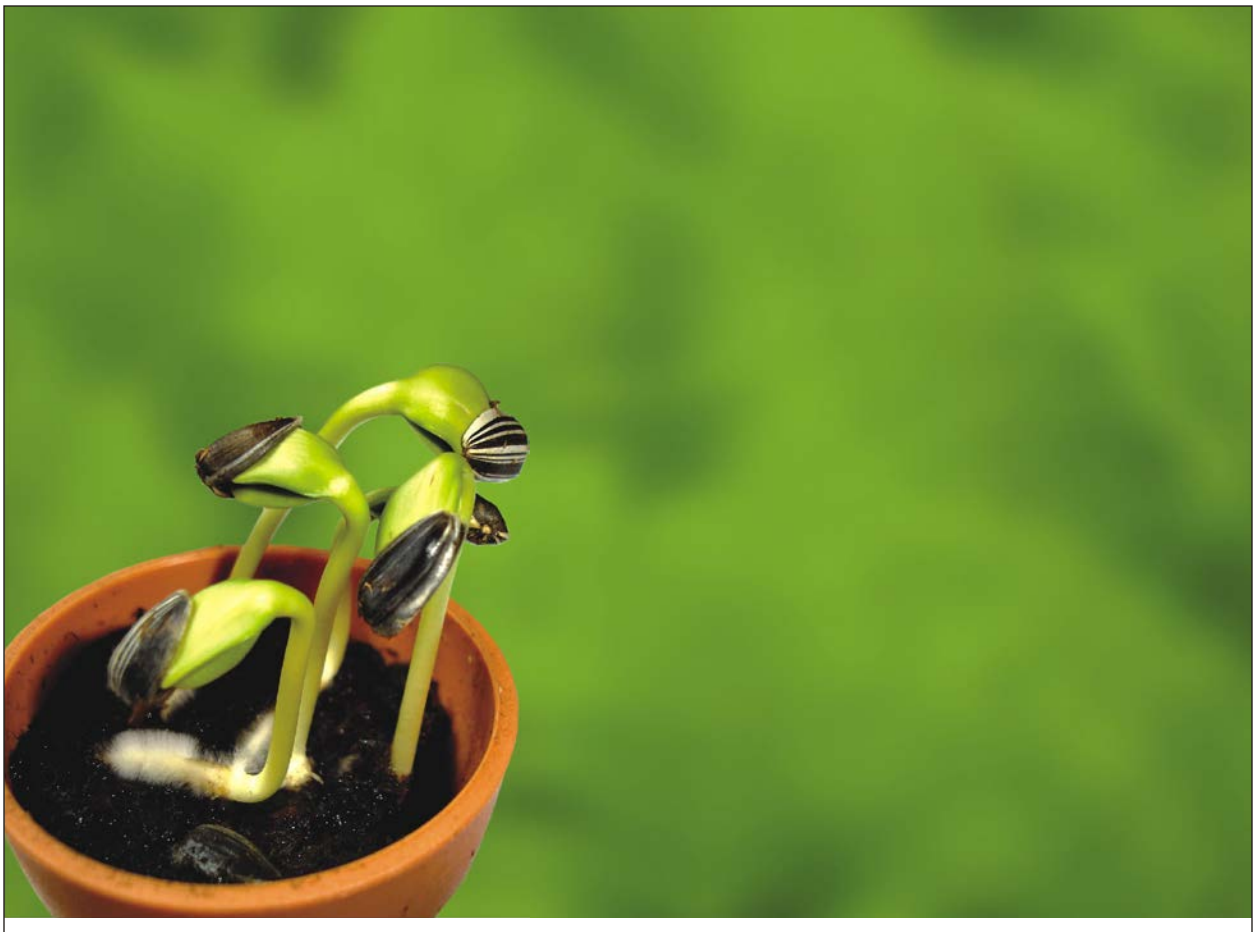

International Journal of Plant Breeding and Genetics

ISSN 1819-3595

Academic Journals Inc.

www.academicjournals.com

International Journal of Plant Breeding and Genetics 2 (2): 93-100, 2008
ISSN 1819-3595

Molecular Mechanism of Sweet and Waxy in Maize

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Abstract: The aim was to uncover the molecular mechanism of sweet and waxy in maize, hopping it to be useful in special-used maize breeding and developing some intragenic selection markers. Sweet maize and waxy maize are popular vegetable food and important materials for processing production. Their sweet and waxy characters are controlled, respectively by recessive genes *sh*$_2$, *bt*$_1$, *bt*$_2$ and *wx* located at different chromosome loci. On molecular level, these recessive genes are mutated from the encoding genes of the key enzymes of starch biosynthesis pathway by the insertion of different transposon elements. The effort to breed double recessive sweet-waxy maize controlled by recessive sweet genes and recessive waxy gene is impractical, because sweet and waxy phenotypes can not be expressed in the same inbred line or hybrid for their exclusion in biochemical metabolism. The backcrossing procedure of the conventional breeding method for sweet maize and waxy maize can be cut down almost to half, if intragenic selection marker is developed on the basis of sequence difference between the dominant genes and the recessive mutated genes and used to identify the recessive genes from the segregating generations.

