**PROJECT CODE**

**BRE0813**

**TITLE**

**Understanding and Improving Lentil and Chickpea Seed Quality - Reduction of Raffinose Family Oligosaccharides (RFO)**

**INVESTIGATORS**

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**STUDY SPONSORS**

Saskatchewan Pulse Growers.
Funding also received from NSERC and Agriculture and Agri-Food Canada Internationalization Program, as well as scholarships to two Ph.D. students.

**TYPE OF STUDY**

**GENETIC IMPROVEMENT – ANTI-NUTRITIONAL FACTORS**

**OBJECTIVES**

The main objective of the research project was to develop strategies to reduce RFO concentration in lentils and chickpeas. This was accomplished by:

1. Characterising the amounts of RFO in lentil and chickpea germplasm;

2. Characterising the synthetic pathway by isolating the principal genes involved in the RFO synthetic pathway;

3. Studying the expression profile of the key genes;

4. Developing genetic maps of selected chickpea and lentil cultivars; and

5. Generating DNA-based molecular markers, to screen germplasm and use in marker-assisted selection to develop genotypes with reduced RFOs in seed.

**WHY STUDY NEEDED**

Compared to grains, legume seeds have higher concentration of soluble carbohydrates belonging to the raffinose family oligosaccharides (RFO), including stachyose and verbascose. The RFO and other non-starch polysaccharides (dietary fibers), contribute up to one-eighth of the seed dry weight in chickpeas. The RFO in human diet and animal feed have both beneficial and undesirable effects. Humans lack the RFO-digesting enzyme alpha-galactosidase, and as a result RFOs pass undigested into the lower gut, where they are fermented by microbes. This produces

gas and flatulence, and in extreme cases diarrhea. This is a major constraint in human pulse consumption in North America and Europe. Seeds with reduced RFO content could lead to increased pulse consumption in the human diet. Knowledge of the RFO content of different genotypes can guide breeders' selections and lead to new cultivars tailored to specific markets.

**HYPOTHESIS**

We hypothesize that:

1. Genotypes of chickpeas and lentils vary in their RFO pathway expression and final concentration, and
2. Markers connected to the pathway can be used to identify lower RFO genotypes for development of new genotypes.

**STUDY DESIGN**

1. The RFO content of mature seed was determined using an HPLC method with pulsed amperometric detection.
2. RNA was prepared from developing seed tissue, and cDNA libraries made. A new RNA method was developed for developing lentil seeds, as existing methods were not efficient for this tissue. Existing databases were used to guide isolation of the lentil forms of galactinol synthase (GS), raffinose synthase (RS), and stachyose synthase (SS) genes.
3. In chickpeas, the accumulation of different sugars during seed development was analysed; while in lentils, the expression of the responsible genes was analysed.
   Association mapping was used to try to associate markers with low and high RFO accumulation. For lentils a biparental set of lines was available for part of the project. High-throughput marker systems, like Diversity Array Technology (DArT®), were applied to statistically analyse association between relatively low and high RFO accumulation and DNA markers.
4. Identification of targeted DNA-based molecular markers to screen germplasm for developing lentil and chickpea cultivars with reduced RFO was assayed. For chickpeas, activity of the pathway enzymes in low/high RFO pairs was assessed with the intent of identifying the limiting enzyme.

**FINDINGS**

1. The RFO content of mature seed of 171 chickpea lines was determined. Stachyose was generally highest, with raffinose and small amounts of verbascose. There was substantial variation; for example, stachyose varied from 0.18 to 2.38 g/100g. However, the major Canadian-grown cultivars showed a limited range, possibly due to the limited diversity in their parents. In chickpea seeds, raffinose, stachyose and verbascose content showed a moderate broad sense heritability (0.25-0.56) when lines were grown at two locations (India field and Saskatoon greenhouse).

Lentils of 20 lines showed less than two-fold variation in content of each RFO, probably also due to limited diversity in their parents. When 168 additional genotypes from ICARDA were examined, however, greater variability was found.

To examine heritability, eleven lentil cultivars (seven green cotyledons and four red cotyledons) grown in ten environments were analyzed by the content and composition of soluble carbohydrates in seeds. The broad sense heritability for RFO was high (0.85).

