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Review Paper

Molecular insights on the origin and development of waxy genotypes in major crop plants

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Abstract

Starch is a significant ingredient of the seed endosperm with commercial importance in food and industry. Crop varieties with glutinous (waxy) grain characteristics, i.e. starch with high amylopectin and low amylose, hold longstanding cultural importance in some world regions and unique properties for industrial manufacture. The waxy character in many crop species is regulated by a single gene known as *GBSSI* (or *waxy*), which encodes the enzyme Granule Bound Starch Synthase1 with null or reduced activity. Several allelic variants of the *waxy* gene that contribute to varying levels of amylose content have been reported in different crop plants. Phylogenetic analysis of protein sequences and the genomic DNA encoding *GBSSI* of major cereals and recently sequenced millets and pseudo-cereals have shown that *GBSSI* orthologs form distinct clusters, each representing a separate crop lineage. With the rapidly increasing demand for waxy starch in food and non-food applications, conventional crop breeding techniques and modern crop improvement technologies such as gene silencing and genome editing have been deployed to develop new waxy crop cultivars. The advances in research on waxy alleles across different crops have unveiled new possibilities for modifying the synthesis of amylose and amylopectin starch, leading to the potential creation of customized crops in the future. This article presents molecular lines of evidence on the emergence of *waxy* genes in various crops, including their genesis and evolution, molecular structure, comparative analysis and breeding innovations.

Keywords: waxy genes; genetics; molecular structure; comparative genomics; waxy mutations; glutinous; cereals; millets; pseudo-cereals; potato

INTRODUCTION

Cereals and other food crop species were first domesticated ~12 000 years ago [1, 2]. However, it was only during the last few centuries that it became understood that it is specifically the carbohydrates in these staple foods that provide the energy that has sustained humanity [3, 4]. Plant carbohydrates consist of sugars, gums, starches and cellulose. The total carbohydrate content in cereal seed/s ranges from 66 to 76%, and starch occupies the major fraction [5]. Starch typically constitutes 55–70% of the total carbohydrates in the seeds, followed by minor constituents such as β-glucans (0.5–7%), arabinoxylans (1.5–8%), cellulose (~2.5%), sugars (~3%) and glucofructans (~1%) [6]. Starch content also varies among grain tissues; for example, the outer and aleurone layer that produces bran on milling contains very little starch compared with endosperm, which contains up to 85% starch.

Since it is the starch that is the desired source of nutritive energy for consumption, its quality has held profound importance throughout human history [6]. Moreover, its importance is not limited to the human diet, as starch has numerous industrial applications such as in making gums, stabilizers and bioethanol production. Starch is chemically constituted of two different polymers of α-D-glucose units, i.e. amylopectin and amylose. In cereals, amylopectin is the major fraction that constitutes 72–75% of the starch, whereas amylose constitutes 20–25%. Unlike amylopectin, which is extensively branched, amylose is a straight chain of α-D-glucose molecules. Variations in branching patterns of amylopectin in terms of distribution of chain length, chains clustering, the capacity to create double-helical conformations confer crystalline features to the amylopectin that affect the overall characteristics and functionality of starch [7].

Multiple enzymes, including AGPase, granule-bound starch synthase (GBSS), starch synthases, starch branching enzyme (SBE), starch debranching enzyme (DBE), starch disproportionation enzyme (DPE) and phosphorylase (PHO), are involved in amylose and amylopectin biosynthesis and modification in plants [8]. Concerted action of these enzymes during seed filling gives rise to the characteristic ratios of amylose and amylopectin in the

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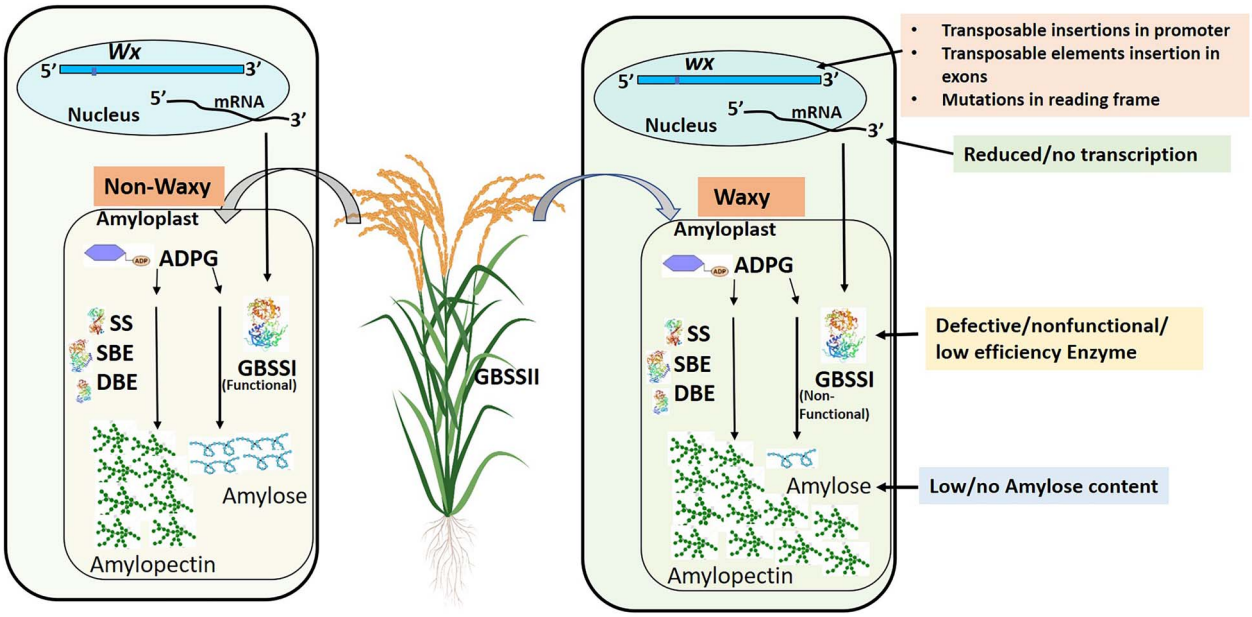

Figure 1. Underlying molecular mechanisms governing Waxy and non-waxy traits in cereals, millets and tuber crops. Plants harbor two types of Granule Bound Starch Synthase genes—*GBSSI* and *GBSSII*. While the *GBSSI* is specifically expressed in the developing seed, the *GBSSII* is expressed in the leaves. The fully functional *GBSSI* gene encodes active enzymes that reside in the amyloplast and uses adenosine diphosphate glucose (ADPG) to generate linear chain of glucose molecules known as amylose. Higher ratios of amylose to amylopectin (branched glucose chain) render the grain non-waxy character. Conversely, when the fully functional *GBSSI* gene is disrupted because of insertions/deletions caused by transposable elements or point mutations in the regulatory or coding region, the *GBSSI* gene produces either defective or nonfunctional enzyme or low-efficiency enzyme, which reduces the overall amylose content in the grain. Lower ratios of amylose to amylopectin or zero amylose in the grain imparts varying degrees of waxiness to the grain.

grains. In general, the amount of amylose is typically three times that of amylopectin. However, if mutations occur, the proportion of amylose to amylopectin can change, which gives rise to altered starch quality. Of the various mutants discovered so far, the '*waxy*' mutants have had the most longstanding cultural importance, especially in Southeast Asia (SEA) where glutinous rice has been an important staple cereal for thousands of years. *Waxy* mutants in cereals and other crops are the result of mutations causing loss-of-function in the *waxy* gene, which regulates amylose synthesis during grain filling. Plants harboring the *waxy* gene exhibit a predominant presence of amylopectin (up to 100%) in their grains, resulting in a sticky and tender texture of the cooked grains in contrast to the prevailing non-waxy counterparts [9]. The waxy types can be easily distinguished from non-waxy types by simple iodine staining; whereas the endosperm starch of waxy types stains red, the non-waxy endosperm stains bluish black in response to the presence of iodine [10–13]. The word 'waxy' describes the appearance of endosperm in the grain when it is sliced or split, not the presence of actual wax. Since they resemble glue when cooked or wet, waxy grains are often known as 'glutinous' grains [14]. As amylose is related to starch content and kernel density, scarcity or absence of amylose may lead to larger space between the starch granules and higher starch and protein digestibility [15]. Because of their exceptional starch quality and stability across multiple applications, waxy grains are experiencing a growing demand in both food and non-food sectors. They are particularly sought after for their suitability in various preparations, including functional foods like noodles and bakery products. Many cereal crops, including different varieties of maize, rice, sorghum, barley, foxtail millet, Job's tears and proso millet, have been found to have waxy types, and a range of allelic variants of the *waxy* gene in these crops have been identified and characterized [16].

It has been suggested that the selection of sticky foods by humans led to the emergence and propagation of waxy and low-amylose varieties of different crops in East Asia and SEA [9]. Hence, the waxy landraces are primarily grown in continental parts of SEA (e.g. Thailand, Laos, Cambodia and Vietnam) and East Asia (e.g. China, Taiwan, Korea and Japan), where glutinous cuisines are popular and associated with the culture of eating by rolling the cooked rice with fingers into small balls for dipping into food [1]. In the Indian subcontinent, by contrast, the mediocre amylose content types are more favored, especially in the case of rice. Besides traditional crop varieties, waxy forms can also be the result of deliberate modifications through modern selective breeding, including the cereals that are largely grown as a source of carbohydrate. One of the primary targets for such alterations has been the loci that determine starch quality [17, 18].

In this review, we focus on the molecular aspects of *waxy* alleles, which have become increasingly important in the cereal industry because of their waxiness, a critical quality trait. We explore the origin and structure of *waxy* alleles, delve into comparative genomics, discuss the recent advancements in breeding techniques and highlight the diverse applications of waxy genotypes across various crops.

Origin of *waxy* alleles: molecular evidence

Ethnological and ethnobotanical studies have revealed that low-amylose waxy grain types evolved through human selection based on cultural preferences for processing and consumption of waxy grains [16, 19]. The waxy trait was likely absent or present at very low frequency in wild ancestors and emerged through selection during domestication [20]. The *waxy* (*Wx*) locus encodes the enzyme Granule-Bound Starch Synthase 1 (GBSS), which, as discussed in the previous section, controls amylose synthesis in the cereal endosperm (Figure 1). Cereal plant genomes studied

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so far have been found to harbor two types of GBSS-encoding genes, i.e. *waxy* (*GBSSI*) and *GBSSII*. While the expression of *GBSSII* is restricted to leaf, culm and pericarp tissues, *GBSS I* is strongly expressed in cereal endosperm. Therefore, loss of function or defective expression of the *GBSSI* gene results in reduced or complete suppression of amylose biosynthesis in cereal grains, giving rise to the characteristic waxy appearance. Molecular genetic analyses carried out to trace the evolution of waxy landraces in different crops have revealed that the waxy types typically had localized geographical origins and were later scattered into their existing regions of dispersion [9, 21, 22]. Waxy types were first discovered in landraces of rice, maize, wheat, sorghum and millets, and more recently, the trait was transferred to improved varieties. For example, the first corn waxy line was reported in Chinese maize landraces and is presently used to produce waxy corn starch all over the world [23].

