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Molecular cloning of protein phosphates and protein kinase genes from crop plants

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Molecular Cloning of Protein Phosphatase and Protein Kinase Genes from Crop Plants

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by

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ABSTRACT

Protein kinases and protein phosphatases are two very large enzyme families functioning in many important cellular processes such as regulation of cell cycle, signal transduction, self-incompatibility and disease resistance in plants. Some members of these two families have been cloned and subjected to detailed studies, but the majority remains unidentified, especially in plants. Barley and maize are two important crops in the world and also excellent experimental organisms particularly in the area of molecular genetics. Genes encoding protein kinase have been cloned from maize and barley, however, genes encoding protein phosphatase have not been cloned from barley. The signal transduction pathways involving the cloned protein kinases and protein phosphatases in maize and barley are not known. Since the amino acid sequences in the catalytic domains of protein kinase and protein phosphatase is conserved within eukaryotes, our approach consisted of using homology based screening to isolate novel protein kinases from maize cDNA library, and protein phosphatases from barley genomic library.

Three degenerate oligonucleotide primers were designed, PPS2C1, PPS2A2 against the conserved catalytic domains of serine-threonine phosphatase PP-2C, and PP-1/PP-2A respectively, and PPY1 against the conserved active-site region within tyrosine phosphatase. The screening of ~160,000 recombinants, using low stringency

hybridization, identified five putative clones for serine-threonine phosphatase but none for tyrosine phosphatase.

*Eco*R I genomic inserts of the isolated clones were excised from EMBL4 λ vector, and the probes localized to the *Eco*R I fragments resulting from internal sites. ~5.5 kb fragment of clone 1-C-2-2-1 (PP-2C), ~2 kb fragment of clone 3-C-1-3-1 (PP-2C), ~3.2 kb fragment of clone 11-A-c-1-1 (PP-1/PP-2A), and ~7.2 kb fragments of the clones 14-A-b-1-3 (PP-1/PP-2A) and 10-A-a-2 (PP-1/PP-2A) were subcloned into pGEM 7Zf (+).

The subclones were restriction mapped, and the fragments screened with the probes. ~0.8 kb *Eco*R I/*Sph* I fragment of the clone 3-C-1-3-1, ~1.2 kb *Xba* I/*Eco*R I fragment of the clone 10-A-a-2, ~1.2 kb *Hind* III/*Eco*R I fragment of the clone 14-A-b-1-3, and ~1.5 kb *Nsi* I/*Eco*R I fragment of the clone 11-A-c-1-1 were identified. *Xba* I/*Eco*R I deletion subclones 14-A-b-1-3 and 10-A-a-2 were sequenced but no readable sequence was obtained. The original *Eco*R I subclones 3-C-1-3-1 and 11-A-c-1-1 were sequenced from either end and a sequence of 201 bp and 276 bp (forward primer), and a sequence of 195 bp and 312 bp (reverse primer) obtained. These only covered small portions of the two clones and the translation in all six reading frames could not identify the characteristic sequences of serine-threonine phosphatases.

A maize cDNA library was screened previously with a PCR amplified 0.5 kb probe (Zhang and Pryor; unpublished results) corresponding to the catalytic subdomain of a known protein kinase gene *Pto* from tomato (Martin *et al* 1993). The heterologous screening identified a 0.8 kb fragment (McPk2.1) which was subsequently cloned in the *Eco*R I/*Xho* I restriction endonuclease sites on the Bluescript SK^- plasmid vector.

The 832 bp DNA sequence of McPk2.1 insert was determined. The 612 bp open reading frame encodes a 204 amino acid protein. The ORF contains seven subdomains characteristic of protein kinases. The absence of subdomains I to IV indicates an incomplete cDNA. The remaining transcript contains all the invariant amino acid residues characteristic of protein kinases. In addition, the transcript contains sequences indicative of serine-threonine specificity that lie in subdomains VIb (DLKPEN), and VIII (G[T/S]XX[Y/F]XAPE). McPk2.1 protein is 64% identical to Pto.

DECLARATION

I certify that this thesis reports an original research project, and does not contain any material which has been accepted for the award of any other degree or diploma in any other institution and no material previously published or written by another person, except where due reference is made in the text.

TRIPTY A. HIRANI

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANGIS	Australian National Genomic Information Service
ATP	adenosine triphosphate
bp	base pairs
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CaMK	calcium-/calmodulin-dependent protein kinase
cAMP-PK	cyclic AMP-dependent protein kinase
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CDPK	calmodulin-like domain protein kinase
CKII	casein kinase II
Ci	Curie
C-terminal	carboxyl-terminal
Co^{2+}	cobalt ion
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
dT	deoxythymidine
EB	ethidium bromide
G2	period between DNA synthesis and mitosis
GMP	guanidine monophosphate
GSK	glycogen synthase kinase
GTP	guanidine triphosphate
H_2O	water
K^+	potassium ion
kb	kilobase pairs
KDa	kilodalton
KV	kilovolt
LB	liquid broth

M	molar
MAPK	mitogen-activated protein kinase
mmole	milli-mole
Mg^{2+}	magnesium ion
Mn2+	manganese ion
msec	milli-second
N-terminal	amino-terminal
OD	optical density
P	phosphate
PCR	polymerase chain reaction
PK	protein kinase
PP	serine-threonine phosphatase
PTK	protein tyrosine kinase
RLK	receptor-like kinase
rpm	rotations per minute
SNF	sucrose non-fermenting
Tyr	tyrosine
UV	ultraviolet
V	volt
α	alpha
β	betta
γ	gamma
μ	micro
Ω	omega

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Chapter 1: Literature Review

1.0 INTRODUCTION

Regulatory proteins and enzymes are often subject to reversible phosphorylation, which can lead to their activation or inactivation. The forward reaction (phosphorylation) is catalysed by protein kinases where, most often, ATP, and sometimes, ADP or GTP may be the donor for the phosphate group. The reverse reaction (dephosphorylation), catalysed by protein phosphatases, is a hydrolysis reaction that yields orthophosphate (Pi) (Budde and Chollet 1988) (Figure 1.1).

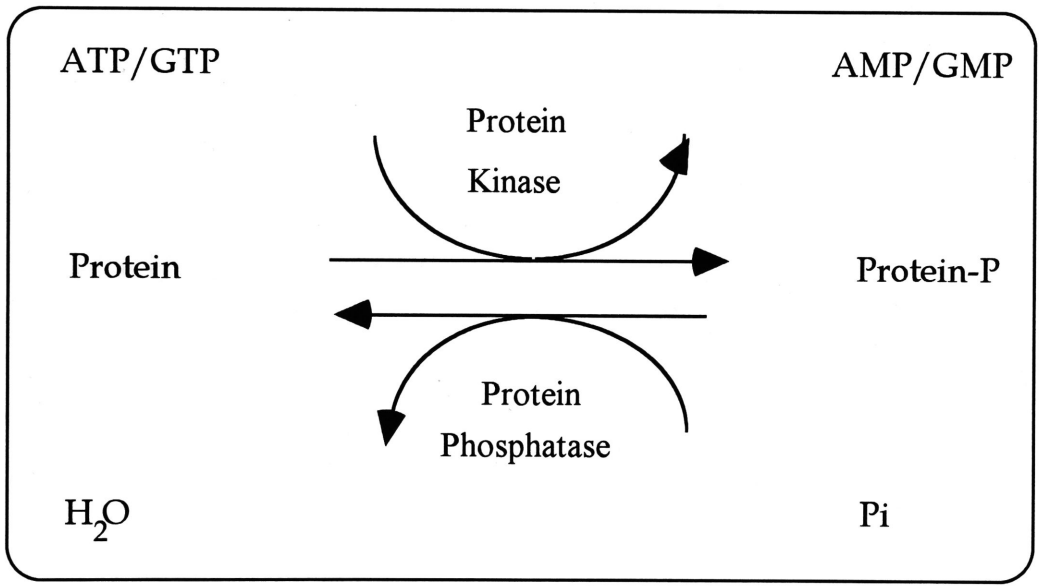


Figure 1.1: Reversible Phosphorylation

Many studies have been directed towards the forward reaction and have led to the identification of protein kinases, primarily from yeasts and animals and a few from plants. Clarification of their physiological roles shows kinases to be highly specific for their substrates where specificity is regulated by a number of second messengers such as cyclic AMP (cyclic AMP-dependent protein kinase), cyclic GMP (cyclic GMP-dependent protein kinase), Ca^{2+} (calcium/calmodulin-dependent protein kinase), and diacylglycerol (protein kinase C) (Cohen *et al* 1990).

Work on protein phosphatases, however, is fairly recent and, as is the case with kinases, has concentrated on yeasts and animals. Biochemical studies have

conventionally been used for their identification, but, more recently, the use of molecular biology has aided in the cloning and sequencing of phosphatase genes from lower and higher eukaryotes. Whereas the biochemical approach can only differentiate among various classes within the phosphatase family, the molecular approach is more specific because it can identify isoforms within a class.

A large number of kinase and phosphatase genes are present in eukaryotic genomes. It is estimated that the human genome has as many as 2000 genes coding for protein kinases, and 1000 genes for protein phosphatases (Hunter 1995). It is possible that the large gene pool contains pseudogenes and is functionally redundant, but this would need to be established through molecular and biochemical studies.

