CLONING AND EXPRESSION STUDIES OF

STARCH PHOSPHORYLASE

FROM *SOLANUM TUBEROSUM*

ANUJ MARKAND BHATT

Thesis Submitted for the Degree of Doctor of Philosophy

Faculty of Science,

University of Glasgow.

January, 1991

ACKNOWLEDGEMENTS

I would like to thank the following people:

Professors M.D. Houslay, C.A. Fewson and J.R Coggins for making the facilities of the Department of Biochemistry available for this research project.

Prof. John T. Knowler, for introducing me to an a-peeling topic of research, for his enthusiastic supervision, 'tips' on the scientific approach and for his patience. Dr. Gareth Jenkins for interesting and helpful discussions.

My sponsors - The CVCP (U.K.) for assistance with my fees in the form of an ORS award, the University of Glasgow, Satmarg Foundation, Maneckji Neterwala Foundation; and above all my parents, and Usha & Ramesh Shukla, for their support and encouragement.

All the past "inmates" of the Sweetie Lab. - Margot, Linda, Ursilla, Colin, Brian, and Mark, for the discussions on scientific and non-scientific matters. Susy and Stewart for their help with some of the techniques. A special thanks to the generous folk of C-30, friends in B4a and to the present members of C-13. I am also grateful to Atip, Misbah, Ranjit and Yasin for their friendship and help.

Shilpa for her support and company, which has made all the difference.

ABBREVIATIONS

The abbreviations used in this thesis are in agreement with the recommendations of the editors of the Biochemical Journal {Biochem. J. (1983) **209**, 1-27.}, except the following:

SDS	Sodium dodecyl sulphate
kb (kbp)	kilo bases (kilo base pairs)
LMP-agarose	low melting point-agarose
SSC	Standard saline citrate
RNase A	Pancreatic ribonuclease A
cpm	counts per minute
poly(A^+)mRNA	polyadenylated mRNA species
SSPE	standard saline phosphate EDTA
IPTG	isopropyl β-D thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indolyl-β-D galactoside
3PGA	3-phosphoglycerate
TP	Triose phosphate
A_{xxx}	Absorbance at xxx nm (xxx=260, or = 280, etc)
SPS	Sucrose phosphate synthase
Fru 2, 6 P2	Fructose 2, 6 bisphosphate
Fru 2, 6-P2ase	Fructose 2, 6-bisphosphatase
Fru 1,6 Pase	Fructose 1, 6 bisphosphatase
GA	Gibberellic acid
ABA	Abscisic acid
GUS	β-glucuronidase
PEG	polyethylene glycol
DAHP synthetase	3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase

CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

The aim of this project was to study starch phosphorylase gene expression in potato tubers during different physiological conditions when starch was mobilised, like wound healing and sprouting. One of the objectives was to clone plastidic potato tuber starch phosphorylase cDNA. cDNA libraries were constructed using mRNA from wounded and unwounded tuber tissue as templates for cDNA synthesis. Based on the amino acid sequence (Nakano & Fukui, 1986) of specific regions of potato tuber starch phosphorylase, two different sets of mixed oligonucleotide probes (called SP1 and SP2) were prepared and subsequently used to screen the cDNA libraries for starch phosphorylase recombinants.

Preliminary screening of 40,000 pfu , each from the wounded and the unwounded tuber cDNA library with ^{32}P end-labelled oligonucleotide SP1, resulted in the identification of two putative starch phosphorylase recombinants from the unwounded tuber library and none from the wounded tuber library. Therefore the wounded tuber cDNA library was not used in further rounds of screening. Finally, on screening another 150,000 pfu from the unwounded tuber library, thirteen recombinants were selected that hybridised to the oligonucleotide probe SP1. Three of these recombinants also hybridised to the 20 mer, SP2. The three (λ2.3, λ2.5, λ2.9) recombinants that were double positives were selected for further investigations. *Eco*RI restriction digests of the recombinants were subjected to Southern blot analysis, and the putative starch phosphorylase cDNA inserts were identified. The ~1.4 kbp cDNA insert of λ2.5 hybridised to both the oligonucleotide probes, and also cross-hybridised with the inserts of λ2.3 and λ2.9. Hence the ~1.4 kbp cDNA insert of λ2.5 was selected for further characterisation and it was subcloned into the *Eco*RI site of pTZ18U (and the recombinant was called pPSP1). During the course of this study two groups independently reported the cloning of potato tuber starch phosphorylase (Brisson *et al.*, 1989; Nakano *et al.*, 1989). The restriction map and the partial nucleotide

sequence of pPSP1 were compared and were similar to the data published for starch phosphorylase cDNA. The ~1.4 kbp insert of pPSP1 was partially sequenced on one strand and its partial sequence showed complete identity with the published nucleotide sequence of starch phosphorylase cDNA. It was concluded that the ~1.4 kbp insert of pPSP1 was a partial cDNA clone of potato tuber starch phosphorylase.

The ~1.4 kbp cDNA insert of pPSP1 was used as a probe in northern blot analysis to demonstrate that phosphorylase mRNA was most abundant in tubers but was also detectable in stolon, root, stem and leaf tissue. Starch phosphorylase mRNA levels were greatly reduced in wounded stem and tuber tissue. The wounding-induced decrease in starch phosphorylase mRNA levels in tuber slices was not reversed in the presence of sucrose or mannitol. Starch phosphorylase and patatin mRNA levels were altered in sprouting tubers and in developing sprouts, there was an increase in the levels of starch phosphorylase and patatin mRNA.

CHAPTER 1. INTRODUCTION

1.1 Starch synthesis

All higher plants produce starch at some stage of their development. Starch is a polymer of glucose and contains two distinct polysaccharides, amylose, a linear α-1, 4 glucan, and amylopectin, a highly branched polymer containing short amylose chains (18-25 glucose units) linked by α-1, 6 branch points. Depending upon their source, starch granules are made up of different proportions of amylose and amylopectin, each having a wide range of molecular sizes. Starch exists as a water-insoluble granule, in the chloroplasts of photosynthetic tissue, and amyloplasts of non-photosynthetic tissue (Dennis & Miernyck, 1982). Each of these plastids has different dynamics and physiology of starch accumulation and based on this, starch can be classified into two types:-

Transitory starch has a rapid turnover and is the form of starch deposited in green leaves. It has the shortest cycle of accumulation and depletion and is subject to diurnal variation. Thus, in the presence of sunlight and during periods of high photosynthetic activity, starch is synthesised. This is then broken down during the hours of darkness and its breakdown products are used to synthesise sucrose, which is transported to other tissues.

Reserve starch, on the other hand, has an extended existence and is predominantly formed in fruits, seeds and tubers. In fleshy fruits like bananas, starch accumulates over a few weeks and is then broken down quickly (Shantha & Siddappa, 1970; Palmer, 1971). Another pattern of reserve starch accumulation is characterised by the accumulation of starch up to a clearly defined stage of development, ripening or maturity, after which starch accumulation ceases. This pattern is typical of seeds and tubers and there may be a considerable passage of time before the mobilisation of starch begins. In the case of seeds it occurs during germination and in tubers, during sprouting (Jenner, 1982). However, this distinction between "assimilatory" starch of leaves and "storage" starch of seeds, fruits and tubers may be invalid, as it appears that starch in mature leaves may serve

a storage function (Dickson & Larwood, 1973).

In amyloplasts, the starch granule displays characteristic morphological features, that depend upon their source. The biochemical basis of this variation in granule morphology is obscure. Visual analysis of the process of starch granule deposition has been performed for a few species (Jenner, 1982). At first, lipid structures form sacs in the stroma of plastids, a particulate material then becomes associated with the sacs. This material resembles starch. The granule arises in place of the particulate material in a swift transition (Banks *et al.*, 1974). By studying the incorporation of ^{14}C into starch granules from potato tubers (Badenhuizen & Dutton, 1956), tobacco leaves (Porter *et al.*, 1959) and beans (Nikuni, 1978) it was demonstrated that growth of starch granules was by apposition.

1.1.1 Route of Starch Synthesis

It was originally believed that the synthesis of α-1, 4 glucosidic linkages in plants was catalysed by phosphorylase (Hanes, 1940) as shown in reaction [1]. However, in 1961, Leloir *et al.* demonstrated the synthesis of α-1, 4 glucosidic linkages in plant extracts using either UDP-glucose or ADP-glucose, the latter was a better substrate, both in terms of V_{max} and affinity (Recondo & Leloir, 1961). It is now agreed that the starch synthase reaction [Reaction 2] is the main reaction involved in the synthesis of α-1, 4 glucosidic linkages in starch. ADP-glucose and UDP-glucose are synthesised by pyrophosphorylase reactions [Reactions 3, 3'] (Espada, 1962) or by reversal of the sucrose synthase reaction [Reaction 4] (Cardini *et al.*, 1955; DeFekete & Cardini, 1964). The α-1, 6 branch point linkages of amylopectin are formed by the action of branching enzyme (Q enzyme) on linear chains of α-1, 4 linked glucose residues [Reaction 5].

$$\alpha\text{-D-glucose-1-P} + \{\text{Glucosyl}\}_n \rightleftharpoons \text{Pi} + \{\text{Glucosyl}\}_{n+1} \qquad [1]$$

$$ADP(UDP)glucose + \{Glucosyl\}_n \rightleftharpoons ADP(UDP) + \{Glucosyl\}_{n+1} \quad [2]$$

$$ATP + \alpha\text{-glucose-1-P} \rightleftharpoons ADP\text{-glucose} + PPi \quad [3]$$

$$UTP + \alpha\text{-glucose-1-P} \rightleftharpoons UDP\text{-glucose} + PPi \quad [3']$$

$$Sucrose + ADP\ (UDP) \rightleftharpoons Fructose + ADP\ (UDP)\ glucose \quad [4]$$

-G-G-G-G-G-G-G-G-G-G- [5]

↓ scission of nonterminal α-1,4 linkage {G=glucose units}

-G-G-G-G-G-G -G-G-G-G-G-G

-G-G-G-G ⟶ formation of α-1,6 linkage -G-G-G-G-G-G-G-G-G-G-G

In plants, enzymatic generation of α-1, 4 glucosidic linkages could take place via three possible routes-

{i} through the action of phosphorylase

{ii} through the incorporation of UDP-glucose

{iii} through the incorporation of ADP-glucose

1.1.2 Evidence for route of starch synthesis

At least in leaves, the pathway of physiological significance appears to be through ADP-glucose incorporation. This view is based on the following observations.

Leaf starch synthases are highly specific for ADP-glucose (Cardini & Frydman, 1966; Ghosh & Preiss, 1965), whereas with UDP-glucose granule-bound or soluble starch synthases from leaves show virtually no activity. Soluble starch synthases of most nonphotosynthetic tissues (Cardini & Frydman, 1966;

Frydman *et al.*, 1966) exhibit a similar specificity for ADP-glucose as a substrate. Only the granule-bound starch synthase of reserve tissue is able to utilize both, UDP-glucose and ADP-glucose. The granule-bound enzyme also has a 15-30 fold higher Km for UDP-glucose compared to its Km for ADP-glucose (Cardini & Frydman, 1966). The concentrations of ADP-glucose and UDP-glucose are roughly equal in rice grains (Murata *et al.*, 1965). Taking into consideration the physiological levels of the two sugar nucleotides and the Km for granule-bound starch synthase, ADP-glucose would be the preferred substrate. The soluble starch synthases from a range of plant species have 10 fold lower Km values for ADP-glucose (0.1 mM-0.3 mM) than the granule-bound form. It is likely, therefore, that the soluble starch synthases are more active than the granule-bound enzyme.

Work on maize mutants also provided evidence that at least 75% of starch was synthesised via ADP-glucose. Two maize endosperm mutants *shrunken-2* (sh-2) and *brittle-2* (bt-2) had starch levels reduced to 25% of that found in normal endosperm (Cameron & Teas, 1954; Creech, 1965). The ADP-glucose pyrophosphorylase activity of the mutants was 10% of that found in normal endosperm (Tsai & Nelson, 1966; Dickinson & Preiss, 1969b). Also, accumulation of starch in the developing endosperm could be correlated to an increase in starch synthase and ADP-glucose pyrophosphorylase activity, whereas an increase in starch phosphorylase and UDP-glucose pyrophosphorylase activity could not be linked to an increase in starch content (Ozbun *et al.*, 1973; Turner, 1969; Moore & Turner, 1969; Sowokinos, 1976).

Lin *et al.* (1988a) have reported an *Arabidopsis* starch deficient mutant. The mutant lacked ADP-glucose pyrophosphorylase activity. Western blotting experiments, using antibodies specific for the spinach ADP-glucose pyrophosphorylase subunits, demonstrated the presence of two cross-reactive polypeptides in the wild type, but these polypeptides were missing in the mutant. These data confirmed that in chloroplasts, starch synthesis was entirely through ADP-glucose pyrophosphorylase.

Recently, Schulman and Ahokas (1990) reported a new endosperm mutant in barley, *shx*, which has 25% of normal starch content. This mutant had reduced sucrose synthase, UDP-glucose pyrophosphorylase and ADP-pyrophosphorylase activities. It was claimed that the mutation *shx* primarily resulted in the decreased activity of the soluble, primer-independent starch synthase.

1.1.3 Enzymes involved in starch synthesis

1.1.3.1 ADP-glucose pyrophosphorylase

As described earlier, this enzyme plays a key role in the biosynthesis of α-1, 4 glucans in plants. ADP-glucose pyrophosphorylase is activated by 3-P-glycerate (3PGA) and inhibited by Pi. The spinach leaf enzyme has been studied extensively (Ghosh & Preiss, 1966; Preiss *et al.*, 1967) and the kinetic properties of leaf extracts of barley, butter lettuce, kidney beans, maize, peanut, rice, sorghum, sugar beet, tobacco and tomato were similar to those observed for the spinach leaf enzyme (Sanwal *et al.*, 1968).

The native molecular weight of the spinach leaf enzyme was found to be about 210,000 (Ribereau-Gayon & Preiss, 1971) and the protein is a tetramer of two polypeptides having molecular weights of 51,000 and 54,000 (Copeland & Preiss, 1981; Morell *et al.*, 1987). Arabidopsis, wheat, rice and maize leaves (Krishnan *et al.*, 1986) had immunologically cross-reactive polypeptides of similar molecular weight.

ADP-glucose pyrophosphorylases from nonphotosynthetic tissues, including maize endosperm (Dickinson & Preiss, 1969a), etiolated peas, wheat germ, potato tuber, carrot roots and avocado mesocarp (Preiss *et al.*, 1967; Sowokinos, 1976), are also activated by 3PGA. The potato tuber enzyme is very sensitive to activation by 3PGA and is practically dependent upon it for the synthesis of ADP-glucose (Sowokinos & Preiss, 1982). It is also inhibited by Pi

(Sowokinos & Preiss, 1982). The purified potato tuber enzyme is also made up of two polypeptides that differ slightly from the spinach leaf enzyme in their net charge and molecular weights. Antiserum specific for the 51 kDa subunit of the spinach leaf ADP-glucose pyrophosphorylase cross-reacts with the small subunit of the potato tuber enzyme, but that specific for the 54 kDa spinach subunit does not cross-react with either of the tuber subunits (Okita *et al.*, 1990). Immunocytochemical localisation shows that ADP-glucose pyrophosphorylase is located exclusively in the amyloplasts of developing tuber cells (Okita *et al.*, 1990). The respective roles of the two enzyme subunits are unknown but both polypeptides bind an analog of the activator 3PGA (Morell *et al.*, 1988; Preiss *et al.*, 1987)

Lin *et al.* (1988b) have described a starch deficient mutant of *Arabidopsis* lacking the 54 kDa subunit of the leaf ADP-glucose pyrophosphorylase. The mutant had leaf ADP-glucose pyrophosphorylase activity that was 5% of that found in the wild type but it accumulated starch to 40% of the wild type levels. The level of the 51 kDa polypeptide was reduced to about 4% of the level found in wild type. With this and similar studies it is difficult to know whether the mutant enzyme allows the accumulation of starch to 40% of the wild type levels or whether other enzymes that are able to catalyse starch synthesis compensate for the mutant enzyme.

1.1.3.2 Starch synthase

As described earlier, starch synthase catalyses the formation of α-1, 4 glycosidic linkages by transferring a glucose unit from ADP-glucose or UDP-glucose to a glucan primer. Multiple forms of soluble starch synthases exist in leaves and reserve tissues (Ozbun *et al.*, 1971a, 1971b; 1972; Hawker & Downton, 1974; Hawker *et al.*, 1972). The soluble and granule-bound starch synthases of maize have been classified, on the basis of their kinetic and physical

properties into two groups - Type I and Type II (Preiss *et al.*, 1985). Type I starch synthases preferred glycogen as a primer and had lower molecular weight (72,000), whereas type II starch synthases had a higher molecular weight (95,000) and preferred amylopectin, over glycogen (Hawker *et al.*, 1974; Preiss *et al.*, 1985). In addition, type I enzymes had (0.5M) citrate dependent, primer independent starch synthase activity (Preiss & Levi, 1980, and references therein). Type II enzymes showed little or no such activity in the presence of 0.5M citrate. As the two types of soluble starch synthases did not show immunological cross-reactivity, they are probably different gene products (MacDonald & Preiss, 1985).

A variety of techniques have been used to solubilise the granule-bound starch synthase activity. Perdon *et al.*(1975) used 50% dimethyl sulfoxide, however the enzyme was still attached to the starch grains. Urea and pullulanase were used by Sasaki and Kainuma (1980) to solubilise sweet potato granule-bound starch synthase. The enzyme from maize was released by treating pulverised maize starch with α-amylase and amyloglucosidase. The activities released were then separated by chromatography and characterised (MacDonald & Preiss, 1983; 1985). Chang (1980a) has isolated a different type of soluble and granule-bound starch synthase, which has greater activity with UDP-glucose than with ADP-glucose. However doubts have been cast on the authenticity of these enzymes, since the products of the reactions were not characterised (Preiss, J., 1988).

1.1.3.3 Branching Enzyme

Branching enzyme catalyses the transfer of a short chain of glucose residues from α(1->4)-linkage to α (1->6)-linkage (Manners, 1974). Potato tuber branching enzyme has been studied extensively (Drummond *et al.*, 1972; Borovsky *et al.*, 1975). The potato tuber branching enzyme has a molecular weight of 85,000 and is composed of only one subunit. Multiple forms of branching enzyme were detected in spinach leaf (Hawker *et al.*,1974), maize

endosperm, developing pea seeds (Matters & Boyer, 1981; 1982), developing sorghum seeds (Boyer, 1985) and in Teosinte seeds (Boyer & Fisher, 1984). Different forms of branching enzyme were purified from maize endosperm by Boyer and Preiss (1978a; 1978b). Enzymes were purified from three different fractions and were called branching enzyme-I (BE-I), branching enzyme-IIa (BE-IIa) and branching enzyme-IIb (BE-IIb). BE-I had a molecular weight of 89,000, whereas BE-IIa and BE-IIb were approximately 80,000; all three enzymes were monomers. Singh and Preiss (1985) characterised the three enzymes by comparing their peptide maps, their amino acid composition and by studying their immunological cross reactivity with an array of monoclonal antibodies. They concluded that there was some homology between all three forms of branching enzyme, although BE-I exhibited some major structural differences when compared with BE-IIa and BE-IIb. BE-IIa and BE-IIb appeared to be very similar.

1.1.4 Multiplicity of enzymes of starch synthesis

*sh*2, *bt*2, and *shx* mutants have already been discussed in Section 1.1.2. The *waxy* (*wx*) mutant of maize has a starchy endosperm that has only amylopectin since it lacks amylose (Sprague *et al.*,1943). In maize, the *waxy* locus regulates the formation of amylose in the endosperm. Nelson and Rines (1962) observed that the granule-bound starch synthase activity was almost absent in waxy mutant endosperm. This result was confirmed for several *wx* alleles by Nelson *et al.*(1978), who also found a small amount of granule-bound starch synthase activity that had a lower Km for ADP-glucose. Tsai (1974) observed that starch synthase activity varied linearly with *wx* gene dosage and proposed that starch synthase was the *wx* gene product.

When solubilised starch granules were subjected to electrophoresis, a major protein band with a molecular weight of 60,000 was found to be missing in most *waxy* mutants (Echt & Schwartz, 1981) as were three minor proteins of

higher molecular weight. However it has not been proved that the 60,000 molecular weight protein is starch synthase (Shure *et al.*, 1983). Recently, Smith (1990) has demonstrated that the *waxy* protein of pea did not have significant starch synthase activity, and that all of the starch-granule-bound starch synthase activity was attributed to a protein which was a different size from, and only very weakly antigenically related to, the *waxy* protein. Smith (1990) has suggested a re-examination of the identity of the starch-granule-bound starch synthase and the role of the *waxy* protein, in different plant species.

The amylose-extender mutant (*ae*) has starch containing a higher proportion of amylose, along with anomalous amylopectin. There are fewer branch points in the amylopectin from the *ae* mutant as compared to the wild type. The dull endosperm mutant, whose starch components have not been extensively characterised, also contains higher proportions of amylose than normal endosperm. In contrast, the *su-1* mutant has a lower starch content with the same proportion of amylose as normal endosperm. In addition, the *su-1* mutant endosperms contained phytoglycogen, which has a greater degree of α-1, 6 branch points than amylopectin.

Extracts from normal and mutant endosperms were analysed for the presence of various starch synthases and branching enzymes. Boyer and Preiss (1978a) and Preiss and Boyer (1980) found that almost all of the branching enzyme activity associated with starch synthase-I was missing in *ae* endosperm extracts. Also, the unprimed, citrate-stimulated activity that is associated with starch synthase-I was reduced. If any of the three branching enzymes from normal maize extracts were added to the *ae* extracts, the citrate-stimulated unprimed-starch synthase activity was restored. It was concluded that the mutation in *ae* endosperm affected the level of BE-IIb activity.

Similar studies with extracts of *dull* maize endosperm showed reduced levels of starch synthase-II and associated BE-IIa activities. As the activities of two enzymes were affected, it was suggested that the mutation might be a regulatory one.

Studies of amylopectin have suggested that it is asymmetric in structure (Gunja-Smith *et al.*, 1970; Marshall & Whelan, 1974). It may therefore be possible that multiple forms of starch synthase and branching enzyme participate in the formation of the asymmetric amylopectin structure. Thus, it is likely that the different isozymes of starch synthase and branching enzyme, each having different specificities for elongation and branching, could interact to synthesise different lengths of chain, thereby conferring asymmetry on amylopectin.

1.1.5 Compartmentation of starch metabolism

In plant tissues, sucrose is metabolised in the cytosol and starch is metabolised in the plastid, and both these compartments have a glycolytic sequence (ap Rees, 1985; Dennis & Greyson, 1987; Dennis & Miernyk, 1982). In nonphotosynthetic tissue, metabolites for starch synthesis must be transported across the amyloplast membrane. Amyloplasts and chloroplasts have many similarities, they both develop from proplastids and appear to be interconvertible under certain conditions during their development (Preiss, 1982). However, they are not identical. Ngernprasirtsiri *et al.*(1988) observed differences in the polypeptide patterns of chloroplast and amyloplast membranes of cultured sycamore cells. They estimated that the amount of the 31 kDa Pi translocator in the amyloplast membrane was 10% of that found in the chloroplast membrane.

Starch synthesis in amyloplasts could either be from triose-phosphates, which could be imported by the phosphate translocator and then be converted to starch via a plastid Fructose 1,6-bisphosphatase (Echeverria *et al.*, 1988; MacDonald & apRees, 1983; Mohabir & John, 1989), or it could be from hexose units imported directly into the amyloplasts (Entwhistle & apRees, 1988; Keeling *et al.*, 1988; Tyson & apRees, 1988).

The uptake and the identity of the major metabolite utilized in plastidic starch synthesis has been elucidated by several approaches. Studies have involved

the isolation and identification of enzymes of amyloplasts, the synthesis of starch from various precursors supplied to isolated amyloplasts, and the uptake and randomisation of radiolabelled glucose incorporated into starch in whole tissues.

Plastids from soybean suspension cells (MacDonald & Preiss, 1983), cauliflower buds (Journet & Douce, 1985), maize endosperm (Echeverria *et al.*, 1988), and potato tuber (Mohabir & John, 1989) contained low activities of Fructose 1,6 bisphosphatase, but this enzyme was not found in guard cells (Hedrich *et al.*, 1985) or wheat endosperm plastids (Entwhistle & apRees, 1988).

Tyson and apRees (1988) have reported that Glucose 1 phosphate was the best precursor for starch synthesis in isolated wheat endosperm amyloplasts. It was also observed that Fructose 1,6-bisphosphate was incorporated into an unidentified insoluble product which was not starch. Other studies with isolated amyloplasts from maize endosperm (Echeverria *et al.*, 1988) and potato tubers (Mohabir & John, 1989), also demonstrated that Fructose 1,6-bisphosphate was incorporated into an insoluble product. It was not proved that this product was starch.

Specifically labelled glucose was supplied to whole tissues and subsequently starch was isolated, in order to determine the randomisation of the label between the C-1 and C-6 position. It was observed that glucose was incorporated into starch without much randomisation in wheat endosperm (Keeling *et al.*, 1988), *Chenopodium* cell cultures, potato tubers, and maize endosperm (Hatzfeld & Stitt, 1990). These results confirmed that the major route of starch synthesis in amyloplasts was not via triose phosphates, but suggested that *in vivo* the uptake of hexose moieties was more important. The contribution of different routes during starch mobilisation is not known.

1.1.6 Regulation of starch synthesis

Ghosh and Preiss (1966) demonstrated that inorganic phosphate (Pi) and

3PGA affected the rate of ADP-glucose synthesis by modulating the activity of ADP-glucose pyrophosphorylase. 3PGA was an activator and Pi was an inhibitor of the enzyme from a variety of plant sources (Sanwal *et al.*, 1968; Preiss, 1982). On the basis of these observations, it was proposed that leaf starch was regulated by the [3PGA]/[Pi] ratio. Confirmation of this control mechanism comes from various studies which correlate changes in 3PGA and phosphate concentrations with the observed changes in rates of starch synthesis. For instance, MacDonald and Strobel (1970) observed that wheat leaves infected with *Puccinia striiformis* had lower concentrations of Pi, and higher levels of starch and ADP-glucose pyrophosphorylase activity. Several groups have studied the effect of Pi and/or 3PGA on starch synthesis in isolated chloroplasts (Heldt *et al.*, 1977; Peavy *et al.*, 1977; Portis, 1982; Stitt *et al.*, 1980). Heldt *et al.*(1977) observed that, change in the Pi concentration of the incubation medium resulted in a decrease in the ratio of [3PGA]/[Pi] in spinach chloroplasts, concomitant with a decrease in the rate of $^{14}CO_2$ incorporation into starch. The addition of 3PGA to the medium was found to reverse the inhibition by Pi and there was no relationship between rate of starch synthesis and the concentration of hexose monophosphates. Based on these observations, Heldt *et al.*(1977) proposed that the [3PGA]/[Pi] ratio regulated starch synthesis at two levels: (i) via the regulation of ADP-glucose pyrophosphorylase activity; (ii) at the level of the phosphate/triose phosphate (TP) translocator.

Fleige *et al.*(1978) have demonstrated that triose phosphate (TP) and 3PGA are the only phosphorylated metabolites that freely permeate the chloroplast membrane. The export of products of CO_2 fixation from the chloroplasts requires exchange with cytosolic Pi (Fleige *et al.*, 1978). Also when leaf discs from different plants were grown in Pi-deficient media, they accumulated higher amounts of starch than when they were grown in media having normal concentrations (Herold *et al.*, 1976). Plants grown in low Pi conditions often have high starch levels (see Herold & Lewis, 1977; Herold & Walker, 1979) as a direct effect of low

[Pi] on enzyme activities and the export of TP from the chloroplasts, although other factors are also involved at the whole plant level. All these observations support the hypothesis that the regulatory kinetics observed *in vitro* for the leaf ADP-glucose pyrophosphorylases reflects the regulation of this enzyme *in vivo*.

1.1.7 Partitioning of carbon

As described earlier, some of the TP and 3PGA generated by CO_2 fixation is exported to the cytosol via the Pi/triose-P translocator. The enzymes catalysing sucrose synthesis are present in the cytosol and Fructose 1, 6-bisphosphatase catalyses the first irreversible reaction in the sucrose synthesis pathway (Robinson & Walker, 1979 ; Stitt *et al.*, 1980). Sucrose can be transported to reserve tissues, where it serves as a carbon source for starch synthesis in amyloplasts (Giaquinta, 1981). TP represents a branch point in partitioning the photosynthate between sucrose and starch synthesis. Sucrose phosphate synthase (SPS) generates most of the cytosolic sucrose in leaves and its activity is altered by the levels of photosynthate intermediates in the cytosol. Under conditions of rapid photosynthesis, sucrose will accumulate in the leaf if the rate of synthesis is higher than the rate of export (Stitt *et al.*, 1984a; 1984b).

Soybean leaf SPS activity changes diurnally, possibly due to an endogenous rhythm. A similar observation was made with SPS in barley leaves, where it may be controlled by post-translational modification (Sicher & Kremer, 1984). Huber (1981a; 1981b) demonstrated that the amount of starch accumulated could be related to the activity of the enzyme in leaves. Species with higher SPS activity accumulated more sucrose and those with lower activities had more starch (Huber, 1981a; 1981b). Photosynthesis would be inhibited unless the rate of sucrose synthesis was controlled to ensure that enough Pi was released to maintain stromal Pi concentrations (Stitt *et al.*, 1987). Photosynthesis would also be inhibited if TP was withdrawn too rapidly, as this would lead to a depletion of

Calvin cycle pools.

Fructose 2, 6-bisphosphate (Fru 2, 6 P2) has been implicated in controlling carbohydrate synthesis in plants (see Stitt, 1990). It is a central factor in coordinating processes in the chloroplast and cytosol and is involved in controlling photosynthate partitioning (Stitt, 1987). It is synthesised by Fructose 6 P 2-kinase and broken down by Fructose 2, 6-bisphosphatase (Fru 2, 6-P2ase). Diurnal changes in these enzymes are seen in spinach leaves (Stitt *et al.*, 1987). The Fru 2, 6 P2 levels increase as sucrose accumulates in leaves. This in turn restricts sucrose synthesis, leads to increased accumulation of TP in the cytosol and to the retention of more of the photosynthate in the chloroplasts for conversion to starch (Stitt *et al.*, 1987). Fru 2, 6 P2 acts by inhibition of Fructose 1, 6-bisphosphatase (Fru 1, 6 Pase) which catalyses an important step in the biosynthetic pathway to sucrose (Stitt *et al.*, 1987). As photosynthesis increases, there is a progressive decrease in Fru 2, 6 P2. This activates Fru 1, 6 Pase and TP can be used for sucrose synthesis. Fru 6 P 2-kinase is inhibited by TP and 3PGA, the depletion of Pi in the cytosol also stimulates Fru 2, 6 P2ase and inhibits Fru 6 P 2-kinase, thereby causing a decrease in Fru 2, 6 P2 levels. Taking into consideration *in vivo* metabolite levels, fluxes and the properties of partially purified enzymes, a model has been developed which predicts that cytosolic Fru 1, 6 Pase remains inactive until a critical threshold of TP is reached. When this level is reached the enzyme is strongly activated by further changes of TP as these lead to a reciprocal increase of the substrate levels and decrease of Fru 2, 6 P2. This would ensure that sucrose synthesis is turned off to protect the metabolite levels in the Calvin cycle and would prevent them from dropping too low. At the same time, this would allow synthesis and recycling of Pi to be strongly activated when photosynthate becomes available (Stitt *et al.*, 1987).

Fru 2, 6 P2 does regulate sucrose synthesis but studies on starch assimilation in different species suggest that an increase in the level of this metabolite does not necessarily lead to an incease in starch synthesis. It is

suggested that starch synthesis could be under complex controls and accumulation rates may not correlate with changes in Fru 2, 6 P2 levels (Sicher *et al.*, 1987).

1.2 Starch mobilisation

As starch exists as a water-insoluble granule within plastids, the enzymes involved in its mobilisation must be compartmentalised with the starch granule and should be able to act on the native granule or its degradation products. The conversion of insoluble polymer to soluble metabolites occurs in three phases -

(i) generation of soluble maltodextrins from the starch granule.

(ii) debranching and breakdown of maltodextrins to glucose and glucose-1-P.

(iii) ultimately the metabolism of glucose and glucose-1-P and the export of the products from the plastids.

1.2.1 Enzymes involved in starch mobilisation

1.2.1.1 α-amylase

α-amylase appears to be the only enzyme able to attack intact starch granules and it probably initiates starch breakdown *in vivo*. α-amylase was present in the leaves of 79 species of plants (Gates & Simpson, 1968), but the localisation of the enzyme was not studied. It catalyses the hydrolytic cleavage of internal α-1, 4 bonds. α-amylase hydrolyses amylose in a biphasic manner (Fischer & Stein, 1961), first producing maltodextrin fragments, which are hydrolysed at a slower rate to produce glucose, maltose and maltotriose. It cannot attack the α-1, 4-D glucosidic linkages adjacent to the α-1, 6-D glucosidic branch points (Thoma *et al.*,1971) in amylopectin. Endoamylases {α-amylases} are usually monomers with molecular weight ranging from 40,000-50,000 (Mundy, 1982; Toda *et al.*, 1982; Tomura &

Koshiba, 1985) but oligomers of the enzyme have also been detected (Okita & Preiss, 1980; Jacobsen *et al.*, 1986). There are differences in the carbohydrate contents of endoamylases isolated from different sources; some have a substantial carbohydrate content (Mitchell, 1972; Mundy, 1982; Toda *et al.*, 1982) while others are not glycosylated (Boston *et al.*, 1982; Tomura & Koshiba, 1985). Endoamylases also ehibit differences in their heat stability and Ca^{+2} dependency for activity. Okita *et al.* (1979) have studied cytosolic and chloroplastic forms of starch biosynthetic and degradative enzymes in spinach leaves. They found that the spinach leaf amylases differed from the endosperm amylases by not being heat stable in the presence of excess Ca^{+2}. Spinach chloroplastic and cytosolic forms of endoamylase differed in their activity with various glucan substrates (Okita *et al.*, 1979). Reserve tissues of wheat or barley or rice seeds have several distinct α-amylase forms, which differ in their immunological properties and/or their peptide maps (Mundy, 1982; Baulcombe & Buffard, 1983; Callis & Ho, 1983; Daussant *et al.*, 1983; Jones & Carbonell, 1984). The amino acid sequences of the different α-amylase isozymes have been derived from cloned cDNA sequences (Miyata & Akazawa, 1982; Baulcombe & Buffard, 1983; Callis & Ho, 1983; Muthukrishnan *et al.*, 1983; 1984; Rogers & Milliman, 1984; Rogers, 1985; Baulcombe *et al.*, 1987).

1.2.1.2 β-amylase

β-amylase catalyses the hydrolysis of α-1, 4 glucosyl linkages in amylose and amylopectin, releasing maltose units from the non-reducing ends. It cannot hydrolyse amylopectin completely as it cannot attack the α-1, 6 glucosyl branch point. β-amylase contains sulphydryl groups, whose oxidation results in enzyme inhibition (Bernfield, 1955; French, 1961) thus, cysteine may be important catalytically or with the structure of the active site. β-amylase cannot attack native cereal starch grains and it is believed that it probably acts on soluble maltodextrins

released by α-amylase catalysed digestion of the native starch grain. In barley seeds, β-amylase exists in the free or latent form - bound to proteins (Ashford & Gubler, 1984; Manners, 1985). It's amino acid sequence has been elucidated from the nucleotide sequence of its cDNA (Kreis *et al.*, 1987) and it has been suggested that the proteolysis of the largest isozyme could result in the release of the bound enzyme and the generation of three isozyme forms (Kreis *et al.*, 1987; Lundgard & Svensson, 1987). Intracellular location of β-amylase in barley seeds depends on the developmental stage. It was found in the cytosol prior to dehydration of the seeds (Nishimura *et al.*, 1987), however following dehydration, it was found associated with the starch granules (Laurière *et al.*, 1986; Hara-Nishimura *et al.*, 1986). A correlation of specific isozyme with a particular stage of development and differentiation has also been observed in rice seeds (Okamoto & Akazawa, 1980). It was observed that, with the onset of germination one isozyme of β-amylase was synthesised *de novo* in the scutellum. At a later stage, a latent β-amylase bound to the periphery of endosperm starch granules was activated. However, the significance of β-amylase enzyme multiplicity in starch mobilisation is not clearly understood. In soy bean, β-amylase is probably not essential for seed development and germination, since genotypes lacking the enzyme or with much reduced levels could still develop and germinate normally. Also, no differences in the starch content and soluble carbohydrate levels were observed in varieties with high and low β-amylase levels (Hildebrand & Hymowitz, 1981; Adams *et al.*, 1981).

