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ISOLATION AND SEQUENCE ANALYSIS OF SOME GENES INVOLVED IN STARCH METABOLISM OF THE COMMON HYACINTH

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Abstract: Studies were conducted at the University of Sussex on the common hyacinth with a view to isolating and sequencing some of the genes that govern starch metabolism in this geophyte. Results indicated that the sequence generated for starch phosphorylase from hyacinth was similar to the sequences of this enzyme from a number of plants, but that of sweet potato was the closest in terms of similarity. In fact, this sequence is well conserved, and is identical at the protein level to that of sweet potato. The sequence generated for starch synthase, however, was similar to the starch synthase sequence from *Sorghum bicolour*. The sequences generated from this study could be used to redesign primers which could have been used to get bigger part of the gene from hyacinth DNA. The inserts could be used to probe a hyacinth genomic library to get the whole genes or to design primers which could be used for RT-PCR analysis to investigate the expression of these genes.

Key words: Hyacinth, Starch metabolism genes, Isolation and sequence analysis, Conserved domains, Primer design, Starch phosphorylase, Starch synthase.

INTRODUCTION

The ornamental hyacinth, *Hyacinthus orientalis*, is a spring-flower bulb and a native of the West and Central Asia. It belongs to the family *Hyacinthaceae* and genus *Hyacinthus*. Hyacinth is cultivated mainly for the production of cut flowers, as potted plants or as landscaping plants. Hyacinth is used in the perfumery industries because an essential oil is extracted from its flowers for the manufacture of perfumes (Usher, 1974). The diploid forms of hyacinth have 16 chromosomes of 5 different types but there are also triploids (3n = 24) and a large number of heteroploids in this species (Rees, 1972). Starch content of hyacinth scales ranged from 214.2 to 306.6 µmol g^{-1} fresh weight, whilst glucose, fructose and sucrose levels ranged from 11.8-14.3, 0.7-4.9 and 12.5-17.4 µmol g^{-1} fresh weight, respectively, at the time of planting. But at senescence, the sugars almost disappeared completely whilst starch accumulated in the scales (Addai, 2010). This implies that starch is the major storage carbohydrate in hyacinth.

Starch is the end product of photosynthesis, and it occurs primarily in the source tissues of plants but stored in the sink tissues. The polysaccharide is not only a source of dietary carbohydrates but it has many industrial uses. As for example, starch is used for the manufacture of packaging materials and for making ethanol. Martin and Smith (1995) stated that, there is a relationship between the basic characteristics of starch and its architectural organisation. Therefore, the organised arrangements of amylopectin and amylose into higher order molecular structures gives rise to granule formation, and this makes the molecule resistant to degradation. In general, plants are very sensitive and responsive to their surroundings even though they are immobile. The inability of plants to move from one place to another place implies that they have few strategies for survival other than acclimatisation. In recent years, the interest in

the genes that are involved in starch metabolism has increased because the genes involved in the regulation and metabolism of this carbohydrate in plants provide important mechanisms for plants to adjust to various environmental changes (Koch, 1996). Enzymes of starch metabolism in plants are grouped into two: starch synthesising enzymes and those responsible for degradation of starch. Enzymes of starch synthesis include ADP-glucose pyrophosphorylase (AGPase), the enzyme that is involved in the formation of ADP-glucose and pyrophosphate from ATP and glucose-1-phosphate; starch synthase, the enzyme involved in the linking of ADP-glucose to the non-reducing end of the growing starch chain by α-(1, 4) bond and which act on both amylose and amylopectin; starch branching enzyme (SBE), the enzyme responsible for the production of the (1, 6) branch points in amylopectin molecules (Myers *et al*., 2000; Nakamura, 2002; Smith, 1990). The degrading enzymes include starch phosphorylase, the enzyme that catalyses a reversible reaction producing glucose-1-phosphate from the non-reducing ends of the starch molecules (Duffus, 1984; Steup, 1990); and the amylases, enzymes that catalyse the hydrolysis of α-(1, 4)-linked glucans directly, thereby yielding sugars and oligosaccharides (Davies, 1990). In general, research work on molecular biology especially on isolation and characterisation of starch metabolising enzymes in hyacinth or flower bulbs in general is either limited or non-existent, according to the available literature, and this needs to be addressed. Miller (1992) also reported that the biochemistry of carbohydrate metabolism, particularly in relationship to the synthesis and/ or breakdown of starch in plants is poorly understood. Ohdan *et al*. (2005) also stated that the metabolic pathways involved in the biosynthesis and degradation of starch are different between source and sink tissues, and the mode of gene expression of enzymes involved in carbohydrate metabolism was dependent on the type of tissues concerned as well as the developmental stage of the plant. Beck and Ziegler (1989) also concluded that starch degradation in plants occurs as a result of the joint action of phosphorolysis and hydrolysis. It has also been established that (Sowokinos, 2007) the characteristics of enzymes involved in the metabolism of carbohydrates influence the process of regulation and partitioning of compounds in the plants. Having a good knowledge about the isolation and sequencing of starch metabolism genes is important because it will provide understanding not only into the basic principles of the biochemistry of starch partitioning and regulation, but will also throw light on growth and physiology of hyacinth in particular, and flower bulbs in general. Being able to study the level of transcription of a particular gene has become by the use of techniques such as RT-PCR, and advanced techniques such as realtime PCR can be used to quantify the level and control of gene expression in individual structures in the plant under different conditions. For these techniques to be used, specific probes need to be generated and for this a degree of sequence information is needed. A molecular level studies were therefore initiated to generate sequence information from some of the enzymes that are involved in the synthesis and degradation of starch in hyacinth.

