Increasing energy demand, concerns over security of oil supplies and energy independence, and the unprecedented increase in greenhouse gases from burning fossil fuels have generated worldwide interest in alternative, carbon-neutral, renewable energy sources. As such, biofuels from biomass are expected to help meet the demand for renewable transportation fuels. In the U.S., starch is the feedstock used for first generation biofuel, which is mostly obtained from corn, but other sources are being considered. Sweetpotato \([Ipomoea batatas (L.) Lam.]\) is a low impact crop mostly produced in the southeastern US, and it possesses several agronomical advantages such as low fertilizer requirements, high adaptability and tolerance to water stress. It accumulates starch in storage roots and gives comparable yields of ethanol as corn, thus constituting an attractive alternative feedstock for bioethanol production in the southeast U.S.

For the industrial conversion of starch to fermentable sugars, starch is partially hydrolyzed to maltodextrins by an \(\alpha\)-amylase during liquefaction, and it is hydrolyzed to monomeric sugars during saccharification by further enzymatic activity. Liquefaction is carried out at elevated temperatures to increase starch solubility, and therefore a thermoactive \(\alpha\)-amylase is required. Enzymes for starch processing are obtained from heterologous sources and add to the overall process economics. We hypothesized that by introducing genes of hyperthermophilic glycosidases into sweetpotato, costs associated with enzyme addition would be reduced, making the process more cost effective.

Towards this end, the production of a hyperthermophilic \(\alpha\)-amylase from \textit{Thermotoga maritima} was initially evaluated in a tobacco NT1 cell culture, as model plant system. Functional enzyme was produced yielding a product with enhanced thermostability, but otherwise identical biochemical properties compared to the recombinant enzyme produced in \textit{Escherichia coli}. The enhanced stability of the plant-made enzyme was shown to be due to the intrinsically provided calcium in plant cells. Given that calcium is a cofactor needed for the activity and stability of most \(\alpha\)-amylases and is typically added to the starch mixture
during liquefaction, these results opened the prospect of cost reduction by eliminating the need for calcium addition along with the thermoactive enzyme.

The gene for *T. maritima* α-amylase tested in tobacco cells was subsequently introduced into the genome of sweetpotato cv ‘Jewel’ by stable transformation with *Agrobacterium tumefaciens*. The transformation procedure was validated by introduction of a reporter gene, and a transgenic line producing functional hyperthermophilic enzyme was subsequently generated. Starch in the transgenic storage roots was readily hydrolyzed at 80°C, while starch in wild type roots remained unchanged. No recombinant enzyme activity was detected at ambient temperatures and transgenic storage roots developed normally. These results demonstrated the viability of producing hyperthermophilic α-amylases in starch-accumulating crops, and further supported the prospect of engineering crops for biomass conversion. A similar transformation procedure was evaluated in two other sweetpotato varieties, along with the identification of a root specific promoter; these results are presented as appendices to this document.

Finally, in vitro regeneration studies were performed in novel, industrial-type sweetpotato genotypes that exhibit higher dry-matter in the storage roots than table-stock varieties. Due to the higher starch content, these novel genotypes may yield higher amounts of fermentable sugars and thus appear promising for enhanced bioethanol production. By testing an array of hormone combinations, regimes were optimized that successfully promoted adventitious shoot regeneration in selected industrial sweetpotato varieties. Such procedures could be used in stable transformation strategies, and represent background work for further studies.
Studies for the Genetic Engineering of Sweetpotato (*Ipomoea batatas* L.) for starch Bioconversion

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University In partial fulfillment of the Requirements for the degree of Doctor of Philosophy

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“To my family and friends, for their constant love and support.”
Biography

Monica Santa-Maria was born on June 8, 1978, in Lima, Peru, and grew up in Trujillo, a city in the Peruvian northern coast. She is the second daughter of 4 children. During high school, she developed great interest in the Sciences and further on in the field of Biotechnology, for all the potentials it offered. For this reason she traveled to Lima to attend college at the National Agrarian University where she obtained her BS degree in Biology under the curricula of Biological Engineering.

Upon graduating from her Bachelors Degree, Monica worked for several months at the Peruvian Association for the Conservation of Nature (APECO) where she became aware of the problems facing our society, the deterioration of the environment, and the need to come up with substantial solutions for day-to-day situations. Soon after, she received a fellowship to work at the CGIAR International Potato Center (Lima, Peru), a first-class research center where she was further trained in the fields of molecular biology and genetics. Here, she completed her thesis research to obtain the accreditation in Biology in 2003. That same year, she was presented with the opportunity to pursue a doctoral degree in Horticultural Science with an emphasis on biotechnology at North Carolina State University. Throughout the application process, she became recipient of the Fulbright Scholarship in 2003. During her doctoral studies, Monica has kept a perfect academic record being awarded membership of several honor societies.

After completion of her doctoral program, Monica will be heading west to undertake a new challenge as Post-doctoral researcher in the laboratory of Dr. Tina Jeoh at the University of California at Davis.
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Chapter I

Introduction

Increasing research is underway and encouraged to devise and utilize alternative forms of energy from renewable sources, as a consequence of the current energy crisis and environmental degradation (EERE, 2008b). The current energy crisis stems from increasing local and worldwide energy demand, the limited and nonrenewable nature of major energy sources, and the restricted locations where the majority of these resources are obtained. Total energy consumption in the US was estimated at 100.2 quadrillion (1x10^{15}) Btu for 2005, and it is predicted to increase in 50% by 2030 (EIA, 2008). While diverse energy sources are used, liquid fuels are by far the major energy source for all uses including transportation (EIA, 2008). At the same time, the world use of liquid fuels is expected to grow from 83.6 million barrels of oil equivalents in 2005 to 95.6 million barrels per day in 2015, and 112.5 million barrels per day in 2030 (EIA, 2008). The fact that oil reserves are not renewable and that the majority of world oil production is restricted to just a few countries creates a strain upon a dwindling supply and concerns over politically motivated allocation of resources and energy security. In response, the US government has adopted a series of strategies to ensure its energy independence and security of supply. Among wind, solar, hydro, nuclear, and clean coal technologies, government investment and incentives for the development of next generation biofuels for transportation are well established (EERE, 2008b).

Besides the need for renewable fuel production a biobased economy is envisioned where various biobased products are obtained from renewable biomass. Biobased chemicals and materials are commercial or industrial products other than food and feed that are obtained from newly harvested biological materials that can replace petroleum-based feedstocks for the manufacturing of a variety of products including green chemicals, renewable plastics, natural fibers, and natural structural materials (Carole et al., 2004; EERE,
Market targets for such products include industries manufacturing polymers, lubricant solvent, adhesive, herbicides and medicines. While many biobased alternatives to petroleum-derived products are already in the market, considerable research is underway to broaden their spectrum and reduce production costs (Carole et al., 2004).

Along with concerns over energy independence and security of supply, the detrimental effect of using fossil fuels on the environment has become a key argument for the pursuit of alternative, renewable, carbon-neutral energy sources. The use of fossil fuels (coal, crude oil, and natural gas) as main energy source results in an increase in atmospheric concentrations of greenhouse gases. Current CO$_2$ and methane (CH$_4$) concentrations in the atmosphere far exceed pre-industrial values found in ice core records dating back to 650,000 years, and multiple lines of evidence confirm that this rise does not stem from natural mechanisms (Solomon et al., 2007). Burning fossil fuels is the primary source of anthropogenic CO$_2$ emissions that contribute to global warming, and, although the consequences of such phenomena are difficult to predict, the effect on the biosphere and world climates could be quite dramatic (Solomon et al., 2007).

**Biofuels**

Due to their compatibility with current engine technology, liquid biofuels are the preferred alternative carbon-neutral energy source for transportation (Fortman et al., 2008). They are obtained from the conversion of plant materials or biomass residues. Two types of biofuels are currently in the market: ethanol-based fuels and biodiesel (EERE, 2008b). Biodiesel is the most common biofuel in Europe, and it is produced from oils or fats by transesterification resulting in a liquid of similar properties as mineral diesel obtained from fossil sources. Biodiesel can be used in conventional diesel engines mixed with petroleum diesel, and most car manufacturers recommend a 15% biodiesel blend. In the US, biodiesel is mostly obtained from soybeans, and raw materials including virgin oil, recycled restaurant
grease, and other feedstocks are readily available to provide feedstock for about 1.7 billion gallons of biodiesel per year, representing roughly 5% of on-road diesel being used. While the US biodiesel industry is still small, it is growing rapidly with production tripling from 2004 to 2005 and again from 2005 to 2006 (EERE, 2008b).

Alternatively, alcohol-based fuels or bioalcohols are obtained by microbial fermentation of monomeric sugars, which are obtained directly from the plant (e.g. sugarcane or sugar beet) or by hydrolysis of starch or cellulosic materials. Ethanol in its pure form can be used as fuel for cars or for direct-ethanol fuel cells (DEFCs), but it is more commonly used as gasoline additive to increase octane ratings and improve vehicle emissions. As such, it is blended with gasoline in varying quantities acting as oxygenate additive and replacing methyl t-butyl ether (MTBE), a ground-water pollutant. Most car engines today can use an E10 blend (10% ethanol in gasoline), however, flex fuel vehicles (FFV) have engines modified to accept higher ethanol concentrations and can use an E85 blend (85% ethanol in gasoline) otherwise corrosive to standard engines (EERE, 2008b). Although ethanol use as direct fuel or fuel additive is well established, microbial engineering could allow the development of biomolecules for improved renewable transportation fuels through fermentation. These include synthetic diesel molecules, butanol or hydrogen (H₂), all of which are gaining increased consideration (Fortman et al., 2008). Besides fermentation products, microorganism can also be engineered to utilize different types of monomeric sugars including hexoses and pentoses (Fortman et al., 2008), of relevance when using lignocellulose as biofuel feedstock.

Currently, bioethanol dominates the world and US biofuel market, and ethanol or alcohol-based fuels are expected to be the dominant renewable fuel for transportation in the near future (Hahn-Hägerdal et al., 2006; Himmel et al., 2007; Fortman et al., 2008). US bioethanol production in 2007 was estimated to be 5.4 billion gallons (EERE, 2008b). However, the US Energy Policy Act of 2005 will require the oil industry to blend 7.5 billion gallons of renewable fuel to gasoline by 2012. This renewable fuel standard was boosted by the Energy Independence Security Act of 2007, which requires that 36 billion gallons of
renewable fuel are used annually by the year 2022 (EERE, 2008a). As of July 2008, the national average price for E85 was $3.27 per gallon, compared to $3.91 per gallon of gasoline (regular) (EERE, 2008a). However, the lower energy density of ethanol fuel compared to gasoline results in a higher average price for a gallon of E85, resulting in $4.62 per gallon of E85 versus $3.91 per gallon of gasoline when compared on an energy basis (EERE, 2008a). In order to meet the mandates specified in both the US Policy Act of 2005 and the Energy Independence Security Act of 2007 a significant increase in US production would be required that should not compromise competitive market prices.

The limiting factors for the increased production of bioalcohols are the price of feedstock and/or the economical conversion of polysaccharides to component sugars for fermentation. Currently, most bioethanol in the US is produced from corn (EERE, 2008b). However, the limited supply along with environmental and economical concerns associated to extensive corn utilization require the implementation of alternative feedstocks for next generation biofuel production (EERE, 2008b; Simpson et al., 2008). Feedstock alternatives to corn, as well as the advantages and challenges for their utilization are discussed in the following sections.

**Lignocellulose feedstock**

The low market price, potential yields in ethanol and environmental attributes of lignocellulose make it an attractive biomass feedstock capable of meeting the increased demand for renewable fuel for transportation (Hahn-Hägerdal et al., 2006; Himmel et al., 2007; Lynd et al., 2008; EERE, 2008b). Lignocellulosic materials such as grasses or forest and agricultural residues are available nationwide, and their even geographical distribution could allow for a domestic energy production and provide security of supply.

Lignocellulosic materials that are obtained from agricultural and forest harvest residues are regarded as second- generation feedstocks. They show promise for near-term adoption if efficient and economical cellulosic conversion technologies are developed.
The third generation feedstock for biofuel production encompasses the development of ‘energy crops’ with cultivation would be devoted to biofuel production, ideally not competing with land used for food production. These crops may include perennial grasses, fast growing trees, and algae. Although energy crops are promising in terms of cost, potential ethanol yields, and environmental attributes, technological breakthroughs are required to overcome the biomass recalcitrance to its utilization (EERE, 2008b; Himmel et al., 2007; Lynd et al., 2008).

Lignocellulose is composed of cellulose (20-30%), hemicellulose (20-30%) and lignin (20-30%), assembled into a complex interconnected network within plant cell walls. Both cellulose and hemicellulose are carbohydrates and can be broken down to component sugars for fermentation. Crystalline cellulose is the main polysaccharide target, being composed of chains of β-linked glucose units that are precisely arranged. Due to strong inter-chain hydrogen bond networking between glucose residues, cellulose fibers are highly packed and very resistant to chemical and biological hydrolysis (Himmel et al., 2007). The access of enzymes to crystalline cellulose is further restricted by its coating of hemicellulose and amorphous cellulose. Hemicellulose is a group of branched heteropolymers composed by different sugar monomers, primarily xylose, that are not readily utilized by fermentative microorganisms. In contrast to crystalline cellulose, hemicellulose and amorphous cellulose are readily digestible by enzymes (Himmel et al., 2007).

According to current biomass conversion technology, a biorefinery will comprise four major sections: feedstock harvest and storage, thermo-chemical pretreatment, enzymatic hydrolysis, and sugar fermentation to ethanol or other fuels (Himmel et al., 2007). The pretreatment step is conducted to reduce the recalcitrance of the material by depolymerizing and solubilizing hemicellulose. Thermo-chemical pretreatments comprise acid and high temperature hydrolysis, such as dilute sulfuric acid and 140°C to 200°C, or schemes based on alkaline explosive decompression and organic solvent extraction such as the ammonia fiber expansion (AFEX) (Himmel et al., 2007). In any case, such pretreatments will break down the macroscopic rigidity of the fiber, decreasing the physical barriers to mass transport and
making crystalline cellulose more accessible to enzymatic saccharification (Hahn-Hägerdal et al., 2006). In the enzymatic degradation, crystalline cellulose, as well as partially depolymerized hemicellulose, are hydrolyzed by the synergistic action of different types of endo- and exo-acting enzymes (endoglucanases and exoglucanases), commonly referred to as ‘cellulases’, as well as hemicellulases and accessory enzymes (Hahn-Hägerdal et al., 2006; Himmel et al., 2007). While saccharification of cellulose releases glucose molecules, depolymerization of hemicellulose releases a mixture of 5 carbon and 6 carbon sugars, primarily xylose, but also glucose, mannose, galactose, rhamnose, and arabinose (EERE, 2008b).

The challenges for the economical utilization of lignocellulose for biofuel production arise from the high costs of the thermo-chemical pretreatment, the slow kinetics of cellulose hydrolysis, and the high cost of enzymes, as well as the lack of an efficient fermentation system for a mixed sugar hydrolysate containing hexoses and pentoses and fermentation inhibitory compounds (Hahn-Hägerdal et al., 2006; Himmel et al., 2007). At the same time, the effective removal and utilization of lignin is another issue to be addressed in a successful biorefinery.

**Starch-based feedstock**

Starch is the most abundant carbon reserve in plants and it is widely used in the food and chemical industries. Starch is organized as granules inside plant cell plastids, and it is composed of various ratios of amylose (15-25%), a linear polymer of $\alpha$-1,4-linked residues, and amylopectin (75-85%), a polymer with $\alpha$-1,6-linked branch points (Bertoldo and Antranikian, 2001). In its native form, amylose and amylopectin are organized as alternating semicrystalline and amorphous layers forming growth rings. The semicrystalline layer is composed by ordered regions of double helices formed by short amylopectin branches, which are further ordered into a crystalin structures known as the crystalline lamellae. Alternatively,
the amorphous layer and the amorphous regions of semi-crystalline layers are composed of amylose, arranged as single helical structures, and non-ordered amylopectin branches that are readily hydrolyzed by starch degrading enzymes (Zhang and Oates, 1999).

As opposed to production from cellulosic fibers, starch hydrolysis to obtain fermentable sugars is straight forward, and the industrial processing of starch is well established. The industrial utilization of starch involves four main steps: milling, liquefaction, saccharification, and isomerisation to produce sugar syrups or fermentation to ethanol or other value-added compounds (Crabb and Mitchinson, 1997). In liquefaction, starch is solubilized at high temperature (gelatinization) and partially hydrolyzed by a thermostable α-amylase (EC 3.2.1.1), an endo-glucano hydrolase, to allow an easier access to saccharifying enzymes. After liquefaction, pH and temperature are adjusted for the saccharification step, in which starch is completely hydrolyzed to monomeric sugars by the synergistic action of enzymes including glucoamylase (EC 3.2.1.3) and pullulanase (EC 3.2.1.41). In addition, other glycoside hydrolyses with various specificities can also be used (Crabb and Mitchinson, 1997; Bertoldo and Antranikian, 2001).

First generation biofuels can be obtained from sugars, such as from sugarcane and sugar beet, but also from starch (Hahn-Hägerdal et al., 2006). In the US, cornstarch is the major feedstock for biofuel production (>95%), and most of the bioethanol is produced in the grain growing states of the Midwest (EERE, 2008b; Simpson et al., 2008). While market competitive prices are achieved, total corn production in the US is not sufficient to meet the growing demand of renewable transportation fuel. In addition, the extensive use of grain to meet ethanol demands could have negative impacts on water quality and the environment (Simpson et al., 2008). Corn is a high input crop that requires extensive fertilizer application, and since it is a relatively inefficient user of nitrogen, as much as 40% to 60% of the nitrogen applied is not utilized by the crop and leaches into the groundwater (Simpson et al., 2008). Corn cultivation for ethanol production increased 15% from 2006 to 2007, with the additional acreage coming from replacing land historically employed for soybeans and land currently in the conservation reserve program for hay and pasture (Simpson et al., 2008). An
increase in acreage for corn cultivation may result in an increase in nitrogen and phosphorous loss to surface and ground water, creating problems of eutrophication that results in algal bloom and oxygen depletion (hypoxia) in the water environment (Simpson et al., 2008). In addition to the environmental concerns, the dramatic increase in ethanol production and proposals for further increases have fueled a rise in corn prices, raising concerns of food security (Simpson et al., 2008). All these elements bring into question the sustainability of widespread use of corn for biofuel production in the US, creating the need for the identification of additional feedstocks for near-term adoption.

**Sweetpotato as biofuel feedstock**

Sweetpotato is an important crop worldwide, with an estimated 126 million metric tons produced annually (FAOSTAT, 2008). It is primarily produced in China, which accounts for 80.9% of the world production, but also in Africa and the Americas. Due to its ease of cultivation, low fertilizer requirements, high adaptability, and drought tolerance sweetpotato is considered a security crop and a major staple food crop in subsistence and rural economies (Woolfe, 1992). In addition to its direct use as table and feedstock for animals, sweetpotato is also a candidate for the production of renewable plant products such as ethanol, high-grade starches, stable natural dyes (purple dye), and vitamin precursors (beta-carotene for vitamin A) (Woolfe, 1992). In the U.S., the majority of sweetpotato production occurs in the southeast where North Carolina is the nation’s leading producer with 302.26 thousand metric tons produced in 2007 (37% of total U. S. supply). North Carolina, along with Mississippi, Louisiana, and Alabama accounted for over 80% of total US sweetpotato production in 2007 (USDA-NASS, 2008).

Sweetpotato is one of the most important starch-producing crops worldwide (Katayama et al., 2004). The dry matter content in sweetpotato storage roots ranges from 21 to 30%, of which about 80% is starch (Zhang and Oates, 1999; Shin et al., 2005). Starch in
the storage roots of sweetpotato is stored in amyloplasts as smooth, round or polygonal starch granules with sizes ranging from from 2.5 to 30.5 µm among different cultivars (Zhang and Oates, 1999; Peroni et al., 2006). Concerning its molecular composition, sweetpotato starch is constituted by 16 to 23% amylose, and 74 to 80% amyllopectin. Differences in the amylose/amyllopecting ratio, as well as starch granule size among sweetpotato cultivars will result in different physico-chemical properties such as gelatinization temperatures and enzyme digestibility (Zhang and Oates, 1999; Ishiguro et al., 2000; Katayama et al., 2004; Peroni et al., 2006). Gelatinization is the heat-moisture induced transition of starch from a crystalline to a more amorphous state that is more susceptible to enzyme digestion (Zhang and Oates, 1999). Depending on the amyllopecting content, gelatinization temperatures of sweetpotato starch can vary from 78 to 83°C, with higher amyllopectin content resulting in higher gelatinization temperatures and lower susceptibility to enzyme attack (Zhang and Oates, 1999). Alternatively, the presence of a higher number of short outer chains in amyllopectin results in a lower gelatinization temperatures for sweetpotato starches due to a reduced packing efficiency of double helices within the crystalline region (Katayama et al., 2004).

In any case, the high percentage of fermentable biomass in the sweetpotato roots makes it a potential feedstock for ethanol production and an attractive alternative to corn in the southeastern U. S. (Wilson et al., 2007). Even more, the suitability of sweetpotato as biofuel feedstock was recently demonstrated; enzymatic hydrolysis followed by fermentation produced an estimated 703 gallons of ethanol per acre of sweetpotato cultivated in the southeast, considerably higher than the estimated 404 gallons per acre for corn (Duvernay, 2008). Another study performed by researchers in Maryland and Alabama showed two to three times as much yields in carbohydrates for fuel ethanol production when using sweetpotatoes compared to field grown corn, further supporting the prospect of sweetpotato as viable feedstock for biofuel production (Cornis, 2008). Furthermore, sweetpotato is a low input crop that requires lower fertilizer and pesticides application than, for example, corn. For use in biofuel production, sweetpotato cultivation is expected to be implemented in
marginal lands, reducing the pressure on lands currently devoted to food production while becoming an alternative sugar feedstock (Duvernay, 2008; Cornis, 2008).

**Sweetpotato transformation**

Biotechnology offers the possibility of expanding the use of crops by genetic engineering leading to metabolic changes. While stable integration of traits into plant genomes is well established in many species, sweetpotato transformation remains challenging. The reduced success in sweetpotato transformation arises from its recalcitrance and genotype-specific response to *in vitro* regeneration and stable transformation procedures (Sihachakr et al., 1997; Moran et al., 1998; Yu et al., 2007). Although stable transformation of sweetpotato has been achieved in recent years, efficiencies are generally low (Newell et al., 1995; Gama et al., 1996; Cipriani et al., 1999), and success is limited to a small number of cultivars (Table 1). For instance, stable transformation of sweetpotato is preferably done in the cultivar ‘Kokei’ (Kimura et al., 2001; Wakita et al., 2001; Otani et al., 2003; Berberich et al., 2005) and ‘Beniazuma’ (Song et al., 2004; Song et al., 2007) in Japan; ‘Yulmi’ in Korea (Min et al., 2006; Yi et al., 2007), ‘Lixiang’ in China (Yu et al., 2007), and ‘Jewel’ in the Americas (Newell et al., 1995; Cipriani et al., 1998; Moran et al., 1998; Santa-Maria, 2003).

The majority of stably-transformed sweetpotatoes have been generated using the bacterium *Agrobacterium tumefaciens*, but *Agrobacterium rhizogenes* (Otani et al., 1993) and particle bombardment (Okada et al., 2002) have also been used as methods for gene delivery (Table 1). While procedures based on embryogenic calli regeneration are generally preferred, they depend upon the establishment of methods for embryonic calli formation from apical meristem cultures, which are not readily available for all desirable genotypes (Yu et al., 2007). Alternatively, stable transformation through the induction of somatic embryos, as well as adventitious shoot regeneration using leaves, petioles and root discs as explant source have been reported for some sweetpotato cultivars (Newell et al., 1995; Cipriani et al., 1998; Moran et al., 1998; Garcia et al., 1999; Santa-Maria, 2003; Song et al., 2004). In order
to further advance genetic engineering in sweetpotato, additional information on the response of different genotypes and explant types to hormone regimes for *in vitro* regeneration, as well as the evaluation of current or novel transformation procedures, need to be developed.

**Hyperthermophilic amylolytic enzymes**

Hyperthermophilic enzymes are obtained from microorganisms that grow optimally above 80°C that are known as *hyperthermophiles*.Hyperthermophiles are generally found in archaea, but also in bacteria of the genera Thermotogales and Aquifex (Bruins et al., 2001; Unsworth et al., 2007). These microorganisms have been isolated from marine and terrestrial ecosystems (including some man-made) with temperatures between 80-110°C, and some were recently discovered that can grow at temperatures as high as 121°C (Vieille and Zeikus, 2001; Unsworth et al., 2007). Enzymes from hyperthermophiles have developed unique structure-function properties that make them extremely thermostable and thermoactive, exhibiting optimal temperatures for catalysis above 80°C (Bruins et al., 2001; Vieille and Zeikus, 2001). Alternatively, thermophilic organisms or *thermophiles* grow optimally between 50 and 80°C, and their enzymes are also adapted for high temperature catalysis with optimal activities generally between 60 and 80°C (Vieille and Zeikus, 2001).

Protein stability is the result of a delicate balance between large stabilizing and destabilizing forces, and relatively small changes in these two forces can result in large changes in protein stability (Bruins et al., 2001). While there are no systematic differences in the structure of extremely thermostable and mesophilic proteins, a series of molecular adaptations have been attributed as contributors for their gain in thermostability. (Bruins et al., 2001; Unsworth et al., 2007). These modifications have been concisely summarized in a recent review by Unsworth et al. (2007), and they include changes in amino acid composition (e.g. with lower content of thermo labile residues such as Asn and Cys), hydrophobic and/or aromatic interactions, metal binding, ion pair networks, high packing and reduction of solvent-exposed surface area, among others. While there are no clear traffic rules mandating
protein adaptation to higher temperatures, hyperthermophilic proteins will show one or more of these modifications when compared to mesophilic orthologs (Vieille and Zeikus, 2001). Additionally, extrinsic factors such as macromolecular crowding and the presence of small stabilizing molecules (e.g. sorbitol, thermamine, cyclic polyphosphates) in the cellular environment can further aid in protein stabilization (Bruins et al., 2001; Unsworth et al., 2007).

