

Comparison of RF3 *B. juncea* to RF3 *B. napus*

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Abstract

RF3 *Brassica napus* L. (InVigor™ Canola) has been in commercial cultivation since 1997. RF3 *B. napus* was produced by *Agrobacterium*-mediated transformation to provide glufosinate ammonium herbicide tolerance to the crop. Canola quality (CQ) varieties of *Brassica juncea* L. (Czern & Coss), a close relative of *B. napus*, have been available in Canada for more than 20 years. Currently, BASF Agricultural Solutions is developing CQ RF3 *B. juncea* via conventional breeding with RF3 *B. napus* to provide similar herbicide tolerance in the *B. juncea* species. The insertion event RF3 contains the *bar* gene (origin *Streptomyces hygroscopicus*) coding for phosphinothricin acetyl transferase (PAT/*bar*) protein which confers tolerance to glufosinate-ammonium herbicides. RF3 also contains the *barstar* gene (origin *Bacillus amyloliquefaciens*), coding for the Barstar protein, which is an inhibitor of the Barnase protein. In the absence of Barnase, there is no impact of Barstar. Many safety studies have been conducted during the development of the canola quality lines of RF3 *B. napus* and RF3 *B. juncea*. This report seeks to compare the two species based on the scientific data generated during the safety assessments of RF3 *B. napus* and RF3 *B. juncea* and discusses the utility of using studies with one species to support the safety of the other species both containing the same insertion event.

Keywords

Canola, brassica, juncea, napus, RF3, Composition, Protein Expression, Molecular Characterization

1. Introduction

Oilseed rape (OSR), rapeseed or canola, is a bright-yellow flowering member of the family Brassicaceae, cultivated mainly for its oil-rich seed. Production and trade statistics do not distinguish between the different sources of oilseeds (*i.e.* *Brassica* species) and its products. Currently rapeseed is the second most impor-

tant oilseed crop in the world, following soybean (**Table 1**) [1]. This paper 1) presents a comparison of the biology and use of canola quality (CQ) *B. napus* and *B. juncea*, 2) includes the definitions of canola used by various agencies around the world; 3) provides a history of the safe use of these Brassica species, 4) describes the development of RF3 *B. juncea* from RF3 *B. napus* and 5) presents the safety studies conducted as part of the risk assessment of these products.

1.1. Biology of Brassica Species

The Triangle of U (**Figure 1**) demonstrates the theory developed by Nagaharu [2] about the evolution and relationships among members of the plant genus *Brassica*. The genomes of three ancestral species of *Brassica* combined to create three of the common contemporary vegetable and oilseed crop species. This theory has since been confirmed by molecular studies. It shows how three of the *Brassica* species were derived from three ancestral genomes, denoted by the letters

Table 1. World Production of major oilseed crops [1] (in million metric tons).

	Production (MMt)	2020/2021
Soybean		362.05
Rapeseed		68.87
Cottonseed		41.80
Peanuts		47.79
Sunflower seed		49.46
Palm Kernel		19.96
Copra		5.75
Total		595.68

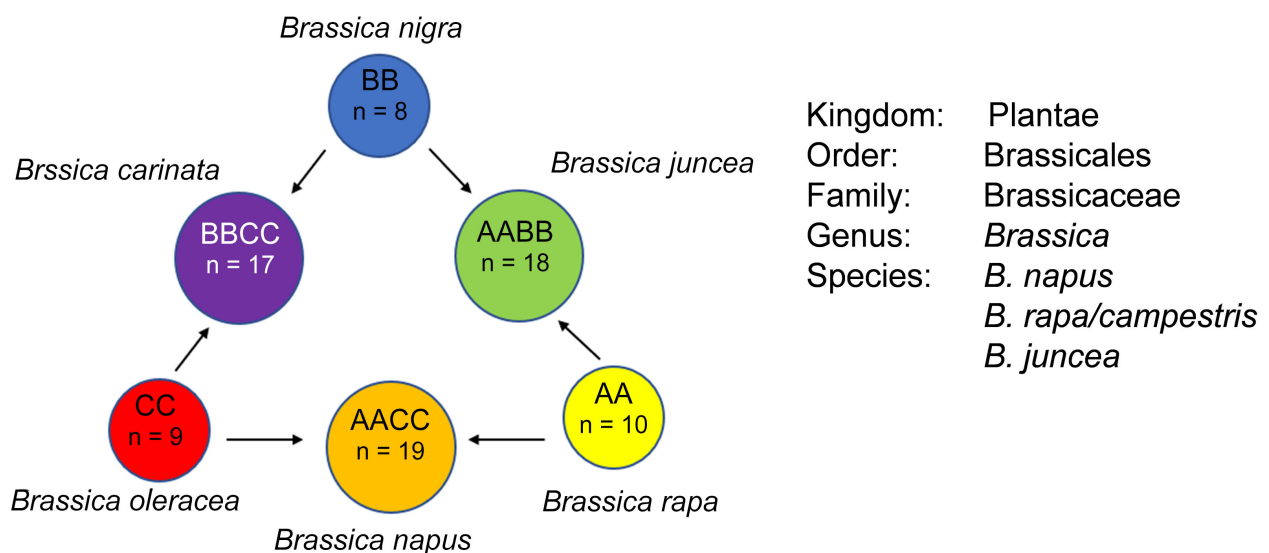


Figure 1. The Triangle of U. AA = *B. rapa*—turnip, Chinese cabbage; BB = *B. nigra*—Black mustard; CC = *B. oleracea*—cabbage, kale, broccoli, Brussel sprouts, cauliflower, kohlrabi.

AA, BB, or CC. Alone, each of these diploid genomes produces a common *Brassica* species. The letter n denotes the number of chromosomes in each genome.

These three species exist as separate species, but because they are closely related, it is possible for them to interbreed, allowing for the creation of three new amphidiploid species of *Brassica*: *Brassica carinata*, *Brassica napus*, and *Brassica juncea*. *Brassica napus* and *Brassica juncea* share the A-genome.

1.2. Definitions of Canola

To qualify as a canola variety, the oil derived must contain less than 2% erucic acid (C22:1) and meal produced from the grain must contain less than 30 $\mu\text{mol/g}$ glucosinolates (oil-free basis). The first canola variety was developed from rapeseed by conventional breeding by Dr. Keith Downey (Agriculture and Agri-Food Canada) and Dr. Baldur R. Steffansson (University of Manitoba) in the early 1970's. The oil content of the seed is about 44% by weight, and the protein content of the remaining meal is about 36% by weight. The name "Canola" was selected to represent an oilseed crop grown primarily in Canada that produces an edible oil (oil + low + acid (ola)).

In North America, canola oil is generally not referred to as rapeseed oil. "Rapeseed" or "High Erucic Acid Rapeseed (HEAR)" are terms typically used when referring to an inedible oil used for industrial purposes such as lubricants, hydraulic fluid, and plastics. This rapeseed oil has high erucic acid content and is not of canola quality.