1.2.1.3 Debranching enzyme

Debranching enzyme catalyses the exclusive hydrolysis of α-1, 6 bonds in glucans containing α-1, 4 and α-1, 6 linkages. Debranching enzymes have been purified from a variety of plant tissues like seeds (Maeda & Nikuni, 1978; Yellowlees, 1980; Iwaki & Fuwa, 1981), leaves (Ludwig *et al.*, 1984) and potato tubers (Ishizaki *et al.*, 1983). Cytosolic and chloroplastic forms of debranching

enzyme have been found in leaves (Okita *et al.*, 1979; Kakefuda *et al.*, 1986) and the chloroplastic form has been purified and characterised (Ludwig *et al.*, 1984). The activity of debranching enzyme increases during germination of seeds of rice and *Araucaria* (Palmiano & Juliano, 1972; Cardemil & Varner, 1984). Conversely, the activity decreased in germinating peas (Yellowlees, 1980). Yamada (1981c) has proposed that activation of a preformed inactive debranching enzyme occurs in germinating rice seeds. Indeed, the catalytically inactive form purified from rice seeds could be converted to the active form *in vitro*, by incubation with reducing agents or a protease (Yamada, 1981a). Since the purified debranching enzymes from barley and pea had very ltttle hydrolytic activity on isolated starch granules, it seemed likely that the physiological function of this enzyme was the hydrolysis of α-1, 6 linkages of soluble glucans.

1.2.1.4 α-glucan phosphorylase

α-glucan phosphorylase (starch phosphorylase) catalyses the following reaction.

$$\alpha\text{-D-glucose 1-phosphate} + G_n \rightleftharpoons Pi + G_{n+1}$$

where G_n and G_{n+1} represent an α-glucan containing n and n+1 α-1, 4-linked glucosyl units. The enzyme acts on the non-reducing end of the α-glucan. It is generally believed that phosphorylase is not involved in starch biosynthesis, mainly because the calculated physiological concentrations of glucose-1-phosphate are far too low to drive the reaction in this direction (see Preiss & Levi, 1980). However the concentration of glucose-1-phosphate in the amyloplast has not been measured directly. Starch phosphorylase is attributed with a role in starch mobilisation. Maize seed and potato tuber starch phosphorylases have been extensively studied and several isozymes from both sources have been described (Frydman & Slabnik, 1973; Gerbrandy & Doorgest, 1972; Gerbrandy *et al.*, 1975; Tsai & Nelson, 1968; 1969). Potato tuber phosphorylases have been studied in detail by Fukui's

group (1987). The 916 amino acid sequence of the major form of potato tuber starch phosphorylase, which is located in the amyloplast, has been determined directly (Nakano & Fukui, 1986). α-glucan phosphorylases of higher plants exist as dimers or tetramers, each subunit having a molecular weight of approximately 100,000 (see Fukui *et al.*, 1987). Potato tuber has two types of starch phosphorylase, the major form (plastidic) and the minor form (cytosolic). The major form of starch phosphorylase from potato tubers exists in only the active form and is not under allosteric control (Lee, 1960). Fukui *et al.* (1987) have classified potato tuber starch phosphorylases into two types based on their molecular weights, localisation, affinity for glycogen and susceptibility to proteases. The major enzyme of potato tubers has a molecular weight of 104,000 and has very low affinity for glycogen. It is located within amyloplasts and is susceptible to thermolysin. The minor form of starch phosphorylase has a molecular weight of 90,000, a high affinity for glycogen, is located in the cytosol and is not very susceptible to cleavage by thermolysin. The two forms of tuber starch phosphorylase are also immunologically distinct. It seems very likely that these two "isozymes" of starch phosphorylase are different gene products.

Steup and Latzko (1979) have studied the intracellular localisation of starch phosphorylases in spinach and pea leaves. Spinach leaf extracts contained two forms of starch phosphorylase, of which one was chloroplastic. Pea leaf extracts had three forms of starch phosphorylase, of which two were chloroplastic. A surprising observation was that the extrachloroplastic form of phosphorylase represented a considerable amount of the total phosphorylase activity in both spinach and pea leaves. Steup and Schächtele (1981) found that the spinach chloroplast starch phosphorylase had a high affinity for linear glucans as compared to branched polymers like glycogen or amylopectin. Their data suggested that the chloroplast starch phosphorylase was not involved in initiation of starch mobilisation.

1.2.1.5 α-glucosidase

α-glucosidases catalyse the hydrolysis or transfer of the terminal α-D-glucosyl residues of α-D-glucosidically linked derivatives. They can hydrolyze maltose, maltotriose, isomaltose, soluble starch and glycogen (Chiba & Shimomura, 1975; Hutson & Manners, 1965; Manners & Rowe, 1969; Sun & Henson, 1990; Takahashi *et al.*,1971). α-glucosidases have been extracted from several different plant sources including: the seeds of barley (Sun & Henson, 1990), buck-wheat (Takahashi & Shimomura, 1968), and sugar beet (Yamasaki & Suzuki, 1980); cultured cells of sugar beet (Yamasaki & Kono, 1989), carrot (Parr & Edelman, 1975) and soybean (Yamasaki & Kono, 1985); and pea seedlings (Beers *et al.*, 1990). α-glucosidase has been attributed with a role in hydrolysis of dextrins released by the action of α-amylases on starch granules (Beck & Ziegler, 1989). Recently, Sun and Henson (1990) demonstrated that α-glucosidase isolated from germinating barley seeds was capable of initiating hydrolysis of native barley starch granules in the absence of α-amylase, and also that there was a synergistic effect in the hydrolysis of granular starch by α-amylase and α-glucosidase. As these α-glucosidases could hydrolyse several different α-glucosidic bonds, it has been suggested that in addition to the removal of maltose, α-glucosidase may act on non-α-1,4 glucosidic linkages on the granule surface, which act as barriers to α-amylosis (Sun & Henson, 1990).

1.2.1.6 D-enzyme

D-enzyme catalyses the reversible condensation of donor and acceptor α-1,4 glucans with the release of free glucose. The enzyme was first isolated from potato tuber (Peat *et al.*, 1956) and has also been found in broad bean, carrot and tomato (Manners & Rowe, 1969). It was also found in leaves (Lin & Preiss, 1988; Okita *et al.*, 1979). Lee & Whelan (1971) have suggested that D-enzyme was

probably involved in the condensation of short oligosaccharides to form larger chains, which were more suitable substrates for starch phosphorylase.

1.2.2 Cereal seed starch mobilisation

Cereal seeds contain large amounts of starch, which is compartmentalised within the non-living cells of the endosperm. The mobilisation of reserve starch in cereal seeds is thought to be predominantly hydrolytic (Preiss, 1982). During germination of wheat seeds two groups of α-amylase isozymes are produced, which differ in their pI values; only one group of α-amylase isozymes can attack native starch granules (Sargeant, 1979). Usually α-amylase activity is not detectable in dormant cereal seeds, however in maize and sorghum endosperm the enzyme is present within lytic bodies (Adams *et al.*, 1975). It has recently become apparent that besides α-amylase, α-glucosidase also plays an important role in cereal seed starch mobilisation (Sun & Henson, 1990).

After barley seeds have germinated, α-amylases, limit dextrinases and α-glucosidase are synthesisied *de novo* and secreted by the cells of the aleurone layer (Filner & Varner, 1967; Bilderback, 1974; Hardie, 1975). It is also now apparent that the scutellum, previously thought to be primarily involved in the uptake of degraded reserves (Nomura *et al.*, 1969; Humphreys, 1975), is a major source of secreted hydrolases (Akazawa & Miyata, 1982; Mundy *et al.*, 1985).

Gibberellic acid (GA) has been shown to elicit α-amylase mRNA synthesis in aleurone tissue of barley (Akazawa & Hara-Nishimura, 1985), wheat (Baulcombe & Buffard, 1983) and wild oats (Zwar & Hooley, 1986). The hormone also regulated the transcription of α-amylase genes in barley aleurone protoplasts (Jacobsen & Beach, 1985). It was also shown that abscisic acid (ABA) reversed the GA-promoted changes in the barley aleurone layer (Jacobsen, 1983) and the inhibitory effect was also at the transcriptional level (Jacobsen & Beach, 1985). Recent studies have indicated that ABA preferentially inhibits production of

one group of α-amylase isozymes and may affect α-amylase mRNA stability (Nolan *et al.*, 1987). Although the stimulatory effects of GA in barley are evident, it is doubtful whether hormonal regulation of α-amylase production represents a classical hormone response (Akazawa & Hara-Nishimura, 1985; Halmer, 1985; Trewavas, 1982).

Akazawa and Hara-Nishimura (1985) have discussed the importance of calcium in the differential induction of α-amylase isozymes, their subsequent intracellular transport and release in barley aleurone and rice scutellum. In barley, the production of high-pI α-amylase required calcium, while that of low-pI isozymes did not. However, the site of regulation by calcium is still obscure (Deikman & Jones, 1986). The α-amylase synthesised in the aleurone layer and the scutellum is secreted via the golgi apparatus (Fernandez & Staehelin, 1985; Gubler *et al.*, 1986). It was also observed that cell wall channels, through which α-amylases and other secreted proteins are released from the aleurone layer, are generated by hydrolases which are directed by the plasmodesmata-lined tubes (Gubler *et al.*, 1987).

Dormant barley seeds contain β-amylase in a free (salt-soluble) and a latent form that is complexed with proteins (Ashford & Gubler, 1984; Mannners, 1985). During germination, the latent form of β-amylase is probably converted to its active (free) form by the action of proteolytic enzymes (Roswell & Goad, 1964). However, as discussed earlier, the physiological role of β-amylase in starch mobilisation is not clear.

The cells of the aleurone layer also synthesise, *de novo*, other hydrolytic enzymes like α-glucosidase and limit dextrinases, which are released in the endosperm (Hardie, 1975). Yamada (1981b) has reported the proteolytic activation of debranching enzyme in germinating barley.

Besides hydrolytic enzymes, proteases and glucanases are produced during germination. They breakdown the cell walls, membranes and proteins which compartmentalise starch, thus making the starch accesible to hydrolytic attack

(Ashford & Gubler, 1984; Halmer, 1985; Halmer & Bewley, 1982; Manners, 1985). As described earlier, no known factors such as phosphorylation and allosteric control influence the activity of the hydrolytic enzymes involved in starch mobilisation. It may be, however, that when the products of starch breakdown - maltose and glucose - accumulate in the endosperm, hydrolytic activity could be inhibited. Indeed, soybean cotyledon β-amylase was inhibited by maltose and glucose (Nomura *et al.*, 1986), but the importance of β-amylase to starch mobilisation in soybean cotyledons is doubtful.

Many cereal seeds have proteins that inhibit endogenous α-amylase (Weselake & MacGregor, 1985). The bifunctional α-amylase/subtilisin inhibitor from barley seeds has been extensively characterised (Mundy *et al.*, 1983; Svendsen *et al.*, 1986) and has recently been cloned (Leah & Mundy, 1989). During barley seed development the inhibitor is synthesised and deposited in the endosperm tissue (Mundy & Rogers, 1986). It is loosely associated with the starch granules and disappears during germination (Lecommandeur *et al.*, 1987). As this inhibitor can interact with the endogenous α-amylase (Weselake *et al.*, 1983; Halayko *et al.*, 1986), it may inactivate any prematurely synthesised α-amylase during precocious germination.

Much less is known about reserve starch mobilisation in plant storage tissues (tubers, corms, etc.), but what is known of starch mobilisation in potato tubers is considered in Section 1.3.5.

1.2.3 Transitory starch mobilisation

Starch assimilated in leaves is regarded as a buffer to the metabolism of sucrose (Stitt, 1984; Stitt & Steup, 1985). The metabolism of starch in leaves differs from the process in cereal endosperm in that, the transitory leaf starch is compartmentalised within intact chloroplasts of living cells. It is subject to diurnal fluctuations and it is likely that factors involved in the regulation of transitory starch

metabolism are different from those for endosperm starch metabolism.

Unlike amyloplasts, intact chloroplasts can be isolated with relative ease and subsequently can be used to study the regulation and route of transitory starch mobilisation (Stitt & apRees, 1980; Stitt & Heldt, 1981). The major products of starch breakdown identified in chloroplasts isolated from spinach leaves are, TP, 3PGA, CO_2, glucose and maltose, identical products are also found during starch mobilisation in pea chloroplasts, except, glucose is a minor product (Stitt, 1984). From these studies it appears that chloroplastic starch breakdown involves both hydrolytic and phosphorolytic pathways.

Studies on the activities and subcellular localisation of various enzymes involved in starch mobilisation may indicate their relative contribution towards the breakdown of starch. Chloroplasts of many species contain substantial activities of starch phosphorylase, which could contribute significantly towards the phosphorolytic degradation of starch (Echeverria & Boyer, 1986; Levi & Preiss, 1977; Lin *et al.*, 1988c; Robinson and Preiss, 1987; Steup & Latzko, 1979). However, spinach (Preiss *et al.*, 1980; Shimomura *et al.*, 1982; Steup & Schätele, 1981) and pea (Steup *et al.*, 1987) chloroplastic phosphorylases do not prefer substrates that have a high degree of polymerisation and branching. The chloroplastic phosphorylase is more effective, if the starch granule is pretreated with hydrolase (Beck *et al.*, 1981; Chang, 1982; Steup *et al.*, 1983).

α-amylase is widely distributed in leaves (Gates & Simpson, 1968), and endoamylolytic activity has been reported in pea and wheat leaf mesophyll chloroplasts (Ziegler, 1988; Ziegler & Beck, 1986), chloroplasts from spinach (Okita *et al.*, 1979), *Arabidopsis* leaf mesophyll (Lin *et al.*, 1988c) and maize leaf bundle sheath cell (Echeverria & Boyer, 1986). These chloroplastic endoamylases are calcium independent and heat labile (Lin *et al.*, 1988c; Ziegler, 1988; also see Stitt, 1984; Preiss, 1982; Stitt & Steup, 1985), unlike the classical α-amylase which is heat stable in the presence of excess calcium. Beck (1985) has shown that the hydrolytic activity of crude spinach chloroplast extracts can dissolve

the "pasty" outer layer of transitory starch granules. Unfortunately since no chloroplastic endoamylase has yet been purified, it is not possible to confirm whether it can attack native starch granules.

β-amylase activity in leaf mesophyll of *Arabidopsis* (Lin *et al.*, 1988c), of barley (Jacobsen *et al.*, 1985) and in pea and wheat protoplasts (Ziegler & Beck, 1986) appears to be mainly extrachloroplastic.

Spinach leaves also contain α-glucan transferase (Linden *et al.*, 1974) and α-glucosyl transferase, which can catalyse the rearrangement of glucose units between glucose, maltose and glucans. Okita *et al.* (1979) found that though spinach leaf chloroplasts had associated D-enzyme activity, most of it was extrachloroplastic. In contrast, pea (Kakefuda *et al.*, 1986) and *Arabidopsis* (Lin *et al.*, 1988c) leaf chloroplasts had substantial D-enzyme activity. There is very little information about how ubiquitous these transferases are in plant leaves and so it is very hard to evaluate their significance in starch metabolism.

Debranching enzymes were present in spinach leaves (Ludwig *et al.*, 1984; Okita & Preiss, 1980) and could be detected in chloroplasts of pea and wheat leaf mesophyll (Kakefuda *et al.*, 1986; Ziegler, 1988; Ziegler & Beck, 1986), and maize leaf bundle sheath cells (Echeverria & Boyer, 1986).

In conclusion, based on the products of starch breakdown in isolated chloroplasts and the enzyme activities associated with chloroplasts, it seems that both, the hydrolytic and phosphorolytic pathway are operational during mobilisation of starch in leaves. It seems likely that the endoamylase attacks the starch granule, while phosphorylase attacks linear glucans generated by endoamylase and debranching enzyme.

An intriguing observation is the high level of several extrachloroplastic enzymes that are capable of participation in starch mobilisation.

1.3 Potato tuber metabolism

1.3.1 Tuber development.

Tuberisation involves differentiation of the underground stem into a storage organ by its radial expansion and accumulation of starch, and a characteristic set of proteins (Artschwager, 1924). The formation of a tuber on a potato plant involves the following developmental steps (see Vreugdenhil & Struik, 1989), which are not necessarily in their chronological order.

1. An axillary bud on the base of the stem is activated and begins to grow horizontally, forming the stolon;
2. the stolon continues to grow and undergoes branching;
3. the longitudinal growth of the stolon ceases;
4. and in response to tuber inducing signal(s), the stolon tip grows radially and alters its metabolic status.

1.3.2 Hormonal regulation of tuberisation.

The onset of tuberisation is controlled by a variety of environmental and genetic factors. Tuber formation is favored by short days, low temperature or low nitrogen supply (Ewing, 1985). Phytohormones also play an important role in tuberisation, and the effect of various plant-hormones on individual steps during the development of a tuber have been reviewed recently by Vreugdenhil and Struik (1989).

Cytokinins were believed to be involved in tuber initiation, as they promoted tuberisation in isolated stolons, cultured *in vitro* (Palmer & Smith, 1969; Forsline & Langille, 1976; Mauk & Langille, 1978) and also because, the levels of endogenous cytokinins in roots and stolons increased upon transfer of plants to short days (Forsline & Langille, 1975; Obata-Sasamoto & Suzuki, 1979; Koda, 1982). However, Jameson *et al.* (1985) found that increases in cytokinin-like activity occurred after tuber formation and they concluded that the tubersation signal(s) is (are) not likely to be cytokinin-like in nature.

GA, on the other hand, acts in a negative manner causing cessation of tuber growth (Hannapel *et al.*, 1985; Melis & Van Staden, 1984). Mares *et al.* (1981) showed that application of GA to developing tubers induced a drastic alteration in potato tuber metabolism, shifting it to a pattern typical for the termination of tuberisation.

Ethylene has been reported to have a positive effect on tuber initiation (Catchpole & Hillman, 1969; Garcia-Torres & Gomez-Campo, 1973), however, the swellings formed after ethylene treatment differed from normal tubers and did not contain starch. In contrast, Mingo-Castel *et al.* (1974,1976) have shown that ethylene inhibits tuber initiation. Vreugdenhil and Struik (1989) have suggested an explanation for the action of ethylene. They suggested that ethylene primarily inhibits elongation of stolons and this subsequently allows tuber initiation; although, the initiation of tubers is inhibited by ethylene.

Wareing and Jennings (1980) showed that ABA could stimulate tuberisation (of the axillary bud) in stem cuttings from a short day induced plant. This seemed to indicate that ABA could have a role in tuber initiation. Its role is questionable, since a wilty mutant of potato (Quarrie, 1982), which has greatly reduced levels of ABA, forms tubers.

Gregory (1956) demonstrated the presence of a graft-transmissible stimulus which controlled tuber initiation in potato plants. In 1982, Wareing proposed the presence of an unknown positive regulator, which was involved in the initiation of tubers and, whose production was promoted by exposure of the plant to short days. Later, a factor that stimulated tuber formation in single-node leaf cuttings was isolated from the apoplast of potato leaves (Struik *et al.*, 1987). It was suggested that the factor was continuously produced in leaves and exposure to short days stimulated the export of this factor to other parts of the plant.

Recently, a substance was isolated (Koda & Okazawa, 1988) and purified (Koda *et al.*, 1988) from potato leaves. This substance was active in inducing tubers on etiolated shoots and its levels in leaves increased under tuberisation-

inducing conditions. It has been identified as 3-oxo-2(5-?-D-glucopyranosoloxy-2-*cis*-pentenyl)-cyclopentane-1-acetic acid, which is closely related to jasmonic acid (Yoshihara *et al.*, 1988).

As tuberisation is accompanied by a deposition of starch, it is likely that phytohormones also influence the levels of various enzymes involved in starch synthesis. Indeed, Mingo-Castel *et al.* (1976) reported significant increases in the activities of phosphorylase and ADP-glucose pyrophosphorylase during kinetin-induced tuberisation in potato sprout sections. Studies on changes in starch and protein contents, and the activities of enzymes involved in starch synthesis during tuberisation of stolon tips, showed changes during early stages of tuber development. These included increases in the activities of starch phosphorylase and granule-bound starch synthase along with the starch content (Obata-Sasamoto & Suzuki, 1979). Furthermore, soluble starch synthase and ADP-glucose pyrophosphorylase activities could not be detected in stolon tips during the early stages of tuber development, but the activities of these enzymes increased at later stages. Hawker *et al.* (1979) examined the activities of enzymes of starch metabolism in relation to tuberisation, and tuber growth rate. During tuber development, the activities of starch synthase, ADP-glucose pyrophosphorylase, UDP-glucose pyrophosphorylase and starch phosphorylase increased. Increases in the activities of ADP-glucose pyrophosphorylase and starch phosphorylase were particularly pronounced. Hawker *et al.* (1979) suggested that enzyme levels may be influenced by sugar levels and concluded that the observed changes were a consequence of tuberisation, and that starch accumulation was partly controlled by the activities of ADP-glucose pyrophosphorylase and starch phosphorylase. The close correlation between tuber starch biosynthesis and the activity of ADP-glucose pyrophosphorylase, observed by Sowokinos (1976), emphasises its major role in tuber starch synthesis.

1.3.3 Gene expression associated with tuberisation

1.3.3.1 Patatin.

Although the physiology and biochemistry of tuberisation has been extensively studied, the molecular mechanisms involved in the process remain incompletely understood.

A characteristic set of proteins are associated with the process of tuberisation. Patatin is the trivial name given to a family of immunologically related glycoproteins with a molecular weight of 40,000, that constitute up to 30-40% of the total soluble protein of potato tubers (Park, W. , 1983; Racunsen & Foote, 1980). Paiva *et al.* (1983) showed that in addition to patatin several other proteins accumulated with tuberisation. Unlike other storage proteins it has been shown to have an esterase activity with numerous lipid substrates (Andrews *et al.*, 1988; Höfgen & Willmitzer, 1990; Racunsen, 1986; Rosahl *et al.*, 1987). The physiological role of this esterase activity has not been defined, but it has been speculated that it could either be involved in wound responses (Andrews *et al.*, 1988), or it could play a role in transition of the tuber from dormancy to vegetative growth (Rosahl *et al.*, 1987). The post-translational modifications, targetting and localisation of patatin have been extensively studied (Sonnewald *et al.*, 1989a; 1989b) and a number of patatin cDNAs and genes have been isolated and characterised (Bevan *et al.*, 1986; Mignery *et al.*, 1984, 1988; Pikaard *et al.*, 1986; Rosahl *et al.*, 1986b; Twell & Ooms, 1988). Depending upon the potato cultivar, there are about 10-18 members of the patatin gene family per haploid genome (Twell & Ooms, 1988) and they are classified into two groups, which are distinguished by the presence (class II) or absence (class I) of a 22 bp insertion in the 5' untranslated region (Mignery *et al.*, 1988). Approximately 98-99% of patatin mRNA in tubers is of class I type, but, under normal conditions this class of patatin mRNA is not expressed in leaves, roots or stems. The remaining 1-2% of tuber patatin mRNA is class II type, which is also present at low levels in roots

(Pikaard *et al.*, 1987).

Normally, the expression of class I patatin gene is restricted to tubers, and stolons associated with growing tubers (Rosahl *et al.*, 1986a). However under certain conditions, patatin can be expressed in other somatic tissue. Paiva *et al.* (1983) demonstrated that patatin could accumulate in detached stems and petioles at levels comparable to those found in tubers; besides patatin, other tuber-specific proteins and massive amounts of starch also accumulated in the detached stems and petioles. This induction of tuber-specific proteins was independent of the tuberisation process.

In an attempt to understand the factors involved in patatin gene expression, the 5'-upstream regulatory regions of class I and class II patatin genes were fused to the *E.coli* β-glucuronidase (GUS) gene and these constructs were analysed in transformed potato plants (Köster-Töpfer *et al.*, 1989; Rocha-Sosa *et al.*, 1989; Twell & Ooms,.1987; Wenzler *et al.*, 1989a). Gene fusion derivatives of both class I (Rocha-Sosa *et al.*, 1989; Wenzler *et al.*, 1989a) and class II (Twell & Ooms,.1987) promoters were actively transcribed in tubers of transgenic plants. Class I genes could also be activated in isolated leaves of transgenic plants, when these were placed in medium containing high levels of sucrose (Rocha-Sosa *et al.*, 1989; Wenzler *et al.*, 1989a). Sucrose-inducible expression of the class I patatin-GUS gene constructs in leaf explants was restricted to mesophyll and epidermal cells (Rocha-Sosa *et al.*, 1989). As a combination of high mannitol concentration and low sucrose concentration did not induce patatin expression in explants, it seemed that the induction by sucrose was not due to an osmotic effect (Wenzler *et al.*, 1989b).

Recently the class I patatin gene promoter region has been subjected to deletion analysis (Jefferson *et al.*, 1990; Liu *et al.*, 1990) to define regions that confer tuber specific expression and sucrose inducibility. Jefferson *et al.* (1990) have identified enhancer like sequences between -369 bp and -40 bp of the class I gene, that, when employed in chimeric GUS gene constructs, specifically promote

GUS expression in tubers but not leaves. It appears that the source-sink relationship of sucrose also influences the distribution of patatin transcripts in the plant (Jefferson *et al.*, 1990; Liu *et al.*,1990).

1.3.3.2 Proteinase Inhibitors.

Considerable amounts of protease inhibitors often accumulate in plant storage organs like seeds or tubers (Ryan, C. A. ,1977). As these proteins preferentially inhibit proteinases from microorganisms or insects (Richardson, M. , 1977), they probably play a role in defense of the plant against microbial and insect attack. The level of proteinase inhibitor II in potato tubers depends on the tuber variety (Ryan, C. A. ,1977) and it constitutes about 5% of the total soluble proteins of Russet Burbank tubers. Proteinase inhibitor II expression is controlled by both developmental and environmental factors. In unwounded potato plants, proteinase inhibitor II is expressed only in tubers and young floral buds (see Prat *et al* , 1990), but on wounding of leaves, the protein accumulates in the aerial organs. The response to wounding is systemic and it results in the accumulation of the inhibitor in unwounded organs away from the wound site. Such a response to wounding suggests the possible involvement of an inducing factor or wound hormone which could be released from the wound site and transported to other parts of the plant, where it would induce the expression of the proteinase inhibitor II genes. Recently, Peña-Cortes *et al.* (1989) have obtained evidence that ABA is involved in the signal transduction pathway of the wounding reaction in leaves.

Proteinase inhibitor II encoding cDNAs and the corresponding genomic clones have been isolated and characterised (Keil *et al.*, 1986; Thornburg *et al.*, 1987). Sánchez-Serrano *et al.* (1987) and Thornburg *et al.* (1987) showed that chimeric proteinase inhibitor II promoters fused to a reporter gene exhibited wound-inducible expression in transgenic tobacco plants. Subsequently, Keil *et al.* (1989) observed that proteinase inhibitor II promoter activity led to expression

in the vascular tissue of wounded and systematically induced leaves, petioles and stems, and developing tubers. Thus, they demonstrated that one member of the proteinase inhibitor gene family contained *cis* elements essential for its response to both, developmental and environmental signals. The progressive deletion of the proteinase inhibitor II promoter, highlighted the importance of a far upstream region (-1300 bp to -700 bp) in high-levels of expression (Keil *et al.*, 1990).

Steikema *et al.* (1988) have cloned four tuber mRNAs from a potato tuber cDNA library by differential hybridisation. Two of the clones represented patatin and proteinase inhibitor II cDNAs, whereas the other two encoded polypeptides that exhibited homology with the soybean Bowman-Birk proteinase inhibitor and with the Kunitz trypsin inhibitor respectively.

Recently Suh *et al.* (1990) have purified three abundant proteins of approximate molecular masses 22, 23, and 24 Kd from potato tubers. These proteins are immunologically related and are present in tubers and, as higher molecular mass forms in leaves, but are absent in stems, roots and stolons. The amino-terminal sequences of the three proteins are identical and match the deduced amino acid sequence of the putative Kunitz trypsin inhibitor encoding cDNA clone isolated by Steikema *et al.* (1988).

1.3.3.3 Enzymes of tuber starch metabolism

The accumulation of starch is another biochemical marker for tuberisation and increases in starch synthesis probably reflect increase in the activity of enzymes involved in starch metabolism. Over the last three years, a large number of cDNAs and genomic clones of various enzymes involved in tuber starch metabolism have been isolated and characterised.

Sucrose synthase is present in the cytoplasm of potato tuber cells, where it breaks down incoming sucrose to UDP-glucose and fructose. Salanoubat and Belliard (1987) have cloned potato tuber sucrose synthase cDNA, using maize

sucrose synthase cDNA as a probe. Wounding results in a decrease in sucrose synthase mRNA levels, which are overcome by anaerobic incubation of tuber slices. Sucrose synthase mRNA levels increase in leaf and petioles with an increase in the sucrose concentration (Salanoubat & Belliard, 1989).

Potato tuber UDP-glucose pyrophosphorylase is localised in the cytoplasm (MacDonald & apRees, 1983) and breaks down UDP-glucose to glucose-1-P and UTP. Glucose-1-P is transported into the amyloplasts and used for starch synthesis (Keeling *et al.*, 1988). Katsube *et al.* (1990) have isolated potato tuber UDP-glucose pyrophosphorylase cDNA using oligonucleotide probes derived from the amino acid sequence. As yet, no data has emerged on the expression of the gene during tuberisation, wounding and sprouting of tubers.

Recently, Müller-Röber *et al.* (1990) and Chauhan *et al.* (1990) isolated cDNA clones encoding two different ADP-glucose pyrophosphorylase polypeptides using heterologous probes from maize and wheat respectively. Müller-Röber *et al.* (1990) demonstrated by northern blot experiments that the two genes differ in their expression pattern in different organs. One of the ADP-glucose pyrophosphorylase genes was strongly inducible by sucrose. Visser *et al.* (1989) have cloned and partially characterised the gene for granule-bound starch synthase from a wild type and an amylose-free potato mutant. Also, two different cDNAs encoding the soluble starch synthase of potato tuber have been isolated (Smith & Martin, personal communication). The isolation of starch phosphorylase cDNA, which forms the results section of this thesis, was paralleled by isolation in two other laboratories (Brisson *et al.*, 1989; Nakano *et al.*, 1989). The data from all three laboratories will be compared in the discussion.

1.3.4. Manipulation of starch metabolism.

Unfortunately, pioneering attempts to manipulate potato tuber starch metabolism, by selectively blocking granule-bound starch synthase expression

using heterologous antisense constructs from maize, have provided results that are difficult to assess(in Visser, 1989). The supression of granule-bound starch synthase activity in starch from minitubers ranged up to 88%, but the most extreme reduction in the amount of granule-bound starch synthase or granule-bound starch synthase-like protein measured immunochemically was 50%! Even more surprising was one case in which a decrease in granule-bound starch synthase activity was associated with a substantial increase in the amount of granule-bound starch synthase or granule-bound starch synthase-like protein. Hopefully future attempts at the manipulation of starch content in potato tubers using homologous antisense constructs should be more productive.

1.3.5 Potato tuber starch mobilisation.

Starch represents up to 70% of tuber dry weight (Ohad *et al.*, 1971) and in mature tubers it is separated from the cytosol by the plastidic membrane (Isherwood, 1976; Sowokinos *et al.*, 1985). Starch granules are essentially degraded intracellularly and lead to an accumulation of products like sucrose, glucose and fructose(Isherwood, 1976; Sowokinos *et al.*, 1985). Several factors can induce the mobilisation of starch into soluble sugars.

"Low temperature sweetening" occurs when tubers are stored at temperatures below 5°C. If tubers subjected to low temperature sweetening are transferred to elevated temperatures, the process is reversed and soluble sugars are rechanneled towards starch synthesis. "Senescent sweeetening", which is distinct from low temperature sweetening and which is associated with tissue senescence, occurs with prolonged storage of tubers at moderate temperatures and results in a slow and essentially irreversible conversion of starch into sucrose (Burton, W. G. , 1965; Halmer & Bewley, 1982). Starch degradation is also associated with defects like tissue translucency (Sowokinos *et al.*, 1985).

Injury to potatoes was reported to bring about a rapid breakdown of

starch, such that there was almost a complete depletion of starch in the cells adjacent to the wound periderm (Barckhausen, 1978), but these observations do not appear to have been followed up.

In sprouting tubers, starch is mobilised to provide the developing sprout with soluble sugars for its structural and metabolic processes. The biochemistry of starch mobilisation during sprouting has been studied extensively (Nowak, 1977; Bailey *et al.*, 1978; Davies, 1984; Davies & Ross, 1984; Davies & Ross, 1986), and has involved analysis of enzyme activities in tuber extracts. Bailey *et al.* (1978) observed that the levels of starch phosphorylase and α-amylase increased at the emergence from tuber dormancy. In contrast, Davies and Ross (1986) showed that activities of starch phosphorylase and α-amylase decreased with starch depletion. They also noted that although the activity of α-amylase was barely detectable, the activities of other enzymes was sufficient to support the observed rates of starch mobilisation.

One serious drawback of these studies was that the enzymes were assayed in crude extracts in which previously compartmentalised enzymes were liberated. It was unclear whether the changes in enzyme levels were due to changes in the levels of cytosolic isozymes or plastidic isozymes.

With tuber senescence, the amyloplast membrane is believed to disintegrate (Schneider *et al.*, 1981), but it is unclear how this is linked to the initiation of starch mobilisation. The damaged amyloplast membranes may well be artefacts of sample preparation, besides, in translucent tissues and during "cold induced sweetening", starch mobilisation occurs inside intact amyloplasts (Isherwood, 1976; Lulai *et al.*, 1986).

In conclusion, despite prolonged physiological and biochemical analysis of the process, neither the route nor the regulation of starch mobilisation in potato tubers is well understood. Also, in contrast to the extensive information available on gene expression during starch mobilisation in germinating cereal seeds, little is known about gene expression during starch mobilisation in potato tubers. We

know little about any effect of phytohormones on genes expressed during this process. The lack of homologous DNA probes has hampered studies on gene expression during potato tuber starch mobilisation, however the availability of cDNAs or genomic clones of various enzymes involved in starch metabolism (described above) should facilitate such studies. It should also be possible to manipulate starch content and composition using homologous antisense constructs in transgenic potato plants. Antisense constructs could also be used to study the interaction and relative contribution of different enzymes in starch metabolism.

CHAPTER 2. MATERIALS AND METHODS

2.1 List of suppliers

All chemicals used were of analytical grade and were obtained from BDH Chemicals, Formachem (Research International) Limited or Fisons Scientific Apparatus. Where chemicals or equipment were obtained from other sources, this is indicated in the text. A list of the suppliers is given below.

Amersham International plc, White Lion Road, Amersham, Bucks HP7 9LL

BDH Chemicals Limited, Poole, Dorset, England.

Boehringer-Mannheim, BCL, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex.

James Burrough Ltd., 70 Eastways Industrial Park, Witham, Essex

Cambridge Biotechnology Labs., 12-14 St. Ann's Crescent, London SW18

Difco Laboratories, Detroit, Michigan, U.S.A.

Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Fluka Fluorochem Ltd., Peakdale Road, Glossop, Derbyshire Sk13 9XE

FSA Lab Supplies, Bishop Meadow Road, Loughborough, Leics.,LE11 0RG

GIBCO-BRL Ltd., P.O.Box 35, Paisley, Scotland

Genetic Research Instrumentation Ltd., Gene House, Dunmow Road, Felsted, Dunmow, Essex CM6 3LD

Pharmacia LKB Biotechnology, Pharmacia House, Midsummer Boulevard, Milton Keynes, MK9 3HP

Sigma Chemical Company, Fancy Road, Dorset, England BH17 7NH

Whatman Ltd., Madstone, Kent, U.K.

2.2 Methods of asepsis

To prevent contamination of glassware, plasticware and solutions by microbes and nucleases, they were autoclaved at 15 p.s.i. for 20 minutes. All

2.3.1 a Plant Material

Mature potato tubers of field grown plants were kindly provided by Dr. Howard Davies of the Scottish Crop Research Institute, Dundee, U.K., and were stored at 4°C in the dark for a minimum period of six months; cv Maris Piper.

solutions used in the preparation and analysis of RNA, except those containing Tris, were made to 0.1% with diethylpyrocarbonate (DEPC), left overnight at room temperature and autoclaved as described above, to remove all traces of DEPC.

Disposable plastic gloves were worn whenever samples of RNA or DNA were handled.

2.3 Manipulation of plant tissue

2.3.1 Wounding of plant tissue

Discs of 24mm diameter and 1mm thickness were cut from the perimedula of tubers. Aerobic ageing was carried out in the dark at 26°C on sheets of Whatman No. 1 filter paper soaked in (ageing buffer) 20mM sodium phosphate, pH6.8, containing 50µg/ml of chloramphenicol, as described by Ishizuka *et al.*(1981). For anaerobic ageing, slices were incubated submerged in the same solution. The effect of various osmotic agents on starch phosphorylase mRNA levels in wounded tubers, was studied by supplementing ageing buffer with sterile solutions of sucrose or mannitol, to the required final concentrations. Stems were wounded by slicing them transversely (1-2mm thick) and incubating them as described above.

2.3.2 Sprouting of tubers

Tubers were placed in cardboard trays in a mono layer and allowed to sprout in the dark at 20°C. Samples were taken from sprouting tubers as follows, a 5mm thick transverse slice of tissue was cut from the middle of the tuber and tissue internal to the vascular ring was excised and frozen in liquid nitrogen - this sample was called 'middle'. Tissue underlying the apical sprout(s) up to a depth of 3cm was removed with a sterile cork borer (10mm diameter) and frozen in liquid

nitrogen - this sample was called 'proximal'. Sprouts were detached from the mother tuber prior to tissue sampling and frozen in liquid nitrogen.

2.4 General cloning procedures

2.4.1 Storage of plasmid and lambda DNA

Plasmid DNA was stored in TE buffer at -20°C. High molecular weight samples i.e.: lambda and genomic DNA were stored in TE buffer at 4°C.