Due to the harsh nature of the extracellular environment of hyperthermophiles (mostly extreme temperatures but also low pH and/or high hydrostatic pressures in some cases), extracellular and cell-bound hyperthermophilic enzymes are usually highly stable, and can be active at temperatures far beyond their hosts’ growth temperature (Vieille and Zeikus, 2001). In order to gain structural stability, these enzymes will face a compromise between two opposing factors: flexibility for the catalytic function, and rigidity to allow for conformational stability (Bruins et al., 2001). As a result, thermophilic enzymes are significantly more rigid than their mesophilic counterparts at moderate temperatures (Bruins et al., 2001; Koutsopoulos et al., 2005). While this rigidity makes them less active at temperatures below 40°C, it protects them from unfolding and preserves their catalytic structure under otherwise denaturing conditions such as extreme temperatures (Vieille and Zeikus, 2001). For instance, hyperthermophilic enzymes are particularly rigid and essentially inactive at moderate temperatures but they gain activity as temperature increases (Unsworth et al., 2007).

In addition to being highly thermostable and thermoactive, hyperthermophilic enzymes are more resistant to chemical denaturants (e.g. organic solvents and guanidinium hydrochloride) and proteolytic attack, and they have higher pH stability (Bruins et al., 2001; Vieille and Zeikus, 2001). These properties allow for their strategic use as improved biocatalysts in many biotechnological and industrial applications (Bruins et al., 2001; Vieille and Zeikus, 2001; Comfort et al., 2004; Unsworth et al., 2007). In general, their use allows for high temperature conversions, which offer several advantages such as higher solubility and mass transfer rates of substrates and products, lower viscosity, fewer risks of bacterial...
Contamination, and frequently result in higher product yields due to an increased amount of soluble substrate (Vieille and Zeikus, 2001; Unsworth et al., 2007). High temperature hydrolysis is particularly desirable in the industrial processing of starch, given the high viscosity of starch and the high risk of bacterial contamination (Wheals et al., 1999).

Due to the difficulties involved in obtaining their native forms, hyperthermophilic enzymes have been produced in mesophilic hosts where they retain their catalytic function and extreme thermostability (Vieille and Zeikus, 2001; Unsworth et al., 2007). This possibility further revolutionized their industrial and biotechnological applications due to the ease of purification (e.g. by heat treatment) and affordability (Vieille and Zeikus, 2001). For any mesophilic enzyme specificity there is almost always a hyperthermophilic equivalent, and novel hyperthermophilic enzymes with known or unknown functions are constantly being discovered (Unsworth et al., 2007). This creates potential for the expanded use of hyperthermophilic enzymes in applications such as bioethanol production, pulp bleaching, leather and textile processing, chemical synthesis, oil and gas well stimulation, and so on (Comfort et al., 2004; Unsworth et al., 2007).

Heterotrophic thermophiles and hyperthermophiles have been identified in recent years that are able to utilize natural polymeric substances such as carbohydrates (Bertoldo and Antranikian, 2001). Several extreme thermophiles that can metabolize α- and β-linked glucosides have been identified in the archaeal order Thermococcales and Sulfolobales (Blumer-Schuette et al., 2008). Alternatively, anaerobic, heterotrophic thermophiles capable of fermenting simple and complex sugars to H₂, CO₂ and acetate have been identified in the bacterial order Thermotogales (Blumer-Schuette et al., 2008). These microorganisms produce intrinsically thermostable and thermoactive enzymes that are able to hydrolyze glycosidic bonds in α- and β-linked glycans. For instance, about 7% of the genome of *Thermotoga maritima*, the type strain and most thermophilic member of the order Thermotogales, is devoted to the metabolism of monosaccharides and polysaccharides (Blumer-Schuette et al., 2008).
Most of the enzymes involved in starch-conversion belong to the α-amylase family or family 13 of glycosyl hydrolases. These proteins share a number of common characteristics such as $(\beta/\alpha)_8$ barrel structure, hydrolysis or formation of glycosidic bonds in the α-conformation, and a number of conserved amino acids in the active site (van der Maarel et al., 2002). There are three groups of such enzymes involved in complete starch hydrolysis: endoamylases, exoamylases, and debranching enzymes. Endoamylases cleave α-1,4 glycosidic bonds in the interior of amyllose and amyllopectin, with α-amylase (EC 3.2.1.1) as the best known and most widespread example in this group. Exoamylases act on external glucose residues of amyllose and amyllopectin, producing only glucose (glucoamylase and α-glucosidase) or maltose and β-limit dextrins (β-amylase). Debranching enzymes exclusively hydrolyze the α-1,6 glycosidic linkage in amyllopectin and include isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41). These two enzymes differ in their substrate specificity; whereas isoamylase can only cleave α-1,6 linkage in amyllopectin, pullulanase can also cleave α-1,6 linkages in pullulan. Another pullulanase, referred to as type II pullulanase or amylopullulanase has been identified that can hydrolyze both α-1,4 and α-1,6 glycosidic bonds (van der Maarel et al., 2002). All of these specificities have been identified in hyperthermophiles, having been characterized in their native or recombinant forms with the ultimate goal of employing them in starch conversion processes (Brown et al., 1990; Brown and Kelly, 1993; Bertoldo and Antranikian, 2001; Vieille and Zeikus, 2001; Unsworth et al., 2007).

**Perspectives on crop engineering for biomass conversion**

The fact that hyperthermophilic enzymes can be produced in mesophilic hosts and can remain inactive until subsequent thermal activation creates the potential for their production directly in the plant tissue that will ultimately provide the substrate for their action, in applications such as starch bioconversion.
There is a few reports on the production of thermophilic and hyperthermophilic glycosidases in transgenic plants. A first report was by Pen et al. (1992) where they produced an α-amylase from *Bacillus licheniformis* in transgenic tobacco plants to be used as bulk enzyme for starch liquefaction. The recombinant enzyme was accumulated in the intercellular space and no apparent affect in the phenotype of transgenic plants was noted. Similarly, a later study by Montalvo-Rodriguez et al. (2000) reported the production of hyperthermophilic α-glucosidase and β-glycosidase from *Sulfolobus solfataricus* in transgenic tobacco plants to serve as bulk enzyme for the hydrolysis of plant polysaccharides. They noted a heat requirement for enzyme activity and no apparent effect in plant growth and development. Another study by Beaujean et al. (2000) reported the expression of a chimeric gene encoding an α-amylase from *Bacillus stearothermophilus* and a glucose-isomerase from *Thermus thermophilus* in transgenic potato plants. They showed that starch in transgenic tubers was processed at 65°C and higher yields in glucose and fructose were obtained compared to wild type tubers. Similar to the former reports, there were no apparent adverse effects on plant development and tuber formation.

However, two later studies by Chiang et al. (2005) and Lin et al. (2008) reported phenotype alteration in transgenic plants expressing thermoactive glycosyl hydrolases. In Chiang et al. (2008) production of a thermostable amylopullulanase from *Thermoanaerobacter ethanolicus* 38E was targeted to the amyloplasts in transgenic rice. While improved starch conversion was measured at high temperatures, alteration of starch composition was noted in rice grains stored at ambient temperatures. A later study by Lin et al. (2008) reported the production of a thermostable β-amylase from *Clostridium thermosulfurogenes* in transgenic potato plants, which was targeted to different cellular compartments including the amyloplasts. They noticed alterations in both the chemical composition and size of tubers from transgenic potatoes grown in the field. In both cases where phenotypic alterations occurred, the thermophilic enzymes were produced in proximity to starch granules and had considerable activity at normal growth temperatures. In addition, both studies reported very high levels of recombinant enzyme accumulation, which
would increase the chance that residual activity at low temperature could contribute to alterations in plant carbohydrate metabolism.

**Research objectives**

In the case of sweetpotato, the addition of thermoactive starch-hydrolyzing enzymes such as α-amylase, glucoamylase and pullulanase to the starch mixture resulted in a significant conversion of starch to simple sugars, and a subsequent fermentation resulted high ethanol yields (Duvernay, 2008). We hypothesized that improved yields in monomeric sugars and subsequent ethanol production could be achieved by a transgenic approach in which genes for hyperthermophilic starch hydrolyzing enzymes are produced directly in the sweetpotato plants. In an industrial setting, using transgenic stroge roots producing active hyperthermophilic glycosidases will eliminate cost associated to enzyme addition, and will probably reduce the processing time and mixing efforts due to an already homogenized enzyme-substrate feedstock. This may results in more cost-effective starch conversion to monomeric sugars and subsequent ethanol production. Several studies were performed in order to test this hypothesis:

- Chapter II reports the production of a hyperthermophilic α-amylase (amyN26) from *Thermotoga maritima* (Tma) in tobacco NT1 cell cultures, used as a model plant system. Active hyperthermophilic enzyme was produced in several transgenic NT1 cell lines, and shown to have the same biological activity as recombinant enzyme produced in *Escherichia coli*. Additionally, the plant-made enzyme benefited from intrinsically provided Ca\(^{2+}\) in plant cells, exhibiting more thermostability at 100°C than its bacterial counterpart. Thus this work demonstrated both the feasibility of producing hyperthermophilic α-amylase in plant cells, as well as the advantage of higher calcium availability in this host.
Chapter III reports the production of active hyperthermophilic α-amylase in sweetpotato plants. A procedure for the stable transformation of sweetpotato using Agrobacterium tumefaciens and a somatic embryogenesis regeneration was first devised using a reporter gene. Transgenic sweetpotato plants producing active hyperthermophilic α-amylase from T. maritima were obtained in a subsequent transformation experiment. Starch in transgenic storage roots producing the hyperthermophilic enzyme was readily hydrolyzed at 80°C, whereas starch in wild type roots remained intact under the same conditions. The viability of engineering sweetpotato for starch self-processing was demonstrated, and the prospect of biomass conversion for improved ethanol production was further supported.

In Chapter IV, prospects for using novel sweetpotato genotypes with higher dry-matter content in storage roots in order to obtain improved bioethanol yields are discussed. Hormone regimes for in vitro regeneration that could be used in stable transformation experiments were tested in selected high starch sweetpotato genotypes. Optimized procedures for adventitious shoot regeneration were established for some promising genotypes.

In Chapter V, the potential use of root-specific promoters to drive expression of hyperthermophilic starch degrading enzymes is discussed. Promoter regions from sporamin, the major protein in sweetpotato storage roots, were identified by sequence analysis of a sweetpotato genomic library. While the putative sporamin clones identified exhibited some variations in reference to a previously reported sporamin B promoter, they retained 100% identity within regulatory elements and conserved regions and their promoter activity was predicted using bioinformatics tools.

Finally, stable transformation experiments were performed in selected industrial type sweetpotato genotypes. Additionally, the potential use of alternative tissues as explant source
for improved sweetpotato transformation in the cultivar Jewel was evaluated. The results from both studies are summarized in the Appendices section.

References


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<th><strong>Cultivar (location)</strong></th>
<th><strong>Explant &amp; regeneration method</strong></th>
<th><strong>Gene delivery &amp; selection method</strong></th>
<th><strong>Inserted trait</strong></th>
<th><strong>Transformation efficiency</strong></th>
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<tr>
<td><strong>White Star</strong> (Americas)</td>
<td>Embryogenic calli from apical meristems (embryogenesis)</td>
<td><em>A. tumefaciens</em> EHA101/ kanamycin&lt;sup&gt;r&lt;/sup&gt;</td>
<td>GUS</td>
<td>2.3% (GUS positive plants)</td>
<td>(Gama et al., 1996)</td>
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<td><strong>Jewel</strong> (Americas)</td>
<td>Root discs (shoots formed from embryogenic calli)</td>
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<td><strong>Jonathan, Jewel, Maria Angola, Huachano (Americas)</strong></td>
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<td><strong>Jewel</strong> (Americas)</td>
<td>Shoot regeneration from full leaf explants</td>
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<td>Gene delivery &amp; selection method</td>
<td>Inserted trait</td>
<td>Transformation efficiency</td>
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<td>3 GUS positive lines</td>
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<td>GUS, followed by several traits</td>
<td>53.1% (GUS positive plants), similar efficiencies in subsequent studies</td>
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<td>Not clearly reported (&lt;20%)</td>
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<td>30.8% (GUS positive plants)</td>
<td>(Song et al., 2004)</td>
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<td>Beniazuma (Japan)</td>
<td>Somatic embryogenesis from stem segments</td>
<td><em>A. tumefaciens</em> EHA101/ kanamycin&lt;sup&gt;r&lt;/sup&gt; and hygromycin&lt;sup&gt;r&lt;/sup&gt;</td>
<td>GUS (under leaf-specific promoter)</td>
<td>110 GUS positive lines from 22 explants</td>
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<td>Cultivar (location)</td>
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<td>Gene delivery &amp; selection method</td>
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<td>Human lactoferrin</td>
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<td>2.8% (GUS positive lines)</td>
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<td>GUS</td>
<td>At least 8 independent lines</td>
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<td>Xu55-2 (China)</td>
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<td>30% (kanamycin&lt;sup&gt;r&lt;/sup&gt; shoots)</td>
<td>(Xing et al., 2008)</td>
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Chapter II

Plant cell calcium-rich environment enhances thermostability of recombinantly produced α-amylase from the hyperthermophilic bacterium *Thermotoga maritima*

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Summary

In the industrial processing of starch for sugar syrup and ethanol production, a liquefication step is involved where the starch is initially solubilized at high temperature and partially hydrolyzed with a thermostable α-amylase. Most amylases require calcium as a cofactor for their activity and stability, such that this divalent cation, along with the thermostable enzyme, are typically added to the starch mixture during enzymatic liquefication, thereby increasing process costs. An attractive alternative would be to produce the enzyme directly in the tissue to be treated. In a proof of concept study, a hyperthermophilic α-amylase (AmyA) from *Thermotoga maritima* was produced in tobacco cell culture yielding a product with enhanced thermostability but with otherwise identical biochemical properties compared to recombinant enzyme produced in *Escherichia coli*. Surprisingly, the plant-made enzyme retained 85% of its initial activity after 3 h incubation at
100°C, whereas the *E. coli*-produced enzyme was completely inactivated after 30 min under the same conditions. The enhanced stability of the plant recombinant enzyme was shown to be due to the intrinsically rich calcium environment in plant cells. Not only do these results indicate that producing *T. maritima* α-amylase in starch-accumulating transgenic plants could reduce the processing cost by avoiding the need for exogenously added enzyme but, at the same time, the need for calcium addition could also be eliminated.

**Introduction**

Soaring oil prices and the contribution of fossil fuels to global warming (Solomon et al., 2007) have triggered interest in alternative energy sources, especially biofuels from renewable resources, to replace conventional transportation fuels (Himmel et al., 2007). Alcohol-based biofuels are obtained from microbial fermentation of monomeric sugars directly produced by the plant or indirectly by the hydrolysis of starch or lignocellulosic materials (Fortman et al., 2008). In the U.S., bioethanol is currently produced from starch, most of which is derived from corn (Hahn-Hägerdal et al., 2006). However, other sources including tuber and root starch are being considered (Kobayashi et al., 1998; Wilson et al., 2007). Alternatively, production of monomeric sugars from starch can also find applications in the food industry, such as for sugar syrups, or through fermentation to amino acids or organic acids (Crabb and Mitchinson, 1997).

Starch is a complex polysaccharide of α-1,4- and α-1,6-linked glucose units with varying amounts of amylose (15-25%), a linear polymer of α-1,4-linked residues, and amylopectin (75-85%), a polymer with α-1,6-linked branch points (Bertoldo and Antranikian, 2001). Industrial starch processing to form monosaccharides involves a liquefaction and a saccharification step in which the starch is solubilized and enzymatically hydrolyzed to simple sugars. During liquefaction, starch granules are gelatinized at approximately 100°C for a short period of time and then partially hydrolyzed by a
thermostable α-amylase. The temperature is then lowered to 55 to 60°C for the saccharification step, where starch is converted to glucose and maltose by further enzymatic activity (Vieille and Zeikus, 2001)

The benefits of utilizing hyperthermophilic enzymes in industrial processes have been discussed (Vieille and Zeikus, 2001; Bruins et al., 2001; Comfort et al., 2004; Unsworth et al., 2007). During industrial starch processing in particular, hyperthermophilic α-glucanases can increase conversion rates through a high-temperature hydrolysis where starch solubility is greater, viscosity is lower, and the diffusion rates of substrate and products are higher. At the same time, bacterial contamination in the sugar mixture can be minimized, a strategic advantage in industrial scale fermentations (Wheals et al., 1999).

Hyperthermophilic enzymes can be obtained from bacteria or archaea that thrive in extreme thermal environments and are most active above 80°C (Bruins et al., 2001). Because of the difficulty in producing them in their native hosts, these enzymes are best generated in mesophilic hosts, where they are properly folded and retain their extreme thermostability and catalytic function (Vieille and Zeikus, 2001). Hyperthermophilic enzymes are essentially inactive at ambient temperatures, presumably due to limited molecular flexibility (Koutsopoulos et al., 2005; Unsworth et al., 2007). The fact that these enzymes can be produced in mesophilic hosts and can remain inactive until subsequent thermal activation creates the potential for production directly in the plant tissue that will ultimately provide the substrate for their action, in applications such as starch bioconversion.

Several hyperthermophilic α-glycosidases have been characterized in their native or recombinant forms, with the ultimate goal of employing them for starch conversion processes (Brown et al., 1990; Brown and Kelly, 1993; Lee et al., 2006; Unsworth et al., 2007). Genes for several hyperthermophilic α-amylases have been identified and their corresponding proteins characterized (Koch et al., 1991; Chung et al., 1995; Dong et al., 1997; Liebl et al., 1997). For example, AmyA, an α-amylase (EC 3.2.1.1) found in *Thermococcus profundus, Pyrococcus furiosus, Pyrococcus Worsei,* and *Thermotoga maritima,* cleaves α-1,4- linkages in the interior of the starch polymer (Bertoldo and Antranikian, 2001). As with their
mesophilic and thermophilic counterparts, hyperthermophilic α-amylases generally require calcium as a cofactor for activity and thermostability, consistent with the fact that cation binding is a common molecular mechanism for protein stabilization (Unsworth et al., 2007). Calcium addition was observed to protect against enzyme deactivation at higher temperatures in several thermophilic and hyperthermophilic α-amylases (Dong et al., 1997; Igarashi et al., 1998). For this reason, calcium is added to processes involving α-amylases, and isoforms that are calcium-independent are highly desirable (Crabb and Mitchinson, 1997; Richardson et al., 2002).

Calcium is abundant in plant cells, where it is present in millimolar and micromolar concentrations in the apoplast and cytoplasm, respectively, and constitutes 0.5% (w/w) of plant dry matter. Calcium is primarily accumulated in the cell wall and central vacuole, and is released to the cytosol to act as secondary messenger in signal transduction pathways in response to a wide range of environmental and hormonal signals (Taiz and Zeiger, 2002). However, the effect of intrinsic calcium in plant cells on the activity and stability of recombinant α-amylases has not been considered.

Only a few reports exist on the production of thermophilic and hyperthermophilic glycosidases in plants (Pen et al., 1992; Beaujean et al., 2000; Montalvo-Rodriguez et al., 2000; Chiang et al., 2005;). Here, a cytosolic version (signal peptide removed) of the amylA gene from Thermotoga maritima (Tma) was expressed in E. coli and in immortalized tobacco ‘NT1’ cell suspension culture, as an initial step towards its production in sweetpotato (Ipomoea batatas L.) and other starchy plants. While most of the biochemical properties of the enzyme produced from either host were comparable, the thermostability of the plant-produced α-amylase benefited significantly from the high intrinsic calcium levels in the tobacco cells. The addition of CaCl₂ or plant cell extracts from tobacco and sweetpotato to the E. coli-produced enzyme resulted in a similar stabilization, demonstrating the importance of a calcium-rich environment for thermostability, as well as the advantage of producing this enzyme in plant cells where calcium is readily available.
Results

Cloning of Tma α-amylase ‘amyN26’

To facilitate the recovery of recombinant enzyme, the signal peptide for translocation to the outer membrane in the original ‘amyA’ gene from Thermotoga maritima (Tma) was removed by appropriate PCR primer design when amplifying from genomic DNA. Sequence analysis of the resulting gene ‘amyN26’ confirmed a 78-nucleotide truncation at the 5’ terminus as compared to the native version of the gene (gb locus tag TM1840); its in-silico translation lacked the corresponding 26 amino acids at the N-terminus when aligned with Tma α-amylase (gb NP 229636) (not shown). Liebl et al. (1997) first described Tma AmyA as a lipoprotein based on the characteristics of its amino terminus, predicting a signal peptide for the initial 18 amino acids, with a cleavage site at positions 16 to 19. Upon removal of the signal peptide in AmyN26, α-amylase activity was found only in soluble protein fractions of cell extracts. Codon optimization is one of several molecular strategies to boost recombinant protein expression in plant cells (Streatfield, 2007), however, codon optimization for amyN26 was not necessary here given its high compatibility with Nicotiana tabacum codon usage. amyN26 was used without further modification, under the control of the cauliflower mosaic virus 35S promoter (35S P) and the nopaline synthase terminator (nos T) in a pBIN20-derived vector that carries the neomycin phosphotransferase gene (nptII) for plant kanamycin resistance (Hennegan and Danna, 1998). The structure of the T-DNA in the plant expression vector is described in Figure 1.

Production of Tma α-amylase in transgenic tobacco NT1 cells

Transgenic NT1 cell lines were generated using Agrobacterium tumefaciens strain ‘AGL1’, carrying the binary vector ‘pBlamy2’ (Figure 1). Over 70 independent kanamycin-resistant transgenic lines were obtained, 40 of which were screened for the amyN26 transcript
by RT-PCR. No amyN26 transcripts were detected in 10% of the lines due to gene silencing or incomplete integration of the T-DNA (Stam et al., 1997; Podevin et al., 2006). Hyperthermophilic α-amylase activity was determined by standard assay using 100 µl of heat purified protein extract (150-190 µg/ml) for a random subset of 26 lines that produced amyN26 transcripts. Significant variation in amylase specific activity was observed among NT1 lines, with only 15 having rates significantly higher than those observed for the wild type (WT) and ‘empty vector’ controls (Dunnett’s t-test with p < 0.05) (Figure 2A). The empty vector control (TC) carried only the nptII gene for plant kanamycin resistance. Background activity in the WT and TC was low and likely arises from endogenous, heat-resistant starch hydrolases. Transgenic lines L25 and L29, the lines with the highest amylase activity, were selected for further analysis.

Real-time quantitative RT-PCR (QPCR) analysis of the amyN26 transcript confirmed that variations in enzymatic activity were partially associated with different ratios of mRNA accumulation. Correlation (r = 0.7; p < 0.0001) was observed between QPCR data for the amyN26 transcript and hyperthermophilic amylase activity in the transgenic NT1 lines (Figure 2B). The QPCR data also showed that the low amylase activity observed in some NT1 lines was related to low levels of amyN26 transcripts.

Purification of recombinant Tma α-amylase

Tma α-amylase was purified from E. coli BL21 (pet21TMamyN26) and transgenic NT1 cells by a heat treatment step. For purification from E. coli, SDS-PAGE revealed one major protein band at approximately 62 kDa (Figure 3A), consistent with the 62.058 kDa predicted from the AmyN26 primary sequence (Vector NTI® software; Invitrogen). In the case of the plant protein extracts, many proteins remained soluble after the heat treatment step, and were evident on SDS-PAGE.

The molecular mass and activity of recombinant α-amylase from plant cells and from E. coli were determined by substrate-SDS-PAGE, using potato starch (electrophoresis grade;
Sigma) co-polymerized with acrylamide. Substrate gels showed hyperthermophilic amylase activity in both *E. coli* and transgenic NT1 cells, but not in the WT and TC lines (Figure 3B). In both cases, the zone of starch hydrolysis was visualized as a ‘clearing’ over dark background after KI/I₂ staining, and was centered at the same approximate molecular weight of 62 kDa (Figure 3B).

Approximately 71,900 units of recombinant amylase activity were obtained from 500 ml of *E. coli* BL21 (pet21TMamyN26) growing at late-log phase, with an apparent specific activity of 1,237.5 ± 83.5 (U/mg), measured under standard assay conditions. In the transgenic NT1 lines, approximately 4.0 ± 0.16 and 4.62 ± 0.13 units of hyperthermophilic α-amylase activity were determined per gram of tissue (fresh weight), for L25 and L29, respectively (average from 10 replications). Assuming identical enzyme activity per unit of recombinant protein produced in tobacco and *E. coli*, the amount of recombinant enzyme produced in plant cells was between 0.16% and 0.21% of total soluble proteins, and 1.4% and 1.5% of proteins in the heat purified fractions for NT1L25 and NT1L29, respectively.

Enzymatic properties of recombinant Tma α-amylase

The activity of Tma α-amylase was measured at temperatures between 22-100°C and at pH values from 5.6 to 8, using 50 µl of heat-treatment purified protein from transgenic NT1 lines L25 and L29 (150-190 µg/ml) and recombinant *E. coli* (4 µg/ml). Plant- and *E. coli*- made enzyme displayed maximal activities at 90°C and 85°C, respectively (Figure 4A). In all cases, Arrhenius plots were linear between 22 and 100°C, implying that the functional conformation of the enzyme remained unchanged (Vieille and Zeikus, 2001) (Figure 4B). Activation energies (Eₐ) of 22.5, 26.5, and 47.1 kJ mol⁻¹ K⁻¹ for Tma α-amylase produced in NT1L25, NT1L29, and *E. coli* respectively, were calculated from the Arrhenius equation ln k = B – Eₐ / RT [where k is the rate constant, B is a constant, Eₐ is the activation energy, R is the molar gas constant (8.314 J mol⁻¹ K⁻¹), and T is the absolute temperature]. The plant-made enzyme was catalytically inactive at temperatures below 37°C, when compared to the
WT and empty vector controls (Figure 4C). Maximum activity was observed at pH 7 in phosphate buffer for both *E. coli* and plant-made Tma α-amylase (Figure 4D).