Canola oil comes from one of three species: *Brassica napus*, *Brassica rapa* or *Brassica juncea*. The total saturated fats are below 7% as well. The oil profile of CQ *B. juncea* is similar to canola oil from *B. napus* and can be used in food, bio-diesel etc. Based on these quality requirements, *B. juncea* has been specified in the definition for "canola" from several global authorities or sources: For example, the canola data available for the Version 5 release of the ILSI Crop Composition Database [3] were derived from *Brassica napus* containing erucic acid content of less than 2%. However, for this database the common name "canola" also refers to *Brassica rapa* and *Brassica juncea* varieties, as well as low erucic acid and low glucosinolate rapeseed and conventionally bred varieties with modified oil profiles

Furthermore, according to the Codex Alimentarius [4] rapeseed oil—low erucic acid (low erucic acid turnip rape oil; low erucic acid colza oil; canola oil) is produced from low erucic acid oil-bearing seeds of varieties derived from the *Brassica napus* L., *Brassica campestris* L. and *Brassica juncea* L., species. Similarly, according to the OECD, the term "canola" has been registered and adopted by many countries to describe the oil (and seeds and plants) obtained from the species *B. napus*, *B. rapa* and *B. juncea*. Canola must contain less than 2% erucic acid in the oil and less than 30 $\mu\text{mol/g}$ glucosinolates (anyone or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate) in the air-dried, oil-free meal.

Likewise in the Korean Food Standard Codex (No. 2020-3) Section 7.1.4.3, rape-seed oil (canola oil) refers to crude oil obtained from rape and treated to be fit for human consumption. Rape contains *Brassica campestris* L., *Brassica campestris* var. *chinensis* T. Ito, *Brassica napus* L. and *Brassica juncea* L.

1.3. Why Canola Quality *B. juncea*?

B. juncea has a number of advantages over *B. napus* including more vigorous seedling growth and quicker ground covering ability. *B. juncea* is better adapted to hot, dry areas than *B. napus* so it can tolerate heat and drought stress [5]. *B. juncea* can tolerate high temperatures during flowering, which has become even more important recently for areas impacted by climate change. It effectively expands potential oilseed acreage to semi-arid zones, including the prairies of Western Canada [6]. *B. juncea* can be grown in more marginal and low rainfall areas and has potential as an oilseed crop in the semi-arid regions of Australia [7]. Additionally, *B. juncea* can naturally be grown in spring canola markets in the USA and Russia.

B. juncea is more resistant to pod shattering than *B. napus* and matures more uniformly, allowing for direct combining which reduces the number of passes through fields and results in less seed loss at harvest [8] [9].

Another advantage of *B. juncea* is its inherent blackleg resistance. *Brassica* species containing the B genome, such as *B. juncea*, have high levels of resistance to blackleg disease, a fungal disease caused by *Leptosphaeria maculans*, which is a major concern of canola producers worldwide [10].

From a grain processing perspective, the key differentiator between these two oilseeds is the seed color, with *B. napus* appearing black, while *B. juncea* can be light brown to bright yellow. The yellow seed of *B. juncea* also provides a low fiber, high quality meal for feed applications for mono-gastric animals. Benefits of *B. juncea* meal for animal feed include:

- Heavier seeds with a thinner coat resulting in a higher percentage of oil and protein
- Lower fiber content resulting in better digestibility of the meal for livestock
- Yellow seed coat blends better with other feedstuffs so the appearance of the final feed is not altered [11]

In addition, the low seed chlorophyll content is an advantage for oil refining, making the oil from yellow-seeded varieties lighter in color.

1.4. Weed Management in Canola Production

In the early days of canola production (approx. 1965), canola was seeded on land relatively free of weeds. Land was often fallow the year before canola production. Weed control in commercial production relied on a mixture of numerous selective herbicides targeting monocot weeds, dicot weeds, and volunteer cereals. Tillage was often combined with herbicide applications.

Since the mid-1990s, herbicide tolerant canola from *B. napus* has been widely

adopted by farmers, greatly reducing the number of herbicide applications needed to control weeds. Currently, >99% of canola (*B. napus*) cultivated in Canada is herbicide tolerant: 34% is Roundup Ready canola with tolerance to glyphosate, 62% is LibertyLink[®] canola with tolerance to glufosinate-ammonium, and the remaining 4% is Clearfield canola with tolerance to imazethapyr/imazamox, along with some conventional canola.

Adoption of herbicide tolerant traits has resulted in the application of less herbicide active ingredient. In 2013, the use of genetically modified herbicide tolerant canola resulted in a 2.1 million kg reduction in the amount of herbicide active ingredient used (-17.1%) [12].

The use of herbicide tolerant canola has reduced fuel usage and altered tillage practices, resulting in soil conservation and related environmental benefits such as carbon sequestering. It is estimated that in Canada in 2013 the use of genetically modified canola resulted in a fuel saving of 69 million litres and reduced carbon dioxide emissions by 185 million kg [12]. Farmers use less tillage and more direct-seeding. The reduction of tillage reduces soil erosion, contributes to less air pollution from dust, improves soil moisture retention, and reduces soil compaction.

Because of these advantages, herbicide-tolerant varieties have quickly grown in popularity since their introduction in 1995.

The first herbicide-tolerant cultivars of *B. juncea* were developed by a process which included the transfer of a resistance gene from *B. napus* [13] [14]. The objective of producing RF3 *B. juncea* was to bring the LibertyLink[®] glufosinate-ammonium herbicide tolerance trait to CQ *B. juncea* lines by conventional breeding. This would capture all the aforementioned advantages of CQ *B. juncea* with an herbicide tolerance trait. The incorporation of the *bar* gene for glufosinate-ammonium tolerance provides broad spectrum weed control in *B. juncea* and also provide growers with an additional tool for their weed resistance management strategy.

1.5. History of Safe Use

Humans have been using *Brassica* oilseeds for cooking and illumination since 2000-1500 B.C. [13] [15]. Canola oil is used in the manufacture of margarine and shortening, salad and cooking oil, mayonnaise, sandwich spreads, creamers and coffee whiteners, confectionery products, low-fat foods, pharmaceuticals, and nutritional supplements.

With the recent development of CQ *B. juncea* varieties, seeds are used and the refined oil from CQ *B. juncea* lines are consumed in the same way as other canola quality rapeseed oils. The fatty acid profiles of the oil from CQ *B. juncea* are within the normal range of other canola quality rapeseed oils. The development of CQ *B. juncea* has not resulted in any change in the consumption pattern for these products. CQ *B. juncea* meal is used as a high protein animal feed for cattle, swine, and poultry. The animal feed can contain up to 35% of this processed

commodity.

For *B. juncea*, Bra j 1 is the major food allergen present in Indian or Oriental mustard. It belongs to the storage protein 2S albumin family, which are abundant proteins in seeds with homologues in *B. napus* and *B. rapa*. However, oil obtained from Canola Quality grain is not considered to be an allergenic food, because protein is absent in the refined oil. Food allergy to canola quality oil has not been reported in the scientific literature [16] [17] [18] [19].

