Diversity of Granule Bound Starch Synthase I Coding Sequence within Selected wild and Cultivated *Triticeae*

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Abstract: Starch is one of the main components of wheat flour. Its relative content and chemical composition affect the quality of the products obtained from wheat. Mutations in genes encoding granule-bound starch synthase I (GBSSI) result in amylose-free starch endosperm of wheat and related cereals. Up to now GBSSI alleles from several species have been sequenced and polymorphisms have been found. We designed PCR markers allowing amplification of GBSSI sequences from *T. aestivum* and wild related species (*Elytrigia, Thinopyrum, Aegilops* and *Triticum*) obtained from the Gene Bank in Prague-Ruzyně. Amplification products were purified and digested with 8 different restriction endunucleases. Data from fragment scoring were processed. The resulting dendrogram showed the similarities among the analyzed species. These generally reflect the known phylogenetic relation among species with exception of *Thinopyrum junceum*. Diploid *Triticum* species with A genome form one group together with *T. araraticum*. Sequencing usually provides more comprehensive data. We amplified, purified, cloned and sequenced first 4 exons and introns of GBSSI genes of *T. urartu*, *T. monococcum*, *T. boeoticum* and *T. dicoccoides*. Sequences were compared with known sequences of wheat and related species and new SNPs were identified. It was proved, that new variability can be discovered and perhaps used in the breeding process.

Keywords: granule-bound starch synthase; waxy; variability; DNA; GBSSI; restriction endonuclease

Starch is an α-glucan, it serves as source of energy for plant itself, but also for potential consumers. There are two types of starch found in mature grains, amylose and amylopectin. Amylose contains up to several thousand of α-glucosyl units linked almost exclusively in α(1 → 4) linkage with very few branches of α(1 → 6) linkage. Amylose accounts for 30% of starch. Amylopectin, on the other hand is a much more branched molecule and contains up to several million glucosyl residues. Amylopectin accounts for 70% of starch (Eliasson 2004).

Starch is synthesized in plastids, including chloroplasts in photosynthetic tissues and amyloplasts in non-photosynthetic tissues such as seeds and roots (Vrinten & Nakamura 2000). Starch synthesized in chloroplasts of photosynthetic tissues is degraded to hexoses during the dark period. Biosynthetic pathway starts with Calvin cycle. Polymerisation of glucosyl molecules is catalysed by soluble and granule bound starch synthases. Four basic types of soluble starch synthases (SS) are recognized: SSI (Baba *et al*. 1993; Knight *et al*. 1998), SSII (Dry *et al*. 1992; Edwards *et al*. 1999; Harn *et al*. 1998) SSIII (Abel *et al*. 1996; Marshall *et al*. 1996; Gao *et al*. 1998), SSIV (Dian *et al*. 2005) and two classes of granule bound starch synthases (GBSS): GBSSI and GBSSII (Kloesgen *et al*. 1986; Okagaki 1992).

GBSSI is exclusively located in the starch granules and cannot be detected in soluble extracts.

GBSSI or *Waxy* is responsible for amylose biosynthesis. In *Triticeae*, GBSSI is encoded by a single copy gene per genome.

Reduction or loss of GBSSI function results in starch with a decreased or absent amylose fraction, which is desired for its improved freeze-thaw

stability and resistance to staling compared to conventional starch. Allelic series of variants in the granule-bound starch synthase I gene in hexaploid and tetraploid wheat have been identified. Many partial sequences have been published and used for genetic resources evaluation (Murai *et al*. 1999; Yan *et al*. 2000).

The search for natural knock-out alleles of GBSSI in bread wheat is complicated by the presence of A, B, and D genomes in allohexaploid bread wheat and A and B genomes in tetraploid pasta wheat. GBSSI is encoded by a locus found on the 7A and 7D homoeologous chromosomes, and, due to a trans- location, on the 4A chromosome.

MATERIAL AND METHODS

Plant material. Several Gene bank accessions were used. They belonged to *Elytrigia pycnantha* (Godron) Love, *Thinopyrum junceum, Aegilops tauschii, Ae. cylindrica, Ae. geniculata, Ae. speltoides, Triticum urartu, T. monoccoccum, T. boeoticum, T. boeoticum, T. araraticum (*wild form of *T. timopheevi*, 2*n* = 28, *AAGG),* and *T. dicoccoides*. Two cultivars of hexaploid wheat (*T. aestivum* L.), Stepowa (obsolete old cultivar from Poland) and Nela (Czech modern cultivar registered at 1998) were used as a control material.

DNA isolation. DNA was isolated from (1) individual plants and (2) bulked plants using 10 plants per accession in both cases. Protocol based on selective precipitation in CTAB as described by Saghai-Maroof (1984) was used. DNA quality and quantity were measure spectrophotometrically and electrophoretically.