Thermostability of recombinant enzyme

The liquefaction step in the conversion of starch to monosaccharides is generally carried out between 80°C and 100°C (Crabb and Mitchinson, 1997). Sufficient thermostability of the recombinant amylase within this temperature range is needed to maximize monosaccharide yields and to minimize processing costs. The recombinant heat purified versions of Tma α-amylase were pre-incubated at 80°C and 100°C, prior to determining the residual starch hydrolyzing activity under standard assay conditions. No major differences in the thermostability of Tma α-amylase were observed at 80°C; after 6 h pre-incubation, 97.2% ± 3.9, 97.4% ± 3.2, and 89.3% ± 3.4 of initial activity was noted for NT1L25, NT1L29, and *E. coli*, respectively (Figure 5A). The stability of Tma α-amylase from *E. coli* at 80°C was not significantly altered when using either the pre-incubation buffer (100 mM Tris-HCl pH 8, 10 mM KCl, and 5% Glycerol) or 20mM Tris pH 8 (not shown). In contrast, the difference in the thermostability of the plant and *E. coli*-made enzyme at 100°C was pronounced. The plant-made Tma α-amylase retained 89.6% ± 2.4 (NT1 L25) and 80.6% ± 2.2 (NT1 L29) of its initial activity after 3 h pre-incubation at the higher temperature, while the *E. coli*-made enzyme was essentially inactive after 30 min (Figure 5B). The half-lives (t_{1/2}) of Tma α-amylase at 100°C obtained from tobacco cells were 9.6 h and 19.2 h for NT1L25 and NT1L29, respectively, compared to 12.8 min for the *E. coli*-made enzyme (Figure 5C).

The possibility of intrinsic factors, such as improper folding, accounted for the lower stability of the *E. coli*-AmyN26 was discarded, given that *E. coli*-AmyN26 was stabilized if pre-incubated in the wild type NT1 heat soluble protein fraction (Figure 6A,B). Prior reports that calcium at 10mM was detrimental to *T. maritima* AmyA activity and stability at 80°C
(Liebl et al., 1997) suggested, at least initially, that factors other than calcium in the plant extracts could be involved in AmyN26 stabilization at high temperatures.

To determine if specific proteins, perhaps thermostable plant chaperones, were involved in AmyN26 stabilization, discrete protein fractions from the WT tobacco heat soluble extract were separated by fast protein liquid chromatography (FPLC) and tested to see if any improvement in E. coli- AmyN26 stability at 100°C could be induced. A few protein fractions, when used in the pre-incubation buffer, were able to reduce the inactivation rate of AmyN26 at this temperature, with the extent of stabilization directly correlating with the magnitude of their corresponding FPLC peak (data not shown). Subsequent analysis by mass spectrometry identified tryptic peptides corresponding to a plant chaperonin protein in all the active fractions (Table 1). However, dialyzed protein samples from WT tobacco (3.5 kDa cut-off) did not improve E. coli Tma AmyN26 stability at 100°C (Figure 6A).

To rule-out the possibility of a small peptide being responsible for Tma α-amylase stabilization, the tobacco WT protein sample was filtered through a 3 kDa cutoff membrane (Microcon YM-3; Millipore), and through a 1 kDa cutoff ultrafiltration disc (Millipore). In both cases, the ‘flowthrough’ and the ‘retentate’ were collected and evaluated. The flowthrough from both the 3kDa and 1kDa size exclusion filtration were able to stabilize E. coli- made Tma α-amylase (Figure 6A), and autoclaving did not diminish the extent of stabilization. Taken together, these results pointed to a cofactor, such as a metal ion, as the cause of AmyN26 thermostabilization.

Indeed, contrary to a previous report by Liebl et al. (1997), calcium significantly stabilized Tma α-amylase at high temperatures. Total calcium concentrations in heat soluble protein fractions from WT and transgenic tobacco cells were determined to be between 0.4 and 0.7 mM. Consequently, CaCl₂ and MgCl₂ at concentrations from 0.01 to 100 mM in the preincubation buffer were evaluated, revealing that CaCl₂ above 0.1 mM significantly reduced the inactivation rate of E. coli-produced Tma α-amylase at 100°C, while magnesium had no effect at all the concentrations tested (Figure 6A, B). In the presence of 10 mM CaCl₂, the enzyme retained 95% of its initial activity after a 3 h incubation at 100°C. Moreover,
addition of calcium or soluble protein extracts from tobacco cells and sweetpotato leaves to the pre-incubation buffer of *E. coli*-produced Tma α-amylase resulted in comparable thermostabilization to the plant-made enzyme with no added cation at 100°C (Figure 6B). This observation supports the premise that the calcium-rich plant cell environment was responsible for the enhanced thermostability of Tma α-amylase.

**Discussion**

In the industrial processing of starch to yield monomeric sugars, the liquefaction step is the point at which starch is initially solubilized at 100°C and then partially hydrolyzed by a thermostable α-amylase. Current methods for starch liquefaction involve the addition of a thermostable enzyme (generally obtained from bacterial systems) as well as calcium, to the starch mixture (Crabb and Mitchinson, 1997). By directly producing the thermostable α-amylase in the target plant for starch self-processing, costs associated with enzyme addition could be reduced, potentially making the process more cost efficient.

This prospect is supported by the results reported here for expression of a modified *amyA* gene, *amyN26*, from *Thermotoga maritima* (Tma) in tobacco NT1 cell cultures. Hyperthermophilic α-amylase activity was detected in 15 transgenic tobacco cell lines, with starch conversion rates significantly higher than those observed in wild type and transformation controls (Figure 2A). Significant variation in AmyN26 activity was observed among transgenic lines, likely the consequence of varying levels of recombinant protein and/or mRNA accumulation. Variations in the activity of recombinant glycosidases produced in transgenic plants were also reported previously (Beaujean et al., 2000; Montalvo-Rodriguez et al., 2000). Through QPCR analysis of the *amyN26* transcript, the variation in AmyN26 activity was determined to be associated with different levels of mRNA accumulation (Figure 2B), possibly a consequence of genomic positioning effects (Stam et al., 1997; Beaujean et al., 2000). It is known that the number of transgenes, the orientation of
multicopy transgenes, and the chromosomal environment of the insertion locus (e.g., heavily methylated), as well as homologies with endogenous genes are some of the factors that affect transgenic mRNA accumulation in plants (Stam et al., 1997).

For economical viability, recombinant amylases must be produced in starch accumulating plants at protein levels high enough to be competitive with existing approaches. In our study, recombinant AmyN26 protein levels in two transgenic tobacco lines were approximately 0.2% of total soluble protein. Pen et al. (1992) found that the production of Bacillus licheniformis α-amylase (BLA) in tobacco leaves (accumulating in the apoplast) was as high as 0.3% of soluble protein, with an average of 0.1% among 59 plants of a same transgenic line. Moltalvo-Rodriguez et al. (2000) reported protein yields of 0.15 and 0.04% of total soluble protein for recombinant hyperthermophilic β-glycosidase and α-glucosidase, respectively, when produced in tobacco plants. In any case, higher recombinant protein yields in plants can be obtained via a combination of methods, by enhancing transcription, mRNA stability, higher translation rates, and targeting expression to cellular compartments with reduced proteolytic degradation for a particular protein (Dai et al., 2005, Streatfield, 2007). Although it has been observed that hyperthermophilic enzymes are generally more resistant to chemical denaturation as well as proteolitic cleavage (Vieille and Zeikus, 2001; Cowan, 1992), evaluation of alternative localization for plant-produced T. maritima α-amylases should be considered.

* T. maritima* AmyA (TMA) has one of the highest specific activities reported for α-amylases, between 3,800 and 5,600 U/mg protein using soluble starch and amylose as substrate respectively, and measured at 80°C and pH 7 (Liebl et al., 1997). Recombinant TMA AmyN26 produced here had comparable biochemical properties as the full length AmyA protein (Liebl et al. 1997). Amylase activity was maximal at pH 7, 90°C and 85°C for the plant- and E. coli- made enzymes, respectively. The lower activation energy (Ea) observed for the plant- made enzyme (22.5, 26.5 kJ mol⁻¹ K⁻¹) compared to E. coli- enzyme (47.1 kJ mol⁻¹ K⁻¹) was likely the consequence of intrinsically supplied calcium in the plant protein samples. The plant-made AmyN26 was significantly more stable at 100°C than its
bacterial counterpart under the same conditions. Plant-made AmyN26 retained 85% of initial activity after 3 h pre-incubation, while \textit{E. coli} AmyN26 was inactivated within the initial 30 min at 100°C (Figure 5B). The half-life ($t_{1/2}$) of plant-made enzyme at 100°C (9.6 to 19.2 h) was comparable to that measured by Dong et al. (1997) for \textit{Pyrococcus furiosus} $\alpha$-amylase (PFA) at 98°C ($t_{1/2} = 13.2$ h) in the absence of calcium. The $t_{1/2}$ of both enzymes was superior to that reported for \textit{Bacillus licheniformis} $\alpha$-amylase (BLA), widely used in industrial starch liquefaction, which was completely inactivated after one hour of incubation at 98°C, even in the presence of 5 mM Ca$^{2+}$ (Dong et al., 1997). Given that the liquefaction step is generally carried out at temperatures between 90-105°C for about 90 minutes (Crabb and Mitchinson, 1997), the intrinsic thermostability of the plant recombinant $\alpha$-amylase, without exogenous calcium addition, is a distinct advantage.

It was initially hypothesized that higher protein concentrations in the plant heat soluble fractions (154 to 164 $\mu$g/ml compared to 4 $\mu$g/ml for \textit{E. coli}), as well as the presence of protective agents, could be responsible for the increased stability of the plant-made enzyme. Higher protein concentrations and other protective agents have been found to stabilize enzymes (Sadana, 1991). Alternatively, the presence of cofactors, such as metal cations, can accelerate re-folding rates and enhance thermostability (Bushmarina et al., 2006). Group I and Group II chaperonins have been observed to enhance thermostability of enzymes (Kohda et al., 2006). While mass spectral analysis of FPLC-separated protein fractions with AmyN26-stabilizing activity, obtained from the WT tobacco heat soluble extract, revealed tryptic peptides with homology to a plant chaperonin (Table 1), dialyzed fractions from the same plant extract did not stabilize AmyN26 at 100°C.

Amino acid sequence analysis of TMA indicated highly conserved polypeptide regions with residues presumably involved in Ca$^{2+}$ coordination; in fact, calcium as well as other divalent cations were able to reactivate TMA after EDTA treatment (Liebl et al. 1997). However, it was also reported that 10 mM CaCl$_2$ reduced TMA activity by 50% and had a destabilizing effect on TMA at 80°C (Liebl et al., 1997). Here, calcium at concentrations from 0.1 mM to 100 mM in the pre-incubation buffer significantly decreased the deactivation
rate of AmyN26 at 100°C. Moreover, in the presence of 10 mM CaCl₂, AmyN26 retained 95% of its initial activity after a 3 h pre-incubation. The addition of calcium or plant cell heat soluble fractions from wild type tobacco cells and sweetpotato leaves to the pre-incubation buffer resulted in an equivalent stabilization at 100°C, with similar enzyme inactivation rates to those observed for the plant-made AmyN26 (Figure 6B). Calcium concentration in the WT and transgenic tobacco heat soluble fractions were between 0.4 to 0.7 mM. Given that the dialyzed plant protein samples did not stabilize AmyN26 produced in *E. coli*, the intrinsic calcium present in the plant cell heat soluble fractions, and not proteins, was concluded to be responsible for AmyN26 thermostabilization. These observations are consistent with the stabilizing role of calcium in many enzymes at various temperatures, and α-amylases in particular (Daniel et al., 1996; Vieille and Zeikus, 2001).

Only a few reports exist on the production of thermostable glycosidases in plants for starch processing (Pen et al., 1992; Beaujean et al., 2000; Montalvo-Rodriguez et al., 2000; Chiang et al., 2005) and, although the enzymes were expressed in active form, long-term thermostability at high temperatures was not examined. In addition, to produce starch-hydrolyzing enzymes in starch-accumulating plants, it is critical that the recombinant enzyme does not interfere with normal carbohydrate metabolism and not adversely impact sink tissue functions. This could be an issue for less thermoactive enzymes including the thermostable BLA that still retained over 20% of its activity at 40°C (Dong et al., 1997). While AmyN26 from both *E. coli* and tobacco cells were only functional at elevated temperatures (Figure 4A), it was noted that the plant-made enzyme was inactive at temperatures below 40°C (Figure 4C). Dong et al. (1997) also observed that the hyperthermophilic α-amylase from *P. furiosus* (PFA) was inactive below 40°C. Previous reports indicate that production of thermostable glycosidases in plants did not affect normal plant metabolism, either because of physical separation or minimal activity at plant growth temperatures (Pen et al., 1992; Beaujean et al., 2000; Montalvo-Rodriguez et al., 2000). However, alteration of amyllose content in starch granules was observed in transgenic rice producing a highly thermophilic amylopullulanase (APU) localized in the amyloplasts (Chiang et al., 2005). Although the
authors reported APU to be hyperthermophilic, this enzyme was isolated from *Thermoanaerobacter ethanolicus* 39E, a thermophilic bacterium with an optimal growth temperature around 65°C, and was rather a thermophilic form with significant relative activity at plant growth temperatures (Saha et al., 1988; Mathupala et al., 1993).

Results here suggest that the hyperthermophilic α-amylase from *Thermotoga maritima* posed minimal risk to normal plant function, although this issue needs to be investigated for specific plant-enzyme combinations. In any case, the results reported here are promising and offer the potential for exploiting transgenic plants, the calcium-rich plant cytoplasm and enzyme thermophilicity as an attractive alternative for processing starch to fermentable sugars.

**Experimental procedures**

Cloning of *T. maritima* amyN26

*Thermotoga maritima* (DSM 3109) was obtained from the German Collection of Microorganisms and Cell-cultures (DSMZ, Braunschweig, Germany). Growth conditions and genomic DNA extraction from *T. maritima* were performed according to previously described by Parker et al. (2001). The gene *amyN26* was isolated by polymerase chain reaction amplification from genomic DNA using Pfu polymerase (Promega, Madison, WI, USA). The primers used, forward ‘TMAmyAF5’ (5’-GGCGCACCATATGTAGCAATCCCTCGTTTCA-3’) and reverse ‘TMamyARv’ (5’-CGCCGCTGGATTCTCAGTTTTTGAAAATGT-3’), introduced restriction sites ‘Nde I’ and ‘Eco RI’ (underlined) and amplified the *amyA* ORF (gb TM1840) starting 78 nucleotides downstream from the original initiation of translation site. A new ‘ATG’ start codon was introduced (bold letters in forward primer), and the signal peptide for export to the outer membrane (Liebl et al., 1997) was removed. The resulting gene *amyN26*, was cloned into vector pet21b (Novagene, Darmstadt, Germany), generating plasmid ‘pet21TMamyN26’,
which was then introduced into *E. coli* strain BL21(DE3) (Novagen) for recombinant protein expression. Codon usage for *amyN26* ORF was compared to the codon usage for *Nicotiana tabacum* using the ‘countcodon’ program and the ‘codon usage database’ at [http://www.kazusa.or.jp/codon/](http://www.kazusa.or.jp/codon/). No major incompatibilities were found. For gene expression in plant cells, *amyN26* was placed under the control of the cauliflower mosaic virus 35S promoter and the nopaline synthase ‘nos’ terminator in a pBIN20-derived vector (Hennegan and Danna, 1998), which also carries the *nptII* gene for plant kanamycin resistance. The resulting plasmid ‘pBlamy2’ (Figure 1) was introduced into *Agrobacterium tumefaciens* strain ‘AGL1’ (Lazo et al., 1991) by electroporation using a BTX Electro Cell Manipulator™ 600.

**NT1 cell culture maintenance and transformation**

Wild type tobacco NT1 cell culture was maintained in 50 ml liquid NT1 medium [MS basal salts (Sigma-Aldrich, St. Louis, MO, USA), sucrose 30 mg/L, myo-inositol 100 mg/L, thiamine-HCl 1 mg/L, KH₂PO₄ 180 mg/L, 2, 4-dichlorophenoxyacetic acid 0.2 mg/L, and pH 5.6] in 250 ml flasks. Cells were agitated in a rotary shaker (150 rpm) at 26°C in the dark and were sub-cultured weekly with a 6% (v/v) inoculum. Transformation of NT1 cells with *A. tumefaciens* AGL1 carrying the binary vector pBlamy2 was performed similar to described by Persson et al. (2001): 4 ml of a 4-day cell culture was co-cultivated with 100 µl of *Agrobacteria* (OD<sub>600</sub> 0.7-0.9) and incubated for 48 h at 26°C in the dark. Co-cultivated cells were re-suspended in 2 ml liquid NT1 media, of which 0.5 ml was spread onto selection plates [NT1 medium with 8 mg/L Phytablend (Caisson Laboratories, North Logan, UT, USA), kanamycin 50 mg/L, and timetin 100 mg/L]. Kanamycin resistant cell clusters were collected after 3 weeks, and transferred to individual selection plates. Transgenic cell lines were then maintained on selection plates at 26°C in the dark, and were sub-cultured every 14 days. Selected lines were transferred to liquid media as previously described (Persson et al., 2001) and maintained as above.
Production of *amyN26* transcript in kanamycin resistant lines was evaluated by RT-PCR. Total RNA was isolated using the RNeasy Kit (QIAGEN, Valencia, CA, USA). Reverse transcription was performed using oligo(dT)$_{20}$ primers and Omniscript$^\text{®}$ reverse transcriptase (QIAGEN). PCR was performed using the primers ‘amyFw1’ (5’-GATGGTACTAGCTCCAACCTTG-3’) and ‘amyRv1’ (5’-GATGGGAGAAGAACCTGTGG-3’) for *amyN26*, and ‘nptFw1’ (5’-CTGGGCACAACAGACAATCG-3’) and ‘nptRv1’ (5’-ACCGTAAAGCACGAGGAAGC-3’) for *nptII*, which amplified fragments of 886bp and 667bp, respectively.

**Protein purification**

*E. coli* BL21(pet21TMamyN26) cells were grown in 500 ml LB medium in a 2 L flask until mid-exponential phase (OD$_{600}$ 0.4-0.6). Expression of *amyN26* was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, QIAGEN), and the cells were grown for an additional 4 h. Cells were harvested by centrifugation (10 min at 10000 g and 4°C), and the cell pellets were washed once with 20 mM Tris-HCl pH 8, re-centrifuged, and then re-suspended in 5 ml of 20 mM Tris-HCl pH 8. Cells were digested with lysozyme (1 h at 37°C) and lysed by sonication for 5 min (10 s burst, 10 s pause).

Tobacco NT1 cells grown on semisolid medium were collected while avoiding any media carry-over. NT1 cells grown in liquid medium were collected by filtration with 3 mm Whatman paper using a vacuum pump, and rinsed thoroughly with distilled water to remove all remaining media. Cells were then frozen with liquid nitrogen and homogenized by grinding with a mortar and pestle. Plant protein extraction buffer ‘PPEB’ [100 mM Tris-HCl pH 8, 10 mM KCl, 5% Glycerol, 10 mM DTT, 1% (v/v) protease inhibitor cocktail ‘PIC’ (P9599, Sigma-Aldrich)] was added immediately (3 ml per gram of tissue) and mixed thoroughly. Protein samples from *E. coli* and tobacco cell cultures were subjected to a heat-precipitation treatment consisting of a 25-30 min incubation at 85°C followed by centrifugation (20 min at 19000 g and 4°C) to remove denatured proteins and cell debris. The
supernatant was collected and stored at 4°C. Total soluble protein samples from plant cells were similarly obtained, but avoiding the heat precipitation step. Calcium concentrations in heat-soluble protein samples were estimated at the Analytical Laboratory directed by Dr. Wayne Robarge (Soil Science Department, North Carolina State University).

When necessary, dialysis of soluble protein fractions was conducted in 10K and 3.5K MWCO dialysis cassettes (Pierce Biotechnology, Rockford, IL, USA) against 100 mM Tris-HCl pH 8. Dialysis was carried out overnight at room temperature with agitation, with buffer replacement at least 3 times during the dialysis. FPLC was performed in a Biologic DuoFlow System (BioRad, Hercules, CA, USA) using a Q-Sepharose packed column (GE Healthcare, Piscataway, NJ, USA), which was coupled to a UV280 detector for continuous scanning of the flowthrough. The mobile phase was 50 mM Tris pH 8 (Buffer A), with a linear gradient of 0 to 2 M NaCl for 55 min, obtained by injection of Buffer B (50 mM Tris pH 8, 2 M NaCl). The flow through the column was 2 ml/min, and 36 fractions of 3 ml each were collected.

Mass spectral analysis

Mass spectral analysis was performed at the Genomic Sciences laboratory at North Carolina State University. Samples were concentrated in a 5 kDa cutoff ultrafiltration device (Millipore) and washed twice with 50 mM ammonium bicarbonate pH 7.8. Proteins were then reduced (1 hour, 55°C) with dithiothreitol and alkylated with iodoacetamide (1 h, 20°C), followed by trypsin digestion (16 h, 37°C). Five microliter tryptic samples were analysed on a Thermo LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA, USA) with an IonMax electrospray ionization source (Thermo) interfaced to a Phenomenex Jupiter 4 µm diameter Proteo C18 reverse phase column (Phenomenex, Torrance, CA, USA). Peptides were eluted with a linear buffer gradient in 30 min with a 28 µl/min flow rate. The linear buffer gradient was as follows: 5% buffer A/ 95% buffer B to 40% buffer A/ 60% buffer B [buffer A – 50 mM acetic acid d$_2$H$_2$O; buffer B - acetonitrile]. The ion source was operated at 4.5 kV. The peptides were analyzed as a big 4 experiment (four most abundant ions observed
in MS-only mode), utilizing Dynamic Exclusion (repeat count, 1). A survey scan (MS-only mode) was followed by four rounds of data-dependent MS/MS scans on the four most abundant ions in the survey scan. Ions chosen for MS/MS were then placed onto an exclusion list for a total of 180 s. The approach frequently allows for the detection and characterization of minor components within the sample. Database searches were made in Bioworks Browser 3.3 (Thermo). The initial database searches were against a Viridiplantae custom local database file downloaded from NCBI (download date, 21 September 2007).

Protein quantification

Total protein was estimated according to the ‘Protein Assay’ (BioRad) that is based on the method by Bradford (Bradford, 1976), in microtiter plates using bovine serum albumin (Sigma-Aldrich) as a standard. Denaturing sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of heat-purified protein extracts was performed in 8-16% precision protein gels (Pierce Biotechnology) and stained with coomassie blue (BioRad).

Enzyme activity assays

Starch hydrolyzing activity was determined by measuring the enzymatic release of reducing sugars from starch (Bernfeld, 1955). One unit of enzyme activity (U) was defined as the amount of protein producing 1 µmol of reducing ends as maltose equivalents per minute. Specific enzyme activity was defined as the enzymatic units per milligram of protein (U/mg). The accumulation of reducing ends in the enzymatic reaction was determined by the dinitrosalicylic acid (DNS) assay (Chaplin and Kennedy, 1994) using a maltose standard curve. All enzymatic reactions were incubated 10 min, using 0.5% substrate (soluble potato starch, Sigma-Aldrich) in a total volume of 500 µl. Our standard-assay conditions were 50 mM phosphate buffer pH 7 and 84°C, using 50 µl of heat purified protein extracts as the enzyme source. Reaction controls (a sample with only substrate and a sample with only
enzyme) were placed in parallel, and each reaction was replicated 2 to 3 times for each assay. The data for enzyme activity reported is the average of the 2 to 3 technical and 3 to 4 biological replications for each assay. To test the effect of pH in recombinant α-amylase activity, pH values from 5.6 to 8 were obtained with phosphate buffer (temperature coefficient -0.0028) adjusted according to the guidelines outlined in Sambrook and Russell (2001). Protein concentrations in the plant extracts were in the range of 150-190 µg/ml. Serial dilutions of E. coli protein were conducted to determine a suitable concentration for the assays. It was determined that protein concentrations from 0.6 to 6 µg/ml gave a linear increase in enzyme activity (data not shown), and had activities comparable to those obtained with the transgenic plant protein samples. A 4 µg/ml dilution of E. coli protein was used for the majority of the assays, and 10 µg/ml to test stabilization at 100°C, using different plant protein fractions and CaCl₂. To test stability of AmyN26 at 80°C and 100°C, protein samples from recombinant E. coli and tobacco cells were pre-incubated at the indicated temperatures and aliquots were taken at discrete time intervals. Residual amylase activity was measured under our standard assay conditions. The pre-incubation buffer composition was 100 mM Tris-HCl pH 8, 10 mM KCl, and 5% Glycerol.

Substrate-SDS-PAGE

Heat-purified plant protein extracts were concentrated with a microcon YM-30 column (Millipore). Protein samples from NT1 wild type (12.9 µg), empty vector control (13.6 µg), NT1L25 (13.7 µg), NT1L29 (13.2 µg), and three ten-fold dilutions of E. coli 1x=2.69 µg were mixed with sample buffer (2x: 0.125 M Tris-HCl pH 6.8, 20% glycerol, 0.04% bromophenol blue, and 2% SDS) and loaded into a 12% acrylamide gel with copolymerized soluble potato starch (electrophoresis grade, Sigma-Aldrich) as previously described (Martinez et al., 2000). Electrophoresis was performed at 4°C, and otherwise according to previously described (Martinez et al., 2000). Following electrophoresis, the gel was rinsed thoroughly with distilled water, and placed in phosphate buffer at pH7. In-gel
starch hydrolysis by recombinant amylase was performed by incubation at 85°C for 1.5 h. After incubation, the gel was rinsed with distilled water, covered with 12% trichloroacetic acid (TCA; Sigma-Aldrich), and shaken gently for 10 min at room temperature. The gel was then stained with KI/I$_2$ solution (BioRad). Regions of amylase activity appeared as cleared bands over a dark blue background (Figure 3B). The gel was subsequently rinsed with distilled water and stained overnight with biosafe coomassie blue stain (BioRad). After rinsing with water, KI/I$_2$ staining gradually dissipated and coomassie stained bands were then visible (Figure 3B).