MATERIALS AND METHODS

Extraction of hyacinth DNA

To isolate genes fragments from enzymes involved in starch metabolism of hyacinth, the Qiagen DNEasy plant mini kit was used to extract DNA from hyacinth. Approximately 0.1 g of the leaves was placed in a 1.5 ml microfuge tube and kept in a bucket containing dry ice. The leaves were ground using a sterile plastic pestle until no lumps remained and 400 µl of Buffer AP 1 was added. An amount of 4 µl of RNase A stock solution (100 mg/ml) was added. The tube was incubated for 10 min at 65 ^{o}C and 130 µl of Buffer AP 2 added and incubated on wet ice for 5 min. This allowed detergents, proteins and polysaccharides to be precipitated. The mixture was centrifuged at at 13,000 rpm for 5 min. The supernatant in a QIA shredder column was centrifuged for 2 min at 13,000 rpm. The flow through was transferred to a new 1.5 ml tube, and 1.5 volumes of Buffer AP 3 was added, mixed, followed by the addition of 650 µl of the lysate, applied in a DNeasy mini spin column and centrifuged for 1 min at 8000 rpm. The flow through was discarded but the collection tube was kept. The DNeasy column was placed in a new 2 ml collection tube and 500 µl of Buffer AW added to the column and centrifuged again at 8000 rpm for 1 min. The flow through was discarded, the collection tube was kept and another 500 ul of Buffer AW added to the column and centrifuged again for 2 min at 13,000 rpm to dry the membrane. The column was transferred to new 1.5 ml tube and 40 µl of Buffer AE added onto the membrane. This was incubated for 5 min at room temperature

followed by centrifugation at 8000 rpm for 2 min to elute the DNA.

Identification of conserved domains and PCR primer design

Multiple DNA alignments were carried out using the Entrez website of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/gquery), the protein sequence database for the enzymes in question were searched for. Then, conserved domains for proteins of a number of plants, ranging from monocotyledonous species to the dicotyledonous plants, were identified using the Multiple Alignment Viewer. Degenerate primers were designed to conserved domains of enzymes using 'Translator' (http://www.justbio.com/translator/index.php). The Tm of primer pairs were matched by adjusting the length of the primer.

Polymerase chain reaction (PCR)

PCR uses a thermostable Taq polymerse to ampify DNA sequences by thermo-cycling. In general, DNA is kept in a test tube, sealed, and the tube placed in a thermal cycler. The cycler is programmed to cycle between three different temperatures, first a high temperature (about 95 ^{o}C) to separate the DNA strands; then a relatively low temperature (about 40 ^{o}C) to allow the primers to anneal to the temperate DNA strands; and then to a medium temperature (about 72 ^{o}C) to allow the DNA synthesis (Weaver, 1999). In this study, the composition of the reaction mixture was as follows: 18.5 µl of distilled water, 5 µl of 10 x PCR buffer, 7 µl of dNTPs, 6.5 µl of $MgCl_2$, 1 µl each of the forward and reverse primers, 0.25 µl of taq polymerase and 1.25 µl of the hyacinth DNA, in a total volume of 40 µl per reaction. The PCR cycling conditions were 94 ^{o}C for 4 min, 94 ^{o}C for 60 sec, 50 ^{o}C for another 60 sec, 72 ^{o}C for another 60 sec, then 72 ^{o}C for 7 min and 4 ^{o}C to hold the reaction. In all, the thermal cycler was programmed for 35 cycles.

Agarose Gel Electrophoresis

Genomic DNA and PCR products were resolved using 1% TBE agarose gel electrophoresis. Ethidium bromide was incorporated into the gel matrix (0.4mg/ml (w/v) and gels were run at 100V and visualised on a transilluminator (= 365nm).

Isolation of DNA fragments from the gel slices

The DNA was recovered from the PCR product (band) by cutting out with a scapel from the gel and purified prior to cloning using the Qiagen Gel purification Kit. The gel slice containing the PCR product was weighed and 3x volume of buffer QG added and incubated at 50 ^{o}C for 10 min. The mixture was applied to column and spun at 1 min, discarding the flow through. An amount of 0.5 ml of buffer QG was added and spun at 1 min, discarding the flow through. An amount of 750 µl of buffer PE was then added and spun at 1 min and the flow through discarded. Spinning was again done at 13,000 rpm for 1 min and the samples placed into a new tube. The DNA was eluted by adding 50 µl of buffer EB, left for 1 min, followed by spinning for another 1 min.