RF3 *B. napus* has a history of safe use since commercialization in 1996. The Food & Feed safety conclusion of RF3 *B. napus* was extended to RF3 *B. juncea* in countries like Canada, the USA, Australia, and China. The FDA granted the oil produced from low erucic acid rapeseed varieties “Generally Recognized as Safe” (GRAS) status, and Health Canada approved the food use of oil derived from “Canola Quality” *B. juncea* varieties.

Based on the data available, CQ *B. juncea* varieties are considered to have a demonstrated history of safe use for consumers and domesticated animals.

Within the *Brassicaceae*, related species have often been used to transfer desired traits which are not available within the primary gene pool of the species [20]. *B. juncea* has acquired traits through interspecific crossing and has been used as a source of traits such as yellow seed color [21] and blackleg resistance [22] in breeding *B. napus*.

Canola Quality characteristics, primarily low erucic acid content in the oil and low glucosinolate content in the meal, were first developed in the rapeseed species *B. napus* and *B. rapa* (*campestris*). These two species were used to develop CQ *B. juncea*. Love *et al.* [23] reported on the successful transfer of low glucosinolate content from *B. rapa* to *B. juncea*. Raney *et al.* [24] further reduced the glucosinolate content of *B. juncea* lines through an interspecific cross with *B. napus*. Malode *et al.* [25] utilized *B. napus* as a source of both low erucic acid and low glucosinolate content. *Brassica napus* was used to further improve the fatty acid profile of *B. juncea* by raising the oleic fatty acid and lowering the polyunsaturated fatty acid content [24] [26] [27].

The success of these efforts to change the quality of *B. juncea* by interspecific crossing led to the commercial release of CQ *B. juncea* cultivars in Canada [28] and Australia [29].

B. napus has been used as a source of other traits in *B. juncea*, such as resistance to white rust [30] [31]. The Barnase and Barstar hybridization system was also transferred to *B. juncea* from *B. napus* [32].

The first herbicide-tolerant cultivars of *B. juncea* were developed by a process which included the transfer of a resistance gene from *B. napus* [13] [14].

2. Production and Characterization of RF3 *B. juncea*

RF3 *B. napus* was produced by *Agrobacterium*-mediated transformation using the transforming plasmid pTHW118. The plasmid derived T-DNA sequences were inserted in the RF3 *B. napus* insertion locus. RF3 contains the *bar* gene

(origin *Streptomyces hygroscopicus*) coding for phosphinothricin acetyl transferase (PAT/*bar*) protein which confers tolerance to glufosinate-ammonium [33]. The *bar* gene is driven by the PssuAt plant promoter that is active in all green tissues of the plant [34]. RF3 also contains the *barstar* gene (origin *Bacillus amyloliquefaciens*), coding for the Barstar protein, which is an inhibitor of the Barnase protein [35]. The *barstar* gene is driven by the Pta29 promoter that restricts gene expression to the tapetal cells during anther development [36]. In the absence of Barnase, there is no impact of Barstar. The OECD identifier of RF3 *B. napus* is ACS-BNØØ3-6. RF3 *B. juncea* was developed by conventional breeding to cross an elite CQ *B. juncea* line with RF3 *B. napus* following the path shown in **Figure 2**.

The inserted T-DNA sequences, together with the 5' and 3' flanking genomic regions, were transferred into *B. juncea* through backcross breeding, the applied procedure to generate RF3 *B. juncea* did not involve any novel transformation. The underlying mechanism through which the RF3 *B. napus* was transferred into *B. juncea* was by a homologous recombination during meiosis. Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two very similar or identical DNA molecules. Through this mechanism, the previously defined sequence of the RF3 *B. napus* transgenic locus (*i.e.* T-DNA and the defined 5' and 3' flanking genomic sequence), together with large stretches of *B. napus* sequence originating further downstream and upstream of the RF3 transgenic locus, were transferred from the *B. napus* sister chromatid to the *B. juncea* sister chromatid. A simplified breeding tree is provided in **Figure 2**:

RF3 *B. napus*

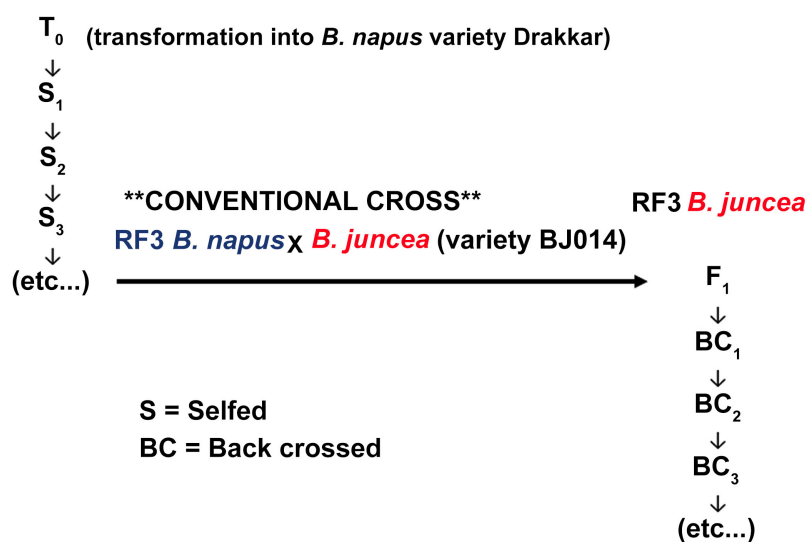


Figure 2. Simplified Breeding Tree depicting the introgression using conventional breeding techniques of the RF3 insertion from *B. napus* to *B. juncea*.

3. Comparative Safety Studies

3.1. Molecular Characterization

To confirm the RF3 *B. juncea* insert organization as determined in RF3 *B. napus*, genomic DNA from ten individual RF3 *B. juncea* plants was digested with restriction enzymes *Nsi*I and *Eco*RV, hybridized with five different vector backbone probes and subsequently with the T-DNA probe. Only the expected fragments, based on the insert organization as determined in RF3 *B. napus* [37] were obtained. The membranes also contained *Nsi*I and *Eco*RV digestions of RF3 *B. napus* DNA, which resulted in the same restriction pattern as the RF3 *B. juncea* plants. Therefore, the Southern blot analyses confirmed the RF3 *B. juncea* insert organization as determined in RF3 *B. napus* and the absence of vector backbone sequences.

To assess the structural stability of RF3 *B. juncea*, three individual plants from five generations were digested with the *Nsi*I restriction enzyme and hybridized with the T-DNA probe. Only the expected fragments, based on the insert organization as determined in RF3 *B. napus*, were obtained for all individual RF3 *B. juncea* plants from the different generations, demonstrating the structural stability of the RF3 *B. juncea* transgenic locus. The membrane also contained a *Nsi*I digestion of RF3 *B. napus* DNA, which resulted in the same restriction pattern as the RF3 *B. juncea* plants [37].