Primer designing. DNA sequence No. AB019624 (NCBI database) was used to design two primer pairs for waxy gene amplification with the help of PRIMER3 program to cover whole genomic sequence. Final primer sequences were as follows:

M1 Forward: 5'-GCCGTCAACTACGACATCACCA-3

M1 Reverse: 5'-CTCGACACCCAGTTCCAGAAGC-3

M2 Forward: 5'-GCTCTGGTCACGTCCCAGCT-3

M2 Reverse: 5'-TGGTGATGTCGTAGT TGACGGC-3

Reaction optimisation. For both primer pairs reaction profiles and reaction mix composition were optimised. Proofreading polymerase Promega Taq DNA Polymerase was used for primer pair M1. Reaction mixture consisted of 9.3 μl H_2O, 1.5 μl Promega buffer, 1 μl 25mM Mg^{2+}, 1 μl 10mM dNTPs, 0.5 μl 5μM forward primer, 0.5 μl 5μM reverse primer, 0.2 μl Promega Taq polymerase and 1 μl template DNA, reaction profile: initial denaturation (94°C, 2 min), denaturation (94°C, 30 s), annealing (60°C, 40 s), extension (72°C, 1 min), final extension (72°C, 10 min), 35 times from denaturation to extension.

Proofreading polymerase Phusion High-Fidelity DNA Polymerase was used for primer pair M2. Reaction mixture consisted of 14.75 μl H_2O, 5 μl Phusion HF buffer, 0.25 μl 25mM Mg^{2+}, 0.5 μl 10mM dNTPs, 1.25 μl 5μM forward primer, 1.25 μl 5μM reverse primer, 0.75 μl DMSO, 0.25 μl Phusion polymerase and 1 μl templateDNA, reaction profile: initial denaturation (98°C, 30 s), denaturation (98°C, 10 s), annealing (66.8°C, 30 s), extension (72°C, 45 s), final extension (72°C, 10 min), 35 times from denaturation to extension.

Product purification. PCR products were separated in 1% agarose gel, stained by ethidium bromide. Amplification products were cut off the gel and purified by QIAquick Gel Extration Kit according to manufacturer instructions. .

Restriction polymorphism of amplified fragments. Purified fragments were cut separately by 7 different restriction enzymes listed: AluI (5'AG/CT 3'), RsaI (5'GT/AC 3'), Hin6I (5'G/CGC 3'), TasI (5'/AATT 3'), MboI (5'/GATC 3'), TaqI (5'T/CGA 3'), TaiI (5'ACGT/3'). Manufacturer's instructions were followed. The resulting fragments were separated on 2% agarose gel and fragments were scored according to their presence or absence.

Sequencing. Purified amplification products resulting *T. aestivum* L. Stepowa and Nela and *T. diccocoides* were cloned into pCR2000, transformed into *E. coli*, plasmids were isolated and purified. Sequencing was done by Applied Biosystem BigDye terminator kit and Applied Biosystem ABI instrument.

Data analysis. For each accession, a binary matrix reflecting specific band presence (1) or absence (0) was generated. Pairwise distances between the accessions based on Hamman dissimilarity metrics were calculated. UPGMA-clustering was conducted using the SYN-TAX 2000 program package (Podani 2001).

RESULT AND DISCUSSION

Characterisation of genetic resources has been based for a long time on evaluation of morpho-