Real Time Quantitative RT-PCR (QPCR)

Total RNA was extracted using an RNeasy kit (QIAGEN) and treated with DNase I (NEB, Ipswich, MA, USA). Reverse transcription was performed using oligo-d(T)$_{20}$ primers and Omniscript® Reverse Transcriptase (QIAGEN). A negative control lacking reverse transcriptase was set for each sample. cDNA samples were quantified with a NanoDrop™ spectrophotometer and diluted to 100 ng/µl with ultra-pure water. The *Nicotiana tabacum* translation initiation factor 4A10 (NeIF-4A10) was used as internal ‘normalizer’ control due to its homogeneous expression across cell types and tissues in tobacco (Mandel et al., 1995). For each QPCR run, two ‘no-template’ controls (NTC), a ‘no-reverse transcriptase’ control (NoRT), and 4-serial dilutions of plasmid DNA containing the gene of interest (GOI) and the normalizer were placed alongside the samples. ‘SYBR green’ (Stratagene, La Jolla, Ca, USA) was used for detection and ‘ROX’ (Stratagene) served as the reference dye. Amplification was performed in a 3000Mx Thermocycler (Stratagene) using the primers ‘amyFw5’ (5’-GGGATAACCTTTGGAAACGCTGA-3’) and ‘amyRv5’ (5’-CGGATTCAAGACCACCAACAGC-3’) for the *amyN26* transcript, and ‘IF4AFw2’ (5’-CGTGATGACGTTACGCTTAAGG-3’) and ‘eIF4ARv’ (5’-ACGACCCAGATCGGAACCTCTCG-3’) for the internal control. Both primer sets have an optimized annealing temperature of 58°C, and amplified fragments of 246 bp and 256 bp
respectively. The QPCR data was analyzed using MxPro-QPCR software (Stratagene) setting
the experiment type to ‘comparative quantitation’, using the wild-type NT1 sample as the
‘calibrator’ and the ‘eIF4A10’ transcript as the ‘normalizer’. Threshold fluorescence was set
manually to optimize amplification efficiencies for either standard curve, according to the
manufacturer’s guidelines (Stratagene, 2007).

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Kelly acknowledges support from the U.S. National Science Foundation.

References

Beaujean, A., Ducrocq-Assaf, C., Sangwan, R.S. et al. (2000) Engineering direct fructose
production in processed potato tubers by expressing a bifunctional alpha-


Table 1. Identity of tryptic peptides possibly involved in Tma α-amylase stabilization

Tryptic peptides were identified by mass spectral analysis and show similarity to chaperonin protein CPN10. These peptides were present in all the wild type tobacco protein fractions (separated by FPLC) that were able to decrease Tma α-amylase inactivation at 100°C.

<table>
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<tr>
<th>Reference</th>
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<td>10 kDa chaperonin (Protein CPN10) (Protein groES)</td>
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† Similarity scores were obtained from BLink (NCBI)
Figure 1. Plasmid vector used in NT1 cells transformation

Figure 2. Expression and activity of recombinant Tma α-amylase in multiple transgenic NT1 cell lines

A. Variation in amylase activity (U/mg protein) among transgenic lines. Asterisks above the bars indicate a significant difference as compared to the wild type (Dunnett’s t-test; p<0.05). Data for starch-hydrolyzing activities were obtained under standard conditions [0.5% substrate (soluble potato starch), pH 7 and 84°C] and means are of 4 biological replicates. NT1 lines L25 and L29 (underlined) had the highest amylase activity and were selected for further analysis. B. Tma α-amylase ‘amyN26’ transcript levels in transgenic NT1 lines relative to the wild type, determined by real time QPCR. Data shown are fold-changes in normalized and baseline corrected relative fluorescence units (dRn) in each sample. There was significant correlation (r = 0.7; p < 0.0001) between enzyme activity and amyN26 transcript level among transgenic lines.
Figure 3. Active recombinant α-amylase was produced in *E. coli* and transgenic tobacco NT1 cells

**A.** Recombinant α-amylase in heat-purified protein sample from *E. coli* visualized by coomassie stain of the SDS-PAGE gel. S, Protein sample. M, mass molecular marker. **B.** Substrate-SDS-PAGE of recombinant α-amylase. Co-polymerized substrate (starch from potato) was hydrolyzed by immobilized enzyme at 85°C. 1-4, 10-fold serial dilutions (1X = 2.69 to 10⁻³ µg/lane) of protein sample from *E. coli* harboring plasmid pet21bTMamyN26. 5-8, heat-purified protein from: 5 and 6, transgenic lines NT1L29 (13.2µg) and NT1L25 (13.7µg); 7, NT1 empty vector control (13.6µg); 8, wild type NT1 (12.9µg). Above, gel after KI/I₂ staining showing area of local starch hydrolysis (white bands over dark background). Below, same gel after coomassie stain. Arrow indicates the position of recombinant Tma α-amylase from *E. coli* (1X sample).
Figure 4. Effect of temperature and pH on recombinant *T. maritima* α-amylase activity
Activity was measured with 0.5% substrate (soluble potato starch) in 10 min reactions. A. Heat activation of recombinant α-amylase from *E. coli* and plant cells. Data points are means of 4 biological replicates. B. Arrhenius plot to estimate activation energy (*E_a*) of *T. maritima* α-amylase. *E_a* was 47.1, 22.5, and 26.5 kJmol⁻¹ for AmyN26 obtained from *E. coli*, NT1L25, and NT1L29, respectively. C. Heat activation of recombinant α-amylase from transgenic tobacco lines. No difference in starch hydrolyzing activity was observed between transgenic lines and the wild type (WT) and empty vector (TC) controls below 37°C. D. Activity of *T. maritima* α-amylase at different pH values measured at 84°C. Data points are means of 2 biological replicates.
Figure 5. Thermostability of recombinant α-amylase at 80°C and 100°C

Recombinant enzyme was obtained from transgenic tobacco cell lines (L25 and L29) and from *E. coli*. Residual amylase activity was measured under our standard assay conditions after preincubating protein samples for different time intervals. Data points are means of 3 to 4 biological replicates. A. Residual amylase activity after preincubation at 80°C. B. Residual amylase activity after preincubation at 100°C. C. Plot for estimating the half-life ($t_{1/2}$) of AmyN26 at 100°C. $t_{1/2}$ of AmyN26 at 100°C was 9.6 h, 19.2 h, and 12.8 min for NT1L25, NT1L29, and *E. coli*, respectively.
Figure 6. Thermostabilization of *T. maritima* α-amylase by Ca$^{2+}$ and plant cell heat-soluble fractions

Residual amylase activity was measured under standard assay conditions after pre-incubation at 100°C for different times. The basal pre-incubation buffer composition was 100 mM Tris-HCl pH8, 10 mM KCl, and 5% Glycerol. A. Residual activity of recombinant α-amylase from *E. coli* in the presence of buffer (○); dialyzed heat soluble protein from tobacco >3.5kDa (X); flowthrough from filtration of heat soluble protein from tobacco <1kDa (◆); 0.01 mM CaCl$_2$ (△); 0.1 mM CaCl$_2$ (▲); and 10 mM MgCl$_2$ (+). Enzyme thermostabilization was achieved with the flowthrough (◆) and CaCl$_2$ concentrations above 0.1 mM (▲). B. Residual activity of recombinant α-amylase from *E. coli* in the presence of buffer (○), heat soluble protein fractions from WT NT1 cells (□), from WT sweetpotato leaves (▷), 10 mM CaCl$_2$ (+), 100 mM CaCl$_2$ (X), and residual activity of recombinant α-amylase from a transgenic tobacco cell line (◼) after preincubation at 100°C. Thermostabilization of recombinant α-amylase from *E. coli* was obtained by coincubation with WT plant cell heat soluble fractions and CaCl$_2$, and was comparable to the thermostability of plant-made enzyme without calcium addition.
Chapter III

Starch self-processing in transgenic sweetpotato roots expressing a hyperthermophilic α-amylase

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Summary

Sweetpotato is an important crop in the southeastern U.S. It requires few inputs, grows well on marginal land, accumulates large quantities of starch in the storage roots, and has been shown to give comparable or superior ethanol yields to corn per cultivated acre in the southeast. Starch conversion to fermentable sugars for ethanol production is carried out at high temperatures, and requires the action of thermostable and thermoactive amylolytic enzymes to convert starch to monomeric sugars. These enzymes are added to the starch mixture, impacting overall process economics. To address this shortcoming, a gene encoding a hyperthermophilic α-amylase from *Thermotoga maritima* was cloned and expressed in transgenic sweetpotato, generated by *A. tumefaciens*-mediated transformation, to create a plant with the ability to self-process starch. While no significant enzyme activity could be detected below 40°C, starch in the transgenic sweetpotato storage roots was readily hydrolyzed at 80°C. The transgene did not affect normal storage root formation in plant
under greenhouse conditions. The results presented here indicate the viability of engineering plants with hyperthermophilic glycoside hydrolases for cost effective starch conversion to fermentable sugars. Furthermore, they also support the potential use of sweetpotato as an alternative energy crop.

Introduction

Increasing energy demand, concerns over security of oil supplies and energy independence, as well as the unprecedented increase in greenhouse gases from burning fossil fuels (Solomon et al., 2007), have all generated considerable interest worldwide in alternative energy production from renewable, carbon-neutral resources. In the near future, ethanol or alcohol-based fuels are expected to be the dominant renewable fuel for transportation (Hahn-Hägerdal et al., 2006; Himmel et al., 2007). For instance, the US Energy Policy Act of 2005 requires blending 7.5 billion gallons per year of renewable fuel by 2012. This will require a substantial increase in bioethanol production, which currently is approximately 1.4 billion gallons per year (EERE, 2008).

Given its low purchase price, potential fuel yield, and environmental attributes, lignocellulosic biomass is viewed as the longer-term solution to renewable energy needs (EERE, 2008; Lynd et al., 2008). However, the high compactness and the presence of lignin reduce the accessibility of enzymes to polysaccharides and not all sugars within the complex chemical structure of lignocellulose are readily fermentable. In addition, the relatively slow kinetics of cellulose hydrolysis, the difficulty of lignin removal, and the high costs of thermochemical pretreatment and enzyme costs are barriers to economical recovery of fermentable sugars from lignocellulosic biomass (Himmel et al., 2007).

Improved starch-based ethanol production is an economically viable near-term technology to meet the increasing demand of renewable transportation fuel. In the US, most bioethanol currently comes from corn. However, problems associated with limited supply,
environmental concerns and input intensity of corn cultivation, as well as competition with food production and the rise in commodity prices due to its use as biofuel feedstock, makes it necessary to identify additional sugar feedstocks for ethanol production (Simpson et al., 2008). Sweetpotato (*Ipomoea batatas* L.) is an important crop in the southeastern US (USDA, 2008). It accumulates starch in storage roots and has several agronomical advantages, including low fertilizer requirements, high adaptability, tolerance to water stress, and the ability to grow on marginal lands (Woolfe, 1992). The potential of sweetpotato to become an alternative energy crop was recently reported; enzymatic hydrolysis followed by fermentation produced an estimated 703 gallons of ethanol per acre of sweetpotato, considerably higher than the estimated 404 gallons per acre for corn (Duvernay, 2008). Additionally, the fact that sweetpotato is able to grow and yield well in marginal land can minimize competition for land that is useful for food production and provide an alternative sugar feedstock in corn-deficient states (Duvernay, 2008).

Starch, the predominant carbon reserve in plants, is composed of amylose (15-25%), a linear polymer of α-1,4-linked residues, and amylopectin (75-85%), a polymer with α-1,6-linked branch points. These saccharides can be readily hydrolyzed to monomeric sugars by the synergistic action of glycosyl hydrolases with different specificities (Bertoldo and Antranikian, 2001). The industrial processing of starch involves a liquefaction step, where starch is gelatinized and solubilized by partial hydrolysis with a thermostable α-amylase, followed by a saccharification step, where monomeric sugars are produced by further enzymatic hydrolysis (Crabb and Mitchinson, 1997). Starch-degrading enzymes, such as α-amylase, glucoamylase and pullulanase have been added to sweetpotato starch improving starch hydrolysis and resulting in higher yields of fermentable sugars and ethanol production (Wang et al., 1998; Johnson et al., 2005; Duvernay, 2008).

The strategic use of hyperthermophilic enzymes to improve biocatalyst-based processes has been discussed (Bruins et al., 2001; Comfort et al., 2004; Unsworth et al., 2007). These enzymes are obtained from bacteria or archaea that thrive in extreme thermal environments, typically above 80°C. Their use in starch processing could facilitate higher
conversion temperatures, where solubility of starch is greater, as well as higher mass transfer rates of substrates and products. In addition, risks of bacterial contamination of the sugar mixture are minimized at high temperatures. α-Amylases (α-1,4 glucanohydrolase; EC 3.2.1.1) are endo-acting glucans that hydrolyze α-1,4 linkages in the starch polymer. Hyperthermophilic α-amylases have been identified in several hyperthermophiles, including Pyrococcus worsei, Thermococcus profundus, Pyrococcus furiosus, and Thermotoga maritima (Koch et al., 1991; Chung et al., 1995; Dong et al., 1997; Liebl et al., 1997). While their hydrolytic specificities are comparable to mesophilic and moderately thermophilic α-amylases, they are especially thermostable and thermoactive (Vieille and Zeikus, 2001). The feasibility of producing hyperthermophilic enzymes in mesophilic hosts, together with their lack of activity at ambient temperatures (Unsworth et al., 2007), raises the prospect for their direct production in transgenic plants, for applications such as starch bioconversion.

The production of thermostable and hyperthermophilic glycosidases in plants has been demonstrated for tobacco (Pen et al., 1992; Montalvo-Rodriguez et al., 2000), rice (Chiang et al., 2005), potato (Beaujean et al., 2000; Lin et al., 2008), and a tobacco model cell system (Santa-Maria et al., 2008). Here, an α-amylase gene from the hyperthermophilic bacterium Thermotoga maritima was cloned into the sweetpotato genome using Agrobacterium tumefaciens-mediated transformation. The resulting transgenic plants produced functional enzyme with no detectable activity below 40°C. The transgenic storage roots appeared normal. However, starch in the transgenic plant’s storage roots could be self-processed at 80°C, while the starch in WT roots remained intact. This result illustrates the potential of transgenic sweetpotato as a source of first generation liquid biofuel, and further supports the prospect of self-processing biomass to meet future energy needs.
Results

Sweetpotato transformation

Transgenic sweetpotato plants of cultivar ‘Jewel’ were generated using *A. tumefaciens* strain EHA105 (Hood et al., 1993) carrying the binary plasmids pBI95 and pBlamy2 (Figure 1). Plasmid pBI95 carries the gene *gfp7* for a modified, intron-bearing, red-shifted version of the GFP protein from *Aequorea victoria* (Mankin and Thompson, 2001). Plasmid pBlamy2 carries a modified (signal peptide removed) version of the AmyA gene from *Thermotoga maritima* that codes for a thermoactive α-amylase with optimal temperature for catalysis at 85-90°C (Santa-Maria et al., 2008). Both genes were put under the control of the CaMV 35S promoter in a pBIN20- derived vector that also carries the neomycin phosphotransferase gene (*nptII*) for plant kanamycin resistance (Hennegan and Danna, 1998).

Initially, transformation with the GFP cassette was performed to allow for non-destructive monitoring of transformed tissue throughout regeneration, and to facilitate the establishment of a transformation procedure. A month after infecting sweetpotato leaves (lamina with petiole), many transgenic calli developed along the surface of petioles under kanamycin selection. These were carefully separated from the surrounding tissue, and placed onto embryo induction medium with kanamycin (G24D; Table 1). While most calli exhibited GFP expression and were resistant to kanamycin, some areas necrotized and were separated in every subculture. After approximately 4 months, embryonic structures developed and were transferred to abscisic acid medium to induce embryo maturation (ABA; Table 1). Initially, the ABA medium did not contain kanamycin to facilitate embryo development. However, chimeric GFP expression was observed among different calli, and some calli did not exhibit green fluorescence at all. These calli, either silenced or untransformed, were discarded. After approximately 2 months in ABA medium, embryos matured and shoot-like structures
emerged from regenerating callus (Figure 2A). While only a few calli differentiated embryonic structures, even fewer ultimately produced transgenic plants (Santa-Maria, 2003). Calli that were differentiating further, were transferred to a zeatin medium without kanamycin for shoot formation (F9; Table 1). After approximately two months, shoots that initially had an unusual appearance (e.g. thickened) reverted to a normal phenotype (Figure 2B), and were transferred onto sweetpotato propagation medium. Putative transgenic lines that were resistant to kanamycin in their culture medium were constitutively expressing GFP, while untransformed shoots regenerated in the same way had no green fluorescence (Figure 2C-F). Twenty-five groups of transformants were obtained, however, Southern blot analysis of 18 individuals taken at random showed the same restriction pattern, indicating that only one independent ‘GFP’ transgenic line was available for analysis (Figure 3A).

A similar transformation procedure was followed to obtain transgenic sweetpotato lines with the Tma α-amylase gene. Given that the pBlamy2 construct did not contain a GFP gene to aid in the selection of transformed tissue, kanamycin at 40 mg/L was added to the ABA medium to prevent regeneration of untransformed events. The addition of kanamycin caused an evident stress to the embryos, but still allowed the recovery of putative transgenic lines. Three putative lines were recovered, and one line was confirmed by Southern blot hybridization and recombinant enzyme activity (Figure 3B).

In both cases, about 40 leaf explants (lamina with petiole) were infected with recombinant A. tumefaciens, generating over 200 independent kanamycin resistance calli, from which one independent transgenic event was verified after several months of somatic embryogenesis regeneration. A similar transformation strategy was assayed for two other high-starch, industrial-type, sweetpotato genotypes, DM01-158 and FTA-94; however, transgenic events were only obtained in the sweetpotato cultivar ‘Jewel’.
Protein purification and enzyme activity

Recombinant Tma α-amylase was produced in *E. coli* strain BL21 (pet21TMamyN26) (Santa-Maria et al., 2008), and was purified by a heat precipitation treatment followed by anion exchange chromatography in a Q-sepharose packed column. Fractions were tested for hyperthermophilic amylase activity, and a protein peak correlated with the recombinant enzyme. Protein purity was verified by denaturing SDS-PAGE and coomassie staining, where a single band of the predicted molecular weight (∼62kDa) was observed (Figure 4A). The specific activity of Tma α-amylase was estimated to be 825.1 ± 13.2 (U/ mg protein), measured under standard conditions using 3.4 μg/ml of purified protein.

Approximately 3.5 units of enzyme activity were obtained per g of leaf tissue (fresh weight) from transgenic sweetpotatoes. Assuming that the specific activity of the plant- and bacterial- made enzymes were comparable, the recombinant protein was estimated to be approximately 0.06% of total soluble protein in transgenic sweetpotato leaves. The sweetpotato-made enzyme had maximal activity at 90°C. Below 40°C, there was no significant increase in activity when compared to the WT or a transgenic line with the GFP construct (Figure 5).

The molecular mass and activity of recombinant Tma α-amylase from *E. coli* and sweetpotato were determined by substrate-SDS-PAGE, where potato starch (substrate) was embedded in the acrylamide matrix. Hyperthermophilic amylase activity, visualized as a clearing zone over dark background upon KI/I₂ staining, was observed in the heat-stable soluble protein fraction from transgenic ‘TM-amy’ sweetpotato and recombinant *E. coli*, but not in the corresponding fraction from WT sweetpotato (Figure 4B). The relative molecular weights were estimated by coomassie staining.
Starch hydrolysis in sweetpotato storage roots

Transgenic sweetpotato lines were transferred from in vitro culture to the greenhouse (20-26°C day/ 17-20°C night, 69% RH, no supplementary lighting). Sweetpotato lines carrying the GFP gene grew vigorously in the greenhouse, with no difference in yield from the WT (Figure 6 A,B). The transgenic ‘TM-amy’ sweetpotato line was initially smaller than the wild type, but a normal phenotype was recovered after a round of nodal cutting propagation (Figure 6C). Storage root formation in both transgenic lines was comparable to the wild type, and medium-sized storage roots were harvested approximately 5 months after introduction to the greenhouse (Figure 6 D-E).

To test starch self-processing in transgenic roots, 20 g of storage roots were homogenized with water (1:2 [w/v]) and were incubated overnight at 80°C. Maltodextrins produced by the enzymatic hydrolysis of root starch were visualized in a thin layer chromatography, using 8 µl of cleared samples. Transgenic sweetpotato storage roots carrying the Tma α-amylase gene readily hydrolyzed starch at 80°C, as well as wild type roots when mixed with vines from transgenic plants or with recombinant enzyme from E. coli (Figure 7). No enzymatic hydrolysis of starch occurred in the wild type sample under the same conditions. Addition of phosphate buffer instead of water to transgenic roots caused inhibition of the thermoactive enzyme (Figure 7), probably due to the removal of the available calcium (Sambrook and Russell, 2001).

Discussion

Sugars derived from starch have many applications as sugar syrups or organic acids, or alternatively as fermentable sugars for ethanol production (Crabb and Mitchinson, 1997). Currently, cornstarch is the primary feedstock for first generation biofuel production in the US (Hahn-Hägerdal et al., 2006), but other sources are being considered and encouraged
(Wilson et al., 2007; Simpson et al., 2008). In the industrial processing of starch to fermentable sugars, enzymatic hydrolysis by glycoside hydrolysins is carried out in two steps: liquefaction and saccharification. In liquefaction, high temperature treatment is applied for starch gelatinization and solubilization is done by partial hydrolysis with a thermostable and thermoactive α-amylase. Under optimal conditions, the cost of enzyme addition in the industrial processing of grain starch is about 3 cents per gallon of ethanol, based on 1.2 and 1.8 cents for the α-amylase and for the glucoamylase, respectively (personal communication, representative of Novozymes, Franklinton, NC). Producing starch-hydrolyzing enzymes directly in plants that contain starch offers a route for significant reduction in large-scale processing costs, and may also allow reduced processing times due to the intrinsic homogenization of enzyme in the starch mixture. The viability of this concept is demonstrated here for transgenic sweetpotato.

Transgenic sweetpotato plants were generated that produced functional hyperthermophilic α-amylase from *Thermotoga maritima* (Tma). The gelatinization or peak transition temperature of sweetpotato starches ranges between 78 and 83°C (Zhang and Oates, 1999). Therefore, in a transgenic approach, a highly thermostable and thermoactive enzyme is desirable, whose functional conformation would not be compromised through gelatinization and will have high conversion rates above 80°C. In a previous report, plant-made Tma α-amylase was demonstrated to be highly thermostable, with a half-life at 100°C of up to 19h (Santa-Maria et al., 2008). Here, the recombinant enzyme produced in sweetpotato leaves exhibited highest activity at 90°C, while no activity at ambient temperatures (< 40°C) was detected (Figure 5). These findings are consistent with previous reports for this protein (Liebl et al., 1997; Santa-Maria et al., 2008).

Starch in transgenic storage roots producing hyperthermophilic enzyme was readily hydrolyzed at 80°C. Processing of starch by recombinant thermostable glycosidases was previously reported in rice, producing an amylopullulanase from *Thermoanaerobacter ethanolicus* 39E (Chiang et al. 2005), and in potato, producing a chimeric α-amylase/glucose isomerase from *Bacillus stearothermophilus* and *Thermus thermophilus* (Beaujean et al.
2000) or a thermostable β-amylase from *Thermoanaerobacter thermosulfurigenes* (Lin et al., 2008). Additionally, the production of bulk enzyme was reported in tobacco, producing a *Bacillus licheniformis* α-amylase (Pen et al., 1992), or a β-glycosidase from *Sulfolobus sulfactarius* (Montalvo-Rodriguez et al. 2000). Besides the auto-hydrolysis of starch in transgenic sweetpotato roots, we also showed that vines from transgenic plants were a viable source of bulk enzyme for starch liquefaction. Recombinant α-amylase in transgenic leaves was estimated to be 0.06% of total soluble protein. While this protein level was enough to liquefy starch in transgenic storage roots, the economic viability of starch self-processing in transgenic roots as well as the use of transgenic vines as source of bulk enzyme will need to be tested at a larger scale, and compared to current processing methods. In any case, it is likely that increased yields of recombinant protein can be obtained by screening a larger number of transgenic lines, and/or by modification of the *amyN26* expression cassette (Streatfield, 2007).

Most α-amylases require Ca$^{2+}$ for their activity and stability (Vieille and Zeikus, 2001). The α-amylase from *T. maritima* used in this study binds Ca$^{2+}$, which helps stabilize the protein at high temperatures (Liebl et al., 1997; Santa-Maria et al., 2008). It was previously demonstrated that intrinsically available Ca$^{2+}$ in plant cells enhanced the thermostability of this enzyme (Santa-Maria et al., 2008). Furthermore, calcium addition was not necessary for prolonged starch liquefaction at 80°C in storage root homogenates. We also observed that using phosphate buffer instead of water reduced starch hydrolysis, possibly because the phosphate scavenges available divalent cations (Sambrook and Russell, 2001), leading to accelerated thermal inactivation of the recombinant protein.

The amylase expressing line grew and produced storage roots similarly to wild type and to the GFP-expressing control. This indicates that expression of the Tma α-amylase gene did not cause major changes in sweetpotato development. We noted, however, that even after rooting of nodal cuttings of the original amylase transformants, the leaves in the amylase expressing clones were lighter in color and had darker-green veins, compared to both wild type and the GFP transgenic control. Additional amylase lines will have to be produced and
evaluated in further research in order to determine whether this small phenotypic difference arose from somaclonal variation (James et al., 2007), or from an effect of expression of a foreign amylase. Possible transgene effects include interference with normal intracellular trafficking or competition with endogenous amylase in terms of binding to substrate. Since the foreign amylase does not have enzymatic activity at plant growth temperature, foreign enzyme activity is not expected to cause any phenotypic differences.