Subcloning of the PCR fragments using the TOPO TA vector

TOPO TA vector system (Invitrogen) was used to subclone the PCR fragments. The TOPO TA cloning reaction was made by adding 2 µl of the PCR product to 1 µl salt solution, 1.5 µl of sterile water and 0.5 µl of TOPO vector. The reaction mixture was gently mixed and incubated for 5 min at room temperature to allow the inserts to ligate into the plasmid.

Plasmid Transformation

A vial of competent Top 10 (Invitrogen) cells were quickly thawed to 4 ^{o}C and kept on ice. The contents of the cloning reaction were added to the competent cells, mixed gently by flicking the tube and incubated on ice for 15 min. The cells were heat shocked in a 42 ^{o}C water bath for 30 seconds and immediately transferred to ice and 250 µl of room temperature SOC medium was added; tubes were capped and shaken horizontally but gently at 37 ^{o}C for 1 hour. Colonies containing recombinant plasmids were selected/identified by plating 50µl of transformed cells onto LB Amp X Gal plates and incubating at 37ºC for at least 8 hours. Then, 500 ml LB agar was melted in the microwave and allowed to cool to approximately 55 ^{o}C and 500 µl ampicillin (50 mg/ml) was added, gently shaken to mix, and 25 ml poured into each petri dish in laminar flow hood, and were allowed to set and dry. Then Xgal solution was made by combining 0.2 g Xgal in 9 ml dimethyl formide and 0.02 g IPTG in 1 ml water. An amount of 100 µl of the Xgal solution was then spread onto each of the 5 LB amp plates and was allowed to dry in the laminar flow hood. Colonies containing plasmids with cloned inserts were identified by white colour. These were purified by streaking on LB amp plates.

E. *coli* plasmid miniprep DNA preparation

The alkaline method was used for this preparation. An amount of 100 µl of ampicillin (50 mg/ml) was added to 100 ml LB broth; then 2 ml of the media was pipetted into sterile tubes. Tiny amounts of the bacterial cells (colonies identified from above) were transferred into the tubes using a wire. The bacterial cells were allowed to grow for a period of 48 hours at 37 ^{o}C with shaking. Then 1 ml of the culture was taken into a clean 1.5 ml eppendorf tube, spun at maximum speed for 1 min, and the supernatant discarded. Then 100 µl of solution I (a mixture of 50 mM glucose, 25 mM Tris HCl @ pH of 8 and 10 mM EDTA) and 200 µl of solution II (a mixture of 0.2 M NaOH and 1 % SDS) were added. The tube content was thoroughly mixed and incubated for 5 min. An amount of 150 µl of solution III (a mixture of 5M KOAc @ pH of 4.8) was added and incubated on ice for 5 min followed by centrifugation in a microfuge at 13000 rpm for 5 min. The supernatant was poured into new tubes leaving all white precipitate behind. Then 100 µl of phenol (lower phase) was added, mixed by inversion, centrifuged for 5 min, and the upper phase taken into fresh tubes. 50 µl of chloroform was added, mixed by inversion, centrifuged for 5 min at 13000 rpm, and the upper phase taken into fresh tubes, 1 ml of 100 % ethanol was added, mixed and centrifuged for 10 min. The supernatant was discarded and 100 µl of 70 % ethanol added, followed by centrifugation for 2 min at 13000 rpm. The supernatant was again discarded. Pellets were dried at 37 ^{o}C in a heating block for 30 min with lid open but covered loosely with a piece of aluminium foil to prevent contamination by any foreign particles. Then, distilled water (45 µl) was added, resuspended gently by flicking the tube and 0.2 µl of RNase A added followed by incubation at 37 ^{o}C for 30 min. Then, 5 µl of 3M NaOH and 3x volume of 100 % ethanol were added and spun for 10 min. The supernatant was discarded followed by the addition of 500 ml of 70 % ethanol, spun for 2 min and the supernatant discarded. Pellets were dried at 37 ^{o}C and 20 µl of distilled water added and kept for sequencing.

Purification of the PCR product prior to sequencing

Buffer PB (5 vol) were added to one volume of the PCR samples and placed in a spin column in 2 ml collection tube and spun for 1 min at 13000 rpm, and the flow through discarded. Then, the sample was washed using 0.75 ml of buffer PE, spun for 1 min at 13000 rpm, and the flow through discarded. The mixture was spun for another 1 min before placing in a new tube. Buffer EB (50 µl) was added on the membrane and spun for 1 min. The sample was then collected and kept for sequencing.

RESULTS

Design of PCR primers

Primers were designed for starch phosphorylase and starch synthase for their use in PCR reactions, with a view to isolating the gene fragments of these enzymes. The domains that were highly conserved across a number of plants, or were common to all the plants considered were recorded along side their positions at which they were located on the total alignment length of the whole protein. Using these domains, the DNA sequences of the various accessions of the plant species were cut and pasted into the programme (Translater), and by translating in 3 reading frames, the DNA (base nucleotides that code for the specific amino acids in the protein domain) for the various domains were recorded as shown in Tables 1-2).