The RF3 *B. juncea* transgenic locus sequence, including 1 kb of each flanking genomic sequence was determined and compared with the RF3 *B. napus* transgenic locus sequence. Both loci were found to consist of identical sequences.

In conclusion, the molecular characterization data obtained for both RF3 species convincingly show that the RF3 *B. juncea* variety is fully comparable to RF3 *B. napus*. This is to be considered as an expected result, since RF3 *B. juncea* originated from RF3 *B. napus* through conventional breeding practices and not out of a novel transformation event. All molecular analyses of RF3 *B. juncea* demonstrate the *B. napus* nature of the RF3 T-DNA region and its flanking genomic sequences.

3.2. Protein Studies

Since it has been determined that the insertion sequences remain the same in RF3 *B. juncea* as in RF3 *B. napus*, existing PAT/*bar* and Barstar studies may be used to demonstrate the safety of the proteins. Studies were conducted using bacterially-produced proteins. The safety assessment for the PAT protein has been described in depth by Hérouet *et al.* [38]. This assessment included examining the stability of PAT to heat, pepsin in simulated mammalian gastric fluid, and pancreatin in simulated mammalian intestinal fluid as well as a bioinformatics assessment and comparison to known allergens and toxins. In summary, PAT protein encoded by the *bar* gene showed no significant changes after heat treatment at up to 90°C for 60 minutes when examined by SDS-PAGE. The PAT/*bar* protein was degraded very rapidly in human simulated gastric fluid, *i.e.*

more than 90% of test protein disappeared within 0.5 minutes of incubation in the presence of pepsin at pH 1.2. PAT/*bar* protein was degraded very rapidly with no protein fragment visible in less than 30 seconds of incubation with simulated intestinal fluid in the presence of pancreatin at pH 7.5. No homology was found to known toxins or allergens.

Similar studies have been conducted for Barstar. Barstar protein was incubated for 30 minutes at 4°C, 25°C, 37°C, 55°C, 75°C, and 95°C followed by assessment by SDS-PAGE and western blot analyses, ELISA analyses and activity assays. The samples treated at 4°C were used for comparison of the other temperature treated samples. These studies demonstrated the stability of the Barstar protein up to 55°C, with some loss of stability at 75°C, and complete loss at 95°C. Barstar degraded within 30 seconds in presence of pepsin at pH 1.2. Greater than 90% of Barstar protein was degraded within 10 minutes in the presence of pancreatin at pH 7.5. No homology was found to known toxins or allergens.

Neither PAT nor Barstar protein when dosed at 2000 mg/kg body weight via the oral route produced any signs of systemic toxicity in male and female C57BL/6J mice.

A protein equivalency study comparing the structural and functional characteristics of PAT/*bar* protein purified from RF3 *B. juncea* with bacterially-produced PAT/*bar* protein was conducted. The identity of the RF3 *B. juncea* plant-purified PAT/*bar* protein was confirmed by intact molecular mass determination, peptide mapping and N-terminal sequence determination. The N-terminal sequence was shown to be acetylated which is a common post-translational modification in plant proteins. The apparent molecular mass of PAT/*bar* protein was confirmed and the immuno-reactivity of the plant-purified PAT/*bar* protein to an anti-PAT/*bar* antibody was demonstrated. The plant-purified PAT/*bar* protein was not glycosylated. Finally, the activity of the plant-purified PAT/*bar* protein was demonstrated. Therefore, the plant-purified and the bacterially-produced PAT/*bar* proteins were considered to be structurally and functionally equivalent.

Likewise, a protein equivalency study comparing the structural and functional characteristics of Barstar protein purified from RF3 *B. juncea* with bacterially-produced Barstar protein was conducted. The identity of the RF3 *B. juncea* plant-purified Barstar protein was confirmed by N-terminal sequence determination. The apparent molecular mass of Barstar protein was confirmed and the immuno-reactivity of the plant-purified Barstar protein to an anti-Barstar antibody was demonstrated. The plant-purified Barstar protein was not glycosylated. Finally, specific activity of the plant-purified Barstar protein was demonstrated. Therefore, the plant-purified and the bacterially-produced Barstar proteins were considered to be structurally and functionally equivalent.

3.3. Composition

Composition data between RF3 *B. napus* and RF3 *B. juncea* were reviewed to

assess similarities or major differences in nutrient content between the two species. Composition data between the non-GM conventional *B. napus* and non-GM conventional *B. juncea* were similarly assessed.

RF3 *B. napus* and the non-GM conventional variety were grown in 2008 in five field trials in the *B. napus* growing regions of Saskatchewan and Alberta, Canada. RF3 *B. juncea* and the non-GM conventional variety were grown in 2017 in eight field trials in the *B. juncea* growing regions of Canada and the USA. Entries were replicated four times in a randomized complete block design (RCBD) in both studies. RF3 *B. napus* and *B. juncea* entries received one application of Liberty® herbicide (glufosinate-ammonium) at 500 g ai/ha. One canola grain sample was harvested from each plot for compositional analysis.

Composition analyses were conducted to determine levels of nutrients and anti-nutrients in the canola grain. Each sample was analyzed for proximates and fiber (Table 2), amino acids (Table 3), fatty acids (Table 4), minerals (Table 5), tocopherols (Table 6), and anti-nutrients (Table 7). Since RF3 *B. napus* has a long history of safe use, composition of RF3 *B. juncea* grain was compared to RF3 *B. napus* grain. Composition of the non-GM conventionals from *B. juncea* and *B. napus* were also compared. Composition data from non-GM *B. napus* was accessed on April 22, 2019, from the ILSI Crop Compositional Database (ILSI-CCDB) Version 7 [3], to provide ranges for reference.

Table 2. Proximates and fiber compounds.

Proximates and Fiber (% DW)	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Moisture (% FW)	5.58	0.32	4.99	6.09	8.53	2.15	4.94	13.7	NA
Crude fat	43.5	2.6	37.3	48.4	42.9	3.5	35.2	49.3	24.6 - 55.2
Crude protein	25.1	2.8	19.9	29.4	26.2	2.0	22.9	29.4	15.6 - 35.7
Ash	3.81	0.22	3.43	4.18	4.28	0.54	3.39	5.17	2.6 - 10.6
Total Carbohydrate	27.5	2.1	24.5	33.1	26.6	2.6	23.1	32.9	17.7 - 47.4
Acid Detergent Fiber	35.6	2.5	29.7	40.6	30.8	1.6	28.1	34.5	8.94 - 42.3
Neutral Detergent Fiber	39.3	2.1	34.8	44.4	33.9	1.8	31.1	37.6	10.9 - 53.7

Proximates and Fiber (% DW)	Non-GM <i>B. napus</i>				Non-GM <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Moisture (% FW)	5.72	0.28	5.13	6.12	7.59	1.34	4.75	9.92	NA
Crude fat	43.1	2.2	39.4	47.2	45.0	3.6	35.0	49.4	24.6 - 55.2
Crude protein	26.1	2.2	21.8	29.8	24.3	1.9	20.2	27.2	15.6 - 35.7
Ash	3.79	0.22	3.27	4.15	4.10	0.55	3.06	5.01	2.6 - 10.6
Total Carbohydrate	27.0	1.6	24.0	29.4	26.6	3.1	23.8	37.1	17.7 - 47.4
Acid Detergent Fiber	34.8	3.2	29.2	40.5	32.1	1.7	28.4	36.3	8.94 - 42.3
Neutral Detergent Fiber	38.4	3.5	33.7	44.1	35.0	1.8	31.5	38.7	10.9 - 53.7

Table 3. Amino acids.

