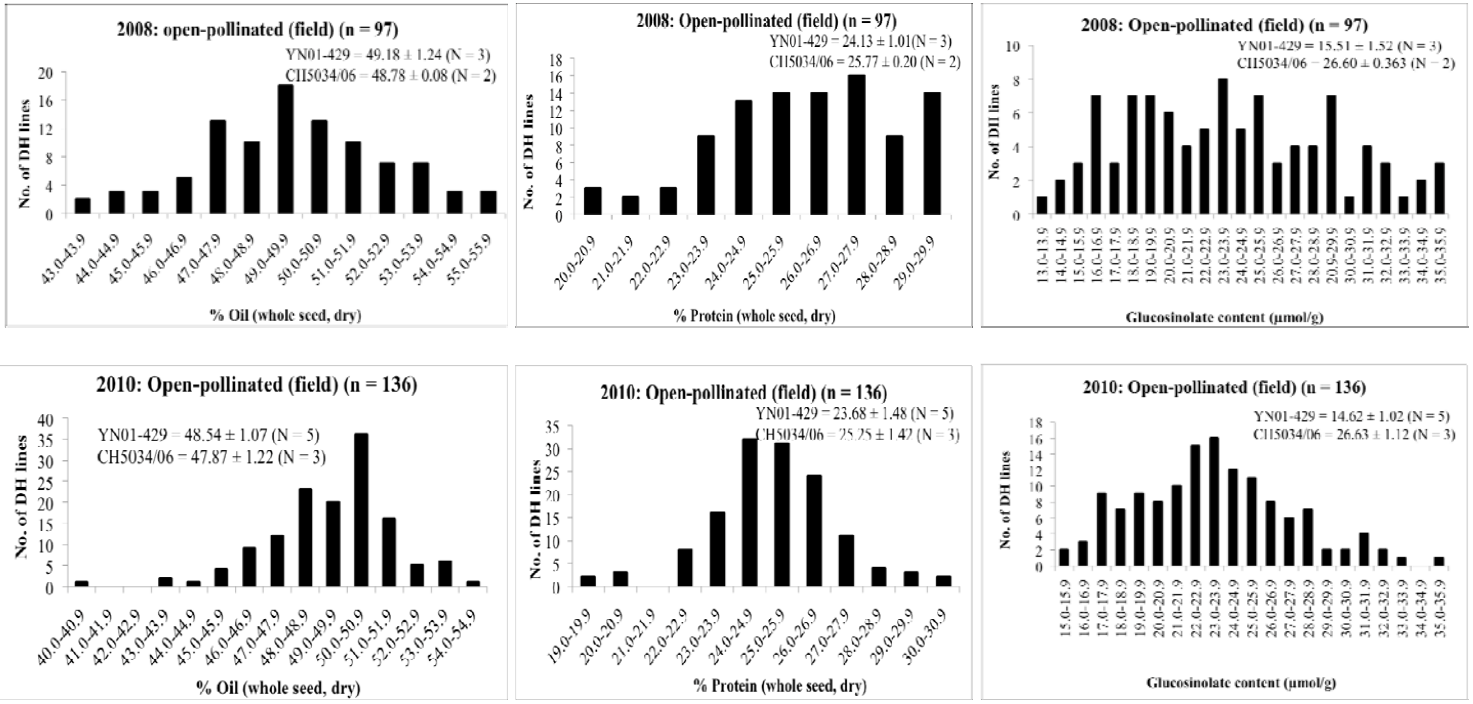


Fig 3.11: Frequency distribution of the doubled haploid lines of YN01-429 x CH5034/06 cross of *Brassica napus* for oil, protein and glucosinolate contents in open-pollinated seeds from 2008 and 2010 field trials. Parental values are also given in the Figures.