Key words: Special-used maize, introgenic selection marker, sweet, waxy

INTRODUCTION

Sweet maize and waxy maize are popular vegetable food and important materials for processing production. Breeding of sweat hybrid and waxy hybrid is distinguished from breeding of ordinary hybrid for introduction of recessive sweat genes or waxy gene into parent inbred line by backcrossing. Although many studies in the early 20th century demonstrated influence of endosperm mutants to type and quantity of carbohydrates in kernels, there are still some misunderstandings about the inheritance of sweet character and waxy character in practice of maize breeding. In recent years, research advances in biochemical pathway of starch biosynthesis in endosperm and sequence mutation of the related genes explained this problem on molecular level and provided reference for breeding practice.

Chromosomal Loci and Phenotypes of Sweet Genes and Waxy Gene in Maize

In classical theory of maize breeding, inheritance of sweet character and waxy character are identified as endosperm mutants determined by recessive genes, which have been located at different loci of maize chromosomes by cytogenetic approach (Table 1) (Cameron and Teas, 1953; Collins, 1909; East and Hayes, 1911; Laughnan, 1953; Weatherwax, 1922). According to the sweeter of kernels determined by the content of reducing sugars and sucrose and the texture of kernels determined by water-soluble polysaccharides, starch content and seed capsule thickness, the varieties with genotypes susu, *sh*$_2$*sh*$_2$, *bt*$_1$*bt*$_1$ and *bt*$_2$*bt*$_2$ are, respectively called sweet, super sweet, brittlely sweet and

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brittlely sweet maize in production. Varieties with genotype *wxwx* produce kernels that contain starch consisting of nearly all amylopectin and are called waxy (sticky) maize (Alexander and Creech, 1977; Creech, 1965; Li, 2002, 2003).

Pathway of Starch Biosynthesis in Maize Endosperm

Referring to MuForster (1996), Okita (1992) and Smith *et al*. (1997), the pathway of starch biosynthesis in maize endosperm can be shown in Fig. 1. At first, sucrose transported from leaves and bracts is catalyzed by sucrose synthase and uridine diphosphate (UDP) glucose pyrophosphorylase and hydrolyzed into 1-phosphoglucose. Under the catalysis of adenosine diphosphate (ADP) glucose pyrophosphorylase, 1-phosphoglucose reacts with adenosine triphosphate (ATP) to form ADP-glucose, which is then transported into amyloplasts in the endosperm by adenylate transport protein and used as substrate of starch biosynthesis. Granule-bound starch synthase as well as isoenzymes of soluble starch synthase transfers the glucopyranosyl from ADP-glucose to oligosaccharide precursor to form amylose linked by α-1,4 glycosidic bond. Starch branching enzyme transforms amylose into amylopectin by cleaving α-1,4 glycosidic bonds of amylose or amylose fragments of amylopectin and linking the cleaved fragments of polysaccharide by α-1,6 glycosidic bonds. Monille *et al*. (1996) discovered the starch debranching enzyme that hydrolyzes specifically α-1,6 glycosidic bonds of amylopectin and transforms amylopectin into amylose. In ordinary maize, granule-bond starch synthase is highly active and its product (amylose) cannot be entirely transformed into amylopectin by starch branching enzyme. Approximately a quarter of the starch accumulated in mature endosperm remains as amylose and the phenotype is non-waxy. In fact, the pathway of starch biosynthesis in maize endosperm is more complex than it is summarized in Fig. 1. Interaction among different starch synthases, starch branching enzyme and debranching enzyme is necessary for detail research.

