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Transgenic Wheat with Improved Agronomic Performance and Value-Added Traits*.

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Wheat: an important crop

Wheat is one of the major staple crops of the world with production reaching close to 600 million tons in the crop year 1997-98. It is grown in most of the countries of the world and in several climatic and edaphic zones. Traditional plant breeding has resulted in the development of an estimated 25,000 different wheat cultivars with increased yield, enhanced grain quality, or improved resistance to abiotic and biotic stresses (Feldman et al., 1995). Wheat was introduced in to Canada at Port Royal (now Annapolis Royal), Nova Scotia, in about 1605 (Campbell and Shebeski, 1986). The subsequent spread of wheat cultivation to other parts of Canada coincided with the movement of settlers westward. The history of Canadian wheat production is closely tied to the development of Western Canada (DePauw et al., 1995). Extensive efforts by the plant breeders have lead to the development of numerous cultivars of wheat for Canadian producers. The first transgenic wheat in Canada was produced at the National Research Council of Canada's Plant Biotechnology Institute (Nehra et al., 1994). During the past fifty years, Canadian wheat production has accounted for almost five percent of the world wheat production and about twenty percent of world wheat trade. In the last three years (1995-98), wheat has been grown on over 11.1 to 12.2 million hectares in Canada, producing about 25 to 30 million metric tons of grain. Eighty percent of the wheat grown in Western Canada belongs to the hard red spring class of wheat. The wheat industry has

been a major foreign exchange earner for Canada. Grain exports contributes about five billion dollars annually to the Canadian economy.

Transgenic plants – Vehicles for application of biotechnology.

Since the mid-fifties, a significant increase in yield of wheat has been achieved world-wide. Average increases in wheat yields during the seventies, eighties, and nineties have been four and a half, three, and two percent. These increases so far have been able to keep pace with the demands of the growing world population. Despite the increase in world wheat production, the world wheat stocks hit record lows in 1996-97. To support the growing global population and shrinking area of arable land, world wheat production has to be significantly increased. Therefore, the emergence of biotechnology in mid-eighties and nineties was a significant event for crop improvement. Biotechnology has contributed to the development of novel and exploitable methods to genetically control and alter plant development, performance, and products, in concert with traditional plant breeding. Genetic engineering is a key component of biotechnology, and it is defined as the production of targeted novel gene combinations by laboratory techniques. Genetic engineering allows the insertion of genes into plant cells, which can subsequently be regenerated into transgenic plants. The selected transgenic plants transmit the inserted genes to their progeny in a predictable manner and exhibit the phenotype conferred by the inserted gene(s).

Interaction of technologies to produce transgenic plants

Transgenic plants result from the successful interaction of three technologies: (i) DNA delivery techniques, (ii) techniques to regenerate fertile plants from undifferentiated cells, and (iii) recombinant DNA techniques to isolated and characterize genes and to construct gene expression vectors for regulated expression of inserted genes. Among all the important crops including cereals, wheat was one of the last crops to be genetically transformed (Vasil et al, 1992; Weeks et al., 1993; Nehra et al., 1994; Becker et al., 1994). Recently, Bommineni et al. (1997) has produced glufosinate-ammonium resistant transgenic durum wheat. The most important limitation was the lack of a DNA-delivery technique. *Agrobacterium tumefaciens,* a soil-borne bacterium, is the most commonly used vector to deliver DNA into plant cells. However, until recently *Agrobacterium* was

not able to infect cereal cells (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Tingay et al., 1997), and thus, could not be used to deliver genes into cereals. The genetic transformation of cereal cells was made possible by the development of a biolistics technique by Sanford and co-workers at Cornell university, USA (Sanford et al., 1987). Biolistics is a technique by which gold or tungsten particles (1-2 micron microprojectiles) coated with DNA are propelled with high pressure into plant cells. The DNA from the microprojectiles is then integrated into the genome of the plant cell. Microprojectile bombardment is the most succesful DNA delivery method to produce transgenic cereals (Chibbar and Kartha, 1994).

The next obstacle was to exploit the totipotency of plant cells to regenerate a fully fertile plant from the cell carrying the inserted gene. In wheat immature zygotic embryos are the most commonly used explants for *in vitro* culture and the regeneration of fertile plants (Nehra et al., 1995; Bommineni et al., 1997). We also demonstrated that immature embryos can accept and express biolistically delivered genes (Chibbar et al., 1991). Researchers in the Cereal Biotechnology Group dissected the various parts of the immature zygotic embryos and found that the removal of the embryo axis and subsequent culture of isolated scutella considerably increased the regeneration efficiency of wheat and other cereals. By using this technique, we could obtain from a single scutellum as many as fifteen fully fertile wheat plants. This regeneration system is called the Enhanced Regeneration System (ERS®) (Nehra et al., 1996). The ERS enriches for embryogenic callus, enhances the production of somatic embryos, expedites the development of plantlets and is genotype-independent. We have successfully used this system to produce transgenic wheat belonging to many classes. In addition to culture of isolated scutellum, we have used immature inflorescences and calli derived from culture of immature anthers, as explants to produce transgenic wheat.