Previous studies on thermophilic and hyperthermophilic glycosidases in plants reported production of active enzyme without compromising normal plant metabolism and resulting phenotype, either because the enzymes had minimal activity at ambient temperatures (Beaujean et al., 2000; Montalvo-Rodriguez et al., 2000), or because they were targeted to a different cellular compartment, for example, the apoplast (Pen et al., 1992). Here, the gene encoding a hyperthermophilic enzyme lacking a signal peptide was expressed, and was likely localized in the cytosol. These two factors likely account for no observed deleterious effect on the host plant. However, two former reports indicated phenotype alteration in transgenic plants. In one case, a thermostable amyllopullulanase was produced in rice amyloplasts (Chaing et al. 2005), while in the other a thermostable β-amylase was targeted to various cellular compartments, including amyloplasts in potato tubers (Lin et al., 2008). In both of these cases, the enzymes had significant activity at ambient temperatures and were produced in proximity to the starch granules, presumably affecting proper starch and sugar metabolism. They also reported higher yields of recombinant protein, which in the case of recombinant amyllopullulanase being accumulated in rice seeds, was estimated to be 5% of total soluble protein in the highest expressing lines (Chiang et al., 2005). In Lin et al (2008), a 10-fold increase in β-amylase activity in field-grown tubers was reported for the highest expressing lines compared to the untransformed controls. The difference in the amount of recombinant protein produced in the transgenic plants could also be a factor for phenotype alteration.

Sweetpotato is generally considered a recalcitrant species for regeneration and transformation (Newell et al., 1995; Song et al., 2004; Yu et al., 2007). Although methods to
generate transgenic sweetpotato plants have been reported in recent years, transformation efficiencies are generally low, and success is limited to a small number of varieties (Gama et al., 1996; Cipriani et al., 1998; Otani et al., 2001; Wakita et al., 2001; Yi et al., 2007; Yu et al., 2007). Genotype-dependence for the effectiveness of regeneration and transformation in sweetpotato has also been previously reported (Sihachakr et al., 1997; Cipriani et al., 1998, Yu et al., 2007). In this work, transgenic sweetpotato plants from the cultivar ‘Jewel’ were obtained by A. tumefaciens-mediated transformation and somatic embryogenesis regeneration. While we were able to repeat the transformation procedure, the efficiencies were still low and higher explant numbers will be required to increase the number of transgenic lines obtained per transformation experiment. A similar transformation and regeneration procedure was assayed in two other high-starch sweetpotato varieties, obtaining transgenic GFP expressing calli, but which failed to regenerate into plantlets under the present regime (see Appendix A). The addition of kanamycin in the abscisic acid medium to induce embryo maturation and further differentiation had a notorious detrimental effect in regeneration efficiency. However, the lack of kanamycin selection at this stage without the aid of a reporter gene resulted in high frequency regeneration of untransformed lines. Inherent kanamycin resistance in sweetpotato callus has been previously observed (Prakash and Varadarajan, 1992). Alternatively, the use of hygromycin has been shown to exert a much stronger selective pressure than kanamycin in regenerating sweetpotato tissues (Song et al., 2004). Therefore, kanamycin concentrations at different regeneration stages, as well as the use of alternative selective agents should be further evaluated.

In conclusion, the results presented herein demonstrate the feasibility of genetically engineering sweetpotato for starch self-processing at high temperatures. Sweetpotato is a major starch-producing crop worldwide (Katayama et al., 2004), and has several agronomical advantages, including the ability to grow in marginal land. The streamlining of the starch processing through this transgenic approach could result in a reduction of the processing costs for starch to fermentable sugars, as well as the potential of sweetpotato to become a
major energy crop in the southeast US. Plans are in place to do field-testing to further advance the feasibility of using transgenic sweetpotato lines for starch self-processing.

**Experimental procedures**

Reagents used for tissue culture and plant transformation were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

**Plant transformation vectors**

Standard cloning techniques (Sambrook and Russell, 2001) were used to create plant transformation vectors ‘pBI95’ and ‘pBIAmy2’, described in Figure 1. The gene *gfp7* in pBI95 codes for a soluble- modified, red-shifted GFP protein with ER targeting and retention signal, and an intron to avoid production in bacterial cells (Mankin and Thompson, 2001). The gene *amyN26* in pBIamy2 is a modified version (signal peptide removed) of the *amyA* gene from *Thermotoga maritima* encoding a hyperthermophilic α-amylase (Santa-Maria et al., 2008). The cloning procedure of *amyN26* into plasmid pet21TMamyN26 for recombinant expression in *E. coli* strain BL21 (DE3) was described previously (Santa-Maria et al., 2008). For expression in plant cells, both *gfp7* and *amyN26* were placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (nos) terminator in a pBIN20- derived vector (Hennegan and Danna, 1998), which also carries the *nptII* gene for plant kanamycin resistance. Given its bacterial origin, the compatibility of *amyN26* ORF with *Ipomoea batatas* codon usage was verified using the ‘countcodon’ program and the ‘codon usage database’ at [http://www.kazusa.or.jp/codon/](http://www.kazusa.or.jp/codon/). No major incompatibilities were found. Binary plasmids were introduced into *Agrobacterium tumefaciens* strain ‘EHA105’ (Hood et al., 1993) by electroporation in a BTX Electro Cell Manipulator™ 600.
Sweetpotato transformation

*In vitro* sweetpotato plants were maintained in magenta boxes with SP propagation medium (MS with minimal organics (cat. no. M6899, Sigma-Aldrich), sucrose 30 g/L, phytagel 3 g/L, pH 5.8), and were kept in an incubation room at 25-27°C, 16 h light photoperiod, 3000 lux, and 70% RH. The composition of all media used for transformation and regeneration is summarized in Table 1. The MS basal medium was obtained from Sigma-Aldrich (cat. no. M5519).

The transformation procedure followed was modified from Cipriani et al. (1998) and Santa-Maria (2003). An isolated colony of *A. tumefaciens* carrying the binary plasmid was grown in 4 ml LB medium with antibiotics for 30 h at 28°C and agitated at 200 rpm. 30 ml of LB medium were inoculated with 100 µl of grown broth in 250 ml flasks, and incubated under the same conditions until late log phase (OD<sub>600</sub> 0.6-0.8) (Peña, 2005). Cells were collected by centrifugation (10 min, 3000 g, and 4°C), resuspended in an equal volume of bacterial infection medium (BIM; Table 1), and incubated for 2 additional hours until infection. About 40 leaf explants (lamina with petiole) were taken from the upper half of 5 week-old *in vitro* grown plants, and were put in explant suspension medium (ESM; Table 1) until infection. For infection, explants were placed in 30 ml BIM with the bacteria, tips of lamina were cut, and small wounds were carefully introduced along the petioles. Explants were left to stand in the bacterial suspension for 2 h in the dark, and were then blotted onto sterile filter paper and placed on semi-solid co-culture medium (SCM; Table 1). Co-culture was carried out in the dark for 2 days at 26°C. Regeneration of transformed plants was performed in an incubator room at 25-27°C, 16 h light photoperiod, 2500 lux, and 70% RH. Upon co-culture, explants were transferred to F15 medium with antibiotics (kanamycin 50 mg/L, cefotaxime 200 mg/L; Table 1), to induce development of transformed cell clusters. After 5 days in F15 medium, explants were transferred to zeatin medium (F9-Km; Table 1) to promote the formation of transformed calli along the petioles and leaves. After 3 weeks, over 200 transgenic calli that formed along the petioles’ surface were excised under a dissecting
scope and placed onto embryo induction medium with kanamycin (G24D; Table 1). Transgenic calli were maintained in G24D medium until differentiation into embryo-like structures was observed (4 to 5 months). At that point, somatic embryos were transferred to embryo maturation medium (ABA; Table 1), which initially did not contain kanamycin, but kanamycin 40mg/L was added in subsequent transformation experiments due to the lack of a reporter gene. In ABA medium, embryos elongated and differentiated into shoot-like structures (Figure 2A). Mature embryos were then transferred to zeatin medium without kanamycin (F9; Table1) and propagated once every 4 weeks. In F9 medium, embryos became shoots and eventually reverted to a normal phenotype (Figure 2B). Transgenic shoots were transferred to SP propagation medium and maintained as described above.

Southern blot analysis

Genomic DNA was purified by a cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991) using 0.5 g of leaf tissue. Plant tissue was pulverized with a mortar and pestle in liquid nitrogen, transferred to 2 ml centrifuge tubes containing 700 µl CTAB buffer (0.2 M Tris-HCl pH8, 0.04 M EDTA pH8, 2.8 M NaCl, 0.11 M CTAB), and incubated 20 min at 65°C. Then, 900 µl of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly. The DNA containing fraction was separated by centrifugation (5 min, 13000 rpm), with the upper fraction being carefully transferred to a new centrifuge tube. DNA was precipitated with 0.6 volume of ice-cold isopropanol and the DNA pellet was collected by centrifugation (5 min, 13000 rpm). DNA was washed once with 500 µl of 70% ethanol and pellets were dried completely at room temperature overnight. DNA was resuspended in 100 µl H2O with RNAse A (0.01 mg/ml). The quality and quantity of genomic DNA was verified in a Nanodrop™ spectrophotometer and agarose gel electrophoresis (Sambrook and Russell, 2001).

For southern blot hybridization, 60 µg of genomic DNA was completely digested with Hind III or Eco RI (NEB, Ipswish, MA, US). Digested genomic DNA was loaded onto a
0.8% agarose gel and separated at low voltage overnight. Dioxigenin- labeled DNA molecular marker II (Roche, Mannheim, Germany) was loaded next to samples, and 1kb DNA ladder (NEB) was loaded in the outermost lane. Southern transfer of DNA to a Hybond\textsuperscript{TM}-N\textsuperscript{+} membrane (Amersham Biosciences, Piscataway, NJ) was performed by downward capillary transfer according to established protocols (Sambrook and Russell, 2001). Depurination of DNA with 0.25 N HCl was necessary for a complete transfer. The capillary transfer buffer was 10X SSC. DNA was cross-linked to nylon membrane in a UV Stratalinker 1800 (Stratagene, La Jolla, CA, US) according to the manufacturer’s specifications. Probes homologous to genes \textit{gfp7} or \textit{amyN26} were synthesized by PCR from plasmids pBI95 or pBIamy2, respectively, (Figure 1) using PCR DIG Probe synthesis kit (Roche). The primers ‘#5GFP fw38’ (5’- AAGAACTTTTCACTGGACTTGTCC -3’) and ‘#5GFP Rv699’ (5’- CAGCTGTTACAAACTCAAGAAGGA -3’) amplified \textit{gfp7} CDS, generating a 666 bp probe, and the primers ‘amyFw4’ (5’- ACGACAGAGACGGGAACGGAG -3’) and ‘amyRv6’ (5’- TCCGCTGAACACCTCTCCCAC -3’) amplified \textit{amyN26} CDS, generating a 650 bp probe. Hybridization and detection of hybridized probe was performed using DIG luminescent Detection Kit (Roche), according to the manufacturer’s specifications. Chemioluminescence signal from the hybridized probe and marker was detected using a Kodak Biomax Light film screen (purchased from Sigma-Aldrich).

Protein purification

Recombinant Tma \(\alpha\)-amylase was obtained from \textit{E. coli} BL21 (pet21TMamyN26) as previously described (Santa-Maria et al., 2008), using a heat treatment (25 min, 80°C) followed by centrifugation (19000 g, 15 min). The heat- soluble protein fraction was further separated by fast protein liquid chromatography (FPLC) in a Biologic DuoFlow System (BioRad, Hercules, CA) using a Q-Sepharose packed column (GE Healthcare, Piscataway, NJ) coupled to a UV\textsubscript{280} detector for continuous scanning of the flowthrough. The flow rate
was 1ml/min. The mobile phase was as follows: after sample injection (6 ml), 30 ml isocratic flow was passed through the column. The isocratic flow consisted of 95% buffer A (50mM Tris pH8) and 5% buffer B (50mM Tris pH8, 2M NaCl), and was followed by 2 linear gradients of 75ml each. The first linear gradient consisted of 95% buffer A/ 5% buffer B to 50% buffer A/ 50% buffer B, and the second linear gradient consisted of 50% buffer A/ 50% buffer B to 0% buffer A/ 100% buffer B. 1 ml fractions were collected and assayed for hyperthermophilic α-amylase activity under our standard assay conditions. Protein bands were visualized by coomassie staining (Bio-safe coomassie stain; BioRad) after denaturing sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% acrylamide gel (BioRad).

Extracts from wild type and transgenic sweetpotato plants were prepared following guidelines described by Gegenheimer (1990). Leaves were rinsed with tap water and submerged in 0.1% sodium hypochlorite (1:50 commercial bleach solution) for 15 s, and then were rinsed thoroughly with distilled water and patted dry. Plant tissue was pulverized with a mortar and pestle in liquid nitrogen and transferred immediately to cold extraction buffer [50mM Tris-HCl pH 8, 0.1M KCl, 5% glycerol, 1.5% PVPP, 5% DMSO, 5mM DTT, and protease inhibitor cocktail (cat. no. 11873580001, Roche)], using 3ml of buffer per g of tissue (fresh weight). Cell debris, as well as phenolics and alkaloids complexed with polyvinylpolypyrrolidone (PVPP), were removed by low centrifugation (10 min, 1500 g), and the supernatant was passed through a Berkshire polx® 1200 wiper. The resulting extract was separated in two batches, one of which was exposed to a heat precipitation treatment (25 min at 80°C followed by centrifugation at 19000 g, 15 min, and 4°C) whereas the other was immediately centrifuged to obtain total soluble proteins. Total protein in plant and E. coli fractions was estimated according to the ‘Protein Assay’ (BioRad) that is based on the method by Bradford (Bradford, 1976), in microtiter plates, using bovine serum albumin (Sigma-Aldrich) as a standard.
Enzyme activity assays

Starch hydrolyzing activity was determined by measuring the enzymatic release of reducing sugars from starch (Bernfeld, 1955). One unit of enzyme activity (U) was defined as the amount of protein producing 1 µmol of reducing ends (measured as maltose equivalents) per minute. Specific enzyme activity was defined as the enzymatic units per mg of protein (U/mg). The accumulation of reducing ends in the enzymatic reaction was determined by the dinitrosalicylic acid (DNS) assay (Chaplin and Kennedy, 1994) using a maltose standard curve. All enzymatic reactions were incubated 10 min, using 0.5% substrate (soluble potato starch, cat. no. S5651, Sigma-Aldrich) in a total volume of 500 µl. Our standard-assay conditions were 50 mM phosphate buffer pH 7 and 85°C, using 50 µl of heat purified protein extracts as the enzyme source. Reaction controls (a sample with only substrate and a sample with only enzyme) were run in parallel, and each reaction was replicated 3 times for each assay. The data for enzyme activity reported is the average of the 3 technical and 2 to 4 biological replications. Temperature activation of recombinant enzyme from sweetpotato was measured by 10 min incubations at different temperatures in a water bath, and otherwise standard assay conditions.

Substrate-SDS-PAGE

Heat soluble protein samples from wild type SP (10 µg), transgenic ‘TM-amy’ SP (10 µg), and three ten-fold dilutions of E. coli 1x= 0.093 µg were mixed with sample buffer (2x: 0.125 M Tris-HCl pH 6.8, 20% glycerol, 0.04% bromophenol blue, and 2% SDS) and loaded into a 12% acrylamide gel, polymerized in the presence of soluble potato starch (cat. no. S5651, Sigma-Aldrich) as previously described (Martínez et al., 2000). Electrophoresis was performed at 4°C, and was otherwise according to previously described conditions (Martínez et al., 2000). Following electrophoresis, the gel was rinsed thoroughly with distilled water, and placed in phosphate buffer (pH7). In-gel starch hydrolysis by recombinant amylase was
performed at 85°C for 1.5 h. After incubation, the gel was rinsed with distilled water, fixed with 12% trichloroacetic acid (TCA), and stained with KI/I₂ solution (BioRad). Regions of amylase activity appeared as cleared bands over a dark blue background (Figure 4B). The gel was subsequently rinsed with distilled water and stained overnight with bio-safe coomassie stain (BioRad). After rinsing with water, KI/I₂ staining gradually dissipated and coomassie-stained protein bands became visible.

Hydrolysis of starch in storage roots

Storage roots from transgenic ‘TMamy’ and wild type sweetpotatoes were harvested from 5-month old greenhouse grown plants. Greenhouse conditions were 20-26°C day/17-20°C night and 69% relative humidity. To test the hydrolysis of starch in the storage roots, 20 g (fresh weight) of storage root was mixed with 40 ml water and 50 µl NaOH 1N, in a blender. Homogenates were placed in 250 ml flasks and incubated in an oil bath at 80°C overnight (approximately 14 h). In addition to the negative control (WT storage roots with water), 20 and 40 g (fresh weight) of transgenic vines were pulverized with a mortar and pestle in liquid nitrogen and added to 2 wild type storage root samples. Also, approximately 250 units of E. coli- produced enzyme was added to a wild type storage root sample to serve as positive control for starch hydrolysis. Homogenized samples were kept at 4°C until all samples were ready, to avoid sample-to-sample variability due to endogenous activities at room temperature. After the high temperature incubation, samples were transferred to centrifuge tubes and debris was separated by centrifugation (10000g, 15 min). The products of hydrolysis were visualized by thin layer chromatography (TLC) in silica gel plates (High performance TLC plates; Whatman, Florham, NJ) with n-butanol-ethanol-water (5: 3: 2 by volume) as the mobile phase. A total of 8 µl per sample were loaded, along with 12 µl of the wild type sample (negative control). The maltooligosaccharides standard consisted of a 0.5% (w/v) mix of maltose (G2) to maltoheptose (G7). Carbohydrate bands were visualized by
dipping the TLC plate in ethanol with sulfuric acid (10% [v/v]) followed by heating it briefly onto a hot plate (Lee et al., 2006).

Acknowledgements

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References


Table 1. Culture media used in sweetpotato transformation and regeneration

<table>
<thead>
<tr>
<th>Initials</th>
<th>Stage</th>
<th>Basic composition ¹</th>
<th>Hormones and antibiotics (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERM</td>
<td>Pre-infection</td>
<td>MS with minimal organics, sucrose (30 g/L), MES (0.5 g/L) – pH 5.5</td>
<td>NAA (0.1), GA₃ (0.01), BAP (1.0)</td>
</tr>
<tr>
<td>BIM</td>
<td>Infection</td>
<td>MS with minimal organics, sucrose (30 g/L), acetosyringone (20 mg/L) – pH 5.5</td>
<td>--</td>
</tr>
<tr>
<td>SCM</td>
<td>Co-culture</td>
<td>MS with minimal organics, glucose (20 g/L), MES (0.5 g/L), Phytigel (3 g/L) – pH 5.5</td>
<td>NAA (0.1), GA₃ (0.01), BAP (1.0)</td>
</tr>
<tr>
<td>F15</td>
<td>Promotion of transformed calli I</td>
<td>MS basal medium, sucrose (30 g/L), Phytigel (3 g/L) – pH 5.7-5.8</td>
<td>Zeatin (0.2), 2,4-D (0.05), kanamycin (50.0), cefotaxime (200.0)</td>
</tr>
<tr>
<td>F9-Km</td>
<td>Promotion of transformed calli II</td>
<td>MS basal medium, sucrose (30 g/L), Phytigel (3 g/L) – pH 5.7-5.8</td>
<td>Zeatin (0.2), kanamycin (50.0), cefotaxime (200.0)</td>
</tr>
<tr>
<td>G24D</td>
<td>Embryo development</td>
<td>MS basal medium, sucrose (30 g/L), Phytigel (3 g/L) – pH 5.7-5.8</td>
<td>GA₃ (0.1), 2,4-D (0.05), kanamycin (50.0), cefotaxime (200.0)</td>
</tr>
<tr>
<td>ABA</td>
<td>Embryo maturation</td>
<td>MS basal medium, sucrose (30 g/L), Phytigel (3 g/L) – pH 5.7-5.8</td>
<td>Abscisic acid (1.0), cefotaxime (200)</td>
</tr>
<tr>
<td>F9</td>
<td>Shoot induction</td>
<td>MS basal medium (SIGMA), sucrose (30 g/L), Phytigel (3 g/L) – pH 5.7-5.8</td>
<td>Zeatin (0.2), cefotaxime (200.0)</td>
</tr>
</tbody>
</table>
Figure 1. Plasmid vectors used in sweetpotato transformation

RB, right border of T-DNA; nosP, nopaline synthase promoter; nptII, neomycin phosphotransferase gene for plant kanamycin resistance; nosT, nopaline synthase 3’UTR; 35S, cauliflower mosaic virus 35S promoter; gfp7, soluble-modified, red-shifted green fluorescence protein (GFP) from Aequorea Victoria; amyN26, N-truncated α-amylase gene from Thermotoga maritima; LB, left border of T-DNA; H, Hind III; B, Bam HI; S, Sac I; E, Eco RI; X, Xba I; Probe, used for southern blot hybridization.
Figure 2. **Transgenic sweetpotato lines obtained by somatic embryogenesis regeneration**

**A.** Regenerating tissue on abscisic acid medium. **B.** Transgenic shoots obtained through somatic embryogenesis, developing on zeatin medium. **C, D.** Transgenic sweetpotato line constitutively expressing GFP protein, viewed under UV (C) or bright field illumination (D). **E, F.** An untransformed line showed no signal under UV illumination (E); the same area is shown with bright field illumination (F).
Figure 3. Southern blot hybridization of transgenic sweetpotato lines

Genomic DNA was hybridized with 666pb and 650bp DNA probes homologous to transgenes gfp7 (A) and amyN26 (B), respectively. A. Transgenic line with the GFP gene. Lane M, DIG-labeled markers; lane 1, wild type genomic DNA digested with Hind III; lanes 6,7, transgenic genomic DNA digested with Eco RI (6) and Hind III (7). B. Transgenic line with the Tma α-amylase gene. Lane M, DIG-labeled markers; lane 1; transgenic genomic DNA digested with Hind III; lane 2, wild type genomic DNA digested with Hind III. The stable insertion of both transgenes in the sweetpotato genome was verified, and at least two copies of each transgene were inserted in each case.
Figure 4. Activity gel demonstrated the production of active Tma α-amylase in *E. coli* and transgenic sweetpotato

**A.** Coomassie stain of SDS-PAGE of purified Tma-α-amylase protein from *E. coli*. Lanes 1-3, 10-fold serial dilution of 1x = 31 \(\mu\)g purified protein; M, mass molecular marker. **B.** Substrate-SDS-PAGE of recombinant amylase; Lane 1, heat-stable soluble proteins from wild type sweetpotato (10 \(\mu\)g); B, blank lane; Lane 2, heat-stable soluble proteins from transgenic ‘TM-amy’sweetpotato (10 \(\mu\)g); M, molecular weight marker; Lanes 3-5, 10-fold serial dilution of 1x = 0.093 \(\mu\)g (lane 5) of purified Tma-α-amylase protein from *E. coli*. 
Figure 5. Temperature activation of recombinant Tma α-amylase from sweetpotato

Starch hydrolyzing activity was measured as the enzymatic release of reducing ends (maltose equivalents) by the dinitrosalicylic acid (DNS) method in 10 min reactions, which were carried out at different temperatures using 0.3 mg/ml heat-soluble protein extracts. Tma α-amylase from transgenic sweetpotato (SP TMamy) showed peak activity at 90°C. In contrast, two controls lines, wild type sweetpotato (SP WT) and transgenic sweet potato with the GFP gene (SP GFP) showed much lower activity without a high temperature peak. The low, background activity observed in the control samples might result from endogenous starch-hydrolyses that resisted heat-denaturation. No difference in activity between the transgenic ‘TMamy’ line and control lines was observed below 40°C. Error bars represent standard error (n=4).
Figure 6. Wild type and transgenic sweetpotato plants grew normally in the greenhouse. A,D, wild type; B,E, transgenic sweetpotato line expressing green fluorescence protein (GFP) from *Aequorea Victoria*. C,D, transgenic sweetpotato line producing functional hyperthermophilic α-amylase from *Thermotoga maritima*. Greenhouse plants were approximately the same age, with the GFP-line being the oldest. Storage roots were harvested 5 months after re-potting transgenic and wild type plants. Both storage roots and vines from transgenic plants appear normal.
Figure 7. Starch self-processing in transgenic storage roots from sweetpotato

Products of starch hydrolysis were visualized in a thin layer chromatography (TLC). Storage roots from wild type and transgenic sweetpotato producing hyperthermophilic $\alpha$-amylase were homogenized with water 1:2 (w/v), and incubated at 80°C. Samples were cleared by centrifugation and 8 $\mu$l of hydrolyzed samples and 12 $\mu$l of wild type sample were loaded to silica plates. M; maltooligosaccharides mix standard (0.5% [w/v] each) consisting in maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7); 1, wild type storage roots; 2, 3, wild type storage roots with 10 g (lane 2) and 20 g (lane 3) of pulverized leaves from transgenic ‘TMamy’ sweetpotato; 4, transgenic sweetpotato storage roots; 5, wild type storage roots with 250 units of recombinant enzyme from *E. coli*; 6, 7, TLC replicate were 6, transgenic sweetpotato storage roots with water, and 7, transgenic storage roots with phosphate buffer. Starch was readily hydrolyzed at 80°C in the transgenic storage roots, and in the wild type storage roots when either bulk enzyme from transgenic vines or purified protein from *E. coli* were added.
Chapter IV

Fast shoot regeneration in industrial ‘high starch’ sweetpotato (*Ipomoea batatas* L.) genotypes

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Abstract

Sweetpotato (*Ipomoea batatas* L.) is an important crop in North Carolina with annual production of 0.33 million tons, accounting for 37% of total US supply (USDA, 2008). To target industrial use, novel high starch, industrial-type, varieties that contain more than 30% dry matter were developed by conventional breeding methods. *In vitro* cultures from selected genotypes were established using meristem culture. To establish regeneration procedures that could be coupled with transformation experiments, conditions for the induction of fast shoot-organogenesis in leaf explants were compared using varying concentrations of the auxins ‘NAA’, ‘IAA’, ‘2,4-D’, and ‘4-FA’ either alone or in combination with zeatin riboside. Regeneration efficiencies, defined as the number of explants developing shoots out of the total number tested, were as high as 57% for the best genotypes, with a significant genotype-dependent response observed in all the hormone regimes evaluated. In all treatments, shoot regeneration was observed within 2 months. Our results led to optimized *in vitro* regeneration
protocols for the high-starch SP genotypes ‘DM01-158’, ‘FTA94’, ‘FT489’, and ‘PDMP4’ that are rapid and reliable.

Abbreviations:
BAP- 6-benzylaminopurine; IAA- indole-3-acetic acid; NAA- naphthaleneacetic acid; SP - sweetpotato; 2,4-D- 2,4-dichlorophenoxyacetic acid; 4-FA- 4-fluorophenoxyacetic acid.