Amino Acids (% DW)	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Alanine	1.12	0.11	0.92	1.29	1.15	0.06	1.02	1.24	0.73 - 1.45
Arginine	1.75	0.21	1.40	2.06	1.64	0.10	1.42	1.81	0.97 - 2.34
Aspartic Acid	1.80	0.22	1.41	2.13	1.85	0.14	1.64	2.16	1.15 - 2.68
Cystine	0.66	0.07	0.53	0.76	0.64	0.12	0.39	0.87	0.19 - 0.96
Glutamic Acid	4.73	0.54	3.78	5.53	4.76	0.38	4.02	5.45	2.37 - 7.31
Glycine	1.30	0.14	1.05	1.49	1.30	0.07	1.13	1.41	0.86 - 1.75
Histidine	0.71	0.07	0.58	0.81	0.74	0.05	0.62	0.80	0.47 - 1.05
Isoleucine	1.06	0.12	0.85	1.27	1.04	0.05	0.94	1.13	0.65 - 1.40
Leucine	1.82	0.21	1.46	2.14	1.81	0.09	1.64	1.96	1.14 - 2.42
Lysine	1.56	0.14	1.32	1.75	1.58	0.14	1.28	1.87	1.07 - 2.09
Methionine	0.52	0.06	0.41	0.61	0.46	0.07	0.30	0.57	0.19 - 0.71
Phenylalanine	1.06	0.11	0.86	1.24	1.03	0.06	0.91	1.14	0.69 - 1.52
Proline	1.57	0.19	1.27	1.90	1.54	0.12	1.30	1.76	1.01 - 2.28
Serine	1.07	0.10	0.87	1.21	1.12	0.07	0.98	1.33	0.66 - 1.53
Threonine	1.08	0.10	0.90	1.21	1.07	0.05	0.96	1.16	0.72 - 1.40
Tryptophan	0.27	0.04	0.20	0.33	0.30	0.04	0.21	0.37	0.17 - 0.50
Tyrosine	0.72	0.07	0.59	0.82	0.63	0.06	0.52	0.74	0.41 - 1.03
Valine	1.34	0.15	1.08	1.59	1.23	0.07	1.11	1.34	0.82 - 1.70

Amino Acids (% DW)	Non-GM <i>B. napus</i>				Non-GM <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Alanine	1.14	0.10	0.98	1.31	1.10	0.08	0.94	1.26	0.73 - 1.45
Arginine	1.78	0.18	1.49	2.07	1.53	0.13	1.34	1.77	0.97 - 2.34
Aspartic Acid	1.83	0.19	1.51	2.12	1.78	0.17	1.53	2.15	1.15 - 2.68
Cystine	0.68	0.06	0.55	0.77	0.54	0.11	0.32	0.79	0.19 - 0.96
Glutamic Acid	4.86	0.49	4.07	5.58	4.40	0.43	3.69	5.39	2.37 - 7.31
Glycine	1.32	0.12	1.11	1.51	1.23	0.08	1.12	1.39	0.86 - 1.75
Histidine	0.72	0.06	0.62	0.82	0.68	0.05	0.59	0.79	0.47 - 1.05
Isoleucine	1.08	0.11	0.90	1.27	0.98	0.07	0.88	1.12	0.65 - 1.40
Leucine	1.85	0.18	1.56	2.16	1.72	0.13	1.53	1.95	1.14 - 2.42
Lysine	1.60	0.11	1.40	1.77	1.46	0.13	1.22	1.87	1.07 - 2.09
Methionine	0.54	0.04	0.44	0.60	0.45	0.06	0.33	0.57	0.19 - 0.71
Phenylalanine	1.07	0.10	0.91	1.25	0.96	0.07	0.84	1.12	0.69 - 1.52
Proline	1.63	0.17	1.37	1.89	1.39	0.11	1.21	1.62	1.01 - 2.28
Serine	1.09	0.09	0.95	1.22	1.07	0.06	0.98	1.21	0.66 - 1.53
Threonine	1.09	0.09	0.95	1.22	1.03	0.06	0.96	1.17	0.72 - 1.40
Tryptophan	0.27	0.03	0.20	0.33	0.28	0.04	0.19	0.35	0.17 - 0.50
Tyrosine	0.73	0.06	0.62	0.81	0.60	0.06	0.51	0.70	0.41 - 1.03
Valine	1.37	0.13	1.15	1.59	1.17	0.09	1.06	1.34	0.82 - 1.70

Table 4. Fatty acids.

Fatty Acids (% Total)	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
C14:0 Myristic	0.06	0.00	0.05	0.07	0.07	0.00	0.06	0.08	<LOQ - 0.09
C16:0 Palmitic	4.35	0.11	4.1	4.51	3.90	0.15	3.56	4.18	3.53 - 5.70
C16:1 Palmitoleic*	0.26	0.01	0.23	0.28	0.16	0.01	0.14	0.18	0.16 - 0.40
C18:0 Stearic	1.95	0.24	1.59	2.36	2.83	0.55	2.08	3.71	1.50 - 2.89
C18:1 Oleic	61.0	1.74	57.6	63.7	54.9	1.6	51.4	58.3	53.2 - 69.5
C18:2 Linoleic*	18.3	0.63	17.1	19.2	23.6	1.1	21.7	26.1	14.1 - 25.7
C18:3 Linolenic	10.8	1.34	9.00	13.34	11.1	0.7	9.86	12.6	5.79 - 13.1
C20:0 Arachidic*	0.64	0.04	0.57	0.70	0.51	0.02	0.48	0.55	0.49 - 0.95
C20:1 Eicosenoic	1.35	0.07	1.26	1.51	1.42	0.29	1.17	2.42	0.93 - 3.33
C20:2 Eicosadienoic*	0.07	0.01	0.06	0.08	0.12	0.02	0.10	0.18	0.04 - 0.86
C22:0 Behenic*	0.32	0.02	0.29	0.36	0.22	0.01	0.20	0.24	<LOQ - 0.52
C22:1 Erucic	0.02	0.01	<LOQ	0.03	0.29	0.36	<LOQ	1.54	<LOQ - 1.96
C24:0 Lignoceric	0.19	0.02	0.14	0.23	0.24	0.02	0.21	0.28	<LOQ - 0.32
C24:1 Nervonic*	0.17	0.04	0.13	0.26	0.41	0.04	0.35	0.48	<LOQ - 0.40