Molecular Mechanism of Sweet Maize

In 1911, East and Hayes discovered sweet gene *su* and located it on the short arm of chromosome 4. Recent studies showed that sweet gene *su* is a mutant of starch debranching enzyme encoding gene. For example, mutant *su1-st* is caused by the insertion of a transposon-like

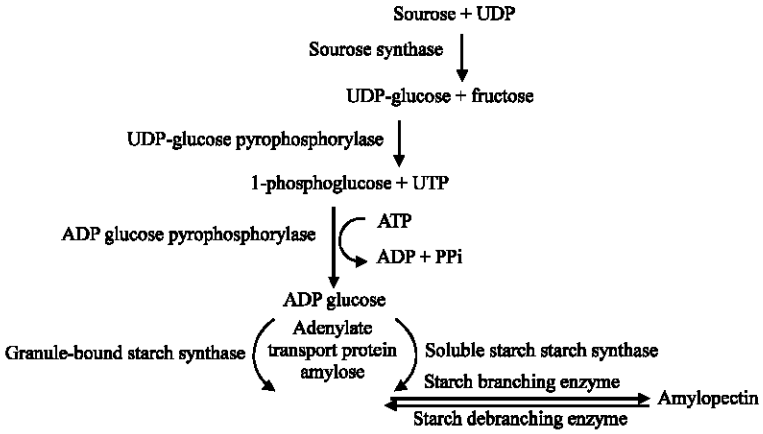

Fig. 1: Pathway of starch biosynthesis in maize endosperm UDP, uridine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PPi, pyrophosphatic acid; UTP, uridine triphosphate

Table 1: Chromosomal loci and phenotypes of sweat and waxy genes in maize

Gene	Chromosomal locus	Phenotype
su	4S-66	At milky ripening stage, the content of reducing sugars and sucrose in kernels is about 3 times of ordinary maize and the content of water-soluble polysaccharides in kernels is about 10 times of ordinary maize. The mature kernels are wrinkled. The starch content is less than half of ordinary maize
sh2	3L-127	At milky ripening stage, the content of reducing sugars and sucrose in kernels is about 6 times of ordinary maize and the content of water-soluble polysaccharides in kernels is as much as ordinary maize. The mature kernels are collapsed shrunken. The starch content is less than 1/3 of ordinary maize
bt1	5L-12	Similar to gene *sh2*
bt2	4S-67	Similar to gene *sh2*
wx	9S-56	The starch in endosperm and pollen consists of nearly all amylopectin, while the starch in endosperm and pollen of ordinary maize consists of 3/4 amylopectin

sequence; mutant *su1-R4582::Mu1* is caused by the insertion of transposon *Mu1* into the first exon and mutant *su1-Ref* is caused by the substitution at sites 576, 1100 and 1819 from bases C, A, T to bases G, G, C respectively, resulting in the change of amino acid residues at sites 163, 338 and 578 of the debranching enzyme protein from Phe, Glu, Trp to Leu, Val, Arg (James *et al*., 1995; Dinges *et al*., 2001). These mutations inhibit the activity of starch debranching enzyme, increase the content of water-soluble polysaccharides as well as reducing sugars and sucrose in milky ripening stage and decrease starch accumulation in mature kernels, resulting in the phenotype of sweet kernels. The mature kernels become wrinkled, because of the lower content of starch accumulated in endosperm.

ADP-glucose pyrophosphorylase is an isotetramer allosteric regulatory enzyme consisting of two big subunits and two little subunits. Its activity is promoted by 3-phosphoglycerol and inhibited by inorganic phosphoric acid. ADP-glucose pyrophosphorylase is a key enzyme to limit the rate of the whole pathway of starch biosynthesis (Greene and Hannah, 1998; Morell *et al*., 1987; Muller-Rober *et al*., 1992; Sanwall *et al*., 1968). In 1953, Laughnan discovered recessive monogenic mutant of shrunk kernel, denominated as *shrunken-2* and located it on the long arm of chromosome 3 (Table 1). Smith *et al*. (1997) revealed that gene *shrunken-2* encodes the big subunit of ADP-glucose pyrophosphorylase. Transposon insertion causes the mutation from dominant gene *shrunken-2* to recessive gene *shrunken-2*, decreases the activity of ADP-glucose pyrophosphorylase and results in inhibition of starch biosynthesis. For example, mutated gene *sh-m5933* is caused by the insertion of a 30 kb transposon element. Because the ADP-glucose pyrophosphorylase catalyzes a single-channel step of starch biosynthesis pathway (Fig. 1), the mutation of recessive gene *shrunken-2* has significant inhibition to starch biosynthesis and dramatic accumulation of reducing sugars and sucrose, resulting in the phenotype of super sweet kernels. The mature kernels become collapsed shrunken, because of the lower content of water-soluble polysaccharides and starch accumulation in endosperm.