1.1 PROTEIN PHOSPHATASES

Protein phosphatases are divided into two groups based on the substrates they dephosphorylate ie. tyrosine type, and serine-threonine type.

Tyrosine phosphatases show no similarity, in their amino acid sequence, to the serine-threonine phosphatases. They may be cytosolic or receptor-like. The first gene was sequenced in 1988 (Pot and Dixon 1992) and, since then, at least 130 genes have been sequenced from animals and yeast (Genbank database via ANGIS; January 31, 1996). A tyrosine phosphatase gene has been cloned in the unicellular green alga *Chlamydomonas eugametos* (Haring *et al* 1995). However, tyrosine phosphatase has not yet been cloned from higher plants, although it has been identified biochemically in pea extracts (Guo and Roux 1995). The functions of tyrosine phosphatases range from cellular differentiation to receptor signaling to cell cycle regulation (for a review see Brady-Kalnay and Tonks 1994).

In the following section, I will focus on serine-threonine phosphatase family, especially in plants.

1.1.1 Serine-Threonine Phosphatases

The serine-threonine phosphatases (PP) identified for almost all the phosphatases studied are divided into four groups according to the four catalytic subunits (reviewed in Cohen 1989): PP-1, PP-2A, PP-2B and PP-2C. Their classification is based on the biochemical properties of their catalytic subunits, of which the most commonly cited are:

- their ability to dephosphorylate the α or the β subunit of the enzyme phosphorylase kinase which functions in glycogen metabolism;
- their degree of sensitivity to two small heat- and acid-stable proteins viz: inhibitor-1 (I-1), and inhibitor-2 (I-2);
- their degree of sensitivity to okadaic acid (a marine sponge toxin); and
- their dependence on divalent cations.

Mammalian PP-1 dephosphorylates the β subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of both I-1 and I-2. PP-2 dephosphorylates the α subunit of phosphorylase kinase and is insensitive to I-1 and I-2 (Cohen 1989).

Depending on its requirement for Ca^{2+} and Mg^{2+}, PP-2 is further classified into three enzymes, viz: 2A, 2B and 2C. PP-2B and PP-2C have absolute requirements for Ca^{2+} and Mg^{2+} respectively while PP-2A is not sensitive to these cations (Cohen 1989). However, its activity may be regulated by Co^{2+} (Cai *et al* 1995).

PP-1 may be inhibited by okadaic acid with an inhibitory concentration (IC_{50}) of 10 nanomoles (Cohen and Cohen 1989; Mackintosh *et a l* 1991), or of 100 nanomoles

(Bialojan and Takai 1988; Ishihara *et al* 1989). PP-2A is highly sensitive to okadaic acid, with an IC_{50} of 1 nanomole (Bialojan and Takai 1988; Ishihara *et al* 1989). PP-2B is more than 1000-fold less sensitive, and PP-2C shows a complete resistance to okadaic acid (Bialojan and Takai 1988; Mackintosh *et al* 1991). The response of serine-threonine phosphatases to the mammalian inhibitors and to okadaic acid is conserved in organisms as diverse as mammals, fruit flies, starfish, yeast and higher plants (Cohen and Cohen 1989). This indicates an evolutionary functional conservation (Cohen 1989) that can be employed for identifying phosphatases from different sources. The exception is *Paramecium* in which PP-2A-like activity is resistant to okadaic acid (Cohen and Cohen 1989).

Okadaic acid is most commonly used for the in vitro study of phosphatases. However, the inhibitory response it generates generally depends on the concentrations of PP-1 and PP-2A in the assay. Therefore, okadaic acid can not be considered an absolute indicator of either one of the two phosphatases (Carter *et al* 1990; Huber and Huber 1990).

In order to substantiate their results, many laboratories have used micryocystin LR – a hepatotoxin from cyanobacteria (Mackintosh *et al* 1991; Siegl *et al* 1990), and calyculin A (Li *et al* 1994; Smith *et al* 1994; Takeda *et al* 1994). Of these, calyculin A inhibits PP-1 and PP-2A with almost equal potency (the IC_{50} for PP-1 is 2 nanomoles, and the IC_{50} for PP-2A is 0.5–1 nanomole) and it is a stronger inhibitor of PP-1 than is okadaic acid (Ishihara *et al* 1989).

Matsuzawa *et al* (1994) describe a novel probe for distinguishing the activity of PP-2A in crude cell extracts. The probe, thyrsiferyl 23-acetate, is a cytotoxic compound derived from marine red alga, and it inhibits PP-2A with an IC_{50} of 4–16 μM but does not affect other phosphatases up to 1 mM.

PP-2B is inhibited by two immunophilin-immunosuppressant complexes viz: cyclophilin-cyclosporin A, and FK 506 - FK 506-binding protein (Liu *et al* 1991; Schreiber 1992; Luan *et al* 1993). These complexes may also alter the substrate specificity of PP-2B; one study indicated that PP-2B is activated towards p-nitro phenyl phosphate in the presence of immunophilin-immunosuppressant complexes (Liu *et al* 1991).

1.1.2 Identification by Molecular Cloning

Several animal and plant serine-threonine phosphatases have been identified by amino acid sequences after cDNA cloning and sequencing. In all cases, the plant phosphatases have been found to be homologous in their amino acid sequence to animals, indicating an evolutionary conservation. So far 26 genes have been identified in plants, including two genes for PP-2C, four genes for the 65 KDa, and one gene for the 55 KDa regulatory units of PP-2A respectively (Arino *et al* 1993; Casamayor *et al* 1994; Evans *et al* 1994; Ferreira *et al* 1993; Leung *et al* 1994; Mackintosh *et al* 1990; Meyer *et al* 1994; Nitschke *et al* 1992; Pay *et al* 1994; Pirck *et al* 1993; Rundle and Nasrallah 1992; Rundle *et al* 1995; Slabas *et al* 1994; Smith and Walker 1991; Smith and Walker 1993; Stone *et al* 1994) (Table 1.1). PP-2B has not been cloned from plants although its presence has been established through biochemical studies (Luan *et al* 1993). At least 116 genes encoding serine-threonine phosphatases have been sequenced in animals and yeast (Genbank database via ANGIS; January 31, 1996).

PP-1, PP-2A, and PP-2B share substantial homology in the amino-acid sequence of their catalytic domains. Their substrate specificity, however, does not overlap since it is determined by the regulatory units that bind to it. Comparison of the amino acid sequences among the mammalian serine-threonine phosphatases reveals that PP-1 (~37 KDa) and PP-2A (~36 KDa) are 50% identical to each other in their catalytic domain. This indicates that they belong to the same gene family (Cohen *et al* 1990). PP-2B (~61 KDa) is a distantly related member of this family; its catalytic domain is 40% identical

to PP-1/ PP-2A (Cohen 1991). However, no similarity is seen between PP-2C (~42–65 KDa) and PP-1/ PP-2A/ PP-2B.

The molecular cloning and sequencing of cDNAs have also identified isoforms for serine-threonine phosphatases in animals and plants. Animal isoforms have been cloned from mammalian brains, rabbit skeletal muscles and liver for the catalytic subunit of PP-2C and PP-2B (Cohen and Cohen 1989), from bovine adrenal cells, human, rat and rabbit liver for the catalytic subunit of PP-2A (Cohen 1989), and from porcine kidney, skeletal muscle (Hemmings *et al* 1990), *Drosophila* and human tissues (Evans *et al* 1994 and references therein) for the 65 KDa regulatory subunit of PP-2A. In plants, at least three isoforms for the 65 KDa regulatory subunit (Slabas *et al* 1994), and one isoform for the 55 KDa regulatory subunit of PP-2A have been identified (Rundle *et al* 1995).

In the case of animal serine-threonine phosphatases the α and β isoforms show a high degree of identity to each other. For instance, the α and β isoforms of human PP-2C are 76% identical to each other in their amino acid sequence. Similarly, the α isoform is 90% identical to the β isoform in the case of rabbit PP-1, 97% in rabbit PP-2A, and 82% in rat PP-2B (Mann *et al* 1992).