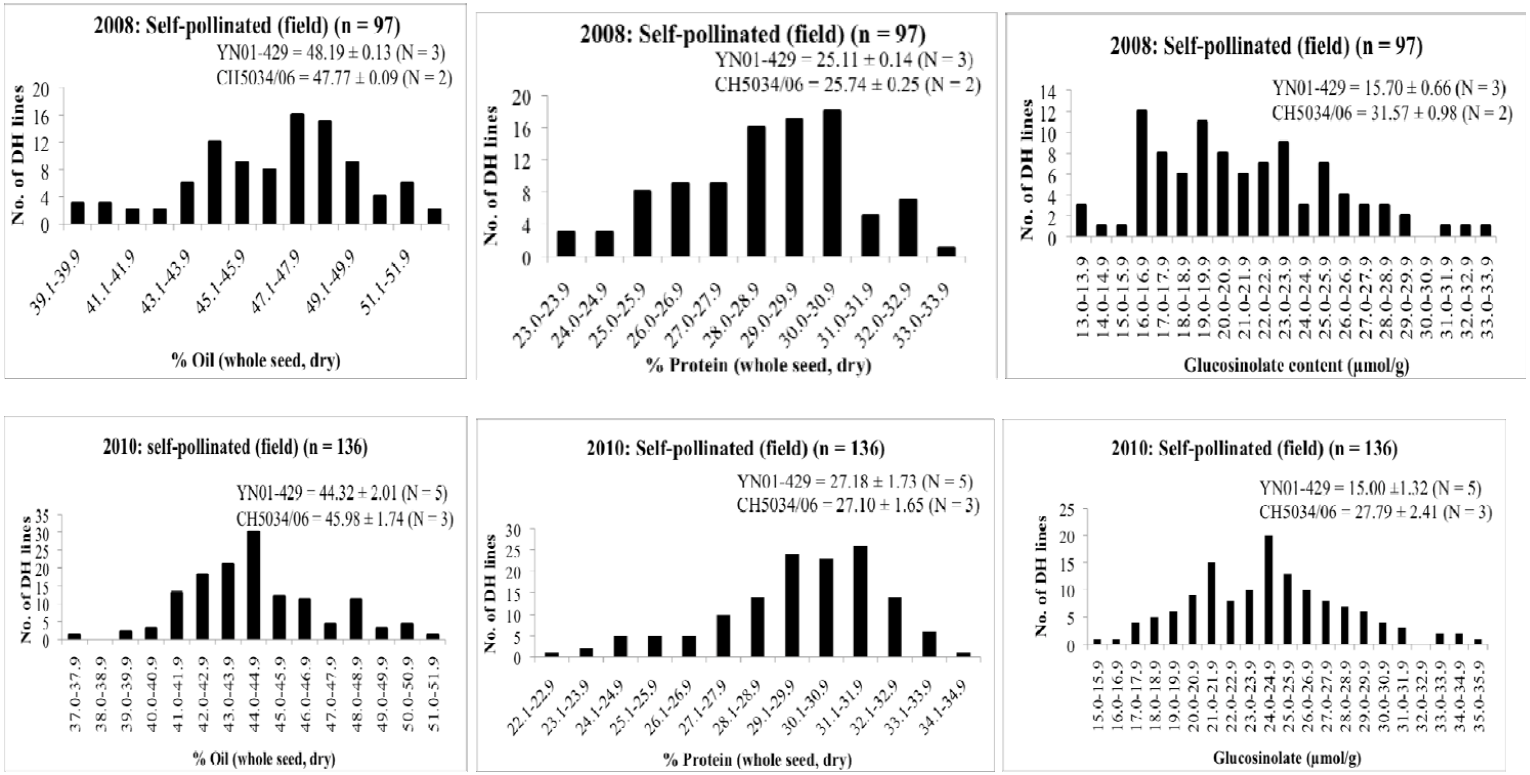


Fig 3.12: Frequency distribution of the doubled haploid lines of YN01-429 x CH5034/06 cross of *Brassica napus* for oil, protein and glucosinolate contents in self-pollinated seeds from 2008 and 2010 field trials. Parental values are also given in the Figures.