The use of suitable gene expression vectors is also important in order to produce transgenic plants. In a genetic transformation experiment, irrespective, of the technique used to deliver the genes, only a few cells receive the DNA and a small proportion of these cells integrate the DNA into their genomes. Selectable marker genes are introduced that will confer resistance to chemicals, that are lethal to plant cells. Antibiotic and/or herbicide resistance genes are commonly used in genetic transformation protocols. In

addition, the genes should also express at a high level in order to impart tolerance to high levels of chemicals in the culture medium. Our results showed that the rice actin promoter with its first intron/exon (McElroy et al., 1990) was highly active in cereal cells (Chibbar et al., 1993).

Production of transgenic wheat

Scutella isolated from spikes harvested at 12 to 14 days post-anthesis (dpa) and pre-cultured for one, two, or four days were bombarded with gold particles (1 micron diameter) coated with the requisite plasmid DNA (gene expression vector). To demonstrate genetic transformation, we used a monocot expression vector, pRC-62, that contains a gus::nptII fusion gene under the control of the rice actin promoter (Nehra et al., 1994). The bombarded scutella were cultured in the dark for one week and in diffuse light for another week, on a selection medium containing an aminoglycosidic antibiotic such as Geneticin or Paromomycin. After the scutella were transferred to light, the green spots that developed on cultured scutella were carefully isolated and nurtured into plantlets. The plantlets were subsequently transferred to soil. The leaves were collected for biochemical and molecular assays to confirm the presence of the gene and its functionality. Active β-glucuronidase (GUS) and neomycin phosphotransferase (NPT II) enzyme was demonstrated by enzyme assays. Histochemical assays revealed GUS activity in pollen, ovaries and seeds. Southern blot hybridization was used to detect the insertion of *gus* and *nptII* genes into the wheat genome and to follow the transmission of transgene into the progeny.

Transgene stability and inheritance in spring wheat

Inheritance of the transgene in two independent T_4 transgenic lines of the cultivar Fielder (transformed with the plasmid pRC62) was assessed by making reciprocal crosses to the untransformed Fielder and molecular analyses of the F1 and F2 generations. Histochemical Gus staining of reciprocal seeds gave similar result to the parents, indicating the lack of a cytoplasmic effect on inheritance. The transgene was stably inherited. However, there was variation in the inheritance of the transgene in F_2 seeds, but overall there was a tendency towards a two gene insertion ratio. Methylation of DNA was observed in one of the crosses as revealed by southern analysis of genomic DNA restricted with methylation-sensitive enzymes. Plants with a methylation problem had

low NPTII and GUS activities, even though PCR and Southern analyses showed the presence of gus and nptII genes. Northern and RT-PCR analyses will reveal the effect of methylation at the transcript level.

Agronomic and field performance of transgenics

Three transgenic wheat lines carrying marker genes have been field-tested at Saskatoon for four years (1994-97). Results indicate that the three lines differed in their agronomic performance. One line, A1, was very similar to the non-transgenic control in phenotype as well as yield. However, another line, A3, had slightly lower yield, while the third line, A6, showed some sterility problems and consequently reduced grain yield. These variations in performance are commonly observed in transgenic plants, but the absence of a significant yield reduction in one of the three lines is nevertheless encouraging, since it suggests that the presence of marker genes per se do not have a negative effect on wheat growth and development.

Applications of transgenic technology

Transgenic technology can be used to produce wheat that is resistant to biotic and abiotic stresses. Examples of abiotic stresses are drought, heat, and cold. Transgenic technology has been very successful in developing crops that are herbicide-tolerant allowing efficient weed control, or plants that are insect or disease resistant. For example pests and diseases cause an annual loss of 100 million tons of grain, an amount of grain large enough to feed all of South America. Transgenic technology has been able to produce a large number of herbicide, insect and disease resistant cereals. A major application of transgenic technology is to improve grain quality. The most obvious targets for transgene technology to improve grain quality include: (i) increasing the protein content, (ii) increasing the essential amino acids such as lysine, (iii) increasing the high molecular weight glutenins to improve bread making properties of wheat flour (Altpeter et al., 1996; Blechl and Anderson, 1996; Barro et al., 1997), (v) increasing heat stability of β-glucanase in barley (Jensen et al., 1996), (vi) modifying starch structure (Chibbar et al., 1997), (vii) producing pharmaceuticals in grains, and (viii) producing neutraceuticals. We have focused our research to modify starch structure in wheat in order to meet the demands of consumers of Canadian wheat.