Fatty Acids (% Total)	Non-GM <i>B. napus</i>				Non-GM <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
C14:0 Myristic	0.06	0.00	0.05	0.06	0.07	0.00	0.06	0.08	<LOQ - 0.09
C16:0 Palmitic*	4.37	0.14	4.17	4.64	3.75	0.15	3.48	4.06	3.53 - 5.70
C16:1 Palmitoleic*	0.26	0.01	0.24	0.28	0.15	0.01	0.13	0.17	0.16 - 0.40
C18:0 Stearic	1.96	0.21	1.68	2.41	2.81	0.53	2.24	3.93	1.50 - 2.89
C18:1 Oleic	61.0	1.39	59.3	63.5	56.0	1.7	51.9	59.5	53.2 - 69.5
C18:2 Linoleic*	18.2	0.55	17.3	19.2	23.5	1.1	21.2	26.5	14.1 - 25.7
C18:3 Linolenic	10.9	1.16	9.22	13.0	10.5	0.8	8.19	11.9	5.79 - 13.1
C20:0 Arachidic*	0.64	0.04	0.57	0.69	0.50	0.03	0.44	0.54	0.49 - 0.95
C20:1 Eicosenoic	1.35	0.06	1.27	1.44	1.37	0.17	1.17	1.83	0.93 - 3.33
C20:2 Eicosadienoic*	0.07	0.01	0.06	0.08	0.12	0.01	0.10	0.15	0.04 - 0.86
C22:0 Behenic*	0.33	0.02	0.29	0.37	0.22	0.02	0.19	0.25	<LOQ - 0.52
C22:1 Erucic	0.03	0.01	<LOQ	0.03	0.17	0.17	<LOQ	0.71	<LOQ - 1.96
C24:0 Lignoceric	0.18	0.03	0.13	0.24	0.25	0.02	0.19	0.29	<LOQ - 0.32
C24:1 Nervonic*	0.15	0.03	0.08	0.20	0.40	0.03	0.33	0.46	<LOQ - 0.40

*Ranges between *B. napus* and *B. juncea* do not overlap. However, the ranges of *B. juncea* do overlap with the ILSI Crop Composition Database ranges for *B. napus*. This is true for both the RF3 (GM) and non-GM conventional entries.

Table 5. Minerals.

Minerals (% DW)	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Calcium	0.41	0.02	0.37	0.45	0.50	0.08	0.37	0.72	0.25 - 1.41
Phosphorus	0.62	0.05	0.55	0.72	0.86	0.18	0.59	1.19	0.41 - 1.85
Potassium	0.68	0.05	0.59	0.74	0.69	0.11	0.54	0.91	0.46 - 0.40
Magnesium	0.29	0.01	0.27	0.31	0.37	0.04	0.30	0.43	0.22 - 0.53
Sodium	NA	NA	<LOQ	0.01	0.001	0.0005	0.0008	0.002	<LOQ - 0.14
(mg/kg DW)									
Iron	61.7	11.9	50.5	90.5	98.5	72.1	53	393	34.2 - 844
Manganese	41.8	1.32	38.9	43.9	29.9	5.7	20	48.4	15.5 - 108
Copper*	3.33	0.22	2.89	3.75	6.27	0.86	4.49	9.08	<LOQ - 9.84
Zinc	40.1	2.9	34.3	45.5	45.4	5.3	36.6	55.9	22.2 - 155

Minerals (% DW)	Non-GM RF3 <i>B. napus</i>				Non-GM RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Calcium	0.41	0.03	0.36	0.45	0.45	0.07	0.30	0.59	0.25 - 1.41
Phosphorus	0.65	0.05	0.55	0.73	0.83	0.17	0.56	1.14	0.41 - 1.85
Potassium	0.69	0.06	0.59	0.79	0.75	0.14	0.55	1.06	0.46 - 1.40
Magnesium	0.30	0.02	0.28	0.34	0.35	0.04	0.28	0.43	0.22 - 0.53
Sodium	NA	NA	<LOQ	0.02	0.001	0.0004	0.0008	0.002	<LOQ - 0.14
(mg/kg DW)									
Iron	61.0	3.8	56.6	70.4	93.8	40.9	48.9	235	34.2 - 844
Manganese	42.7	2.3	39.4	47.2	29.5	5.0	21	37.6	15.5 - 108
Copper	3.58	0.27	2.99	4.04	5.63	0.90	3.93	7.75	<LOQ - 9.84
Zinc	41.4	2.4	36.7	46.1	42.2	6.1	31.2	53.7	22.2 - 155

*Ranges between *B. napus* and *B. juncea* do not overlap. However, the ranges of *B. juncea* do overlap with the ILSI Crop Composition Database ranges for *B. napus*.

Table 6. Tocopherols.

Tocopherols (mg/kg DW)	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Alpha	68.9	9.5	55.5	87.3	54.5	8.7	47.2	78.1	9.57 - 180
Beta	<LOQ	NA	NA	NA	<LOQ	NA	NA	NA	<LOQ - 2.88
Delta	6.90	1.43	<LOQ	9.48	3.16	0.53	2.35	4.24	<LOQ - 15.1
Gamma*	237	27	186	287	146	11	124	171	25.0 - 274
Total*	312	37	242	381	204	10	179	225	35.8 - 389

Continued

Tocopherols (mg/kg DW)	Non-GM <i>B. napus</i>				Non-GM <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Alpha	73.3	10.6	56.2	92.3	53.2	4.9	45.8	65.8	9.57 - 180
Beta	<LOQ	NA	NA	NA	<LOQ	NA	NA	NA	<LOQ - 2.88
Delta	6.95	1.27	<LOQ	8.81	3.49	0.76	1.81	5.47	<LOQ - 15.1
Gamma*	245	27	175	280	153	7	138	165	25.0 - 274
Total*	325	38	232	368	210	9	190	228	35.8 - 389

*Ranges between *B. napus* and *B. juncea* do not overlap. However, the ranges of *B. juncea* do overlap with the ILSI Crop Composition Database ranges for *B. napus*. This is true for both the RF3 (GM) and non-GM conventional entries.

Table 7. Anti-nutrients.

Anti-nutrients	RF3 <i>B. napus</i>				RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Phytic Acid (% DW)	1.73	0.20	1.33	2.19	2.14	0.61	1.02	3.3	0.94 - 3.88
Total Glucosinolates ($\mu\text{mol/g}$ DW)	9.06	1.60	7.01	12.9	12.2	3.1	7.89	17.7	0.41 - 32

Anti-nutrients	Non-GM RF3 <i>B. napus</i>				Non-GM RF3 <i>B. juncea</i>				ILSI-CCDB
	Mean	SD	Min	Max	Mean	SD	Min	Max	Range
Phytic Acid (% DW)	1.81	0.24	1.43	2.26	2.11	0.53	1.16	3.1	0.94 - 3.88
Total Glucosinolates ($\mu\text{mol/g}$ DW)	9.43	1.57	7.31	14.1	10.4	2.8	6.05	16.2	0.41 - 32

An assessment of the proximates and fiber data demonstrated that the ranges for RF3 *B. juncea* for all analytes overlapped with the ranges determined for RF3 *B. napus* and overlapped with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for RF3 *B. juncea* for each analytical parameter also fell within the ILSI Crop Composition Database range. The same observations were made when comparing the non-GM *B. napus* to the non-GM *B. juncea*. Therefore, it can be stated that the proximate and fiber data for RF3 *B. juncea* are essentially comparable to that of RF3 *B. napus*.