2.4.2 Quantitation of DNA and RNA

The concentration of a DNA sample or a RNA sample was estimated by measurement of its absorbance (A) at 260nm (A_{260}) in a spectrophotometer. An A_{260} of 1 corresponds to 50µg/ml of double stranded DNA and 40µg/ml of RNA. All RNA samples were checked for purity by measurement of their A_{260} and A_{280}. The ratio A_{260}/A_{280} for pure RNA was 2.0.

2.4.3 Bacterial strains

Table 2.1 shows the different bacterial strains used during the course of this study and their genotypes.

2.4.4 Storage of bacterial strains

For short term storage, bacterial colonies on the appropriate agar plates were stored up to 2 weeks at 4°C. For long term storage, 0.15 ml of sterile glycerol was added to 0.85 ml of an overnight culture in LB medium. The mixture was then snap frozen in a dry ice-methanol bath and stored at -20°C or -80°C. The bacteria were viable for several years, as long as the samples did not thaw. Agar

stab cultures of different strains were maintained at room temperature as a backup to frozen glycerol stocks.

2.4.5 Composition of growth media

The composition of various media is shown in Table 2.2. All media were autoclaved at 15 p.s.i for 20 minutes. If required, antibiotics or vitamins were added aseptically to medium after it had cooled to 50°C.

2.4.6 Supplements to growth media

Stock solutions of antibiotics and vitamins were made up in sterile water, filtered through a Millipore filter (0.2 μm) and stored at -20°C.

Ampicillin and kanamycin were prepared as stocks of 50mg/ml. Working concentration for ampicillin was 50μg/ml and that for kanamycin was 70μg/ml. Chloramphenicol stocks were made at 50mg/ml in absolute ethanol, stored at -20°C and used at a final concentration of 50μg/ml.

2.4.7 Commonly used solutions

The composition of solutions used frequently is listed in Table 2.3. All the solutions, except 50X Denhardts were sterilised by autoclaving at 15 p.s.i. for 20 minutes.

2.4.8 Phenol extraction and ethanol precipitation of DNA

Crystalline phenol was redistilled at 160°C, equilibrated twice with equal volumes of 1M Tris.HCl pH8.0 and stored in aliquots at -20°C under nitrogen. Before use, the phenol was melted at 65°C, 8-hydroxyquinoline was added at a

Table 2.1 Genotype of the bacterial strains

The genotype of bacterial strains used for the maintainance of plasmids, the propagation of lambda phage and the production of single stranded DNA are shown on the opposite page .

Strain	Genotype	Source
E. coli L87	*Sup*E, *Sup*F, *h s d* R (Ecok r⁻ m ⁻) *trp* r, *met* D, *ton*A	Amersham
E. coli NM514	*hsd*R514 (Ecok r⁻m-) *Arg*H, *gal*E, *gal*X, *str*A, *lyc*B7 (hfl⁻)	Amersham
E. coli DS941	AB1157, *rec*F⁻, *lac*Z M15,*lac*I^q	Sherrat,D Dept. of Genetics, Glasgow Univ.
*E. coli mv*1190	Δ(*lac-pro*), *thi*, *sup*E, Δ(*sr*1-*rec*A)::Tn10(*tet*^{r}), F':*tra*D36, *pro*AB, *lac*Iq ZΔM15	Biorad

Table 2.2 Composition of growth media

The composition of various media used during the course of the project are shown opposite. All media were autoclaved after preparation (Section 2.2) and, if required, antibiotic or vitamin supplements were added when the media had cooled to 50°C.

[A] COMPLEX MEDIA-

MEDIUM	COMPOSITION PER 1000ml
(1) L-broth (LB)	10g bacto tryptone 5g yeast extract 5g NaCl
(2) 2X TY	16g bacto tryptone 10g yeast extract 5g NaCl
(3) Top Agar	10g bacto tryptone 5g yeast extract 5g NaCl 2.5g $MgSO_4$ 7g Agarose
(4) L-Agar (LB)	L-broth 15g Agar

[B] DEFINED MEDIA-

(1) M9 medium The following M9 salts were autoclaved in a 100 ml volume

6g Na_2HPO_4
3g KH_2PO_4
0.5g NaCl
1g NH_4Cl
pH 7.4

After cooling, the following sterile solutions were added to 885ml of sterile distilled water together with 100ml of M9 salts

2ml 1 M $MgSO_4$
10ml 20% glucose
1ml 0.1 M $CaCl_2$
2ml thiamine HCl

(2) M9 Agar 1000ml M9 medium
15g Agar

Table 2.3 Commonly used solutions

The solutions used frequently and their compositions are outlined on the opposite page. All solutions were sterilised by autoclaving as described in Section 2.2. Solutions used in analysis of RNA were treated with 0.1% DEPC for 16 hours and then autoclaved.

Phage buffer (SM):
50mM Tris.HCl pH7.5
100mM NaCl
10mM $MgSO_4$
0.01% (w/v) Gelatin

TE buffer:
10mM Tris.HCl pH8.0
1mM EDTA.$2H_2O$

STE buffer:
100mM NaCl
10mM Tris.HCl pH7.5
1mM EDTA.$2H_2O$

20X SSC:
3M NaCl
0.3M Tri-sodium citrate pH7.0

20X SSPE:
3.6M NaCl
200mM NaH_2PO_4 pH7.0
20mM EDTA.$2H_2O$

10X TBE:
0.89M Tris
0.89M boric acid
0.02M EDTA.$2H_2O$

50X Denhardt's solution:
1% (w/v) BSA fraction IV
1% (w/v) Ficoll type 400
1% (w/v) Polyvinylpyrrolidone 40

50X Denhardt's solution was made up in sterile DEPC treated distilled water, filtered through a Millipore membrane and stored at -20°C .

final concentration of 0.1% (w/v) and it was equilibrated with an equal volume of 0.1M Tris.HCl pH8.0 and stored under the same buffer at 4°C in a dark bottle. For RNA preparation an equal volume of a mixture of chloroform and isoamyl alcohol (24:1) was added to the phenol, which was equilibrated and stored as described above.

DNA solutions were extracted with phenol:chloroform and finally chloroform:isoamyl alcohol (24:1) as follows. The sample was extracted with an equal volume of phenol:chloroform saturated with TE buffer (pH8.0), vortexed for 30 seconds and centrifuged in a microfuge for 3-5 minutes at room temperature. The upper aqueous layer was carefully removed and transferred to a fresh tube. The sample was extracted with chloroform:isoamylalcohol, as described for phenol extraction and the aqueous extract was precipitated with absolute ethanol.

DNA was purified from low melting point-agarose (LMP-agarose) gel slices as follows. Excess agarose was trimmed off and two volumes of TE buffer were added to the gel slice. The tube was incubated at 65°C for 5 minutes, vortexed with an equal volume of hot phenol (65°C), the phases were separated after centrifugation and the aqueous phase was extracted with hot phenol (65°C) in the same manner. The aqueous phase was collected and extracted two times with hot phenol:chloroform (24:1; at 65°C). Finally, the aqueous phase was extracted with chloroform and precipitated with ethanol.

DNA was routinely precipitated with absolute ethanol as follows. One-tenth volume of 2.5M sodium acetate pH5.2 was added to the DNA sample Two volumes of absolute ethanol (-20°C) were added and the contents were mixed, and precipitated at -20°C for at least one hour. The DNA was pelleted after centrifugation for 10 minutes in a microfuge at 4°C, the supernatant was discarded and the pellet was washed with 70% ethanol (-20°C) and DNA was pelleted as described above. The pellet was dried briefly in a vacuum desiccator and dissolved in TE buffer.

2.4.9 Plasmid purification

2.4.9.1 Plasmid mini preps

10ml of LB containing ampicillin (50µg/ml) was inoculated with a single colony of bacteria carrying the required plasmid. Cells were grown overnight with aeration at 37°C. Around 1.0ml of the cell suspension was centrifuged in an Eppendorf microfuge at 12,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 100µl of lysis solution (25mM Tris.HCl pH8.0, 50mM glucose, 10mM EDTA and 2mg/ml lysozyme). The tube was left on ice and, after 5 minutes, 200µl of freshly prepared alkaline SDS (0.2M NaOH and 1% SDS) was added and the contents of the tube were mixed gently. At this stage lysis of cells had occurred and as a result the suspension became viscous. Chromosomal DNA was selectively precipitated by adding 150µl of high salt solution (3M sodium acetate pH5.0). The suspension was left on ice for 5 minutes and the precipitate of chromosomal DNA was removed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was saved and 1ml of cold absolute ethanol (-20°C) was added to it. Precipitation was carried out at -20°C for 30 minutes and plasmid DNA was pelleted by centrifugation at 12,000 rpm for 2 minutes. The pellet was resuspended in 100µl of 0.1M sodium acetate pH6.0 and 200µl of ethanol was added to it. Precipitation was carried out at -20°C for 10 minutes and plasmid was pelleted by centrifugation at 12,000 rpm for 2 minutes. The pellet was dried and dissolved in 20µl of TE buffer.

2.4.9.2 Plasmid midi preps

As described above, 10-40 ml of LB containing ampicillin (50µg/ml) was inoculated with a colony of bacteria containing the required plasmid and grown with agitation at 37°C overnight. The cells were harvested by centrifugation in sterile

30ml corex tubes at 10,000 rpm for 5 minutes in a Sorvall RC-5 Superspeed Centrifuge (HB-4 rotor). The supernatant was discarded and the pellet was resuspended in 1.0ml of lysis solution (25mM Tris.HCl pH8.0, 50mM glucose, 10mM EDTA and 2mg/ml lysozyme). The tube was left on ice and after 30 minutes, 2.0ml of freshly prepared alkaline SDS (0.2M NaOH and 1% SDS) was added to it; the contents of the tube were mixed gently. After incubation on ice for 5 minutes, 1.5ml of high salt solution (3M sodium acetate pH5.0) was added, the suspension was mixed gently by inversion and was left on ice for 60 minutes. The suspension was centrifuged at 10,000 rpm, 4°C for 10 minutes (HB-4 rotor) and 4.0ml of the clear supernatant was collected in a fresh 15ml corex tube. Nucleic acids were precipitated by adding 8.0ml of absolute ethanol (-20°C) and incubating at -20°C for 15 minutes. The precipitated nucleic acids were pelleted by centrifugation at 10,000 rpm for 5 minutes at 4°C (HB-4 rotor), the supernatant was discarded and the pellet was resuspended in 2.0ml of TE buffer. Deproteinisation was carried out by extraction with an equal volume of phenol:chloroform as described in section 2.4.8. The aqueous phase was collected in a fresh 15ml corex tube and the organic phase was back extracted with 1.0ml of TE buffer as described above. The second aqueous phase was combined with the aqueous phase from the first extraction, two volumes of ethanol (-20°C) was added and precipitation was carried out at -20°C for 15 minutes. After the sample was centrifuged at 10,000 rpm for 5 minutes at 4°C, the supernatant discarded, and the pellet resuspended in 400µl of TE, it was transferred to a 1.5ml microfuge tube and precipitated with 60µl of 1M sodium acetate pH8.0 and 1.0ml absolute ethanol (-20°C) at -20°C for 10 minutes. Centrifugation was carried out at 12,000 rpm in an Eppendorf microfuge for 2 minutes, after which the supernatant was discarded and the pellet resuspended in 200µl of TE. 30µl of 1M sodium acetate pH8.0 and 500µl of ethanol (-20°C) was added to it and it was incubated at -20°C for 10 minutes. The sample was centrifuged for 2 minutes in a microfuge, the supernatant was discarded and the pellet was resuspended in 200µl of TE buffer. The

resuspended pellet was then incubated with 20µl of RNAse (1mg/ml; heat treated at 100°C for 10 minutes to inactivate contaminating DNAses) at 37°C for 30 minutes. 7.5µl of 4M sodium acetate pH6.0 and 300µl of ethanol were added to the digested sample and precipitation was carried out at room temperature for 10 minutes. The sample was centrifuged for 2 minutes, the supernatant discarded and the pellet was resuspended in 200µl TE buffer. Yields of 100µg/10ml of culture were usually obtained for derivatives of pBR322. The plasmids could then be used for purification of inserts.

2.4.10 Restriction endonuclease digestion of DNA

Restriction enzyme digestion of DNA was carried out routinely in a reaction volume of 20µl. A typical reaction contained up to 10µg of DNA, 1µl of the appropriate restriction enzyme (10 units/µl), 2µl of the corresponding 10X reaction buffer (provided by the manufacturer) and distilled water to make the final volume 20µl. The digestion was carried out at 37°C for 2-3 hours. The digested sample was extracted twice with phenol (Section 2.4.8) and precipitated with ethanol as described in Section 2.4.8.

2.4.11 Agarose electrophoresis

Agarose gels were prepared as in Maniatis *et al.*(1982). Routinely 0.8-1.5% agarose gels were used depending upon the size range of DNA fragments to be separated. The agarose was dissolved in Tris-borate electrophoresis buffer (0.089M Tris base, 0.089M Boric acid, 0.002M EDTA) by autoclaving at 15 p.s.i. for 5 minutes. After the agarose solution had cooled to 55°C, it was poured in a casting tray (GIBCO-BRL, H5 apparatus) and allowed to set. The precast gel was completely submerged in Tris-borate electrophoresis buffer containing ethidium bromide (0.5µg/ml). 0.1 volumes of sample buffer (0.25% [w/v] bromophenol

blue in 40% [w/v] sucrose) was mixed with the DNA sample, which was then applied to the preformed wells and electrophoresis was carried out at 20 volts for 16 hours or until the bromophenol blue had migrated to the opposite end of the gel. DNA was visualised by ethidium bromide fluorescence under UV illumination from a transilluminator. Gels were photographed with a Polaroid CU-5 camera (Genetic Research Instrumentation) using 665 positive/negative film.

LMP-agarose gels were prepared essentially as described above, with the following modifications. The gels were cast and allowed to set at 4°C. The electrophoresis of samples was carried out as usual at room temperature. DNA was visualised under long wavelength UV illumination and the bands of interest were cut out with a scalpel and purified as described in Section 2.4.8.

2.4.12 Labelling of probes with radioisotopes

2.4.12.1 Endlabelling of oligonucleotide probes

100-200ng of dephosphorylated oligonucleotides were end-labelled with 100µCi of [γ-^{32}P]ATP (5000Ci/mmole; Amersham) in 10mM $MgCl_2$, 100mM Tris.HCl pH7.6, 0.02M β–mercaptoethanol using 10 units of T4 polynucleotide kinase in a final volume of 50µl, at 37°C for 1 hour. The reaction was stopped by the addition of 2µl of 0.5M EDTA pH8.0 and 2µl of 20% SDS. The oligonucleotide probes were used immediately or were stored at -20°C up to 1 week. The amount of ^{32}P incorporated was measured as described in Section 2.5.2.1.

2.4.12.2 Labelling of cDNA inserts using random primers

The random priming reaction was carried out as described by Feinberg and Vogelstein (1983). 50ng of cDNA insert in 10µl TE buffer was denatured in a

boiling water bath for 5 minutes and cooled rapidly on ice. 10µl of random priming cocktail (Table 2.4) was added to the denatured DNA together with 1µl of molecular biology grade BSA (20mg/ml; Boehringer) which was added to stabilise Klenow polymerase. 25µl of distilled water, 30µCi of [α-^{32}P]dCTP (Amersham) and 1 unit of Klenow polymerase (Boehringer) were added to the mix to get a final volume of 50µl. After an overnight incubation at R.T., the reaction was stopped with 2µl of 0.5M EDTA pH8.0 and 2µl of 20% SDS. The amount of ^{32}P incorporated was measured as described in Section 2.5.2.1, and the cDNA probes were used immediately or were stored at -20°C up to 2 weeks.

2.4.12.3 Labelling of sticky ends

1µl of [α–^{32}P] dATP (for *Eco*R1 ends) and 1 unit of Klenow polymerase were added to a 20µl restriction digestion reaction, and the reaction was incubated at 30°C for 30 minutes. The samples were subjected to electrophoresis (Section 2.4.11), the gel was fixed in 7% trichloroacetic acid for 30 minutes and dried. Labelled digestion products were detected by autoradiography of the gel at -80°C for the appropriate period.

2.4.13 Southern blotting

DNA, separated by electrophoresis on agarose gels, was visualised under UV illumination on a transilluminator and the gel was photographed as described in Section 2.4.11. The gel was then soaked in denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 minutes and subsequently in neutralisation buffer (0.5M Tris.HCl pH7.6, 1.5M NaCl) for a further 30 minutes. Blotting of DNA was on to Hybond N nylon membrane (Amersham) using 20X SSC for the transfer over 16 hours as described by Maniatis *et al.*(1982) The membrane was then air dried and baked at 80°C for 2 hours.

Table 2.4 Compostion of hexanucleotide labelling solution

The hexanucleotide solution described in Section 2.4.12.2 is outlined on the opposite page. The hexanucleotide labelling solution was prepared as described, divided into 12µl aliquots and stored at -20°C.

Solution A: 0.5mM deoxynucleotide triphosphates (dATP, dGTP, dTTP) in 1.25M Tris HCl pH8, 0.125M $MgCl_2$, 10mM β-mercaptoethanol.

Solution B: 2M Hepes pH6.6

Solution C: 50 units hexadeoxynucleotides (Pharmacia) resuspended in 556µl distilled water.

Solutions A, B and C were mixed in the ratio 1.0:2.5:1.5, to prepare the hexanucleotide labelling solution.

Sandwich blots were done essentially in the same way, except that after the neutralisation step the gel was soaked in 20X SSC for 15 minutes, removed from the 20X SSC wash and placed inbetween two Hybond N nylon membranes. Absorbent tissues were placed on either side of the "sandwiched" gel and the transfer of DNA was allowed to proceed from 4-8 hours, as described by Maniatis *et al.*(1982).

2.4.14 Northern Blotting

Denatured RNA was separated on an agarose-formaldehyde gel in MOPS electrophoresis buffer. Usually 1.5% agarose-formaldehyde gels were prepared in 1X (20mM) MOPS (20mM MOPS, 5mM sodium acetate pH7.0, 1mM EDTA) containing 6.8% (w/v) formaldehyde. Formaldehyde was added to molten agarose in 1X MOPS after it had cooled to 65°C. Electrophoresis was in 1X MOPS buffer, with buffer recirculation.

RNA in 4.8µl of distilled water was mixed with 10µl deionised formamide, 2µl 10X MOPS buffer and 3.2µl 38% (w/v) formaldehyde. RNA was denatured in this solution at 65°C for 5 minutes, cooled on ice and then made 12.5% (w/w) glycerol, 0.025 mg/ml bromophenol blue. The samples were loaded on to the gel, subjected to electrophoresis for 16 hours and the gel was blotted overnight on to Hybond-N nylon membrane using 20X SSC for the transfer (Maniatis *et al.*,1982). After the filter was dried in air, the transferred RNA was cross-linked to the filter by exposing it (RNA side down) to ultraviolet light from a transilluminator for 1 minute and 45 seconds. The filter was then ready for hybridisation.

2.4.15 Hybridisation of nucleic acids

Depending upon the type of probe used (see Sections 2.4.12.1-2),

different hybridisation conditions were used.

2.4.15.1 Oligonucleotide probes

2.4.15.1.1 Hybridisation of plaque impressions

Hybridisation was carried out as described by Woods (1984). Duplicate filters of plaque impressions were always used, in order that genuine hybridisation could be discriminated from spurious signals. The filters were prewashed in 3X SSC/0.5% SDS at 65°C for approximately 3 hours prior to prehybridisation. The filters were pre-hybridised in 5X SSC, 0.5% SDS, 200 µg/ml heparin, 0.05% sodium pyrophosphate at 37°C for 2 hours, after which (200ng/50ml) ^{32}P end-labelled oligonucleotide probe was added to fresh hybridisation buffer (5X SSC, 200 µg/ml heparin, 0.05% sodium pyrophosphate) and hybridisation was carried out at 37°C for 16 hours. Filters hybridised with ^{32}P end-labelled oligonucleotide SP1 and oligonucleotide SP2 were subjected to post-hybridision washes of different stringencies. Filters hybridised with oligonucleotide SP1 and oligonucleotide SP2 were washed in a large volume of 6X SSC, 0.05% sodium pyrophosphate at 37°C for 1 hour, after which, those hybridised with oligonucleotide SP1 were transferred to prewarmed 6X SSC, 0.05% sodium pyrophosphate at 47°C and washed for approximately 10 minutes, whereas those hybridised with oligonucleotide SP2 were washed in prewarmed 6X SSC, 0.05% sodium pyrophosphate at 50°C for approximately 10 minutes. The filters were removed, drained of excess buffer and exposed to X-ray film between two intensifying screens at -70°C for 7 days.

2.4.15.1.2 Hybridisation of blots

Hybridisation of Southern blots with oligonucleotide SP1 or

oligonucleotide SP2 were essentially done as described above, except that, the filters were not subjected to a preliminary washing step.

Northern blots were hybridised with 100 µg of ^{32}P end-labelled 28S rRNA oligonucleotide probe (Barbu & Dautry, 1989) in 4X SSPE, 0.2% sodium pyrophosphate, 0.2% SDS, 500 µg/ml heparin at 42°C for 16 hours and were washed in 2X SSPE, 0.1% sodium pyrophosphate, 0.1% SDS at 37°C for 30 minutes. The moist filter was exposed to preflashed X-ray film between two intensifying screens at -70°C for approximately 24 hours.

2.4.15.2 cDNA probes

Hybridisation was carried out in hybridisation chambers (Boulnois, 1987) in 20-30ml of hybridisation buffer, which contained 50% formamide, 5X SSPE, 0.1% SDS, 100µg/ml of denatured salmon sperm DNA and 5X Denhardts solution. The filters were prehybridised with gentle shaking in the hybridisation buffer at 42°C for 4 hours.

The cDNA probes were denatured by heating in a boiling water bath for 5 minutes, quenched on ice for 2 minutes, after which 10^6 cpm/ml of the denatured ^{32}P-labelled cDNA probe was added to the prehybridisation solution and hybridisation was carried out at 42°C for 16 hours. The filters were then removed from the hybridisation solution, were rinsed briefly and washed twice in 2X SSPE/0.1% SDS (prewarmed) at 42°C for 15 minutes. The filters were further washed twice in 0.5X SSC/0.1% SDS (prewarmed) at 65°C for 15 minutes and subsequently in 0.1X SSC/0.1% SDS.(prewarmed) at 65°C for 20 minutes. After excess moisture was drained from the wet filters, they were covered in Saran wrap and exposed to preflashed X-ray film with intensifying screens at -70°C.

The radioactive probe was routinely removed and the filters reused for hybridisation as follows. Wet Hybond N nylon membranes were washed in (prewarmed to 65°C) 5mM Tris HCl pH8.0, 2mM EDTA and 0.1X Denhardts

solution at 65°C for 2 hours. The filter was then processed as described earlier, with a fresh probe.

2.4.16 Isolation of total RNA

Total RNA was prepared from plant tissues as described by Logemann *et al.*(1987). Wherever possible fresh tissue was used, alternatively tissue was frozen in liquid nitrogen and stored at -80°C until required. Tissue was frozen in liquid nitrogen and ground to a fine powder in a precooled (-80°C) sterile mortar and pestle. The powdered tissue was transferred to another precooled (-20°C) sterile mortar, in which it was swiftly homogenised with 2 volumes of guanidine buffer (8M guanidine hydrochloride, 0.02M 4-morpholine-ethansulfonic acid, 0.02M EDTA and 0.05M β–mercaptoethanol pH7.0). The guanidine extract was immediately centrifuged at 10,000 rpm for 10 minutes at 0°C in a precooled centrifuge (Sorvall RC-5, HB-4 rotor). The RNA containing supernatant was filtered through one layer of sterile mira cloth to remove debris and the filtrate was extracted with 1 volume of phenol/chloroform/isoamylalcohol (25:24:1) to remove proteins. After extraction the mixture was centrifuged at 10,000 rpm for 15 minutes at 15°C to separate the phases. The aqueous phase was collected and mixed with 0.7 volumes of precooled (-20°C) ethanol and 0.2 volumes of 1M acetic acid.. This step is reported to selectively precipitate RNA, leaving DNA and residual proteins in solution (Logemann *et al.*, 1987). The precipitation was carried out at -70°C for 1 hour and the precipitated RNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The RNA pellet was washed twice with 3M sodium acetate pH 5.2 at room temperature. This step removes contaminating polysaccharides and low molecular weight RNAs, which remain in solution, leaving intact RNA as a pellet after centrifugation at 10,000 rpm for 5 minutes at 15°C. Residual salt was removed from the pellet by a 70% ethanol wash. The RNA was stored as a pellet under 70% ethanol at -20°C until required.

2.4.17 Purification of poly(A$^+$)mRNA

Poly(A$^+$)mRNA was purified by batch adsorption to oligo dT cellulose (GIBCO-BRL) according to Maniatis *et al*(1982). 10ml of 1X loading buffer (20mM Tris.HCl pH7.6, 500mM NaCl, 1mM EDTA) was added to 1g of oligo dT cellulose in a sterile glass universal. The slurry was left at room temperature for 60 minutes, transferred to a sterile column, and washed with three column volumes each of:

1) Distilled water

2) 0.1M NaOH and 0.005M EDTA

3) Distilled water, until the pH of the column effluent was less than 8.

The packed column was washed with five column volumes of loading buffer and the slurry of activated oligo dT cellulose and loading buffer was transferred to a sterile bijou. After oligo dT cellulose had settled, excess loading buffer was siphoned off. Total RNA (0.5mg/ml) in distilled water was heated at 65^{o}C for 5 minutes, an equal volume of 2X loading buffer was added and the mix was cooled to RT. The RNA sample was then added to the oligo dT cellulose in the bijou and the slurry was gently mixed at RT for 10 minutes. Once the oligo dT cellulose had settled, the supernatant which contained unbound RNA was removed. The unbound RNA sample was reheated at 65^{o}C for 5 minutes, cooled to RT and mixed with the same batch of oligo dT cellulose as described earlier. After the unbound RNA had been siphoned off, the oligo dT cellulose was very gently resuspended in 3 volumes of loading buffer and carefully loaded into the glass column. Oligo dT cellulose was then washed with 5 column volumes of loading buffer, followed by 4 column volumes of loading buffer containing 0.1 M NaCl. Poly(A$^+$)mRNA was then eluted with 2-3 column volumes of 10mM Tris HCl pH7.5 and 1mM EDTA. Poly(A$^+$)mRNA was precipitated with 3M sodium acetate pH5.2 and absolute ethanol at -70^{o}C overnight. The batch adsorption method was

selected as the problem of column clogging was avoided, as a large bulk of the total RNA that was unbound was siphoned. Yields of poly(A^+)mRNA obtained were comparable to those by the standard method.

2.5 Construction of potato tuber cDNA libraries

2.5.1 cDNA synthesis

cDNA synthesis was carried out using a commercial kit from Amersham (cDNA synthesis system plus RPN.1256), essentially by the method of Gubler and Hoffmann (1983). First strand cDNA synthesis reaction contained 5µg poly(A^+)mRNA (potato tuber), 2.5 units of human placental ribonuclease inhibitor, 1.0mM dATP, 1.0mM dGTP, 1.0mM dTTP, 0.5mM dCTP and 12.5µCi of [α^{32}-P]dCTP. The reaction was carried out with 4µg of oligo dT primer and 100 units of AMV reverse transcriptase in a final volume of 50µl. The concentration of sodium pyrophosphate and the buffer constituents was not provided by the manufacturer.

The mRNA was heated at 70°C for 1 minute, quenched on ice and then added to the reaction. First strand cDNA synthesis was carried out at 42°C for 90 minutes, after which the reaction was placed on ice and a 1µl aliquot was removed for analysis (Section 2.5.2).

125µCi of [α^{32}-P]dCTP, 4 units of *E.coli* ribonuclease H and 115 units of *E.coli* DNA polymerase I were added to the first strand reaction mix, and the buffer concentration was adjusted (details of constituents not provided) to give a final volume of 250µl. The contents were mixed gently and sequentially incubated at 12°C for 60 minutes, at 22°C for 60 minutes and an inactivation step was carried out at 70°C for 10 minutes. 10 units of T4 DNA polymerase was added to the cDNA synthesis mix, which had been cooled on ice and the mix was incubated at 37°C for 10 minutes. Finally, 10µl of 0.2M EDTA pH8.0 was added to terminate the reaction, and 1µl aliquot was removed for analysis (Section 2.5.2).

The cDNA was purified by phenol/chloroform extraction (Section 2.4.8) and ethanol precipitation in the presence of 20μg glycogen (Boehringer).

2.5.2 Analysis of cDNA products

The incorporation of [α^{32}-P]dCTP provided information about the efficiency of the cDNA synthesis reaction, the mass of the cDNA synthesised and its size distribution.

2.5.2.1 Incorporation of radioisotope

1μl sample of the first strand reaction was added to 20 μl of TE buffer and mixed. This was called sample I. Two Whatman DE81 paper discs (2.4 cm) designated A and B had 2μl of sample I spotted on each of them. Filter B was washed 6 times in 0.5M $Na_2HPO_4$for 5 minutes, two times in distilled water for 1 minute each and two times in 95% ethanol for 1 minute each. Both filters, A and B, were dried under a lamp, were put into separate scintillation vials containing 5 mls of Ecoscint and the amount of radioactivity was measured using a LKB 1209 Rackbeta scintillation counter. The unwashed filter A represented the total radioactivity in the sample while the washed filter B represented the radioactivity incorporated into cDNA.

Similarly 1μl sample of the second strand reaction was added to 20μl of TE buffer and mixed. This was called sample II. Two Whatman DE81 paper discs (2.4cm) designated C and D were each spotted with 2μl of sample II. Filter D was washed as described above for filter B. The amount of radioactivity on both filters was measured in a scintillation counter as described above.

2.5.2.2 Calculation of the efficiency of cDNA synthesis

The efficiency of cDNA synthesis reaction in terms of both the mass synthesised and the product size distribution could be determined by including [α–^{32}P]dCTP in the synthesis reaction.

The monitoring procedure is outlined below:

[i] The percentage incorporation of [α-^{32}P]dCTP in the first and second strand of cDNA was determined by liquid scintillation of radioactivity bound to DE81 discs (Section 2.5.2.1).

[ii] the quantity of first and second strand cDNA synthesised was calculated from the values of percentage incorporation of [α-^{32}P]dCTP.

[iii] The size of the first and second strand cDNA synthesised was examined on an alkaline agarose gel (Section 2.5.2.3).

The total cpm. in the sample of the first strand reaction was A cpm (on filter A) and radioactivity on filter B represented B cpm incorporated into nucleic acid. Also cpm on filter C represented the cpm in the sample of the second strand reaction and the cpm incorporated into nucleic acid was represented by radioactivity on filter D.

The percentage incorporation of [α-^{32}P]dCTP in the first strand was calculated as follows:

$$\text{Percentage incorporation (first strand)} = \frac{B}{A} \times 100\%$$

However before calculating the percentage incorporation for the second strand reaction, the amount of radioactivity incorporated in the first strand reaction must be subtracted. Since the first strand reaction mix is 1/5.33 of the volume of the

stopped second strand reaction mix [First strand reaction volume was 49µl; second strand reaction volume was 260.5µl], B must be divided by 5.33 to compensate for this and the resultant value subtracted from C and D.

The percentage incorporation in the second strand was calculated as follows:

$$\text{percentage incorporation (second strand)} = \frac{D - \frac{B}{5.33}}{C - \frac{B}{5.33}} \times 100\%$$

The percentage incorporation values were used to estimate the amount of cDNA synthesised for both, the standard (50µl) first strand reaction and the (250µl) second strand reaction as follows:

1) amount of cDNA synthesised = (% incorporation x 350 ng)

2) total amount of double stranded cDNA = (2 x amount of second strand cDNA synthesised)

The yield of the first and second strand synthesis was calculated using the following formulae:

Yield 1.

$$\%\ \text{of mRNA transcribed} = \frac{\text{ng of 1st strand cDNA synthesised}}{\text{ng of input RNA}} \times 100\%$$

Yield 2.

$$\%\ \text{of 2nd strand cDNA transcribed from 1st. strand cDNA} = \frac{\text{ng of 2nd strand cDNA}}{\text{ng of 1st strand cDNA}} \times 100\%$$

Yields 1 and 2 gave an estimate of the success of the first and second strand synthesis.

2.5.2.3 Alkaline agarose gel electrophoresis

The cDNA products were analysed on an alkaline agarose gel (Mac Donnell *et al.*, 1977). cDNA samples, each containing 10,000-30,000 cpm of radioactivity incorporated into nucleic acid, were taken from the aliquots for analysis of cDNA products. They were placed in a microfuge tube and traces of RNA were hydrolysed with alkali as follows:- 20µl of 100µg/ml salmon sperm DNA was added to the cDNA sample and to this cDNA-carrier DNA mixture was added one third the volume of 1M NaOH. Hydrolysis was carried out at 46°C for 30 minutes. A volume of 1M HCl, equal to the volume of 1M NaOH was added and the samples were buffered with an equal volume of 1M Tris buffer pH8.0. This mixture was extracted with phenol/chloroform and precipitated with ethanol. The cDNA pellet was resuspended in 10µl of alkaline agarose loading buffer (50mM NaOH, 1mM EDTA, 2.5% Ficoll type 400, 0.025% bromocresol green), and the denatured samples were loaded on an 1.5% alkaline agarose gel cast in 50mM NaCl, 1mM EDTA that had been equilibrated in alkaline electrophoresis buffer (30mM NaOH, 1mM EDTA) at least 30 minutes prior to loading cDNA samples. Electrophoresis was carried out in alkaline electrophoresis buffer at 150mA with buffer circulation for three hours. The gel was fixed in 7% trichloroacetic acid (two changes) for 30 minutes, dried in a vacuum drier and exposed to X-ray film at -70°C for the appropriate period.

2.5.3 Cloning of cDNA

2.5.3.1 Methylation, linker ligation and *Eco*R1 digestion of cDNA

1µg of double stranded, blunt ended cDNA in TE buffer was methylated using 20 units of *Eco*R1 methylase and S-Adenosylmethionine in methylation buffer (Amersham) at 37°C for 60 minutes. The enzyme was inactivated at 70°C for 10 minutes.

Methylated cDNA was added to a tube containing the ligation buffer, *Eco*R1 linkers and 5 units of T4 DNA ligase, and the ligation reaction was carried out at 15°C for 16-24 hours. T4 DNA ligase was inactivated at 70°C for 10 minutes.

Linkered cDNA was added to *Eco*R1 digestion buffer containing 100 units of *Eco*R1 restriction endonuclease. The digestion was carried out at 37°C for 5 hours and the enzyme was inactivated at 70°C for 10 minutes.

2.5.3.2 Separation of linkered cDNA from free linkers

A prepacked column provided with the kit was rinsed with 3 ml of STE and equilibrated with a further 3ml of STE. The amount of radioactivity in the *Eco*R1 digested cDNA sample was measured by Cerenkov counting and it was loaded on the column. After the sample had entered the column, the column was washed with 100µl of STE and the column eluate (~200µl) was collected in an Eppendorf tube. Subsequently, STE was used to wash the column in 200µl aliquots and 10 fractions were collected. The leading two fractions that contained most of the radioactivity were detected using a hand held geiger counter. The amount of radioactivity was measured by Cerenkov counting. Only linkered cDNA was present in the leading two fractions, whereas the trailing fractions contained cDNA contaminated with free linkers. The two major fractions with highest dpm were pooled and the linkered cDNA was precipitated with 20µl of glycogen (GIBCO-BRL) as described in Section 2.4.8. cDNA was pelleted in a microfuge at 14,000 rpm, at 4°C for 30 minutes. The cDNA was resuspended in STE buffer.

2.5.3.3 Ligation to λgt10 arms

The cDNA, with added *Eco*R1 sticky ends, was ligated to λgt10 *Eco*R1 arms. Three crucial control ligations were included at this stage. These performed two functions, they were tests of the efficacy of the entire cloning process and they provided background data about the composition of the library (recombinants : non-recombinants)-{see Section 2.5.4.3}. All ligations were carried out in 1X ligation buffer with 2.5 units of T4 DNA ligase in a total volume of 10μl at 15°C for 20 hours.

The ligation products were precipitated with 1μl of 3M sodium acetate pH 5.2 and 27μl absolute ethanol in an ethanol/dry-ice bath for 30 minutes. The ligation products were pelleted by centrifugation in a microfuge for 15 minutes, the pellet was dried and resuspended in 2.5μl of TE buffer.

2.5.3.4 *In vitro* packaging

Packaging extracts were provided by Amersham. For each packaging reaction one tube of extract A and one tube of extract B was used. The extracts were thawed on ice, and were used immediately. 10μl of extract A was added to the ligation products (Section 2.5.3.3) and subsequently 15μl of extract B was added to the same tube. The contents were mixed gently using a plastic pipette tip, the reaction tube was briefly centrifuged and incubated at 20°C for 2 hours. 0.5 ml of SM buffer, followed by 10μl of chloroform was added to the packaging reaction. Packaged phage extracts were stored at 4°C.

