Review paper

Genetic modification for designer starch from cassava

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Abstract

Starch is a basic raw material for many food and non-food industries, the products ranging from frozen foods to dextrin, adhesives, biodegradable plastics, gypsum binders and more. Modified starches are extensively used as fillers, emulsion stabilizers, consistency modifiers etc. Starch contains two chemical compounds, amylose and amylopectin, and varying the proportion of these results in modified starch with enhanced suitability for specific use in various industries. Such modified starch, in this context, is referred to as 'designer starch'. Chemical modification is the usual procedure for making starch suitable for industrial use but is expensive and less eco-friendly. Through the varied expression of genes involved in starch metabolism, a stable supply of modified starch could be assured and *waxy* cassava developed by CIAT, using the antisense RNA technology, stands as a practical example for designer starch. Waxy starch from cassava has improved paste clarity, low retro-gradation and better freeze thaw stability. Further, the pasting temperature of this waxy starch from cassava is better than cereal derived waxy starch. Efforts for making waxy starch from cassava are undertaken in various parts of the world including Netherlands, China, Thailand and India. Genetic modification through silencing the RNA is the method adopted in all the cases and by far Netherlands have reached the field trial stage of genetically modified waxy cassava. This paper reviews the method adopted, progress and challenges involved in developing waxy starch from cassava.

Key Words: Amylopectin, Amylose, Gene silencing, *gbssI*, *Manihot esculenta*, Designer Starch, Waxy Cassava

Introduction

Starch is a major component of cassava that elevates its status from a backyard crop with only a secondary food value, to an industrial raw material. Right from its inception as a backyard crop, starch extraction from cassava has been an activity for small household units. Recently, large industries have emerged to extract starch from cassava, after identifying the potential of cassava as a major source of starch, next to corn and maize.

Starch

Starch is a polymer of two glucans, amylose and amylopectin. Industrial use depends on the proportions of amylase to amylopectin. Amylose is a liner polymer of α 1-4 lined glucosyl residues and amylopectin is a branched polymer with α1-6 glycosidic bonds. The key enzymes in starch biosynthetic pathway are ADP-glucose pyrophosphorylase and starch synthases. The starch synthase enzymes add glucose units to the non-reducing end of glucan chains via α1-4 linkages. There are at least nine isoforms of starch synthases located at the surface of the granule and are responsible for amylopectin synthesis. Starch branching enzymes are also involved in amylopectin synthesis. The granule bound starch synthase is found exclusively within granules and is the key

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enzyme for amylose synthesis (Jobling, 2004). Genetic modifications in these enzymes are the mode of producing designer starch – waxy or amylose - free starch or high amylose starch. Such modifications could also affect the structure and properties of the end product, starch (Jobling, 2004; Edwards et al., 1999).

The major sources of starch in the world are maize, wheat, rice, potato and more recently, cassava and sweet potato (Akpa and Dagde, 2012). Maize or corn is the major source all over the world, whereas cassava and potato starches are utilised in Asian countries (Jobling, 2004). Tuber and root derived starches have additional advantage over cereal derived starch in having lower protein and lipid content and larger granule size.

Depending upon the final product, some industries require starch completely devoid of amylose or 'waxy cassava', whereas some others require high amylose starch. Waxy cassava starch has high viscosity, high clarity and low synerisis, so is advantageous for making those products that require gel with high clarity and viscosity. Amylopectin synthesis involves many enzymes and therefore altering a single gene could not silence amylopectin. However Edwards et al. (1999) have reported that simultaneous inhibition of starch synthases SSII and SSIII resulted in starch with altered structure, that is, increase in number of short chains in its amylopectin, which forms starch with easy gelatinizing property and therefore useful in food industry.

Usually, the amylose content varies from 20 to 25 per cent. The prevailing practice to get waxy starch is through chemical treatment and amylose removal after starch extraction. Chemical modification of starch involves the treatment of native starch with specific chemical reagents and this includes acetylation, oxidation, lintnerization, pyro-dextrinization, hydroxypropylation, cross-linking, etc. (Kaur et al., 2004). These methods carry the great disadvantage of being unfriendly to the environment (Raemakers et al., 2005). Creation of natural waxy starch receives credibility in this situation. In this paper, the different strategies adopted by various countries to develop waxy cassava are reviewed and the possible methods for Indian conditions are discussed.