With the growing importance of waxy starch and the parallel rise in the availability of cereal genome sequences, the molecular genetic basis of the waxy trait has been characterized in multiple crops [24, 25]. Information about the sequence variations present in these alleles is given in Table 1 and summarized below. In waxy barley, the synthesis of GBSSI protein is restricted to low levels in comparison to the wild type because of impaired transcription caused by a 413-bp deletion in the 5' UTR and promoter region of *GBSSI* [26, 27]. In sorghum, waxiness is caused by a non-synonymous substitution in the *GBSSI* gene sequence and an SNP, which causes a replacement of glutamic acid to histidine at 268 and activates the GBSSI protein [28, 29].

In the case of rice, several *Wx* alleles such as Wx^{a}, Wx^{b}, Wx^{op}, Wx^{in}, Wx^{mw}, Wx^{mq}, Wx^{lv} and *wx* have been reported carrying mutations and insertions of transposable elements into the *waxy* gene, resulting in a range of waxy phenotypes. Studies on the *waxy* gene of wild and cultivated rice genotypes have revealed that the allele Wx^{lv} is the ancestral allele of the *Wx* gene; it mainly exists in Aus cultivars that might have evolved from the $Wx^{lv\text{-}w}$ haplotype of wild rice. The Wx^{lv} allele evolved from $Wx^{lv\text{-}w}$ subsequently spread into different haplotypes through artificial selection resulting in different *Wx* alleles in cultivated rice [22]. Recent studies utilizing the nucleotide sequence data from the 3 K rice genomes project (3KRGP) revealed that Wx^{a}, Wx^{b}, Wx^{in} and Wx^{lv} are the four main alleles and are distributed across diverse rice-growing regions of the globe. Their geographical distributions are coherent with the eating habits of different regions. The Wx^{b} allele is widely dispersed in the high latitude, Wx^{in} and Wx^{a} is extensively dispersed in the low-latitude tropical region and mid-latitude region, respectively. Whereas the *wx* allele was frequently discovered in SEA and East Asia, the Wx^{lv} allele was found to be mostly dispersed in South Asia-Central (SAC) and South Asia-East (SAE) (EAS) [30]. Similarly, the rice *waxy* allele Wx^{op}, which is associated with very low (<10%) amylose content is found in the *indica* group only and is extensively dispersed in China, Indonesia, Nepal and India [31]. The single nucleotide substitution of A/G at position +714 in the exon 4 of the *GBSSI* results in the substitution of amino acid Asp to Gly and is distinct to the Wx^{op} allele [32].

In foxtail millet, several independent transposable elements insertions in the *Wx* gene have resulted in reduced or entire loss of gene activity that associates with reduced-amylose and waxy trait [9, 25, 33–35]. Thirteen alleles have been identified through PCR amplification, which arise because of various transposable elements insertions in the gene. An examination of the structural components of transposable elements indicated that the shift from non-waxy to waxy happened on three occasions, whereas the change from non-waxy to low amylose, low amylose to waxy and waxy to low amylose each occurred once [9, 25, 36]. Studies on non-waxiness of the foxtail millet progenitor (green foxtail millet) and high diversity in *Wx* DNA sequences of non-waxy genotypes further showed that the waxy types evolved from non-waxy types after domestication of foxtail millet [37].

In contrast to the single *waxy* gene present in diploid crop species, multiple copies are present across the homologous chromosomes of polyploid cereals. In the case of tetraploid broomcorn millet (*Panicum miliaceum*), multiple origins of *waxy* alleles have been suggested by Hunt *et al.* [38]. Sequencing of the *GBSSI* (*waxy*) gene unraveled two unique variants (L and S), which differed in their gene sequences and intron length. While the L-type exhibits two sequence variations, all waxy accessions of the S-type have a 15-bp deletion in exon 10 that causes the loss of five amino acids from glucosyltransferase domain 1 (GTD1). The first of these is a frameshift mutation, which is caused by the insertion of a adenine nucleotide in exon 9, whereas the other sequence variant has guanine to adenine substitution in the exon 7 that results in cysteine to tyrosine amino acid substitution. Since all the three L-type alleles are derived from a single allele, it was suggested that the three *waxy* alleles might have originated in parallel from this ancestral allele.

In Japanese barnyard millet, which is hexaploid ($2n = 6x = 54$), molecular investigations revealed three functional *GBSSI* alleles labeled as *EeWx1*, *EeWx2* and *EeWx3* in the wild-type cultivars. However, in the low amylose genotypes, only one functional *GBSSI* gene product (*EeWx1*) was present. The other alleles, i.e. *EeWx2* and *EeWx3*, were detected to have spontaneous deletions of entire and terminal segments of the gene, respectively. Further, inducing a premature stop codon in *EeWx1 via* mutagenesis (creating a 1-bp frameshift deletion) was sufficient to generate a fully waxy cultivar, indicating that *EeWx1* is the only functional gene copy [39].

Unlike in other cereals and millets, *waxy* forms in bread wheat (*Triticum aestivum* L.) are not found in landraces because of its hexaploid nature, which means that co-occurrence of mutations in all three homologous *waxy* genes is required to produce the waxy phenotype. Therefore, for modern applications, waxiness has been incorporated through hybridization and mutagenesis. In some cases where hybridization was used to develop waxy wheat, genotypes that have single or double null *waxy* alleles were combined to generate genotypes that produce none of the Wx-A1, Wx-B1 and Wx-D1 proteins or functional GBSS enzyme [40, 41]. In the case of mutants, independently originated null *Wx-1* alleles in all polyploid wheat species have been identified, which have distinct large or single-nucleotide insertions and deletions in the coding region that cause frameshift mutations resulting in loss of *GBSSI* activity [42].

In addition to the various cereals surveyed earlier, the waxy trait has also been molecularly characterized in some non-cereal crop species. While analyzing the *waxy* genes of three grain amaranth genotypes (*Amaranthus caudatus* (*Wx-ca*), *A. cruentus* (*Wx-cr*) and *A. hypochondriacus* (*Wx-hy*), Park *et al.* [43] revealed that the *waxy* mutation in each species arose from a single but different mutational event in the wild type. The *waxy* allele *wx-ca* was the result of an insert of T base in exon 8, whereas the alleles *wx-cr* and *wx-hy* were the outcomes of G-to-T base substitution in exon 10 and G-to-A base substitution in exon 6, respectively. These nonsense mutations were unique events in the evolution of *waxy* types in *Amaranthus*.

In the root crop cassava, a single nucleotide deletion in the exon 6 (MeWxEx6-del-C), which generates a premature termination codon, as well as two SNPs one in exon 11 and another

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Table 1. Waxy alleles in different crops

Crop	Allele variants	Molecular change happened in Wx gene during domestication	Remark	Reference
Rice	Wx^{a}	G at Int1–1 and T at Exon10–115	High amylose content	[142]
	Wx^{b}	T at Int1–1 and C at Exon10–115	Low amylose content	[142]
	Wx^{in}	A single nucleotide polymorphism on exon 6 (exon 6 SNP A/C) was found that associates with intermediate AC	Intermediate-type amylose content	[31, 61]
	Wx^{lv}	G at Int1–1 and C at Exon10–115	High amylose content and low viscosity	[21]
	$Wx^{op/hp}$	G in intron 1 like the Wx^{a}	Very low AC	[63, 143]
	Wx^{mq}	G-to-T mutation intron 1 (similar to Wx^{b})	Very low AC	[60]
	Wx^{mp}	T in Int1–1 and A at Exon4–53	Very low AC	[66]
	wx	23-bp duplication inserted in exon 2 or 7764-bp insertion in exon 9	Very low or no AC	[62, 144]
	Wx^{mw}	single A-to-C substitution on exon 6 (EX6–62)	Low AC	[66]
Maize	*wx-m1*	409 bp long *Ds* insertion in the 9th exon sequence	–	[145]
	wx-m5	2-kb *Ds* element at −470 bp relative to the start of *waxy* transcription	–	[146–148]
	wx-m6	2.1 kb insertion	–	[149]
	wx-m7	4.3 kb transposable element in the promoter region	–	[46]
	wx-m9	4.3 kb transposable element in the exon 10	Intermediate phenotype	[150]
	wx-m8	Integration of a receptor part of Spm	–	
	wx-m844	Insertion of En-I element 8.4 kb long, had a 13 bp long perfect inverted repeat at its termini and generated a 3 bp target site duplication	–	[151, 152]
	wx-stonor	4.5 kb insertion in junction of intron 5-exon 6	Waxy	[150]
	wx-B5	5.0 kb insertion in the intron 8	–	[150]
	wx-G	6.1 kb insertion in the intron 2	–	[150]
	wx-M	Retrotransposons insertions	–	[150]
	wx-I	Retrotransposons insertions	–	[150]
	wx-K	Retrotransposons insertions	–	[150]
	wx-Reina	5.4-Kb retrotransposon Reina inserted in the tenth intron	–	[153]
	wx-Cin4	466 bp insertion in Exon6	Waxy	[153]
	wx-124	116 bp insertion in Exon 7	Waxy	[153]
	wx-B3	4.5Kb insertion Exon10–Intron10 boundary	Waxy	[153]
	wx-B2	128 bp insertion in Exon 11	Waxy	[153]
	wx-B4	1.5 Kb insertion in Exon13	Waxy	[153]
	wx-B1	Deletion	Waxy	[153]
	wx-B	Deletion	Waxy	[153]
	wx-B6	Deletion	Waxy	[153]
	wx-B4	Deletion	Waxy	[153]
	wx-B7	Deletion	Waxy	[153]
	wx-C34	Deletion	Waxy	[153]
	wx-c	Deletion	Waxy	[153]
	wx-1240	Deletion	Waxy	[153]
	wx-B12	Deletion	Waxy	[153]
	wx-D7	Deletion	Waxy	[153]
	wx-c2	Deletion	Waxy	[153]
	wx-D10	Deletion	Waxy	[153]
Wheat	*Wx-A1*	Wild type	Non-waxy	[154]
	Wx-B1	Wild type	Non-waxy	[154]
	Wx-D1	Wild type	Non-waxy	[154]
	Wx-A1a	Deletion of part of exon 9 through exon 12	Waxy	[154]
	Wx-B1l	Frame shift because of deletion of a cytosine residue in exon 2 resulting in the premature stop codon in the exon4	Waxy	[154]
	Wx-B1b	Complete deletion	Waxy	[155]
	Wx-A1b	Deletion of 23 bp in the second exon–intron junction and the presence of filler DNA	Waxy	[156]
Sorghum	wx^{a}	4 kb large insertion in the third exon	Waxy	[16]
	wx^{b}	Missense mutation that changes glutamine 268 to a histidine	Waxy	[16]
	wx^{c}	+1G-to-C mutation in the 5′ splice site at the intron 10-exon 11 boundary	Waxy, found in a Taiwanese landrace	[118]
Cassava	Wx	A single base substitution at position 78–79 of exon 11, causing GG to change to AT; another base substitution at position 14 of intron 11, causing G to change to C	Non-waxy	[44]

(continued)