3.3.8 Correlation analysis between chemical traits and seed color

Seed color showed negative correlation with oil content in both open- and self-pollinated seeds, implying that darker the seed color lower the oil content in seed. On the other hand, seed color was positively correlated with neutral detergent fibre (NDF). This means that, dark seeds contein higher NDF compared to light color seeds. NDF on whole seed basis is positively correlated with NDF on fat free basis. Correlation between oil and protein content was negative (Table 3.11) – as normally occur in *Brassica* oilseeds.

Table 3.11: Pearson correlation coefficient between seed color, oil, protein and neutral detergent fibre (NDF) content in open-pollinated (OP) and self-pollinated (SP) seeds of the DH lines of YN01-429 x CH5034/06 cross of *Brassica napus* field tested in 2008 (OP, N = 62; SP, N = 41)

	Oil		Meal protein		NDF whole seed		NDF fat free	
	OP	SP	OP	SP	OP	SP	OP	SP
Seed color	-0.3*	-0.2	-0.2	-0.1	0.7***	0.7***	0.6***	0.7***
Oil			-0.6***	-0.4**	-0.3*	-0.3	0.1	-0.02
Meal protein					-0.1	-0.1	-0.3**	- 0.2
NDF whole seed							0.9***	1.0***

3.4 Discussion

The yellow-seeded *B. napus* line YN01-429 was developed by AAFC, Saskatoon, from interspecific crosses involving yellow-seeded *B. carinata,* yellow-seeded *B. rapa*, yellow-seeded *B. juncea* and black-seeded *B. napus* (Rakow et al. 2011). From the crossing design reported by the authors (Rashid et al. 1994, Rakow et al. 2011), it is difficult to infer which species contributed yellow seed color in YN01-429. The second yellow-seeded line CH5034/06 was developed through introgression of yellow seed color gene(s) of *B. rapa* var. 'yellow sarson' into the C-genome of *B. napus* (AAC^yC^y) followed by crossing of the line with a Resynthesized *B. napus* line carrying A^yA^yCC genome created from black-seeded *B. alboglabra* (C^BC^B) and 'yellow sarson' (A^yA^y) (Rahman 2001b). Progeny of this cross, i.e. $A^yA^yC^BC^B$ x AAC^yC^y yielded yellow-seeded *B. napus*, which genome composition in respect to seed color is expected to be $A^yA^yC^yC^y$. According to Rahman et al. (2008), introgression of the yellow seed color allele of 'yellow sarson' in this yellow-seeded *B. napus* occurred in C3 (≅ N13) of the C-genome. Thus, it is apparent that genetic control of yellow seed color in YN01-429 and CH5034/06 is very different. Occurrence of brown- to black-seeded lines, as well as lines with brighter yellow seed color than the parents in the DH popualtion of YN01-429 x CH5034/06 suggests that the genetic background of the two yellow-seeded parents are different in respect to seed color genes. Rahman (2001b) developed a trigenomic hexaploid (AABBCC) from *B. rapa* var 'yellow sarson' x yellow-seeded *B. carinata* crosses, and demonstrated that combining yellow seed color genes of these two species results brown seed.

Yellow-seeded *B. carinata* was involved in the parentage of YN01-429. Therefore, it can be assumed that YN01-429 carry the yellow seed color gene of *B. carinata*, and this might be the reason of occurrence of brown/black seeded lines in the DH population of YN01-429 x CH5034/06 cross. The DH lines with brighter yellow seed color than the parents apparently resulted from favourable combination of yellow seed color alleles from the two parents