Primers design for starch phosphorylase

The forward primer for the starch phosphorylase enzyme, STPH IF, was designed using the domains GRLASCF that occurs at position 1 of the total alignment length of the domains conserved for starch phosphorylase (Table 1a). Comparing the base nucleotides that code for the specific amino acids in the protein of the domains across the various plant species, the forward primer, STPH 1F was obtained as: 5'GGNAGBCTHGCHTCNTGYTT (Tm = 62.9^{o}C, Mol. Wt = 643 µg), where N = A, C, G, T; B = A, G; H = C, A, T; and Y = C, T (Table 1a). The reverse primer for the starch phosphorylase enzyme, STPH 564R, was designed from the domains, GGKAFATY which are located at position 564 of the total alignment. To get the STPH 564R, first the forward primer (STPH 564F) needed to be designed and the reverse primer was simply obtained by reading the nucleotide backwards (reversing it). Thus comparing the nucleotides across the list of the plant species as shown in Table 1b, then, the forward primer of starch phosphorylase, that is primer STPH 564F, was thus obtained as: 5'GGWGGDAARGCDTTYGCNACNTAY, where W= A, G; D = A, G, T; B = A, G; Y = C, T; N = A, G, C, T. By reversing the above, the reverse primer, that is primer STPH 564R was therefore obtained as shown below:

5'YTANGTNGCYAADGCRTTDCCWCC (Tm = 64.7 °C, Mol. Wt = 943 µg).

Table 1: Conserved protein domain of starch phosphorylase enzyme
(a) Position 1 of the total alignment

Position 1	G	R	L	A	S	C	F
Ipomoea batata	GGA	AGG	CTT	GCT	TCT	TGC	TTT
Vitis vinifera	GGA	AGG	CTT	GCT	TCA	TGC	TTT
Vitis vinifera	GGA	AGG	CTT	GCT	TCC	TGC	TTC
Ricinus communis	GGA	AGA	CTT	GCT	TCA	TGC	TTT
Populus trichocarpa	GGA	AGA	CTT	GCT	TCA	TGT	TTT
Citrus hybrid cultivar	GGC	AGG	CTA	GCT	TCA	TGC	TTC
Cucurbita maxima	GGA	AGG	CTT	GCT	TCA	TGT	TTT
Arabidopsis thaliana	GGG	AGA	CTT	GCC	TCG	TGT	TTC
Arabidopsis thaliana	GGG	AGA	CTT	GCC	TCG	TGT	TTC
Arabidopsis thaliana	GGG	AGA	CTT	GCC	TCG	TGT	TTC
Arabidopsis thaliana	GGG	AGA	CTT	GCC	TCG	TGT	TTT
Vicia faba	GGT	AGG	CTT	GCT	TCT	TGC	TTT
Spinacia oleracea	GGG	AGG	CTC	GCT	TCG	TGC	TTT
Oryza sativa	GGT	AGG	CTC	GCA	TCT	TGC	TTT
Oryza sativa	GGT	AGG	CTC	GCA	TCT	TGC	TTT
Sorghum bicolar	GGT	AGG	CTT	GCA	TCT	TGC	TTT
Zea mays	GGT	AGG	CTT	GCA	TCT	TGC	TTT
Zea mays	GGT	AGG	CTT	GCA	TCT	TGC	TTT
Triticum aestivum	GGC	AGG	CTT	GCA	TCT	TGC	TTT

(b) Position 564 of the total alignment

Position 564	G	G	K	A	F	A	T	Y
Ipomoea batata	GGG	GGA	AAA	GCA	TTT	GCG	ACC	TAT
Vitis vinifera	GGA	GGA	AAA	GCA	TTC	GCT	ACA	TAC
Vitis vinifera	GGA	GGA	AAA	GCA	TTC	GCT	ACA	TAC
Ricinus communis	GGA	GGA	AAA	GCA	TTT	GCG	ACA	TAC
Populus trichocarpa	GGA	GGA	AAA	GCA	TTT	GCA	ACA	TAT
Citrus hybrid cultivar	GGA	GGG	AAA	GCA	TTT	GCA	ACA	TAT
Cucurbita maxima	GGA	GGA	AAA	GCA	TTT	GCT	ACA	TAT
Arabidopsis thaliana	GGG	GGT	AAA	GCA	TTT	GCC	ACC	TAT
Arabidopsis thaliana	GGG	GGT	AAA	GCA	TTT	GCC	ACC	TAT
Arabidopsis thaliana	GGG	GGT	AAA	GCA	TTT	GCC	ACC	TAT
Arabidopsis thaliana	GGG	GGT	AAA	GCA	TTT	GCC	ACC	TAT
Vicia faba	GGA	GGA	AAG	GCA	TTT	GCA	ACG	TAC
Spinacia oleracea	GGA	GGA	AAA	GCT	TTT	GCC	ACA	TAT
Oryza sativa	GGG	GGA	AAA	GCA	TTC	GCG	ACT	TAC
Oryza sativa	GGG	GGA	AAA	GCA	TTC	GCG	ACT	TAC
Sorghum bicolar	GGA	GGG	AAA	GCA	TTT	GCA	ACA	TAC
Zea mays	GGA	GGG	AAA	GCG	TTT	GCA	ACA	TAC
Zea mays	GGA	GGG	AAA	GCG	TTT	GCA	ACA	TAC
Triticum aestivum	GGA	GGG	AAA	GCA	TTT	GCA	ACA	TAC