Herbicide Resistant Wheat

Glufosinate-ammonium-resistant wheat was produced by microprojectile bombardment of isolated scutella with a gene expression cassette carrying the bar gene. The transgenic wheat survived spraying with herbicide (400 and 800g active ingredient per hectare), while all the weeds were killed. In a limited number of field trials conducted by AgrEvo, an approximate 40% yield advantage was observed in sprayed transgenic wheat as compared to control non-sprayed wheat under weed pressure. Molecular analysis of the transgenic wheat revealed that the inserted gene was stably imherited over the six generations studied. Using a similar strategy, we also produced glyphosate-resistant wheat that survived applications of Roundup while all the weeds present were killed.

Improvement of grain quality – Modification of starch structure in wheat

Seventy percent of Canadian wheat is exported accounting for one-fifth of the global wheat trade. Customers of Canadian wheat have changed over the years. Bacon (1995) predicted that the Pacific rim, the Middle East and South America are the future major consumers of Canadian wheat. In 1997, Iran, China, Indonesia, and Japan were the major customers for Canadian wheat (Grain Matters, Jan-Feb, 1998). In Iran wheat is mostly consumed as flat breads, while noodles are the most common mode of wheat consumption in the other three countries (McGregor 1997). Starch structure and quantity are important determinants for noodle quality (Miura and Tanii, 1994; Wang and Seib, 1996).

About two-thirds to three-quarters of the wheat grain is composed of starch (Hucl and Chibbar, 1996), which can be fractionated into two types of glucan polymers: (i) the almost linear amylose molecules, and (ii) the heavily branched amylopectin. The ratio of amylose to amylopectin in starch determines its physico-chemical properties and thereby its end-use. Starch is stored in the form of discrete granules known as starch granules. In wheat there are two types of starch granules, the large A type (15 microns or more) and the small B type (less than 10 microns) granules. The A granules account for 30% by number but 90% of starch weight. The remainder granule portion is made up of small B type starch granules. Starch biosynthesis in cereals is accomplished by the concerted action of several enzymes that include adenosine glucose pyrophosphorylase (ADPGPP),

starch synthases (SS and GBSS), starch branching enzyme enzymes (SBE) and starch debranching enzymes (DBE). Inactivation of any of the starch biosynthetic genes by natural mutations in maize, barley, and rice results in an altered starch structure. Such mutants are not likely to develop spontaneously in wheat due to its hexaploid genome (2n=6X=42 AABBDD). We have employed two strategies to change the starch structure in wheat. In the first strategy, we used molecular biology techniques to identify wheat germ plasm with mutations in genes encoding granule bound starch synthase (GBSS), which is the enzyme responsible for amylose synthesis. Traditional plant breeding techniques were thereafter used to recombine the mutant lines to produce prototype waxy wheat with an endosperm starch with only amylopectin. In the second strategy, we used genetic engineering to regulate the starch branching enzyme (SBE) activity in wheat kernels and these experiments resulted in altered starch structure.

Production of waxy wheat

Granule-bound starch synthase1 (GBSS I) is the key enzyme involved in amylose production. In cereals, GBSS1 is encoded by the waxy locus (*wx*) and is termed waxy protein (Wx). Fully waxy wheat lacks all three GBSS I isoproteins. Canadian and other wheats have been evaluated for mutations at the waxy loci (Demeke et al., 1997). Null alleles for GBSS1 A, B, and D isoproteins have been identified from screening of germplasm by one and two-dimensional sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE). Prototype waxy wheat (amylose-free) has been identified by conventional crossing of lines with null alleles. Wheat grain from fully waxy wheat, when stained with iodine solution gave brown colour as compared to normal wheat that stained blue. Spring type waxy wheat has also been identified and awaits detailed starch analysis. The partially and fully waxy wheat lines have been crossed to Canadian wheat cultivars.