The brittlely sweet genes *brittle-1* and *brittle-2* discovered by Cameron and Teas (1953) are also transposon insertion mutations of key enzymes to starch biosynthesis. Gene *brittle-1* is caused by the insertion of transposon into adenylate transport protein encoding gene. For example, the inserted fragment of mutated gene *brittle-1m* is an incomplete *Spm* transposon. This insertion mutation obstructs ADP-glucose from transporting into amyloplasts (Cao *et al*., 1995; Sullivan and Kaneko, 1995). Gene *brittle-2* is mutated from the encoding gene of the little subunit of ADP-glucose pyrophosphorylase. Because they obstruct starch biosynthesis pathway at the next or the same step with gene *shrunken- 2*, genes *brittle-1* and *brittle-2* express the similar phenotype to gene *shrunken-2*. At milky ripening stage, the content of reducing sugars and sucrose is higher and the content of water-soluble polysaccharides and starch in endosperm is lower than genotype *sususu*, resulting in collapsed shrunken kernels at mature stage.

Molecular Mechanism of Waxy Maize

In 1909, Collins discovered recessive waxy gene *wx* from a waxy cultivar introduced from China to USA and located it on the short arm of chromosome 9. Bates *et al*. (1943), Greenwood (1956), Sprague *et al*. (1943), Weatherwax (1922) and Whelan (1961) demonstrated by iodine staining that almost all the starch accumulated in endosperm of genotype *wxwxwx* is amylopectin, while in endosperm of ordinary maize only 75% of the starch is amylopectin and the other 25% is amylose. It was revealed later that gene *wx* is mutated from the encoding gene of granule-bound starch synthase. Under the mutation inhibition to the activity of granule-bound starch synthase, all the amylose synthesized by soluble starch synthase isoenzymes of low activity is transformed into amylopectin by starch branching enzyme. Only amylopectin is accumulated in endosperm and the kernel phenotype is waxy (Nelson, 1968; Nelson and Rines, 1962; Tsai, 1974).

The encoding sequence of granule-bound starch synthase gene is 3718 bp long and consists of 14 exons and 13 introns. The insertion of *Ac-Ds*, *Spm* and some other transposons at different sites of the exons change the protein structure of granule-bound starch synthase and inhibit the synthesis of amylose (Klosgen *et al*., 1986). More than 50 different insertion mutations have been found during the encoding sequence of granule-bound starch synthase gene. The activity of granule-bound starch synthase is decreased by 5 to 95% (McClintock, 1951, 1952, 1963, 1964; Wessler and Varagona, 1985). Some of these mutations, that decrease the enzyme activity significantly and result in waxy phenotype, are denominated as recessive gene *wx*, because they are allelic on cytogenetic level. For example, mutants *wx-m7* and *wx-m9* are caused by the insertion of transposon *Ac-Ds* beyond of the first exon and into the tenth exons respectively, while mutant *wx-m8* is caused by the insertion of transposon *Spm* into the eleventh exon of granule-bound starch synthase encoding gene.

Breeding of Sweet Maize and Waxy Maize

The recessive mutated sweet genes and waxy gene only determined the content of reducing sugars and sucrose of sweet maize and starch component of waxy maize. As vegetable food, the quality of sweet maize and waxy maize is also influenced by protein content, amino acid component, seed capsule thickness and many other characters. The conventional method of parent line breeding for sweet maize and waxy maize hybrids is backcrossing. After hybridization between current parent and donor parent containing the recessive sweet genes or waxy gene, backcrossing and selfing are conducted alternately to select individual plants or lines combining synthetic agronomic characters of the recurrent parent and the sweet or waxy character of the donor parent from the segregating populations.

The above analysis tells us that the starch content in sweet endosperm is decreased significantly either because of the starch biosynthesis obstruction controlled by recessive genes *sh2*, *bt1* and *bt2*, or because of the starch hydrolysis controlled by recessive gene *su*. Whereas, in waxy endosperm controlled by recessive gene *wx*, what it is changed is only starch component, but the starch content is similar to ordinary maize with the same genetic background. Although sweet genes *su*, *sh2*, *bt1* and *bt2* can be introduced by backcrossing into the same inbred line together with waxy gene *wx* because they are separated on different chromosomes (Table 1), their sweet and waxy phenotypes can not be expressed in the same inbred line or hybrid because the starch biosynthesis obstruction or starch hydrolysis prevent from the accumulation of amylopectin, that is the basis of waxy phenotype. Therefore, the effort to breed double recessive sweet-waxy maize controlled by recessive sweet genes and recessive waxy gene is impractical. Garwood and Creech (1972) demonstrated that only sweet phenotype can be expressed in double recessive or triple recessive genotypes with different combinations of sweet genes and waxy gene, such as *susuwxwx*, *sh2sh2wxwx*, *bt1bt1wxwx* and *bt2bt2wxwx*. The phenotype of waxy gene *wx* is covered by the epistatic action of the sweet genes. In the absence of the sweet genes, waxy maize with genotype *aeae* or *wxwx*, as well as ordinary maize, tastes a little sweetish if harvested appropriately earlier. However, this is caused by the reducing sugars and sucrose retained to be transformed to starch in endosperm at milky ripening stage, but not the phenotype of double recessive sweet-waxy genotype.