Primers design for starch synthase

The forward primer of the starch synthase enzyme, SS 13F was designed from the domain KTGGLGDV, located at position 13 of the multiple alignment of the proteins conserved for starch synthase. Thus comparing the nucleotides specific for the domain KTGGLGDV, across the plant species, primer SS 13F of starch synthase was derived as: 5'AAAACAGGTGGBCTBGGWGATG (Tm =

57.7°C, Mol. Wt = 489 µg), where, B = C, T or G; W = A or T (Table 2a). To get the reverse primer 109R, the forward primer (i.e. primer 109F) was designed from position 109 of the conserved domains, FCKAAVE (Table 2b). Comparing the nucleotides specific for the domain FCKAAVE (position 109) primer 109F was obtained as: primer 109F = 5'TTRTGCAAYGCNGCWGTVGAG (Table 2b), where R= C, T; W= A, G; N= C, T, A, G; Y= A, T, G; V= C, T, and G. Then, reversing the above, primer 109 R of starch synthase was obtained finally as: 5'CTCVACWGCNGCYTTGCARAA (Tm = 71°C, Mol Wt = 553 µg).

Table 2: Conserved protein domain of starch synthase enzyme
(a) Position 13 of the total alignment

Position 13	K	T	G	G	L	G	D	V
Triticum aestivum	AAA	ACA	GGT	GGT	CTG	GGA	GAT	GTT
Triticum aestivum	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Aegilops geniculata	*	*	GGT	GGT	CTG	GGA	GAT	GTT
Hordeum vulgare	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Zea mays	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Zea mays	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Oryza sativa	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT
Oryza sativa	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT
Vitis vinifera	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTC
Manihot esculenta	AAA	ACA	GGT	GGC	CTT	GGT	GAT	GTC
Glycine max	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Glycine max	AAA	ACA	GGT	GGT	CTT	GGA	GAT	GTT
Ricinus communis	AAA	ACA	GGT	GGC	CTC	GGA	GAT	GTT
Lotus aborigines	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT
Populus trichocarpa	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT
Amaranthus cannabinus	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT
Vigna unguiculata	AAA	ACA	GGT	GGG	CTT	GGT	GAT	GTA
Vigna unguiculata	AAA	ACA	GGT	GGG	CTT	GGT	GAT	GTA
Pisum sativum	AAA	ACA	GGC	GGG	CTT	GGA	GAT	GTT
Arabidopsis thaliana	AAA	ACA	GGT	GGC	CTT	GGA	GAT	GTA
Arabidopsis thaliana	AAA	ACA	GGT	GGC	CTT	GGA	GAT	GTA
Phaseolus vulgaris	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTA
Solanum tuberosum	AAA	ACA	GGT	GGG	CTT	GGA	GAT	GTT

PCR reactions

Two PCR reactions were carried out in all. The first reaction (reaction 1), involved the use of the primers designed for the starch phosphorylase enzyme (STPH 1F and STPH 564R) whilst the second reaction (reaction 2), made use of the primers designed for the starch synthase enzyme (SS 13F and SS 109R). The annealing temperature for reaction 1 was 55 °C, and at this temperature, two PCR bands, one being the required band of about 563 bp, were obtained (Fig 1). The DNA fragments were isolated from the band containing the PCR product, as already described above, prior to cloning. Reaction 2 was maintained at annealing temperature of 50 °C, producing a PCR product (band) of about 96 bp as anticipated, and the DNA fragments from this PCR product were also isolated prior to cloning.