Production of transgenic wheat with altered starch structure

We have used genetic engineering to regulate the starch branching enzyme activity in wheat. The starch branching enzymes (SBE) catalyze formation of α1,6-branches on the glucan polymer and together with starch synthases control the amount of amylopectin produced. At least two types of SBE, SBEI, and SBEII, are encoded by the wheat genome and cDNA corresponding to both isoforms have been isolated and characterized

(Båga et al., 1998b; Nair et al. 1997; Repellin et al. 1997). To alter the amount of amylopectin accumulated in wheat starch, we have constructed expression vectors carrying monocot promoters controlling expression of wheat *Sbe1* cDNA in the sense and anti-sense orientation. These expression cassettes have been stably introduced into various wheat cultivars from different classes. Analysis of branching enzyme (BE) activity, in transformed hard red spring wheat carrying the anti-sense *Sbe1* gene cassette, revealed different BE activity levels in 14 dpa kernels. One of the transgenic wheat lines exhibited a ten-fold lower BE activity level as compared to non-transgenic wheat. The low BE activity during kernel development correlated with a higher amount of high-molecular weight glucan in extracted starch from mature kernels. The modified starch was also found to be less crystalline than normal starch when analyzed by X-ray crystallography. Differential scanning calorimetry analysis revealed that transgenic starch gelatinized at a lower temperature and had a significantly lower enthalpy value as compared to starch extracted from kernels of non-transgenic wheat plants. The transgenic wheat (cv Columbus and cv Fielder) with altered starch structure will be field-tested in the spring/summer of 1998.

Challenges and opportunities

Genetic transformation protocols have been developed for most of the cereals including wheat. We have produced transgenic wheat belonging to four major Canadian classes of wheat. To successfully employ transgenic technology for wheat improvement, factors controlling the stability and expression of transgenes needs to be studied (Båga et al., 1998a). A distinct advantage of transgenic technology is that it can incorporate genes from diverse origins into wheat to generate improved agronomic performance or incorporation of value-added traits. Transgenic technology can be used to reduce producer inputs, for example: (i) inserting nitrate transporter gene that can increase the efficiency of nitrogen up-take, (ii) inserting disease and insect resistance genes that will give inherent resistance and exclude the application of expensive chemicals that are also harmful to the environment, or (iii) improving the abiotic stress tolerance of wheat to increase the area under wheat cultivation. With starch as an example, we have demonstrated the value-added products that can be produced in wheat using the transgenic technology. A multitude of carbohydrate, protein, or other useful products

can be produced in wheat grains by the regulated expression of inserted genes isolated from other organisms. A dialogue and close interaction between the wheat industry and the research community is needed in order to formulate specific objectives and action plan to insure the continued success and maintenance of the competitive edge of the Canadian grain industry in the global market place.

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Literature cited

Altpeter F, Vasil V, Srivastava V, and Vasil IK. (1996) Expression of a novel high-molecular-weight glutenin subunit 1Ax1 gene into wheat. Nature Biotechnology 14: 1155-1159.

Bacon GB. (1995) Canadian wheat and barley market opportunities. Value-Added Cereals Through Biotechnology. June 10-13, 1995 Saskatoon, Canada pp 30.

Båga M, Chibbar RN and Kartha KK. (1998a) Expression and regulation of transgenes for selection of transformants and modification of traits in cereals. In: Advances in Cellular and Molecular Biology of Plants Vol. 5: Molecular Improvement of Cereal Crops. Ed. I.K. Vasil, Kluwer Publishers (in press).

Båga M, Mallard CS and Chibbar RN. (1998b) A starch branching enzyme in wheat is encoded by mRNA transcribed from alternative promoters. Plant J.

Barro, F, Rooke L, Bekes F, Gras P, Tatham AS, Fido R, Lazzeri PA, Shewry PR, and Barcelo P. (1997) Transformation of wheat with high molecular weight subunit genes result in improved functional properties. Nature Biotechnology 15: 1295-1299.

Becker D, Brettschneider R and Lörz H. (1994). Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. The Plant J 5: 299-307.

Blechl AE and Anderson OD. (1996) Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnology 14: 875-879.

Bommineni VR and Jauhar PP. (1997) An evaluation of target cells and tissues used in genetic transformation of cereals. Maydica 42: 107-120.

Bommineni VR, Jauhar PP and Peterson, TS. (1997) Transgenic durum wheat by microprojectile bombardment of isolated scutella. Jl. of Heredity 88: 475-481.

Campbell AB and Shebeski LH. (1986) Wheat in Canada-past and present. In Wheat Production in Canada- A review. Eds. AE Slinkard and DB Fowler. Univ. of Saskatchewan, Saskatoon, SK. pp 1-14.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, and Wan Y. (1997). Genetic transformation of Wheat Mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115: 971-980.