2.5.4 Determination of phage titre

2.5.4.1 Preparation of bacteria for phage plating

10 ml of LB supplemented with 0.4% maltose was inoculated with a single colony of the appropriate *E.coli* strain (NM514 or L87) and incubated with aeration at 37°C overnight. 1 ml of the overnight culture was added to 50 ml of prewarmed LB supplemented with 0.4% maltose. The cells were incubated at 37°C with vigorous aeration until they reached an A_{600} of 0.5 ($2.5x10^8$ cells/ml). After the culture was cooled on ice, the cells were pelleted at 3,000 rpm for 10 minutes at 4°C (Beckman bench top). The cells were resuspended in 15 ml of ice cold 10mM $MgSO_4$, mixed thoroughly and stored at 4°C. Cells were usually used on the same day.

2.5.4.2 Plating of the phage

10 fold serial dilutions of the λ phage stock were prepared in SM phage buffer (Section 2.4.7). *E.coli* cells were infected as follows, each sample was prepared by mixing 100µl of the phage plating cells (Section 2.5.4.1) with 100µl of the dilution to be assayed. The samples were incubated at 37°C for 15 minutes, to allow for the adsorption of the phage particles on bacteria. 4 ml of molten phage top agar at 45°C was added to each sample, mixed gently and poured onto a dry 90 mm plate containing approximately 20ml of solidified LB agar. The top agar was allowed to set at RT and then the plates were incubated at 37°C overnight. The plaques were counted and the titre was determined for each sample.

2.5.4.3 Analysis of phage titre

The titre of the cDNA containing λgt10 recombinants was determined on *E.coli* L87 (wild type) and *E.coli* NM514 (*hfl*$^+$). In addition to the cDNA reactions, several controls were included in the ligation and packaging reactions.

1) Control λgt10 DNA - the titre on *E.coli* L87 reflected the efficiency of the *in vitro* packaging reaction.

2) λgt10 arms only - this crucial control provided several items of information. Its titre on L87 checked that the λgt10 arms and T4 DNA ligase were working efficiently.
Two other values were obtained from this control, the ratio of the titre on L87:NM514 (called the arms selective ratio) and the background titre on NM514 (number of non-recombinants).

3) λgt10 arms plus control *Hae*III fragments - this sample was used to monitor the performance of the whole cloning system.

4.λgt10 arms plus cDNA sample - the most important figure was the increase over the background level of plaques on NM514. A true background figure for individual reaction was derived as follows: The arms selective ratio derived from control 2 was an invariant property of each batch of λgt10 arms and the host cells in combination, this ratio was directly applicable to the cDNA samples since the same batches of arms and cells were used. The L87 titre represented total products of the ligation/packaging reaction, which were mainly religated λgt10. Dividing the L87 titre by the arms selective ratio, provided a value for the true background on *E.coli* NM514 if no recombinants had been formed. The number of recombinants obtained could be accurately determined for each cDNA sample by subtracting its background titre from the actual titre obtained on *E.coli* NM514 cells.
The cloning efficiency per μg of cDNA was then obtained using the formula -

$$\text{Cloning efficiency (per } \mu\text{g cDNA)} = \frac{\text{number of recombinants} \times 1000}{\text{ng cDNA used}}$$

2.5.4.4 Screening the cDNA library

E. coli NM514 plating cell (Section 2.5.4.1) were infected with phage from the appropriate cDNA library at a ratio of 10^5pfu/10^9cells. *E. coli* infected with 10^5pfu were plated on to 23x23 cm petridishes in 50 ml of 0.7% top agarose and the top agarose was allowed to set. The plates were incubated upside down at 37°C for 12-16 hours and transferred to 4°C for atleast one hour. Hybond-N nylon membrane was used to take replica impressions and was placed carefully onto the surface of the plate. The filter and the plate were marked by making three asymmetrically placed holes through both with a hypodermic syringe needle. After 30 seconds, the filter was peeled off with a blunt ended forcep. Up to five replica filters could be taken from the same plate and with each successive filter the contact time was increased by 30 seconds. Filters were laid plaque side up for 5 minutes on a sheet of Whatman 3MM paper soaked in 0.5M NaOH, 1.5M NaCl. The filters were neutralised for 5 minutes in 0.5M Tris HCl pH7.0, 1.5M NaCl, rinsed in 2X SSC and placed on filter paper to dry. DNA was fixed to the filters by baking at 80°C for 2 hours.

The filters were hybridised as described in section 2.4.15.1.1

2.5.4.5 Rescreening of plaques

Geniune hybridisation signals on the autoradiographs were aligned with the petridishes and the growth on the plate corresponding to them was aseptically removed with the broad end of a pasteur pipette into 1.0 ml of SM buffer. Phage dilutions were prepared and 100-300 pfu were plated out as described earlier (Section 2.5.4.2). These plaques were screened (Section 2.5.4.4) until 100% of the plaques hybridised to the probe.

2.5.5 High titre phage lysate of λgt10 clones

High titre phage lysate was prepared by small scale liquid culture. A single isolated plaque was cored out from a LB agar plate using a sterile pasteur pipette. The plaque was added to 0.5ml *E.coli* NM514 plating cells (Section 2.5.4.1) in a 50ml Falcon tube. The tube was left at room temperature for 15 minutes to allow the cells to adsorb the phage. 5ml of LB broth containing 5mM $CaCl_2$ was added to the phage infected cells and the culture was incubated at 37°C for 4-5 hours with aeration. A few drops of chloroform were added to the tube and the contents were mixed for 5 minutes. Bacterial debris was pelleted at 3,000 rpm for 10 minutes in a Beckman benchtop centrifuge, the supernatant containing phage particles was removed and stored at 4°C until required. The supernatant contained a high titre of phage particles and was used to prepare small amounts of λgt10 DNA.

2.5.6 Phage DNA purification

An equal volume of 20% polyethylene glycol (PEG), 2M NaCl in SM buffer was added to the phage lysate (usually 4ml), the sample was mixed and incubated on ice for 60 minutes. Phage particles were pelleted at 3,000rpm for 20 minutes in a Beckman benchtop centrifuge. The pellet was drained and traces of polyethylene glycol were wiped from the inside of the tube. The pellet was resuspended in 750µl of LB broth, transferred to a microfuge tube and mixed with 750µl of DE-52 in LB. The contents were mixed gently by inverting the tube 30 times and centrifuged for 5 minutes in a microfuge. The supernatant was transferred to a fresh microfuge tube and mixed with DE-52 as described above.

750µl of the supernatant was treated with 13µl of Proteinase K (0.1mg/ml) and 32µl of SDS (10%) at room temperature for 5 minutes. 150µl of 3M potassium acetate was added to the sample and the mix was incubated at 88°C for 20 minutes, cooled on ice for 10 minutes, and spun in a microfuge at 14,000

rpm for 10 minutes. 800µl of the supernatant was transferred to a fresh tube and DNA was precipitated with an equal volume of isopropanol (-70°C) at -70°C for 10 minutes. The tube was warmed to room temperature and centrifuged in a microfuge at 14,000 rpm for 10 minutes. The pellet was washed in 70% ethanol and dried in vacuo. Phage DNA was resuspended in 20µl TE buffer and stored at 4°C.

2.5.7 Subcloning into pTZ18

The clones selected from the λgt10 cDNA library were subcloned into the multiple cloning site of phagemid pTZ18U (Biorad). This allowed both the production of single stranded DNA for use in sequencing reactions and the production of double stranded DNA, which was used to purify insert DNA.

pTZ phagemids contain the *lacZ* gene and within this gene is the polylinker region from pUC18 or pUC19, into which DNA fragments can be cloned. When pTZ is introduced into *lacZ*$^-$ *E.coli* strains, the transformed cells give rise to blue colonies on agar plates supplemented with IPTG (Sigma) and X-gal (GIBCO-BRL) i.e.β-galactosidase activity is restored to the bacterial strain due to α-complementation. β–galactosidase cleaves the chromogenic substrate X-gal to produce a blue chromophore, when transformed cells are grown in the presence of non metabolisable inducer IPTG. When DNA fragments are cloned in the polylinker region, the *lacZ* gene is disrupted and thus will not complement *lacZ*$^-$ *E.coli* strains. Cells transformed with recombinant plasmids will not be able to cleave X-gal and hence the transformant colonies harbouring recombinant pTZ will remain white in the presence of X-gal and IPTG. The transformation mix is spread on plates containing ampicillin, which prevents the growth of untransformed cells, while the cells transformed with pTZ are able to grow as the phagemid contains an ampicillin resistance gene.

The phagemid pTZ18 contains two origins of replication, one from the bacteriophage f1 for the generation of single stranded DNA and the other from

pBR322 for the generation of double stranded DNA. The production of single stranded DNA requires the pTZ transformed cells to be superinfected with M13KO7 helper phage. M13KO7 provides the enzymes and packaging proteins to synthesise and package single stranded pTZ DNA. In cells containing pTZ the replication and packaging of M13KO7 is not efficient, such that the ratio of single stranded pTZ DNA isolated from pTZ and M13KO7 cotransfected cells is at least 50:1.

2.5.7.1 Alkaline Phosphatase Treatment of Plasmid DNA

After digestion with a restriction enzyme, vector DNA was treated with calf intestinal alkaline phosphatase as described in Maniatis *et al.* (1982). The removal of terminal 5' phosphate groups prevented the re-circularisation of the vector during ligation reactions.

Approximately 10µg of digested DNA was precipitated in ethanol (Section 2.4.8) and resuspended in 50µl of Phosphatase buffer (0.05M Tris HCl pH9.0, 1mM $MgCl_2.6H_2O$ and 0.1mM $ZnCl_2$). Calf intestinal alkaline phosphatase (0.05 units) was added and the reaction was carried out at 37°C for 30 minutes. The sample was diluted to 100µl with TE buffer and extracted with phenol:chloroform (Section 2.4.8) three times. Traces of phenol were removed from the sample by extracting it twice with ether (saturated with TE) and DNA was precipitated with ethanol as described in Section 2.4.8.

2.5.7.2 Ligation of cDNA inserts excised from recombinant λgt10 into pTZ

Vector (pTZ) and insert DNA (or recombinant λgt10 DNA) were digested separately with the appropriate restriction enzyme, extracted with phenol:chloroform (Section 2.4.8) and precipitated with ethanol. If necessary,

vector DNA was treated with alkaline phosphatase after restriction enzyme digestion (Section 2.5.7.1).

The ligation reaction was set up in such a way, that the molar ratios of total clonable ends of vector:insert were 3:1. The ligation mix contained 2.2µg of lambda DNA (recombinant), 0.3µg plasmid DNA, 3µl (5mM) ATP, 3µl 10X ligase buffer (GIBCO-BRL), 1 unit of T4 DNA ligase (GIBCO-BRL). Prior to addition of ligase, the reaction volume was made up to 30 µl with sterile distilled water. The ligation was carried out at 15°C for 16 hours.

2.5.7.3 Preparation of competent cells.

The two strains of *E.coli* - DS941 (for propagation and characterisation of recombinant plasmids) and *mv*1190 (for preparing single stranded DNA) were transformed with the recombinant plasmids. The details of these strains are given in Section 2.4.3.

A single colony of *E.coli* DS941 was used to inoculate 10ml LB medium and incubated overnight at 37°C with aeration. 100ml LB medium was inoculated with 1 ml of the overnight culture and incubated at 37°C with agitation until the A_{600} reached 0.3. After the cells were chilled on ice for 10 minutes, they were harvested by centrifugation at 6,000 rpm (Sorvall GSA rotor) for 5 minutes at 4°C. The supernatant was discarded and the pelleted cells were gently resuspended in 6ml ice-cold 100mM $CaCl_2$ in 10mM Tris.HCl pH8.0. The suspension was incubated on ice for 30 minutes and then centrifuged at 6,000 rpm (Sorvall HB-4 rotor) for 5 minutes at 4°C. Again the supernatant was discarded, and the bacterial pellet was gently resuspended in 2ml of ice-cold 100mM $CaCl_2$ in 10mM Tris.HCl pH8.0. Bacteria were rendered competent by this treatment and could be used for transformation. Fresh competent cells were used for all transformations.

*E.coli mv*1190 was made competent as described above, with the following modifications- colonies of *E.coli mv*1190 were first grown on minimal

agar and later a single colony was propagated in 2X TY medium.

2.5.7.4 Transformation of competent *E.coli*

15µl of the ligation mix (Section 2.5.7.2) was added to 100µl of competent cells (Section 2.5.7.3) and then incubated on ice for 30 minutes. The cells were transferred to 42°C for 3 minutes, 1ml of LB medium (Section 2.4.4) was added and the transformed cells were allowed to recover at 37°C for 60 minutes. 0.5% X-gal (GIBCO-BRL) in dimethyl formamide and 0.5% (w/v) IPTG (Sigma) in water were spread on the surface of LB agar plates supplemented with 50µg/ml ampicillin. 100µl of the transformation mix was plated on LB/ampicillin/X-gal/IPTG agar plates. After the suspension had been adsorbed, the plates were inverted and incubated at 37°C for 16 hours. The positive (white) colonies were subcultured on LB/ampicillin/X-gal/IPTG agar plates and incubated at 37°C overnight to confirm that they were not false positives. Colonies that stayed white after the second round of plating were used to purify plasmid DNA (Section 2.4.9) or to prepare single stranded DNA for use in DNA sequence analysis (Section 2.5.8.2).

2.5.8 DNA sequencing

2.5.8.1 Propagation of M13KO7 helper phage

M13KO7 helper phage was kindly provided by Dr. L. M. Anderson and was propagated as follows: Phage were streaked on a LB agar plate, 4ml of top agar containing an overnight culture of *E.coli mv*1190 was poured across the agar surface, allowed to solidify and the plates were incubated overnight at 37°C. The top agar was scraped into a 250ml flask containing 50ml 2X TY medium with 70µg/ml kanamycin and incubated at 37°C with agitation for 10 hours. The

bacteria were pelleted by centrifugation at 8,000 rpm (Sorvall HB-4 rotor) and the supernatant fraction which contained M13KO7 was retained, recentrifuged to remove residual bacteria, and stored at 4°C.

2.5.8.2 Preparation of single stranded DNA

*E.coli, mv*1190 transformed with phosphorylase subclones, (in pTZ) were grown at 37°C overnight in 10ml minimal medium with 100µg/ml ampicillin. 10ml of 2X TY medium containing 100µg/ml ampicillin was then inoculated with 100µl of the overnight culture and grown with aeration at 37°C until the A_{660} was 0.5. 2ml of this culture was transferred to a 50ml Falcon tube and inoculated with M13KO7 at a multiplicity of infection of 10, i.e. 10 pfu of M13KO7 -checked by standard dilution- per *E.coli mv*1190 cell (assuming an A_{660} of 1.0 is $8x10^8$ cells/ml) and incubated at 37°C for 1 hour with good aeration. 10ml of 2X TY, supplemented with 70µg/ml kanamycin was inoculated with 400µl of infected cells and incubated at 37°C overnight with vigorous agitation. The bacteria were pelleted by centrifugation at 6,000 rpm (Sorvall HB-4) and the supernatant was retained. The supernatant which contained packaged single stranded pTZ DNA, was repeatedly centrifuged at 6,000 rpm until there was no bacterial pellet. The phage particles were precipitated from the supernatant with 1ml of 25% PEG in 3M NaCl, at room temperature for 15 minutes. The precipitated phage were recovered by centrifugation at 8,000 rpm (Sorvall HB-4), resuspended in 350µl of 20mM Tris.HCl pH7.5, 20mM NaCl, 1mM EDTA and transferred to an Eppendorf tube. The phage suspension was extracted with phenol:chloroform and DNA was precipitated with ethanol as described in Section 2.4.8. The DNA pellet was resuspended in 50µl of TE buffer.

2.5.8.3 DNA Sequencing by the Sanger dideoxy chain-termination method.

The cDNA clones were sequenced by the Sanger chain termination method as described in the 'M13 Cloning and Sequencing Handbook' provided in the sequencing kit (Amersham).

2.5.8.3.1 Annealing primer to template

The reverse sequencing primer was used when sequencing inserts in pTZ18R and the universal sequencing primer was used when sequencing inserts in pTZ18U/pTZ19U.

5µl (approximately 1µg) of single stranded DNA template (Section 2.5.8.2) was annealed to 1µl of the appropriate primer (1.2mg/ml) in a final volume of 10µl containing 1.5µl of 10X Klenow reaction buffer (100mM Tris HCl pH8.0, 50mM $MgCl_2$). The samples were placed in a boiling water bath, the heat turned off and the samples left in the water bath for 45 minutes.

2.5.8.3.2 Chain elongation with Klenow polymerase

The annealed primer/template mixture was spun briefly to the bottom of the Eppendorf tube, 15µCi of [α-^{35}S]dATP and 1 unit of Klenow polymerase were added and the contents of the tube were mixed gently.

Four Eppendorf tubes, labelled A, C, G,and T were placed in an Anderman 4515 microfuge and 2.5µl of the above primer/template/label/enzyme mix was added to each tube. 2µl of the relevant dNTP/ddNTP mix (Table 2.5) was added to the corresponding tube and the reaction was started by spinning the contents of the tube down. After 20 minutes, 2µl of the chase solution (0.5mM of all 4 dNTPs) was added, the samples mixed and incubated for a further 15 minutes. The samples were then stored at -20°C until required.

Prior to electrophoresis, 2µl of formamide dye mix (0.1% w/v xylene

cyanol FF, 0.1% w/v bromophenol blue, 20mM EDTA in deionised formamide) was added to each sample. The samples were heated in a boiling water bath for 2 minutes, loaded onto a sequencing gel and subjected to electrophoresis as described below.

2.5.8.3.3 Polyacrylamide gel electrophoresis to analyse DNA sequence

DNA samples were subjected to electrophoresis and separated on polyacrylamide/urea denaturing gels [containing 6% acrylamide/bis acrylamide (19:1) and 7M urea] in TBE buffer. The notched gel plate was siliconised with Repelcote (dimethyldichlorosilane in 1, 1, 1 trichlorosilane; BDH) and assembled with 0.4mm thick spacers. The wells for loading the samples were cast by inserting the comb in the acrylamide prior to polymerisation.

The gel was prerun at 30mA for 15 minutes, after which the denatured samples were immediately loaded and subjected to electrophoresis for 1.5 to 3 hours.

The gel was fixed in 10% methanol/10% acetic acid for 20 minutes, dried using a vacuum gel drier and the dried gel was exposed to X-ray film, overnight at room temperature.

Table 2.5 Composition of dNTP/ddNTP mixes

The composition of deoxy (d) NTP/dideoxy (dd) NTP mixes used in Section 2.5.8.3.2 are shown. Stock solutions from the Amersham sequencing kit were used to make the mixes as per the manufacturers instructions in the "M13 Cloning and Sequencing Handbook".

The deoxy (d)/dideoxy (dd) nucleotide mixes contained:

d/ddA 62.5μM of dCTP, dGTP and dTTP and 50μM of ddATP

d/ddC 82μM of dGTP and dTTP, 4μM of dCTP and 50μM of ddCTP

d/ddG 82μM of dCTP and dTTP, 4μM of dGTP and 150μM of ddGTP

d/ddT 82μM of dCTP and dGTP, 4μM of dTTP and 250μM of ddTTP

CHAPTER 3. RESULTS

There are three subdivisions to this section, the first deals with the construction of cDNA libraries prepared from unwounded and wounded tuber mRNAs respectively, the second part deals with the isolation and characterisation of starch phosphorylase cDNA(s), and the last part covers the study of starch phosphorylase mRNA levels in different organs of *Solanum tuberosum* L. cv. Maris Piper and in potato tubers responding to different physiological conditions.

3.1 Preparation of potato tuber RNA

Many different methods were tried in early attempts to purify potato tuber mRNA. These included methods based on, 1) Phenol extraction from guanidinium salts (Chirgwin *et al.*, 1979; Logemann *et al.*, 1987), 2) Preparation of polysomes (Jackson & Larkins, 1976), 3) Selection of poly (A^+)mRNA after phenol extraction of homogenate (Huttly, A.K., personal communication). One of the major problems encountered was starch contamination of the RNA. Even a variant of method 1, that was specifically developed for plant tissues (Logemann *et al.*, 1987) and particularly potato tubers, occasionally gave samples with some starch contamination. However, this residual starch was very insoluble and could be largely eliminated by dissolving the RNA in water and discarding the starch pellet after centrifugation. Using this slight modification to the method, total RNA was routinely prepared with an $A_{260/280}$ of 1.85-2.00 and a yield of 60-80μg/g of tuber tissue. poly (A^+)mRNA was purified from total RNA by oligo dT cellulose as described in the methods section.

3.2 Construction of cDNA libraries

3.2.1 cDNA Synthesis

The use of high quality mRNA is important in the synthesis of double

stranded cDNA, as it will affect the representation of mRNA sequences in the cDNA library. However, before initiating a cDNA cloning program, the strategy for detecting the clone of interest must be considered as this will affect both the strategy of priming first strand cDNA synthesis and the choice of vector used for cDNA cloning. As the complete amino acid sequence of potato tuber starch phosphorylase (major type or L-type) was published (Nakano & Fukui,1986), it was possible to devise oligonucleotide probes based on specific regions of the protein. Subsequently, these oligonucleotides could be used to isolate starch phosphorylase cDNA from a potato tuber cDNA library. Two oligonucleotides probes were prepared complementary to amino acids 666-672 and 761-766 of the published amino acid sequence of starch phosphorylase (Nakano & Fukui, 1986). These sequences were selected because of the low redundancy of their coding DNA. The oligonucleotides were synthesised on an Applied Biosystems Synthesiser. The sequence of the oligonucleotides is shown in Figure 3.1. As the oligonucleotides were derived from regions near the C terminus of the protein, the cDNA synthesis was primed by oligo dT to ensure that the 3' end of the mRNAs would be present in most of the cDNAs. A published method, suitable for isolation of intact and good quality RNA from plant tissues was used (Logemann *et al.*, 1987). Unwounded developing tubers, which have high starch phosphorylase activity, were used as a source of poly (A^+) mRNA to generate the unwounded tuber cDNA libraries. For reasons outlined in Section 1.3.5, it was hoped that wounding would cause an increase in the starch phosphorylase activity, as a consequence of higher mRNA levels. Therefore, poly (A^+) mRNA purified from wounded tubers was used to prepare the wounded tuber cDNA libraries.

A cDNA synthesis kit (Amersham) was used to prepare oligo dT-primed cDNA from potato tuber poly (A^+)mRNA (Section 2.5). The scheme for cDNA synthesis is outlined in Fig.3.2 [A]. Briefly, first strand cDNA is primed with oligo dT and synthesised by reverse transcriptase. The mRNA/cDNA hybrid is used as a substrate for second strand synthesis as follows. *E.coli* ribonuclease

Figure 3.1 Oligonucleotide probes used to isolate starch phosphorylase recombinants

The sequence of the oligonucleotide probes SP1 and SP2, and the corresponding amino acid sequence of potato tuber starch phosphorylase (Nakano & Fukui, 1986) from which they were devised is shown opposite. Only the first two nucleotides (which are underlined) of the threonine codon were included in the sequence of oligonucleotide SP2, inorder to reduce the redundancy of the probe sequence .

Oligonucleotide probe SP1: 18 mer; 32 variants.

```
761                    766

-met-lys-phe-ala-met-asn-


              A
3'    C  A  G     A 5'
 TACTT AA CG TACTT
      T  G  C     G
            T
```

Oligonucleotide probe SP2: 20 mer; 16 variants.

```
666                          672

-lys-lys-met-lys-glu-met-thr

 3'    C  C     C  C       5'
   TT TT TACTT CT TACTG
     T  T     T  T
```

Figure 3.2 Scheme for cDNA synthesis and structure of λgt10

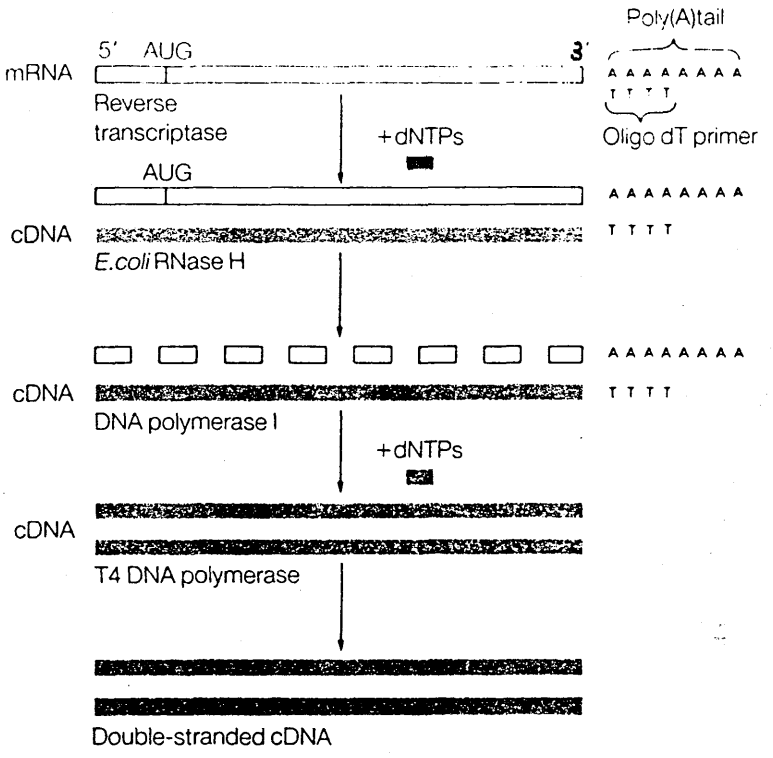
Panel A:

An outline of the procedure for oligo dT-primed cDNA synthesis is shown. The process of cDNA synthesis is described in detail in Section 3.2.1.

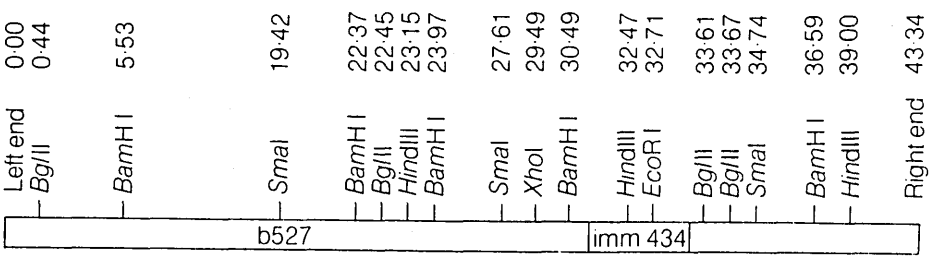
Panel B:

The structure of the vector (λgt10) used to clone cDNA is shown opposite. λgt10 has an unique *Eco*RI restriction site, which is present in the cI gene, and into which the cDNA was cloned. The salient features of the vector are described in Section 3.2.3

A



B



Map of λgt10. Restriction endonuclease cleavage sites are designated in kilobase pairs from the left end.

H produces nicks and gaps in the mRNA strand of the mRNA/cDNA hybrid. This provides primers with 3'OH ends which are used by *E.coli* DNA polymerase I to replace the mRNA strand by DNA in a nick translation type reaction. Finally, T4 DNA polymerase is used to remove any small 3' overhangs on the first strand cDNA.

This method does not depend on the hairpin loop generated by reverse transcriptase as a means of priming the synthesis of the second strand. It thus avoids the need for S1 nuclease digestion of the hairpin loop. Not only is the hairpin loop a relatively inefficient primer, but second strand synthesis from the hairpin loop is at best a poorly controlled step (Okayama & Berg, 1982). Also the S1 nuclease digestion of the hairpin loop is difficult to control and often results in significant loss of cDNA sequences (Okayama & Berg, 1982).

The above method does however have one drawback. A small proportion of the second strands in cDNAs are primed from the hairpin loop, but as the protocol does not include a S1 nuclease digestion step, these cDNAs, which have a hairpin loop at one end, cannot be cloned.

Several poly (A^+)mRNA samples (1μg each) from either wounded or unwounded tuber tissue were used as templates in cDNA synthesis. The yield and size of the cDNA synthesised from different samples were compared (data not shown) and subsequently unwounded and wounded tuber tissue poly (A^+)mRNA (5μg) samples, which gave optimum size and yield of cDNAs in these preliminary experiment, were used to prepare oligo dT primed double stranded cDNA as described in Section 2.5.1. Control cDNA was synthesised from oligo dT primed rat globin mRNA (1μg; supplied by Amersham). 1μl aliquots were removed from the first and second strand synthesis reactions and the efficiency of cDNA synthesis was determined as described in Section 2.5.2.2. The yields of various cDNAs synthesised are given in Table 3.1.

The yield of 1st strand cDNA prepared from tuber mRNA and globin mRNA were equivalent to the expected values. However, there was a discrepancy

in the 2nd strand cDNA yields of both tuber cDNAs and globin cDNA. All three samples had yields above the expected value of >90% presumably because of inaccuracies in aliquoting different cDNA samples. The higher than normal values for 2nd strand cDNA yields were not due to the mRNAs used, as the 2nd strand cDNA yield of control globin mRNA was 150%. When 1μg of the same potato poly (A^+)mRNA was used for cDNA synthesis, rather than 5μg as above, the yield of 2nd strand cDNA exceeded 120% (data not shown).

The cDNA products from the 1st and 2nd strand synthesis reaction were subjected to electrophoresis on an alkaline agarose gel as shown in Fig. 3.3. 2nd strand cDNA products ranged from 0.125 kb to 9.6 kb in size. Globin cDNA migration in the gel gave a size profile of >0.56 kb and <2.0 kb as expected. As a major proportion of the cDNA products were in the range of 0.5-2.3 kb, it seemed likely that the sequences corresponding to the 3' end of the mRNA would be well represented (since the cDNA synthesis was primed with oligo dT). Finally as the electrophoretic analysis of the cDNA products showed no abnormalities in the cDNA, it was decided that it was reasonable to clone the cDNA and proceed with the screening of recombinants for potato tuber starch phosphorylase-encoding inserts.

3.2.2 Purification of cDNA

Double stranded cDNA was purified by phenol extraction and precipitated with absolute ethanol in the presence of molecular biology grade glycogen (Boehringer) as described in Section 2.4.8. The amount of double stranded cDNA synthesised was estimated by assuming that 100% of the 1st strand cDNA was transcribed into 2nd strand cDNA. The radioactivity incorporated into the cDNA (Section 2.5.2) was used to monitor its progress throughout the cloning procedure.

Table 3.1 Efficiency of cDNA synthesis

Samples were removed from the cDNA reactions after the appropriate time (Section 2.5.1). Duplicate dilutions of the samples were spotted on DE-81 filters and the amount of radioactivity incorporated into cDNA was calculated as described in Section 2.5.2.1. The mean of the two values obtained was used in the calculations.

Yield I represents the percentage mRNA transcribed into 1^{st} strand cDNA and Yield II represents the percentage of 1^{st} strand cDNA transcribed into 2^{nd} strand cDNA.

Filter A represents the total radioactivity in the 1^{st} strand cDNA synthesis

Filter B represents the radioactivity incorporated into the 1^{st} strand cDNA

Filter C represents the total radioactivity in the 2^{nd} strand cDNA synthesis

Filter D represents the radiactivity incorporated into 2^{nd} strand cDNA ($+1^{st}$ strand)

* estimated by assuming that 100% of the 1^{st} strand of cDNA was transcribed into the 2^{nd} strand.

Source of mRNA	Filter	Mean cpm (n=2)	%incorporation		Yield		Amount of double stranded cDNA *
			I strand	II strand	I	II	
1. Unwounded tubers (5µg)	A	48818.6	4.38	7.7	31	175	2697 ng
	B	2053.7					
	C	166132.6					
	D	13154.5					
2. Wounded tubers (5 µg)	A	47934.5	4.66	9.9	33	211	3446 ng
	B	2234.9					
	C	89108.45					
	D	9147.3					
3. Globin control (1 µg)	A	30368.6	2.02	3.1	28	151	429 ng
	B	616.0					
	C	62855.6					
	D	2039.2					

Figure 3.3 Analysis of cDNA on an alkaline agarose gel

cDNA, synthesised as described in Section 2.5.1 was denatured and subjected to electrophoresis on an 1.5% alkaline agarose gel (Section 2.5.2.3). The gel was fixed in 7% TCA for 30 minutes, dried and exposed to X-ray film at -70°C for 48 hours. The autoradiograph is shown opposite.

Lane M corresponds to ^{32}P-labelled λ *Hin*dIII fragments

Lane UI corresponds to unwounded tuber 1st strand cDNA.

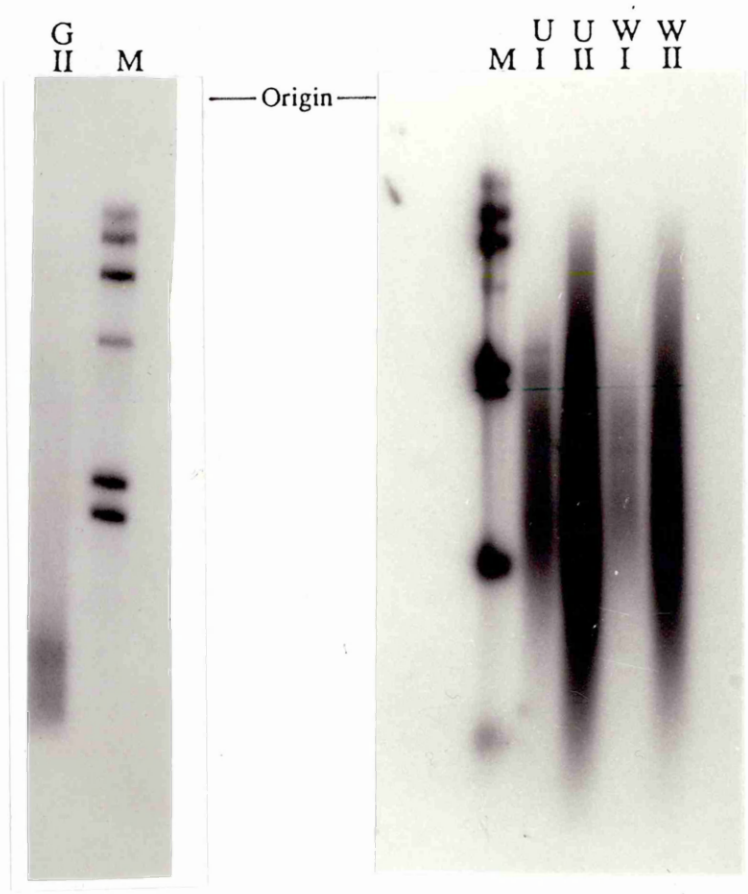
Lane UII corresponds to unwounded tuber 2nd strand cDNA.

Lane WI corresponds to wounded tuber 1st strand cDNA.

Lane WII corresponds to wounded tuber 2nd strand cDNA.

Lane GII corresponds to globin 2nd strand cDNA.

The size of the different λ *Hin*dIII fragments correspond (in kb) to 23.13, 9.41, 6.55, 4.36, 2.32, 2.02, 0.56 and 0.125 respectively.


G
II
M
Origin
M
U
I
U
II
W
I
W
II

3.2.3 Cloning rational

λgt10, whose structure is shown in Figure 3.1 [B], was used as a vector to clone cDNA because the high efficiency and reproducibility of *in vitro* packaging could be exploited to introduce cDNA sequences into *E.coli*. Also, λgt10 could accept inserts up to 7.6 kbp (Huynh *et al.*, 1985), the size range of most mRNA and hence cDNA. Recombinant phage could be detected easily by the use of biological selection.

λgt10 contains a single *Eco*R1 site within the phage repressor gene {cI} (Huynh *et al.*, 1985), cDNA, inserted into this site inactivates the cI gene and thus prevents the entry of recombinant phage into the lysogenic pathway forcing it to enter the lytic pathway. Parental λgt10 favour the lysogenic pathway because the cI gene is active. Nevertheless they produce turbid plaques on wild type *E.coli* and the *E.coli* L87 strain because some phage have entered the lytic pathway. If however the parental λgt10 infects *E.coli* strains with a high frequency of lysogeny (*hfl*$^+$) mutation (such as *E.coli* NM514), it is forced into the lysogenic pathway and does not produce phage particles or cause lysis (and hence no plaques). In contrast, the recombinant phage (cI$^-$) are not forced into lysogeny and lyse the cells, producing plaques. Thus, plating λgt10 cDNA libraries on *hfl*$^+$ strains allows the unambiguous detection of recombinants. The availability of a commercial λgt10 cDNA cloning kit (Amersham), minimises the need for optimisation of conditions for cloning and allows for the quick and efficient generation of a λgt10 cDNA library.

3.2.4 cDNA cloning

As a component of the cDNA cloning kit, *Hae*III digested M13 mp18 DNA was provided as a control to monitor the entire cloning process.

1μg aliquots of double stranded, blunt ended cDNA representing

unwounded and wounded tuber poly (A^+)mRNAs, were used for cloning. The radioactivity in the cDNA was used to monitor progress through various stages of the cloning process.

*Eco*RI methylase, an enzyme that methylates the A residue in the *Eco*RI recognition site, was used to protect any internal *Eco*RI restriction sites in the cDNAs. This ensured that even after subsequent *Eco*RI restriction endonuclease digestion, the full cDNA sequences were cloned.