Cassava Biotechnology

Cassava (*Manihot esculenta* Crantz.) has its origin in South America, from where it was introduced to Kerala through the foresight of the King of the erstwhile State of Travancore, His Highness Vishakam Thirunal. The king had foreseen the suitability of this crop as a saviour at times of famine and subsequently, this crop has emerged as a major food crop in the state.

Various traits of cassava such as low protein, high cyanogenic glucosides, starch content and constitution are amenable to be altered. Conventional breeding in cassava has a lot of limitations like low production of flowers, apomixis, long reproduction cycle, limited seed set, ploidy variation and inbreeding depression (Mistika et al., 2006), necessitating transgenic approaches in cassava improvement programmes. Gene transfer methods in cassava have already been established and Mistika et al. (2006) have speculated that *Agrobacterium* mediated method is preferable over biolistic method since the number of gene copies integrated at transcriptionally active domains will be more in the former case.

Waxy cassava

As already mentioned, cassava is the largest starch source in tropical regions with easily extractable pure white starch, with low protein and fat (Ceballos et al., 2007). The non-cereal taste of cassava starch makes it particularly desirable for food industries. Although waxy starch can be from different sources like maize, wheat, potato or cassava, each has its own distinct chemical properties which make it uniquely suitable for different industrial applications. Waxy starch from cassava has excellent freeze thaw stability, forms clear gel and has increased viscosity, low synerisis and therefore, is highly suitable for industrial applications like frozen food and paper making. Moreover, cassava starch has been reported to be least resistant to enzymatic break down compared to other non-cereal starch, and hydrolic curves have been found on par with normal maize starch (Rickard et al.,1991), the most used one in the world market (Jobling, 2004). The average starch content in the present day cassava cultivars is 26 to 28 per cent, of which 14-24 per cent is contributed by amylose (Moorthy and Ramanujan, 1986). However, two recent high starch industrially important cassava varieties, Sree Athulya and Sree Apoorva, released by Central Tuber Crops Research Institute (CTCRI), contains more than 30 per cent extractable starch (CTCRI, 2013.)

Cassava with high amylose content

High amylose starches are developed through inhibition of genes encoding starch branching enzymes. In rice, mutation in gene encoding SBEIIb could give high amylose starch, but in potato, only slight increase in amylose was seen. However, amylose level of more than 60% was achieved through inhibition of SBEI and SBEII (Schwall et al., 2000). This starch is commercially valuable with its high gelling strength, and its film forming capability makes it suitable in fried snack industry as this renders low fat uptake in snacks. It also has high nutritional value. This starch can be processed into resistant starch that is not digested in the small intestine, but is fermented in the large intestine by gut bacteria, producing butyric acid beneficial for colon health (Jobling, 2004). Another modification in the enzymes of starch pathway, starch synthases II and starch synthases III, results in short chains in amylopectin and has altered chemical properties like gelatinisation at very low temperature.

Amylose content in starch is reported to be solely influenced by genetics, and factors such as age of the plant or environment have no significant role (Ceballos et al., 2007) and thus, by genetically nullifying the amylose content, it is possible to get waxy starch from cassava. The starch biosynthesis pathway is well studied and it has been inferred that the silencing of granule bound starch synthase enzyme leads to amylose free starch production (Visser and Jacobsen, 1993; Chakraborty et al., 2004; Raemakers et al., 2005).

Starch Biosynthesis pathway

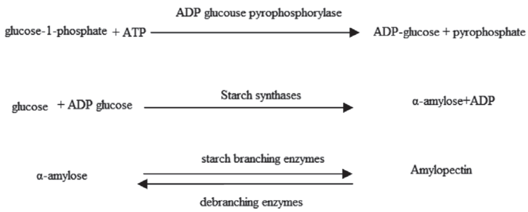

Image courtesy: http://www.andrewgray.com/essays/starch.htm

At the International Centre for Tropical Agriculture (CIAT) waxy mutants of cassava in which the (GBSS) granule bound starch synthase enzyme was absent have been discovered (Ceballos et al., 2007). The properties of the waxy cassava were comparable with waxy maize starch.