2. Lentils have two major isoforms of GS gene, one of RS and one of SS.
3. GS was expressed until late in seed development, while RS and SS were mostly expressed during the period of rapid seed maturation and desiccation, when the products of these enzymes also accumulated most rapidly. Association mapping showed some connection between particular genome regions/markers and RFO traits. DArT markers were used to analyze population structure and genetic relationships within a specifically designed chickpea germplasm collection including wild, cultivated, and some advanced breeding lines, for seed quality traits. Association mapping identified genomic regions associated with variation in total RFO, raffinose, stachyose, and verbascose concentrations, although associations were not very strong. These potential genomic regions need to be dissected further to develop diagnostic markers for use in chickpea breeding programs. The DArT markers provided significant information about population structure, and association mapping is a useful approach to identify important genomic regions associated with RFO related traits in chickpea, although this is affected by the narrow genetic base in the domestic forms of both species, particularly the Canadian cultivars. This technique suggests marker-trait associations which then need to be verified independently. Further work is in progress.
4. The analysis of chickpea enzyme activity during development showed an association of RFO enzyme activity with the period of seed desiccation, for which it may be necessary. High RFO genotypes expressed about 2- to 3-fold higher activity of all RFO biosynthetic enzymes compared with low RFO concentration genotypes; GS is suggested as the most likely limiting factor. In lentils, two forms of GS peaked in expression at different times, and two SS forms also showed different expression profiles, with one barely detectable. Sequencing of multiple genotypes showed some variants in sequence, but they could not be associated with differences in RFO concentration.

**SIGNIFICANCE OF STUDY**

This project has identified natural variation in RFO in chickpea and lentil germplasm, improved our understanding of RFO biosynthesis during seed development, identified and characterized major RFO biosynthetic genes and their expression during seed development. We also identified some key genomic regions associated with RFO concentration in chickpeas and identified allelic variation in two RFO biosynthetic genes.

**PUBLICATIONS, PRESENTATIONS, EDUCATIONAL MATERIALS PRODUCED**

**Peer-Reviewed Papers**

Gangola, M.P., Jaiswal, S., Kannan, U., Gaur, P.M., Båga, M., and Chibbar, R.N. (2016) Galactinol synthase enzyme activity influences raffinose family oligosaccharides (RFO) accumulation in developing chickpea (*Cicer arietinum* L.) seeds. Phytochemistry 125: 88-98.

Kannan, U., Ganeshan, S. and Chibbar, R.N. (2014) A hexa decyl trimethyl ammonium bromide (CTAB) based protocol to isolate high quality RNA in adequate quantities for gene expression analyses in developing seeds of lentils (*Lens culinaris* Medik). Gene Expression to Genetical Genomics 7: 7-16.

Gangola, M.P., Jaiswal, S., Khedikar, Y.P. and Chibbar, R.N. (2014) A reliable and rapid HPAECPAD method for soluble sugar and RFO analysis in chickpea. Food Chemistry 154: 127-133.

Gangola, M.P., Khedikar, Y.P., Gaur, P.M., Båga, M. and Chibbar, R.N. (2013) Genotype and growing environment interaction shows a positive correlation between substrates of raffinose family oligosaccharides (RFO) biosynthesis and accumulation in chickpea (*Cicer arietinum* L.) seeds. J. Agric. Food Chem 61: 4943 – 4952.

Jukanti, A.K., Gaur, P.M., Gowda, C.L.L. and Chibbar, R.N. (2012) Chickpea: Nutritional Properties and Its benefits. British J Nutrition 108(S1):11-26.

Tahir, M., Båga, M., Vandenberg, A. and Chibbar, R.N. (2012) An assessment of raffinose family oligosaccharides (RFO) and sucrose concentration in the genus *Lens*. Crop Science 52: 17131720.

Tahir M, Lindeboom N, Båga M, Vandenberg A and Chibbar RN. (2011) Composition and correlation between major seed constituents in selected lentil (*Lens culinaris* Medik) genotypes. Can. J. Plant Sci. 91: 825-835.

Tahir, M, Vandenberg, A, and Chibbar, RN. (2011) Influence of environment on seed soluble carbohydrates in selected lentil cultivars. J. Food Composition Analysis 24:596-602.

Chibbar, R.N., A. Priyatharini, and R. Hoover 2010. Molecular diversity in pulse seed starch and complex carbohydrates and their role in human nutrition and health. Cereal Chemistry 87(4): 342352.