Plant	PP-1	PP-2A	PP-2C
Alfalfa	***pp1Ms*** Pay *et al* 1994	***pp2aMs*** Pirck *et al* 1993	
Arabidopsis thaliana	***TOPP1*** , ***TOPP2*** , ***TOPP3***, ***TOPP4***, ***TOPP5*** Smith and Walker 1993 ***PP1-At*** Nitschke *et al* 1992 ***PP1A-At1*** , ***PP1A-At2*** Ferreira *et al* 1993	***2A-1*** , ***2A-2***, ***2A-3*** ***AP-1*** Arino *et al* 1993 ***2A-4*** Casamayor *et al* 1994 ***AtB*** Rundle *et al* 1995 ***pDF1*** , ***pDF2*** , ***regA*** Slabas *et al* 1994	***ABI1*** Leung *et al* 1994; Meyer *et al* 1994 ***KAPP*** Stone *et al* 1994
Brassica napus	***PP-1*** Mackintosh *et al* 1990	***PP-2A*** Mackintosh *et al* 1990	
Brassica oleracea	***BoPP1*** Rundle and Nasrallah 1992		
Maize	***ZmPP1*** Smith and Walker 1991		
Pea		***pME1*** Evans *et al* 1994	

Table 1.1: List of Serine-Threonine Phosphatase Genes Cloned from Plants

Although research indicates that the isoforms are generally products of distinct genes, there is some evidence that they may also arise as a result of alternative splicing. Six different regulatory subunits of PP-2A have been described from studies in human, rabbit and *Xenopus* tissues (Hendrix *et al* 1993a; Hendrix *et al* 1993b). The subunits are of 65 KDa, 54 KDa, 55 KDa, 62 KDa, 72 KDa and 74 KDa. Recently, a 130 KDa protein

was identified in fetal brain libraries and found to have the same carboxyl terminal sequence as the 72 KDa protein, and an amino terminal extension not present in the 72 KDa protein, implying that the two transcripts arose from different promoters on the same gene, where the promoters were expressed in a tissue-specific manner (Hendrix *et al* 1993a). Similar results were noted for the PP-2B catalytic subunit in mammalian brain that had two forms, 58- and 59 KDa, generated by alternative splicing (Cohen and Cohen 1989). The physiological significance of these isoforms needs to be determined, and it remains to be established if they are functionally significant or redundant.

The use of PCR and genomic Southern studies have also identified multigene families for PP-1 and PP-2A in animals (Chen *et al* 1992), and in plants (Arino *et al* 1993; Smith and Walker 1993). PP-2B and PP-2C are not so abundant. The multigene families, identified in plants, by the latter technique have yet to be sequenced to determine their degree of sequence homology. To date, the largest multigene family for PP-1 in plants has been identified in *Arabidopsis* (Smith and Walker 1993). It comprises five distinct genes encoding the catalytic subunit. A multigene family for PP-1 has also been found in *Brassica oleracea* (Rundle and Nasrallah 1992), and in alfalfa (Pay *et al* 1994).

Similarly multigene families for both PP-2A catalytic and regulatory subunits have been identified in pea and *Brassica napus* (Evans *et al* 1994), and for the PP-2A catalytic subunit in *Arabidopsis* (Arino *et al* 1993; Casamayor *et al* 1994)), and alfalfa (Pirck *et al* 1993).

Amino acid sequences of the serine-threonine phosphatases are conserved between higher and lower eukaryotes. Such a comparison has identified several conserved domains, in the catalytic regions, for PP-1, PP-2A, and for PP-2C (Figure 1.2).

Eight domains are conserved in PP-1 and PP-2A, while three domains are conserved in PP-2C. The domains for PP-1 and PP-2A were determined by aligning amino acid

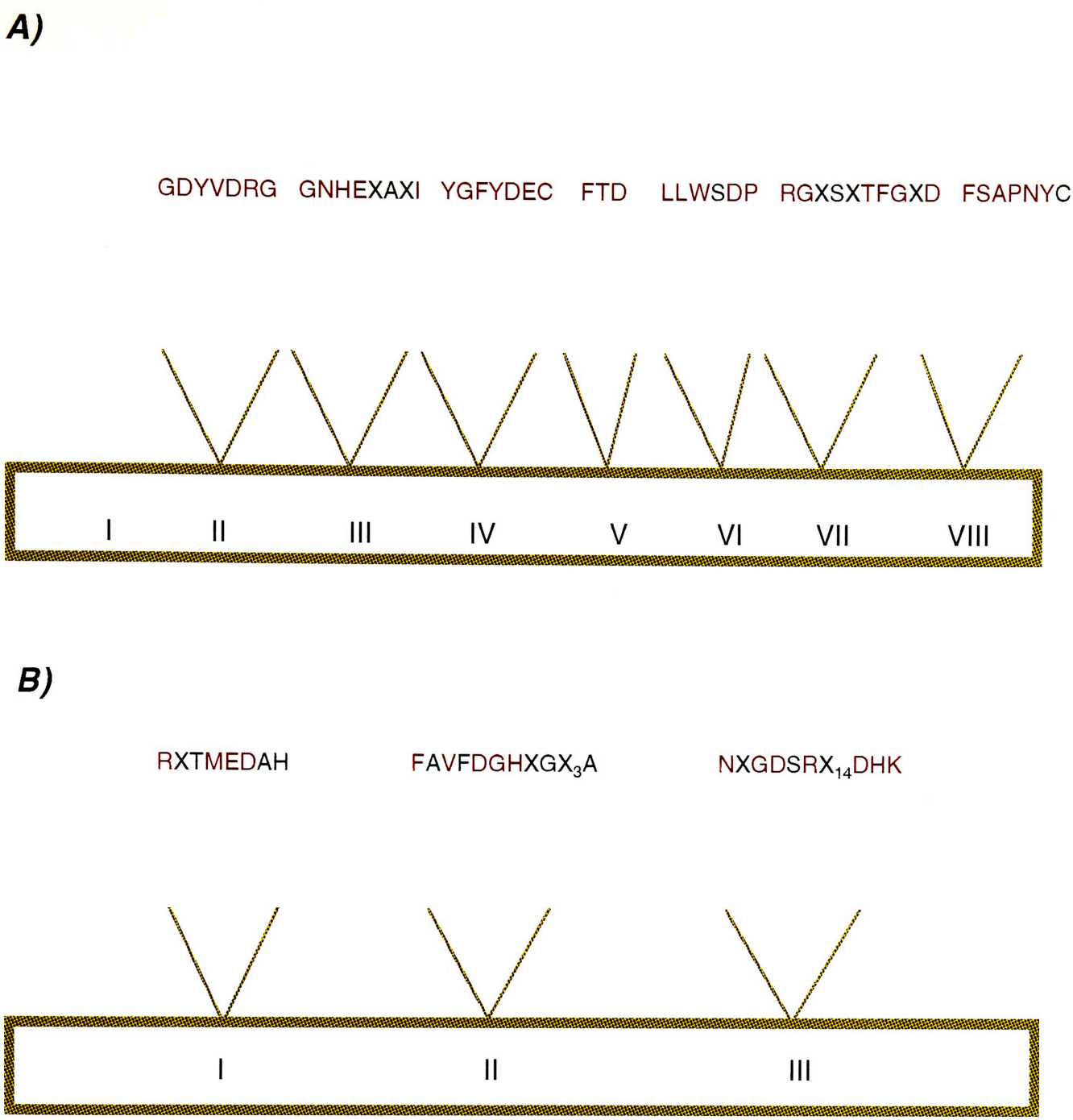


Figure 1.2: Conserved Amino Acid Sequence Domains in the Catalytic Regions of Serine-Threonine Phosphatases. A. *Conserved amino acid sequence domains of PP-1 and PP-2A.* ***B.*** *Conserved amino acid sequence domains of PP-2C (Adapted from Klumpp et al 1994). Identical residues are shown in red. The most conservative replacements are shown in green.* X_n *represents number of variable residues.*

sequences from plants (combined GenBank/Swissprot protein database, ANGIS, February 1995) and comparing them with PP-1α from rabbit skeletal muscle and liver, and PP-2Aα from rabbit skeletal muscle (Cohen 1991). Similarly, amino acid sequences for PP-2C from plants and animals were aligned (combined GenBank/Swissprot protein database, ANGIS, February 1995) and were further compared with the consensus sequence by Klump *et al* 1994. Amino acid sequences were aligned using the program PILEUP.

PP-1 has been conserved between mammals and *Aspergillus* even though the animal and the fungal kingdoms diverged a billion or so years ago (Cohen 1989). For instance, the amino acid sequences of PP-1 from mammals and *Drosophila* show an overall homology of 90%, while mammalian PP-1 is 80% identical to the corresponding enzymes in *Aspergillus* and yeast (Cohen *et al* 1990). In addition, an open reading frame (orf 221) in bacteriophage lambda encodes a Mn^{2+}-stimulated casein phosphatase which is 35% identical to PP-1/PP-2A and contains 22 identical and 19 conserved residues between residues 45 and 130 of PP-1α (Cohen and Cohen 1989), suggesting that these residues may be important for catalytic function of PP-1/PP-2A (Cohen and Cohen 1989). More specifically, regions very likely to be responsible for the catalytic activity have been identified as residues 63–67, 90–97, and 121–126 of PP-1α (Cohen *et al* 1990). There is no evidence that these phosphatases are present in *Escherichia coli* (Chen *et al* 1992; Mackintosh *et al* 1991), although this bacterium contains over 100 phosphoproteins (Mackintosh *et al* 1991).

It is suggested that PP-1 and PP-2A are the most conserved enzymes within eukaryotes: two catalytic subunits isolated from a *Brassica napus* cDNA library show 72% and 79% overall homology to rabbit muscle PP-1 and PP-2A respectively (Mackintosh *et al* 1990). A partial cDNA isolated from an early developing pea (*Pisum sativum* L) embryo library encodes a 65 KDa regulatory subunit of PP-2A that shows a 54.7% homology to the regulatory subunit of human PP-2A (Evans *et al* 1994) (see table 1.2 for similar

examples). Similarly, in *Drosophila*, three genes encoding the isoforms of PP-1 have been identified, two of which: PP1α_1 and PP1α_2 are 94% identical to the mammalian PP-1α while the third PP-1β is 94% identical to the mammalian PP-1β (Cohen *et al* 1990).