T4 DNA ligase was used to ligate phosphorylated *Eco*RI linkers (GGAATTCC) to both ends of the cDNA. This resulted in cDNA molecules with multiple linkers ligated to both ends of the cDNA. Subsequent *Eco*RI digestion produced cDNA having only one *Eco*RI 'sticky' end on each terminus and free linker molecules. The excess linker molecules were separated from linkered cDNA by chromatography of the sample on a prepacked column (composition of packing material not specified by the manufacturer). Prior to loading on the column, the total cpm of each cDNA sample was measured by Cerenkov counting. The passage of linkered cDNA through the column was monitored using a Geiger counter and the two fractions (typically fractions 3 and 4) that contained most of the eluted radioactivity were selected for further analysis. The amount of linkered cDNA recovered in these fractions was determined by Cerenkov counting. Around 95% of the linkered cDNAs were recovered in these fractions, which were pooled and precipitated with absolute ethanol in the presence of molecular biology grade glycogen (Boehringer).

Ligation of the linkered cDNA into the *Eco*RI site of λgt10 was carried out according to the manufacturers recommendations (Section 2.5.3.3). Three different concentrations of cDNA; 50ng, 100ng, and 150ng, each from the wounded tuber cDNA and unwounded tuber cDNA populations, were ligated to 1.0μg λgt10 *Eco*RI arms. 50ng of linkered *Hae*III control DNA was also ligated to 1.0μg λgt10 arms to monitor the performance of the whole cloning system and to confirm cloning of inserts. Intact λgt10 DNA (0.5μg) was included as a control to

monitor the performance of the cloning system, and λgt10 arms (1.0μg) were included as a control to check the efficiency of the ligation reaction and the biological selection. The ligation products were precipitated with ethanol and resuspended in TE buffer.

3.2.5 *In vitro* packaging & titre of the libraries

The ligation products were packaged *in vitro* using extracts provided by the manufacturer, and then stored at 4°C in 0.5 ml of SM buffer till required.

The titre of the packaging reactions determined by plating on *E.coli* L87 (the non selective host) and NM514 (the selective host) is shown in Table 3.2. From the titre it was possible to determine the arms selective ratio and the total number of recombinants in the library.

Control 1. The titre of intact λgt10 packaged extracts on *E.coli* L87 was 9×10^6 pfu/μg DNA, which was much lower than the expected value of 2×10^8 pfu/μg. Thus, the efficiency of *in vitro* packaging was less than that recommended. However, the ratio of L87:NM514 titre was within the expected range, which was between 100 and 1000.

Control 2. The titre of λgt10 *Eco*RI arms packaged extract on *E.coli* L87 was also lower than the recommended value of 10^7 pfu/μg. However, the arms selective ratio exceeded 100, as was recommended.

Control 3. The blunt end control DNA sample gave perfect results and had the highest percentage of recombinants, clearly demonstrating the efficiency of the cloning system.

The number of recombinants per μg cDNA for all of the experimentally packaged extracts (wounded and unwounded cDNA libraries) ranged from 5.7×10^6 to 2.5×10^7, again, this confirmed that the cDNAs were efficiently cloned and packaged.

Table 3.2 Titre of recombinants in each of the cDNA libraries

The titre of each packaged reaction was determined on permissive (L87) and non-permissive hosts (NM514) inorder to estimate the number of recombinants in each cDNA library. The details of the calculations are described in Sections 2.5.4.3 and 3.2.5.

No.	λDNA	Insert DNA	L87 titre (pfu/µg) arms	NM514 titre (pfu/µg) arms	L87:NM514 ratio	Adjusted background	Recombinants		
							Total	%	per µg cDNA
1.	λgt10 arms	-	$9x10^6$	$4x10^4$	225.00	-	-	-	-
2.	λgt10 arms	-	$1.87x10^6$	$9.85x10^3$	190.00	-	-	-	-
3.	λgt10 arms	*Hae*III control	$3.02x10^6$	$2.08x10^6$	1.45	$1.6x10^4$	$2.06x10^6$	99	-
UNWOUNDED TUBER cDNA									
4.	λgt10 arms	50ng cDNA	$6.48x10^6$	$1.26x10^6$	5.14	$3.4x10^4$	$1.23x10^6$	97	$2.5x10^7$
5.	λgt10 arms	100ng cDNA	$2.7x10^6$	$9.65x10^5$	2.79	$1.4x10^4$	$9.5x10^5$	98	$9.5x10^6$
6.	λgt10 arms	150ng cDNA	$2.36x10^6$	$7.8x10^5$	3.02	$1.2x10^4$	$7.7x10^5$	98	$5.1x10^6$
WOUNDED TUBER cDNA									
7.	λgt10 arms	50ng cDNA	$3.54x10^6$	$4.96x10^5$	7.13	$1.8x10^4$	$4.8x10^5$	96	$9.6x10^6$
8.	λgt10 arms	100ng cDNA	$6.82x10^6$	$1.28x10^6$	5.33	$3.6x10^4$	$1.24x10^6$	97	$1.24x10^7$
9.	λgt10 arms	150ng cDNA	$5.57x10^6$	$8.81x10^5$	6.33	$2.9x10^4$	$8.51x10^5$	96	$5.7x10^6$

3.3 Isolation & characterisation of putative starch phosphorylase cDNA(s)

3.3.1 Screening the cDNA libraries

Prior to screening, the libraries produced from 50ng, 100ng and 150ng of wounded tuber cDNA were pooled. Similarly, the three unwounded cDNA libraries were pooled. The total number of recombinants in the pooled unwounded tuber cDNA library was 2.95×10^6 and that in the pooled wounded tuber cDNA library was 2.57×10^6.

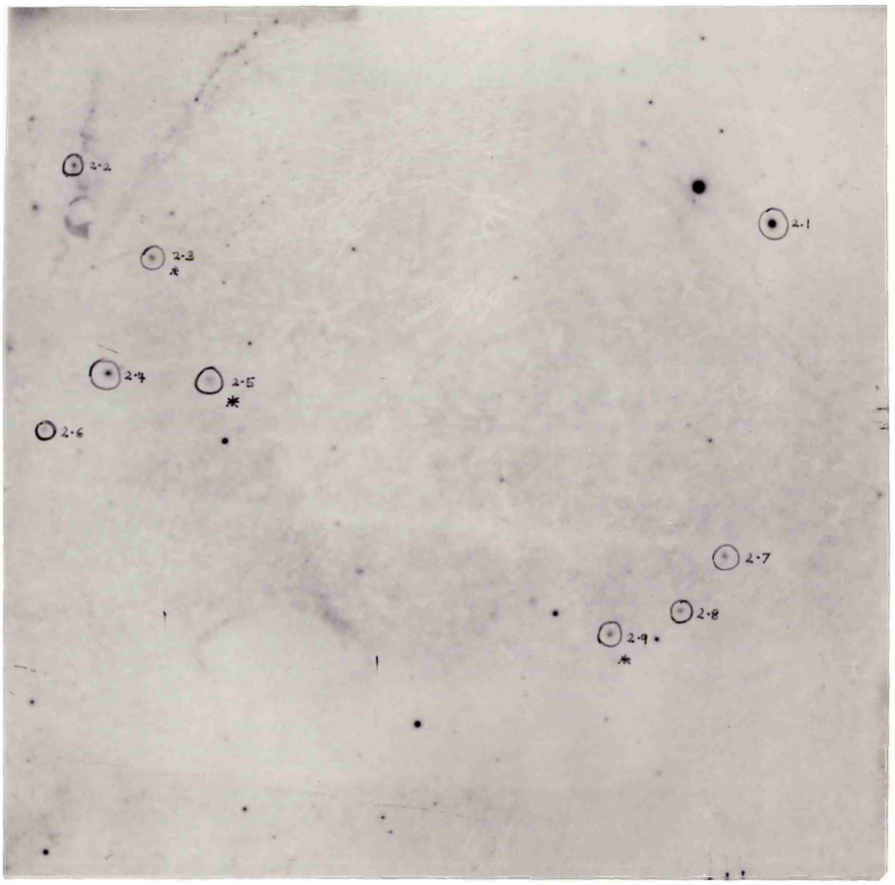
Duplicate impression filters of the cDNA libraries were hybridised to ^{32}P end-labelled oligonucleotide probes in aqueous hybridisation buffer at 37°C (Section 2.4.15.1.1). Later, the filters were washed in buffer at temperatures that removed non-specific hybrids. In order to eliminate false positives, duplicate filters were always used while screening. As it was not clear which cDNA library would have a higher representation of phosphorylase cDNAs, initially approximately 40,000 pfu each, from the wounded tuber cDNA library and the unwounded tuber cDNA library, were screened (Section 2.5.4.4) using the probe oligonucleotide SP1. Two clones in the unwounded tuber cDNA library that hybridised to oligonucleotide SP1 were isolated. No positives were selected from the wounded tuber cDNA library and so it was decided not to continue screening for recombinants from the wounded tuber cDNA library. The reason that there were no detectable phosphorylase encoding recombinants in the wounded tuber cDNA library becomes obvious in subsequent analysis (see Section 3.4.3)

A further 150,000 pfu from the unwounded tuber cDNA library were screened using oligonucleotide SP1 (Fig 3.4). The combined results of this first round of screening, produced 16 plaques that hybridised to oligonucleotide SP1. With subsequent purification and rescreening of these plaques, only 11 of these 2nd

Figure 3.4 Isolation of potato tuber starch phosphorylase clones from the unwounded tuber cDNA library.

Duplicate impression filters representing approximately 75,000 plaques (Section 2.5.4.4) were hybridised to [γ-^{32}P] labelled oligonucleotide SP1 (Section 2.4.15.1.1) and the filters were exposed to X-ray film, in between two intensifying screens at -70^{o}C for 7 days.

The encircled spots on the autoradiograph represent authentic hybridisation signals which were coincident on duplicate filters, and the corresponding plaques were selected for further analysis. Each of the corresponding plaques was randomly given a code number (2.1, 2.2, etc.). At a later stage of screening, it was discovered that those spots marked with an asterix (*), also hybridised with oligonucleotide SP2.


2·2
2·1
2·3
2·4
2·5
2·6
2·7
2·8
2·9

batch clones remained positive and these were taken through further rounds of purification and screening as described in Section 2.5.4.5; these 11 clones were selected in addition to the 2 isolates described earlier. Finally, plaque impressions of the 13 clones that hybridised to oligonucleotide SP1, were hybridised to oligonucleotide SP2. Only 3 clones - λ2.3, λ2.5, and λ2.9 - of the 13 clones hybridised to oligonucleotide SP2. Figure 3.5 shows duplicate filters of plaque impressions of clone λ2.5 hybridised to oligonucleotide SP1 and oligonucleotide SP2. Oligonucleotide SP2 gave a stronger hybridisation signal than oligonucleotide SP1, probably because of its lower complexity (i.e. there was more of each oligonucleotide in the mixture).

To summarise the result of the screening, 13 recombinants were selected that hybridised to the probe SP1 complementary to amino acids 761-766 of potato tuber starch phosphorylase. Three of these also hybridised to the 20mer, SP2, that was prepared against amino acids 666-672. These were selected as most worthy of further investigation (a) because they were double positives and (b) because they were likely to contain the longest inserts.

3.3.2 Characterisation of the inserts of clones selected by SP1

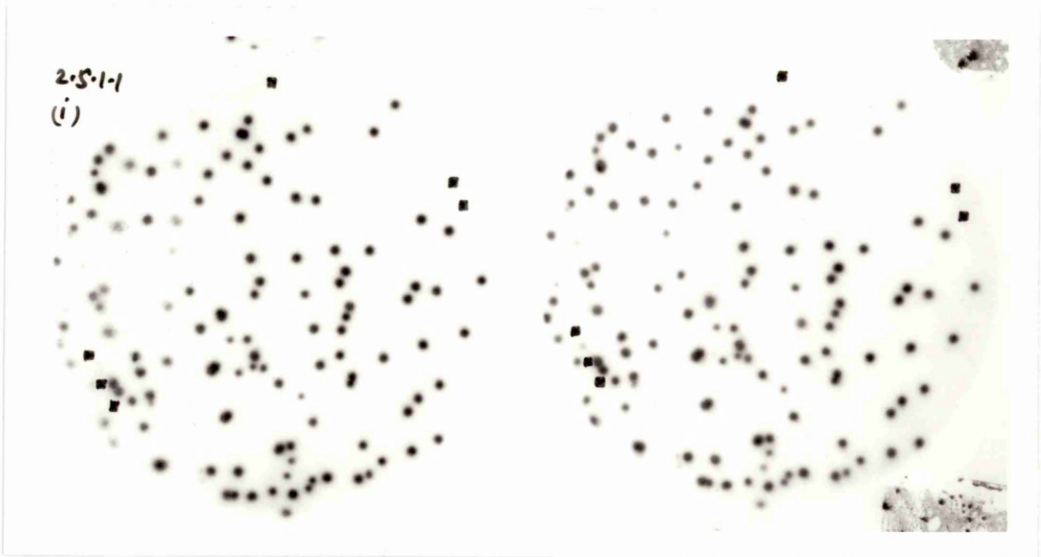
In order to compare the cDNA inserts of different clones selected by hybridisation, DNA prepared from each of the recombinants was digested with *Eco*RI to release the insert. The digestion products were labelled with [α–^{32}P] dATP (Section 2.4.12.3), subjected to electrophoresis and the dried gel was exposed to X-ray film at -70^{o}C. The autoradiograph is shown in Figure 3.6. The size of the fragments released from each of the clones differed considerably. However, some of the clones could be placed into groups on the basis of the number and the approximate size of the fragments, which were released on *Eco*RI digestion. Group A included clones λ2.3, λ2.5 and λ2.9, each of which released two fragments on *Eco*RI digestion of their DNA, the molecular size of one of

Figure 3.5 Hybridisation of purified plaques of λ2.5 to oligonucleotide SP1 and oligonucleotide SP2.

Duplicate filters (approximately 130 plaques) of λ2.5 plaque impressions were hybridised with [γ-^{32}P] labelled oligonucleotide SP1 (Panel A) and oligonucleotide SP2 (Panel B) as described in Section 2.2.4.15.1.1. The filters were exposed to X-ray film, in between two intensifying screens at -70°C for 3 days.

The autoradiographs are shown on the opposite page.

A



B

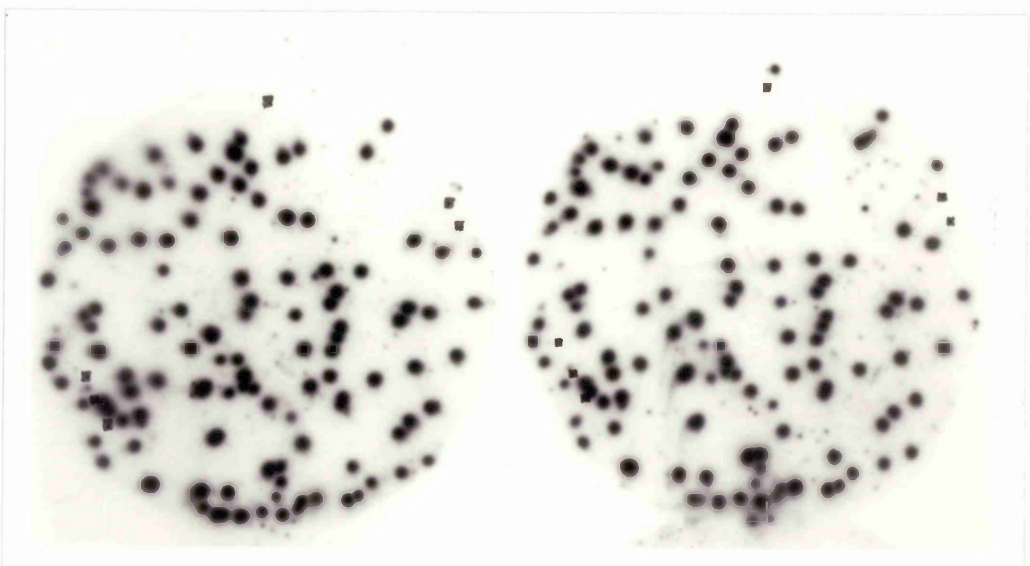


Figure 3.6 Analysis of cDNA inserts of putative potato tuber starch phosphorylase clones.

Panel A:

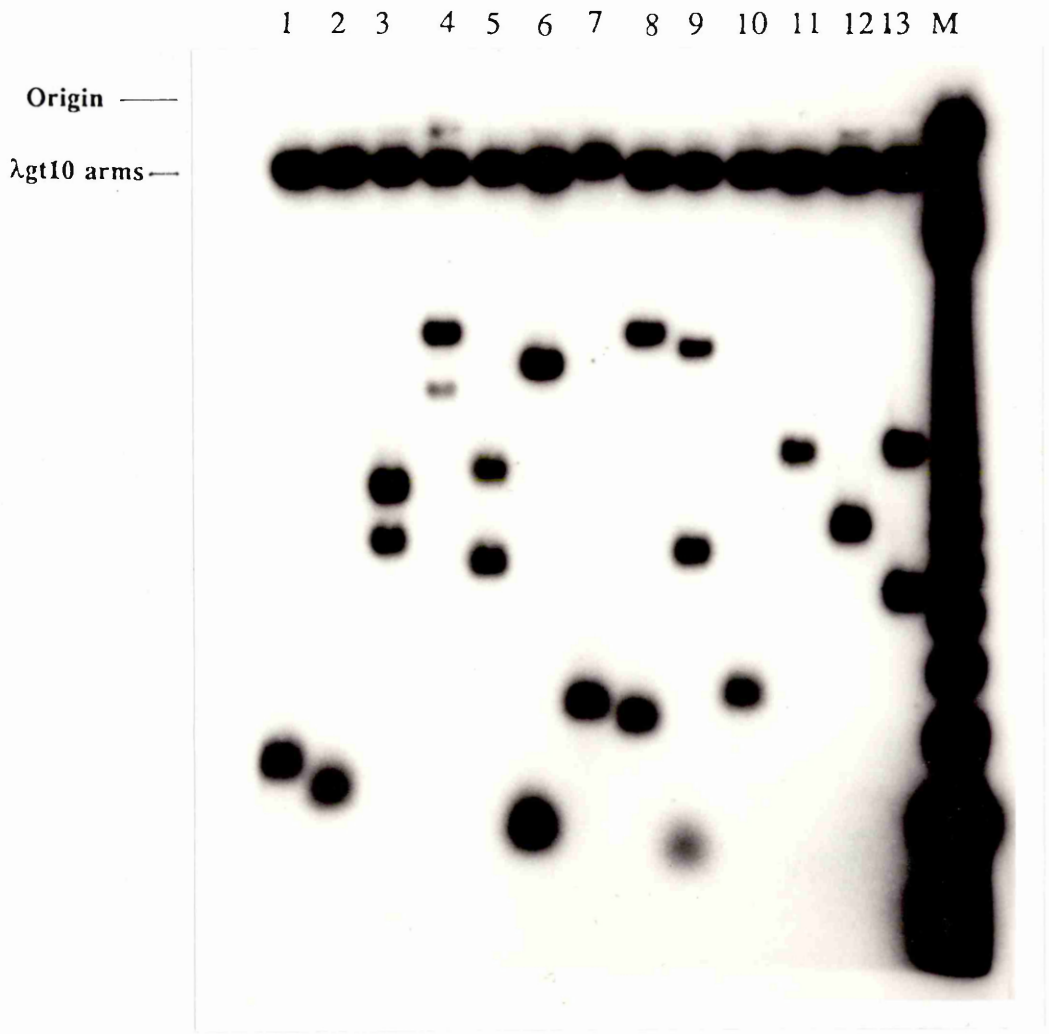
*Eco*RI digested (Section 2.4.10), ^{32}P-labelled DNA prepared (Section 2.4.12.3) from the following putative potato tuber starch phosphorylase clones -

λ1.6 (lane 1), λ1.7 (lane 2), λ2.1 (lane 3), λ2.3 (lane 4), λ2.4 (lane 5), λ2.5 (lane 6), λ2.7 (lane 7), λ2.9 (lane 8), λ3.2 (lane 9), λ3.3 (lane 10), λ3.5 (lane 11), λ3.6 (lane 12), and λ3.7 (lane13) was subjected to electrophoresis on an 1.5% agarose gel as described in Section 2.4.11. Lane M corresponds to the [γ-^{32}P] end-labelled 123 bp DNA ladder (GIBCO-BRL).

Panel B:

Panel B shows the approximate molecular size of the cDNA inserts shown in panel 3.6 [A].

A



B

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
Molecular size	0.21	0.19	0.7	1.35	0.62	0.12	0.32	0.27	0.1	0.31	1.1	0.74	0.55
of inserts (kbp)			0.92	>1.9	1.0	1.55		>1.9	0.65				1.1
-approximate.									>1.8				

the fragments was between 1.4-1.8 kbp, and more important, these three clones hybridised to both the oligonucleotide probes. Group B included clones λ2.1, λ2.4 and λ3.7, again *Eco*RI digestion of their DNA released two fragments, the molecular size of one of the fragments ranged from 0.9-1.1 kbp, also the sum of the molecular size of both fragments in all these clones was ~1.6 kbp. The other clones λ1.6, λ1.7, λ2.7, λ3.2, λ3.3, λ3.5 and λ3.6 were all different and could not be classified.

The presence of multiple fragments could be interpreted in two ways. Either the cDNA had an internal *Eco*RI site, or multiple cDNA fragments were cloned into the same vector. As the restriction fragments of each clone are present in equimolar amounts, they should appear as bands of equal intensities on an autoradiograph. Clone λ2.3 has a fragment (see Figure 3.6), which produced a weak signal. It is likely that this lower molecular weight fragment of λ2.3 is an artefact produced by *Eco*RI* activity.

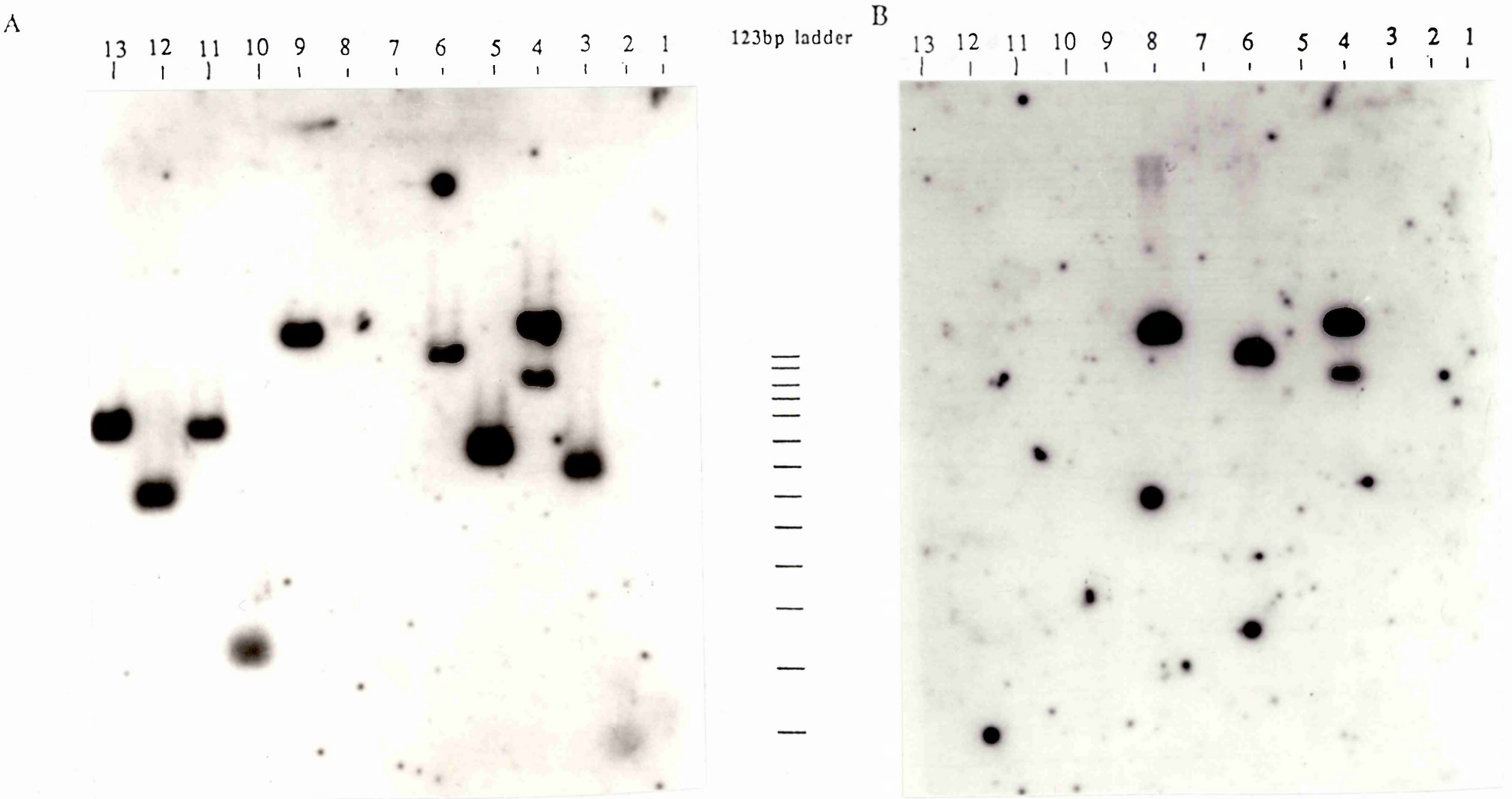
3.3.3 Southern blot analysis of *Eco*RI digested recombinant DNA.

It was essential to demonstrate that the oligonucleotide probes hybridised to the cDNA inserts and not to the vector. To confirm this, *Eco*RI digests of DNA from different clones were subjected to Southern blot hybridisation with oligonucleotide SP1 and oligonucleotide SP2 respectively (Figure 3.7). Oligonucleotide SP1, which had hybridised to plaques of all 13 clones, hybridised to one cDNA fragment from all the clones, with the exception of clone λ2.3 that had two cDNA fragments that hybridised to the probe. Oligonucleotide SP2 on the other hand hybridised to cDNA inserts from clones λ2.3, λ2.5 and λ2.9 only. Again two cDNA fragments of λ2.3 hybridised to oligonucleotide SP2. As both, oligonucleotide SP1 and oligonucleotide SP2 hybridised to the same cDNA fragments from λ2.3, λ2.5 and λ2.9, it seemed likely that these cDNA fragments

Figure 3.7 Sandwich Southern blot hybridisation of putative starch phosphorylase clones to oligonucleotide probes SP1 and SP2.

*Eco*RI digests of DNA from λ1.6 (lane 1), λ1.7 (lane 2), λ2.1 (lane 3), λ2.3 (lane 4), λ2.4 (lane 5), λ2.5 (lane 6), λ2.7 (lane 7), λ2.9 (lane 8), λ3.2 (lane 9), λ3.3 (lane 10), λ3.5 (lane 11), λ3.6 (lane 12), and λ3.7 (lane13) were subjected to electrophoresis on an 1.5% agarose gel, blotted onto Hybond N nylon membranes (Section 2.4.13) and hybridised to [γ-^{32}P] end-labelled oligonucleotide SP1 (Panel A) and oligonucleotide SP2 (Panel B) as described in Section 2.4.15.1.2.

The position of the molecular size markers (123 bp DNA ladder; GIBCO-BRL) is also shown.


A
13 12 11 10 9 8 7 6 5 4 3 2 1
123bp ladder
B
13 12 11 10 9 8 7 6 5 4 3 2 1

could encode sequences of phosphorylase. To study the relationship between these clones, the following experiment was done. The ~1.4 kbp insert from λ2.5 was purified from a LMP-agarose gel (Section 2.4.11), labelled by random priming (Section 2.4.12.2) and hybridised to Southern blots of *Eco*RI digests of the different clones. Figure 3.8 shows the resultant autoradiograph. The ~1.4 kbp insert of λ2.5 hybridised to itself and the ~1.8 kbp inserts of λ2.3 and λ2.9, a second hybridising fragment (~1.3 kbp) was also present in *Eco*RI digests of λ2.3. No cross hybridisation was seen with inserts from the other λ recombinants. It was concluded that the ~1.8 kbp inserts of λ2.3 and λ2.9 were similar to the ~1.4 kbp insert of λ2.5.

Further characterisation of λ2.3 was abandoned because of the anomalies observed during its initial characterisation. Instead, as the ~1.4 kbp insert of λ2.5 had been purified, it was cloned into the *Eco*RI site of pTZ18U; this recombinant called pPSP1, was used for further characterisation.

At this stage of the project, Brisson *et al.* (1989) and Nakano *et al.* (1989) independently reported the cloning of potato tuber starch phosphorylase cDNA. As the complete nucleotide sequence of starch phosphorylase was published (Nakano *et al.*, 1989), it was decided that the *Eco*RI insert of pPSP1 would be sequenced only in part, and compared with the published starch phosphorylase cDNA sequence to confirm its identity.

3.3.4 Construction of a partial restriction map of pPSP1

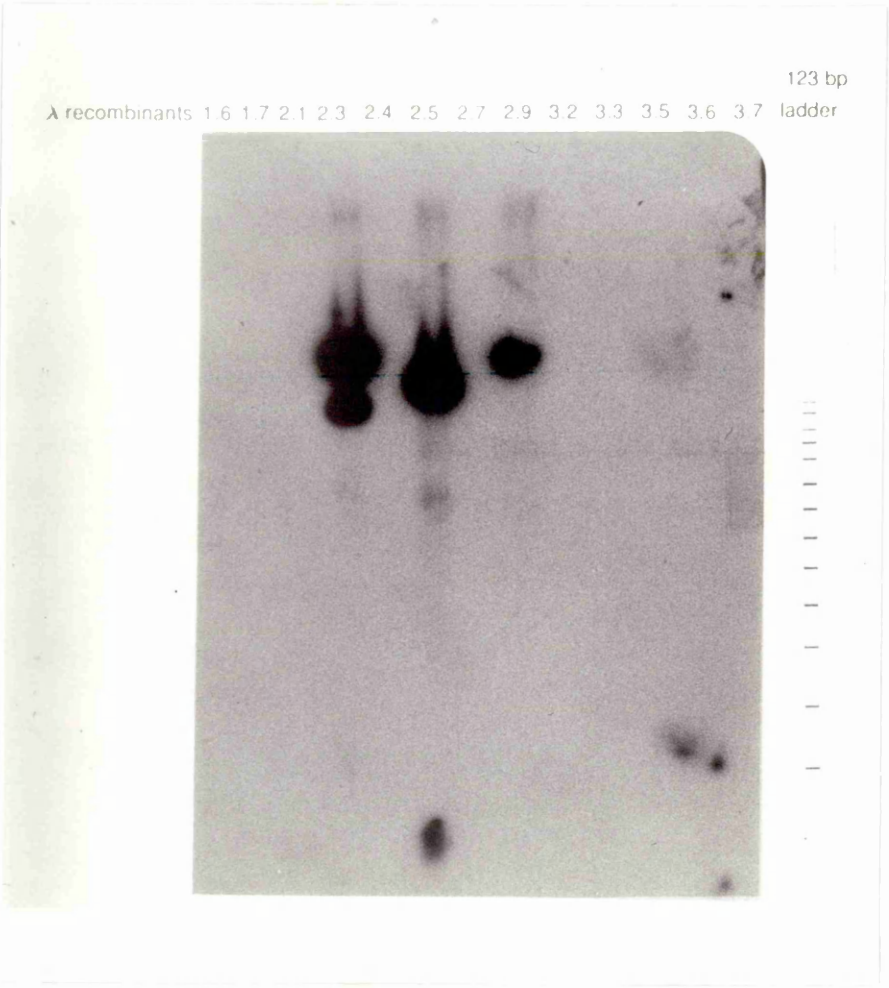
A restriction map of pPSP1 was essential to proceed with the subcloning and sequencing of the restriction fragments. It could also be compared with the published restriction map of starch phosphorylase cDNA.

pPSP1 was digested with *Eco*RI and later was separately digested with restriction endonucleases *Bgl*II, *Hin*dIII, *Pst*I and *Sst*I, which were known to cut starch phosphorylase cDNA (Nakano *et al.*, 1989), and with *Kpn*I, which

Figure 3.8 Southern cross-hybridisation of the ~1.4 kbp insert of λ2.5 to *Eco*RI digests of different λ clones

*Eco*RI digests of DNA from λ1.6, λ1.7, λ2.1 , λ2.3, λ2.4, λ2.5, λ2.7, λ2.9, λ3.2, λ3.3, λ3.5, λ3.6, and λ3.7 were subjected to electrophoresis on an 1.5% agarose gel, blotted onto Hybond N nylon membranes (Section 2.4.13) and hybridised to ^{32}P-labelled (~1.4 kbp) insert of λ2.5 as described in Section 2.4.15.2.

The position of the molecular size markers (123 bp DNA ladder; GIBCO-BRL) is also shown.


123 bp
λ recombinants 1.6 1.7 2.1 2.3 2.4 2.5 2.7 2.9 3.2 3.3 3.5 3.6 3.7 ladder

does not have any sites in the starch phosphorylase cDNA fragment that represents the 3' end of the mRNA sequence. Double digestion of the *Eco*RI insert of pPSP1 with *Hin*dIII-*Pst*I, *Hin*dIII-*Sst*I, and *Pst*I-*Sst*I was used to locate the position of the restriction sites on its map. The size of the different restriction fragments of the *Eco*RI insert of pPSP1 shown in Figure 3.9 [A] were estimated, a partial restriction map was constructed (Figure 3.9 [B]) and compared with the map of starch phosphorylase cDNA (Figure 3.9 [C]) (Nakano *et al.*, 1989).

3.3.5 Identification of restriction fragments of pPSP1 hybridising to the oligonucleotide probes

Examination of the published sequence of Nakano *et al.*(1989) revealed that the sequences having homology to the oligonucleotide probes (SP1 and SP2) were on either side of the *Hin*dIII and *Pst*I sites. To confirm whether this was also true for pPSP1, single and double restriction endonuclease digests were carried out using the appropriate enzymes and the digestion products were subjected to Southern blot hybridisation with oligonucleotide SP1 and oligonucleotide SP2 as probes, in separate experiments (Figure 3.10 [A]). The size of the restriction fragments of pPSP1 which hybridised with oligonucleotide SP1 and oligonucleotide SP2 was calculated and they were located on the restriction map of pPSP1 as shown in Figure 3.10 [B].

3.3.6 Determination of the nucleotide sequence of pPSP1

3.3.6.1 Subcloning strategy

On the basis of the restriction map of pPSP1, it was decided to clone the *Eco*RI-*Hin*dIII fragments of pPSP1 into pTZ18. *Eco*RI-*Hin*dIII fragments of pPSP1 were purified from a 0.6% LMP- agarose gel (Section 2.4.11) and cloned

Figure 3.9 Construction of a restriction map of pPSP1

Panel A:

Panel A shows an 1.0% agarose gel loaded with pPSP1 that had been digested with

Lane 1. *Eco*RI + *Pst*I + *Sst*I

Lane 2. *Eco*RI + *Hin*dIII + *Sst*I

Lane 3. *Eco*RI + *Hin*dIII + *Pst*I

Lane 4. *Eco*RI + *Sst*I

Lane 5. *Eco*RI + *Pst*I

Lane 6. *Eco*RI + *Hin*dIII

Lane 7. *Eco*RI + *Kpn*I

Lane 8. *Eco*RI + *Bgl*II

Lane 9. *Eco*RI

Lane M. was loaded with λ*Hin*dIII molecular size markers. The size of the different λ *Hin*dIII fragments correspond to (in kbp) 23.13, 9.41, 6.55, 4.36, 2.32, 2.02, 0.56 and 0.125 respectively.

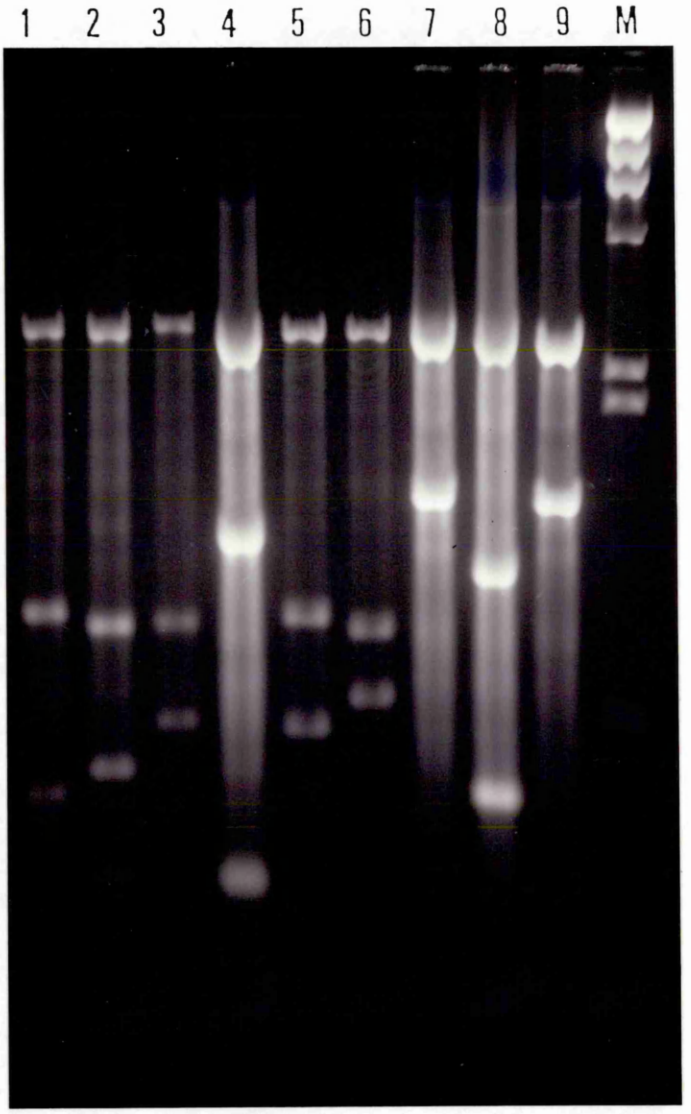
Panel B:

Panel B shows the partial restriction map of pPSP1 .