(b) Position 109 of the total alignment

position 109	F	C	K	A	A	V	E
Triticum aestivum	TTC	TGC	AAG	GCC	GCT	GTT	GAG
Triticum aestivum	TTC	TGC	AAG	GCC	GCT	GTC	GAG
Aegilops geniculata	TTC	TGC	AAG	GCC	GCT	GTT	GAG
Hordeum vulgare	TTC	TGC	AAG	GCC	GCT	GTC	GAG
Zea mays	TTT	TGC	AAG	GTT	GCT	GTT	GAG
Zea mays	TTT	TGC	AAG	GTT	GCT	GTT	GAG
Zea mays	TTT	TGC	AAG	GTT	GCT	GTT	GAG
Oryza sativa	TTT	TGT	AAG	GCT	GCT	GTT	GAG
Oryza sativa	TTT	TGT	AAG	GCT	GCT	GTT	GAG
Oryza sativa	TTT	TGT	AAG	GCT	GCT	GTT	GAG
Vitis vinifera	TTT	TGC	AAG	GCA	GCT	ATT	GAG
Manihot esculenta	TTT	TGC	AAA	GCT	GCT	GTT	GAG
Glycine max	TTT	TGC	AAG	GCA	GCT	GTT	GAG
Glycine max	TTT	TGC	AAG	GCA	GCT	GTT	GAG
Ricinus communis	TTT	TGC	AAA	GCA	GCT	ATT	GAG
Lotus aborigines	TTT	TGC	AAG	GCA	GCT	GTC	GAG
Populus trichocarpa	TTT	TGC	AAA	GCA	GCT	GTT	GAG
Amaranthus cannabinus	TTC	TGT	AAG	*	GCA	GTT	GAG
Vigna unguiculata	TTT	TGC	AAG	GCA	GCA	GTG	GAG
Vigna unguiculata	TTT	TGC	AAG	GCA	GCG	GTT	GAG
Pisum sativum	TTT	TGC	AAG	GCG	GCG	GTT	GAG
Arabidopsis thaliana	TTT	TGC	AAG	GCT	GCT	GTT	GAG
Arabidopsis thaliana	TTT	TGC	AAG	GCT	GCT	GTT	GAG
Phaseolus vulgaris	TTT	TGC	AAG	GCA	GCA	GTT	GAG
Solanum tuberosum	TTT	TGC	AAA	GCA	GCG	ATT	GAG

Colony purification of bacterial strains and sequencing

White colonies from reactions 1 and 2 were involved in PCR reactions using the same reaction conditions and the original primers, but the number of cycles was reduced from 35 to 15. The colonies that produced the right band size were thus identified. Other PCR reactions, involving the use of M13 primers instead of the original primers, were also carried out, using colonies identified from this reaction. The PCR products produced from reactions with the original primers were compared with the bands produced with the M13, and the samples that gave the right size of band from the two sets of primers (original primers and the M13) were noted (Fig 2). The PCR samples of reaction 1 containing the M13 primers, which yielded the required band size (563 bp) were purified using the PCR purification kit as detailed above (section 2.6.3), prior to sequencing. The *E. coli* plasmid miniprep DNA preparation (section 2.6.2) was conducted on colonies identified from reaction 2. The sequences generated for starch phosphorylase and starch synthase (Fig 3) were similar to the sequences of these enzymes in sweet potato and sorghum, respectively. Figure 4 shows the sequence alignments of these enzymes as found in hyacinth and the other plant species.

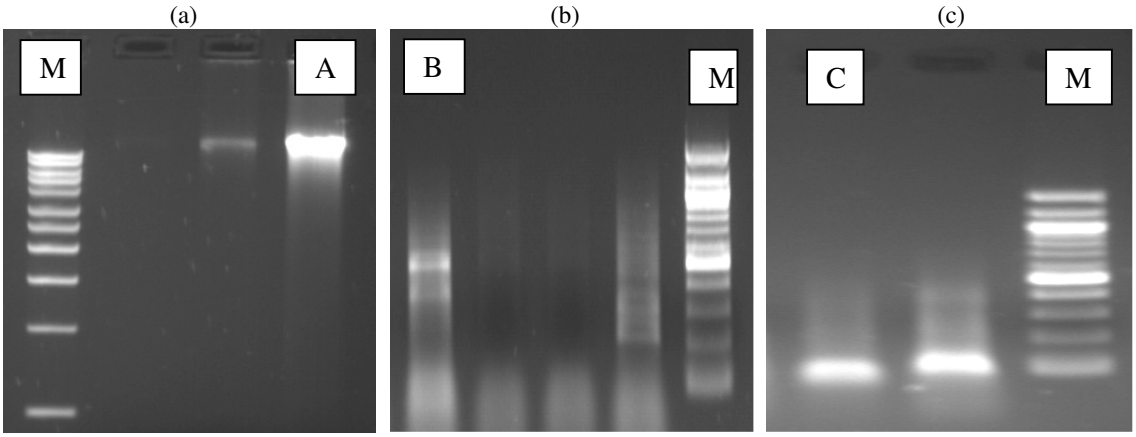
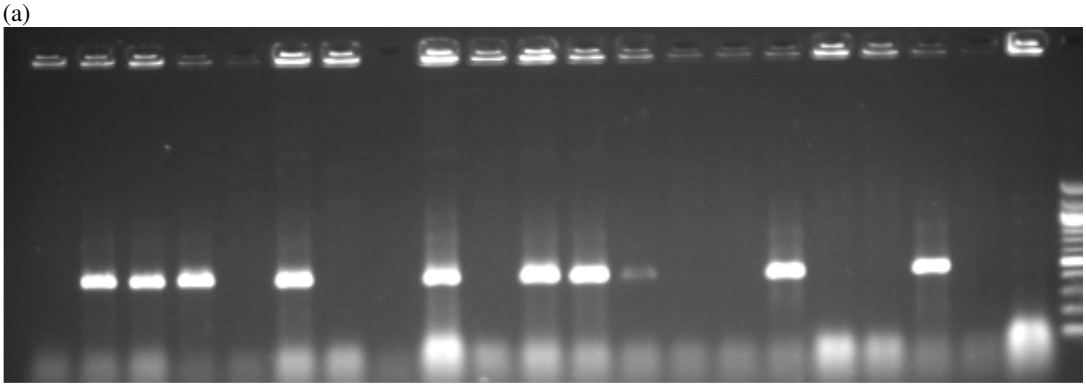
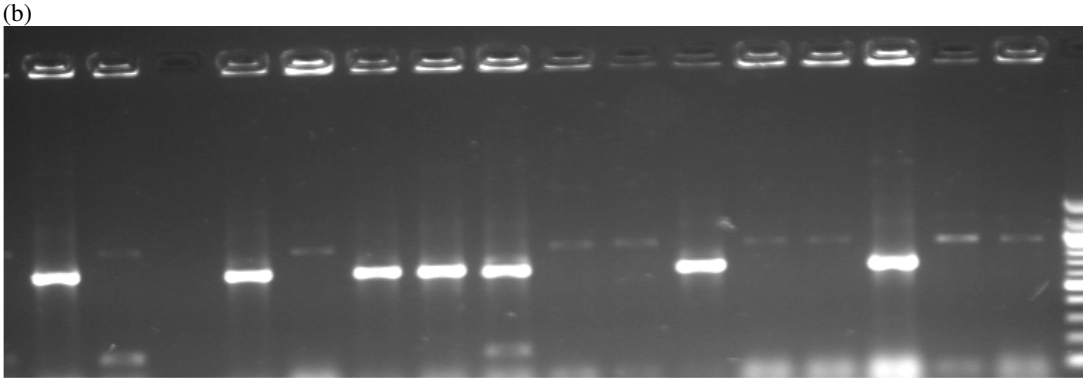