Interspecific hybridization for the development of yellow-seeded *B. napus* has been attempted by various researchers, as no yellow-seeded forms are naturally available in *B. napus* (Chen et al. 1988 and Badani et al. 2006). But stability of yellow seed color has always been a major issue due to environmental influence on seed color. Seed color is affected by growth condition − especially temperature. High temperture results less pigmentation in seed coat and produces lighter color seed (Van Deynze et al. 1993). In this study, analysis of data from 2008 and 2010 shows significant effect of year on seed color. Furthermore, DH lines grown in greenhouse produced brighter color seeds than the plants grown in field trials. Temperature in greenhouse generally maintained between 20-25°C; however, in summer this can raise to about 30°C. The skewed distribution of the DH lines in greenhouse towards lighter color seed is apparently due to the effect of temperature on seed color. Thus, results from this study support the results of earlier observation of temperature influence on seed color in *Brassica*.

Genetic studies on seed color in *B. napus* have shown involvement of two or more genes in the control of this trait (Henderson and Pauls 1992, Baetzel et al. 1999, Rahman et al. 2001). In the present study, seed color variation in the DH

population was continuous. It was difficult to group the DH lines into distinct classes based on seed color. Therefore, χ^2-test was done including the DH lines of brighter yellow seed color than the parents (transgressive segregants) into one class and the remaining DH's into another class. The test indicated that three to six gene loci might be involved in the control of this trait in *B. napus*. However, greenhouse data showed two to three genes involved in the control of this trait – apparently due to effect of environment on the seed color genes.

In addition to seed color genes, the two parents were also found to differ for oil, protein and glucosinolate contents, and transgressive segregation also occurred for these traits in the DH population. Seed oil and protein contents in *B. napus* are quantitative traits, controlled by many genes (Burns et al. 2003, Zhao et al. 2005, Qiu et al. 2006), and the trait is affected by genotype x environment interactions (Zhao et al. 2005, Qiu et al. 2006). Seed glucosinolate content is controlled by at least four gene loci, where recessive condition in all loci result low glucosinolate (Rahman et al. 2001). Therefore, it is possible that positive and negative alleles for these seed quality traits are dispersed between the parents, and this might be reason of occurrence of transgressive segregation for these traits in the DH population of YN01-429 x CH5034/06 cross. The positive correlation between seed color and fibre content observed in the DH population implies that the darker the seed color, the greater the fibre content; and lighter color seed tend to have higher oil content. The results of the present study also agree with the results reported by Rahman et al. (2001) that yellow seed color in *B. napus* is associated with reduced fibre and higher oil content.

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Chapter 4

General Discussion and Conclusions

4.1 General discussion

Yellow-seed in Canola, pertaining to high oil, high protein and low fibre contents, is advantageous over brown/black seeds. The low fibre in yellow-seed meal is important for digestibility of this protein-rich meal in animal feed.

Attempts have been made by different breeders and researchers to develop yellow-seeded *Brassica napus* as yellow-seeded forms do not occur naturally in *B. napus*. Various interspecific crosses involving *B. alboglabra* and *B. rapa* (Chen et al. 1988), *B. carinata*, *B. napus* and *B. juncea* (Rashid et al. 1994), *B. rapa* var 'yellow sarson', *B. alboglabra* and *B. carinata* (Rahman 2001), *B. napus* and *B. rapa* ssp. *oleifera* accessions (Badani et al. 2006) have been performed to develop yellow-seeded *B. napus*. The genetic control of seed color in different *Brassica* species is different; therefore, genetic control of seed color in the yellow-seeded *B. napus* lined derived from these interspecific crosses expected to be different. In this study, two yellow-seeded *B. napus* lines, developed from two different design of interspecific crosses, were crossed and doubled haploid (DH) lines were produced from F_1, and these DH lines were studied to understand the genetic control of seed color and other seed quality traits, eg. oil, protein and glucosinolate content. Seed color in the DH population varied from bright yellow (brighter than the parents) to black. Similar transgressive segregation was observed for oil and other seed quality traits are very distinct in the two parental yellow-seeded *B. napus* lines. The yellow-seeded *B. napus* lines YN01-429 was