An assessment of the amino acid data demonstrated that the ranges for RF3 *B. juncea* for all amino acids overlapped with the ranges determined for RF3 *B. napus* and overlapped with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for RF3 *B. juncea* for each amino acid also fell within the ILSI Crop Composition Database range. The same observations were made when comparing the non-GM *B. napus* to the non-GM *B. juncea*. Therefore, it can be stated that the amino acid data for RF3 *B. juncea* are essentially comparable to that of RF3 *B. napus*.

An assessment of the fatty acid data demonstrated that the ranges for RF3 *B.*

juncea for myristic, palmitic, stearic, oleic, linolenic, eicosenoic, erucic, and lignoceric fatty acids overlapped with the ranges determined for RF3 *B. napus*. The ranges for RF3 *B. juncea* for palmitoleic, linoleic, arachidic, eicosadienoic, behenic, and nervonic fatty acids did not overlap with the ranges determined for RF3 *B. napus*. A similar trend was observed between the non-GM conventionals for *B. napus* and *B. juncea*, with the addition that the palmitic fatty acid range for non-GM *B. juncea* did not overlap with that of non-GM *B. napus*. Therefore, the observation that some of the fatty acid minor components of RF3 *B. juncea* did not overlap with RF3 *B. napus* may be attributed to differences among the two species, since a similar trend was apparent in the non-GM conventional comparison. The ranges for RF3 *B. juncea* for all fatty acids did overlap with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for RF3 *B. juncea* for each fatty acid also fell within the ILSI Crop Composition Database range. Therefore, it can be stated that the fatty acid profile for RF3 *B. juncea* is essentially comparable to that of RF3 *B. napus*.

An assessment of the mineral data demonstrated that the ranges for RF3 *B. juncea* for calcium, phosphorus, potassium, magnesium, sodium, iron, manganese, and zinc overlapped with the ranges determined for RF3 *B. napus*. The range for copper in RF3 *B. juncea* was higher than the range determined for RF3 *B. napus*. A similar trend was observed between the non-GM conventionals of *B. napus* and *B. juncea* in that copper was consistently higher in *B. juncea*, although the ranges in the non-GM conventional did overlap slightly. The ranges for all minerals for RF3 *B. juncea* overlapped with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for RF3 *B. juncea* for all minerals also fell within the ILSI Crop Composition Database range. Therefore, it can be stated that the mineral content for RF3 *B. juncea* is essentially comparable to that of RF3 *B. napus*.

An assessment of the tocopherol data demonstrated that the ranges for RF3 *B. juncea* for alpha-, beta- and delta-tocopherols overlapped with the ranges determined for RF3 *B. napus*. The ranges for RF3 *B. juncea* for gamma- and total tocopherols did not overlap with the ranges determined for RF3 *B. napus*. A similar trend was observed between the non-GM conventional counterparts of *B. napus* and *B. juncea*. Therefore, the observation that gamma- and total tocopherol did not overlap with RF3 *B. napus* may be attributed to differences among the two species, since a similar trend was apparent in the non-GM conventional comparison. The ranges for RF3 *B. juncea* for all tocopherols did overlap with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for RF3 *B. juncea* for each tocopherol also fell within the ILSI Crop Composition Database range. Therefore, it can be stated that the tocopherol content for RF3 *B. juncea* is essentially comparable to that of RF3 *B. napus*.

An assessment of the anti-nutrient data demonstrated that the ranges for RF3 *B. juncea* for phytic acid and total glucosinolates overlapped with the ranges determined for RF3 *B. napus* and overlapped with the ranges established within the ILSI Crop Composition Database for non-GM *B. napus*. The mean values for

RF3 *B. juncea* for each anti-nutrient also fell within the ILSI Crop Composition Database range. Therefore, it can be stated that anti-nutrient data for RF3 *B. juncea* is essentially comparable to that of RF3 *B. napus*.

3.4. Protein Expression Data

PAT and Barstar protein expression data obtained from RF3 *B. napus* and RF3 *B. juncea* samples collected from different field trials were visually inspected and compared to assess similarities or major differences between the two species.

Tissue samples for protein expression analyses were harvested from plants grown in the USA and Canada. RF3 *B. napus* samples were produced in 2014, while RF3 *B. juncea* samples were produced in 2017. Replicate samples of whole plant, root, raceme, and grain matrices were collected from single plots at multiple developmental stages from untreated and treated with glufosinate ammonium herbicide RF3 *B. napus* and RF3 *B. juncea*. Specifically, the treated RF3 *B. napus* and *B. juncea* entries received one application of Liberty® herbicide (glufosinate-ammonium) at 500 g ai/ha at the 2 - 6 leaf growth stage.

Protein expression levels of PAT/*bar* and Barstar were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as described by Privalle *et al.* [39]. The mean, standard deviation, and range of analyte concentrations in RF3 *B. juncea* and RF3 *B. napus* are presented PAT/*bar* and Barstar in **Table 8** and **Table 9**, respectively.

Table 8. PAT/*bar* protein ($\mu\text{g/g DW}$) Levels in RF3 *B. napus* and RF3 *B. juncea*.

Growth Stage	Matrix	RF3 <i>B. juncea</i> (Untreated)				RF3 <i>B. napus</i> (Untreated)			
		Mean	SD	Min	Max	Mean	SD	Min	Max
BBCH 14-16	Whole Plant	121.50	38.55	71.9	230.31	62.70	21.39	22.10	92.77
BBCH 30-39	Root	3.41	1.49	1.19	6.23	1.61	0.79	0.27	2.94
BBCH 30-39	Whole Plant	106.69	28.79	59.74	169.3	49.12	19.58	13.77	85.09
BBCH 57-65	Root	2.46	0.98	1.01	4.02	2.03	2.97	0.59	12.56
BBCH 57-65	Whole Plant	67.10	15.83	38.79	93.96	33.06	10.98	14.12	52.95
BBCH 57-65	Raceme	66.18	10.99	51.76	85.41	43.27	8.26	24.44	55.38
BBCH 87-99	Grain	2.66	2.21	1.07	6.75	0.75	0.10	0.51	0.92
(n = 12)					(n = 15)				
Growth Stage	Matrix	RF3 <i>B. juncea</i> (Treated)				RF3 <i>B. napus</i> (Treated)			
		Mean	SD	Min	Max	Mean	SD	Min	Max
BBCH 14-16	Whole Plant	117.06	28.47	71.34	167.49	63.71	37.53	23.91	181.94
BBCH 30-39	Root	2.94	1.87	0.28	5.47	2.56	2.33	0.95	10.57
BBCH 30-39	Whole Plant	99.12	35.00	41.11	141.04	56.84	22.66	28.11	107.38
BBCH 57-65	Root	1.90	1.03	0.47	3.15	1.62	0.85	0.36	3.53
BBCH 57-65	Whole Plant	58.89	20.27	28.56	84.15	43.20	20.15	6.49	89.33
BBCH 57-65	Raceme	62.58	12.01	41.05	87.75	40.59	13.30	12.54	62.63
BBCH 87-99	Grain	2.00	1.38	0.96	5.12	0.83	0.25	0.57	1.39
(n = 12)					(n = 15)				

¹Two of 15 data points missing. ²One of 15 data points missing.