PP-2C is not so highly conserved, which indicates a functional diversity (Klumpp *et al* 1994). A comparison of the sequences in the conserved domains of PP-2C from rabbit, rat, *Arabidopsis*, yeast and *Leishmania* reveals an overall identity of 40% (Klumpp *et al* 1994), whereas an individual comparison of the complete sequence from *Arabidopsis* and rat reveals a 35% identity (Leung *et al* 1994).

1.1.3 Structure

In general, the mammalian PP-1, PP-2A and PP-2B are heterodimers consisting of catalytic and regulatory subunits, the latter controlling the activity and targeting of these molecules (Evans *et al* 1994). PP-2C (31–60 KDa) is the only serine-threonine phosphatase to exist in a monomeric form.

A catalytic unit may also be complexed with more than one regulatory subunit, for example dimeric and trimeric forms of PP-2A have been purified from rabbit and *Xenopus* tissues (Hendrix *et al* 1993b). In all cases, the core of PP-2A is composed of a 36 KDa catalytic unit and a 65 KDa regulatory unit (Hemmings *et al* 1990; Hendrix *et al* 1993a; Kamibayashi *et al* 1992). Recently, the 65 KDa subunit has been found in pea plants, indicating its conservation in higher plants (Evans *et al* 1994).

PP-1 has primarily been studied in muscle cells where it appears to exhibit five to ten times more activity when its catalytic subunit (37 KDa) is complexed to a 161 KDa glycogen-binding subunit that directs it to the sarcoplasmic reticulum and to the enzymes of glycogen metabolism (Cohen and Cohen 1989). In addition, its activity

Pair	Percentage amino acid identity	Type	Reference
Maize (*ZmPP1*): ***Arabidopsis*** (*TOPP1, TOPP5*)	80	**PP-1**	Smith and Walker 1993
Maize (*ZmPP1*): **Rabbit** (*PP-1*)	80	"	"
Alfalfa (*pp1Ms*): ***Arabidopsis*** (*TOPP3*)	85	**PP-1**	Pay *et al* 1994
Alfalfa (*pp1Ms*): **Rabbit** (*PP1Rb*)	73.5	"	"
Arabidopsis (*PP2A3*): ***Brassica*** (*PP-2A*)	95	**PP-2A**	Arino *et al* 1993
Arabidopsis (*PP2A3*): **Man** (*PP-2A*)	81	"	"
Arabidopsis (*ABI1*): **Rat** (*PP-2C*)	35	**PP-2C**	Leung *et al* 1994; Meyer *et al* 1994
Arabidopsis(*KAPP*): **Mouse** (*PP-2C*)	15	**PP-2C**	Stone *et al* 1994

Table 1.2: Percentage of Amino Acid Identity within the Conserved Domains of Catalytic Region of Plant and Animal Serine-Threonine Phosphatases

towards myosin is due to its being bound to another protein distinct from the glycogen-binding subunit (Cohen and Cohen 1989). Other regulatory subunits for animal and plant PP-1 have yet to be identified. Furthermore it has been suggested that the substrate specificity of PP-1 may be influenced by the variability in the catalytic subunit that flanks a conserved core of 300 amino acids (Smith and Walker 1993).

PP-2B or calcineurin (Leung 1994), is a Ca^{2+}-dependent calmodulin-stimulated enzyme that was first identified in bovine brain where it constitutes 1% of total brain protein (Cohen 1989). PP-2B is composed of two subunits: calcineurin B, a 15–19 KDa regulatory subunit with a calcium-binding site; and calcineurin A, a 57–61 KDa catalytic subunit with a calmodulin-binding site (Klee *et al* 1988; Liu *et al* 1991).

1.1.4 Physiological Roles

PP-1 and PP-2A appear to have broad substrate specificities, while PP-2B and PP-2C are highly specific. The broad substrate specificity of PP-2A could be accounted for by the choice of regulatory subunits that are available, and by the several isoforms of both the catalytic and regulatory subunits, the latter revealed by cDNA cloning and chromatographic analysis (Hendrix *et al* 1993a,b). This suggests that the substrate specificity of serine-threonine phosphatases, in general, may be determined by the regulatory subunit and the isoform. Recent evidence from the molecular cloning of plants indicates that serine-threonine phosphatases also have multifunctional domains that may participate in signal transduction pathways (Leung *et al* 1994; Meyer *et al* 1994; Stone *et al* 1994).

There is evidence from biochemical studies in plants of protein phosphatase cascades whereby one class of serine-threonine phosphatase is involved in regulating the activity of another class of serine-threonine phosphatase. For instance, the protein inhibitor of PP-1 found in mammals and discovered recently in the plant *Brassica oleraceae* (Rundle and Nasrallah 1992) is activated via phosphorylation by cAMP-dependent protein kinase, and is deactivated via dephosphorylation by the Ca^{2+}/calmodulin dependent PP-2B (Klee *et al* 1989; Rundle and Nasrallah 1992) (Figure 1.3). Hence the activity of PP-1 is indirectly regulated by PP-2B.

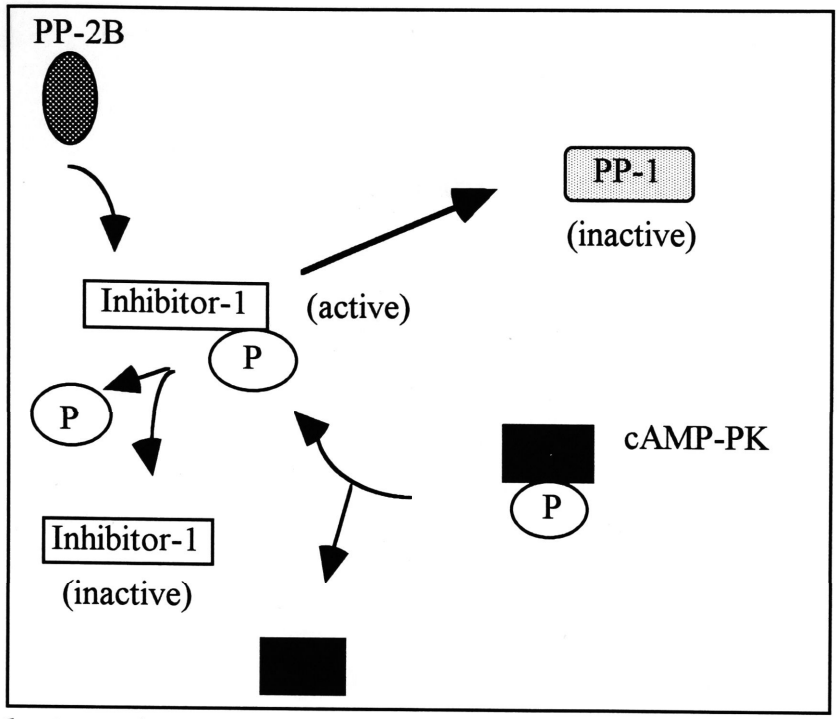


Figure 1.3 : Regulation of PP-1 Activity by PP-2B via cAMP-PK and Inhibitor-1. *In its phosphorylated state, Inhibitor-1 continues to hinder the activity of PP-1. Dephosphorylation of Inhibitor-1 by PP-2B inactivates Inhibitor-1 which can no longer inhibit PP-1.*

Serine-threonine phosphatases may be involved in regulating the enzymes of metabolic pathways, or regulating the signal transduction cascade. In addition, studies in animals and yeast have implicated them in the control of the cell cycle. In signal transduction studies and in cell cycle studies, the substrates of serine-threonine phosphatases have yet to be identified.

The physiological functions of PP-1 and PP-2A have been investigated using structurally different toxins that can easily penetrate eukaryotic cells and inhibit PP-1 and PP-2A activities. Though used to indicate in vivo activity, these toxins cannot distinguish reliably between the two classes (Huber and Huber 1990; Li *et al* 1994; Smith *et al* 1994), thereby requiring cell extracts or purified proteins to be treated with inhibitors (Carter *et al* 1990; Mackintosh *et al* 1991).

1.1.4.1 *The Cell Cycle*

Evidence for the role of PP-1 in the regulation of the cell cycle has come from the genetic studies of *Aspergillus nidulans* and *Schizosaccharomyces pombe* (reviewed in Cyert and Thorner 1989). Two genes have been identified to play a role in mitotic defects. These are the *bimG11* ("blocked in mitosis") (Doonan and Morris 1989), and the *dis2-11* ("defective in sister chromatid disjoining") (Cyert and Thorner 1989, and references therein).

The *bimG* gene product has 86% homology to the catalytic subunit of rabbit muscle PP-1 (Doonan and Morris 1989), while the *dis2* gene product is 82% homologous to the mammalian PP-1 (Cyert and Thorner 1989, and references therein). Mutations in both the genes cause defective chromosome separation. The two gene products therefore appear to play a role during mitosis.

Genetic studies in yeast have also revealed another gene called *bws1+* ("bypass of wee suppression") that is 81% homologous to the amino acid sequence of the catalytic subunit of mammalian PP-1α. This gene is thought to function in the initiation of mitosis (Booher and Beach 1989).