Introduction

Sweetpotato is an important crop worldwide, with an estimated 126 million metric tons produced annually (FAOSTAT, 2008). Sweetpotato is primarily produced in China, which accounts for 80.9% of the world production, but also in Africa and the Americas. Due to its ease of cultivation, low fertilizer requirements, drought tolerance and high adaptability, sweetpotato is considered a security crop and a major staple food crop in subsistence and rural economies. Besides its direct use as table and feed stock, sweetpotato is also a candidate for the production of renewable plant products such as ethanol, high-grade starches, stable natural dyes (purple dye), and vitamin precursors (beta-carotene for vitamin A) (Woolfe, 1992). About 80% of the dry matter in sweetpotato storage roots consist of carbohydrates, which are principally type C starches that are quite susceptible to α-amylase digestion (Shin et al., 2005). The high percent of fermentable biomass in the sweetpotato roots makes it a potential biofuel feedstock and an attractive alternative to corn in the southeastern U. S. (Wilson et al., 2007).

Biotechnology offers the possibility of expanding and optimizing the use of crops by means of genetic engineering. However, in order to develop new transgenic crops, robust transformation and regeneration methods must be in place. Sweetpotato has long been considered a recalcitrant species for plant regeneration (Sihachakr et al., 1997). Nevertheless, a variety of methods for in vitro regeneration of diverse sweetpotato genotypes have been
published within the last 30 years (Dessai et al., 1995; Liu and Cantliffe, 1984; Liu et al., 2001; Zheng et al., 1996). Different tissues have been used as explants, which undergo either direct shoot organogenesis or embryogenesis. Shoot tips were preferred for embryogenic procedures (Cavalcante et al., 1994; Padmanabhan et al., 1998) but leaves, petioles, roots and stems have also been used (Cheng and Yeh, 2003; Gong et al., 2005). Alternatively, fast shoot-organogenesis has been obtained from leaf explants (Dessai et al., 1995; Garcia et al., 1999; Gosukonda et al., 1995). Besides tissue regeneration procedures, methods to produce transgenic sweetpotato plants have also been reported. Strably transformed plants have been obtained by embryogenic regeneration from callus obtained from apical meristems (Gama et al., 1996; Otani et al., 2001), somatic embryogenesis regeneration from petioles and stem segments (Cipriani et al., 1999; Song et al., 2004), and adventitious shoot regeneration from leaf explants (Moran et al., 1998; Garcia et al., 2000; Luo et al., 2006).

One of the most problematic aspects of developing transgenic sweetpotatoes is that novel or modified in vitro regeneration procedures must be developed for each desirable genotype, because of the significant variability in the response to hormone combinations. For instance, Dessai et al. (1995) found significant differences in the regeneration frequency among 27 genotypes from a wide geographical distribution when they were put under a 2,4-D (0.2 mg l⁻¹) and zeatin (0.2 mg l⁻¹) regime to induce shoot organogenesis. Among these 27 genotypes, eighteen exhibited shoot regeneration with frequencies ranging from 10% to 83.3%, and nine were recalcitrant to regeneration. Variation among the genotypes was found both in shoot regeneration ability, shoot number, and the time required for regeneration. Similarly, Sihachakr et al. (1997) found differences in the response of 10 sweetpotato cultivars to an auxin treatment (10 µM 2,4-D) to induce embryogenesis. Frequencies ranged from no response at all to 17% when using lateral buds as explants. Many other observations of genotype dependence for in vitro regeneration in sweetpotato have been reported (Al-Mazrooei et al., 1997; Thinh et al., 1997).

In this study, we optimized hormone regimes to induce fast shoot regeneration in novel, conventionally bred, high-starch sweetpotato genotypes. Optimized regimes promoted
shoot regeneration of 2 to 4 shoots per explant within 6 to 8 weeks from initiation of the shoot induction treatment. Regeneration frequencies were between 40 and 57% for the most responsive genotypes, which are comparable to previously reported frequencies for SP shoot organogenesis (Dessai et al., 1995; Gong et al., 2005). The methods for in vitro regeneration developed in this work represent an initial step towards developing an efficient transformation strategy for these promising high-starch sweetpotato varieties.

Materials and methods

Reagents used for tissue culture were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Plant material

Industrial-type sweetpotato genotypes (ISPs) ‘BM85-42’, ‘DM01-074’, ‘DM01-158’, ‘FTA-94’, ‘FT4-89’, ‘PDM P4’; the cultivar ‘Suwon’, and the table-stock variety ‘Jewel’ were used in this study. The ISP-genotypes were developed by the ‘Sweetpotato Breeding and Genetics Program’ at North Carolina State University (NCSU), they exhibit higher dry matter and starch content than their tabletop counterparts, and are adapted to the Southeast U.S. All plants brought from the field were kept in the greenhouse (27°C to 37°C night/day, full sun, and 70-100% RH) in isolated screen cages with regular pesticide spraying.

In vitro culture initiation

Stem segments containing the shoot apex and 2 to 3 lateral buds were collected from greenhouse-grown plants. All the branches and leaves were cutoff and the stems were surface-sterilized by submerging them for 15 min in a 15% (v/v) commercial bleach (6%
sodium hypochlorite) solution with 1 to 2 drops of Tween 20 (Sigma-Aldrich). The stems were then rinsed twice with sterile distilled water. Apical meristems were excised with the aid of a Leica dissecting microscope and placed onto meristem culture medium (MCM) in 25mm diameter glass culture tubes. MCM composition was Murashige and Skoog (MS) with minimal organics (cat. no. M6899, Sigma-Aldrich), sucrose (30 g l\(^{-1}\)), myo-inositol (0.1 g l\(^{-1}\)), BAP (0.3 mg l\(^{-1}\)), NAA (0.03 mg l\(^{-1}\)), Phytagel (3 g l\(^{-1}\)), with pH adjusted to 5.7 (Love and Rhodes, 1985). Meristem-derived plantlets were propagated in magenta boxes containing SP propagation medium, which contains MS with minimal organics, sucrose (3 g l\(^{-1}\)), ascorbic acid (0.2 g l\(^{-1}\)), L-arginine (0.1 g l\(^{-1}\)), putrescine (0.02 g l\(^{-1}\)), gibberelic acid (0.01 mg l\(^{-1}\)), calcium panthotenate (2 mg l\(^{-1}\)), Phytagel (3 g l\(^{-1}\)), with pH adjusted to 5.7 (Cipriani et al., 1999). \textit{In vitro} sweetpotato plants were incubated at 26°C, 16/8h (light/dark) photoperiod at a fluorescent light intensity of 3000 lux.

To determine if the tissue was virus-free, meristem-derived plants, or ‘mericlones’, were transferred to the greenhouse in 4-inch plastic pots filled with moist Metromix® soil. Stem segments containing 2 lateral buds (scion) were cut and grafted onto indicator plants (rootstock). The rootstock included either \textit{Ipomoea nil} for geminivirus (e.g. \textit{Sweet Potato Leaf Curl Virus}), or \textit{Ipomoea setosa} for potyvirus and crinivirus. Three replicates per pot and one repetition were done for each indicator plant (rootstock) and mericlon (scion) combination. Symptoms in the indicator plant were recorded at 4 and 8 weeks from grafting. \textit{In vitro} cultures corresponding to asymptomatic scions were then used in the regeneration studies.

\textit{In vitro} shoot regeneration studies

Leaves (lamina with petioles of 0.5-1.5 cm) from the first third of 4 to 6 week old \textit{in vitro} grown plants were used as explants, as recommended by Dessai et al. (1995). The regeneration medium contained MS with minimal organics (Sigma-Aldrich), sucrose (30 g l\(^{-1}\)), phytagel (3 g l\(^{-1}\)), with pH adjusted to 5.7. Different types of growth regulators at various
concentrations were added as indicated in any particular treatment. The plants were regenerated in a growth room at 26°C, 16/8h (light/dark) photoperiod, and 2500 lux. Initially, all the genotypes were grown on medium containing IAA (0.5 mg l⁻¹). The IAA treatment was then repeated using different concentrations (0.2, 0.5, 0.8, and 1.5 mg l⁻¹) for DM01-158, the ISP genotype that had the highest regeneration frequency in the first experiment. A subset of genotypes was then tested on the auxins IAA (0.5 mg l⁻¹) and NAA (0.5 mg l⁻¹), and the cytokinin BAP (0.5 mg l⁻¹) for comparison (Table 2). The NAA treatment was then repeated at two concentrations (0.3 and 0.7 mg l⁻¹) for FTA-94, the only ISP genotype showing shoot regeneration with this auxin. A second strategy was also tested in which all the genotypes were grown using a two-stage procedure consisting of an initial exposure to an auxin, either 2,4-D (0.2 mg l⁻¹) or 4-FA (0.2 mg l⁻¹), which was followed by 4 to 5 weeks in zeatin-riboside at three different concentrations (0.05, 0.2, and 0.4 mg l⁻¹). A similar two-stage procedure, consisting of 4-FA (0.1 and 0.2 mg l⁻¹) followed by zeatin (0.2 mg l⁻¹) was repeated for DM01-158, which had one of the highest regeneration frequencies. Finally, a treatment consisting of 4-FA (0.05 mg l⁻¹) in combination with three zeatin concentrations (0.2, 0.4, and 0.6 mg l⁻¹) was tested for DM01-158, one of the most responsive ISP genotypes.

An average of 25 explants were used in each treatment, and treatments that promoted shoot regeneration were repeated at least twice. The tissue was examined periodically for shoot organogenesis and other morphological changes, and the regeneration frequencies were recorded after 8 weeks of growth.
Results

In vitro culture initiation

In vitro culture stocks of selected high starch sweetpotato genotypes were established by meristem culture using guideleines and procedures previously described (Love and Rhodes, 1985; Kuo, 1991). Excised apical meristems typically had one and occasionally two leaf primordia. We were able to establish in vitro stocks of all our selected genotypes using the BAP (0.3 mg l⁻¹) and NAA (0.03 mg l⁻¹) regime, albeit with different efficiencies (Table 1). In addition, zeatin riboside (2 mg l⁻¹) was used to establish in vitro stocks of the genotype ‘FTA-94’ with a higher efficiency (data not shown). Meristem culture efficiencies were determined as the number of plants that were established from the total starting number of meristems used. Meristem-derived plantlets were grown in magenta boxes on MPB medium for one month prior to transferring them to pots. All the plants exhibited normal phenotype and grew vigorously in the greenhouse (Figure 1A). Virus indexing results showed most grafted indicator plants did not show disease symptoms (Figure 1B). However, a few grafted Ipomoea nil showed minor leaf curling, which might indicate virus in the scion (possibly SPLCV). In vitro plantlets corresponding to scions whose inicator plants were asymptomatic were used in subsequent regeneration studies.

In vitro shoot regeneration studies

I. Single IAA, NAA, and BAP

A significant genotype-dependent response to the IAA treatment was observed among the SP genotypes. Shoot organogenesis was observed in the genotypes Jewel, DM01-158, and FT4-89 with mean regeneration frequencies of 80%, 35%, and 39%, respectively (Figure
2A-C; Table 2). In all cases, shoots developed from the cut-site of petioles and from leaf-derived roots (Figure 2A). FTA-94 developed roots and had foliar growth, but no shoot organogenesis. All of the other genotypes tested, which included Suwon, PDMP4, BM85-42, and DM01-074, were recalcitrant to regeneration under these conditions. Further optimization of the IAA concentrations for the genotype DM01-158 gave shoot regeneration frequencies of 13%, 33%, 57% and 30% for IAA concentrations of 0.2, 0.5, 0.8 and 1.5 mg l-1, respectively (Table 2). In addition, shoots appeared a week earlier in 0.5 mg l-1 IAA and 0.8 mg l-1 IAA, whereas some inhibition was found in the rooting and foliar growth in 1.5 mg l-1 IAA. Although explants were taken 1.5 weeks later, the regeneration frequency of 33% at 0.5 mg l-1 IAA was not significantly lower than the average from the former assays (37.9%).

When 0.5 mg l-1 NAA was used alone, only FTA-94 and Jewel showed regeneration, at frequencies of 37.5% and 29.2% respectively (Table 2). All of the other genotypes tested were unresponsive and developed callus and/or roots, but no shoot organogenesis (Figure 2D-E). Additional tests of FTA-94 on NAA concentrations of 0.3 and 0.7 mg l-1 resulted in regeneration frequencies of 12.5% and 25%, respectively. No shoot regeneration was observed in any of the genotypes in the study when 0.5 mg l-1 BAP was used, and only compact callus formed at the cut site of petioles (Figure 2F).

II. *Multiple hormone experiments: Auxins and cytokinin*

The use of a two-stage hormone regime to alter the hormone treatment as the explant develops has been described in previous studies (Dessai et al., 1995; Santa-Maria, 2003). Here, we tested the effect of an initial exposure to either 2,4-D or 4-FA, followed by three different concentrations of zeatin riboside in the second stage. Petiole swelling was observed after 4 to 5 days under 2,4-D, or 5 to 7 days under 4-FA. Upon swelling, explants were placed in zeatin riboside medium at concentrations of 0.05, 0.2, and 0.4 mg l-1. Clear
differences were found between the 2,4-D or 4-FA used in the initial stage, which was consistent among all the genotypes used in our study.

We found that when 2,4-D was used, after a period in zeatin, the petioles became brittle, and easily fell apart. Callus developed along their surface, and necrotic leaves, and root-like structures formed, with little, or no, shoot organogenesis (Figure 3A-B). Among all the genotypes in the study, only DM01-158 regenerated shoots with 5% frequency at zeatin concentrations of 0.05 and 0.2 mg l⁻¹ (Table 2). However, when 4-FA was used in the initial stage, rooting, foliar growth, and shoot regeneration were observed in all the genotypes. In addition, multiple shoots appeared from callus at the cut site of petioles (Figure 3C-D).

Regeneration frequencies were 6.7%, 26.8%, 10%, 25%, 100%, and 30% for DM01-074, DM01-158, FTA-94, FT4-89, Jewel, and PDMP4, respectively (Table 2). There was not a significant difference in the shoot regeneration frequencies at zeatin concentrations of 0.05 and 0.2 mg l⁻¹ in the second stage (Table 2). However, a trend was observed among all the genotypes with 0.05 mg l⁻¹ zeatin resulting in more rooting, whereas 0.4 mg l⁻¹ zeatin lead to the formation of compact callus at the cut site of the petioles.

Discussion

Genetic engineering techniques are becoming increasingly important to achieve rapid improvements in important sweetpotato cultivars. To accomplish this goal and efficiently produce transgenic plants, improvements in the current time-consuming methods for *in vitro* regeneration need to be established. In this study, optimized procedures for fast shoot regeneration in high starch sweetpotato varieties were established, with potential application in genetic transformation experiments.

In vitro stocks from all selected genotypes were established by meristem culture and the resulting plants were virus free. Meristem culture is a common practice to establish *in vitro* stock of field material while cleansing from virus in sweetpotato (Kuo, 1991; Love and
Rhodes, 1985). In this method, apical buds appear to be the best ex-plant to obtain virus free plantlets with higher regeneration frequencies (Kuo et al., 1985; Shang et al., 1996). Variations in meristem culture efficiency among sweetpotato genotypes observed in our study were also noted in previous studies for sweetpotato shoot tip cultures (Al-Mazrooei et al., 1997; Otani and Shimada, 1996).

In order to establish shoot regeneration procedures that could be used in genetic transformation experiments, we tested hormone regimes whose use was previously reported in successful sweetpotato transformation. We chose shoot organogenesis procedures over somatic embryogenesis because of its rapidity, as well as to avoid prolonged callus stages that can increase risk for somaclonal variation (James et al., 2007). We initially tested the auxin IAA at 0.5 mg l⁻¹, which was previously used for sweetpotato cv Jewel, where it promoted 80 to 100% shoot regeneration and led to the production of stably transformed transgenic plants (Garcia et al., 2000; Santa-Maria, 2003). In our study, shoot-regeneration frequencies for cv Jewel were similar to those previously reported, and the IAA treatment also promoted shoot regeneration in ISP genotypes ‘DM01-158’ and ‘FT4-89’. Similar to previous work (Cheng and Yeh, 2004; Garcia et al., 1999), adventitious shoots emerged from the cut-site of petioles and from leaf-derived roots, which would appear to be advantageous for an Agrobacterium- mediated transformation.

NAA and BAP were also tested since these growth regulators have been used for sweetpotato shoot organogenesis from roots, leaves, or petioles (Pido et al., 1995; Cheng and Yeh, 2003; Cheng and Yeh, 2004). Cheng et al. (2004) compared different auxins, including IAA and NAA, in several SP cultivars and reported that overall higher shoot regeneration frequencies were obtained with the IAA treatments, but that the highest regeneration frequencies (93.4% and 82.4%) in two Chinese varieties were obtained with 0.1 mg l⁻¹ NAA. Our data indicate that both IAA and NAA can promote shoot regeneration in sweetpotato (Figure 2A-E). Genotypes that responded to IAA showed lower, or no shoot regeneration when NAA was used. Interestingly, FTA-94 was the only ISP genotype with shoot regeneration under NAA. Such data suggest that it is important to test several auxin types and
concentration ranges when novel genotypes are being tested, given that responses among
them can be quite different. The BAP treatment produced a phenotype that was recalcitrant to
regeneration, mainly compact calli, a result that was consistent among all the genotypes in
our study. BAP has been previously shown to inhibit secondary root development in
sweetpotato (Pido et al., 1995).

We also tested a two-stage ‘auxin-cytokinin’ regime as an alternative approach.
Dessai et al (1995) first demonstrated the use of the two-stage approach by first exposing
leaves to 2,4-D for 3 to 4 days, which was followed by a prolonged exposure to zeatin in the
second stage. Using this procedure Dessai et al (1995) were able to promote shoot
regeneration in sweetpotato genotypes from a wide geographical distribution. A similar
procedure, where the auxin 2,4-D was replaced by 4-FA in the first stage followed by a
prolonged exposure to zeatin-riboside, was reported for sweetpotato cv ‘Jewel’, yielding
100% shoot regeneration then stably transformed transgenic plants (Luo et al., 2006; Santa-
Maria, 2003). In the present study, we compared the use of either auxin type in the first stage
and found that 4-FA was more effective for promoting shoot regeneration. While most
genotypes failed to regenerate when the 2,4-D/zeatin regime was used, most exhibited shoot
regeneration when 4-FA was used instead of 2,4-D in the first stage. Desai et al. (1995)
previously observed that a slightly longer exposure to auxin in the first stage can cause
profuse callusing at the end of petioles and failure to regenerate adventitious shoots.
Therefore, it is possible that the 4 to 5 days exposure to 2,4-D in the initial stage in our
experiments may have been too long. While this may be the case, we found that 4 to 5 days
were necessary for swelling of the petioles to occur, which is a requirement for the transition
to the second-stage hormone treatment (Dessai et al., 1995). In our study, under the 4-FA/
zeatin regime, explants developed multiple adventitious shoots that emerged from callus at
the cut site of petioles, in accordance to previous observations (Santa-Maria, 2003). The
effect of lower or higher zeatin concentrations for promoting either rooting or compact callus
formation has also been observed in earlier studies (Dessai et al. 1995).
In conclusion, growth regulator regimes were optimized to increase shoot regeneration frequencies in our novel high starch sweetpotato varieties. However, other parameters such as light-intensity, explant age and type, as well as sucrose concentrations could also be tested. For example, higher sucrose concentration in a callus induction media was reported to increase the shoot regeneration frequency in one sweetpotato genotype (Chen et al., 2006). In any case, the optimization of hormone regimes for promotion of shoot organogenesis in this work, represents an initial step towards the development of an efficient transformation and regeneration technology for novel high starch genotypes that are candidates for use in biofuel and sugar syrup production.

Acknowledgements

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References


Cheng HH, Yeh MS (2003) Studies on tissue culture of sweet potato I. The effects of different kinds and concentration of auxin on plant regeneration of explants from different parts. Journal of Agriculture and Forestry 52(3): 63-79


Table 1. Genotypic variation in meristem culture of high starch sweetpotato genotypes

All meristems were cultured in a medium containing BAP (0.3 mg l\(^{-1}\)) and NAA (0.03 mg l\(^{-1}\)).

<table>
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<th>Genotype</th>
<th>Total no. meristems</th>
<th>Plant formation efficiency(^a)</th>
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<td>BM85-42</td>
<td>12</td>
<td>0.26 ± 0.04</td>
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<td>DM01-074</td>
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<td>0.65 ± 0.16</td>
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<td>PDM-P4</td>
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</tr>
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<td>Suwon</td>
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\(^a\) Plant formation efficiencies were calculated as the number of meristems that regenerated into plants from the total number of starting meristems. Mean ± standard error. Means are from 2 to 3 replicates.
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*a* All auxin-cytokinin combinations are a two stages procedure consisting of an initial brief exposure to the auxin followed by a prolonged exposure to zeatin riboside.

*b* Regeneration frequencies were calculated as the number of leaf-explants that regenerated shoots divided by the total number of explants in the experiment, regardless of having multiple shoot per explant.

*c* Confidence intervals were calculated using a binomial model and statistical analysis software (SAS).
Figure 1. Meristem-derived plants or mericlones from high starch sweetpotato genotypes

A. Healthy mericlones growing in the greenhouse. B. Grafted Ipomoea nil with scion from DM01-158. Arrows point to growing scions. Circles show healthy Ipomoea nil. All meristem-derived plants grew normally in the greenhouse, and the majority didn’t promote viral infection symptoms when grafted to indicator plants.
Figure 2. Adventitious shoot regeneration in sweetpotato genotypes under a single hormone regime

A. Jewel in IAA 0.5 mg l⁻¹. Arrows point to shoots regenerating from nodes on leaf-derived roots. B, C. DM01-158 (B) and FT489 in IAA 0.5 mg l⁻¹(C). Arrows point to shoots regenerating from cut site of petiole. D, E. FT4-89 (D) and PDMP4 (E) in NAA 0.5 mg l⁻¹. No shoot regeneration, only roots and callus were observed. F. FT489 in BAP 0.5 mg l⁻¹, compact callus at the cut site of petioles were similarly observed in all the genotypes tested.
Figure 3. Adventitious shoot regeneration in sweetpotato genotypes under a two stages auxin-cytokinin regime

A, B. FT4-89 (A) and DM01-158 (B) under 2,4-D (0.2 mg l\(^{-1}\)) and zeatine (0.05 mg l\(^{-1}\)), note profuse callusing at the end of petioles. C. Jewel under 4-FA (0.2 mg l\(^{-1}\)) and zeatin (0.2 mg l\(^{-1}\)). D. PDM-P4 under 4-FA (0.2 mg l\(^{-1}\)) and zeatin (0.05 mg l\(^{-1}\)). Arrows indicate shoots regenerating from callus at cut site of petioles.
Chapter V

Identification of sporamin promoter regions in a sweetpotato genomic library

Introduction

Sporamin protein is abundant in sweetpotato storage roots accounting for 60 to 80% of total soluble protein (Hattori et al., 1989; Yeh et al., 1997; Wang et al., 2002; Morikami et al., 2005). It has two precursor forms ‘pre-sporamin’ and ‘pro-sporamin’, and its mature form ‘sporamin’ is localized in the vacuolar fraction of the cell (Hattori et al., 1989; Yeh et al., 1997). Sweetpotato sporamin is encoded by a multigene family that, based on nucleotide homologies, have been grouped into 2 subfamilies: sporamin A and sporamin B (Hattori et al., 1989; Yeh et al., 1997). There is extensive homology among sporamin A and sporamin B genes; cDNA sequences show over 90% and 80% nucleotide homology among intra- and inter- subfamily genes respectively (Hattori et al., 1989; Wang et al., 2002). In general, the coding region among sporamin gene variants is very conserved with just a few nucleotide replacements resulting in amino-acid substitutions. However, the sequences in the 5’ and 3’ non-coding regions diverge extensively with most differences being explained by insertions and deletions and a few base pair substitutions (Hattori et al., 1989). Based on dot blot hybridizations, restriction digestion analysis, and RNase mapping analysis, the hexaploid genome of sweetpotato has been estimated to contain approximately 36 and 24 copies of sporamin A and sporamin B genes respectively, and its is possible that the structural differences in the classification analysis might have been derived from allelic variations (Hattori et al., 1989; Ohta et al., 1991). It has been suggested by experimental data that certain members of the sporamin multigene family are preferentially expressed, and it is not known if certain members may constitute pseudogenes (Hattori et al., 1989). In any case, the
gene multiplicity of sporamin appears to be partly responsible for the vast abundance of this protein in the storage roots of sweetpotato (Hattori et al., 1989).

A dual role for sporamin has been proposed both as a somatic storage protein in storage roots and as a natural defense agent in the above ground organs (Yeh et al., 1997). Sporamin functions as a storage protein in roots, where it serves as nutritional resource for storage root sprouting (Wang et al., 2002). Additionally, sporamin belongs to a large family of Kunitz-type trypsin inhibitors (Hattori et al., 1989; Morikami et al., 2005) and as such, it posses strong trypsin inhibitory activity and its insect defense capability has been demonstrated (Yeh et al., 1997; Wang et al., 2002). Under normal conditions a low amount of sporamin is present in the stems (about 1% of its storage-root levels), but none is found in leaves (Hattori et al., 1989; Yeh et al., 1997). However, consistent with its role as natural defense agent, expression of sporamin in aereal organs can be induced by wounding, both locally and systemically, in a fashion typical of the systemic wound-response gene-expression patterns of proteinase inhibitors (Yeh et al., 1997). The wound response is very rapid and restricted to aerial tissues; expression of the β-glucuronidase gene (GUS) under a sporamin promoter in leaves and stems of transgenic tobacco plants showed activity in as little as 15 min from wounding, reaching a maximum level of expression after 12 h, and this level remained up to 48 h after the wounds were inflicted (Wang et al., 2002). The sporsmin gene promoter used in the study was isolated from the sporamin genomic clone gSPOR5-31 (gb U12436; Wang et al., 1995) and belongs to the sporamin A gene family. Wound inducible expression was not observed in roots of the same transgenic tobacco plants (Wang et al., 2002).