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Table 1. Continued

Crop	Allele variants	Molecular change happened in *Wx* gene during domestication	Remark	Reference
	wx	a single base deletion (cytosine) at position 92 of exon 6	Waxy	[44]
Barley	Wx-CDC Candle	397 bp deletion and a 193 bp insertion in the promoter a 15 bp insertion in the transit peptide region and in CDC Candle	Waxy	[157]
	Wx-Bowman	11 bp insertion in the promoter region	Non-waxy	[157]
Foxtail millet	Type I	Wild type	Non-waxy	[9]
	Type II (TSI-1)	343 bp insertion in intron1	Non-waxy	[9]
	Type III (TSI-6)	4050 bp insertion in intron1	Low amylose	[9]
	Type IV (TSI-2)	5250 bp insertion in in intron1	Waxy	[9]
	Type IVa (TSI-4)	5589 bp insertion in intron1	Waxy	[9]
	Type IVb (TSI-5)	5614 bp insertion in intron1	Waxy	[9]
	Type V (TSI-7)	7674 bp insertion in exon-3	Waxy	[9]
	Type VI (TSI-10)	4544 bp insertion in intron-12	Low amylose	[9]
	Type VII (TSI-9)	9331 bp insertion in exon-3	Waxy	[9]
	Type VIII (TSI-11)	1479 bp insertion in intron12	Low amylose	[9]
	Type IX (TSI-3)	2823 bp insertion in intron1	Low amylose	[9]
	Type X (TSI-8)	936 bp insertion in exon3	Waxy	[9]
Barnyard millet	*Ee*Wx1	Wild type	Non-waxy	[39]
	$EeWx1_{CM}$	Insertion in the exon8 causing frameshift	Waxy	[39]
	*Ee*Wx2	Wild type	Non-waxy	[39]
	*Ee*Wx2*null*	Deletion of structural gene	Waxy	[39]
	*Ee*Wx3	Wild type		[39]
	$EeWx3_{NH\&CM}$	Two synonymous T/G substitutions in the 1st Exon; GT bases deletion in the 1st intron; C/A substitution in the 6th intron; complete deletion of the 13th exon.	Waxy	[39]
Proso millet	WxL_C	Wild type	Non-waxy	[38]
	WxL_f	An insertion of adenine residue in exon 9 causing a frame shift (Lf)	Non-waxy	[38]
	WxL_Y	Substitution of G/A in exon 7 resulting in substitution of cysteine with tyrosine (LY)	Non-waxy	[38]
	WxS_0	Wild type	Non-waxy	[38]
	WxS_{-15}	15-bp deletion in 10th exon resulting to the loss of five amino acids	Waxy	[38]
Job's tear	EeWx	275-bp deletion in exons 10–11	Waxy	[158]
Amaranths	Type Ia	Exon 1(39, T), Exon 1(49,G), Exon 1(236,G), Exon6(1439,.G	Non-waxy	[159]
	Type Ib	Exon 1(39, G), Exon 1(49,G), Exon 1(236,G), Exon6(1439,G)	Non-waxy	[159]
	Type IIa	Exon 1(39, T), Exon 1(49,G), Exon 1(236,G), Exon6(1439,G)	Non-waxy	[159]
	Type IIb	Exon 1(39, G), Exon 1(49,C), Exon 1(236,A), Exon6(1439,..)	Non-waxy	[159]
	Type IIc	Exon 1(39, T), Exon 1(49,G), Exon 1(236,G), Exon6(1439,G)	Non-waxy	[159]
	Type IId	Exon 1(39, T), Exon 1(49,G), Exon 1(236,A), Exon6(1439,G)	Non-waxy	[159]
	Type IIIa	Exon 1(39, T), Exon 1(49,G), Exon 1(236,A), Exon6(1439,A)	Waxy	[159]
	Type IIIb	Exon 1(39, G), Exon 1(49,G), Exon 1(236,A), Exon6(1439,A)	Waxy	[159]
	Type IIIc	Exon 1(39, C), Exon 1(49,G), Exon 1(236,A), Exon6(1439,A)	Waxy	[159]

a substitution in intron 11, have all been reported to be linked with waxy genotypes [44]. However, subsequent analysis with an expanded sample of 89 genotypes revealed that the single deletion in exon 6 is alone responsible for conferring the waxy phenotype [45].

Molecular structure and comparative analysis of *waxy* genes across major crops

The sequencing of the *waxy* gene in maize marked the first instance of determining the sequence of a *waxy* gene. Klosgen *et al.* [46] revealed the genomic and cDNA sequence of the wild-type *waxy* (wx^+) locus of maize and reported that the 3718 bp coding region is made of 14 exons and 13 small introns. This was closely followed by the determination of the nucleotide sequence of the *waxy* locus of barley by Rohde *et al.* [47], who revealed that the gene comprises 12 exons and 11 introns. Subsequently, Wang *et al.* [48] revealed that the rice *waxy* gene is composed of 14 exons and 13 introns, encoding a GBSS preprotein of 609 amino acid residues. The determination of the sequences of these genes paved the way to identify the *waxy* genes in other cereals. Presently, the *waxy* gene sequences of most of the major cereals are available in the public databases. To achieve additional insights, we used these known *waxy* gene sequences as queries in blast searches to identify homologous *waxy* sequences in a wider sample of plants, including *Eragrostis curvula*, *E. tef* (tef), *Eleusine coracana* (finger millet), *Digitaria exilis* (fonio), *P. miliaceum* (proso millet), *Secale cereale* (rye) and wild relatives of rice (*Oryza* spp.). Sequence analysis of *waxy* genomic sequences from cereals and pseudo-cereals revealed that the length of *waxy* genes varies considerably from 2782 bp in wheat (*Wx-A1*, located on the 7A chromosome) to 4984 bp in finger millet; the corresponding GBSS protein sequence ranges between 583 amino acids (wheat 4A) to 648 amino acids (foxtail millet). The variation of gene length across the cereals is also reflected in the exon-intron composition of the gene.

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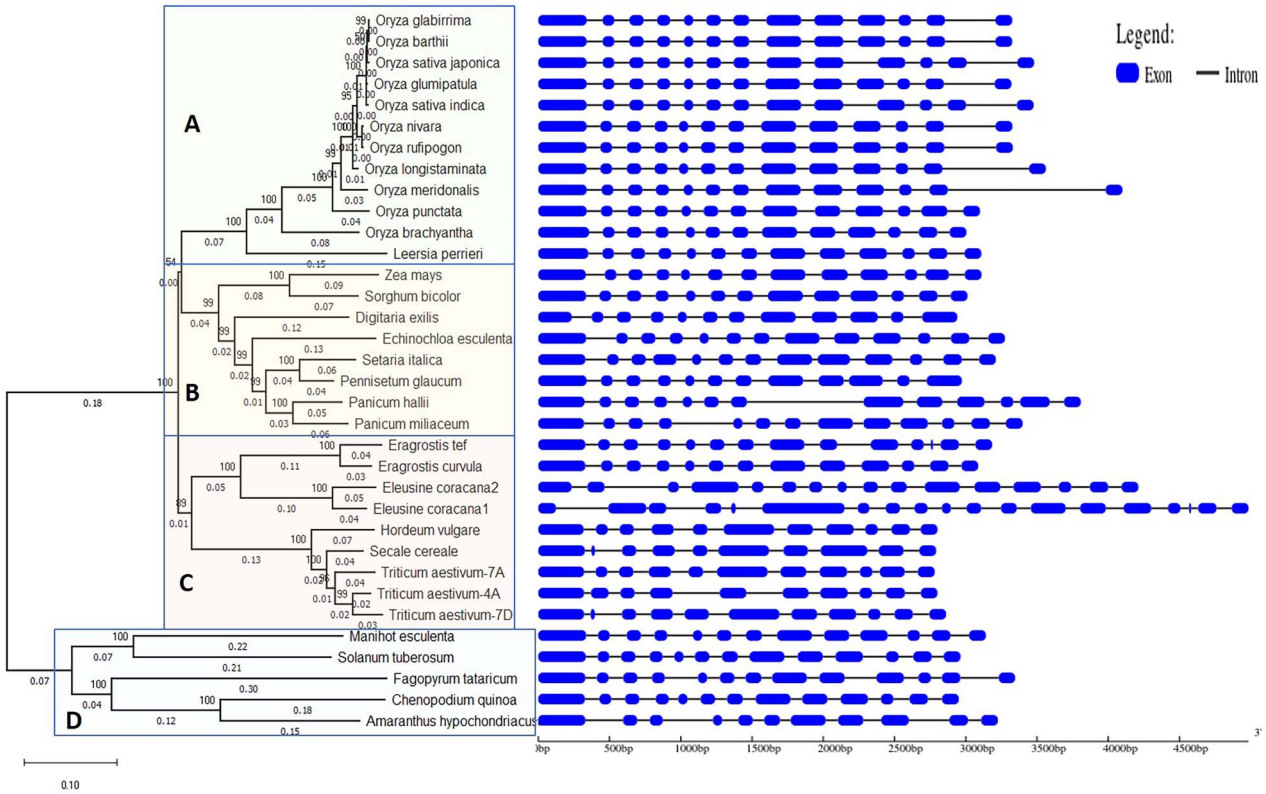

Figure 2. Gene structure and phylogenetic relationship of the genic region of waxy genes of different cereals. The genomic sequences encoding *GBSSI* of major cereals, millets, pseudo-cereals clustered into four major groups. Group A consists of rice and its relatives (*Oryza longistaminata, Oryza nivara, Oryza meridonalis, Leersia perrieri, Oryza glumipatula, Oryza glabirrima, Oryza rufipogon, Oryza brachyantha, Oryza barthii, Oryza punctata, Oryza sativa japonica, Oryza sativa indica*), Group B consists of millets along with sorghum and maize (*Zea mays, Setaria italica, Sorghum bicolor, Pennisetum glaucum, Panicum hallii, Eragrostis tef, Digitaria exilis, Eragrostis curvula, Panicum miliaceum, Eleusine coracana1, Eleusine coracana2, Echinochloa esculenta*) and Group C consists of wheat, barley and rye (*Triticum_aestivum-4A, Triticum_aestivum-7A, Triticum_aestivum-7D, Hordeum vulgare, Secale cereale*), whereas Group D consists of *Fagopyrum tataricum, Chenopodium quinoa, Amaranthus hypochondriacus, Manihot esculenta*).

The representation of gene structure of the *waxy* alleles across different crops would not only help the reader or the researchers working in the field to quickly correlate and visualize the binding sites of the primers listed in the table but also provide firsthand information to develop strategies for preparing constructs for carrying out future genome editing experiments.

Sequence alignment and phylogenetic analysis of the genomic sequences across the cereals and millets show that the *waxy* genes fall into four major clusters that correspond broadly to phylogenetic relationships; we labeled these as A, B, C and D (Figures 2 and 3). Cluster A consists of rice (*Oryza*)species and a close relative, whereas cluster B consists of the *waxy* genes of various millets (*E. tef*, *E. curvula*, *E. coracana*, *D. exilis*, *Setaria italica*, *Pennisetum glaucum*, *P. hallii*, *P. miliaceum*), maize and sorghum. Cluster C consists of *waxy* genes of wheat along with those of barley and rye (*S. cereale*). Beyond the grass family, cluster D consists of *waxy* genes of pseudocereals (e.g. *A. hypochondriacus*, *Chenopodium quinoa*, *Fagopyrum tataricum*), potato (*Solanum tuberosum*) and cassava (*Manihot esculenta*).