Granule bound starch synthase gene

The granule bound starch synthase is the key enzyme in the starch metabolic pathway. It is the enzyme for synthesis of amylose, encoded by the gene *gbssI.* The full length DNA for the enzyme was cloned and sequenced by Salehuzzaman et al. (1993) and this served as the base of all the subsequent *gbssI* silencing studies. *gbssI* exists as a single copy gene in all the plants examined so far (Mason-Gamer et al., 1998) and hence is amenable for easy manipulation and silencing.

Anti sense RNA for waxy cassava

The first amylose free transgenic cassava was developed by the Wageningen University (Raemakers et al., 2001; Taylor et al., 2004). Field testing of genetically modified cassava with low amylase or amylase - free starch has been done and it has been reported that starch from transformants in which amylase was very low or absent showed all physical and rheological properties as expected from amylase free starch. It was also reported that it is possible to obtain commercially relevant low amylose cassava plants with good root yield and starch quality (Putten, 2012). Puentes et al. (2003), have developed waxy starch cassava variety through the anti sense mediated silencing of *gbssI* in CIAT. They have cloned the constructs for full length *gbssI* gene in sense and anti sense orientation in the binary vector *pCAMBIA*1305.2. Friable embryogenic callus (FEC) of cassava genotype TMS6044 was transformed with the constructs and one line was successfully regenerated. The use of FEC ensured that no chimeras were formed. FEC is the secondary embryo developed from callus that has high regeneration potential. Raemakers et al. (2005) have reported the production of amylose free cassava transformants by transformation of Nigerian cultivar TMS60444 through anti sense *gbssI* construct. They also have successfully used an RNAi construct and observed that this strategy is more efficient than the anti sense technology.

RNAi technology

Sequence information of the gene *gbssI* was available through the work done by Salehuzzaman et al. (1993) and Zhao et al. (2011). This information was used to plan a hairpin construct of *gbssI* gene and to transform cassava with the construct in all the further experiments in the field. The expected action was that the hairpin construct of *gbssI* gene when introduced to cassava would produce null expression of the gene through a targeted degradation of the mRNA by an RNA-induced silencing complex that incorporated small RNAs derived from the dsRNA hairpin (Howard-Till and Yao, 2006).

Attempts to silence amylopectin by altering a single gene, as in the case of amylase, is not possible as many enzymes like starch synthases, starch branching enzymes and debranching enzymes, each of which having multiple isoforms are involved in amylopectin synthesis (Jobling, 2004). Moreover there are reports that mutation in the SBEI of maize did not produce any phenotypic change (Blauth et al., 2002). But same enzyme modification in rice showed modification in amylopectin structure. However the structure and properties of starch have been reported to be dramatically altered with simultaneous inhibition of starch synthase II and III (Edwards et al., 1999). The modified amylopectin containing short chains could gelatinize at very low temperature and this could have valuable uses in food industry (Jobling, 2004).

Applications

Starch needs to be modified to make it a more useful industrial raw material. Harsh treatments alter the physio-chemical properties of the native starch and affect retrogradation and viscosity, increase the tendency for syneresis, lower the stability and thus, reduce the shelf life. As of now, to make it suitable for wider industrial applications, native starch is modified through physical, chemical, or biological methods, the chemical being the most frequent (Daramola and Osanyinlusi, 2006). Waxy starch derived from any source is characterized by the high freeze thaw stability compared to native starch. Thus, waxy starch shows great promise for industries in the fields of frozen foods, adhesive making and paper making.

A naturally occurring mutant of cassava has been reported by CIAT (Ceballos et al., 2007). The physio-chemical properties of the waxy starch from the mutant have been well studied and found comparable with other waxy starches developed through other methods including chemical methods. To conclude, Amylose - free waxy starch could be produced *in planta*, through selecting mutants or developing transgenics that have the *gbssI* gene silenced. There is a wide range of commercially valuable starches with altered chemical properties.

By altering the enzymes involved in starch synthesis pathway, more industrially relevant starches could be developed. Silencing *gbssI* gene is a successful method for developing waxy cassava and it is reported that genetically modified cassava with low amylose has high commercial value along with good root yield and starch quality. For Asian countries, starch from waxy cassava is more suitable than from any other source due to its ease of production. So in India, developing waxy cassava through hairpin RNA technology will give a boost to the concept of cassava as an industrial crop. China and Thailand have already initiated gene modifications with this in view, and in India also several works are in the pipe line.

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