The role of plant PP-1 in cell cycle has been determined through complementation studies in yeast. PP-1-At, isolated from *Arabidopsis thaliana* has been shown to successfully complement a cold sensitive *dis2-11* cell cycle mutant of *Schizosaccharomyces pombe* (Nitschke *et al* 1992), while another serine-threonine phosphatase (PP-1A-At1) from the same plant restores the temperature sensitivity of a *Schizosaccharomyces pombe cdc25ts/wee1*-double mutant (Ferreira *et al* 1993). These results suggest that the plant serine-threonine phosphatase can interact with genes that regulate the fission yeast mitotic apparatus, and may therefore play a role in the plant cell cycle. Direct evidence however, comes from studies in the spiderwort plant *Tradescantia*, where the use of

serine-threonine phosphatase inhibitors implicates a protein phosphatase activity during metaphase and in the process that controls sister chromatid separation at the onset of anaphase (Wolniak and Larsen 1992).

It has also been proposed that PP-2A functions in the initiation of the cell cycle (Dunphy 1994, and references therein), and in the transition from metaphase to anaphase (Vandre and Wills 1992). In eukaryotic cells, the transition from G2 to M phase during the cell cycle is influenced by a protein kinase complex called the maturation promoting factor (MPF). MPF is a complex of two proteins called cyclin and p34 ^{cdc2}. A form of PP-2A (called INH) has been shown to negatively regulate this complex in *Xenopus* oocytes (Lee *et al* 1991). PP-2A has also been implicated in controlling the activities of cdc25 (a tyrosine phosphatase involved in activating MPF) and wee1 (a tyrosine kinase that is a negative inhibitor of MPF) (Dunphy 1994) (Figure 1.4).

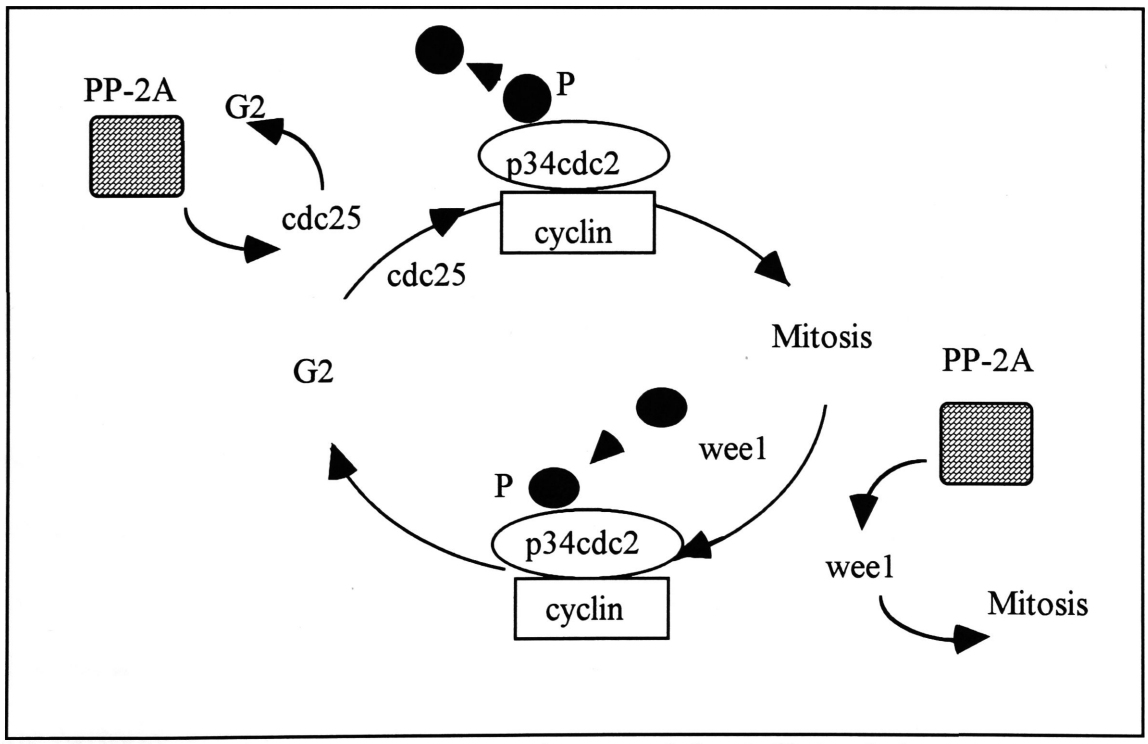


Figure 1.4 : Positive and Negative Regulation of the Cell Cycle by PP-2A in Animals

In addition, the in vivo analysis of the cell cycle of African trypanosomes suggests that PP-1/PP-2A may be involved in mitochondrial DNA division (Das *et al* 1994).

Furthermore, studies with neuronal and non-neuronal cells suggest that PP-2A may regulate cell cycle-dependent microtubule functions, such as karyokinesis and membrane transport (Sontag *et al* 1995).

1.1.4.2 Regulation of Metabolic Pathways

In vitro studies of skeletal muscles and liver extracts has demonstrated that PP-1, and PP-2A are involved in glycogen metabolism, muscle contractility, protein synthesis, cholestrol synthesis, fatty acid synthesis, glycolysis/gluconeogenesis and aromatic amino acid breakdown (Cohen 1989).

PP-2B has not been implicated in the regulation of metabolic pathways, but is thought to be involved in signal transduction (Cohen 1989) although its substrates have yet to be identified.

PP-2C functions in mammals as a 6-phosphofructo-1-kinase phosphatase, as the major protein phosphatase that dephosphorylates and inactivates the cyclic AMP-activated protein kinase, and, is involved in the differentiation of skeletal muscle cells. In the fungus *Blastocladiella emersonii,* PP-2C functions as an L-glutamate-fructose-6-phosphatase transaminase phosphatase (Klumpp *et al* 1994 and references therein).

Few plant metabolic enzymes have been identified as substrates for the endogenous serine-threonine phosphatases. For PP-2A, these include sucrose phosphate synthase (SPS) in spinach leaves (Huber and Huber 1990; Siegl *et al* 1990), quinate dehydrogenase (QDH) in carrot cells (Mackintosh *et al* 1991), and phosphoenol pyruvate carboxylase in *Bryophyllum fedtschenkoi* (a crassulacean acid metabolism plant) (Carter *et al* 1990). It has been shown that dephosphorylation activates SPS and QDH, while it inactivates phosphenol pyruvate carboxylase. It has also been proposed that PP-2C serves as quinate dehydrogenase phosphatase (Mackintosh *et al* 1991).

1.1.4.3 *Signal Transduction*

Studies with animal cells and yeasts indicates that PP-2B and PP-2C may be involved in the transcription of immediate early genes and in the osmoregulatory pathway, respectively.

For instance, in the neuronal and pancreatic cell lines, PP-2B is involved in the regulation of the calcium-dependent transcription of immediate early genes such as *c-fos*, *NF-AT*, *NGF1-A*, and *NGF1-B* (Enslen and Soderling 1994), as well as in the regulation of the cAMP-responsive element mediated gene transcription (Schwaninger *et al* 1993). In addition, PP-2B is involved in the Ca^{2+} stimulation of interleukin-2 expression in T-cells (Enslen and Soderling 1994 and references therein), although the exact site of action remains to be identified.

Similarly, genetic studies in yeasts indicate that the osmosensing signal transmitted via the mitogen activated protein kinase pathway is negatively regulated by PP-2C (Shiozaki and Russell 1995).

Takeda *et al* (1994) have shown that the sucrose-inducible expression of the genes for sporamin, β-amylase, and the small subunit of AGPase (ADP-Glc pyrophosphorylase) in the sweet potato depends on protein dephosphorylation by PP-1/PP-2A. Sporamin and β-amylase are two major storage proteins of the tuberous roots of the sweet potato, whereas AGPase is a key enzyme in starch biosynthesis. It has been postulated that the substrates for phosphatases may be the same as for protein kinases, and that their role may be to transduce carbohydrate metabolic signals to the nucleus.

In addition, the use of okadaic acid to demonstrate the efficient blockage of chlorophyll accumulation in etiolated maize leaves, as well as the suppression of two photosynthetic fusion genes, suggests that PP-1 may be involved as a positive regulator

in the transduction of light signals from photoreceptors to chloroplasts and nucleus (Sheen 1993).

Recent evidence from the use of phosphatase inhibitors as well as whole-cell patch-clamp technique indicates that PP-1, PP-2A and PP-2B are involved in regulating K^+ ion channels in plants. Using immunosuppressants cyclosporin A and FK506, as well as patch-clamp techniques, Luan *et al* (1993) have shown that a PP-2B activity is associated with the Ca^{2+} mediated inactivation of K^+ channels in guard cells of *Vicia faba* (fava bean). Work from the same laboratory in 1994 has indicated that PP-1 and PP-2A activities are also present in *Vicia faba* leaf extract, suggesting their involvement in regulating the voltage-dependant K^+ channel activities in guard cells and mesophyll cells (Li *et al* 1994).