Although the genic region is not well conserved across different crop plants, the waxy proteins encoded by these genes are rather well conserved. This is because of the two highly conserved domains of the GBSSI protein: (i) starch synthase catalytic domain (pfam: Glyco_transf_5) and (ii) glycosyl transferases group 1 domain (pfam: Glyco_trans_1_4). The Glyco_transf_5 represents the main catalytic domain of starch synthases and, in plants, uses ADP-glucose (EC 2.4.1.21) as the glucose donor (Figure 4). Phylogenetic analysis of waxy protein sequences across cereals clearly separated them into five distinct groups: a, b, c, d and e (Figure 5). Group a consisted of only the rice and its related species, group b had only three millet species and group c comprised the remaining millet species along with maize and sorghum, whereas group d consisted of all the wheat *waxy* genes and the barley *waxy* genes. Group E consisted of the GBSSI proteins of the dicot pseudo-cereals, *Amaranthus*, *Chenopodium* and *F. tataricum*, along with potato. The clear separation of GBSSI proteins into the respective monocot and dicot groups correspond to their phylogenetic relationship but might also suggest structural differences in the GBSSI proteins of monocots and dicots that could be associated with differences in catalytic efficiencies.

Molecular breeding advancements in the development of waxy strains

Because of the ethnological preference for waxy crops being geographically restricted to parts of eastern Asia, widespread breeding strategies to introgress *waxy* alleles into non-waxy crop varieties for consumption purposes has been somewhat slow. However, with the emerging application of waxy starch in food processing, brewing, manufacture of adhesives and in new food applications such as emulsions, films and coatings, the focus has shifted away from developing varieties used for direct consumption toward varieties for non-food applications.

The non-food applications of waxy starch include utilizing them as fillers and reinforcing agents in polymer composites,

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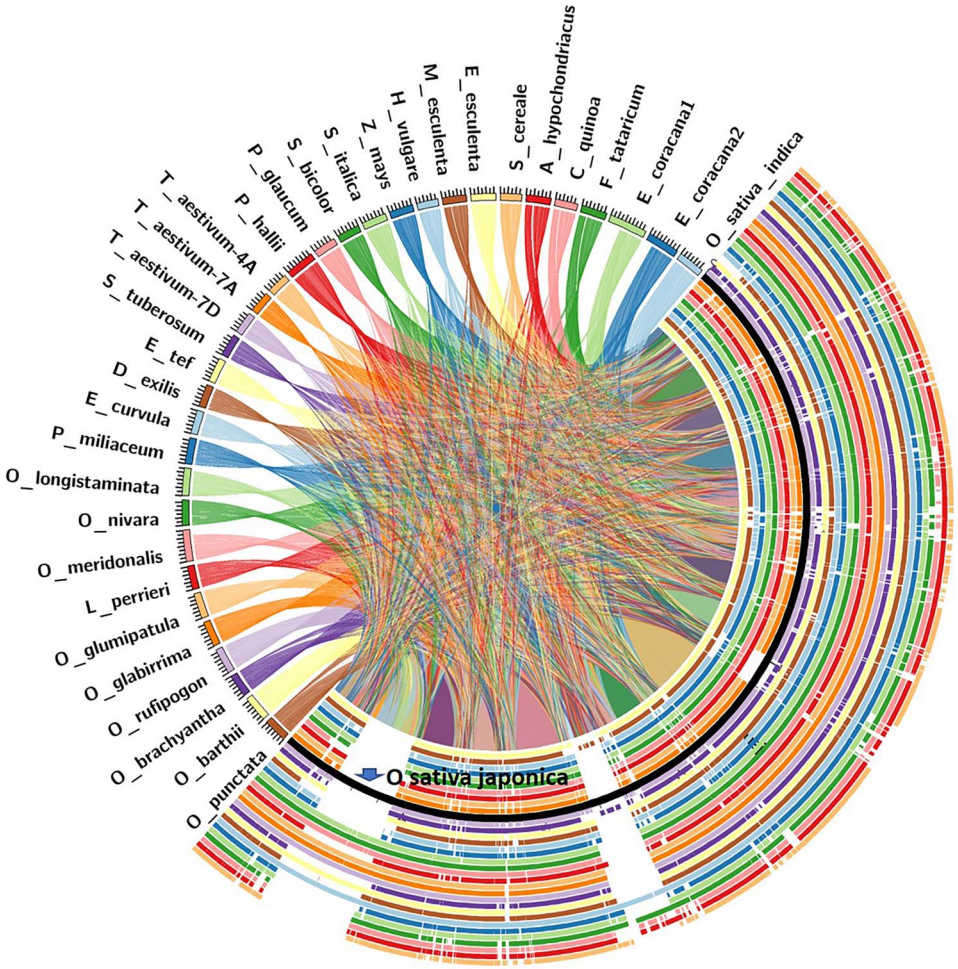

Figure 3. Circos plots showing one-to-one comparative alignment of genomic DNA sequences of major cereals, millets and pseudo-cereals encoding *GBSSI* with the genomic DNA sequence encoding *GBSSI* of rice (*O. sativa* ssp. *japonica*). Genomic DNA of rice encoding *GBSSI* occupies 50% of the circle, whereas the genomic DNA of other plants encoding *GBSSI* is scaled according to their gene length.

as well as in the preparation of nanoparticles [173]. In addition to conventional breeding, which has been the most favored option by breeders for creating elite lines in various crops, more advanced techniques such as RNAi and CRISPR/CAS9 have also been employed develop waxy lines [49–53]. The sections below review insights on waxy genotypic and phenotypic variation in the major cereal crops, the millets and potato.

Rice

In rice, the amylose content in the grains has a profound effect on consumer choice. Higher amylose rice, which on cooking produces firm and separate grains, is popular in the Indian subcontinent along with western Asia and a large part of the western hemisphere, whereas lower amylose rice is preferred by consumers throughout East and Southeast Asia, including China, Japan, Korea, Thailand, Vietnam, Cambodia and neighboring countries [54, 55]. Screening the amylose content across the rice genotypes has revealed that the grain amylose content is not markedly discrete as low and high, but rather displays continuous variation. According to the quality of cooked rice, this variation has been classified as high, intermediate, low very soft or soft and waxy or glutinous with amylose contents of >25%, 20–25%, 10–19%, 3–9% and <2%, respectively [56]. This array of amylose content across rice genotypes is mainly because of the allelic difference at the Wx locus [22, 57, 58]. Initially, Wx^a and Wx^b were the two wild-type alleles identified. The Wx^a allele was found to exist predominantly in the indica rice, whereas the Wx^b allele was detected mainly in japonica rice. Except for a change of one amino acid residue, both the genes share an identical protein sequence, suggesting that their specific activities are also similar. However, there are two crucial differences: (i) compared with the Wx^b allele, the transcription of Wx^a is 10-fold higher, and (ii) the Wx^b transcript undergoes defective splicing resulting in the accumulation of unspliced transcripts that significantly lower the synthesis of the GBSSI protein [54, 55].

After this initial identification of the rice *waxy* alleles, several other alleles of the *waxy* locus have been identified as discussed in the earlier section. The association of these alleles with varying levels of amylose content in rice cultivars has been established, and certain alleles have been successfully incorporated into high-performing rice varieties to enhance their cooking and eating characteristics [22, 31, 59–64]. However, introgressive breeding with some low-amylose-content alleles (<13%), such as $Wx^{op/hp}$, Wx^{mq} and Wx^{mp}, have been reported to confer a dull or opaque appearance to the grains, which is unappealing to the consumers [63, 65]. Recently, another *waxy* allele designated Wx^{mw} was identified from a low amylose–containing cultivar 'Mowanggu', which not only had relatively low amylose content (14.1%) but also the desirable transparent grain appearance. This allele would be useful in developing newer japonica-type rice varieties with the

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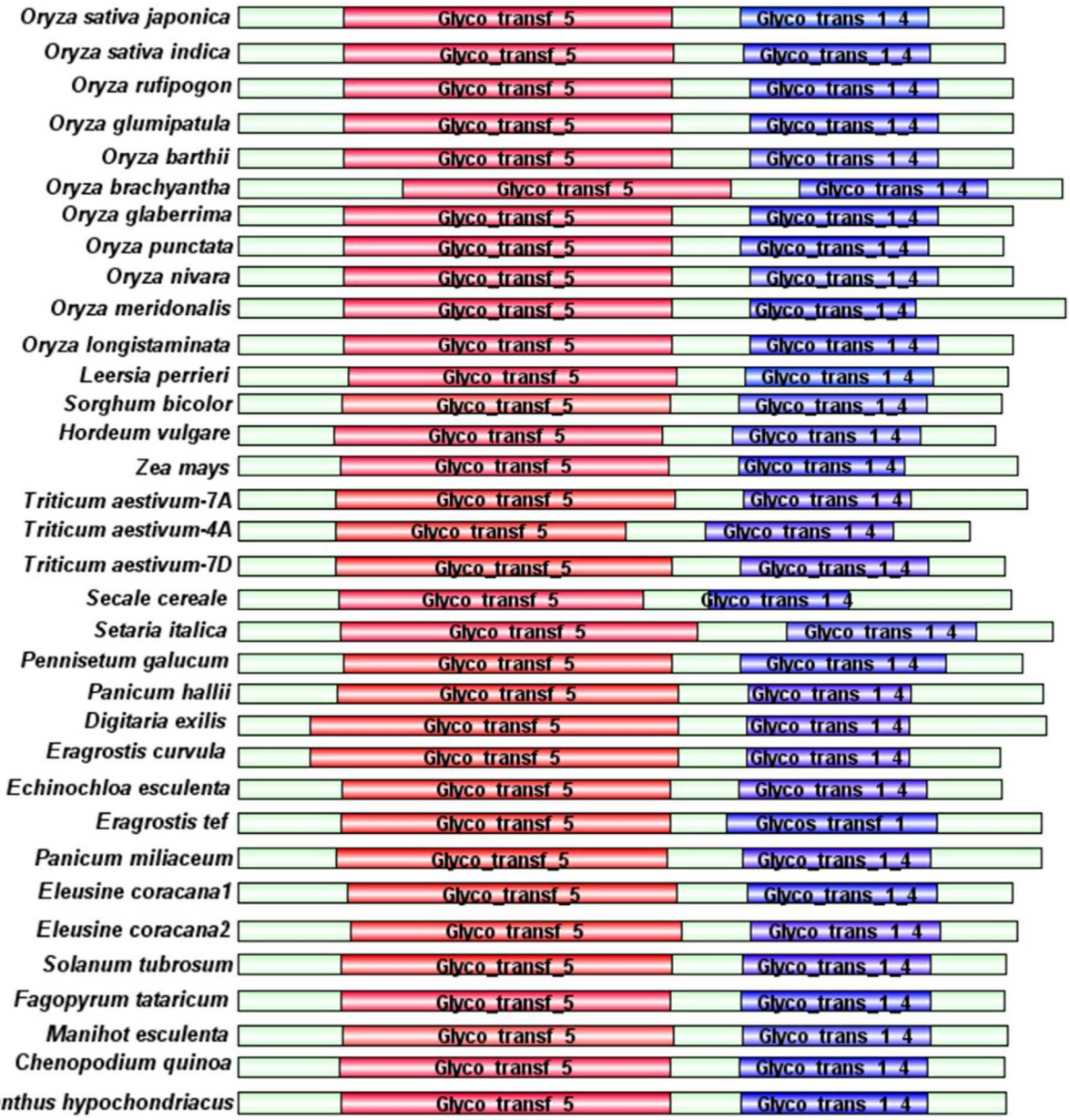

Figure 4. Conserved domains of GBSSI proteins of different cereals, millets and pseudo-cereals. All the GBSSI proteins consist of two highly conserved domains *viz.*, Glyco-transf-5 and Glyco-trans-1-4. 'Glyco-transf-5' represents the main catalytic domain of the GBSSI.

desired level of amylose content as well as transparent grains preferred by consumers [66].