Chibbar RN, Båga M, Repellin A, Demeke T, and Hucl P. (1997). Genetic Engineering of wheat for starch modification. In: Proceedings, International Wheat Quality Conference, (eds. JL Steele and O.K. Chung), Manhattan, Kansas. Grain Industry Alliance, Manhattan, Kansas pp 249-259.

Chibbar RN and Kartha KK (1994). Transformation of plant cells by bombardment with microprojectiles. In Biotechnological applications of plant cell cultures (eds. PD Shargool and TT Ngo). CRC Press Boca Raton, USA pp 37-60.

Chibbar R.N, Kartha KK, Leung N, Qureshi J, and Caswell K. (1991). Transient expression of marker genes in immature zygotic embryos of spring wheat (*Triticum aestivum* L.) through microprojectile bombardment. Genome 34(3): 453-460.

Chibbar RN, Kartha KK, Datla RSS, Leung N, Caswell K, Mallard CS, and Steinhauer L. (1993). The effect of different promoter-sequences on transient expression of gus reporter gene in cultured barley (*Hordeum vulgare* L.) cells. Plant Cell Reports 12: 506-509.

Demeke T, Hucl P, Nair RB, Nakamura T, and Chibbar RN. (1997) Evaluation of Canadian and other wheats for waxy proteins. Cereal Chemistry 74: 442-444.

DePauw RM, Boughton GR, and Knott DR. (1995). Hard Red Spring Wheat. In Harvest of Gold: The history of field crop breeding in Canada. eds. AE Slinkard and DR Knott. University Extension Press, University of Saskatchewan, Saskatoon, Sask. pp 5-35.

Feldman M, Lupton FGH, and Miller TE. (1995) Wheats Triticum spp. (Gramineae-Triticinae) In Evolution of crop plants. (J Smart and NW Simmonds eds) Longman Scientific and Technical pp 184-192.

Hiei Y, Ohta S, Komari T, and Kumashiro T. (1994) Efficient transformation of rice (*Oryza sativa*L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of T-DNA. Plant J. 6: 271-282.

Hucl PJ. and Chibbar RN. (1996). Variation for starch content in spring wheat and its repeatability relative to protein content. Cereal Chem. 73: 756-758.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, and Kumashiro T. (1996) High efficiency transformation of maize (*Zea mays* L) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14: 745-750.

Jensen LG, Olsen O, Kops O, Wolf N, Thomsen KK, and von Wettstein D. (1996) Transgenic barley expressing a protein-engineered, thermostable (1,3-1,4)-ß-glucanase during germination. Proc. Natl. Acad. Sci. (USA) 93: 3487-3491.

McElroy D, Zhang W, Cao J, and Wu, R. (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163-171.

McGregor, K. (1997) Noodles and pasta – an instant hit. Cereals International 2:17.

Miura H and Tanii S. (1994) Endosperm starch properties in several wheat cultivars preferred for Japanese noodles. Euphytica 72: 171-175.

Nair RB, Båga M, Scoles GJ, Kartha KK, and Chibbar RN. (1997). Isolation, characterization and expression analysis of a type II branching enzyme cDNA from wheat. Plant Science 122: 153-163.

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard CS, Steinhauer L, Båga M, and Kartha KK. (1994). Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. The Plant Journal 5 (2): 285 - 297.

Nehra, N.S., Chibbar RN, and Kartha KK. (1995). Wheat transformation: methods and prospects. Plant Breeding Reviews (CAB International) 65(6): 803-808.

Nehra NS, Kartha KK, and Chibbar, RN. (1996) Enhanced Regeneration System. US Patent No. 5,589,617.

Repellin A, Nair RB, Båga M, and Chibbar RN. (1997) Isolation of a Starch Branching Enzyme 1 cDNA from a Wheat Endosperm library (Accession no. Y12320). Plant Gene Register (#PGR97-094). Plant Physiol. 114: 1145.

Sanford JC, Klein TM, Wolf ED, and Allen N. (1987). Delivery of substances into cells and tissues using a particle bombardment process. Particulate Sci. Technol. 5:27-33.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, and Brettel R. (1997). *Agrobacterium tumefaciens* –mediated barley transformation. Plant J.11: 1369-1376.

Vasil V, Castillo AM, Fromm ME, and Vasil IK. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10: 667-674.

Wang L and Seib, PA. (1996). Australian salt-noodle flours and their starches compared to US wheat flours and their starches. Cereal Chem. 73: 167-175.

Weeks JT, Anderson OD, and Blechl AE. (1993). Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). Plant Physiology 102: 1077-1084.