In addition to its wound response, sporamin expression in aereal tissues is also induced by sugars. Sweetpotato plantlets cultivated axenically in 3% sucrose medium exhibited large amounts of sporamin accumulating in the stems (Ohta et al., 1991). Also, sporamin accumulation in leaves and petioles of sweetpotato can be induced by high sucrose concentrations, as well as glucose and fructose (Ohta et al., 1991). Studies of the sporamin ‘gSPO-A1’ promoter controlling GUS expression in transgenic tobacco plants allowed the
identification of conserved cis-acting regulatory sequences within the -305 and -94 promoter regions that are involved in sucrose-inducible expression (Hattori et al., 1990; Ohta et al., 1991; Morikami et al., 2005). GUS expression under the gSPO-A1 promoter could be induced in leaves of transgenic tobacco plants by placing leaf segments in agar plates with 10% sucrose (Morikami et al., 2005). In addition, metabolic regulation of the sporamin promoter by a massive influx of sucrose into the cell was proposed based upon observations of spatial patterns of GUS staining in stems of transgenic tobacco (GUS expression being controlled by the sporamin ‘gSPO-A1’ promoter), which were similar to the spatial pattern of starch accumulation in the stems of sweetpotato growing in a high sucrose medium (Ohta et al., 1991). This indicates that the sporamin gene is expressed in stem cells with a transient carbohydrate sink function (Ohta et al., 1991).

The use of sporamin promoters to drive the expression of heterologous genes has been demonstrated in transgenic tobacco, where reporter genes were used to determine promoter function and identify cis-acting regulatory elements (Ohta et al., 1991; Wang et al., 2002; Morikami et al., 2005). Additionally, a few reports exist where a sporamin promoter was used to control high-level recombinant protein expression in other plants species such as potato (Hong et al., 2008) and Brassica oleracea (Chen et al., 2006).

The purpose of this study was to identify novel sporamin B gene promoters that could be used for targeted high-level recombinant protein expression in the storage roots of sweetpotato. A sweetpotato genomic library was screened to identify putative promoter regions with homology to the sporamin ‘gSPO-B1’ gene (gb X13510; Hattori and Nakamura, 1988) (Figure 1). These sporamin promoter sequences were subsequently cloned into a pBlueScript-derived vector, controlling the expression of gfp7 that encodes a modified GFP protein from Aequorea victoria (Figure 2). Promoter activity was assayed by transient expression of green fluorescence protein (GFP) upon particle bombardment of leaf segments from sweetpotato, placed onto a high sucrose medium. Although expression of gfp7 allowed a clear GFP screening in previous stably transformed sweetpotato tissues (see Chapter III), phenolic compounds released in the healing process after particle bombardment as well as
trichomes strongly fluoresced, interfering with the transient GFP signal and preventing a conclusive determination of promoter activity (Figure 3). For further evaluations, the use of an alternative reporter gene such as GUS that will not be confounded by intrinsic interferences is highly recommended. Alternatively, the use of stems and/or root segments rather than leaves might result in a stronger promoter activity and reporter gene signal.

Methods and Results

Screening of the sweetpotato genomic library

A genomic sublibrary containing putative sporamin clones was generated by PCR amplification from clones derived from a sweetpotato BAC library. The BAC clones were isolated based upon positive hybridization with a sporamin B (gSPO-B1) cDNA probe. Amplified fragments were cloned into the pGEM®-T Easy vector (Promega; Madison, WI) by standard cloning techniques (Sambrook and Russell, 2001) and maintained in E. coli strain DH10B (data not shown). Screening for promoter sequences was performed by double sequencing with T7, Sp6 and M13 primers (QIAGEN; Valencia, CA) in an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems; Foster City, CA). Multiple alignments were performed between the sequencing data and the published sporamin B gene ‘gSPO-B1’ (gb X13510) (Hattori and Nakamura, 1988) using a ‘basic local alignment search tool’ (BLAST) algorithm from GenoMax™ 3.0 (InforMax; Rockville, MD). From the 96 clones evaluated, six were identified that contained similarity with the published gSPO-B1 sequence (Table 1). Clones with homologous sequences were isolated from the library and further sequenced to generate contigs (Figure 1) using the Vector NTI Contig Express tool (Invitrogen; Carlsbad, CA). Additionally, promoter prediction was done using the plant promoter prediction tool (TSSP) at http://www.softberry.com/berry.phtml. Contigs A7, A9, A14 and B1 were predicted to contain a plant promoter region.
Particle bombardment experiments

The putative sporamin promoter sequences were cloned into a plmnc95 vector (Mankin and Thompson, 2001) by directional cloning with Pst I and Eag I (NEB; Ipswich, MA), replacing the original CaMV 35S promoter with the sporamin promoter clone upstream the gfp7 gene (Figure 2). The gene gfp7 in plmnc95 encoded a soluble modified, red-shifted GFP protein from _Aequorea victoria_ that also has an intron and ER retention signal (Mankin and Thompson, 2001).

Particle bombardment of sweetpotato leaves was performed in a PDS-1000/He system (BioRad; Hercules, CA) using 0.6 µm diameter gold particles (BioRad). A day prior to the bombardments, leaf segments from _in vitro_ sweetpotato plants were placed onto MS minimal medium (Sigma-Aldrich; St. Louis, MO) supplemented with mannitol (20 mg/L), sorbitol (20 mg/L), Phytagar (3 g/L), with pH adjusted to 5.8. Gold particles were coated with plasmid DNA as follows: 60 mg gold particles were washed with 1 ml ethanol (100%) for 2 min, pelleted and resuspended in 1 ml distilled water. Suspended gold particles were separated into 50 µl aliquots. During agitation of the particles, 10 µl plasmid DNA (300 ng/µl), 50 µl of 2.5M CaCl₂ and 20 µl of 0.1 M spermidine were sequentially added to each tube, which were further mixed for 3 min. Coated particles were centrifuged 10s at 10,000 rpm, the supernatant was discarded, and 500 µl of ethanol (100%) was added. Particles were then centrifuged for 10 s at 10,000 rpm, the supernatant was again discarded, and 75 µl ethanol (100%) was added to each tube. Immediately afterwards, 10 µl of the coated gold particles were spread onto the center of a macrocarrier (BioRad) in the carrier holder, and were then allowed to dry in a sterile flow chamber. Leaf segments from sweetpotato cv ‘Jewel’ were bombarded at a distance of 6 cm using helium driven pressure of 1100 psi and chamber vacuum of 28 mmHg. Five replicates per plasmid construct were performed. Five plates were bombarded with empty gold particles to serve as negative controls, and 5 were
bombarded with plasmid plmnc95, which has the gfp7 under the control of the CaMV 35S promoter to serve as a positive control for GFP expression.

After bombardment, plates were sealed and put in a growth chamber at 26°C and 16/8 h (light/dark) photoperiod. Next day, plates were transferred to a high sucrose medium (MS minimal medium, sucrose 10%, Phytagel 0.3%, pH 5.8) and screened for GFP expression in a Nikon SMZ-1500 Fluorescence Microscope with a narrow-band pass Endow filter for red-shifted GFP signal. The same exposure settings (10 s) were used to collect GFP fluorescence under blue light illumination in all bombarded tissues including control plates. An initial GFP screening was done after 16 h from bombardments. At this point, background fluorescence in the negative control was still low and 35S-driven GFP expression resulted in GFP fluorescence that was notoriously more intense that background levels (not shown). However, there was not a clear difference in GFP-fluorescence intensity between tissue bombarded with the sporamin promoter constructs and background levels observed in the negative (empty particle) control, possibly due to a weak or uninduced promoter activity at this point. Plates were kept in the incubator chamber in high sucrose medium for 5 days and screened again for GFP expression under the same settings. At this point, significant background fluorescence in the sweetpotato tissue bombarded with the empty particle (negative control) did not allow a conclusive determination of promoter activity (Figure 3).

Discussion

As observed previously, variation in the promoter region and 5’UTR between sporamin promoter clones identified in this work and the published gSPO-B1 gene (gb X13510) consisted primarily of deletions and/or insertions (Hattori et al., 1989). Given the homology of these deletions with adjacent sequences, they may have been generated by slipped missparing of direct repeats during DNA replication, as noted by Hattori et al. (1989). Conserved sequences highlighted in Figure 1 are between sporamin A and sporamin
B genes and were first described by Hattori et al. (1988). In all promoter clones used in this study, direct repeats as well as conserved sequences were 100% identical to the gSPO-B1 sequence. This conservation of discrete sequences may indicate their relevance in sporamin promoter function.

The putative wound- and sucrose- responsive elements previously identified in sporamin A promoters (Wang et al., 2002; Morikami et al., 2005) were not found in any of the sporamin B promoter clones in this study. However, slight variations in these consensus sequences were noted (bp 762-766; Figure 1). While the conserved cis- sucrose responsive element consensus ‘TGGACGG’ described by Morikami et al., (2005) was not found in full, the core sequence ‘GGACG’ (bp 762-766; Figure 1) was identified in all sporamin B promoter clones in this study. Further analysis of promoter activity by sequential 5’-deletions and/or base substitutions could help elucidate the existence of similar or alternative cis-acting regulatory elements in these novel sporamin B promoter sequences.

To determine promoter activity in the sporamin clones A7, A9, A14 and B1, new constructs will need to be generated with an alternative reporter gene such as GUS, instead of GFP, in order to prevent endogenous interference with the reporter gene signal. Additionally, bombardment of other sweetpotato tissues such as storage roots or stems under sucrose medium should be considered; stronger promoter activity is expected in those tissues and this may further facilitate the screening of reporter gene signals.

References


Table 1. Sweetpotato genomic clones with homology to a sporamin B promoter.

Sequences of sporamin clones in pGEM®-T Easy vector were obtained by DNA sequencing with the T7 primer, and were compared to gSPO-B1 sequence using the BLAST algorithm from GenoMax™ 3.0. From the 96 clones evaluated, clones A1, A5, A7, A9, A14 and B1 (in bold) had high similarity scores to the target sequence and were selected for further sequencing and analysis.

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Figure 1. Novel sporamin promoter sequences homologous to gSPO-B1

Contigs were generated using Vector NTI Contig Express with 4 to 11 sequences from each selected sporamin clone in either orientation. Multiple alignments were performed in Vector NTI using the BLAST algorithm. Base-pair numbers correspond to the published gSPO-B1 complete gene sequence (gb X13510). Direct repeats and conserved sequences between type A and type B sporamin promoters (Hattori and Nakamura, 1988), as well as sucrose-responsive element (Morikami et al., 2005) are in black-framed boxes with text highlighted in grey. TATA box and transcription initiation sites (+1) are in bold letters.
sporamin B (201) TCACACAAGTACGAGCCATAGCCCAAACCTGGGAAACGACAAATACTA
Contig A14 (1) -------------------------- TAACGTGCGGTTGTAATTGTC
Contig A7 (1) -------------------------- TAACGTGCGGTTGTAATTGTC
Contig A9 (1) -------------------------- TAACGTGCGGTTGTAATTGTC
Contig B1 (1) -------------------------- TAACGTGCGGTTGTAATTGTC
Consensus (201) TTTCCGTGCGGTTGTGTAATGTGC

sporamin B (251) AACCCGAATTACATTTTTTTG--ACAAACATCAATGCACCTTAAGAA
Contig A14 (25) ATT-----ACAAATGCACTTTAAAGTACAACAAAATCA-TGCACCTTTAAGAA
Contig A7 (25) ATT-----ACAAATGCACTTTAAAGTACAACAAAATCA-TGCACCTTTAAGAA
Contig A9 (25) ATT-----ACAAATGCACTTTAAAGTACAACAAAATCA-TGCACCTTTAAGAA
Contig B1 (25) ATT-----ACAAATGCACTTTAAAGTACAACAAAATCA-TGCACCTTTAAGAA
Consensus (251) ATT ACAATGGGACACTTTAAAGTACAACAAAATCA TGCACCTTTAAGAA

sporamin B (299) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA
Contig A14 (70) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA
Contig A7 (70) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA
Contig A9 (70) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA
Contig B1 (70) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA
Consensus (301) CACAACCCACGCACCTTAAGCATACAAATCAGGCACCTTAAGAAGAAAA

direct repeat 1

sporamin B (349) AAACGCACCTTTAAGATGAAAAATCAGCGACCTTTAAATTAATTAAAGCTT
Contig A14 (120) AAACGCACCTTTAAGATGAAAAATCAGCGACCTTTAATTAAATTAAAGCTT
Contig A7 (120) AAACGCACCTTTAAGATGAAAAATCAGCGACCTTTAATTAAATTAAAGCTT
Contig A9 (120) AAACGCACCTTTAAGATGAAAAATCAGCGACCTTTAATTAAATTAAAGCTT
Contig B1 (119) AAACGCACCTTTAAGATGAAAAATCAGCGACCTTTAATTAAATTAAAGCTT
Consensus (351) AAACGCACATTAAGATGAAAAATCAGCGACCTTTAATTAAATTAAAGCTT

direct repeat 1

133
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**Figure 1. Continued**
Figure 1. Continued

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Contig A14   (369) AATAGAGGTCAAGAGGTCAAAAAATGAATATTTGGAGATGAGAGTAT
Contig A7    (368) AATAGAGGTCAAGAGGTCAAAAAATGAATATTTGGAGATGAGAGTAT
Contig A9    (368) AATAGAGGTCAAGAGGTCAAAAAATGAATATTTGGAGATGAGAGTAT
Contig B1    (369) AATAGAGGTCAAGAGGTCAAAAAATGAATATTTGGAGATGAGAGTAT
Consensus   (601) AATAGAGGTCAAGAGGTAGAAAAATGAATATTTGGAGATGAGAGTAT

651                                            700
sporamin B   (647) TTAGAA----AAAAAAGATTTTCTGTATGGTGTATTTTGC
Contig A14   (419) TTAGAAAAAAGATTTTCTGTATGGTGTATTTTGC
Contig A7    (418) TTAGAAAAAAGATTTTCTGTATGGTGTATTTTGC
Contig A9    (418) TTAGAAAAAAGATTTTCTGTATGGTGTATTTTGC
Contig B1    (419) TTAGAAAAAAGATTTTCTGTATGGTGTATTTTGC
Consensus   (651) TTAGAA AAAAAAAAAAGATTTTCTGTATGGTGTATTTTGC

701
putative sucrose responsive element

751
sporamin B   (693) ATATG-----AAAAACAAATTATAAAATACG
Contig A14   (469) ATATGAAAAAAAATACG
Contig A7    (467) ATATGAAAAAAAATACG
Contig A9    (467) ATATGAAAAAAAATACG
Contig B1    (469) ATATGAAAAAAAATACG
Consensus   (701) ATATGATAATAGGACAATAATCAGCGAAATTCATATCG

751                                            800
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Contig A14   (519) AAACAATTATAAGGACAATAATCAGCGAAATTCATATCG
Contig A7    (517) AAACAATTATAAGGACAATAATCAGCGAAATTCATATCG
Contig A9    (517) AAACAATTATAAGGACAATAATCAGCGAAATTCATATCG
Contig B1    (519) AAACAATTATAAGGACAATAATCAGCGAAATTCATATCG
Consensus   (751) AAACAATTATAAGGACAATAATCAGCGAAATTCATATCG
Figure 1. Continued

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- **Contig A14**: ATATACGGAGTATATGATACATTTTTTGAGTACTATTGACTATGTTACA
- **Contig A7**: ATATACGGAGTATATGATACATTTTTTGAGTACTATTGACTATGTTACA
- **Contig A9**: ATATACGGAGTATATGATACATTTTTTGAGTACTATTGACTATGTTACA
- **Contig B1**: ATATACGGAGTATATGATACATTTTTTGAGTACTATTGACTATGTTACA

Consensus sequences for other regions:

- **Consensus**:
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  - 901-950: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
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Conserved sequences:

- **Sporamin B**: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
- **Contig A14**: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
- **Contig A7**: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
- **Contig A9**: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
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Consensus sequences for other regions:

- **Consensus**:
  - 801-850: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
  - 901-950: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
  - 951-1000: ATATACGGAGTATATGATACATTTCTTTGAGTACTATTGACTATGTTACA
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Figure 2. Plasmid vectors employed in particle bombardment experiments

*bla*, β-lactamase gene for ampicillin resistance; *ColE*₁, replication origin; 35S P, cauliflower mosaic virus 35S promoter; *gfp*₇, soluble-modified and red-shifted GFP gene from *Aequorea victoria*; *nosT*, nopaline synthase terminator; P, Pst I; E, Eag I; Spoprom A7, A9, A14, and B1, putative sporamin promoter sequences.
Figure 3. Transient GFP expression in bombarded leaves from sweetpotato cv Jewel

All frames were taken under 11.25x magnification in a Nikon SMZ-1500 Fluorescence Microscope. In all plates, blue light exposure for image capture was 10 s. Bright field exposure was for 1 s.  

A, B, leaf bombarded with plasmid plmnc95 under blue light (A) and bright field (B); C, D, leaf bombarded with plasmid plmncA7 under blue light (C) and bright field (D); F, G, leaf bombarded with empty gold particles (no DNA) under blue light (F) and bright field (G). Background fluorescence observed in the negative control made difficult a clear determination of 35S and sporamin gene promoter activity. Pictures are representative of observations with all sporamin promoter constructs and replicates.
Chapter VI

Concluding Remarks

Throughout the studies reported in this document, the feasibility of producing hyperthermophilic amylolytic enzymes in sweetpotato storage roots as an alternative approach for a more cost effective conversion of starch to monomeric sugars has been demonstrated. Besides, complementary studies for further optimization of starch bioconversion in sweetpotato storage roots were presented. Novel findings, findings translated from other systems to sweetpotato, and suggested ‘next steps’ are summarized in this section.

Novel findings:

• Contrary to a previous report (Liebl et al., 1997), calcium is required for the stability of Thermotoga maritima (Tma) α-amylase above 80°C. More importantly, available calcium (Ca$^{2+}$) in plant cells exists in a sufficiently high concentration to allow Tma α-amylase stabilization at these temperatures. We found that recombinant production of Tma α-amylase in plant cells benefits from intrinsically supplied Ca$^{2+}$ for an enhanced thermostability at above 80°C, in contrast to purified enzyme from Escherichia coli.

• Starch in the storage roots of transgenic sweetpotato plants producing active hyperthermophilic α-amylase can be self-processed at 80°C when homogenized solely with water. Calcium was intrinsically provided in the sweetpotato cells and was sufficient to prevent an early thermal decay of the protein at high temperatures.

• Novel sporamin promoters regions were identified by a genome sequencing approach, which presumably correspond to sporamin B genes. Conserved elements described in the
gSPO-B1 promoter (gb X13510) were present in the identified, novel sporamin promoter regions sharing 100% identity.

Findings translated from other systems:

- Active recombinant α-amylase from the hyperthermophilic bacterium *Thermotoga maritima* can be produced in plant cells (tobacco NT1 cell cultures and sweetpotato plants), where it retains its biological activity and temperature-dependent catalysis, as compared from recombinant production in *E. coli*.

- Stable transformation of sweetpotato cv ‘Jewel’ was done using an *Agrobacterium tumefaciens*-mediated transformation and regeneration procedure previously described for other sweetpotato cultivars (‘Jonathan’ and ‘Huachano’) with some modifications.

- Adventitious shoot regeneration can be promoted in high starch sweetpotato genotypes using hormone regimes previously described for other sweetpotato cultivars. Such regimes include IAA at concentrations between 0.2 and 1.5 mg/L, and NAA at concentration between 0.3 and 0.7 mg/L. Alternatively, shoot regeneration in selected genotypes can also be promoted by a two-stage hormone regime consisting on an initial brief exposure to the auxin 4-FA, followed by a prolonged exposure to zeatine riboside.

Perspectives from these studies:

- The tobacco NT1 cell culture was proven to be a suitable model plant system for testing recombinant production of hyperthermophilic glycosidases. Therefore, routine testing of additional enzymes involved in starch hydrolysis should be done in this system prior to their cloning into the sweetpotato genome.
• Improvements in the sweetpotato transformation procedure described in Chapter III are required to increase transformation efficiencies and reduced the time required for regeneration. Alternative, different explant sources, modified regeneration regimes, and expansion of methods to high starch cultivars will be required to generate improved starch feedstocks for cost-effective industrial biofuel or sugar syrups production.

• The biomass conversion efficiency in transgenic lines producing hyperthermophilic liquefying enzyme generated in this work needs to be evaluated and compared to established starch conversion methods. The prospect of recombinant α-amylase levels in transgenic storage roots being high enough to signify a more cost-effective conversion compared to exogenous enzyme applications needs to be determined. This could be done by comparing starch conversion efficiencies when using transgenic sweetpotato storage roots versus wild type storage roots mixed with different units of enzyme activity obtained from E. coli and/or liquefying enzyme obtained from commercial sources.

• The promoter activity of the sporamin clones identified in Chapter V needs to be determined. This could be done using an alternative reporter gene, such as GUS, in transient expression experiments. Alternatively, the tissue specificity and promoter strength could be tested by a stable transformation approach with a suitable reported gene in sweetpotato or other plant system.
Appendix A

Stable transformation experiments for industrial-type sweetpotato genotypes

Summary

*Agrobacterium tumefaciens-* mediated transformation was performed for the industrial-type sweetpotato genotypes ‘FT489’, ‘DM01-158’ and ‘FTA94’, which exhibit higher dry matter content than table top varieties and are adapted to the southeast US. These genotypes were described in Chapter III. Selected methods for adventitious shoot regeneration, as well as somatic embryogenesis procedures described in the previous chapters were tested to regenerate transformed tissue. The binary vectors pBI95, pBIA7, pBIamy2 (Figure 1) were used. Plasmids pBI95 and pBIA7 carry the gfp7 gene for a soluble-modified, red-shifted version of the GFP protein from *Aequorea Victoria* (Mankin and Thompson, 2001), under the control of either the cauliflower mosaic virus (CaMV) 35S promoter (pBI95) or the sporamin promoter ‘clone A7’ described in Appendix A (pBIA7). Plasmid pBIamy2 carries the amyN26 gene for a hyperthermophilic α-amylase from *Thermotoga maritima* under the control of the CaMV 35S promoter. All plasmids were cloned into a pBIN20 backbone that also carries the gene nptII for plant kanamycin resistance (Hennegan and Danna, 1998). Transformation procedures and observations from each experiment are summarized in Tables 1, 2 and 3.

Developing transformation technology for these novel high-starch sweetpotato genotypes will allow for the expansion of their use in industrial applications. Of particular interest is their use in first generation biofuel production, for which improved starch conversion is desired, but also for the production of sugar syrups, organic acids through fermentation, or for the production of starch-based polymers (Crabb and Shetty, 1999; Carole et al., 2004). While transgenic plants were not obtained in any of the genotypes under the regimes evaluated, useful information regarding their susceptibility to *Agro* infection, as
well as the response to selectable markers and hormone regimes was generated. We believe
that the results summarized here constitute background work that may pose helpful in further
studies.

**Figure 1. Plasmid vectors used for industrial-type sweetpotato transformation.**

RB, right border of T-DNA; nosP, nopaline synthase promoter; *nptII*, neomycin
phosphotransferase gene for plant kanamycin resistance; nosT, nopaline synthase 3’UTR; 35S, cauliflower mosaic virus 35S promoter; Spoprom A7, sweetpotato sporamin promoter clone A7 (Appendix A); *gfp7*, soluble-modified, red-shifted green fluorescence protein (GFP) from *Aequorea Victoria*; *amyN26*, N-truncated α-amylase gene from *Thermotoga maritima*; LB, left border of T-DNA.
Table 1. Transformation experiments in high-starch sweetpotato cultivar ‘FT489’

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th>Procedure: Shoot organogenesis regeneration</th>
<th>Observations</th>
</tr>
</thead>
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<td>E1</td>
<td>AGL1/ pBIA7</td>
<td>The OD_{600} of the bacterial culture prior to co-culture was 0.5-0.65. Upon co-culture, explants were kept in MO4FA medium for 5 days and then transferred to Zeatin-1 medium. A total of 25, 33, and 29 explants were used for E1, E2, and E3 respectively. All explants were 6.5 weeks old.</td>
<td>Two weeks after infection, no rooting was observed for any explant and the leaves appeared chlorotic. This was also the case for explants infected with WT bacterium. Five weeks after infection, no rooting was observed for any explant, and they all appeared necrotic, including in the WT-agro control. Eventually, the bacteria overgrew in the plates and they were discarded. Although, the explants infected with the WT bacterium were in better condition, they also failed to regenerate adventitious shoots and were discarded.</td>
</tr>
<tr>
<td>E2</td>
<td>AGL1/ pBI95</td>
<td>Same as above but regeneration medium did not contain kanamycin. 14 explants were used.</td>
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<td>E3</td>
<td>AGL1/ pBIamy2</td>
<td>The OD_{600} of the bacterial culture prior to co-culture was 0.4-0.55. Upon co-culture explants were kept in MO4FA medium for 5 days and then transferred to Zeatin-2 medium. A total of 15, 36, and 33 explants were used, in E5, E6, and E7 respectively. Explants were 7 weeks old.</td>
<td>Two weeks after infection, no rooting was observed for any explant, and the leaves appeared chlorotic. Five weeks after infection, the majority of the explants was senescing (necrosis), and explants were discarded.</td>
</tr>
</tbody>
</table>

*a Basic medium composition was MS basal salts with minimal organics (cat. no. M6899, Sigma-Aldrich), sucrose (30 g/L), Phytage (3 g/L), kanamycin (50 mg/L), and timetin (100 mg/L). Additionally, MO4FA medium contained 4-fluorophenoxyacetic acid (4FA, 0.2 mg/L); Zeatin-1 medium contained zeatin (0.2 mg/L); and Zeatin-2 medium contained zeatin (0.1 mg/L).
Table 2. Transformation experiments in the industrial-type sweetpotato cultivar ‘DM01-158’.