In another example of modern rice breeding, Wang *et al.* [67] crossed the high-yielding japonica rice variety 'Wu-xiang-jing 14' with the japonica rice 'Kantou 194' carrying the low-amylose Wx^{mq} allele and developed a new japonica rice variety, 'Nanjing 46', which had both good eating quality and high yield. Liu *et al.* [68] used a CAPS molecular marker to distinguish Wx^{a} *and* Wx^{b} alleles (conferring high and low amylose synthesis, respectively) for improving the cooking and eating quality of the Chinese rice cultivar Teqing. This marker distinguishes the G (functional) or T (intron 1 splice donor mutation) sequence variants that determine GBSSI protein synthesis. Similarly, Shu and Wang [69] used three STS markers for screening the BC_5F_2 population and developed eight *wx* genotypes. Chen [70] crossed the popular japonica variety 'Wuyujing 3' with the japonica rice variety Kanto 194 carrying both the rice stripe disease resistance allele *Stv-bi* and low-amylose *waxy* allele Wx^{mq} through a backcross strategy. Of the 10 improved lines obtained from the BC_3F_4 generation, two lines, K01 and K04, were stripe disease resistant and had an amylose content of <7%.

Genes that modify the expression of the *Wx* gene are known as 'dull genes' and have also been identified and used to develop low amylose cultivars [71–74] first reported that dull gene mutants are low amylose-containing mutants with dull endosperm and are non-allelic to the *Wx* locus. It has been reported that the reduced amylose content observed in dull mutants is controlled by five recessive loci, namely, *du-1*, *du-2*, *du-3*, *du-4* and *du-5*, which act additively to lower the amylose content [75].

The group further reported that the alleles *du-2*, *du-3* and *du-5* are located independently on chromosome 6, whereas two loci, *du-1* and *du-4*, are positioned on chromosomes 7 and 4, respectively. Using mutagens such as ethyl methane sulfonate (EMS), N-methyl-N-nitrosourea (MNU) and gamma radiation,

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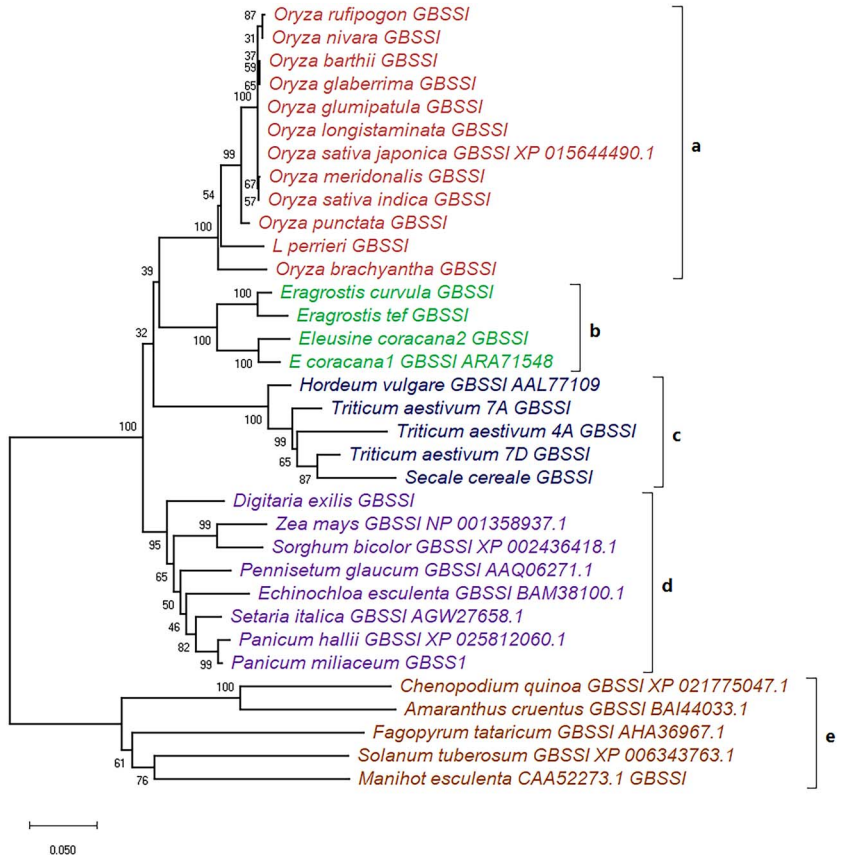

Figure 5. Phylogenetic relationship among the GBSSI proteins of different cereals, millets and pseudo-cereals. The GBSSI proteins clustered into five major groups. Group a consists of rice and its related species, Group b consists of finger millet, Tef and *Eragrostis curvula*, Group c consists of other millets along with maize and sorghum, Group d consists of wheat, barley and rye and Group d consists of pseudo-cereals Amaranthus, Chenopodium and buckwheat along with potato.

several dull mutants have been recovered in rice that have been used to develop low-amylose-content rice. A summary of the rice cultivars developed using dull mutants is provided in Table 2. Pyramiding dull genes along with *waxy* mutant genes has also been attempted. For example, recently Lee *et al.* [76] developed the pyramided rice cultivar 'Milyang319' containing Wx^{mq} and the dull gene *du12(t)* by crossing the parents' cv. Milky-queen (Wx^{mq}) and cv. LGS-soft (du12(t)). In comparison to the donor parents, the amylose content in the pyramided cultivar was much reduced (9.0%).

In addition to the conventional breeding strategies described earlier, modern techniques such as antisense gene silencing technology and genome editing (*e.g.* CRISPR/CAS9) have been successfully used to disable the activity of the *waxy* gene [52, 53, 77]. These techniques obviate the impact of linkage drag caused by the introgression of recessive mutant alleles through backcrossing. In the case of silencing *waxy* genes through antisense technology, molecular techniques such as electroporation and agrobacterium-mediated transformation have been successfully employed [50, 51, 78, 79]. Rice varieties developed through these techniques had reduced amylose contents, up to 2–10% only. As CRISPR/Cas9 gene editing technology offers several advantages, it has become the technology of choice in the last few years to develop waxy rice cultivars. The *waxy* gene was disabled in two widely cultivated japonica rice varieties, XS134 and 9522, using CRISPR/Cas9 technology to produce waxy rice while preserving other favorable agricultural characteristics [52]. Similarly, Yunyan *et al.* [77] knocked out the *Wx* gene of two elite *japonica* rice lines, Huaidao 5 (HD5) and Suken 118 (SK118), using CRISPR/Cas9 and developed 36 wx-HD5 and 18 wx-SK118 homozygous transgene-free Wx-mutant plants in the T1 generation. The amylose content in the seeds of the mutants ranged between 2.6% and 3.2% only. The high yield potential of hybrid rice has also been targeted to produce waxy rice cultivars. Wang *et al.* [80] developed the glutinous CMS line WX209A by first knocking out the *Wx* gene in the maintainer line 209B using CRISPR/CAS9 to produce the homozygous low amylose (<3.5) line followed by one round of hybridization and two rounds of backcrossing (BC_2F_1) with the male and female parents, i.e. WX209B and 209A, respectively. Recently Fu *et al.* [81] developed four waxy rice starches by editing the Wx^a and Wx^b alleles of different rices through CRISPR/Cas9 and reported that it is possible to produce waxy rice starch with different physicochemical properties such as gel consistency and gelatinization temperature. The success of both techniques offers a straightforward method to quickly develop waxy or glutinous rice.

Maize

While maize is a New World cereal, waxy maize varieties have become an important component of the diet in many East Asian and SEA countries where low-amylose cereals are preferred, such as China, Korea Thailand and Vietnam; therefore, waxy maize breeding programs are active in these countries. However, the waxy maize breeding suffers from a limited germplasm base compared with normal maize [81–83]. The waxy maize lines carry various types of mutant alleles, many of which are still

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Table 2. Dull genes identified in rice

Dull genes	Method of development	Type	Target variety	Variety developed	Amylose content	References
du-1	Spontaneous	Recessive	–	–	Low	[75]
du-2						
du-3						
du-4						
du-5						
du-6a(t)	Mutagen N-methyl-N-nitrosourea (MNU)	Recessive	Hwacheong	–	5.9–9%	[160]
du-6b(t)	Mutagen N-methyl-N-nitrosourea (MNU)	Recessive				
Du-7(t)	Mutagen N-methyl-N-nitrosourea (MNU)	Dominant				
duEM47	Mutagen N-methyl-N-nitrosourea (MNU)	Recessive	Kinmaze	eM47	1.9%	[160]
du2120	Ethyl methane sulfonate (EMS)	Recessive	Sasanishiki	2120	4.9%	[161]
du2035	Ethyl methane sulfonate (EMS)	Recessive		2035	4.6%	
du12(t)	Gamma-irradiation	Recessive	Nihonmasari	Aya	16.7%	[162, 163]
				LGS soft	14.5%	[164]
				Ayahime	14.4%	[165]
				Milyang319 (Wxmq+ du12(t))	9.0%	[76]

unknown [81, 84]. So far, more than 50 types of mutations, such as transposon insertions, and insertion and deletion of shorter nucleotide sequences in the *Wx* gene have been reported that result in inhibition of GBSS1 activity. Of these mutants, two deletion mutants, namely, wx-D7 and wx-D10, having a deletion of 30 bp in the 7th exon and 15 bp in the 10th exon of the *GBSSI* gene, respectively, are used as donor alleles for developing waxy cultivars in China. The transposon-derived *waxy* mutations have been classified into two types: (i) DNA transposons and (ii) RNA transposons. The maize mutants carrying the *waxy* alleles of the DNA transposons type are of three types. First are insertion mutants, which include the *waxy* alleles *wx-m1, wx-m5*, *wx-m6, wx-m7, wx-m9, wx-B3* and *wx-B4* caused by the Ac/Ds transposable element types. Second is the dSpm element type that includes the allele *wx-m8*, and third is the En/Spm element type, which includes the allele *wx-844*. The *waxy* maize mutants carrying the mutants *wx-Cin4*, *wx-stonor*, *wx-Reina*, *wx-M*, *wx-B5*, *wx-G*, *wx-I* and *wx-K* have been categorized as RNA transposons. Intragenic selection markers developed from these transposons have been successfully employed to detect individual plants genotypes, i.e. *WxWx*, *Wxwx* and *wxwx* [84].