Abscisic acid is a plant hormone that controls stomatal closure during desiccation, maintanance of seed dormancy and inhibition of plant growth at high concentrations (Giraudat 1995). The study of genetic mutants of *Arabidopsis thaliana,* and the subsequent molecular characterization of the locus *ABI1* (Abscisic acid insensitivity 1 gene) (Armstrong *et al* 1995; Leung *et al* 1994; Meyer *et al* 1994), has revealed that the gene product is a serine-threonine phosphatase with a putative calcium binding site at the amino terminal and a PP-2C sequence at the carboxyl terminal sequence. The fact that mutants of *ABI1* locus are not inhibited by high concentrations of abscisic acid, suggests that this Ca^{2+} modulated phosphatase may be involved in transducing signals from Ca^{2+} and abscisic acid, leading to the control of cell division.

Stone *et al* (1994), have isolated a serine-threonine phosphatase cDNA clone using the interactive cloning technique. This involved using a peptide probe derived from a serine-threonine receptor-like kinase (RLK5) from *Arabidopsis thaliana* to screen an expression library. The serine-threonine phosphatase identified was novel in that it had multifunctional domains viz: an amino terminal signal anchor, a kinase interaction (KI)

domain, and a type 2C serine-threonine phosphatase catalytic region, and was called kinase-associated protein phosphatase (KAPP). The KI domain could bind to RLK5 only when the latter was phosphorylated, suggesting that this binding may regulate the phosphatase activity, and that KAPP may act downstream of the receptor RLKs.

Smith *et al* (1994) have suggested that PP-1 and PP-2A have multiple functions in controlling root growth and development. This is based on their observation that okadaic acid and calyculin-A arrest root hair growth, affect cell shape and inhibit root growth rates in *Arabidopsis*. It is suggested that the serine-threonine phosphatases may control these functions directly through cell cycle regulation and microtubule reorganization, or via light and hormone inducible genes.

Other functions that have been attributed to plant serine-threonine phosphatases include a role in hypersensitive response. Both in vitro and in vivo studies on tobacco plants using okadaic acid and inhibitor-2 have demonstrated that the hypersensitive response of tobacco plants to tobacco mosaic virus infection requires PP-1 (Dunigan and Madlener 1995). In addition, work by Raz and Fluhr (1993) implicates the involvement of PP-1 and PP-2A in the ethylene-activated signal transduction pathway that leads to the production of pathogenesis-related proteins and a hypersensitive response. These results demonstrate that serine-threonine phosphorylation is associated with the hypersensitive response and, together with protein kinases, constitutes the defence mechanism. What remains to be seen is how these phosphatases are regulated and what their target proteins are.

1.2 PROTEIN KINASES

Like protein phosphatases, protein kinases are divided into two families ie: tyrosine type, and serine-threonine type. Whereas both types exist in animals, tyrosine kinases have not been found in plants yet.

The amino acid sequences of protein kinases have been conserved across different phyla. In addition there are eleven conserved regions within the catalytic domain of protein kinases. The domain tends to lie near the carboxyl end in most single subunit enzymes, and contains 250–300 amino acid residues showing alternate regions of high and low conservation (Hanks *et al* 1988) (Figure 1.5).

1.2.1 Plant Serine-Threonine Kinase Families and Signal Transduction

Since 1989 serine-threonine kinases have been cloned from several plants including *Arabidopsis thaliana*, bean, maize, rice, tobacco, tomato, pea, petunia, and two *Brassica* species (Stone and Walker 1995 and references therein). Some are ubiquitously found within eukaryotes while others are restricted to plants.

Based on the general classification of Hanks and Hunter (1995), plant serine-threonine kinases have been classified into five main groups (For a review see Stone and Walker 1995). These groups comprise of:

- the AGC group consisting of the cyclic nucleotide-dependent family (ex. PKA and PKG), the PKC family, and the ribosomal S6 Kinase family,
- the CaMK group, consisting of calcium/calmodulin-dependent kinase and the SNF1/AMP activated protein kinase,
- the CMGC group containing the CDK, the MAPK, GSK-3 and CKII families,
- the conventional PTK group, and
- the 'other' group containing both, protein kinases unique to plants as well as protein kinases widespread in eukaryotes.

SNF1, CKII, MAPK and CDK analogues have been found in plants, while the cyclic nucleotide-dependent protein kinases and conventional PTKs are absent. Higher plants also possess unique protein kinases distinct from those found in most eukaryotes such

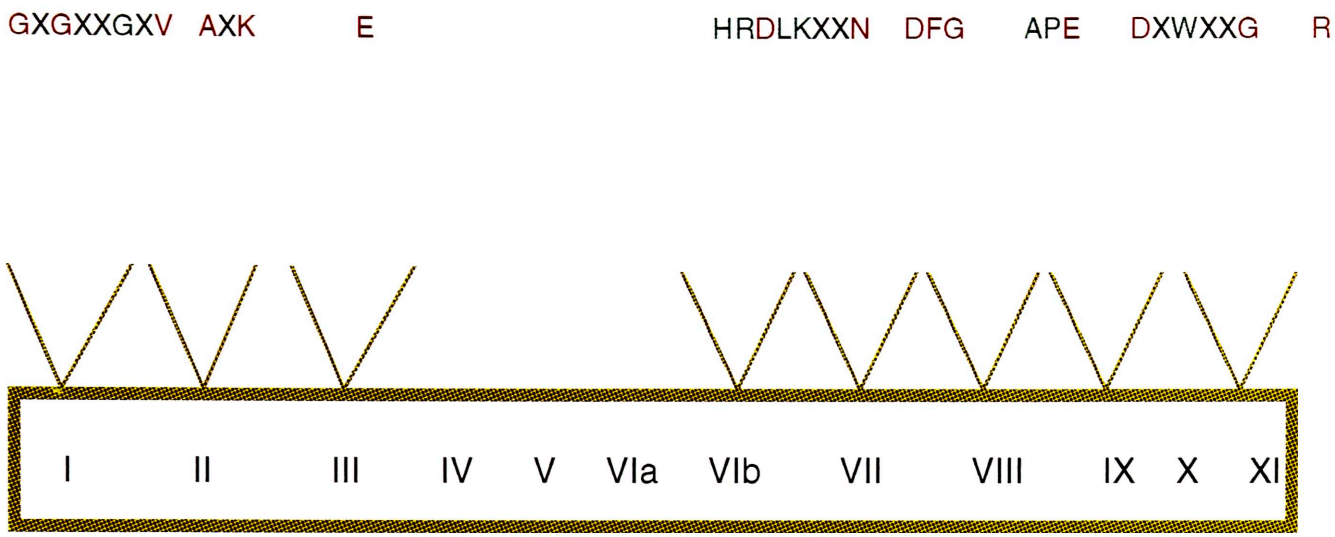


Figure 1.5: Conserved Amino Acid Sequence Domains in the Catalytic Regions of Protein Kinases. *Consensus sequences found in some domains are shown in red (identical), and green (similar). X represents variable residues. Taken from Stone and Walker 1995.*

as RLKs (responsible for signal recognition), and CDPKs (regulated by second messengers) (Stone and Walker 1995).

CDPKs from plants do not require second messengers such as calmodulin, phospholipids, and diacylglycerol. They are encoded by multigene families (Stone and Walker 1995). Their amino terminal half shows homology to the kinase catalytic domains of the CaMK family, while the carboxyl terminal half is homologous to calmodulin and contains four Ca^{2+} -binding sites (Harper *et al* 1991).

RLKs are sub-divided into three types: those having an extracellular S-domain containing an array of ten cysteine residues found in combination with other conserved motifs, the leucine-rich repeat family of proteins, and those that contain an epidermal growth factor-like repeat (Walker 1994). All plant RLKs known to date are serine-threonine kinases.

Some of the functions suggested for plant serine-threonine kinases include: a role in the response to environmental signals, transcriptional regulation, self-incompatibility, disease resistance, and plant morphogenesis (Stone and Walker 1995; Walker 1994). The roles of only a few kinases in cellular processes have been determined. These include CTR1 that is considered a negative regulator of ethylene-response pathway, and SRK6 and SRK910 that are associated with sporophytic self-incompatibility (Loh 1995 and references therein).

1.2.2 Plant Disease Resistance and Serine-Threonine Kinases

One of the ways by which plants ward off pathogenic invasion is by generating a hypersensitive response that involves localized induced cell death in the host plant at the site of infection (Staskawicz *et al* 1995). This response is termed 'incompatibility' and is generalized as 'host resistance: pathogen avirulence' (Lamb 1994).

The genetic basis of incompatibility is explained by the 'gene-for-gene' hypothesis according to which resistance to a particular race of pathogen is conferred in plants via a dominant resistance gene only if the pathogen expresses the corresponding avirulence gene (Lamb 1994). It is thought that the interaction of elicitors (specific signal molecules produced by the pathogen) with receptors (in the host) leads to a hypersensitive response. This interaction may be extracellular or intracellular. It is also speculated that the signal is transmitted to the nucleus via a phosphorylation cascade and involves transcription factors. This may result in an expression of stress-response genes, such as those for the production of salicylic acid. Alternatively, the elicitor-receptor interaction may lead to an oxidative burst in the host resulting in apoptosis, cross-linking of the cell wall and induction of structural proteins like chitinases and glucanases (Martin *et al* 1994, Lamb 1994).