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<th>Procedure I: Shoot organogenesis regeneration</th>
<th>Observations</th>
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<td>AGL1/ pBI95</td>
<td>The OD$_{600}$ of both Agro strains prior to co-culture was 0.3. After co-culture, explants were transferred to IAA-medium.</td>
<td>Four weeks after infection, explants infected with WT-Agro and those that were not infected exhibited foliar growth and some rooting, while the explants under kanamycin selection were senescing (necrosis). After 6 weeks, the Agro overgrew in the plates and they were discarded. Explants infected with WT Agro, and those not infected rooted, but they did not regenerate adventitious shoots.</td>
</tr>
<tr>
<td>E2</td>
<td>AGL1/ WT</td>
<td>a. The regeneration medium for explants infected with WT-Agro did not contain kanamycin. 28 and 20 explants were used in E1, E2 respectively. Explants were 6 weeks old.</td>
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<tr>
<td>E3</td>
<td>No agro</td>
<td>Same as above, but there was no Agro present in the infection and the co-culture, and the regeneration medium did not contain kanamycin. 16 explants were used.</td>
<td></td>
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<tr>
<td>E4</td>
<td>EHA105/ pBI95</td>
<td>The OD$_{600}$ of Agro prior to co-culture was 0.65. Co-culture of explants and bacterium was carried out in liquid co-culture medium for 23h. After co-culture the explants were blotted onto sterile filter paper, and placed onto regeneration medium MO4FA. Seven days after treatment, the explants were transferred to Zeatin-medium.</td>
<td>Seven weeks after infection, a few shoots developed, and were transferred to sweetpotato propagation medium. However, the putative lines were negative in the kanamycin test, and had no green fluorescence so they were discarded.</td>
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</tbody>
</table>

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*a* The OD$_{600}$ of both Agro strains prior to co-culture was 0.3. After co-culture, explants were transferred to IAA-medium. The regeneration medium for explants infected with WT-Agro did not contain kanamycin. 28 and 20 explants were used in E1, E2 respectively. Explants were 6 weeks old. Experiments were performed with explants 6 weeks old. Four weeks after infection, explants infected with WT-Agro and those that were not infected exhibited foliar growth and some rooting, while the explants under kanamycin selection were senescing (necrosis). After 6 weeks, the Agro overgrew in the plates and they were discarded. Explants infected with WT Agro, and those not infected rooted, but they did not regenerate adventitious shoots. The OD$_{600}$ of Agro prior to co-culture was 0.65. Co-culture of explants and bacterium was carried out in liquid co-culture medium for 23h. After co-culture the explants were blotted onto sterile filter paper, and placed onto regeneration medium MO4FA. Seven days after treatment, the explants were transferred to Zeatin-medium.
Table 2. Continued

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th>Procedure II: Somatic embryogenesis</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E5</td>
<td>EHA105/pBI95</td>
<td>The transformation and regeneration procedure described in Chapter II was followed, using 40 leaf explants that were 5 weeks old.</td>
<td>Four weeks after infection, transgenic calli formed on the surface of the petioles and were transferred to G24D medium. Some calli developed embryonic structures 2-3 months after infection, and were transferred to ABA-Km medium for embryo development. Differentiating calli necrotized in ABA-Km medium and they were discarded.</td>
</tr>
<tr>
<td>E6</td>
<td>EH105/pBI95</td>
<td>The transformation and regeneration procedure described in Chapter II was followed, with some modifications. First, calli harvested from petioles were placed in embryo induction medium with 4 different hormone compositions: [1] GA3 (0.1 mg/L) and 2,4D (0.05 mg/L), [2] GA3 (0.1 mg/L) and 2,4-D (0.1 mg/L), [3] GA3 (0.1 mg/L) and 2,4D (0.2 mg/L), and [4] 2,4D (0.05 mg/L). Secondly, embryonic calli were transferred to a medium containing GA3 (0.5mg/L) instead of abscisic acid (ABA-Km). The explants used for transformation were 5 weeks old.</td>
<td>Petioles in F9-Km medium appeared to be more brittle than those from the cultivar ‘Jewel’. Green calli that formed on the surface of petioles after 4 to 5 weeks were harvested and placed onto embryo induction medium. Two months after infection, calli on embryo-induction medium [4] necrotized, possibly due to the higher auxin content. A similar response was observed in the treatment [3]. Calli in the two lower auxin treatments had a yellow appearance. Four months after infection, a few calli under 2,4-D (0.05 mg/L) differentiated embryonic structures and were transferred to GA3, but they necrotized and were ultimately discarded.</td>
</tr>
</tbody>
</table>
Table 2. Continued

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th>Procedure II: Somatic embryogenesis</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>No agro (No Km selection)</td>
<td>The transformation and regeneration procedure described in Chapter II with some modifications was followed. First, coculture was performed in liquid co-culture medium for 14 h, followed by semi-solid co-culture for 30 h, in a medium containing 4FA (0.2 mg/L) and no ASG. Following the co-culture, explants were transferred to MO4FA medium, and after 7 days they were transferred to Zeatin-medium. Calli that formed along the surface of petioles under selection were harvested and placed onto embryo induction medium. 13, 10, and 53 explants were used in E7, E8, and E9 respectively.</td>
<td>Five weeks after co-culture, explants placed under kanamycin selection that were not Agro-infected necrotized and were discarded. Explants that were not infected and were not under kanamycin selection exhibited swelling of petioles, a few green compact calli on the leaves, with thickened roots on a few explants. Infected explants under selection developed calli along the surface of the petioles, which then were harvested and placed onto G24D medium for embryo induction. Four months after infection, only yellowish compact, non-embryogenic calli formed and were eventually discarded.</td>
</tr>
<tr>
<td>E8</td>
<td>No agro (Plus Km selection)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E9</td>
<td>EHA105/ pBI95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 2. Continued**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th><strong>Procedure II: Somatic embryogenesis</strong></th>
<th><strong>Observations</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>E10</td>
<td>EHA105/pBIA7</td>
<td>The transformation and regeneration procedure described in Chapter II was followed, with some modifications. Calli that formed on the surface of the petioles under F9-Km were harvested and placed onto two different embryo induction media: [1] GA₃ (0.1 mg/L) and 4FA (0.05 mg/L), and [2] GA₃ (0.1 mg/L) and 2,4-D (0.01 mg/L). Thirty, 5 week old explants were used in each transformation experiment.</td>
<td>Two months after infection, calli in embryo induction media [1] were green and compact, while those in medium [2] had a yellow appearance and were more friable, and a few calli appeared to be differentiating. After 3 months calli in either treatment were not differentiating further, and after 5 months, all calli were discarded.</td>
</tr>
<tr>
<td>E11</td>
<td>EHA105/pBIN20</td>
<td>The basic composition of the regeneration medium was MS basal salts with minimal organics (cat. no. M6899; Sigma-Aldrich), sucrose (30 g/L), Phytagel (3 g/L), kanamycin (50 mg/L), and pH 5.8; additionally, IAA-medium contained indoleacetic acid (IAA, 0.8 mg/L), MO4FA-medium contained 4-FA (0.2 mg/L), and Zeatin-medium contained zeatin riboside (0.2 mg/L). To remove residual Agrobacteria, Timetin (100 mg/L) was employed for strain ‘AGLI’, and cefotaxime (200 mg/L) was employed for strain ‘EHA105’.</td>
<td></td>
</tr>
</tbody>
</table>

a The basic composition of the regeneration medium was MS basal salts with minimal organics (cat. no. M6899; Sigma-Aldrich), sucrose (30 g/L), Phytagel (3 g/L), kanamycin (50 mg/L), and pH 5.8; additionally, IAA-medium contained indoleacetic acid (IAA, 0.8 mg/L), MO4FA-medium contained 4-FA (0.2 mg/L), and Zeatin-medium contained zeatin riboside (0.2 mg/L). To remove residual Agrobacteria, Timetin (100 mg/L) was employed for strain ‘AGLI’, and cefotaxime (200 mg/L) was employed for strain ‘EHA105’.

b The liquid co-culture medium composition was MS basal salts with minimal organics (Sigma-Aldrich), sucrose (30 mg/L), acetosyringone (ASG, 20 mg/L) and pH 5.5; additionally, the liquid co-culture medium contained the same growth regulator at the same concentration that was used in the subsequent regeneration medium.

c The kanamycin test consisted of placing leaf- segments onto a high kanamycin medium, where leaf-segments from kanamycin-resistant plants will show profuse callusing after 5 weeks and those from non-transgenic plants will become chlorotic and won’t develop calli (Cipriani et al., 1998; Santa-Maria, 2003). The high kanamycin-medium composition was MS basal medium (cat. no. M5519; Sigma-Aldrich), glucose (20 mg/L), mannitol (20 mg/L), MES (0.5 g/L), Phytagel (3 g/L), zeatin riboside (1 mg/L), naphthaleneacetic acid (NAA; 0.1 mg/L), kanamycin (100 mg/L), and pH 5.8.
Table 3. Transformation experiments for industrial-type sweetpotato cultivar ‘FTA94’

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agrostrain</th>
<th>Procedure II: Somatic embryogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>EHA105 / pBI95</td>
<td>The transformation and regeneration procedure described in Chapter II was followed, with some modifications. Calli that formed on the surface of petioles in F9-Km medium were harvested and placed onto embryo-induction medium with 3 different hormone compositions: [T1; GA&lt;sub&gt;3&lt;/sub&gt; (0.1 mg/L) and 2,4-D (0.05 mg/L)], [T2; GA&lt;sub&gt;3&lt;/sub&gt; (0.1 mg/L) and 2,4-D (0.1 mg/L)], and [T3; GA&lt;sub&gt;3&lt;/sub&gt; (0.1 mg/L) and 4FA (2.0 mg/L)]. Approximately forty, 4 week old explants were used.</td>
<td>Five weeks after infection, many calli developed on the surface of petioles on F9-Km medium, and were placed onto embryo induction medium. Two months after infection, calli that were on T1 were green and compact. Calli under T2 appeared more friable and some appeared to be differentiating, but after 6 months no embryonic structures developed and they were ultimately discarded. Calli under T3 appeared green and somewhat compact. Embryonic structures did not develop and the calli were discarded after 8 months.</td>
</tr>
</tbody>
</table>
Table 3. Continued

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th>Procedure II: Somatic embryogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>EHA105 / pBIA7</td>
<td>The transformation and regeneration procedure described in Chapter II was followed, with some modifications: Calli harvested from the surface of the petioles under F9-Km treatment were placed onto embryo induction medium with 4 different hormone compositions: [T1; NAA (0.1 mg/L)], [T2; NAA (0.5 mg/L)], [T3; BAP (1 mg/L) and NAA (0.5 mg/L)], and [T4; GA3 (0.1 mg/L) and NAA (0.5 mg/L)].</td>
<td>Forty, 5 week old explants were used. Four weeks after infection, many calli developed on the surface of the petioles on F9-Km medium, which were then harvested and placed onto embryo-induction medium. Two months later, calli under treatment T1 became necrotic, possibly due to lower auxin, and compact green calli were observed in treatments T2, T3 and T4. After 6 months, calli in either treatment still appeared green, compact and non-embryogenic, and were ultimately discarded.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Procedure description continued...
<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Agro strain</th>
<th>Procedure II: Somatic embryogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3</td>
<td>EH105/ pBI95</td>
<td>The transformation and regeneration procedure followed was similar to that described in Chapter II with some modifications: In treatment 1 [T1], the hormone composition in ERM, SCM and F15 was the same as that described in Chapter II-Table 1. In treatment 2 [T2], the auxin concentration in ERM, SCM and F15 was increased 10-fold: NAA (1.0 mg/L) in ERM and SCM, and 2,4-D (0.5 mg/L) in F15. All explants were transferred to F9-Km medium and calli that developed along the surface of the petioles were harvested and placed onto embryo induction media with different hormone compositions: [T1.1; GA₃ (0.1 mg/L) and 2,4-D (0.2 mg/L)], and [T1.2; 2,4-D (0.2 mg/L)]. Forty, 5 week old explants were used in each treatment.</td>
<td>Four weeks after infection, explants under T1 looked firm and developed many discrete calli along the petioles. However, explants under T2 looked brittle and did not form discrete calli on the petioles, possibly due to the higher auxin. Calli from explants under T1 were harvested and placed onto embryo induction media T1.1 and T1.2. Explants under T2 were discarded. It was noted that a 10-fold increase in auxin concentration in ‘FTA94’ gave a similar response as standard auxin concentration in the genotype ‘DM01-158’ therefore, ‘DM01-158’ may be more susceptible to auxin than both ‘FTA94’ and cv. ‘Jewel’. Six months after infection, calli on embryo induction medium T1.1 appeared green and friable, with little necrosis. Calli on medium T1.2 were also green and friable, but exhibited greater necrosis than those on medium T1.1. In both cases, there was no embryogenic calli differentiation, and the calli were ultimately discarded.</td>
</tr>
</tbody>
</table>

<sup>a</sup> The basic composition of all media used (e.g. ERM, SCM, F15 and F9-Km) is described in Chapter II- Table 1. The only modifications made were in the hormone type and concentrations as described.
References


Appendix B

Regeneration studies in tissues of sweetpotato cv. Jewel

Introduction

For the sweetpotato transformation procedure described in Chapter II there were some concerns regarding the large size of the explants used and the long regeneration time that was necessary to obtain regenerants. Using complete leaves (lamina and petiole) as explant creates space constrains given that each petri plate can accommodate only 4 to 6 explants, limiting the possible number of replicates per transformation experiment. The other major setback was the long time required for regeneration. Prolonged cultures can increase the risk of somaclonal variation in the resulting transgenic plants (Larkin and Scowcroft, 1981; Podwysznyska et al., 2006), and this is in addition to the inconvenience of maintaining cultures for prolonged time intervals. To address these shortcomings, various hormone regimes were tested on different explant types of sweetpotato cv ‘Jewel’, to evaluate the likelihood of using them in similar transformation procedures to those described in Chapter II. The specific aim was to identify regimes able to promote discrete and profuse callusing along the explant surface that may become embryogenic in later stages of regeneration.

Regimes used in previous transformation studies for various sweetpotato cultivars, and modifications thereof were tested (Otani et al., 2001; Otani et al., 2003; Jiang et al., 2004; Yang et al., 2005; Yi et al., 2006; Yu et al., 2007). Leaf discs, petioles and stem segments were used as explants for somatic embryogenesis regeneration, and apical meristems to generate embryogenic calli. The work presented here was carried out at the Plant Biotechnology Laboratories of Bayer CropScience Corporation in Lyon, France, as part of a research internship during the summer of 2007. Several regimes that appear promising
for their use in sweetpotato transformation were identified and are described in the following appendix.

**Calli induction in leaf, petiole and stem explants**

The auxins 2,4- dichlorophenoxyacetic acid (2,4-D) and 4- fluorophenoxyacetic acid (4-FA) alone or in combination with giberelic acid (GA$_3$) at different concentrations were tested to promote profuse callusing on sweetpotato explants. Discrete, non-friable or not too compact calli were regarded as promising for a transformation experiment, showing the potential to become embryogenic in subsequent regeneration steps. Leaf discs, petioles and stem segments from 5-weeks old *in vitro* grown sweetpotato plants cv. ‘Jewel’ were used as explants. The hormone regimes evaluated were the same as or modified from previous reports on sweetpotato transformation (Otani et al., 2001; Otani et al., 2003; Jiang et al., 2004; Song et al., 2004; Yang et al., 2005; Yi et al., 2006). A total of 20 explants per treatment were employed. The regeneration medium basic composition was MS basal medium (cat. no. M5519; Sigma-Aldrich, St. Louis, MO), sucrose (3%), Phytagel (0.3%), and a pH of 5.8, which was supplemented with hormones as described in Table 1. Explants in the regeneration medium were placed in an incubator room at 24°C (shelf)/ 25°C (air), 70% RH, and a 16/8 h (light/ dark) photoperiod.

When using leaf discs as explants, the most promising regimes were 4-FA (1.0 mg/L), GA$_3$ (0.1 mg/L) combined with 2,4-D (0.1 mg/L) and to a lesser degree 2,4-D (0.5 mg/L). All of these treatments promoted callusing of the desired morphology without apparent damage or toxicity to the tissue (Figure 1). Higher 2,4-D concentrations, in particular, caused evident toxicity to the explant resulting in generalized necrosis. When using petioles as explant, similar responses were observed. The best treatments were 4-FA (either 1.0 or 2.0 mg/L), and GA$_3$ (0.1 mg/L) combined with 2,4-D (0.1 mg/L) (Figure 2). Higher auxin concentrations caused toxicity and subsequent necrosis of the explant. When using stem segments, 4-FA at all concentrations tested appeared promising for a transformation experiment. Similarly, GA$_3$
(0.1 mg/L) combined with 2,4-D (0.05 mg/L) also promoted callusing of the desired morphology. A common response among all explant types was the toxicity to 2,4-D above 0.5 mg/L resulting in severe necrosis within a month of treatment. In general, there was a more favorable response to 4FA, which appeared to be a milder auxin than 2,4-D, but lower 2,4-D concentrations should still be tested. Additionally, petioles and stems seemed to be better explants for transformation, given profuse callusing formed along their surfaces and were less damaged by manipulation and would likely better stand co-culture and *Agrobacterium* infection than leaf explants.

**Table 1. Callus induction on sweetpotato tissues.**

Observations were made 5 weeks after experiment initiation. In all cases, a higher auxin concentration lead to profuse callusing, which beyond a threshold resulted in tissue toxicity and generalized necrosis.

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Leaf discs Observations</th>
<th>Petioles Observations</th>
<th>Stem segments Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-FA (0.5 mg/L)</td>
<td>Few calli and some necrosis in leaf blade.</td>
<td>Compact calli at the end of the petiole.</td>
<td>Callusing and root-like structures along surface. A few shoot buds were noted.</td>
</tr>
<tr>
<td>4-FA (1.0 mg/L)</td>
<td>Callusing and root-like structures developed at the leaf base.</td>
<td>Profuse callusing on petiole surface.</td>
<td>Profuse callusing and some root-like structures on stem surface.</td>
</tr>
<tr>
<td>4-FA (2.0 mg/L)</td>
<td>Callusing at cut sites.</td>
<td>Profuse callusing, somewhat embryogenic.</td>
<td>Callusing, somewhat embryogenic.</td>
</tr>
<tr>
<td>Treatment†</td>
<td>Leaf discs Observations</td>
<td>Petioles Observations</td>
<td>Stem segments Observations</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------</td>
<td>-----------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>GA3 (0.1 mg/L) 2,4-D (0.05 mg/L)</td>
<td>Profuse rooting and some callusing at the leaf base.</td>
<td>Large, compact calli at the end of petioles.</td>
<td>Green callusing with some differentiation.</td>
</tr>
<tr>
<td>GA3 (0.1 mg/L) 2,4-D (0.01 mg/L)</td>
<td>Some rooting and callusing at the leaf base.</td>
<td>Calli at the end of the petioles, some necrosis.</td>
<td>Callusing and embryogenic-like differentiation.</td>
</tr>
<tr>
<td>GA3 (0.1 mg/L) 2,4-D (0.5 mg/L)</td>
<td>Profuse callusing at the cut site of the leaf discs.</td>
<td>Necrosis.</td>
<td>Necrosis.</td>
</tr>
<tr>
<td>GA3 (0.1 mg/L) 2,4-D (1.0 mg/L)</td>
<td>Profuse callusing and some necrosis.</td>
<td>Severe necrosis, petioles appeared brittle.</td>
<td>Severe necrosis.</td>
</tr>
<tr>
<td>2,4-D (0.5 mg/L)</td>
<td>Profuse callusing at cut edges of the leaf discs.</td>
<td>Necrosis.</td>
<td>Necrosis.</td>
</tr>
<tr>
<td>2,4-D (1.0 mg/L)</td>
<td>Profuse callusing accompanied by necrosis</td>
<td>Severe necrosis.</td>
<td>Severe necrosis.</td>
</tr>
<tr>
<td>2,4-D (2.0 mg/L)</td>
<td>Severe necrosis.</td>
<td>Severe necrosis.</td>
<td>Severe necrosis.</td>
</tr>
</tbody>
</table>

† In each treatment n=20. The basic medium composition was MS minimal medium (Sigma-Aldrich) sucrose (3%), Phytagel (0.3%), and pH 5.8.
Figure 1. Callus induction on leaf discs of sweetpotato cv. ‘Jewel’.

Pictures were taken under a dissecting scope 5 weeks after experiment initiation. A, B, C, 4-FA at 0.5 mg/L (A), 1.0 mg/L (B) and 2.0 mg/L (C). D, E, F, G, GA₃ (0.1 mg/L) combined with 2,4-D at 0.05 mg/L (D), 0.1 mg/L (E), 0.5 mg/L (F) and 1.0 mg/L (G). F, I, 2,4-D at 0.5 mg/L (F) and 1.0 mg/L (I). Low auxin lead to rooting and increasing 2,4-D concentration above 0.5 mg/L caused profuse callusing and some necrosis.
Figure 2 Callus induction on petioles.

Pictures were taken under a dissecting scope 5 weeks after experiment initiation. A, B, C, 4-FA at 0.5 mg/L (A), 1.0 mg/L (B) and 2.0 mg/L (C). D, E, F, GA₃ (0.1 mg/L) combined with 2,4-D at 0.05 mg/L (D), 0.1 mg/L (E) and 0.5 mg/L (F). G, H, I, 2,4-D at 0.5 mg/L (G), 1.0 mg/L (H) and 2.0 mg/L (I). With both auxin types, lower auxin promoted some rooting, increasing auxin concentration promoted profuse callusing on petiole surface, and 2,4-D above 0.5 mg/L resulted in necrosis of tissue. 4FA is a milder auxin than 2,4-D and promoted discrete callusing along the petiole surface that appear suitable for transformation experiments.
Figure 3. Effect of auxin treatments on stem segments.
Pictures were taken under a dissecting scope 5 weeks after experiment initiation. A, B, C, 4-FA at 0.5 mg/L (A), 1.0 mg/L (B) and 2.0 mg/L (C). D, E, F, GA3 (0.1 mg/L) and 2,4-D at 0.05 mg/L (D), 0.1 mg/L (E) and 0.5 mg/L (F). G, H, 2,4-D at 0.5 mg/L (G) and 1.0 mg/L (H). A more embryogenic-like response is observed in stems segments compared to leaf discs and petioles.
Embryogenic calli

Embryogenic calli have been the preferred explant for sweetpotato transformation in several laboratories (Yu et al., 2007; Otani et al., 2001; Jiang ShengJun et al., 2004; Yi et al., 2007; Zang Ning et al., 2008; Choi HyeJin et al., 2007; Wakita et al., 2001). However, to implement stable transformation procedures for novel cultivars, methods to generate embryogenic calli need to be in place (Yi et al., 2007). Here, several regimes commonly used to generate embryogenic calli from apical meristems and modifications thereof were assayed for the sweetpotato cultivar ‘Jewel’ (Otani and Shimada, 1996; Otani et al., 2003; Jiang et al., 2004; Song et al., 2004; Yang et al., 2005; Yi et al., 2006). Apical meristems were obtained from sweetpotato plants growing in the greenhouse. Vines containing the apical bud and 3 to 4 internodes were harvested, they were then surface sterilized in 2% bleach solution and rinsed with sterile distilled water 3 times. Apical buds with 2 leaf primordia were excised under a dissecting scope and put onto embryogenic calli induction medium (ECM). The ECM basic composition was MS basal medium (Sigma-Aldrich), sucrose (3%), phytagel (0.3%) and pH adjusted to 5.8; and it was supplemented with different growth regulators as described in Table 2. A total of 20 to 25 apical buds were used in each treatment. Calli were incubated at 25°C in the dark, and were sub-cultured every 4 weeks. Results and observations are summarized in Table 2 and Figure 4.

Calli that developed organized structures and eventually exhibited tissue differentiation were regarded as embryogenic, which are in contrast to those calli that were fast-growing and friable. Regimes able to promote embryogenic calli were 2,4-D at 0.05 mg/L, 0.1 mg/L and 0.5 mg/L, and 4FA at 0.5 mg/L and 1.0 mg/L. Higher auxin concentrations resulted in generalized necrosis. In most cases, regimes containing GA3 lead to the formation of friable calli.
Table 2. Promotion of embryogenic calli from apical meristems.
Observations were taken 5 to 6 weeks after experiment initiation. Embryogenic calli are those exhibiting an organized structure and some tissue differentiation, in contrast to friable fast-growing calli.

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>4-FA (mg/L)</th>
<th>2,4-D (mg/L)</th>
<th>GA3 (mg/L)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>A few appeared embryogenic.</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>--</td>
<td>--</td>
<td>Most appeared embryogenic with some tissue differentiation.</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>Growing but no differentiation.</td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td>0.05</td>
<td>0.1</td>
<td>Friable, didn’t appear embryogenic.</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
<td>0.1</td>
<td>0.1</td>
<td>The majority was friable and not embryogenic.</td>
</tr>
<tr>
<td>6</td>
<td>--</td>
<td>0.5</td>
<td>0.1</td>
<td>Little development was observed, and they remained small.</td>
</tr>
<tr>
<td>7</td>
<td>--</td>
<td>0.05</td>
<td>--</td>
<td>Most appeared embryogenic.</td>
</tr>
<tr>
<td>8</td>
<td>--</td>
<td>0.1</td>
<td>--</td>
<td>Most appeared embryogenic.</td>
</tr>
<tr>
<td>9</td>
<td>--</td>
<td>0.5</td>
<td>--</td>
<td>A few appeared embryogenic, some necrosis.</td>
</tr>
<tr>
<td>10</td>
<td>--</td>
<td>1.0</td>
<td>--</td>
<td>Some necrosis.</td>
</tr>
<tr>
<td>11</td>
<td>--</td>
<td>2.0</td>
<td>--</td>
<td>Severe necrosis.</td>
</tr>
</tbody>
</table>

† In each treatment n=20. The basic medium composition was MS minimal medium (Sigma-Aldrich) sucrose (3%), Phytagel (0.3%), and pH 5.8.
Figure 4. Promotion of embryogenic calli from apical meristems.

Different auxin treatments were tested to promote embryogenic calli from apical buds in sweetpotato cv ‘Jewel’. All frames were taken under a dissecting scope 5 to 6 weeks after experiment initiation. A, B, C, 4-FA at 0.5 mg/L, 1.0 mg/L and 2.0 mg/L. D, E, F, GA3 0.1 mg/L combined with 2,4-D at 0.05 mg/L, 0.1 mg/L and 0.5 mg/L. G, H, I, 2,4-D at 0.05 mg/L, 0.1 mg/L and 0.5 mg/L. Embryogenic calli were obtained with 4-FA and 2,4-D regimes.