**Conference Presentations**

Chibbar, R.N. (2014) Genomic strategies to identify and characterize chromosomal regions associated with agronomic and grain quality traits in cereals and pulses. International Conference on Computer applications in manufacturing, food technologies and bio-nano engineering, Universal Institute of Technology, Garhi (Hansi), Distt. Hisar (Haryana), India. February 21-22, 2014.

Chibbar, R.N. (2014) Genetic strategies for in plant modification of storage carbohydrates to add value to cereals and pulses. International conference on New approaches in Food security and value addition: Technological and genetic options. R.B.S. Engineering and Technical campus, Bichpuri (Agra), India. February 17 – 19, 2014.

Chibbar, R.N. (2014) Genetic strategies for cereals and grain legumes improvement to achieve food and nutrition security. Assocom 4th International Grains Conference 2014. India International Centre, New Delhi, India. February 10 – 11, 2014.

Chibbar, R.N. (2013) New genomics markers – how pulses fit – Genetic variation detection and utilization in pulse crops. Pulse Industry Roundtable, Delta Winnipeg, Winnipeg, Manitoba, Canada, December 10-11, 2013

Chibbar, R.N. (2013) Grain carbohydrates digestibility – a critical factor to ameliorate hunger and improve human health. International seminar on "Cutting edge science and technologies towards food, environment and health", Göttingen, Germany, September 2 – 4, 2013.

Chibbar, R.N. and Båga, M. (2013) Biotechnological strategies to improve cereal and pulse grain carbohydrates for innovative food uses to alleviate hunger and malnourishment.1st International conference on Innovations in Food Processing, Value Chain Management & Food Safety (IFpvs), National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonepat, Haryana, India. January 10 – 11, 2013. Keynote speaker.

Gangola, M.P., Khedikar, Y.P., Thudi, M., Wang, R., Jaiswal, S., Gaur, P.M., Båga, M., Varshney, R.K. and Chibbar, R.N. (2012) Genetic analysis of seed carbohydrates in chickpeas (*Cicer arientinum* L.). VI International Conference on Legume Genetics and Genomics, Hyderabad Marriot Hotel & Convention Centre, Hyderabad, India, October 2 – 7, 2012.

Chibbar, R.N. (2012). Collaborative research projects with Indian institutions to improve grains and pulses for improved human health. Engaging India: Human and social dimensions of science and technology, Shastri Indo-Canadian Institute, University of Calgary, Calgary, Canada, June 3 - 4, 2012.

Chibbar, R.N. (2011) Genomics strategies for stress tolerance and grain quality improvement in cereal and pulse crops. ISTP-India Canada Workshop, NRC, PBI Saskatoon, SK, Canada February 13 – 15, 2011.

**Poster Presentations**

Gangola, M.P. Jaiswal, S., Båga, M. and Chibbar, R.N. (2013) Understanding raffinose family oligosaccharide biosynthesis during chickpea seed development.

20th Annual Life & Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada March 15, 2013. (First prize in poster competition in Plant Sciences section).

Kannan, U., Ganeshan, S., Gangola, M.P., and Chibbar, R.N. Characterization of selected raffinose family oligosaccharides biosynthetic genes during seed development in lentil (*Lens culinaris* Medik). 20th Annual Life & Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada March 15, 2013. (Second place prize in poster competition in the Plant Sciences section).

Kannan, U., Ganeshan, S. and Chibbar, R.N. (2012) Expression analysis of raffinose family oligosaccharides (RFO) biosynthetic genes in developing lentil (*Lens culinaris* Medik) seeds. Ninth Canadian Pulse Research Workshop, St. Catharines, Ontario, Canada November 7-9, 2012. (Oral presentation by UK; won the best oral presentation by a student award).

Gangola, M.P., Khedikar, Y.P., Gaur, P.M., Båga, M., Varshney, R.K. and Chibbar, R.N. (2012) A study of natural variation in Raffinose family Oligosaccharides (RFO) in chickpea. Annual meeting, American Association of Cereal Chemists International (AACCI), Hollywood, Florida, USA September 30 to October 3, 2012. (oral presentation by MPG).