Current research in plant pathology is focused on identifying components of the elicitor-recognition mechanism. This is being done using transposon-based gene tagging systems and map-based positional cloning. It has led to the identification of 'R' (resistance) genes from maize, tobacco, tomato, flax and *Arabidopsis*.

Two 'R' genes have been cloned that have intracellular serine-threonine kinase domains. Both conform to the hypothesis of gene-for-gene interaction. *Pto* was cloned from tomato and it imparts resistance to *Pseudomonas syringae* pv *tomato* strains that express the avirulence gene *avrPto* (Martin *et al* 1993). It is cytosolic and possesses a signal anchor at the amino terminal which suggests an interaction with transmembrane proteins.

Xa21 was cloned from rice and it confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6. It has an extracellular domain that contains leucine-rich repeats and is suggested to be involved in protein-protein interaction, and an intracellular serine-threonine kinase domain (Song *et al* 1995). Both genes are thought to be involved in the initial

stages of transduction of the elicitor signals that lead to a hypersensitive response. It is thought that the binding of the pathogen ligand to the extracellular receptor domain may activate the cytoplasmic kinase domain that may in turn phosphorylate a cytosolic kinase. The resulting phosphorylation cascade would lead to the transcription of defence genes or stimulate an oxidative burst that would result in a hypersensitive response.

1.3 AIMS OF THE PROJECT

Protein kinases and protein phosphatases are two very large enzyme families functioning in many important cellular processes. Some members of these two families have been cloned and subjected to detailed studies, but the majority remains unidentified, especially in plants. Molecular characterization of protein kinase and protein phosphatase genes in plants and an understanding of the mechanisms by which they are involved in development, defence and other processes will be of fundamental importance and may also prove beneficial to agriculture.

Barley and maize are two important crops in the world and also excellent experimental organisms particularly in the area of molecular genetics. Based on database searches (combined GenBank/EMBL nucleic acid database, ANGIS; February 31, 1996), there have been 9 serine-threonine kinase and 1 serine-threonine phosphatase genes cloned from maize, while two serine-threonine kinase and no serine-threonine phosphatase genes cloned from barley. Most of the protein kinase and protein phosphatase members in these two plants have yet to be identifed. The signal transduction pathways involving the cloned serine-threonine kinases and serine-threonine phosphatases in maize and barley are not known.

In order to investigate the role of protein phosphatases in the crop plant barley, the first part of the project has consisted of isolating and cloning the genes for serine-threonine

phosphatases, and tyrosine phosphatases using the homology-based method of oligonucleotide screening.

The plant serine-threonine kinases are involved in resistance to bacterial diseases. *Pto* is the first evidence of the involvement of protein phosphorylation in the signal transduction of the defence mechanism. It is likely that by using homology-based methods, genes encoding serine-threonine kinases from other plants may be isolated and their roles in hypersensitivity studied. To test this hypothesis, *Pto* was previously used as a probe to screen a maize cDNA library (Zhang and Pryor; unpublished), and a few putative clones isolated. The present study also includes sequencing one of the cDNA clones from maize and comparing the sequence with *Pto*.

Chapter 2: Materials And Methods

2.1 *HANDLING OF A BARLEY GENOMIC DNA LIBRARY*

A barley (*Hordeum vulgare* cv Himalaya) genomic DNA library, constructed in EMBL4 λ bacteriophage was kindly provided by Dr M. Robertson (Division of Plant Industry, CSIRO, Canberra). Total barley DNA had been partially digested with *Sau*3A I and ligated into the bacteriophage vector that had been digested with *Bam*H I.

2.1.1 *Escherichia coli Cell Culture*

For phage infection, *Escherichia coli* strain KW251 (Promega) was grown in 2YT (see appendix), that contained 0.2% maltose, 10 mM $MgSO_4$ and 12.5 µg/ml tetracycline, for approximately 6–7 hours at 37°C or overnight at 30°C.

2.1.2 *Titrating the Library*

To determine the concentration of bacteriophage in the library, the library was titrated. Serial dilutions of 10^{-1} and 10^{-2} were made in SM solution (see appendix) from a 1 ml master stock of this library. 2 µl and 5 µl of bacteriophage each from the 10^{-1} dilution was incubated with 200 µl of *Escherichia coli* strain KW251. Similar inoculations were made using 5 µl and 20 µl of bacteriophage from the 10^{-2} dilution. Incubation was done at 37°C for 15–30 minutes. Cells were mixed with 1 ml of NZCYM Top Agarose (see appendix) that had been prewarmed to 55°C, and poured onto prewarmed LB agar (see appendix) plates that were 6.5 cm in size and contained tetracycline at a concentration of 12.5 µg/ml. The plates were incubated at 37°C overnight. The number of plaques were counted the next day and the concentration of bacteriophage/µl was determined.

2.1.3 *Amplifying the Library* (Sambrook *et al* 1989)

To increase the concentration of recombinant bacteriophage, the library was amplified. Five µl bacteriophage from the master stock, containing ~10,000–20,000 recombinant bacteriophages (as calculated from previous titration, see method 2.1.2), was incubated with 800 µl KW251 and plated on each of 35 large plates (15 cm in size). Plates were incubated at 37°C overnight. The following day these plates were overlayed with 12 ml of SM solution and left for 24 hours at 4°C with intermittent shaking. Bacteriophage suspension was recovered from each plate and transferred to a sterile centrifuge tube. Each plate was rinsed with 4 ml of SM and the solution decanted into the first tube. Chloroform to a final concentration of 0.2 ml/plate was added to the first tube which was then incubated for 15 minutes at room temperature with occasional shaking. The tube was centrifuged at 4000 g for 5 minutes at 4°C and the supernatant was transferred to a fresh bottle. One to two drops of chloroform was added to the bottle, and the bottle stored at 4°C. Concentration of bacteriophage in the amplified library was determined.

2.2 *DEGENERATE OLIGONUCLEOTIDE PRIMERS*

A set of three degenerate oligonucleotide primers ranging from 17 mer to 20 mer was synthesized (Table 2.1). The amino acid sequence for each primer corresponded to highly conserved regions in the catalytic subunits of protein phosphatases (Figures 2.1-2.3).

Name	Type	Length	Degeneracy
PPY1	Tyrosine Phosphatase	20 mer	32
PPS2C1	Serine-threonine Phosphatase 2C	17 mer	32
PPS2A2	Serine-threonine Phosphatase 1/2A	20 mer	64

Table 2.1 : Degenerate Oligonucleotide Primers

The amino acid sequences were aligned using PILEUP (Genetics Computer Group Inc. sequence analysis software package, version 8, September 1994, Madison, Wisconsin). The degeneracy of primers was based on the codon usage bias for barley (Wada *et al* 1991). The degeneracy ranged from 32 to 64.

As no sequences for plant tyrosine phosphatases could be found in the database (combined GenBank/Swissprot protein database, ANGIS, February 1995), ten animal tyrosine phosphatases, both receptor-like and cytosolic, were aligned in order to design the oligonucleotide sequence (Figure 2.1). The oligonucleotide sequence chosen was part of a conserved active-site region in tyrosine phosphatases viz: HCXAGXXR(S/T)G (Haring *et al* 1995; Pot and Dixon 1992) where X can be any residue.

Among the serine-threonine phosphatase sequences for the catalytic domain available for plants, a set of seven sequences comprising of PP-1, and PP-2A were aligned and a homologous stretch chosen (Figure 2.2). Since there were only two sequences for plant PP-2C available at the time, they were aligned with five animal and one yeast PP-2C sequences (Figure 2.3).

The oligonucleotide sequences designed for PP-1/PP-2A, and PP-2C corresponded to the sequence of the anti-sense strand. This approach was considered to facilitate, in the future, the use of a second primer upstream- corresponding to the sense strand, that could be used in conjuction with current primers for PCR amplification.

A)

HSPTPG	.s..... aarmpetgpv l**vhcsagv**gr tgtyividsm lqqi.....	Human
HSPTPA	.k..... acnpqyagai v**vhcsagv**gr tgtfvvidam ldmm.....	Human
HSPTPE	.k..... tlnpvhagpi v**vhcsagv**gr tgtfividam mamm.....	Human
HSPTPD	.k..... tcnppdagpm v**vhcsagv**gr tgcfividam leri.....	Human
PTPK_MOUSE	.k..... lsnppsagpi v**vhcsag**agr tgcyividim ldma.....	Mouse
HUMSHPTP1A	qr..... qeslphagpi i**vhcsag**igr tgtiividml menis....t	Human
B38189	qr..... qeslphagpi i**vhcsag**igr t.....	Rat
DDIPTPA	he..... krs....gpi v**vhcsag**igr sgtfvaihsi vakfakhyde	*Dictyos*
MUSPTP36	svrrhtnsvl egirtrhppi v**vhcsagv**gr tgvvilselm iycl.....	Mouse
MUSCDC25A	vcvlptvpgk hpdlkyispd t**v**aallsgkf qsvierfyii dcrypyeylg	Mouse

B) Primer PPY1: V- H- C- S- A- G- V

Val- His- Cys- Ser- Ala- Gly- Val

5'> GT(CG) CAC TGC (TA)(CG)C GC(CG) GG(CG) GT <3'