In Thailand, the white kernel variety, 'Sumlee Esarn', was developed by crossing waxy (wx), sweet (su) and super sweet (sh2) corn varieties [85]. 'Sumlee Esarn' was introduced in 1999 and had soft sticky consistency with a sweet flavor. It was appreciated by both consumers and growers. Developing waxy hybrids were also attempted which resulted in the development of two single cross hybrids with medium ear: (i) KKU white waxy-2901 and (ii) two lines of KKU bicolor waxy-2916. All the hybrids had distinct flavor and consistency. Similarly, in Indonesia, Numba and Ibrahim [86] developed waxy corn F_1 and F_2 lines by crossing the corn variety Srikandi Putih (81.92% amylopectin) and corn Local Waxy Corn (Donor Parents) (97.80% amylopectin). Both F_1 and F_2 lines showed increased amounts of amylopectin (92.57 and 91.31%, respectively) compared with Srikandi Putih. The waxy maize development program in Vietnam was initiated in 2003, in which several local waxy maize accessions were collected from different parts of the country and studied along with several exotic and commercial waxy maize varieties. The program generated and evaluated 1683 inbred lines and resulted in the development of five maize hybrids, namely, HUA 601, MH8, VNU16, VNUA 69 and NT141. NT141 is a purple-colored high-quality waxy maize hybrid with high anthocyanin content [87]. Similar to rice and other crops, transgenic modifications through modern genome editing techniques such as CRISPR/CAS9 have also been used to disable the *waxy* gene to develop waxy maize. Dong *et al.* [88], using the *Agrobacterium*-mediated approach, transformed maize inbred line ZC01 with a single construct containing two sgRNAs that targeted the *Wx* gene along with the *SH2* gene (SHRUNKEN2—for more sweetness). PCR amplification revealed that the technique introduced indels in both the genes resulting in loss of function. Of the 22 T0 plants developed, 20 plants carried either of the two alleles (*sh2* or *wx* alleles) or carried both. Recently, Qi *et al.* [89] disabled the *waxy* gene in the same maize inbred line ZC01 and obtained plants with amylopectin content up to 94.9%. Similarly, a *waxy* knock-out high-yielding corn line was introduced by DuPont Pioneer for commercial cultivation [90].

Wheat

In wheat, the allele *Wx-B1a* predominates in amylose synthesis and produces 21–22% amylose. The two other alleles, *Wx-D1a* and *Wx-A1a*, generate 20–21% and 15–18% amylose, respectively. As discussed in the previous section, waxiness in bread wheat has been achieved by combining nonfunctional (null) alleles of the three *waxy* homeologs, *Wx-A1*, *Wx-B1* and *Wx-D1*, through conventional breeding methods [41, 91, 92]. Wheat with no functional copies of the *GBSSI* gene produces waxy starch with little or no amylose. In contrast, when one or two copies of the GBSSI gene are mutated or missing, the resulting starch is partially waxy and contains a reduced amount of amylose [41, 93, 94]. *Wx-A1* and *Wx-B1* null mutations have been found to occur frequently in Japanese wheat cultivars developed for udon noodles [95]. Yasui *et al.* [96] developed two waxy lines through chemical mutagenesis with EMS. Similarly, Nakamura *et al.* [95] identified two partial *waxy* mutants designated as Kanto107 (K107) and K79, with low amylose content. Analysis of the grain proteins of both the mutants through SDS-PAGE revealed that protein was only produced by the locus *Wx-D1*, indicating that either the *Wx-A1* and *Wx-B1* alleles are not transcribed because of mutations in the regulatory regions or that mutations in the reading frame have caused truncation of proteins, resulting in only one functional *GBSSI* gene to produce partial waxiness.

Nullisomic studies on the cultivar 'Chinese Spring' was carried out by Nakamura *et al.* [95] to identify the genomic locations of the three *Wx* loci; this work revealed that *Wx-A1, Wx-B1* and *Wx-D1* are located on chromosome arms 7AS, 4AL and 7DS, respectively.

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As a result of a reciprocal translocation that occurred during the evolution of bread wheat, genetic material from chromosomes 7B and 4A were exchanged, explaining why the *waxy* allele is present on the wheat 4A homologous chromosome rather than 7B. Although being located on the A genome, this region is still known as *Wx-B1*. Identification of the wheat homeologs has enabled further study of their expression in wheat endosperm; this work led to the detection of three types of null mutants among the Japanese and Chinese wheat cultivars, which has further advanced possibilities of breeding waxy wheat [97, 98].

Using the partial *waxy* mutants Bai Huo (*Wx-D1* null) and Kanto107 (K107, *Wx-A1* and *Wx-B1* null), Nakamura *et al.* [41] reported the first genetic modification of wheat starch by developing tetra- and hexaploid *waxy* mutants that showed complete loss of Wx gene activity resulting in reduction of amylose content. To further study the null alleles of three *Wx* loci that lead to the production of fully waxy lines, Miura *et al.* [91] examined eight recombinant lines containing various null alleles (*b*) at the *Wx* loci in the CS genetic background and investigated the amylose production capacity of each *Wx* gene. The waxy CS (Chinese Spring) and CS normal type exhibited a range of 0–25% in their amylose content, respectively. The maximum reduction in effect was observed in the case of *Wx-B1b* for the single null alleles, but it was still relatively small. On the other hand, there was no significant difference observed between *Wx-A1b* and *Wx-D1b*. However, the double null types exhibited reductions in amylose content > 3%. While amylose concentration was not found to be directly associated with the number of the *Wx* genes, a comparison of the double null lines with the single null or normal lines revealed that *Wx* genes work in an epistatic way.

After studying eight wheat genotypes at *waxy* loci, Kim *et al.* [94] revealed that the amylose content decreases, and starch crystallinity increases as the number of null alleles are increased. However, it was observed that the null allele at the *Wx-B1* locus had a higher impact on the starch amylose content than the single null alleles at the other two loci.

Hence, Nakamura *et al.* [99] argued that six different types of partly waxy wheat might be developed utilizing the combination of mutations occurring in three homologous waxy loci.

Millets

As described earlier for rice, maize and wheat, induced mutations have also been widely used for induction of waxiness in grain starch in various millets. Hoshino *et al.* [100] developed a completely waxy cultivar of the hexaploidy Japanese barnyard millet (*Echinochloa esculenta*) by inducing mutations through gamma irradiation. To achieve this, they chose to irradiate known landraces that had approximately half of the amylose content (15–20%) relative to the amount of amylose content found in most of the Japanese barnyard millet genotypes (25–30%). A fully waxy line was obtained from the M_2 plant (4-584), which, according to iodine tests, revealed that it had waxy character. Further, while comparing the *waxy* gene sequences of the developed waxy Japanese barnyard millet lines by analyzing the PCR products generated through the conserved primers, it was confirmed that there exist three separate waxy in the Japanese barnyard millet genome. This observation was consistent with expectations for homeologs in a hexaploid species. The analysis of the PCR products revealed that the low amylose line lacks one of the three *waxy* genes, causing reduced amylose content in the grains.

Likewise, Graybosch and Baltensperger [101] revealed that the stickiness trait in the tetraploid proso millet (*P. miliaceum* L.) is governed by recessive alleles at two loci. They evaluated 650 accessions for the presence of amylose-free starch in the endosperm and identified six waxy accessions. Using the segregating F_2 and F_3 populations derived from the crosses of these waxy accessions, it was found that the waxy character was governed by two loci designated as *wx-1* and *wx-2*. Despite the investigation, the molecular genetic basis of the waxy phenotype was not identified in the study. Subsequently, Hunt *et al.* [38] characterized the two *waxy* loci by analyzing the *waxy* gene sequences of 14 waxy phenotype plants identified after screening 72 plants from 38 landrace accessions. The study divulged two diverse forms of *GBSSI* that was designated as L (for long gene) and S (for short gene). A 15-base pair deletion was detected in the coding region of the S type GBSSI, resulting in the removal of five amino acids from the glucosyltransferase domain 1 (GTD1). The L type, however, shows two types of sequence polymorphism: (i) insertion of A in the exon 9 that results in frameshift mutation and (ii) a G/A substitution in exon 7 that causes cysteine–tyrosine substitution. Hence the L type was detected to carry three types of alleles. The 15 bp deletion in the GBSSI-S gene leads to the loss of enzyme activity, whereas the frameshift mutation in the L-type gene causes the GBSSI protein to be absent from the starch granules, suggesting that the resultant mutated protein might not be targeted to the starch granules. However, the second L allele, which encodes the enzyme with the Tyr residue, was present in the starch granules but was nonfunctional. The functional analysis of the enzyme with the cysteine residue variant was suspected to be of the functional type.

Potato

Among non-cereal crop species, potato (*S. tuberosum*) is an important starch-rich staple crop consumed across the globe. In potato tubers, starch is the major storage compound that has several uses in both culinary and non-food applications. Development of potatoes with unique starch properties has been a longstanding goal. However, the complex tetrasomic inheritance in this tetraploid species has been a major hinderance in genetic modification of traits including starch quality. Despite these challenges, potato has been modified to solely synthesize amylopectin starch by eliminating GBSS activity through mutagenesis, RNAi and gene editing using the CRISPR-CAS system [102–105]. The first induced *waxy* mutation in potato was produced through inactivation of the *waxy* gene through gamma irradiation [106]. Later, EMS mutagenesis was used in di-haploid potato to generate a series of point mutations with altered enzyme function in the *GBSSI* gene [107]. This strategy allowed the identification of 10 missense mutations and one splice site mutation that was found to cause a loss of GBSSI enzyme activity. In addition to this, a potato clone with high-amylopectin starch carrying the waxy splice site mutation in homozygous condition was also generated [107]. A genetically modified potato variety 'Amflora' has been developed by BASF plant science in which >98% of amylopectin starch is produced [108]. However, as high amylose content is also a desirable trait, researchers have also been successful in developing high-amylose potatoes by targeting two starch branching enzymes [109–114].

Besides potato and the cereal crops reviewed earlier, efforts to introduce the waxy trait have also been carried out in many other crops (see Table 1). These studies on the development of waxy and non-waxy genotypes continue to expand as advances in breeding technologies facilitate the increased ability to perform genetic modifications in non-model crop species.

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Identification of waxy types: a molecular screening approach

Molecular markers offer a quick and effective method to screen desirable traits. Before the advent of molecular markers, the waxy trait was detected through morphological features of the starch such as its pasting properties, retrogradation and viscosity characteristics, as well as by the iodine test described earlier. Availability of nucleotide sequence information for *waxy* genes of diverse species, including wheat, maize, rice, foxtail millet, pearl millet, barley, sorghum, pea, *Arabidopsis* and potato has led to the development of molecular markers that enable quick identification of the waxy strains from non-waxy ones.