developed from complex interspecific crosses involving yellow-seeded *B. carinata*, *B. juncea* and *B. rapa* (Rakow et al. 2011), while the line CH5034/06 was developed from a yellow-seeded *B. napus* line carrying yellow seed color allele of 'yellow sarson' (*B. rapa*) in both A and C genomes (Rahman 2001). Therefore, it would be expected that the yellow-seeded line YN01-429 carry yellow seed color gene(s) of *B. carinata* and/or *B. juncea* and/or *B. rapa*. The genetic control of yellow seed color in these three species is very different (Getinet and Rakow 1997, Vera et al. 1979, Rawat 1989, Lionneton et al. 2004, Yan et al. 2009, Stringam 1980, Zaman 1989 and Rahman et al. 2007). Therefore, it can be hypothesized that the genetic control of yellow seed color in YN01-429 and CH5034/06 is different; and occurrence of bright yellow to black seed in the progeny of YN01-429 x CH5034/06 indeed substantiate this hypothesis. The re-shuffling of yellow-seed color genes of the two parents in the DH lines has produced different seed colors, ranging from bright yellow (brighter than both parents) to dark brown or black seed colors. Genetic analysis of seed color in the DH lines and pedigree populations revealed that seed color is controlled by four to five genes in these populations.

Brassica rapa has naturally occurring yellow-seeded forms and several studies have been conducted to understand the genetic control of seed color. It has been established that seed color is controlled by two or more genes (Mohammad et al. 1942, Ahmed and Zuberi 1971, Stringam 1980, Hawk 1982, Schwetka 1982, Zaman 1989, Chen and Heneen 1992, Rahman 2001, Rahman et al. 2007 and Choudhary 2008). Rahman et al. (2007) identified a sequence-related amplified

polymorphism (SRAP) marker for the major seed coat color gene Br_1/br_1 in *B. rapa*. Recently, Xiao et al. (2011) reported that the major seed color gene to be located at A9 of *B. rapa*.

In this study, SSR markers were used for mapping the major seed color gene in backcross derived self-pollinated population of (Sampad x 3-0026.027) x Sampad of *B. rapa* segregating for the major seed color gene. Yellow seed color gene was mapped on linkage group A9 and several SSR markers linked to the yellow seed color allele of 'yellow sarson' were identified for use in marker-assisted breeding. Use of SSR markers provides wider use in plant breeding purposes. SSR markers are user friendly, co-dominant, repeatable markers known to aid in gene transfer and gene pyramiding programmes. Since this type of marker is not affected by PCR conditions, can be used across laboratories and research groups for collaborative projects. The results for this research also agree with mapping results of seed color genes in *B. napus*, where a major seed color gene was mapped at A9 (Rahman et al. 2010).

4.2 Conclusions

The novel findings from my research are listed below:

Brassica napus

- The two yellow-seeded lines YN01-429 and CH5034/06 are genetically distinct for the seed color genes.
- Crossing these yellow-seeded lines resulted transgressive segregants with brighter yellow-seed color than the parents.

Brassica rapa

- The major yellow-seed color gene (Br_1) of the *Brassica* A genome is mapped to the linkage group A9, and several SSR markers for yellow seed color allele identified.

4.3 Future research

Seed color of the DH lines produced in this study ranged from bright yellow to black. Frequency distributions and preliminary genetic analysis suggests that more than four gene loci are involved in the control of seed color difference in this population; therefore, QTL mapping can be performed to detect these loci. The bright yellow-seeded DH lines can be used in breeding to develop bright yellow-seeded *B. napus* cultivars with stable expression of this trait under different environments. Furthermore, the bright yellow-seeded DH lines can be also be crossed with other black seeded *B. napus* lines, and QTL mapping can be done to detect additional seed color loci. Knowledge gained from mapping of the seed color gene(s) can be used for fine mapping and eventually map-based cloning of the gene.

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