Table 9. Barstar protein ($\mu\text{g/g DW}$) Levels in RF3 *B. napus* and RF3 *B. juncea*.

Growth Stage	Matrix	RF3 <i>B. juncea</i> (Untreated)					RF3 <i>B. napus</i> (Untreated)				
		¹ No. <LLOQ	Mean	SD	Min	Max	¹ No. <LLOQ	Mean	SD	Min	Max
BBCH 13-16	Whole Plant	(9)	0.03	0.06	<LLOQ	0.15	(15)	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 30-39	Root	(12)	<LLOQ	NA	<LLOQ	<LLOQ	(13) ²	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 30-39	Whole Plant	(12)	<LLOQ	NA	<LLOQ	<LLOQ	(11)	0.09	0.14	<LLOQ	0.40
BBCH 57-65	Root	(11)	<LLOQ	NA	<LLOQ	0.06	(15)	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 57-65	Whole Plant	(0)	0.17	0.11	0.05	0.46	(10)	0.10	0.13	<LLOQ	0.37
BBCH 57-65	Raceme	(0)	0.61	0.50	0.02	1.67	(0)	1.13	0.67	0.48	3.13
BBCH 87-99	Grain	(12)	<LLOQ	NA	<LLOQ	<LLOQ	(15)	<LLOQ	NA	<LLOQ	<LLOQ
(n = 12)						(n = 15)					
Growth Stage	Matrix	RF3 <i>B. juncea</i> (Treated)					RF3 <i>B. napus</i> (Treated)				
		¹ No. <LLOQ	Mean	SD	Min	Max	¹ No. <LLOQ	Mean	SD	Min	Max
BBCH 13-16	Whole Plant	(9)	0.02	0.03	<LLOQ	0.08	(15)	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 30-39	Root	(8)	0.03	0.05	<LLOQ	0.13	(15)	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 30-39	Whole Plant	(9)	0.02	0.04	<LLOQ	0.09	(14)	<LLOQ	NA	<LLOQ	0.33
BBCH 57-65	Root	(11)	<LLOQ	NA	<LLOQ	0.05	(15)	<LLOQ	NA	<LLOQ	<LLOQ
BBCH 57-65	Whole Plant	(0)	0.19	0.06	0.07	0.27	(14)	<LLOQ	NA	<LLOQ	0.18
BBCH 57-65	Raceme	(0)	0.66	0.44	0.04	1.32	(1)	1.20	1.03	<LLOQ	3.38
BBCH 87-99	Grain	(12)	<LLOQ	NA	<LLOQ	<LLOQ	(15)	<LLOQ	NA	<LLOQ	<LLOQ
(n = 12)						(n = 15)					

¹Number in parenthesis indicates the number of values that were <LLOQ. When a mean was determined, 1/2 the LLOQ was substituted in the calculations. ²Two of 15 data points missing.

Measured expression levels of PAT/*bar* and Barstar exhibited overlapping ranges when comparing RF3 *B. juncea* and RF3 *B. napus* with one exception. The PAT/*bar* range in untreated grain did not quite overlap between RF3 *B. juncea* (1.07 to 6.75 $\mu\text{g/DW}$) and RF3 *B. napus* (0.51 to 0.92 $\mu\text{g/DW}$). However, the PAT/*bar* range did overlap in the grain from the plots treated with Liberty[®] herbicide. This and the fact that the maximum level of PAT/*bar* in untreated *B. napus* (0.92 $\mu\text{g/DW}$) was very close to the minimum level of PAT/*bar* in untreated *B. juncea* (1.07 $\mu\text{g/DW}$) indicate that this difference is insignificant and not biologically relevant. In addition, protein expression has been shown to be highly variable due to many biological, technical, and environmental factors; therefore, interpretation of comparative results should also consider natural variation in protein expression levels [40].

Based on the observed overlapping ranges of measured analyte concentrations, it appears that PAT/*bar* and Barstar are similarly expressed when comparing RF3 *B. juncea* and RF3 *B. napus*.

3.5. Animal Feeding/Wholesomeness Studies

A 90-day rodent feeding study with RF3 *B. napus* found no adverse effects on the growth or health of Sprague Dawley rats fed a diet containing 15% RF3 *B. napus* meal. A 42-day broiler feeding study with RF3 *B. napus* found the growth and health of chickens fed a diet containing 10% RF3 *B. napus* meal were comparable to chickens on a nutritionally equivalent diet containing no RF3 *B. napus*. In addition, a 56-day channel catfish feeding study with RF3 *B. napus* concluded that catfish consuming a diet containing 30% RF3 *B. napus* meal demonstrated health and growth characteristics comparable to catfish consuming the conventional control variety.

After consideration of the factors listed above, new feeding studies were not scientifically justified and were not conducted with RF3 *B. juncea* because of its similar nature to RF3 *B. napus*. The RF3 *B. napus* data could be used as surrogate data for RF3 *B. juncea* since it is the same molecular insert, is similar in composition, and had similar PAT and Barstar expression levels as RF3 *B. napus*.

4. Conclusions

B. napus and *B. juncea* are closely related species within the same genus with a history of safe use. Both meet the definition of “canola” (<2% erucic acid in the oil and <30 $\mu\text{mol/g}$ glucosinolates in the meal). Molecular characterization has established that the CQ RF3 *B. juncea* variety is fully comparable to RF3 *B. napus*. Composition data between RF3 *B. napus* and CQ RF3 *B. juncea* were comparable between the two species. Protein expression analyses demonstrated that PAT/*bar* and Barstar are similarly expressed when comparing CQ RF3 *B. juncea* and RF3 *B. napus*. Since both produce the same proteins, existing PAT/*bar* and Barstar protein studies were applicable in the safety of CQ RF3 *B. juncea*. Animal feeding studies with RF3 *B. napus* demonstrated no adverse effects in rodents, broiler chickens, or channel catfish.

A review of the scientific data generated during the development of RF3 *B. napus* and Canola Quality RF3 *B. juncea* have demonstrated close comparability between the two species.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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