In wheat, quick identification of the genotypes carrying all the three *waxy* loci or combinations of it was achieved by molecular markers that were also utilized in the wheat breeding program. To screen *waxy* mutants in wheat, Nakamura *et al.* [99] designed three PCR primer sets that distinguished between waxy and partial-waxy cultivars. Later, Saito *et al.* [115] discovered a novel *waxy* mutation in 168 common wheat varieties by utilizing DNA-based markers to identify null *waxy* alleles. The mutation involved a 173-nucleotide insertion in exon 4, which was located in a different position from the earlier identified Wx-*A1* mutation. Subsequently, several more null alleles caused by different mutations have been described, and many of them can be detected using molecular markers [42, 116, 117]. In Japanese barnyard millet, Ishikawa *et al.* [39] analyzed the three homoeologous *waxy* alleles using conserved PCR primers and divulged that the genic region encoding the *GBSSI* protein are well conserved across the grass species. In the case of sorghum, Sattler *et al.* [15] and Kawahigashi *et al.* [118] developed allele-specific DNA markers to differentiate between the *waxy* (wx^a, wx^b, wx^c) and non-*waxy* alleles of the *GBSSI* locus. The wx^a allele is associated with no detectable levels of the enzyme GBSS1, whereas the wx^b allele is associated with inactive *GBSSI* in the endosperm. The wx^c allele is linked with suppression of *GBSS1* gene expression.

Similarly, Cho *et al.* [29] employed quantitative real-time PCR (qPCR) assay to detect and measure waxy content in a mixture of waxy and non-waxy sorghum samples. The assay involved the use of separate allele-specific primers for detecting *waxy* (wx^a) and non-*waxy* allele. The primer pairs were highly sensitive and were capable of detecting tiny amounts (>0.5%) of waxy sorghum types in the mixture. The allele-specific primers failed to generate any PCR amplicons from non-target templates. For classifying Chinese sorghum germplasm, Lu *et al.* [119] developed cleaved amplified polymorphic sequence (CAPS) markers based on *waxy* mutations. Sequence variation information caused by transposon insertions have also been used to design primers to detect *waxy* alleles. Jia *et al.* [120] designed six sets of STS primers using sequence variation information resulting from transposon insertions. These primers were utilized to identify 10 allelic variants of the *waxy* gene across 11 different strains of foxtail millet. A summary of different primers used to detect known *waxy* alleles in various crops is presented in Table 3.

Uses and applications of waxy grains

As noted above, waxy crop varieties hold long-standing cultural significance in the food habits and traditions of East and Southeast Asia, such as ease in eating rice with chopsticks because of its stickier texture [9, 16, 19]. However, this textural quality is also important from an industrial point of view, especially in food processing and preparation of special food formulations. The production of a palatable and nutritious food product with desirable flavor and texture through food processing relies on the gelatinization temperature of starch present in the food. Amylose content affects the temperature at which gelatinization occurs; high amylose content prevents granule expansion and raises the gelatinization temperature [121]. Low-amylose (waxy) starch, in contrast, has lower gelatinization temperature because of greater starch grains swelling power and solubility, which causes softness of swelled starch granules that can be easily dissolved at lower temperatures (65–70°C), leading to a thick paste [122]. As waxy strains have a greater amylopectin content, they provide a more solid gel structure, crystallinity and hardness to foods containing starch. As a result, they have several uses in bakery products for delaying staleness, extending bread's shelf life and creating a new texture of bread [123, 124]. Waxy grains have been used for a variety of food uses because of their much stickier texture, including making sticky grains when they are cooked, cakes by hammering steamed grains, spring rolls by boiling or steaming dough or batter, brewing beverages, etc., whereas non-waxy types can only be used for boiling or mixing with other food products, like cooked rice or grain soup [125, 126]. A high level of amylopectin alters the properties of flour and starch because of which waxy wheat flour tends to absorb additional water and requires less time and energy for dough development, which is the desired character in baking and food processing industries [127]. To enhance the texture and functionality of the final products, waxy wheat flour can be blended with regular wheat flour. However, it should not be used alone because of its poor dough strength [124]. Adding <20% of waxy hexaploid wheat flour to bread, cakes, white salted noodles and pasta results in significant enhancements to the products' shelf life. This approach may prove beneficial in the development of grain-based foods that are more tolerant to staling and freezing [128]. However, when waxy wheat flour was added up to 40%, it led to larger loaves and improved the glutinous texture of bread in comparison to non-waxy wheat flour [129]. Slightly lower amylose contents confer desirable phenotypic properties like water uptake power, high peak consistency, a low degree of crystallinity and a high rate of breakdown for the production of noodles [38, 99, 130]. Also, the eating quality of the noodles correlates negatively with the amylose contents of flour [131]. The noodles produced with flours containing reduced amylose exhibit a reduction in firmness, gumminess and chewiness, but an increase in cohesiveness, springiness and resilience properties [124, 132]. Waxy wheat flour is being used more frequently as a viable component for producing direct expanded products with less energy [133].

In brewing industries, waxy strains have profound importance, especially in the case of brewing with waxy sorghum. Characterized by a comparatively weak endosperm and protein matrix, the waxy sorghum has a lower gelatinization temperature (69.6°C) and more susceptibility to hydrolytic enzymes such as proteases and amylases. These features confer waxy sorghum with better qualities for brewing beer in terms of strength of ethanol and fuel oils [134–136]. Total amylose and starch contents ranged from 5.5 to 7.3% and from 65.4 to 76.3%, respectively, in 25 waxy grain sorghum varieties. Fermentation efficiency ranged from 86.0 to 92.2%, producing ethanol yields of 2.6–3.03 gal per bushel, respectively [137]. Jampala [138] integrated the high-digestibility trait with waxy lines (HD-WX) in sorghum, which fermented rapidly (i.e. within 48 h). Waxy wheat strains were also found better for bioethanol production than non-waxy strains with higher conversion efficiency and faster fermentation [139]. Waxy barley starch was observed to have higher levels of β-glucan (6.4%) and extract viscosity, rendering it suitable for use in malting and brewing, as well as in food and feed applications [140]. Similarly,

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Table 3. Molecular markers for identification of waxy types in crops

Crop	Allele	Marker type	Primer sequence	Remark	Reference
Rice	Wx^a	CAPS	WP-CAPS (5′-TGTTGTTCATCAGGAAGAACATCTCCA AG-3′)	Digestion with EcoT14I	[166]
	Wx^b		WP-B (5′-TTAATTTCCAGCCCAACACC-3′)	Wx^a produces two fragments 133 and 29 bp Wx^b one fragment 162 bp	[166]
	Wx^{in}		5´-GAGATCAATTGTAACTCACCAG-3′ 5´-GTTGGAAGCATCACGAGTTT-3	155 bp for detecting exon 6 SNP nucleotide C; no amplification for exon 6 SNP nucleotide A	[166]
	Wx^{lv}	Alle-specific primer CAPS	PAG F: ACCATTCCTTCAGTTCTTTG R: ATGATT TAA CGA GAG TTGAA	Int 1–1 (G/T) (PCR-AccI CAPS)	[21]
		Alle-specific primer	WX10AT1 F: GGCTGGAGGAACAGAAGGACC R: TCGAACTTCTTCTTTCCAGTACCCT WX10AT2 F: CGACGCAACCACGGTAAGAACGAAT R: GCGGCGGCCATGACGTCTGA	Exon 10–115 (C/T)	
	$Wx^{op/hp}$	Allele-specific maker	WXop1 F: GGAGTCGACCGTGTGTTCATTGA R: GTAATCAACTCCAGTGTCAGGTCCG WXop2 F: CCACAGGGTCATGGTGATCTCTC R: CCTTCTCCAGGAATGACGGATAGC	Exon 4–77 (A/G)	[21]
	Wx^{mq}	Allele-specific maker	W1- GGTGAGGTTTTTCCATTGCTACAAGCA W2- GCCCTGGTAGGAGATGTTGTGGAT	G-to-T mutation intron 1	[60]
	Wx^{mp}	Allele-specific maker	WXmp1 F: ATGTTGTGTTCTTGTGTTCTTTGCAGGC R: GTCGATGAACACACGGTCGACTCAAT WXmp2 F: GGGTGAGGTTTTTCCATTGCTACAATCG R: GTAGATCTTCTCACCGGTCTTTCCCCAA	Exon 4–53(G/A)	[167]
	wx		Glu-23F - 5´-TGCAGAGATCTTCCACAGCA-3′ Glu-23R - 5´-GCTGGTCGTCACGCTGAG-3′	Discriminate between varieties with *Wx* and *wx*	[62]
	Wx^{mw}	Allele-specific maker	WXmw1 F: AACAACCCATACTTCAAAGGAACATC R: GTAGATGCCATTGGGCTGGTAGT WXmw2 F: GCTTAGCTTCCACTGGTGATTTCA R:TCTTGAGATCAATTGTAACTCCCCAT	Exon 6–62(A/C)	[167]
Maize	*wx-m1*		Exon (1–4) F: AGAAGTGTACTGCTCCGTCC R: AGAACCTGACCGTCTCGTAC	*Ds* insertion in *wx-m1* exon sequence is 409 bp long	[66]
	wx-m5		Exon (4–8) F: TACGAGACGGTCAGGTTC R: GGTAGGAGATGTTGTGGAT	2-kb *Ds* element at −470 bp relative to the start of *waxy* transcription.	[153]
	wx-m6		Exon (8–12) F: GATTTCATCGACGGGTCTGT R: TCTGTCCCTCTCGTCAGGAT	2.1 kb insertion	[84]
	wx-m7		Exon (12–14) F: ATCCTGACGAGAGGGACAGA R: CACCGAACAGCAGGGATTAT	TE insertion of 4.3 kb in the exon 10 of *waxy gene*	[84]
	wx-m9				
	wx-m8			2 KB insertion in the exon 11	[84]
	wx-m844			Insertion of 8.4 kb long En-I element	[84]
	wx-stonor			4.5 kb, junction of intron 5-exon 6),	[84]
	wx-B5			5.0 kb, intron 8)	[84]
	wx-G			6.1 kb, intron 2)	[84]
	wx-M			5.7 kb insertion	[84]
	wx-I			4.5 kb insertion	[84]
	wx-K				[84]