Development of Intragenic Selection Marker

During the backcrossing procedure of transferring synthetic agronomic characters from the recurrent parent, backcrossing and selfing have to be conducted alternately, to avoid losing the recessive sweet genes or waxy gene introduced from the donor parent. The recessive sweet genes are identified by the homozygous recessive wrinkled or shrunken kernels of the selfed segregating generation. The recessive waxy gene *wx* is identified by luster of the homozygous kernels of the selfed segregating generation or iodine staining detection of the homozygous gametophyte pollens of segregating individual plants (Li, 2002, 2003). This procedure can be cut down almost to half by omitting the selfed segregating generations, if the recessive sweet genes or waxy gene can be identified from the heterozygous individual plants and the heterozygous individual plants containing the recessive sweet genes or waxy gene can be selected from the segregating population of backcrossing and used to backcrossed with the recurrent parent.

Liu *et al*. (2007) detected sequence differences between the dominant gene *Wx* of non-waxy maize and its mutated recessive allele *wx* of waxy maize. On the basis of this difference, two pairs of specific primers were designed and were used as intragenic selection marker to identify individual plants of genotypes *WxWx*, *Wxwx* and *wxwx* by PCR amplification from the segregating population of the F_2 generation crossed between waxy and non-waxy inbred lines. Iodine staining detection and starch component assay showed that all the 35 F_2 plants identified as genotype *WxWx* produced non-waxy kernels of the F_3 generation and that all the 33 F_2 plants identified as genotype *wxwx* produced waxy kernels of the F_3 generation. The waxy phenotype of kernels is expressed at the ripening stage after pollination. Using the intragenic selection marker developed in this study, selection for recessive waxy gene *wx* can be conducted at the seedling stage before its phenotype is expressed at the ripening stage and even from heterozygous genotype, in which recessive waxy gene *wx* cannot be expressed. After eliminating the seedlings without recessive waxy gene *wx*, all the effort of selection can be devoted to other agronomic characters.

DNA molecular marker-assisted selection is a useful tool to obviate interference of environments and for selecting genotypes directly (Knapp, 1998; Lalitha, 1999; Lee, 1995; Ribaut and Hoisington, 1998). The efficiency of selection, however, is always lower than expected, because of unavoidable crossover between the target gene and its linked molecular marker. Most of the molecular markers, for example SSR, RFLP and AFLP, often used in crop breeding are, moreover, non-expression sequences in intergenic regions. It is difficult to use these to distinguish differences during expression sequences of their linked genes. Because only differences during expression sequences can be used for selection of the target traits, intragenic selection markers (functional markers), designed on the basis of sequence differences in the target gene, are much more useful for selection of single genes and major polygenes (Andersen and Lubberstedt, 2003). In medicine, intragenic selection markers are widely used to diagnose genetic diseases in humans (Amikam *et al*., 1997; Dunne and Maselli, 2004; Korytina *et al*., 2003; Munroe *et al*., 1996; Yandell *et al*., 1990). Little information is available on intragenic selection markers in plant breeding other than a small amount on overelaborate RFLP markers (Andersen and Lubberstedt, 2003; Kata *et al*., 1994; Leister *et al*., 1996).

By intragenic selection marker, not only the difficulty of phenotypic selection for recessive genes in cross and backcross breeding procedures can be overcome, but also the effect of genetic crossover to selection response in ordinary molecular marker-assisted selection can be avoided. The high accuracy of intragenic molecular marker selection is due to the low ratio of genetic crossover between selection marker and target gene. If the corresponding relationship between the selection marker and the target gene was broken by intragenic recombination, the target gene itself might have mutated and the selection objective should be changed, because the selection marker is included in the target gene itself. Therefore, intragenic selection marker is not only beneficial to improving selection efficiency of waxy maize breeding but also useful in selection for other single genes and major polygenes.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (30571172 and 30671309), Rockefeller Foundation (2004 FS 047) and Program for Changjiang Scholar and Innovative Research Team in University (PCSIRT, IRT0453).

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