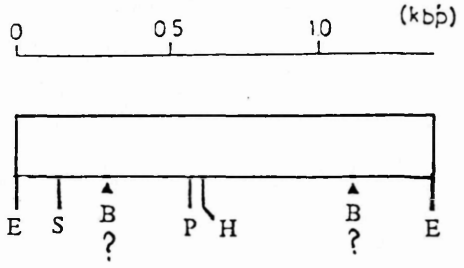
Panel C:

Panel C shows part of the restriction map of potato tuber starch phosphorylase cDNA (Nakano *et al.*, 1989) .

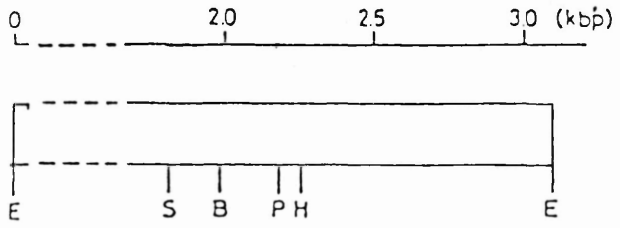
A



B



C



*Eco*RI (E) ; *Bgl*II (B); *Pst*I (P); *Sst*I or *Sac*I (S) ; *Hin*dIII(H)

{▲ B ?} indicates the presence of a *Bgl*II site in the two possible orientations on the partial restriction map of pPSP1.

Figure 3.10 Southern blot analysis of pPSP1 restriction digests

Panel A:

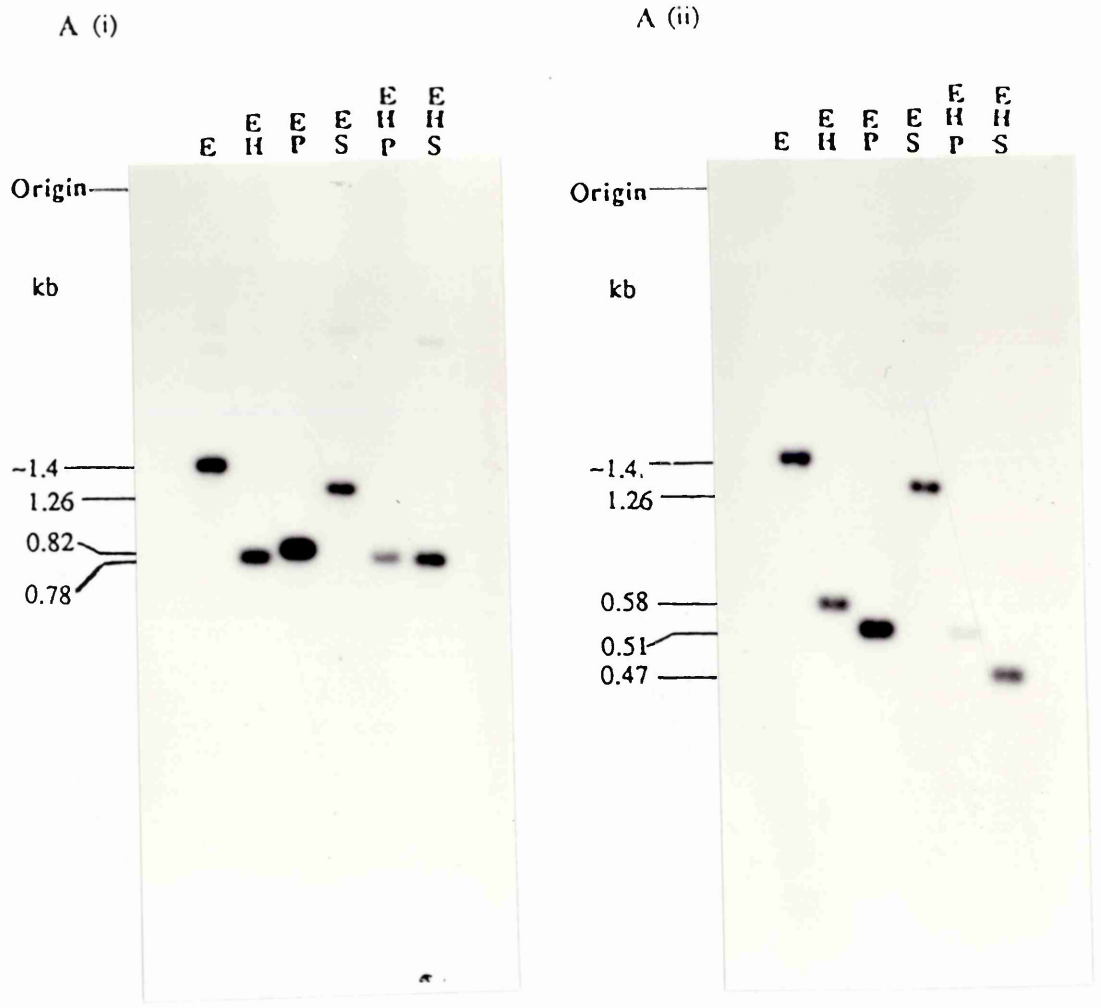
pPSP1 was subjected to single and multiple digestions with the following restriction endonucleases-*Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Sst*I (S), the digestion products were then loaded on a 1.0% agarose gel and subjected to electrophoresis (Section 2.4.11). After electrophoresis, the gel was subjected to Southern blot analysis (Section 2.4.13) and hybridised (2.4.15.1.2) with ^{32}P-labelled oligonucleotide SP1 {Panel A (i)} and oligonucleotide SP2 {Panel A (ii)} respectively.

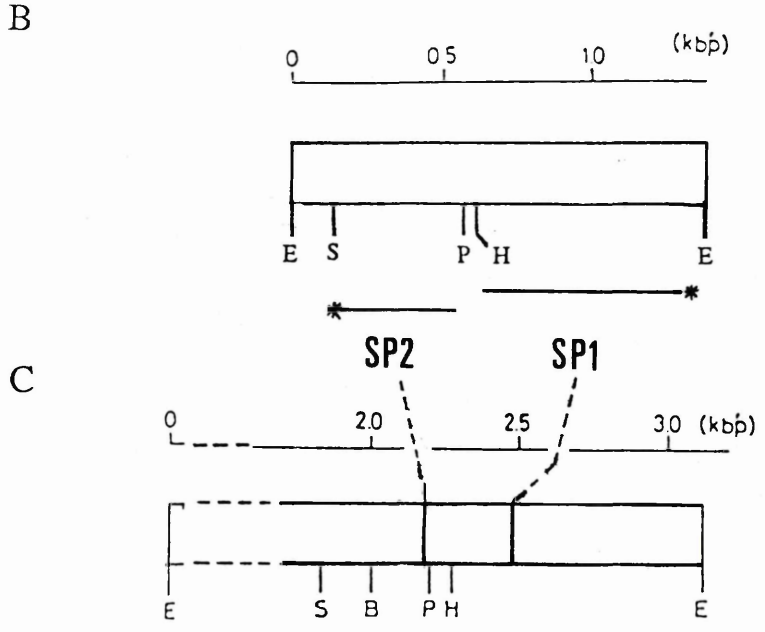
Panel B:

Panel B shows the partial restriction map of pPSP1 and the fragments complementary to oligonucleotide SP1 and oligonucleotide SP2.

Panel C:

Panel C shows the published restiction map of starch phosphorylase cDNA (Nakano *et al.*, 1989) and the regions from which oligonucleotides SP1 and SP2 were derived.





*Eco*RI (E) ; *Bgl*II (B); *Pst*I (P); *Sst*I or *Sac*I (S); *Hin*dIII(H)

into *Eco*RI-*Hin*dIII cut pTZ18 to give the resultant recombinants EH1 (in pTZ18R) and EH3 (in pTZ18U). Additional subcloning strategies employed purified *Eco*RI insert of pPSP1 that had been digested with *Sau*3A. The digestion products were purified by phenol extraction (Section 2.4.8) and shotgun cloned into pTZ18U which had been cut with either *Bam*HI (recombinant B1), or *Eco*RI and *Bam*HI (recombinant EB4). It was expected that EB4 would represent a terminal fragment of the *Eco*RI insert from pPSP1.

3.3.6.2 Sequencing of subclones

Single stranded DNA was prepared from the different subclones (Section 2.5.8.2) and the nucleotide sequence was determined as described in Section 2.5.8.3. The nucleotide sequence was determined on only one strand for all the subclones. Figures 3.11, 3.12 and 3.13 show the nucleotide sequence of the different subclones and the corresponding published potato tuber starch phosphorylase nucleotide sequence (Nakano *et al.*, 1989). All the nucleotide sequence data from the different subclones showed complete identity to the the published sequence. The sequence identity of EB4 and potato tuber starch phosphorylase cDNA extended into the 3' noncoding region up to the putative polyadenylation site (Figure 3.13). However due to limitation of time and a change of priorities brought about by the work of the groups of Fukui and Brisson, it was decided not to complete the sequencing of the 3' end. Approximately 35% of one strand of the insert from pPSP1 had been sequenced and was found to be identical to the published sequence for potato tuber starch phosphorylase cDNA.

On the basis of sequence data, Southern hybridisation and restriction mapping, it was concluded that pPSP1 encoded ~1.4 kbp portion of the potato tuber starch phosphorylase cDNA. The overall nature of the sequence data generated is shown in Figure 3.14. It was decided to use the cDNA insert of pPSP1 as a probe for starch phosphorylase mRNA in northern blot hybridisation experiments.

Figure 3.11 Nucleotide sequence of subclones EH1 and B1

The nucleotide sequence of two subclones EH1 and B1 of putative potato tuber starch phosphorylase cDNA (pPSP1), are arranged above the corresponding published sequence of potato tuber starch phosphorylase {SP} (Nakano *et al.*, 1989). The latter sequence is underlined.

EH1 was produced by cloning the *Eco*RI/*Hin*dIII fragment of pPSP1 into pTZ18R and B1 was produced by shotgun cloning a *Sau*3A digest of the *Eco*RI insert of pPSP1 into the *Bam*HI site of pTZ18U.

The four nucleotide overlap of the two clones is marked ||||, and the *Sac*I (*Sst*I) site is shown in *italics*.

Sequence of EH1:

```
    1||||
EH1: TTGTGTGTTG TGGGCGGCCA TGCTGTTAAT GGAGTTGCTG AGATCCATAG
     ********** ********** ********** ********** **********
 SP: TTGTGTGTTG TGGGCGGCCA TGCTGTTAAT GGAGTTGCTG AGATCCATAG
  1754
    51                                          SacI/SstI
EH1: TGAAATTGTG AAGGAGGAGG TTTTCAATGA CTTCTATGAG CTCTGGCCGG
     ********** ********** ********** ********** **********
 SP: TGAAATTGTG AAGGAGGAGG TTTTCAATGA CTTCTATGAG CTCTGGCCGG
1804

   101 102
EH1: AA
     **
 SP: AA
 1854  1855
```

Sequence of B1:

```
      1
B1: AAGCAGCTGC AGAAAAAGAC ATTGACAAGA AAACTCCCGT GAGTCCGGAA
    ********** ********** ********** ********** **********
SP: AAGCAGCTGC AGAAAAAGAC ATTGACAAGA AAACTCCCGT GAGTCCGGAA
 1665


       51                                        | |||93
B1: CCAGCTGTTA TACCACCTAA GAAGGTACGC ATGGCCAACT TGT
    ********** ********** ********** ********** ***
SP: CCAGCTGTTA TACCACCTAA GAAGGTACGC ATGGCCAACT TGT 1757
 1715
```

Figure 3. 12 Nucleotide sequence of subclone EH3

The nucleotide sequence of the subclone EH3 of putative potato tuber starch phosphorylase cDNA (pPSP1), is arranged above the corresponding published sequence of potato tuber starch phosphorylase {SP} (Nakano *et al.*, 1989). The latter sequence is underlined.

EH3 was produced by cloning the *Eco*RI/*Hin*dIII fragment of pPSP1 into pTZ18U

SEQUENCE OF EH3:

```
        1
   EH3. TACTATAAAC ATGATCCAGA AATCGGTGAT CTGTTGAAGG TAGTCTTTGT
        ********** ********** ********** ********** **********
SP 2323 TACTATAAAC ATGATCCAGA AATCGGTGAT CTGTTGAAGG TAGTCTTTGT

       51
EH3     GCCAGATTAC AATGTCAGTG TTGCTGAATT GCTAATTCCT GCTAGCGATC
        ********** ********** ********** ********** **********
SP 2373 GCCAGATTAC AATGTCAGTG TTGCTGAATT GCTAATTCCT GCTAGCGATC

      101      108
   EH3  TATCAGAA
        ********
SP 2423 TATCAGAA 2430
```

Figure 3.13 Nucleotide sequence of subclone EB4

The nucleotide sequence of the subclone EB4 of putative potato tuber starch phosphorylase cDNA (pPSP1), is arranged above the corresponding published sequence of potato tuber starch phosphorylase {SP} (Nakano *et al.*, 1989). The latter sequence is underlined. The translation termination site is denoted by |||.

EB4 was produced by shotgun cloning a *Sau*3A digest of the *Eco*RI insert of pPSP1 into pTZ18U that had been cut with *Eco*RI and *Bam*HI.

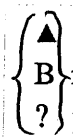
Sequence of EB4:

```
          1
EB4       GATCGTACAA GTTCAGCAGT GACAGAACAA TCCATGAATA TGCCAAAGAC
          ********** ********** ********** ********** **********
SP   2862 GATCGTACAA GTTCAGCAGT GACAGAACAA TCCATGAATA TGCCAAAGAC

         51                                   |||
EB4       ATTTGGAACA TTGAAGCTGT GGAAATAGCA TAAGAGGGGG AAGTGAATGA
          ********** ********** ********** ********** **********
SP   2912 ATTTGGAACA TTGAAGCTGT GGAAATAGCA TAAGAGGGGG AAGTGAATGA

        101
EB4       AAAATAACAA AGGCACAGTA AGTAGTTTCT CTTTTTATCA TGTGATGAAG
          ********** ********** ********** ********** **********
SP   2962 AAAATAACAA AGGCACAGTA AGTAGTTTCT CTTTTTATCA TGTGATGAAG

        151                                                   196
EB4       GTATATAATG TATGTGTAAG AGGATGATGT TATTACCACA TAATAA
           ********** ********** ********** ********** ******
SP   3012 GTATATAATG TATGTGTAAG AGGATGATGT TATTACCACA TAATAA3057
```

Figure 3.14 Comparison of starch phosphorylase, starch phosphorylase cDNA and pPSP1

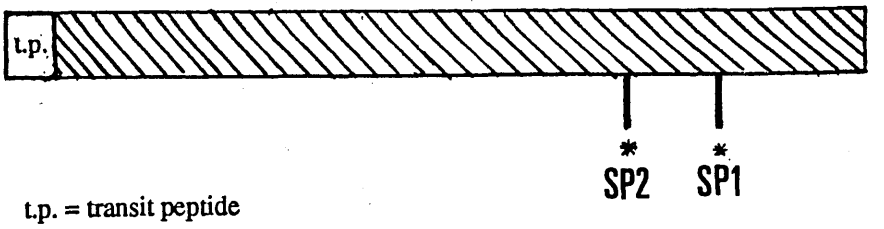
The nucleotide sequence data and the results of the restriction, and hybridisation analysis of pPSP1 were collated and compared with the published data for potato tuber starch phosphorylase.

The structure of starch phosphorylase protein ([A]; modified from Nakano & Fukui, 1986 and Nakano *et al.*, 1989), starch phosphorylase cDNA ([B]; modified from Nakano *et al.*, 1989) and pPSP1 [C] are shown opposite. The regions from which the two oligonucleotide probes were derived are marked (✱) on the protein and cDNA. pPSP1 is aligned with [A] and [B] and the portions of pPSP1 that were sequenced, and the corresponding subclones are shown opposite.



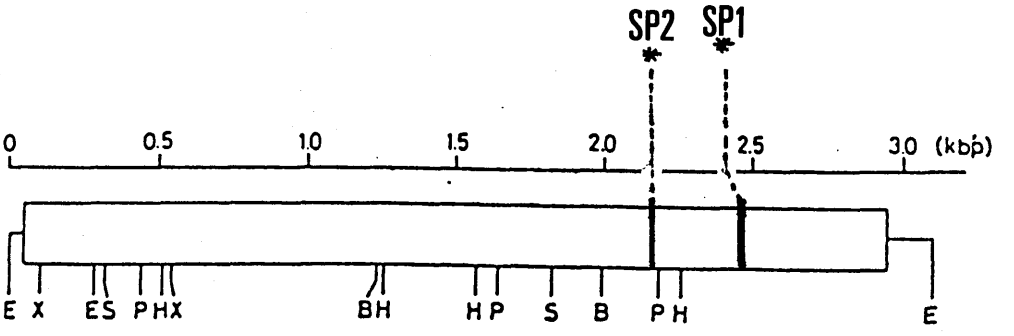
indicates the presence of a *Bgl*II site in the two possible orientations on the partial restriction map of pPSP1.

A



t.p. = transit peptide

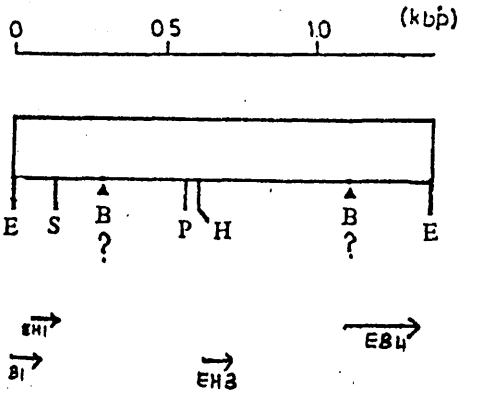
B



The open box corresponds to the coding sequence of starch phosphorylase.

The untranslated sequence is denoted by a horizontal line .

C



*Eco*RI (E) ; *Bgl*II (B) ; *Pst*I (P) ; *Sst*I or *Sac*I (S) ; *Xba* I (X) ; *Hin*dIII (H) .

3.4 Studies on potato tuber starch phosphorylase mRNA levels

The ~1.4 kbp *Eco*RI insert of pPSP1 was routinely used to probe northern blots to study starch phosphorylase mRNA levels. This study focused on the analysis of mRNA levels in potato tubers that were subjected to different conditions. Total RNA was isolated from plant tissue according to Logemann *et al.* (1987), subjected to electrophoresis on a formaldehyde-agarose gel, blotted onto Hybond-N nylon membrane (Amersham) and hybridised with the *Eco*RI insert of pPSP1.

3.4.1 Determination of potato tuber starch phosphorylase transcript size

1 μg of poly (A^+)mRNA prepared from unwounded tuber tissue was subjected to northern blot analysis and hybridised to starch phosphorylase cDNA at high stringency. As shown in Figure 3.15 [A], only one band of hybridisation was detected and the calculated size of the transcript was ~3.2 kb (relative to RNA size markers; GIBCO-BRL). This was in agreement with the published size of potato tuber starch phosphorylase mRNA (Brisson *et al.*, 1989).

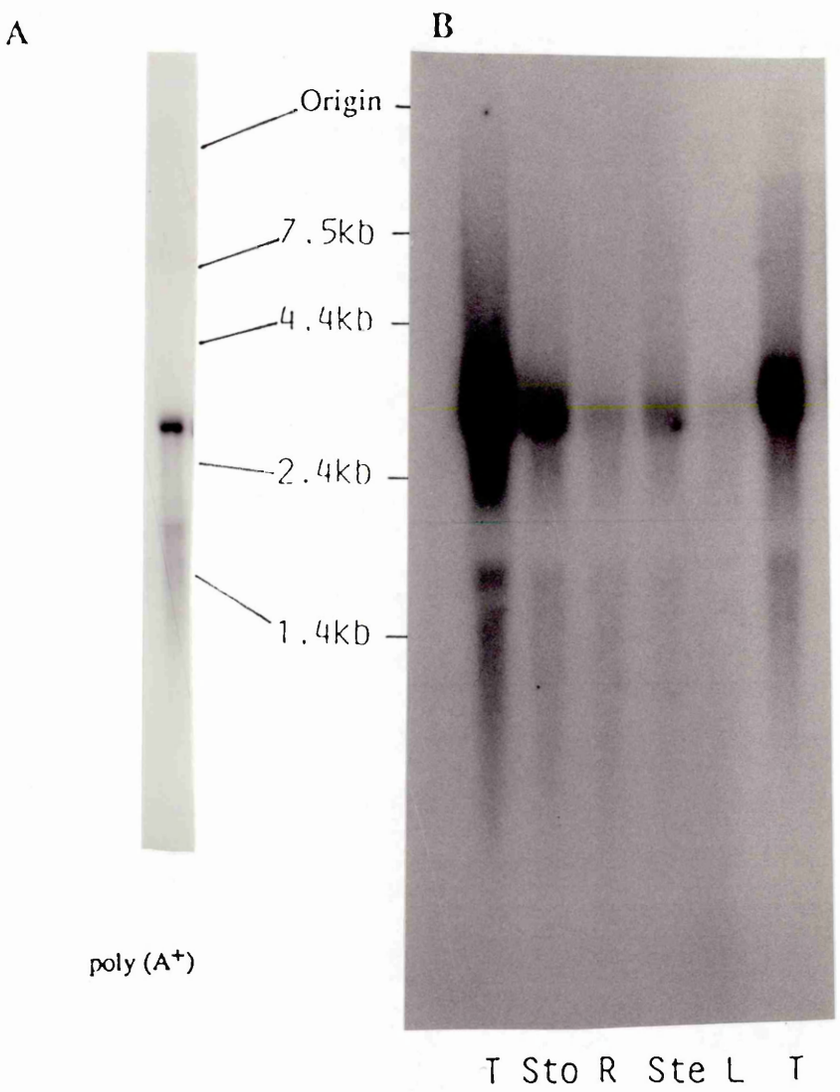
3.4.2 Tissue distribution of phosphorylase mRNA

Comparative analysis of mRNA in various potato tissues was performed using tissue from 8 week-old field grown plants. Figure 3.15 [B] shows that starch phosphorylase transcript was an abundant component of potato tuber RNA. It was also present, at lower levels, in stolons and at much lower levels in roots, stem and leaves.

Fig 3.15 Relative levels of starch phosphorylase mRNA in different potato tissues

Panel A- Northern blot hybridisation of 1μg potato tuber poly(A^+)mRNA to ^{32}P-labelled starch phosphorylase cDNA (~1.4 kbp insert of pPSP1) was carried out as described below.

Panel B- Total RNA, isolated from the different tissues of 8 week-old field grown plants, was analysed on northern blots (Section 2.4.14) for sequences complementary to a ^{32}P-labelled probe (Section 2.4.12.2) prepared using the ~1.4 kbp insert of pPSP-1. Hybridisation was carried out as described in Section 2.4.15. Lanes from left to right contain 50 μg total RNA from tuber (T), stolon (Sto), root (R), stem (Ste) and leaf (L) while the most right hand lane contains 20μg of tuber (T) RNA. Size indicators were derived from RNA size markers (GIBCO-BRL).


A
B
Origin
7.5kb
4.4kb
2.4kb
1.4kb
poly (A+)
T Sto R Ste L T

3.4.3 Wounding induced changes in the amount of phosphorylase mRNA.

Figure 3.16 [A] shows that the wounding of tuber tissue resulted in a decrease in the level of starch phosphorylase-encoding sequences. The loss was rapid, with mRNA levels reduced by 32% two hours after slicing and by six hours, only 16% of the levels of unwounded tissue were observed (Figure 3.16 [B]). Thereafter, there was a steady and reproducible but incomplete recovery in the levels of phosphorylase mRNA, which reached 38% of unwounded levels by 24 hours after slicing but showed no further increase up to 72 hours after wounding (Figure 3.18). The reduction in the levels of starch phosphorylase mRNA was less drastic in 3 mm thick tuber slices than in 1 mm thick tuber slices at 72 hours after wounding. The wounding induced decrease in starch phosphorylase mRNA levels was also observed in tuber slices incubated anaerobically (Figure 3.20 [A]). Wounding also induced a decrease in starch phosphorylase mRNA levels in potato stem tissue (Figure 3.19). No starch phosphorylase mRNA could be detected in wounded stem tissues after 18 hours of ageing.

After autoradiography, the blots were stripped of phosphorylase cDNA probe and were reprobed with patatin cDNA insert (recombinant pPOT301 kindly provided by Ooms, G.; Twell & Ooms, 1988), which served as a positive control, as its mRNA levels were known to decrease in wounded potato tubers (Logemann *et al.*, 1988). Patatin mRNA decreased on wounding as expected, it could be detected up to 6 hours after wounding after which it could not be detected (Figure 3.17 [A]). Furthermore, patatin mRNA was not detected in wounded tuber slices incubated anaerobically (Figure 3.20 [B]). As a further control, the amount of 25S rRNA in each track was checked by probing the stripped filter with a human 28S rDNA oligonucleotide probe (Barbu & Dautry, 1989). Amounts were found to be approximately equal in each lane (Figure 3.17 [B]).

Fig.3.16 Effect of wounding on the levels of starch phosphorylase mRNA in potato tuber

Panel A- Tuber slices were prepared and incubated aerobically for varying lengths of time before the preparation of RNA. 20μg quantities of total RNA were analysed on northern blots in comparison with RNA isolated from unwounded tissues (U) as described in Figure 3.15. The blot was hybridised to ^{32}P-labelled starch phosphorylase cDNA as described in Section 2.4.15.2.

Panel B- Densitometric analysis of the 3.2 kb band in A, employed a Bio-Rad 620 Video densitometer .

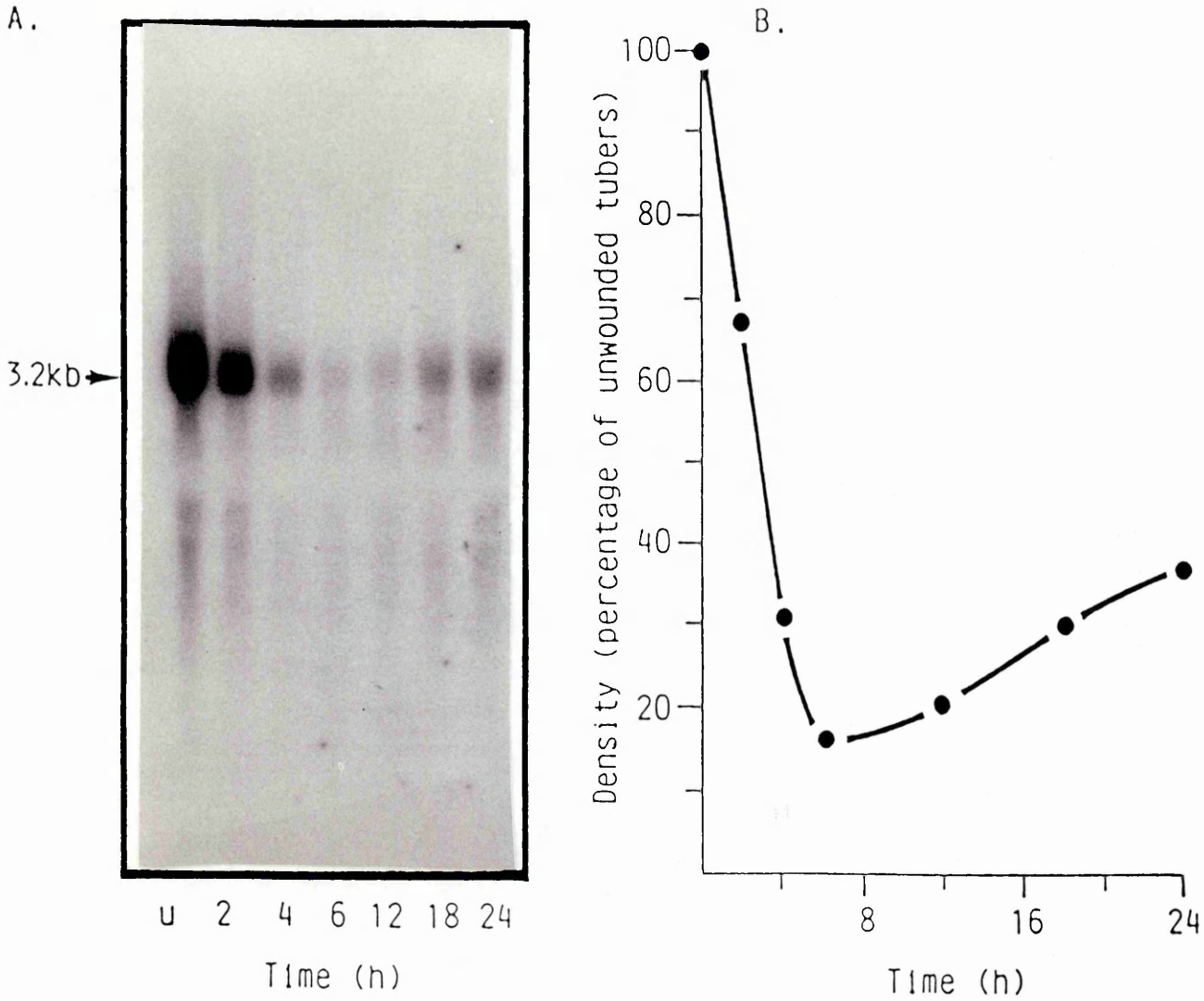
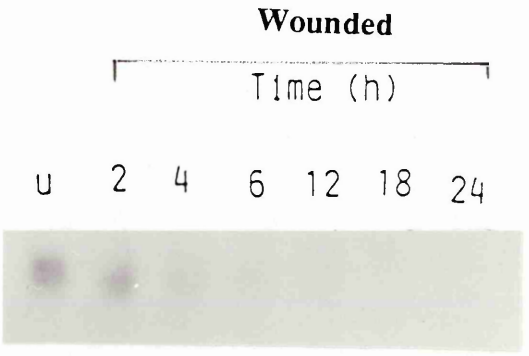

A.
3.2kb
u 2 4 6 12 18 24
Time (h)
B.
100
80
60
40
20
Density (percentage of unwounded tubers)
8
16
24
Time (h)

Fig.3.17 Effect of wounding on the levels of patatin mRNA and 25S rRNA - a control

Panel A- After the probe was removed from the blot shown in Figure 3.16 [A], it was hybridised to a ^{32}P-labelled patatin cDNA insert as described in Section 2.4.15.2 and exposed to X-ray film between two intensifying screens for 3 days at -70^{o}C.

Panel B- the blot shown in [A] was stripped of its probe and rehybridised to ^{32}P end-labelled 28S rRNA oligonucleotide probe as described in Section 2.4.15.1.2. The filter was exposed to X-ray film between two intensifying screens at -70^{o}C for 1 day.

A



B

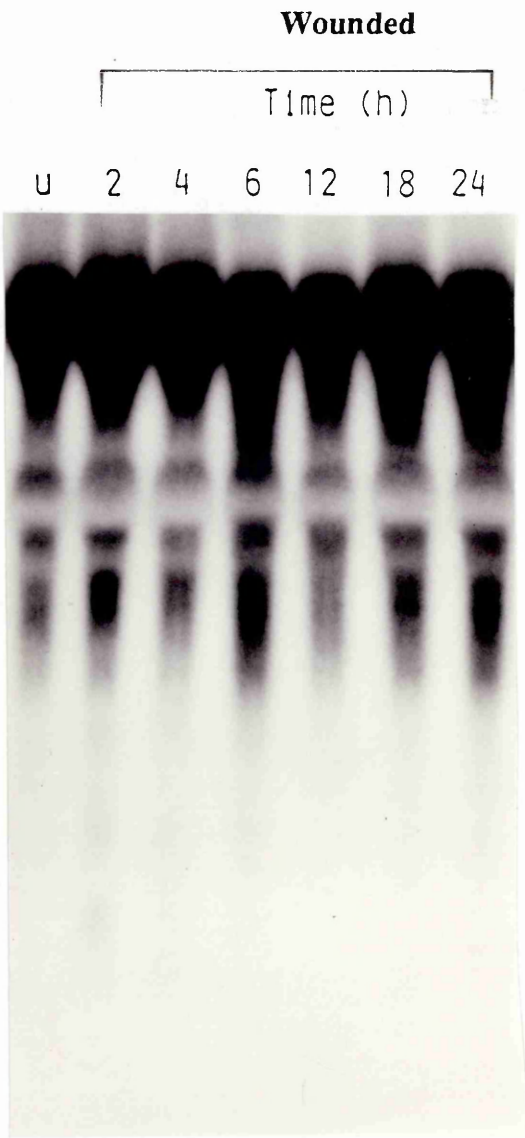


Fig.3.18 Steady state starch phosphorylase mRNA levels on prolonged ageing of tuber slices.

Northern blot analysis was performed as described earlier (Section 2.4.14), using ^{32}P-labelled (Section 2.4.12.2) phosphorylase cDNA insert as a probe. 10μg and 20μg quantities of total RNA from unwounded tuber were loaded in gel lanes 1 and 2 respectively. 20μg quantities of total RNA from 1 mm thick slices of wounded tubers incubated aerobically for 6 hours (lane 3), for 24 hours (lane 4), for 48 hours (lane 5) and for 72 hours (lane 6), and 3 mm thick slices incubated aerobically for 72 hours (lane 7) were analysed in parallel. The blot was hybridised with ^{32}P-labelled starch phosphorylase cDNA insert as described in Section 2.4.15.2.

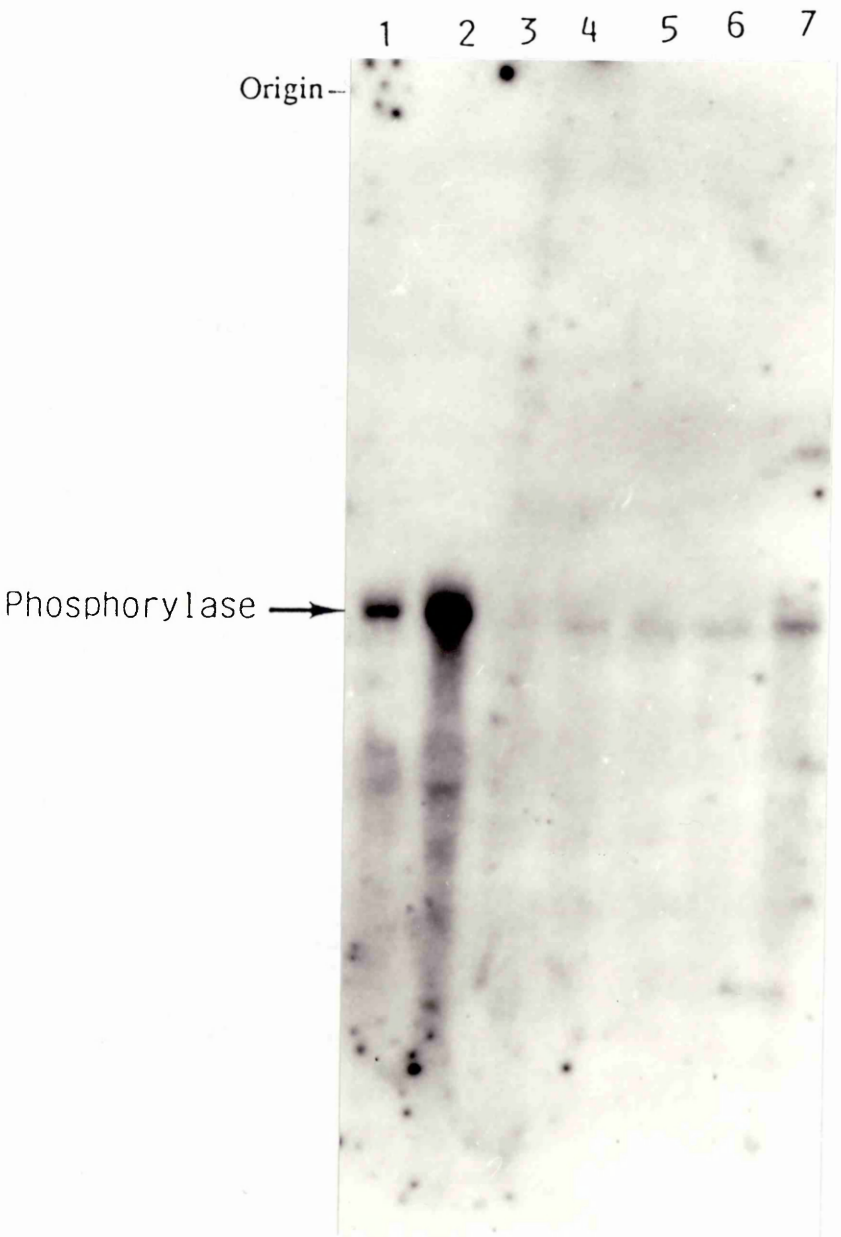
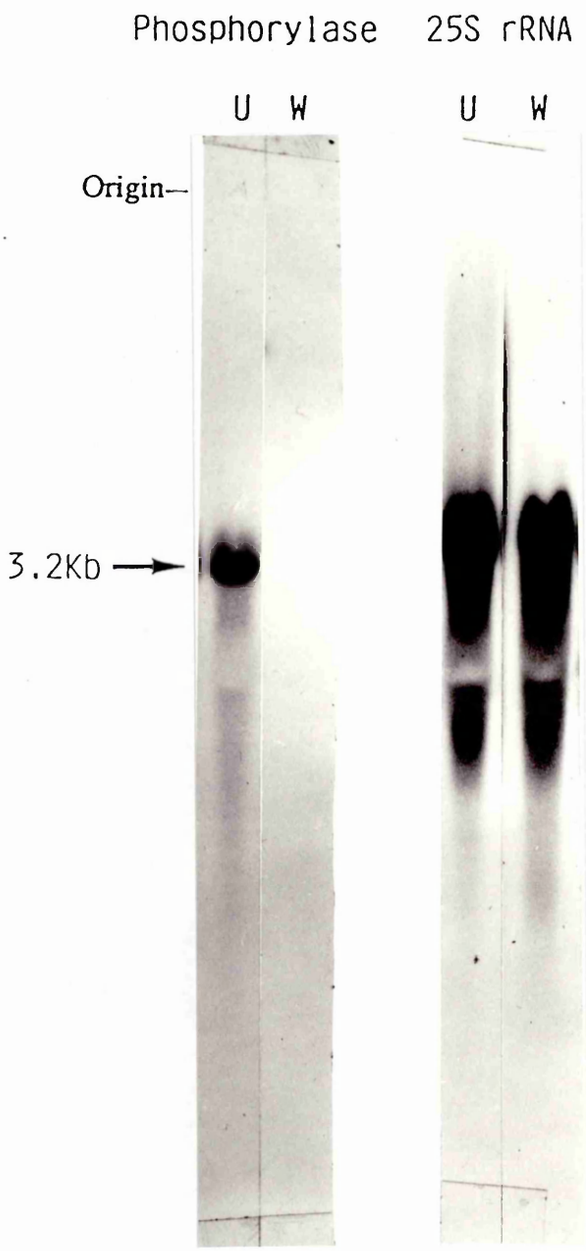

1
2
3
4
5
6
7
Origin
Phosphorylase

Fig.3.19 Effect of wounding on phosphorylase mRNA levels in stem tissue

Northern blot hybridisation (Section 2.4.15.2) of ^{32}P-labelled random primed phosphorylase cDNA insert with 40µg of total RNA purified from unwounded stem (U) and from wounded stem (W) tissue incubated anaerobically for 18 hours. The same blot is shown on the right, after it was stripped and hybridised to an end labelled 28S rRNA oligonucleotide probe as described in Section 2.4.15.1.2.