Fig 1: (a) Hyacinth DNA (A), (b) PCR product (B, 563 bp), and (c) PCR product (C, 96 bp). PCR products B and C were obtained by using the starch phosphorylase and starch synthase primers, respectively. M is the 100 bp marker used

(a)



(b)



(c)

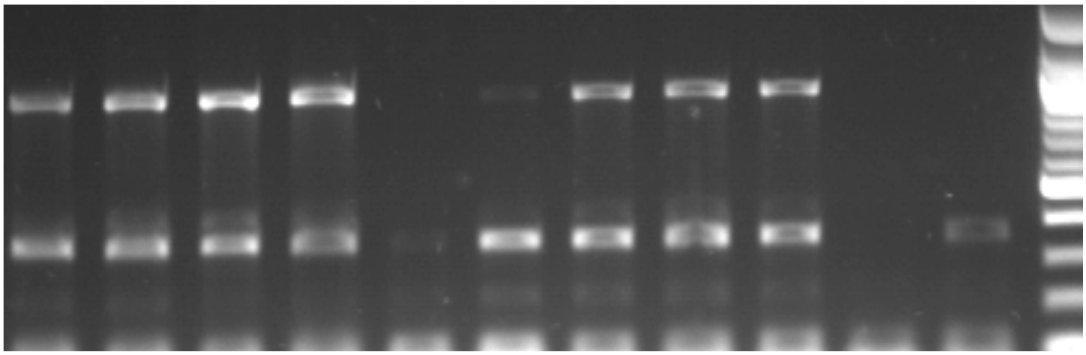


Fig 2: PCR involving (a) clones of starch phosphorylase with the original primers, (b) starch phosphorylase clones with M13 primers, and (c) starch synthase with M13 primers. M is the 100 bp marker.

(a)
GGGAGGCTTGCATCGTGCTTGAAGAAAAGGTGAAGATTTTCACTGATCATAAAAGCTTGAG
GGTTATGGATTGAGGTACAAACATGGACTGTTCAAGCAACGTATCACCAAAGCAGGACAAG
AGGAGATTGCTGAAGATTGGCTGGAGAAATTCAGTCCCTGGGAAGTTGCAAGGCATGATGA
GCAGACTTCCAGCAGTGATGCTTCTTACCTCACAGAGCAGACTTCAGCAGAGCTTGTGCGGG
ATGGACATTGAGGTTATTCTTTCAGGAGAGTCAGCTTCACTGATGAGTTTGCAGATTCAGTC
GACTTTAGTGGATCGGATCAAGGCTGGACAAGCTGGAGATCCGAGATTGCAGAAGATCAGA
GCAGAGGTTCTAGCAGGCCAGAGACCAGAGTTTCAGATTCACGAGGATGGATCGTTGCGCT
ATGGAGTCAGATTGTGTGTTCCTTCTGGTGACATCAGAGATGAGCTTTTGTTGGAGGCAAGG
CATTCGCAACATATA

(b)
CATGCACCGTGTAGTAGCTGATATCACGGTTGAACGTGGTCGTCGTGGCTGCTGAGTGTTCT
CCCTGGTG CAAAACAGGTGGTCTGGGAGTGGCGGTGTACA

Fig 3: Sequencing of the clones identified from reaction 1 (a) and reaction 2 (b). That is sequences generated from hyacinth for starch phosphorylase and starch synthase, respectively.