Khedikar, Y., Gangola, M., Thudi, M., Gaur, P., Varshneya, R., and Chibbar, R.N. (2012) Genome wide association analysis of raffinose family oligosaccharides in chickpea (*Cicer arietinum* L.) Plant Biology 2012, Annual meeting of the American Society of Plant Biologists, Austin, Texas, USA, July 20-24, 2012.

Gangola, M., Khedikar, Y., Gaur, P., Båga, M., Varshney, R. and Chibbar, R. 2012. Variation in important seed constituents among various chickpea genotypes. The 19th Annual Life & Health Sciences Research Day, March 9, 2012, University of Saskatchewan, Saskatoon, Saskatchewan, Canada pp125.

Kannan, U., Ganeshan, S. and Chibbar, R.N. 2012. Selected raffinose family oligosaccharide (RFO) biosynthetic genes show peak expression in mid to late seed developmental stages in lentil (*Lens culinaris* Medik). The 19th Annual Life & Health Sciences Research Day, March 9, 2012, University of Saskatchewan, Saskatoon, Saskatchewan, Canada pp127. (Winner Best Poster award in Plant Sciences section)

Gangola, M., Khedikar, Y., Gaur, P., Båga, M., Varshney, R. and Chibbar, R. (2012) Variation in important seed constituents among various chickpea genotypes. Ist ICC India Grains Conference: Developments in Grain Science and Technology to Ensure High Quality, Safe and Healthy Grainbased Foods. 16 – 18, January, New Delhi, India. pp 71.

Kannan, U., Ganeshan, S. and Chibbar, R.N. (2012) Isolation of selected raffinose

family oligosaccharide biosynthetic genes in lentil (*Lens culinaris* Medik.) 1st ICC India Grains Conference: Developments in Grain Science and Technology to Ensure High Quality, Safe and Healthy Grain-based Foods. 16 – 18, January, New Delhi, India. pp74. (Winner Best Poster Award).

Kannan, U., Ganeshan, S., and Chibbar, R.N. (2011) Expression analysis of selected raffinose family oligosaccharide (RFO) biosynthetic genes in developing lentil (*Lens culinaris* Medik). American Association of Cereal Chemists International, annual meeting, Palm springs, California, USA October 16 – 18, 2011

Khedikar, Y.P., Gangola, M.P., Gaur, P.M., Båga, M., Varshney, R.K., and Chibbar, R.N. (2011) Molecular diversity analysis in a chickpea germplasm collection assembled for grain quality traits. Poster 24 The 18th Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 11, 2011.

Gangola, M.P., Khedikar, Y.P., Gaur, P.M., Båga, M., Varshney, R.K. and Chibbar, R.N. (2011) Variation for seed constituents in chickpea germplasm collection. Poster 91. The 18th Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 11, 2011

Kannan, U., Ganeshan, S. and Chibbar, R.N. (2011) Expression analysis of selected raffinose family oligosaccharides (RFO) biosynthetic genes in lentil (*Lens culinaris* Medik). Poster 93 The 18th Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 11, 2011 The 18th Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 11, 2011

Tahir, M., Båga, M., Vandenberg, A., and Chibbar, R.N. (2011) An assessment of raffinose family oligosaccharides (RFO) and sucrose concentrations in seeds of genus *Lens*. Poster 96 The 18th Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 11, 2011

Kannan, U., Båga, M. and Chibbar, R.N. (2010) Cloning of genes in raffinose family oligosaccharide biosynthesis in legumes. The 17th Annual Life and Health Sciences Research Day, University of Saskatchewan, Saskatoon, SK, Canada, March 17th, 2010, pp 86.

**Book chapters**

Ganeshan S, Gaur P, Chibbar RN. 2013 Biotechnological Strategies to Enhance Abiotic Stress Tolerance in Pulse Crops. In: Improving crop Productivity in Sustainable Agriculture (eds. Tuteja N, Gill SS, Tuteja R) Wiley-VCH Verlag & Co. KGaA, Weinheim, Germany, pp 423 – 450.

**VALUE TO PRODUCERS**

This project has identified lentil and chickpea genotypes that are being used to develop varieties with reduced RFO concentration. The identified DNA markers, when completely characterized, will accelerate development of lentil and chickpea varieties with reduced RFO, thus causing less stomach discomfort. This will promote consumption of lentils and chickpeas or their products. The DNA-based markers identified in this project can also be used for other pulse crops such as peas and beans and reduce the time needed to respond to changing market demands.