Phosphorylase
25S rRNA
U
W
U
W
Origin
3.2Kb

3.4.4 Effect of osmoticum on phosphorylase mRNA levels

The wounding induced decrease in starch phosphorylase mRNA levels was not reversed by inclusion of sucrose (5% and 14%) or mannitol (300mM) in ageing media but the levels were slightly higher in the presence of 14% sucrose, 6 hours after wounding (Figure 3.20). After 18 hours, the level of starch phosphorylase mRNA in all the samples was uniformly low.

When the stripped blot was reprobed with the patatin cDNA insert, it was clear that the presence of osmoticum in the ageing medium did not affect patatin mRNA levels in wounded tuber slices.

Multiple bands of hybridisation were seen when the stripped blot was reprobed with a putative potato 25S rRNA probe. This putative rRNA probe also hybridised to several low molecular weight species in RNA from tubers aged in buffer only. When the blot was washed at high stringency, no signal was observed.

3.4.5 Changes in phosphorylase and patatin mRNA levels in sprouting tubers

As shown in Figure 3.21, one week after the initiation of sprouting, the level of starch phosphorylase mRNA was increased marginally in the middle portion of the tuber, while the level of patatin mRNA was decreased. Patatin mRNA levels which were monitored as a control, continued to decrease and were not detectable in the middle of the tuber 8 weeks after sprouting. After the first week of sprouting, starch phosphorylase mRNA levels also decreased to be barely detectable at 8 weeks. In contrast to these data, mRNA levels for starch phosphorylase and particularly for patatin, were markedly increased 2-3 weeks after sprouting in the "proximal" region of the tuber taken from immediately below the

sprout. However by 8 weeks of sprouting, the levels of both mRNAs had fallen dramatically and again this was more drastic in the case of patatin mRNA. In contrast, 8 week old sprouts had high levels of both phosphorylase and patatin mRNAs. The levels of 25S rRNA were unchanged during sprouting.

Fig.3.20 Effect of osmoticum on phosphorylase mRNA levels in wounded tuber tissue

Panel A- Northern blot hybridisation (Section 2.4.15.2) of ^{32}P-labelled random primed phosphorylase cDNA insert with 20µg quantities of total RNA isolated from unwounded tubers (U) or from wounded tubers which were incubated as follows -

Lane 1 - 6 hours ageing in 20 mM phosphate buffer pH 6.8

Lane 2 - 6 hours ageing in 20 mM phosphate buffer pH 6.8 containing 5% sucrose

Lane 3 - 6 hours ageing in 20 mM phosphate buffer pH 6.8 containing 14% sucrose

Lane 4 - 6 hours ageing in 20 mM phosphate buffer pH 6.8 containing 300 mM mannitol

Lane 5 - 18 hours ageing in 20 mM phosphate buffer pH 6.8

Lane 6 - 18 hours ageing in 20 mM phosphate buffer pH 6.8 containing 5% sucrose

Lane 7 - 18 hours ageing in 20 mM phosphate buffer pH 6.8 containing 14% sucrose

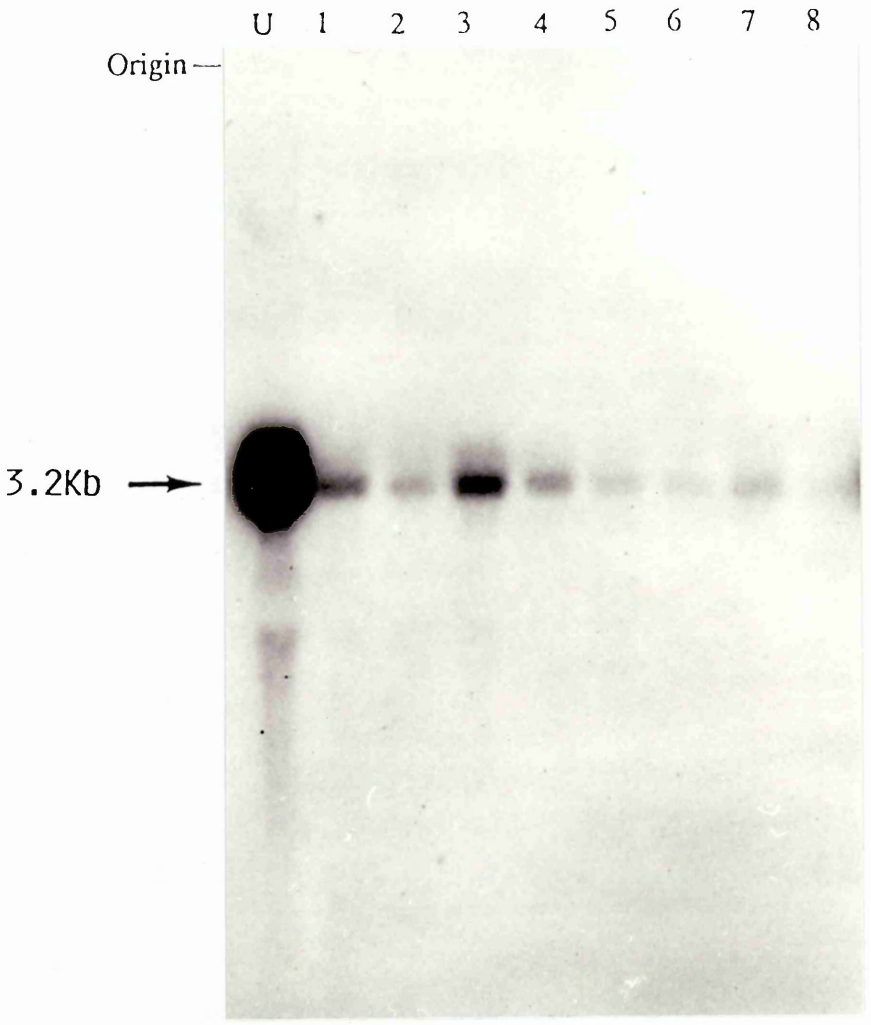
Lane 8 -18 hours ageing in 20 mM phosphate buffer pH 6.8 containing 300 mM mannitol

The filter was exposed to preflashed X-ray film, between two intensifying screens at -70°C for 3 days.

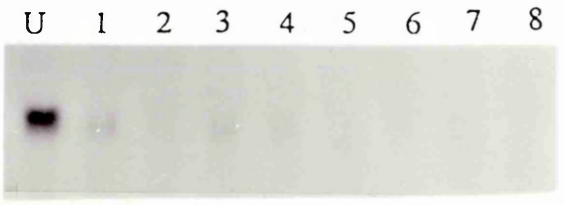
Panel B- The blot shown in [A] was stripped and hybridised to ^{32}P-labelled patatin cDNA probe as described in Section 2.4.15.2. The filter was exposed to preflashed X-ray film, between two intensifying screens at -70°C for 2 days.

Panel C- The blot shown in [B] was stripped and hybridised to a ^{32}P-labelled random primed 25S rRNA probe (Section 2.4.15.2; Logemann, J., personal communication). The filter was exposed to preflashed X-ray film, between two intensifying screens at -70°C for 3 days.

A



B



C

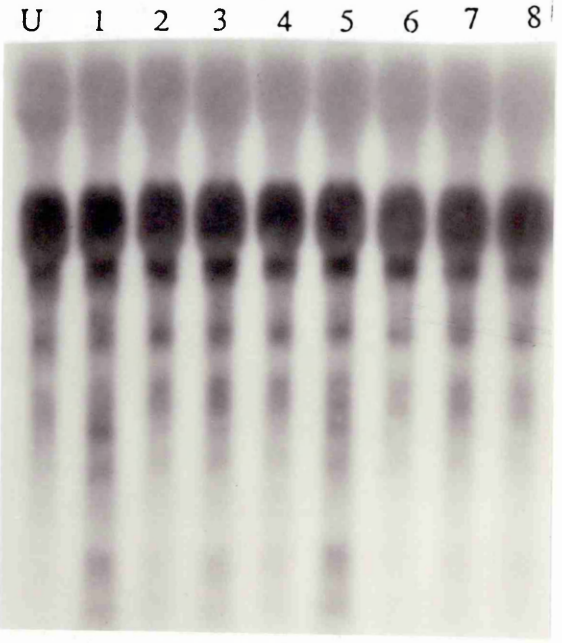
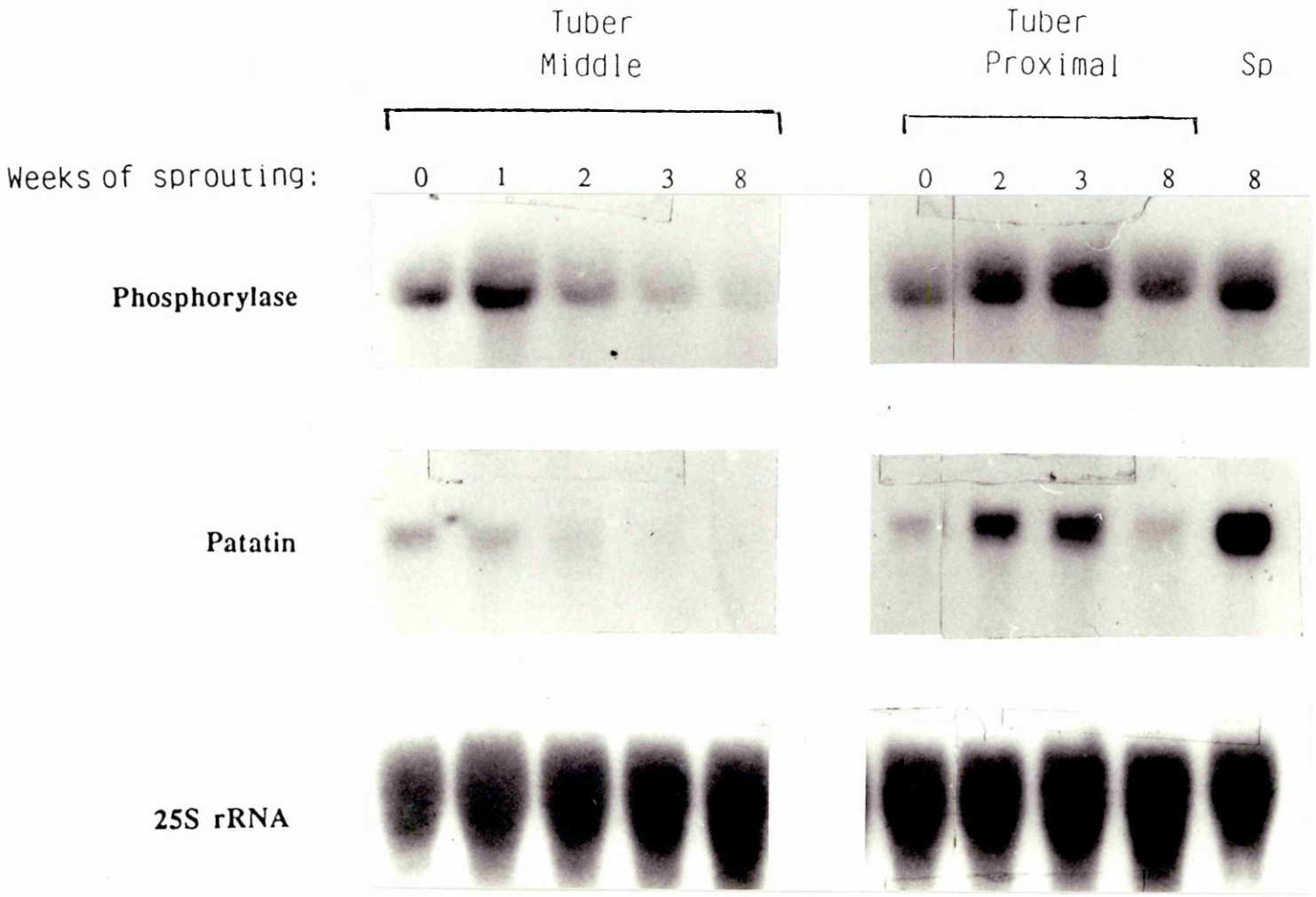


Fig.3.21 Analysis of change in phosphorylase and patatin mRNA levels during sprouting

Northern blot analysis of 20μg total RNA, isolated after 0, 1, 2, 3 and 8 weeks of sprouting from different regions of the mother tuber (middle and proximal) and from sprouts (Sp). The blot was sequentially hybridised to ^{32}P-labelled cDNA inserts of phosphorylase, patatin as described in the legend to figure 3.20, and ^{32}P end-labelled 28S rRNA oligonucleotide probe as described in Section 2.4.15.1.2 .


Tuber
Middle
Tuber
Proximal
Sp
Weeks of sprouting:
0 1 2 3 8
0 2 3 8 8
Phosphorylase
Patatin
25S rRNA

CHAPTER 4. DISCUSSION

DISCUSSION

One of the aims of this project was the construction of potato tuber cDNA libraries, that could subsequently be used to isolate recombinant clones that encoded the different enzymes involved in the process of starch mobilisation. The second aim of this project was the isolation and characterisation of starch phosphorylase clones from the cDNA library. Lastly, the starch phosphorylase cDNA clone was to be used as a probe to study the corresponding mRNA levels by northern blot hybridisation.

It was important to prepare high quality mRNA for use in cDNA synthesis, since any degradation of the mRNA would affect the representation of mRNA sequences in the cDNA library. Good quality total RNA was routinely prepared from tuber tissues by a slight variant of the method of Logemann *et al.*(1987). This method employed guanidine hydrochloride, a strong denaturant that is known to almost instantaneously inactivate cellular ribonucleases. The quality of unwounded tuber mRNA was checked by northern blot hybridisation with a patatin cDNA probe (data not shown) and showed a single intact band of hybridisation having the appropriate molecular size. Patatin mRNA could not be detected in wounded tuber mRNA. This was not surprising as, at that time, it was reported that patatin mRNA was absent (Logemann *et al.*, 1988) in wounded tubers. Therefore, the patatin cDNA probe could not be used to check the quality of wounded tuber mRNA, and subsequently this sample was used directly for cDNA synthesis.

The first aim of this project, the construction of potato tuber cDNA libraries, was achieved. Each of the libraries had a large number of total recombinants, thus increasing the likelihood that specific low abundance sequences could be isolated from them. Although the titres of the two control samples; namely those employing λgt10 and λgt10 arms were less than that expected (Amersham), the titre of the third control in which *Hae*III fragments were cloned and that of the cDNA reactions confirmed that the

DNA was cloned and packaged efficiently. The poor result with the phage controls could indicate that the amounts of λgt10 DNA used were lower than that recommended. Alternatively, these samples may have been damaged when they were cloned and packaged *in vitro*. Granger (1989), used a similar cDNA synthesis and cloning kit (Amersham), and obtained similar results for the control samples.

A common problem encountered when screening cDNA libraries with oligonucleotide probes arises from the nonspecific hybridisation of the probe to the filter impressions. As duplicate impresion filters were used, the identification of genuine hybridisation signals was facilitated. Also, the chances of selection of the authentic phosphorylase recombinants were increased, as the filter impressions could be hybridised with two different oligonucleotide probes (Section 3.3.1). Preliminary screening of ~40,000 pfu from the unwounded tuber and wounded tuber cDNA libraries was carried out using oligonucleotide SP1. Two recombinant clones that hybridised consistently to oligonucleotide SP1 were isolated from this initial screen of the unwounded cDNA library and the analysis of a further 150,000 pfu led to the isolation of a further eleven clones. No putative phosphorylase recombinants were isolated from the wounded tuber cDNA library a finding that was puzzling at the time but was explained when subsequent data revealed that the level of phosphorylase mRNA substantially decreased in wounded tissue. Out of the 13 clones selected by screening with oligonucleotide SP1, 3 putative starch phosphorylase clones - λ2.3, λ2.5, and λ2.9 - were identified, that also hybridised with the second oligonucleotide probe. One puzzling aspect of the 13 selected recombinants was that they contained multiple *Eco*RI fragments. There were at least two possible explanations for this. Firstly, multiple cDNA fragments may have been cloned into the *Eco*RI site of λgt10. This could occur if, for instance, the amount of cDNA used in the ligation reaction was higher than estimated. Since the second strand cDNA yield was only an estimate (see Section 3.2.1) errors of this sort were always possible. Alternatively, the clones could have a single cDNA insert with internal *Eco*RI site(s). In further Southern blot analyses only one *Eco*RI fragment from each of the selected clones λ2.3, λ2.5, and λ2.9 hybridised with both the oligonucleotide probes, confirming that

sequences with homology to the probes were present on these cDNA fragments. No further analyses were performed on the other *Eco*RI fragments and based on the combined data of the Southern hybridisations and Southern-cross hybridisation, a single *Eco*RI fragment of approximately 1.4 kbp was selected for further analysis. It was subcloned into the *Eco*RI site of pTZ18U and subsequently called pPSP1.

As the full length cDNA encoding starch phosphorylase was 3.1 kbp in size (Nakano *et al.*, 1989), the ~1.4 kbp of the pPSP1 insert represented about 45% of the full length sequence. Furthermore, as the cDNA synthesis had been primed with oligo dT and the oligonucleotide probes with which it hybridised were derived from sequences near the C terminus of the protein, it was likely that the cDNA insert of pPSP1 represented the 3' half of the corresponding mRNA. Initial characterisation of pPSP1 involved generation of a restriction map, and the localisation of regions bearing homology to the oligonucleotide probes (SP1 and SP2) on this restriction map. When these data for pPSP1 were compared with that published by Nakano *et al.*(1989), extensive similarities were observed (Figures 3.9 and 3.10). From these studies, it seemed likely that the ~0.78 kbp *Eco*RI/*Hin*dIII fragment of pPSP1 probably represented the poly A tail of starch phosphorylase mRNA. Although the precise location of the *Bgl*II site, relative to the position of the *Sst*I, *Pst*I, and *Hin*dIII sites on the restriction map of pPSP1 was not determined, in one of the possible orientations, the *Bgl*II site of pPSP1 coincided exactly with the corresponding site on the published restriction map.

As previously explained, at this point it became known that two other groups had cloned potato tuber starch phosphorylase. The identity of pPSP1 was therefore confirmed by comparing the published sequence of phosphorylase with the partial nucleotide sequence of pPSP1 subclones EH1, EH3, B1, and EB4, each of which represented a different restriction fragment. There was complete identity between the nucleotide sequence of the different subclones and the published nucleotide sequence of starch phosphorylase mRNA. Approximately 35% of the cDNA insert of pPSP1 was sequenced on one strand. From the nucleotide sequence of B1, it is likely that the 5' end

of the insert of pPSP1 extends up to (at least) amino acid 462 of the 916 amino acid mature protein (Nakano & Fukui, 1986). The sequence of EB4 encoded 25 amino acids which represented the C terminus of starch phosphorylase and the rest of the 3' noncoding region (see Figures 3.11,.3.12, 3.13 & 3.14).

By this time it was clear that further sequencing was an unnecessary duplication of the work of others. From the sequence data collected it was concluded that the ~1.4 kbp *Eco*RI insert of pPSP1 was a partial clone of potato tuber starch phosphorylase cDNA and this insert was subsequently used as a probe to analyse corresponding transcript levels in northern blot hybridisations. Patatin cDNA, and a oligonucleotide probe (based on the human 28S rRNA sequence) specific for 25S rRNA of potato, were routinely used as controls to probe the northern blots.

Besides the major form of tuber starch phosphorylase, for which there is a single gene per haploid genome (Camirand *et al.*, 1990), there is known to be at least one minor form of tuber starch phosphorylase. The substrate specificity, antigenic specificity, molecular weight and peptide map for this enzyme are markedly different from those of the major type starch phosphorylase (Fukui *et al.*, 1987), so it appears unlikely that the probe employed in this study would cross react with mRNA of the minor form of starch phosphorylase. However, until this minor form is better characterised, the possibility of cross-hybridisation in northern blot analyses cannot be totally excluded.

The level of starch phosphorylase mRNA was highest in tubers in keeping with the high starch levels of the tissue. Phosphorylase expression was not however tuber specific and was detected in all tissues examined. This pattern of expression was unlike that observed for tuber storage proteins like patatin and proteinase inhibitor (see Prat *et al.*, 1990) but was similar to that reported for genes encoding enzymes involved in tuber starch metabolism such as, potato granule-bound starch synthase (Visser *et al.*, 1989) and one type of ADPglucose pyrophosphorylase (Müller-Röber *et al.*, 1990).

Wounding of quiescent plant storage organs leads to a variety of metabolic and morphological changes (van Stevinick, 1975; Kahl, 1978). A consequence of the metabolic activation associated with the wounding of potato tubers is an increase in RNA

and protein synthesis (Sato *et al.*, 1978). Within 1 hour mRNA synthesis is stimulated whereas a 2-3 hour lag preceeds the synthesis of ribosomal RNA (Sato *et al.*, 1978). In slices of potato tuber (Ishizuka *et al.*, 1981), an increase in the level of polysomes is noted within 1 hour of wounding and continues for several hours. Wounding of potato tubers also leads to an increase in the activity of phenyl alanine ammonia-lyase (Lamb, 1977), peroxidases (Borchert, 1978), fatty acid synthetase (Willemot & Stumpf, 1967) and DAHP synthetase (Dyer *et al.*, 1989). The increased activities of these enzymes is due to accumulation of the corresponding mRNAs (Lamb, 1977; Willemot & Stumpf, 1967b; Dyer *et al.*, 1989; Roberts *et al.*, 1988). In addition, the wounding of potato tubers also leads to an increased synthesis of specific transcripts - *win*1, *win*2 (Stanford *et al.*, 1989) and *wun*1, *wun*2 (Logemann *et al.*, 1988) - which code for proteins of unknown function.

Wounding potato tubers also causes a mobilisation of starch, ultimately leading to the disappearance of starch granules from the cells of the wound periderm (Barckhausen, 1978). It therefore seemed likely that the activities of enzymes contributing to starch mobilisation would increase in wounded tubers. Starch phosphorylase, which has been assigned a role in the degradation of starch, is not regulated allosterically or by phosphorylation (Section 1.2.1.4). Hence, it appeared possible that starch mobilisation would be associated with an increase in starch phosphorylase activity that would probably be a consequence of an increase in the levels of its mRNA. The results obtained were in total contrast to these expectations. Phosphorylase mRNA levels, which were relatively high in unwounded tubers, fell dramatically in wounded tubers (Figures 3.16, 3.18 & 3.20). This wounding-induced decrease in starch phosphorylase transcript levels was also observed in stem tissue.

The levels of several tuberisation-associated mRNA species including those encoding patatin, proteinase inhibitor II and sucrose synthase, also fall after wounding of tubers (Butler *et al.*, 1990; Logemann *et al.*,1988; Salanoubat & Belliard, 1989). It was observed that this degradation was specific for a subset of the cytoplasmic RNA species, but that there was also a large class of tuber RNA species whose steady state

levels remained unchanged after wounding. These included actin mRNA (Butler *et al.*, 1990).

The work here described assayed patatin mRNA and 25S rRNA levels as controls (Figures 3.17 & 3.20). Patatin mRNA could be detected up to 6 hours after wounding. These data contrasted to the observations of Logemann *et al.*(1988), that patatin mRNA disappeared 1 hour after wounding of tubers. On the other hand, the kinetics of patatin mRNA depletion reported in this study are in agreement with the results of Butler *et al.*(1990). The kinetics of depletion of starch phosphorylase mRNA in tuber slices also depended on the conditions under which the slices were incubated. 6 hours after wounding, tuber slices that were incubated anaerobically had a higher level of starch phosphorylase mRNA than that observed in tuber slices incubated aerobically. However, 18 hours after wounding, aerobically incubated tuber slices had a higher level of starch phosphorylase mRNA than those incubated anaerobically. Once again, this was in contrast to patatin and sucrose synthase mRNA levels, the levels of which were observed to decrease moderately when slices were incubated anaerobically (Logemann *et al.*,1988; Salanoubat & Belliard, 1989). Starch levels were analysed in an attempt to correlate the change in starch phosphorylase mRNA levels with the mobilisation of starch in wounded tubers. In contrast to the findings of Barckhausen (1978) no significant change in the starch levels was detected up to 72 hours after wounding (data not shown).

The fall in phosphorylase mRNA levels in potato tuber slices was hard to equate with other known events associated with wound healing (Kahl, 1978). Many of these fall into three groupings as follows:-

a) events associated with the mobilisation of energy stores in order to feed the repair processes, i.e starch breakdown.

b) the production of anti-microbial compounds that protect the damaged tissue. This includes the induction of the enzymes of the phenylpropanoid pathway, and the production of proteinase inhibitors in aerial tissues.

c) synthesis of wound periderm. In the case of potato tubers this involves the synthesis of suberin and is exemplified by the induction of peroxidases (Borchert,

1978).

Since the balance of evidence suggests that starch phosphorylase functions mainly in starch degradation, it was surprising to see the levels of its mRNA fall dramatically at a time when it was assumed that starch was being mobilised. In an attempt to clarify the data, both the levels of starch and phosphorylase activity were assayed in unwounded and wounded tissue. Time did not permit repeated assays and those that were performed gave poor replicates. For this reason the data are not included in the main results section but it seemed that there was little significant change in either potato slice starch levels or in phosphorylase activity during the first 24 hours of the wounding response.

Except for patatin (Logemann *et al.*,1988), it is not known whether the observed depletion of tuberisation-associated mRNAs reflects changes in transcription or increased turnover. The latter must be considered a possibility as an increase in ribonuclease activity has been observed in wounded potato tubers (Isola & Franzoni, 1981). Unfortunately, attempts to purify potato tuber nuclei, in order to measure phosphorylase mRNA synthesis by run-on transcription, were unsuccessful. It has been observed that when transcription was inhibited in wounded tubers, mRNA which was synthesised during the development of tubers and/or post-harvest storage, was continuously recycled in polysomes because it was not replaced by newly synthesised wound-induced mRNA (Ishizuka & Imaseki, 1989). It seems likely that such a displacement from polysomes could render these mRNAs susceptible to ribonuclease attack. However, it was unclear why only these abundant messages were selectively degraded in wounded tubers (Butler *et al.*, 1990).

Sucrose has been shown to increase the amount of several transcripts. These include patatin mRNA in wounded tuber tissue (Logemann & Schell, 1989), sucrose synthase mRNA and one of the ADP-glucose pyrophosphorylase mRNAs (Müller-Röber *et al.*, 1990) in detached leaves of *S. tuberosum.* No such effect of sucrose (or mannitol) was seen on the amount of starch phosphorylase mRNA in wounded tuber tissue. Indeed in contrast to the observation of Logemann and Schell (1989), neither was

an effect of sucrose seen on patatin mRNA levels. In this study, the tuber slices were wounded by cutting them 1 mm thick, whereas Logemann and Schell (1989) used 3 mm thick tuber slices. It was clear from Figure 3.18, that the decrease in starch phosphorylase mRNA was more drastic in 1 mm thick slices than in 3 mm thick slices. Perhaps the severity of the wounding-induced decrease of starch phosphorylase and patatin mRNA in 1mm thick tuber slices precluded any effect of sucrose. It has also been observed that with an increase in the distance from the wound surface of a tuber, the induction of DAHP synthetase activity was lower (Dyer *et al.*, 1989) .

A sprouting tuber represents an experimental system in which gene expression of the enzymes involved in starch metabolism can be studied in source and sink organs. Although the biochemistry and physiology of sprouting potato tubers has been studied extensively, the relative contribution of various enzymes to starch mobilisation and the regulation of this process are unclear. During the process of sprouting, the mother tuber represents the source, in which starch is mobilised and ultimately translocated as sucrose to the sink organ, represented by the developing sprout. The development and growth of the sprouts depends upon the experimental conditions to which the sprouting tubers are exposed. In this study, sprouting of tubers was carried out on trays in the dark, under such conditions, the sprouts grow slowly and channel sugars towards starch synthesis (Davies, 1984). High levels of starch phosphorylase and patatin mRNA were observed in these sprouts. The high levels of starch phosphorylase were evident in two week old sprouts (data not shown) that had been grown in the dark on trays. Preliminary experiments indicated that illuminating two week old dark-grown sprouts for 72 hours did not affect the levels of starch phosphorlase mRNA (data not shown).

The levels of starch phosphorylase and patatin mRNAs changed during the sprouting of potato tubers but regional differences were observed in the mother tuber at different stages of sprouting. The high levels of starch phosphorylase and patatin mRNA in the 'proximal' sample after 2-3 weeks of sprouting could reflect the position of the proximal sample, which could make it sensitive to translocate from the mother tuber or to

signals diffusing from the sprout. It is also likely that it reflects the heterogeneity of the tissue sample, i.e. it included tissue inner and outer to the vascular ring.

Although the balance of evidence suggests that the major function of starch phosphorylase is in the catalysis of starch degradation (see Preiss, 1982; Morell & ap Rees, 1986), Catz *et al.*(1989) suggest that the loss of the major tuber starch phosphorylase during dedifferentiation is consistent with a synthetic role for the enzyme. Studies in *Chlamydomonas reinhardtii* have shown that with starch accumulation, there is an increase in the starch phosphorylase activity in phosphate starved cells (Ball *et al.*, 1990).

Brisson *et al.* (1989) have observed that the major starch phosphorylase was located inside the amyloplast stroma of young tubers actively involved in starch synthesis, whereas in older tubers the enzyme was found to be in the cytoplasm in the vicinity of the amyloplasts. They claim that their data is suggestive of a role for the enzyme in starch synthesis rather than degradation. However, from the cytochemical evidence it was difficult to interpret conclusively, whether the activity of starch phosphorylase was regulated *in vivo* by compartmentation.

The data presented here, on the abundance of starch phosphorylase transcripts in wounded tissues and in sprouting tubers, does not resolve the issue of whether starch phosphorylase has a role in starch breakdown or starch synthesis. Nevertheless in sink tissues, like developing sprouts and tubers, high levels of starch phosphorylase mRNA were detected, whereas in source tissues starch phosphorylase mRNA levels decreased ultimately. These data can be said to support a role for starch phosphorylase in starch synthesis rather than its degradation. Recently, Müller-Röber *et al.*(1990) have reported that the mRNAs for two different ADP-glucose pyrophosphorylase disappeared from seed tubers after sprout and plant development. Thus a known starch synthesising enzyme gave results that at least partially paralleled those obtained with starch phosphorylase.

FUTURE WORK

Were this study to continue, it would be essential in the first instance to effectively correlate the observed changes in starch phosphorylase mRNA levels with both phosphorylase enzyme activity and with tuber starch levels. It would also be desirable to develop antibodies to the enzyme so that absolute levels of starch phosphorylase could be measured. The combination of all these approaches should allow:-

1. Effective analysis of the role of starch phosphorylase in starch synthesis and breakdown. Mini-tuber development would be a good system in which to study its role in starch synthesis.

2. Analysis of the role of starch phosphorylase in wound healing, sprouting, cold-induced sweetening and senescent sweetening.

3. Analysis of the effect of plant hormones on starch phosphorylase gene expression. Of particular interest would be the effect of jasmonic acid, which is known to regulate the expression of vegetative storage protein genes in soy bean (Mason & Mullet, 1990), and is also closely related to the "tuber inducing" substance isolated from potato leaves (Yoshihara *et al.*, 1988).

4. Perhaps the most rewarding approach to investigating the importance of starch phosphorylase in tuber starch metabolism would be to introduce phosphorylase antisense sequence into transgenic plants. The careful choice of promoters to regulate such constructs would make these studies most meaningful. Thus, if the antisense sequence were linked to a patatin promoter, it would only be expressed in the tubers of transgenic potato plants.

5. It would be interesting to check the effect of sucrose on starch phosphorylase mRNA levels in leaf explants of not only potato, but also of tobacco and tomato, because the genomic DNA of these plants had sequences that had homology to the potato tuber starch phosphorylase cDNA (data not shown).

REFERENCES

REFERENCES

Adams, C.A., Watson, T.G. & Novellie, L. (1975) Phytochem. **14**, 953-956.

Adams, C.A., Broman, T.H. & Rinnie, R.W. (1981) Ann. Bot. **48**, 433-440.

Akazawa, T. & Hara-Nishimura, I. (1985) Annu. Rev. Plant Physiol. **36**, 441-472.

Akazawa, T. & Miyata, S. (1982) Essays in Biochem. **18**, 40-78.

Andrews, B., Beames, B., Summers, M.D. & Park, W.D. (1988) Biochem. J. **252**, 199-206.

ap Rees, T. (1985) in The Encyclopedia of Plant Physiology (Douce, R. & Day, D.A., eds) NS, Vol. 18, pp 390-417, Springer-Verlag, Hiedelberg.

Artschwager, E. (1924) Anal. Biochem. **152**, 304-307.

Ashford, A.E. & Gubler, F. (1984) in Seed Physiology, Germination and Reserve Mobilisation (Murray, D.R., ed) Vol.2, pp 117-162, Academic Press Australia, Sydney.

Badenhuizen, N.P. & Dutton, R.W. (1956) Protoplasma **47**, 156-163.

Bailey, K.M., Phillips, I.D.J. & Pitt, D. (1978) Ann. Bot. **42**, 649-657.

Ball, S.G., Dirick, L., Decq, A., Martiat, J.-C. & Matagne, R.F. (1990) Plant Sci. **66**, 1-9.

Banks, W., Greenwood, C.T. & Muir, D.D. (1974) Stäerke **26**, 46-50.

Barbu, V. & Dautry, F. (1989) Nucl. Acids Res. **17**, 7115.

Barckhausen, R. (1978) in Biochemistry of Wounded Plant Tissue (Kahl, G., ed) pp 1-42, de Gruyter, Berlin.

Baulcombe, D.C. & Buffard, D. (1983) Planta **157**, 493-501.

Baulcombe, D.C., Huttly, A.K., Martienssen, R.A., Barker, R.F. & Jarvis, M.G. (1987) Mol. Gen. Genet. **209**, 33-40.

Beck, C.D. (1985) in Regulation of Carbon Partitioning in Photosynthetic Tissue (Heath, R.L. & Preiss, J., eds) pp 27-44, Am. Soc. of Plant Physiol., Waverly Press, Baltimore.

Beck, E. & Ziegler, P. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 95-117.

Beck, E., Pongratz, P. & Reuter, I. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed) Vol. 4, pp 529-538.

Beers, E.P., Duke, S.H. & Henson, C.A. (1990) Plant Physiol. **94**, 738-744.

Bernfield, P. (1955) Methods Enzymol. **1**, pp149-158.

Bevan, M., Barker, R., Goldsbrough, A., Jarvis, M., Kavanagh, T. & Iturriaga, G. (1986) Nucleic Acids Res. **14**, 4625-4638.

Bilderback, D.E. (1974) Plant Physiol. **53**, 480-484.

Borchert, R. (1978) Plant Physiol. **62**, 789-793.

Borovsky, D., Smith, E.E. & Whelan, W.J. (1975) Eur. J. Biochem. **59**, 615-625.