(a)

Sweet potato STPH	62	GYGLRYKHGLFKQRITKAGQEEIAEDWLEKFSPWEVARHD	181
		GYGLRYKHGLFKQRITKAGQEEIAEDWLEKFSPWEVARHD	
Hyacinth STPH	21	GYGLRYKHGLFKQRITKAGQEEIAEDWLEKFSPWEVARHD	60

(b)

Sorghum bicolor SS	30	LNVVVVAAECSPWCKTGGLGVAV	98
		+NV+VVAAECSPWCKTGGLG V	
Hyacinth SS	252	MNVIVVAAECSPWCKTGGLGDVV	274

Fig 4: Sequence alignments of (a) Hyacinth starch phosphorylase (STPH) with sweet potato starch phosphorylase, and (b) Hyacinth starch synthase (SS) with the starch synthase of *Sorghum bicolour*. Sequences were generated using the blastx on the nr database (NCBI). Identities are highlighted in red colour.

DISCUSSION

The sequences generated for starch phosphorylase and starch synthase from hyacinth were similar to the sequences of these enzymes from a number of plants. As for example, the hyacinth starch phosphorylase sequence produced from the present study was similar to the starch phosphorylase sequences from *Ipomoea batata*, *Ricinus communis*, *Arabidopsis thaliana, Citrus*, *Vitis vinifera*, *Oryza sativa*, *Triticum aestivum*, *Vitis vinifera*

and *Zea mays*, but that of *Ipomoea batata* was the closest in similarity, according to the blast search. The observed similarity of the hyacinth starch phosphorylase sequence produced from this study to that of *Ipomoea batata*, and the other plant species is due to the fact that, it was from these plants species that the conserved domains (Kapitononov and Yu, 1999) were selected during the design of primers for this enzyme (see Table 1a and b). In fact, the hyacinth starch phosphorylase sequence (Hyacinth STPH) generated in the present study is identical at the protein level to that of *Ipomoea batata*. This is not surprising, as the sequence is well conserved. This sequence could be used to redesign primers which could have been used to get a bigger part of the gene from hyacinth DNA. Similarly, the insert could have been used to probe a hyacinth genomic library to get the whole gene. Additionally, this sequence could be used to design a primer which could be used for RT-PCR analysis.

As in the case of the hyacinth starch phosphorylase sequence, the sequence generated for starch synthase in this work was similar to the starch synthase sequences from a number of plant species from which the primers for this enzyme were designed: *Sorghum bicolor, Aegilops tauschii, Hordeum vulgare, Zea mays, Triticum aestivum, Oryza sativa, Amaranthus cruentus*. The starch synthase sequence from *Sorghum bicolor* was the closest in terms of similarity to the starch synthase sequence produced from hyacinth. This observation agrees with that made by Salehuzzaman *et al*. (1993) who also reported that the sequence generated from starch synthase clone constructed from cDNA library from cassava tuber in their study had 74% identity with that of potato, but the percentage identity from other the other plant species varied from 60 to 72 %. In general, even though the hyacinth starch synthase sequence from produced from this study definitely corresponds to starch synthase; it is clearly a short segment. Some of it may be from the primer, but there is a bit which is also from hyacinth. The sequence could be used to make a specific primer with a view to isolating more of the gene from hyacinth DNA. Alternatively, the insert could be used to probe a hyacinth genomic library to get the whole gene (Lutz, 2003; Zhang *et al*., 2002). Designing a specific primer rather than a degenerate one would have a much greater chance of success so even a short sequence which contains no degeneracy will be much more successful.

In this work, though fragments of two of the enzymes involved in starch metabolism of hyacinth have been isolated and sequenced, difficulties were encountered in this study particularly during the design of the primers. There were virtually no close relatives of hyacinth in the databases and conserved domains of a number of plant species ranging from monocotyledonous species to dicotyledonous ones were used. Also, in carrying out the PCR reactions, it was difficult for degenerate primers to prime specifically because at a high annealing temperature reactions did not produce any PCR product, and too low an annealing temperature gave rise to mixed priming. Studies in the future on this area of research could be possible, because the sequences generated can be used to design new primers which are specific to hyacinth. This means that there will be much greater chances of getting more specific products than the present study. Full characterisation of genes can be made by making and probing genomic libraries and isolating clones from cDNA libraries and the gene expression of these enzymes can be studied by using specific probes for RT-PCR analysis (Tangphatsornruang *et al*., 2005).

CONCLUSION

The hyacinth starch phosphorylase and starch synthase sequences generated in this work were similar to those of *Ipomoea batata* and *Sorghum bicolour*, respectively. These sequences could be used to design new primers which are specific to hyacinth with a view to isolating more of the gene from hyacinth DNA. In fact they could be used to probe a hyacinth genomic library to get the whole gene and this will facilitate any future studies on these genes for instance in making and probing genomic libraries, and isolating clones from cDNA libraries. There will also be the need to investigate the expression of these genes by using specific probes for RT-PCR.

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