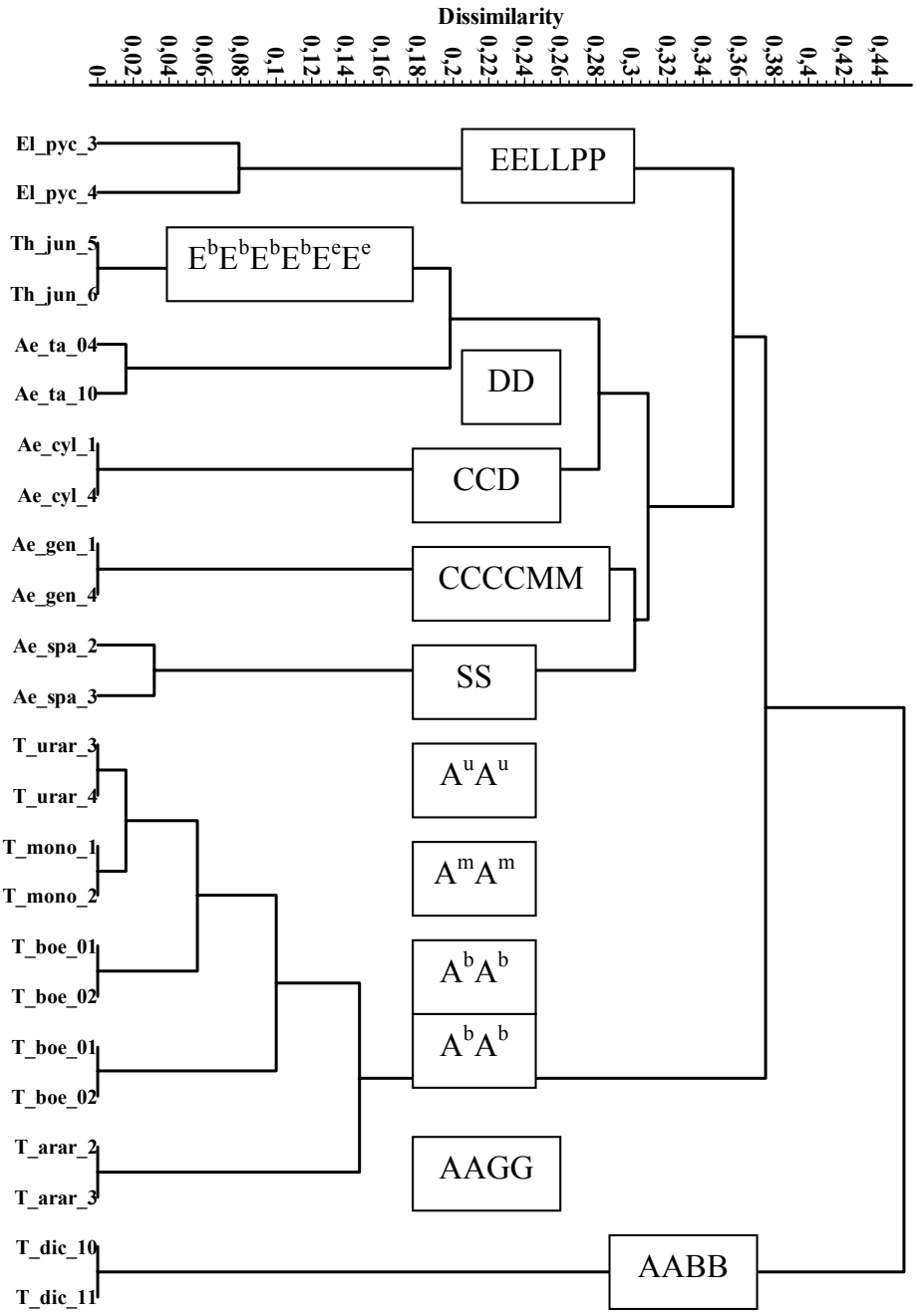

Figure 1.Association among analysed accession based on scored restriction fragment polymorphism of GBSS I.

logical traits and in the case of cultivated species on the results of field trials. Biochemical makers and in the recent years DNA markers have been introduced successfully. The information is used for classification and maintenance of genetic resources and core collection development and perhaps in practical breeding (Ovesná *et al*. 2002).

In our investigation we attempt to describe variability of GBBSI underlying genomic sequence. Primers were designed and PCR reaction optimised. Optimised reaction conditions as described in material and methods were used to amplify GBSSI underlying genomic sequence from accessions listed in material and methods. Purified amplification fragments were subjected to restriction analysis and together 63 different restriction fragments were scored indicating high level of variability in the coding sequences. For each accession two bulks of 10 plants were analysed. Length polymorphisms of amplification products and further restriction

fragment of amplification products were scored. No length polymorphism of amplification products was recorded. More precise techniques than agarose gel electrophoresis, e.g., capillary electrophoresis and fluorescently labelled primers, would be necessary to detect the differences (Sobotka *et al*. 2004) Cluster analysis and resulting dendrogram fully reflects expected relation among analysed species (Kellog 2001 – www.virtualherbarium.org/GPWG/) (Figure 1). Cluster analysis clearly separate *T. diccocoides* L. with AABB genomes. Accessions possessing A genomes (*T. urartu, T. monoccoccum, T. boeoticum, T. boeoticum)* clustered together with *T. araraticum* which has AAGG genomes. Relative genetic distance between *T. urartu* and *T. monoccoccum* was 0.016 and they were identified as the two most closely related species. On the other hand genetic distances were higher in case of two bulked accessions of *T. boeoticum* and two bulks of *Ae. tauschii.* The most distant were *Elytrigia pycnantha* and *T. dicoccoides* with relative genetic distance 0.635. This demonstrates the variability of the sequence within genetic resources of wild species. It is possible to conclude, that wild wheat related species might be potential sources of new allele types. Variability in the sequences amplified from eight *T. aestivum* L. today cultivars were not identified (data not shown here) using restriction endonucleases.

The search for natural knock-out alleles of GBSSI in bread wheat is complicated by the presence of A, B, and D genomes in allohexaploid bread wheat and A and B genomes in tetraploid pasta wheat. GBSSI is encoded by a locus found on the 7A and 7D homoeologous chromosomes, and, due to a trans- location, on the 4A chromosome. Difficulties of finding naturally-occurring knock-out alleles of the waxy locus as a consequence were pointed out by several authors (Graybosch 1998; Moehs 2005).

DNA sequences that we identified in *GBSSI* of modern wheat cultivar Nela were identical with those available in public database (NCBI). One DNA *GBSSI* sequence (A genome specific) identified in old cultivar Stepowa differed from published sequences combining known SNPs and In/Dels from *Wx-1A* and *Wx-1B* specific sequences. DNA sequences identified in *GBSSI* in *T. dicoccoides* differ from published sequences. Several SNPs and In/Del were found in intron and exons.Analysis of a single gene can assist in revealing genetic variability within and between species. Sequencing of a single gene across accessions can reveal in more detail allelic variants of the gene. Sequencing is more powerful than restriction analysis. Different allelic forms could be detected and properties of corresponding protein(s) may be further investigated for their final qualities.

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