Boston, R.S., Miller, T.J., Mertz, J.E. & Burgess, R.R. (1982) Plant Physiol. **69**, 150-154.

Boulnois, G.J. (1987) in Gene Cloning and Analysis. A Laboratory Guide. (Boulnois, G.J., ed) Blackwell Scientific Publications, Oxford.

Boyer, C.D. (1985) Phytochem. **24**, 15-18.

Boyer, C.D. & Fisher, M.B. (1984) Phytochem. **23**, 733-737.

Boyer, C.D. & Preiss, J. (1978a) Biochem. Biophys. Res. Commun. **80**, 169-175.

Boyer, C.D. & Preiss, J. (1978b) Carbohydrate Res. **61**, 321-334.

Brisson, N., Giroux, H., Zollinger, M., Camirand, A. & Simard, C. (1989) The Plant Cell **1**, 559-566.

Burton, W.G. (1965) Eur. Potato J. **8**, 80-91.

Butler, W., Cook, L. & Vayda, M.E. (1990) Plant Physiol. **93**, 264-270.

Callis, J. & Ho, D.T.-H. (1983) Arch. Biochem. Biophys. **224**, 224-234.

Cameron, J.W. & Teas, H.J. (1954) Am. J. Bot. **41**, 50-55.

Camirand, A., St-Pierre, B., Marineau, C. & Brisson, N. (1990) Mol. Gen. Genet. **224**, 33-39.

Cardemil, L. & Varner, J.E. (1984) Plant Physiol. **76**, 1047-1054.

Cardini, C.E. & Frydman, R.B. (1966) Methods Enzymol. **8**, 387-394.

Cardini, C.E., Leloir, L.F. & Chiriboga, J. (1955) J. Biol. Chem. **214**, 149-155.

Catchpole, A.H. & Hillman, J. (1969) Nature **223**, 1387.

Catz, D.S., Moreno, S. & Tandecarz, J.S. (1989) Physiol. Plant. **76**, 221-225.

Chang, C.W. (1980a) Microchem. J. **25**, 179-189.

Chang, C.W. (1980b) Plant Physiol.**65** (Supplement), 114.

Chang, C.W. (1982) Phytochem. **21**, 1263-1269.

Chauhan, G., Nall, C. & Bevan, M. (1990) in Abstracts - Society for Experimental Biology Symposium on Molecular Biology of Plant Development.

Chiba, S. & Shimomura, T. (1975) Agric. Biol. Chem. **39**, 1033-1040.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) Biochem. **18**, 5294-5299.

Copeland, L. & Preiss, J. (1981) Plant Physiol. **68**, 996-1001.

Creech, R.G. (1965) Genetics **52**, 1175-1186.

Daussant, J., Miyata, S., Mitsui, T. & Akazawa, T. (1983) Plant Physiol. **71**, 88-95.

Davies, H.V. (1984) Potato Res. **27**, 209-218.

Davies, H.V. & Ross, H.A. (1984) Potato Res. **27**, 373-381.

Davies, H.V. & Ross, H.A. (1986) J. Plant Physiol. **126**, 387-396.

De Fekete, M.A.R. & Cardini, C.E. (1964) Arch. Biochem. Biophys. **104**, 173-184.

Deikman, J. & Jones, R.L. (1986) Plant Physiol. **80**, 672-675.

Dennis, D.T. & Greyson, M. (1987) Physiol. Plant. **69**, 395-404.

Dennis, D.T. & Miernyk, J.A. (1982) Annu. Rev. Plant Physiol. **33**, 27-50.

Dickson, R.E. & Larwood, P.R. (1973) Plant Physiol. **57**, 185-193.

Dickinson, D.B. & Preiss, J. (1969a) Arch. Biochem. Biophys. **130**, 119-128.

Dickinson, D.B. & Preiss, J. (1969b) Plant Physiol. **44**, 1058-1062.

Drummond, G.S., Smith, E.E. & Whelan, W.J. (1972) Eur. J. Biochem. **26**, 168-176.

Dyer, W.E., Henstrand, J.M., Handa, A.K., Herrmann, K.M. (1989) Proc. Natl. Acad. Sci. **86**, 7370-7373.

Echeverria, E. & Boyer, C.D. (1986) Am. J. Bot. **73**, 167-171.

Echeverria, E., Boyer, C.D., Thomas, P.A., Liu, K.-C. & Shannon, J.C. (1988) Plant Physiol. **86**, 786-792.

Echt, C.S. & Schwartz, D. (1981) Genetics **99**, 275-284.

Entwhistle, G. & ap Rees, T. (1988) Biochem. J. **255**, 391-396.

Espada, J. (1962) J. Biol. Chem. **237**, 3577-3581.

Evers, A.D. & McDermott, E.E. (1970) Stärke **22**, 23-26.

Ewing, E.E. (1985) in Potato Physiology (Li, P.H., ed) pp 153-207, Academic Press, London.

Feinberg, A.P. & Vogelstein, B. (1983) Anal. Biochem. **132**, 6-13.

Fernandez, D.E. & Staehelin, L.A. (1985) Planta **165**, 455-468.

Filner, P & Varner, J. E. (1967) Proc. Natl. Acad. Sci. **58**, 1520-1526.

Fischer, E.H. & Stein, E.A. (1961) in The Enzymes (Boyer, P.D., Lardy, H. & Myrbach, K., eds) Vol 4, pp 313-343, Academic Press, New York.

Fliege, R., Flugge, U.-I., Werdan, K. & Heldt, H.W. (1978) Biochim. Biophys. Acta **502**, 232-247.

Forsline, P.L. & Langille, A.R. (1975) Physiol. Plant. **34**, 75-77.

Forsline, P.L. & Langille, A.R. (1976) Can. J. Bot. **54**, 2513-2516.

French, D. (1961) in The Enzymes (Boyer, P.D., Lardy, H. & Myrbach, K., eds) 2nd edition, Vol.4, pp 345-368. Academic Press, New York.

Frydman, R.B., DeSouza, B.C.& Cardini, C.E. (1966) Biochim. Biophys. Acta **113**, 620-623.

Frydman, R.B. & Slabnik, E. (1973) Ann. N. Y. Acad. Sci. **210**, 153-169.

Fukui, T., Nakano, K., Tagaya, M. & Nakayama, H. (1987) in Biochemistry of Vitamin B_6 (Korpela, T. & Christen, P., eds) pp 267-276, Birkhäuser Verlag, Basel.

Garcia-Torres, L. & Gomez-Campo, C. (1973) Potato Res. **16**, 73-79.

Gates, J.W. & Simpson, G.M. (1968) Can. J. Bot. **46**, 1459-1462.

Gerbrandy, S.J. & Doorgest, A. (1972) Phytochem. **11**, 2403-2407.

Gerbrandy, S.J., Shankar, V., Shiraran, K.N. & Stegemann, H. (1975) Phytochem. **14**, 2331-2333.

Ghosh, H.P. & Preiss, J. (1965) Biochemistry **4**, 1354-1361.

Ghosh, H.P. & Preiss, J. (1966) J. Biol. Chem. **241**, 4491-4504.

Giaquinta, R.T. (1981) in The Biochemistry of Plants (Preiss, J., ed) Vol. 3, pp 271-320, Academic Press, New York.

Granger, S.P. (1989) Ph.D. thesis, University of Glasgow.

Gregory, L.E. (1956) Am. J. Bot. **43**, 281-288.

Gubler, U. & Hoffman, B.J. (1983) Gene **25**, 263-269.

Gubler, F., Jacobsen, J.V. & Ashford, A.E. (1986) Planta **168**, 447-452.

Gubler, F., Ashford, A.E. & Jacobsen, J.V. (1987) Planta **172**, 155-161.

Gunja-Smith, Z., Marshall, J.J., Mercier, C.E., Smith, E.E. & Whelan, W.J. (1970) FEBS Lett. **12**, 101-104.

Halayko, A.J., Hill, R.D. & Svensson, B. (1986) Biochim. Biophys. Acta. **873**, 92-101.

Halmer, P. (1985) Physiol. Vég. **23**, 107-125.

Halmer, P. & Bewley, J.D. (1982) in Encyclopedia of Plant Physiology (Loewus, F.A. & Tanner, W., eds) NS, Vol.13A, pp 748-793, Springer-Verlag, Berlin Heidelberg New York.

Hanes, C.S. (1940) Proc. R. Soc. London Ser. B **129**, 174-208.

Hannapel, J.D., Creighton-Miller, J.C. & Park, W.D. (1985) Plant Physiol. **78**, 700-703.

Hara-Nishimura, I., Nishimura, M. & Daussant, J. (1986) Protoplasma **134**, 149-153.

Hardie, D.G. (1975) Phytochem. **14**, 1719-1722.

Hatzfeld, W.-D. & Stitt, M. (1990) Planta **180**, 198-204.

Hawker, J.S., Ozbun, J.L. & Preiss, J. (1972) Phytochem. **11**, 1287-1293.

Hawker, J.S. & Downton, W.J.S. (1974) Phytochem. **13**, 893-900.

Hawker, J.S., Marschner, H. & Downton, W.J.S. (1974) Aust. J. Physiol. **1**, 491-501.

Hawker, J.S., Ozbun, J.L., Ozaki, H., Greenberg, E. & Preiss, J. (1974) Arch Biochem. Biophys. **160**, 530-551.

Hawker, J.S., Marschner, H. & Krauss, A. (1979) Physiol. Plant. **46**, 25-30

Heldt, H.W., Chon, C.J., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R. & Heber, U. (1977) Plant Physiol. **59**, 1146-

1155.

Herold, A. & Lewis, D.H. (1977) New Phytol. **79**, 1-40.

Herold, A. & Walker, D.A. (1979) in Handbook of Transport Across Membranes (Giebisch, G., Tosteson, D.C. & Ussing, H.H., eds) pp 411-439, Springer-Verlag, Hiedelberg, New York.

Herold, A., Lewis, D.H. & Walker, D.A. (1976) New Phytol. **76**, 397-407.

Hildebrand, D.F. & Hymowitz, T. (1981) Physiol. Plant. **53**, 429-434.

Höfgen, R. & Willmitzer, L. (1990) Plant Sci. **66**, 221-230.

Huber, S.C. (1981a) Z. Planzenphysiol. **101**, 49-54.

Huber, S.C. (1981b) Z. Planzenphysiol. **101**, 443-450.

Humphreys, T.E. (1975) Phytochem. **14**, 333-340.

Hutson, D.H. & Manners, D.J. (1965) Biochem. J. **94**, 783-789.

Huynh, T.V., Young, R.A., Davis, R.W. (1985) in DNA Cloning Techniques. A Practical Approach (Glover, D., ed), Vol. 1, pp 1-49, IRL Press, Oxford.

Isherwood, F.A. (1976) Phytochem. **15**, 33-41.

Ishizaki, Y., Taniguchi, H., Maruyama, Y. & Nakamura, M. (1983) Agric. Biol. Chem. **47**, 771-779.

Ishizuka, M. & Imaseki, H. (1989) Plant Cell Physiol. **30**, 229-236.

Ishizuka, M., Sato, T., Watanabe, H. & Imaseki, H. (1981) Plant Physiol. **68**, 154-157.

Isola, M.C. & Franzoni, L. (1981) Plant Cell Physiol. **27**, 331-335.

Iwaki, K. & Fuwa, H. (1981) Agric. Biol. Chem. **45**, 2683-2688.

Jackson, A.O. & Larkins, B.A. (1976) Plant Physiol. **57**, 5-10.

Jacobsen, J.V. (1983) in The Biochemistry and Physiology of Gibberellins (Crozier, A., ed) Vol.2, pp 157-187, Praeger, New York.

Jacobsen, J.V. & Beach, L.R. (1985) Nature **316**, 275-277.

Jacobsen, J.V., Hanson, A.D. & Chandler, P.C. (1986) Plant Physiol. **70**, 1647-1653.

Jameson, P.E., McWha, J.A. & Haselmore, R.M. (1985) Physiol. Plant. **63**, 53-57.

Jefferson, R., Goldsbrough, A. & Bevan, M. (1990) Plant Mol. Biol. **14**, 995-1006.

Jenner, C.F. (1982) in Encyclopedia of Plant Physiology NS (Loewus, F.A. & Tanner, W., eds) Vol 13A, pp 700-747, Springer-verlag, New York, Heidelberg, Berlin.

Jones, F.T. & Bean, M.M. (1972) Microscope **20**, 333-340.

Jones, R.L. & Carbonell, J. (1984) Plant Physiol. **76**, 213-218.

Journet, E.-P. & Douce, R. (1985) Plant Physiol. **79**, 458-467.

Kahl, G. (1978) in Biochemistry of Wounded Plant Tissue, deGruyter, Berlin.

Kakefuda, G., Duke, S.H. & Hostak, M.S. (1986) Planta **168**, 175-182.

Katsube, T., Kazuta, Y., Mori, H., Nakano, K., Tanizawa, K. & Fukui, T. (1990) J. Biochem. **108**, 321-326.

Keeling, P.L., Wood, J.R., Tyson, R.H. & Bridges, I.G. (1988) Plant Physiol. **87**, 311-319.

Keil, M., Sánchez-Serrano, J., Schell, J. & Willmitzer, L. (1986) Nucleic Acids Res. **14**, 5641-5650.

Keil, M., Sánchez-Serrano, J.J., Schell, J. & Willmitzer, L. (1989) EMBO J. **8**, 1323-1330.

Keil, M., Sánchez-Serrano, J.J., Schell, J. & Willmitzer, L. (1990) The Plant Cell. **2**, 61-70.

Koda, Y. (1982) Plant Cell Physiol. **23**, 843-849.

Koda, Y. & Okazawa, Y. (1988) Plant Cell Physiol. **29**, 969-974.

Koda, Y., Omer, E.A., Yoshihara, T., Shibata, H., Sakamara, S. & Okazawa, Y. (1988) Plant Cell Physiol. **29**, 1047-1051.

Köster-Töpfer, M., Frommer, W., Rocha-Sosa, M., Rosahl, S., Schell, J. & Willmitzer, L. (1989) Mol. Gen. Genet. **219**, 390-396.

Kreis, M., Williamson, M., Buxton, B., Pywell, J., Hejgaard, J. & Svendsen, I. (1987) Eur. J. Biochem. **169**, 517-525.

Krishnan, H.B., Reeves, C.D. & Okita, T.W. (1986) Plant Physiol. **81**, 642-645.

Lamb, C.J. (1977) Planta **135**, 169-175.

Laurière, C., Laurière, M. & Daussant, J. (1986) Physiol. Plant. **67**, 383-388.

Leah, R. & Mundy, J. (1989) Plant Mol. Biol. **12**, 673-682.

Lecommandeur, D., Laurière, C. & Daussant, J. (1987) Plant Physiol. Biochem. **25**, 711-715.

Lee, E.Y.C & Whelan, W.J. (1971) in The Enzymes (Boyer, P.D., ed) Vol. 5, pp 191-234, Academic Press, New York.

Lee, Y.P. (1960) Biochim. Biophys. Acta **43**, 18-24.

Leloir, L.F., de Fekete, M.A.R. & Cardini, C.E. (1961) J. Biol. Chem. **236**, 636-641.

Lin, T.-P. & Preiss, J (1988) Plant Physiol. **86**, 260-265.

Lin, T. -P., Caspar, T., Sommerville, C. & Preiss, J. (1988a) Plant Physiol. **86**, 1131-1135.

Lin, T. -P., Caspar, T., Sommerville, C. & Preiss, J. (1988b) Plant Physiol. **88**, 1175-1181

Lin, T.-P., Spilatro, S.R. & Preiss, J. (1988c) Plant Physiol. **86**, 251-259.

Linden, J.C., Tanner, W. & Kandler, O. (1974) Plant Physiol. **54**, 752-757.

Liu, X.J., Prat, S., Willmitzer, L. & Frommer, W.B. (1990) Mol. Gen. Genet. **223**, 401-406.

Logemann, J., Schell, J. & Willmitzer, L. (1987) Anal. Biochem. **163**, 16-20.

Logemann, J., Mayer, J.E., Schell, J. & Willmitzer, L. (1988) Proc. Natl.Acad. Sci. **85**, 1136-1140.

Logemann, J. & Schell, J. (1989) Mol. Gen. Genet. **219**, 81-88.

Ludwig, I., Ziegler, P. & Beck, E. (1984) Plant Physiol. **74**, 856-861.

Lulai, E.C., Sowikinos, J.R. & Knoper, J.A. (1986) Plant Physiol. **80**, 424-428.

Lundgard, R. & Svensson, B. (1987) Carlsberg Res. Commun. **52**, 313-326.

MacDonald, F.D. & apRees, T. (1983) Biochim. Biophys. Acta **755**, 81-89.

MacDonald, F.D. & Preiss, J. (1983) Plant Physiol. **73**, 175-178.

MacDonald, F.D. & Preiss, J. (1985) Plant Physiol. **78**, 849-852.

MacDonald, P.W. & Strobel, G.A. (1970) Plant Physiol. **46**, 126-135.

MacDonnel, M.V., Simon, M.V. & Studier, F.W.J. (1977) J. Mol. Biol. **110**, 119.

Maeda, I. & Nikuni, Z. (1978) Carbohydr. Res. **61**, 309-320.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) in Molecular Cloning. A Laboratory Manual, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Manners, D.J. (1974) Essays in Biochem. **10**, 37-71.

Manners, D.J. (1985) in Biochemistry of Storage Carbohydrates (Day, P.M. & Dixon, R.A., eds) pp 149-204, Academic, London.

Manners, D.J. & Rowe, K.L. (1969) Carbohydr. Res. **9**, 441-450.

Mares, D.J., Marschner, H. & Krauss, A.(1981) Physiol. Plant. **52**, 267-274.

Marshall, J.J. & Whelan, W.J. (1974) Arch. Biochem. Biophys. **161**, 234-238.

Mason, H.S. & Mullet, J.E. (1990) The Plant Cell **2**, 569-579.

Matters, G.L. & Boyer, C.D. (1981) Phytochem. **20**, 1805-1809.

Matters, G.L. & Boyer, C.D. (1982) Biochem. Genet. **20**, 833-848.

Mauk, C.S. & Langille, A.R. (1978) Plant Physiol. **62**, 438-442.

Melis, R.J.M. & Van Staden, J. (1984) Z. Pflanzenphysiol. **113**, 271-283.

Mignery, G., Pikaard, C., Hannapel, D. & Park, W. (1984) Nucleic Acids Res. **12**, 7989-8000.

Mignery, G., Pikaard, C. & Park, W. (1988) Gene **62**, 27-44.

Mingo-Castel, A.M., Negmn F.B. & Smith, O.E. (1974) Plant Physiol. **53**, 798-801.

Mingo-Castel, A.M., Smith, O.E. & Kunamoto, J. (1976) Plant Physiol. **57**, 480-485.

Mitchell, E.D. (1972) Phytochem. **11**, 1673-1676.

Miyata, S. & Akazawa, T. (1982) Plant Physiol. **70**, 147-153.

Mohabir, G. & John, P. (1989) Plant Physiol. **88**, 1222-1228.

Moore, C.J. & Turner, J.F. (1969) Nature **223**, 303-304.

Morell, S. & apRees, T. (1986) Phtochem. **7**, 1579-1585.

Morell, M., Bloom, M., Knowles, V. & Preiss, J. (1987) Plant Physiol. **85**, 182-187.

Morell, M., Bloom, M. & Preiss, J. (1988) J. Biol. Chem. **263**, 633-637.

Müller-Röber, B.T., Koßmann, J., Hannah, C., Willmitzer, L. & Sonnewald, U. (1990) Mol. Gen. Genet. **224**, 136-146.

Mundy, J. (1982) Carlsberg Res. Commun. **47**, 263-274.

Mundy, J. & Rogers, J.C. (1986) Planta **169**, 51-63.

Mundy, J., Svendsen, I. & Hejgaard, J. (1983) Carlsberg Res. Commun. **48**, 81-94.

Mundy, J., Brandt, A. & Fincher, G.B. (1985) Plant Physiol. **79**, 867-871.

Murata, T., Sugiyama, T. & Akazawa, T. (1965) Biochem. Biophys. Res. Commun. **18**, 371-376.

Muthukrishnan, S., Chandra, G.R. & Maxwell, E.S. (1983) J. Biol. Chem. **258**, 2370-2375.

Muthukrishnan, S., Gill, B.S., Swegle & M., Chandra, G.R. (1984) J. Biol. Chem. **259**, 13637-13639.

Nakano, K. & Fukui, T. (1986) J. Biol. Chem. **261**, 8230-8236.

Nakano, K., Mori, H. & Fukui, T. (1989) J. Biochem. **106**, 691-695.

Nelson, O.E. & Rines, H.W. (1962) Biochem. Biophys. Res. Commun. **9**, 297-300.

Nelson, O.E., Chourey, P.S. & Chang, M.T. (1978) Plant Physiol. **62**, 383-386.

Ngernprasirtsiri, J., Harinasut, P., Macherel, D., Strzalka, K., Takabe, T., Akazawa, T. & Kojima, K. (1988) Plant Physiol. **87**, 371-378.

Nikuni, Z. (1978) Stäerke **30**, 105-111.

Nishimura, M., Hara-Nishimura, I., Bureau, D. & Daussant, J. (1987) Plant Sci. **49**, 117-122.

Nolan, R.C., Lin, L.-S. & Ho, T.-H.D. (1987) Plant Mol. Biol. **8**, 13-22.

Nomura, T., Kono, Y. & Akazawa, T. (1969) Plant Physiol. **44**, 765-769.

Nomura, K., Mikami, B. & Morita, Y. (1986) J. Biochem. **100**, 1175-1183.

Nowak, J. (1977) Z. Pflanzenphysiol. Bd. **81**, 125-140.

Obata-Sasamoto, H. & Suzuki, H. (1979) Physiol. Plant. **45**, 320-324.

Ohad, I., Friedberg, I., Ne´eman, Z. & Schramm, M. (1971) Plant Physiol. **47**, 465-477.

Okamoto, K. & Akazawa, T. (1980) Plant Physiol. **65**, 81-84.

Okayama, H. & Berg, P. (1982) Mol. Cell. Biol. **2**, 161-170.

Okita, T.W. & Preiss, J. (1980) Plant Physiol. **66**, 870-876.

Okita, T.W., Greenberg, E., Kuhn, D.N. & Preiss, J. (1979) Plant Physiol. **64**, 187-192.

Okita, T.W., Nakata, P.A., Anderson, J.M., Sowokinos, J., Morell, M. & Preiss, J. (1990) Plant Physiol. **93**, 785-790.

Ozbun, J.L., Hawker, J.S. & Preiss, J. (1971a) Biochem. Biophys. Res. Commun. **43**, 631-636.

Ozbun, J.L., Hawker, J.S. & Preiss, J. (1971b) Plant Physiol. **48**, 765-769.

Ozbun, J.L., Hawker, J.S. & Preiss, J. (1972) Biochem. J. **126**, 953-963.

Ozbun, J.L., Hawker, J.S., Greenberg, E., Lammel, C., Preiss, J. & Lee, E.Y.C. (1973) Plant Physiol. **51**, 1-5.

Paiva, E., Lister, R.M. & Park, W.D. (1983) Plant Physiol. **71**, 161-168.

Palmer, C.E. & Smith, O. (1969) Nature **221**, 279-280.

Palmer, J.K. (1971) in The Biochemistry of Fruits and their Products (Hulme, A.C., ed) Vol.2, pp 65-105, Academic Press, London, New York.

Palmiano, E.P. & Juliano, B.O. (1972) Plant Physiol. **49**, 751-756.

Park, W. (1983) Plant Mol. Biol. Reporter **1**, 61-68.

Parr, D.R. & Edelman, J. (1975) Planta **127**, 111-119.

Peat, S., Whelan, W.J. & Rees, W.R. (1956) J. Chem. Soc. Part 20, 44.

Peavey, D.G., Steup, M. & Gibbs, M. (1977) Plant Physiol. **60**, 305-308.

Peña-Cortés, H., Sánchez-Serrano, J.J., Mertens, R., Willmitzer, L. & Prat, S. (1989) Proc. Natl. Acad. Sci. **86**, 9851-9855.

Perdon, A.A., Del Rosario, E.J. & Juliano, B.O. (1975) Phytochem. **14**, 949-951.

Pikaard, C.S., Mignery, G.A., Ma, D.P., Stark, V.J. & Park, W.D. (1986) Nucleic Acids Res. **14**, 5564-5566.

Pikaard, C.S., Brusca, J.S., Hannapel, D.J. & Park, W.D. (1987) Nucleic Acids Res. **15**, 1979-1994.

Porter, H.K., Martin, R.V. & Bird, I.F. (1959) J. Exp. Bot. **10**, 264-276.

Portis, A.R. Jr. (1982) Plant Physiol. **70**, 393-396.

Prat, S., Frommer, W.B., Höfgen, R., Keil, M., Koßmann, J., Köster-Töpfer, M., Li, X.J., Müller, B., Peña-Cortés, H., Rocha-Sosa, M., Sánchez-Serrano, J.J., Sonnewald, U. & Willmitzer, L. (1990) FEBS Lett. **268**, 334-338.

Preiss, J. (1988) in The Biochemistry of Plants (Preiss, J., ed) Vol. 14, pp 181-254, Academic Press, New York.

Preiss, J. (1982) Annu. Rev. Plant Physiol. **33**, 431-454.

Preiss, J. & Boyer, C.D. (1980) in Mechanisms of Polysaccharide Polymerisation and Depolymerisation (Marshall, J.J., ed) Academic Press, New York.

Preiss, J. & Levi, C. (1980) in The Biochemistry of Plants (Preiss, J., ed) Vol. 3, pp 371-423, Academic Press, New York.

Preiss, J., Ghosh, H.P. & Wittkop, J. (1967) in Biochemistry of Chloroplasts (Goodwin, T.W., ed.) Vol 2, pp 131-153, Academic Press, London New York.

Preiss, J., Okita, T.W. & Greenberg, E. (1980) Plant Physiol. **66**, 864-869.

Preiss, J., MacDonald, F.D., Singh, B.K., Robinson,N. & McNamara,K. (1985) in "Proc. Int. Conf. on New Approaches to Research on Cereal Carbohydrates" (Munck, L. & Hill, R., eds) pp 1-11, Elsevier Science Publishers, Amsterdam.

Preiss, J., Bloom, M., Morell, M., Knowles, V.I., Plaxton, W.C., Okita, T.W., Larsen, R., Harmon, A.C. & Putnam-Evans, C. (1987) in Tailoring Genes for Crop Improvement (Bruening, G., Harada, J., Kosuge, T. & Hollaender, A., eds) pp 133-152, Plenum Publishing Corp., New York.

Quarrie, S.A. (1982) Plant Cell Environ. **5**, 23-26.

Racunsen, D. (1986) Can. J. Bot. **64**, 2104-2106.

Racunsen, D. & Foote, M. (1980) J. Food. Biochem. **4**, 43-52.

Recondo, E. & Leloir, L.F. (1961) Biochem. Biophys. Res. Commun. **6**, 85-88.

Ribereau-Gayon, G. & Preiss, J. (1971) Methods Enzymol. **23**, 618-624.

Richardson, M. (1977) Phytochem. **16**, 159-169.

Roberts, E., Kutchan, T. & Kolattukudy, P.E. (1988) Plant Mol. Biol. **11**, 15-26.

Robinson, N.L. & Preiss, J. (1987) Plant Physiol. **85**, 360-364.

Robinson, S.P & Walker, D.A. (1979) FEBS Lett. **107**, 295-299.

Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratman, M., Schell, J. & Willmitzer,

L. (1989) EMBO J. 8, 23-29.

Rogers, J.C. (1985) J. Biol. Chem. **260**, 3731-3738.

Rogers, J.C. & Millman, C. (1984) J. Biol. Chem. **259**, 12234-12240.

Rosahl, S., Eckes, P., Schell, J. & Willmitzer, L. (1986a) Mol. Gen. Genet. **202**, 368-373.

Rosahl, S., Schmidt, R., Schell. J. & Willmitzer, L. (1986b) Mol. Gen. Genet. **203**, 214-220.

Rosahl, S., Schell, J. & Willmitzer, L. (1987) EMBO J. **6**, 1155-1159.

Roswell, E.V. & Goad, L.J. (1964) Biochem. J. **90**, 12.

Ryan, C.A. (1977) Annu. Rev. Plant Physiol. **24**, 173-196.

Salanoubat, M. & Belliard, G. (1987) Gene **60**, 47-56.

Salanoubat, M. & Belliard, G. (1989) Gene **84**, 181-185.

Sánchez-Serrano,J.J., Keil, M., O'Connor, A., Schell, J. & Willmitzer, L. (1987) EMBO J. **6**, 303-306.

Sanwal, G.G., Greenberg, E., Hardie, J., Cameron, E.C. & Preiss, J. (1968) Plant Physiol. **43**, 417-427.

Sargeant, J.G. (1979) in Recent Advances in the Biochemistry of Cereals (Laidman, D.L. & Wyn Jones, R.G., eds) pp 339-343, Academic Press, New York.

Sasaki, T. & Kainuma, K. (1980) Biochem. J. **189**, 381-383.

Sato, T., Watanabe, A. & Imaseki, H. (1978) Plant Cell Physiol. **19**, 609-616.

Schneider, E.M., Becker, J.-U. & Volkmann, D. (1981) Planta **151**, 124-134.

Schulman, A.H. & Ahokas, H. (1990) Physiol. Plant. **78**, 583-589.

Schwartz, D. & Echt, C.S. (1982) Mol. Gen. Genet. **187**, 410-413.

Shantha, H.S. & Siddappa, G.S. (1970) J. Food Sci. **35**, 74-77

Shimomura, S., Nagai, M. & Fukui, T. (1982) J. Biochem. **91**, 703-717.

Shure, M., Wessler, S. & Federoff, N. (1983) Cell **35**, 225-233.

Sicher, R.C. & Kremer, D.F. (1984) Plant Physiol. **76**, 910-912.

Sicher, R.C., Baysdorfer, C. & Kremer, D.F. (1987) Plant Physiol. **83**, 768-771.

Singh, B. & Preiss, J. (1985) Plant Physiol. **79**, 34-40.

Smith, A.M. (1990) Planta **182**, 599-604.

Sonnewald, U., Studer, D., Rocha-Sosa, M. & Willmitzer, L. (1989a) Planta **178**, 176-183.

Sonnewald, U., Sturm, A., Chrispeels, M.J. & Willmitzer, L. (1989b) Planta **179**, 171-180.

Sowokinos, J.R. (1976) Plant Physiol. **57**, 63-68.

Sowokinos, J.R. & Preiss, J. (1982) Plant Physiol. **69**, 1459-1466.

Sowokinos, J.R., Lulai, E.C. & Knoper, J.A. (1985) Plant Physiol. **78**, 489-494.

Sprague, G.F., Brimhall, B. & Hixon, R.M. (1943) J. Am. Soc. Agron. **35**, 817-822.

Stanford, A., Bevan, M. & Northcote, D. (1989) Mol. Gen. Genet. **215**, 200-208.

Steikema, W.J., Heidekamp, F., Dirkse, W.G., van Beckum, J., deHaan, P., ten Bosca, C. & Louwerse, J.D. (1988) Plant Mol. Biol. **11**, 255-269.

Steup, M. & Latzko, E. (1979) Planta **145**, 69-75.

Steup, M. & Schächtele, C. (1981) Planta **153**, 351-361.

Steup, M., Robeneck, H. & Melkonian, M. (1983) Planta **158**, 428-436.

Steup, M., Conrads, J. & VanBerkel, J. (1987) in Progress in Photosynthesis Research

Stitt, M. (1984) in Storage Carbohydrates in Vascular Plants (Lewis, D.H., ed) pp 205-229, Cambridge Univ. Press, Cambridge.

Stitt, M. (1987) Plant Physiol. **84**, 201-204.

Stitt, M. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**, 153-185.

Stitt, M. & apRees, T. (1980) Biochim. Biophys. Acta **627**, 131-143.

Stitt, M. & Heldt, H.W. (1981) Plant Physiol. **68**, 755-761.

Stitt, M. & Steup, M. (1985) in Encyclopedia of Plant Physiology NS (Douce, R. & Day, D.A., eds) Vol. 18, pp 347-389, Springer-Verlag, Berlin, Hiedelberg, New York, Tokyo.

Stitt, M., Wirtz, W. & Heldt, H.W. (1980) Biochim. Biophys. Acta **593**, 85-102.

Stitt, M., Herzog, B. & Heldt, H.W. (1984a) Plant Physiol. **75**, 548-553.

Stitt, M., Kurzel, B. & Heldt, H.W. (1984b) Plant Physiol. **75**, 554-560.

Stitt, M., Huber, S. & Kerr, P. (1987) in The Biochemistry of Plants (Hatch, M.D. &

Boardman, N.K., eds) Vol. 10, pp 327-409, Academic Press, New York.

Struik, P.C., Boon, E.J. & Vreugdenhil, D. (1987) Plant Physiol. **84**, 214-217.

Suh, S.-G., Peterson, J.E., Steikema, W.J. & Hannapel, D.J. (1990) Plant Physiol. **94**, 40-45.

Sun, Z. & Henson, C.A. (1990) Plant Physiol. **94**, 320-327.

Svendsen, I., Hejgaard, J. & Mundy, J. (1986) Carlsberg Res. Commun. **51**, 43-50.

Takahashi, M. & Shimomura, T. (1968) Agric. Biol. Chem. **32**, 923-928.

Takahashi, N., Shimomura, R. & Chiba, S. (1971) Agric. Biol. Chem. **35**, 2015-2024.

Thoma, J.A., Spradlin, J.E., Dygert, S. (1971) in The Enzymes, 3rd edition (Boyer, P.D., ed) Vol. 5, pp115-189, Academic Press, New York.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. & Ryan, C.A. (1987) Proc. Natl. Acad. Sci. **84**, 744-748.

Toda, H., Kondo, K. & Narita, K. (1982) Proc. Jpn. Acad. **58**, 208-212.

Tomura, H. & Koshiba, T. (1985) Plant Physiol. **79**, 939-942.

Trewavas, A.J. (1982) Physiol. Plant. **55**, 60-72.

Tsai, C.Y. & Nelson, O.E. (1966) Science **151**, 341-343.

Tsai, C.Y. & Nelson, O.E. (1969) Plant Physiol. **44**, 159-167.

Tsai, C.Y. (1974) Biochem. Genet. **11**, 83-96.

Tsai, C.Y. & Nelson, O.E. (1968) Plant Physiol. **43**, 103-112.

Turner, J.F. (1969) Aust. J. Biol. Sci. **22**, 1145-1151.

Twell, D. & Ooms, G. (1987) Plant Mol. Biol. **9**, 365-375.

Twell, D. & Ooms, G. (1988) Mol. Gen. Genet. **212**, 325-336.

Tyson, R. H. & ap Rees, T. (1988) Planta **175**, 33-38.

van Stevinick, R.F.M (1975) Annu. Rev. Plant Physiol. **26**, 237-258.

Visser, R.G.F. (1989) Ph.D. thesis, Rijksuniversiteit Groningen.

Visser, R.G.F., Hergersberg, M., Van Der Liej, F.R., Jacobsen, E., Witholt, B. & Feenstra, W.F. (1989) Plant Sci. **64**, 185-192.

Vreugdenhil, D. & Struik, P.C. (1989) Physiol. Plant. **75**, 525-531.

Wareing, P.F. (1982) in Chemical Manipulation of Crop Growth and Development

(MacLaren, J.S., ed) pp 129-138, Butterworth, London.

Wareing, P.F. & Jennings, A.M.V. (1980) in Plant Growth Substances (Skoog, F., ed) pp 293-300, Springer-Verlag, Berlin.

Wenzler, H., Mignery, G., Fisher, L. & Park, W. (1989a) Plant Mol. Biol. **12**, 41-50.

Wenzler, H., Mignery, G., Fisher, L. & Park, W. (1989b) Plant Mol. Biol. **13**, 347-354.

Weselake, R.J. & MacGregor, A.W. (1985) Cereal Chem. **62**, 120-123.

Weselake, R.J., MacGregor, A.W. & Hill, R.D. (1983) Plant Physiol. **72**, 809-812.

Willemot, C. & Stumpf, P.K. (1967) Plant Physiol. **42**, 391-397.

Woods, D. (1984) Focus **6** (3), 1-2.

Yamada, J. (1981a) Carbohydr. Res. **90**, 153-157.

Yamada, J. (1981b) Agric. Biol. Chem. **45**, 1013-1015.

Yamada, J. (1981c) Agric, Biol. Chem. **45**, 747-750.

Yamasaki, Y. & Kono, H. (1985) Agric. Biol. Chem. **49**, 849-850.

Yamasaki, Y. & Kono, H. (1989) Phytochem. **28**, 2583-2585.

Yamasaki, Y. & Suzuki, Y. (1980) Planta **148**, 354-361.

Yellowlees, D. (1980) Carbohydr. Res. **83**, 109-118.

Yoshihara, T., Omer, E.A., Shibata, H., Koshimo, H., Sakamara, S., Koda, Y., Kikuta, Y. & Okazawa, Y. (1988) Abstract 13th International Conference on Plant Growth Substances, Calgary.

Ziegler, P. (1988) Plant Physiol. **86**, 659-666.

Ziegler, P. & Beck, C.D. (1986) Plant Physiol. **82**, 1119-1121.

Zwar, J.A. & Hooley, R. (1986) Plant Physiol. **80**, 459-463.