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Table 3. Continued

Crop	Allele	Marker type	Primer sequence	Remark	Reference
	wx-B3			4.5 kb insertion in exon 10	[84]
	wx-B2			0.15 kb insertion between exon 2 and 3	[84]
	wx-B4			Deletion	[84]
	wx-B1			Deletion	[84]
	wx-B5			6.1 kb insertion	[84]
	wx-B			Deletion	[84]
	wx-B6			Deletion	[84]
	wx-B4			Deletion	[84]
	wx-B7			Deletion	[84]
	wx-C34			Complete deletion	[84]
	wx-D7			30-bp deletion in the seventh exon–intron region	[84]
	wx-c2			Deletion	[84]
	wx-D10			15-bp deletion in the 10th exon region	[84]
	wx-Reina		Reina_1R TGTTCCTCTTCTTCGCCTGTA Reina_1F TCGATTTGTCGCTCACTACG Reina_2F GTTGCTTACCCTGCTTCTGC Reina_2R CGTCGGTTTCCACTACGAAT Reina_3R CTACTGGGGTTTTGACAGTGG	5.4-Kb retrotransposon *Reina* inserted in the 10th intron	[153]
	wx-Cin4		F- TACGAGACGGTCAGGTTC R: GGTAGGAGATGTTGTGGAT	*wx-Cin4* mutation is a 466-bp retrotransposon inserted into exon six.	[168]
	wx-124			The *wx-124* mutation is a 116-bp miniature inverted-repeat transposable element inserted into exon seven.	
	wx-c		F: CGCGTGTTCGTTGACCACCC R: GCGGAGGAACAAGTCGCGGA (amplicon digested with *SstI*)	A 30-bp deletion within the gene. Wild type produces a band of size 632 bp, whereas mutant produces a band of 602 bp	[169]
	wx-1240				
	wx-B12				
	wx-hAT		Exon(1–4) F: AGA AGT GTA CTG CTC CGT CC Exon(1–4) R: AGA ACC TGA CCG TCT CGT AC WaxyF2: AGT ATT GCT TCT ACC TGT GGCA	Amplify an 884-bp product for the wild-type *waxy* gene, a 608-bp product for the mutant type	[84]
Wheat	Wx-*A1*		5′-TTGCTGCAGGTAGCCACACCCTG-3′ 5′-AGTTGCTCTTGAGGTAGC-3′		[156]
	Wx-*B1*		Wx-B1 (wild type) F: 5′ CTGGCCTGCTACCTCAAGAGCAACT 3′ R: 5′ CTGACGTCCATGCCGTTGACGA 3′ Wx-B1 (null) F: 5′ CGTAGTAAGGTGCAAAAAAGTGCCACG 3′ R: 5′ ACAGCCTTATTGTACCAAGACCCATGTGTG 3′		[156]
	Wx-*D1*		5′-TAGTGCGTCCAGACTCACAG-3′ 5′-GAGATGGTCAAGAACTGCAT-3′		[156]
	Wx-*A1b*		AFC - TCGTGTTCGTCGGCGCCGAGATGG AR2- CCGCGCTTGTAGCAGTGGAAGTACC		[156]
	Wx-*B1b*		BDFL- CTGGCCTGCTACCTCAAGAGCAACT BRD- CTGACGTCCATGCCGTTGACGA		[156]
	Wx-*D1b*		Forward 5′-CCTTGCGTCATTTGTTGTGTGG-3′ Reverse-5′-TTGCTGCAGGTAGCCACACCCTG-‘		[156]

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Table 3. Continued

Crop	Allele	Marker type	Primer sequence	Remark	Reference
Sorghum	Wx	Allele specific	Wx-F-GCTGGTTCTGAGTGCAACAA Wx-R, ACTTCTTCTTGCCAGTGACC	Wild	[15]
	wx^a	Allele specific	wx-a-F 5-CGTGGCGAGATCAAACTCTA-3 Wx-R 5-GCAGCTGGTTGTCCTTGTAG-3	Amplified a 615 bp fragment containing the junction between wxa insertion and exon 5.	[15]
	wx^b	CAPS (NcoI)	Wx, forward 5' CGACCGTGTGTTCATTGACCAC-3' Reverse 5'-TTGTTCAGTGCCTTTGCCTCG-3'	NcoI and analyzed by agarose gel electrophoresis. Wxb PCR product contained a single NcoI restriction site following the digest, generating 745 and 537 bp DNA fragments. The wild-type PCR product did not contain a NcoI restriction site and a single 1281 bp fragment was observed.	[15]
	wx^c	Allele-specific primer	Wx-F-GCTGGTTCTGAGTGCAACAA Wxc-R-ACTTCTTCTTGCCAGTGACG	–	[15]
Potato					
Cassava	Wx		*F2: [GC]ATGTTGAAGTAAGTAAAGATGC* *RN: TGCTCAAGGCGTGGGAAGGT*	–	[45]
	wx		*F4: [GG]ATGTTGAAGTAAGTAAAGATGG* *RN: TGGTGAAGGGGTGGGAAGGT*	–	
Barley	Wx-CDC Candle Wx-Bowman		Promoter-wx-F/R F:ACAGACGACAAGCGGAGAA R:ACGCACCACCACGGAGA Wx-F1/R1 F:CTCACTGCAGGTAGCCAC R:GCACCTTTGAGTTTGGAA Wx-F2/R2 F:ACCGCGTGTTCATCG R:CTTGTCGTAGCCGTCAA Wx-F3/R3 F:CTTCATTGACGGCTACGAC R:AGATGCTCCATGCACCAG	Differentiates the alleles	[155]
Foxtail millet	Type II (TSI-1)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +300 bp (Non-waxy)	[9]
	Type III (TSI-6)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +4 kbp (Low Amylose)	
	Type IV (TSI-2)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +5 kbp (Waxy)	
	Type IVa (TSI-4)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +5.5 kbp (Waxy)	
	Type IVb (TSI-5)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +5.5 kbp (Waxy)	
	Type V (TSI-7)		ex2int2: (F) CATGGCCGTAAGTCCCATCGATCGATCATC (exon 2-intron2) ex4r: (R) TAGCAGTGGAAGAACCTCACCCTCTCGTAC (exon 4)	Wild type +7.5 kbp (Waxy)	
	Type VI (TSI-10)		M7: (F) CACCAGCCGCTTCGAGCCCT (exon 11) R10: (R) GCCGGCGTGCCGACCACCTT (exon 13)	Wild type +400 bp (Low Amylose)	
	Type VII (TSI-9)		M5: (F) GGACGTCAGCGAGTGGGACC (exon 9) R7: (R) ACGAGTCCACCGGTGGACGC (exon 12)	Wild type +9 kbp (Waxy)	
	Type VIII (TSI-11)		M7: (F) CACCAGCCGCTTCGAGCCCT (exon 11) R10: (R) GCCGGCGTGCCGACCACCTT (exon 13)	Wild type +1.5 kbp (Waxy)	
	Type IX (TSI-3)		ex1:(F) TGCAAGCCAGTGACGGATCGACGACGACA (Exon1) ex2: (R) ATGCCGGTGACCAGCGTGGAGGGCTAGCTA (exon 2)	Wild type +3 kbp (Low Amylose)	

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Table 3. Continued

Crop	Allele	Marker type	Primer sequence	Remark	Reference
	Type X (TSI-8)		ex2int2: (F) CATGGCCGTAAGTCCCATCGATCGATCATC (exon 2-intron2) ex4r: (R) TAGCAGTGGAAGAACCTCACCCTCTCGTAC (exon 4)	Wild type +1 kbp(Waxy)	[9]
Barnyard millet	*Ee*Wx1		Wx1F: ACAGCTCACTCACTGTCTACTCTGGATTA Wx1R: CGATACATGCACTGAATACTTGCACAC	Wild 348 bp	[39]
	$EeWx1_{CM}$			Waxy 262 bp	
	*Ee*Wx2		Wx2F CCATGGCTGTAAGTCCATCACTTCT Wx2R TGACACACATGACACGTCATGTATGC	Wild type 170 bp	
	*Ee*Wx2*null*			–	
	*Ee*Wx3		Wx3F: TTCCTCCCTCTCTTCACAGTCCAAT Wx3R: TAGATGTTCACTTGGGGGTCCATATC	Wild type 460 bp	
	$EeWx3_{NH\&CM}$			Waxy 548 bp	
Proso millet	WxL_f	Allele-specific primer	CGTGACCATCTCTTCCTGTA CGACGACGAACTCTCAACAC	Amplifies the 632-bp region including the A insertion site	[38]
	WxL_Y	Allele-specific primer	ATGTTTGAATGAATGCTCC TGGTAGTTGCTCTTGAGGTA	Amplifies the 251-bp region including the SNP	
	WxS_0	STS marker	GGACGTCAGCGAGTGGGACC CAGGCACACTGCTCCCAATG	Amplifies the 391-bp region including the deletion site	
	WxS_{-15}		GGACGTCAGCGAGTGGGACC CAGGCACACTGCTCCCAATG		
Job's tear	EeWx	STS marker	M5-F GGACGTCAGCGAGTGGGACC (Exon 9) R7-R ACGAGTCCACCGGTGGACGC (Exon 12)	Waxy allele produces an amplicon 275 nt less than the wild type	[158]
Amaranths	Type Ia	STS primers for sequencing	GBSS-F20 ATGGAAACAGTAACATCTTCTCACT GSP2 CATCTTTTCATAGAATAGCCAAGTCA	Exon 1 to partial intron 4 (1098 bp)	[43]
	Type Ib				
	Type IIa		Wx-F1 ACATGGTTTATTCTATGTCATTTATT Genomic R AGGCAAATCTTCCTTGATATACAATA	Partial intron 3 to partial exon 7 (925 bp)	
	Type IIb		GSP 2–2 CAAAGGTATTGGCTGAAATGTAACTA GBSS-R12 AAGAATGTCAGAACCTTTTTGCTCTT	Partial intron 4 to partial exon 9 (1053 bp)	
	Type IIc		Wx-F2 ATTGATCAGTTATGTTTTACAGGTAT GBSS-R13 GATATATCAGCAGGATCTACCATGTC	Partial intron 7 to partial exon 12 (1166 bp)	
	Type IId		Wx-F3 ATGTTAATTCCTAGCAGATTTGA Wx-R1 CTTTGTGAATTTGTTGTTGAATA	Partial exon 10 to partial 3′-UTR (914 bp)	
	Type IIIa				
	Type IIIb				
	Type IIIc				

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the waxy varieties of proso millet were also found to be better in fermentation efficiencies than their non-waxy counterparts [141]. Waxy starch can also be utilized in the production of adhesives, as it has been deemed superior to regular starch because of its ability to create a fluid paste using considerably less water[170] Consequently, waxy-starch gums can be employed on paper without significantly dampening the paper [14].

CONCLUSION

Waxy starch, with already much-renowned importance, immense future industrial potential and many unexplored aspects, has provided researchers with possible avenues for research in diverse strains of different crops. The *waxy* gene is one of the most widely studied genes in crop plants, and numerous natural variants in the *Wx* locus have been identified across diverse crop species. Genes and their genetic organization in controlling waxy character have already been deciphered through the use of molecular techniques in many crops. Enhanced understanding of the genetics that regulate starch biosynthesis has enabled the cultivation of crops with unique starch characteristics through selective breeding. Despite the significant strides made in enhancing the quality of starch and its various uses in the food, papermaking, pharmaceutical and biofuel sectors, the presence of linkage drag has hindered efforts to overcome the yield barrier, thereby limiting overall success. Integrating advanced genetic engineering techniques such as RNAi and gene editing with breeding methods to accurately target and generate advantageous *Wx* alleles seems to be a promising approach for creating healthier and future ready superior breeding lines.

Key Points

- High-amylopectin and low-amylose-content starches have cultural importance and are well suited for use in industrial manufacturing processes.
- Waxy trait is determined by a solitary gene known as GBSSI (or Waxy), which is responsible for encoding the enzyme Granule Bound Starch Synthase 1.
- Several waxy alleles have been identified in diverse crops.
- Phylogenetically, the GBSSI orthologs form distinct clusters that correspond to specific lineage of crops.
- Modern genome editing techniques as well as molecular breeding techniques have been successfully utilized to develop waxy cultivars in different crops.

AUTHOR CONTRIBUTIONS

S.S.: Conceptualization, Original Draft Preparation and Editing; V.S.G.: Data Collection, Analysis and Original Draft Preparation; CG: Editing; K.M.O.: Guidance and Editing and Revision.

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Conflict of interest

The authors declare no conflict of interest.

Data availability

Authors have used and analyzed the already available data of waxy genes and proteins in the NCBI database. No new data was generated in this study.

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