Figure 2.1: A. Partial Alignment of Amino Acid Sequence from Animal Tyrosine Phosphatases. *Sequences were aligned using PILEUP program of Genetics Computer Group Inc. Sequence in the bold and underline represents the consensus sequence for which the oligonucleotide primer was designed. Protein names are on the far left, their sources are on the far right. Abbrev. Dictyos: Dictyostellium.* ***B. Degenerate Oligonucleotides Used for Screening Barley Genomic Library***

A)

ATPP1ATM	penffllrgn hesasinri**y** **gfydec**krrf .nvrlwkift dcfnclpvaa	*Arabid* (1)
BOBOPP1	penffllrgn hecasinri**y** **gfydec**krrf .nvrlwkift dcfnclpvaa	*B.oler* (1)
ATPPHOS1	penffllrgn hecasinri**y** **gfydec**krrf .svrlwkvft dsfnclpvaa	*Arabid* (1)
BNPP1	pskiyllrgn hedakinri**y** **gfydec**krrf .nvrlwkift dcfnclpvaa	*B nap* (1)
MVPP1MS	kenffllrgn hecasinri**y** **gfydec**krry .nvrlwktft dcfnclpvaa	*Alfal* (1)
MSPP2AMS	pqritilrgn hesrqitqv**y** **gfydec**lrky gsanvwkift dlfdffplta	*Alfal* (2A)
BNPP2A	pqritilrgn hesrqitqv**y** **gfydec**lrky gnanvwkyft dlfdylplta	*B nap* (2A)

B) Primer PPS2A2: Y- G- F- Y- D- E- C

Tyr- Gly- Phe- Tyr- Asp- Glu- Cys

3'> AT(AG) CC(CG) AA(AG) AT(GA) CT(AG) CT(CT) AC <5'

Figure 2.2: A. Partial Alignment of Amino Acid Sequences from Catalytic Domains of Plant Serine-Threonine Phosphatase 1, and 2A. *Sequences were aligned using PILEUP program of Genetics Computer Group Inc. Sequence in the bold and underline represents the consensus sequence of PP-1 and PP-2A for which the oligonucleotide primer was designed. Protein names are on the far left, their sources are on the far right. Abbrev. Arabid: Arabidopsis, B.oler: Brassica oleraceae, B.nap: Brassica napus, Alfal: Alfalfa.* ***B. Degenerate Oligonucleotides Used to Isolate Genomic Clones from Barley.*** *Nucleotide sequence corresponding to the antisense strand was used (see text).*

A)

BOVPHOS	disleaqvgd pnsflnylvl rvafsgatac vahvdgvdlh vantgdsram	*Bovine*
P2CA_HUMAN	hmrvmsekkh gadrsgstav gvlis..... pqh.....ty fincgdsrgl	*Human*
P2CA_RABIT	hmrvmsekkh gadrsgstav gvlis..... pqh.....ty fincgdsrgl	*Rabbit*
P2CB_RAT	ymrnfsdlrn gmdrsgstav gvmis..... pth.....iy fincgdsrav	*Rat*
MUSPP2C2	ymrnfsdlrn gmdrsgstav gvmvs..... pth.....my fincgdsrav	*Mouse*
A54588	lalridqewm dsgreggstg tffvalkegn kvh.....lq vgnvgdsrvv	*Arabid*
ATABI1G	eiesvapetv g...stsvva vvfpsh.... .....if vancgdsrav	*Arabid*
YSPPTC1X	kiakathndi c...gctaav affryeknrt rrv.....ly tanagdariv	*Yeast*

B) Primer PPS2C1: N- C- G- D- S- R

Asn- Cys- Gly- Asp- Ser- Arg

3'> TTG ACG CC(CG) CT(AG) (TA)(CG)G (TG)C <5'

Figure 2.3: A. Partial Alignment of Amino Acid Sequence from Catalytic Domains of Plant and Animal Serine-Threonine Phosphatase 2C. *Sequences were aligned using PILEUP program of Genetics Computer Group Inc. Sequence in the bold and underline represents the consensus sequence of PP-2C for which the oligonucleotide primer was designed. Protein names are on the far left, their sources are on the far right. Abbrev. Arabid: Arabidopsis.* ***B. Degenerate Oligonucleotides Used to Isolate Genomic Clones from Barley.*** *Nucleotide sequence corresponding to the antisense strand was used (see text).*

2.2.1 Primer Synthesis

The primers were synthesized, by standard phosphoramidate chemistry, on an Applied Biosystems 391 DNA Synthesizer PCR-Mate (Chemistry Department, University of Wollongong). The oligonucleotides were eluted from the columns using 28% fresh ammonia, and deprotected by heating the solution for one hour at 65°C. The solution was aliquoted and the contents dried under vacum. The oligonucleotide was dissolved in 100 mM Tris-HCl pH 7.0, and the DNA concentration measured at an absorbance of 260 nm using a UV spectrophotometer (Pharmacia). The stock solution ranging in

concentration from 1.9 µg/µl to 2.9 µg/µl was diluted such that 100 ng could be drawn for making the probe. The oligonucleotides were stored at -20°C.

2.2.2 *Labelling* (Woods 1984)

The labelling mix consisted of 5 µl of 10 x Polynucleotide Kinase (PNK) buffer (Promega), 100 ng of oligonucleotide primer, 150 µCi of γ ^{32}P-ATP (4000 Ci/mmole) (Bresatac), 20 units of T_4 Polynucleotide Kinase (Progen), and water added to the final volume of 50 µl. The mix was incubated at 37°C for 1 hour.

Oligonucleotide PPS2C1, PPS2A2, or PPY1 was labelled, and purified using Sephadex G-25 column (Pharmacia Biotech). The sample was made upto 100 µl by adding 1 x STE buffer pH 8.0 (see appendix), and applied to the column which was centrifuged, and the eluant collected in a decapped Eppendorf tube. Radioactivity in the eluate and the column was determined roughly using a β-Geiger monitoring device (Mini-instruments LTD). The eluted DNA was stored at -20°C or used immediately.

2.3 *SCREENING FOR PHOSPHATASE GENES*

2.3.1 *Plating for Screening*

Plating was done using the protocol of Zhang and Walker 1993 with modifications on the volume of phage used. Aliquots of the amplified barley DNA library containing ~20,000 pfu (phage forming units) in a volume of 20 µl were mixed with 800 µl of *Escherichia coli* KW251, and incubated at 37°C for 15-30 minutes. A total of eight phage innoculations were made. After incubation the cells were mixed with 8 ml of prewarmed Top Agarose and poured onto eight prewarmed 15 cm plates. Plates were incubated at 37°C until the plaques began to touch each other and a web-like pattern

could be observed. The plates were chilled at 4°C for one hour to allow the Top Agarose to harden before making plaque membrane blots.

The autoradiograph result from the primary screening was aligned with each primary plate and the area on the plate corresponding to a positive signal, containing ~20 plaques, was picked up using the wide mouth of a sterilized pasteur pipette. It was resuspended in 500 µl of SM solution and 20 µl of chloroform. After titrating on 6.5 cm plates, the plates containing ~200-500 plaques were chosen for preparing the membrane blots for secondary screening. Plates were incubated until plaques were 1 mm in diameter.

A single plaque from the secondary plate, corresponding to the positive signal on the autoradiograph, was resuspended in a solution containing 100 µl SM and 20 µl chloroform. It was titrated to give ~50-100 plaques on each 6.5 cm plate. Where plaques were adjacent and could not be isolated without contamination from neighbouring plaques, another round of screening was repeated using the same conditions as for tertiary screening.

2.3.2 *Plaque Blotting*

Plaque membrane blots were prepared according to the Hybond ^{TM} N^{+}, Positively Charged Nylon Membrane Version 2.0 product booklet by Amersham Life Science. Briefly, the labelled nylon membrane was placed on the agar surface and the orientation of plaques was noted by marking the membrane and the agar using a needle dipped in india ink. The membrane was removed from agar plate after 1 minute and placed, plaque side up, in a tray containing just enough denaturing solution (see appendix), to soak the membrane.

The membrane was treated with denaturing solution for 7 minutes and then transferred, plaque side up, to another tray containing neutralizing solution (see appendix). Neutralization was done for 3 minutes. Denaturation and neutralization was repeated after which the membrane was washed in 2 x SSC and air-dried plaque side up. The membrane was fixed, DNA side up, in a tray containing 0.4 M NaOH enough to wet it, for 30 minutes. It was rinsed by immersing in 5 x SSC with gentle agitation for 1 minute.

The membrane blots were washed in 3 x SSC/0.1% SDS at 65°C for 16–20 hours. Large volumes of buffer ~500 ml or less were used and the buffer was changed at least twice. The filters were wrapped in a plastic sheet and stored at room temperature or used immediately for hybridization.

2.3.3 *Hybridization* (Woods 1984)

Membrane blots were wet in 3 x SSC, and placed in a hybridization bottle (Hybaid), containing 20–100 ml of pre-hybridization buffer (see appendix), depending on the number of membrane blots used. Pre-hybridization was carried out at 37°C to 39°C for 4–6 hours in a Mini Oven MKII Hybaid hybridization oven. The probe was mixed with the buffer and transferred back to the bottle containing the blots. The volume of the buffer was reduced to 20–80 ml. Hybridization was carried out at 37°C to 39°C for 16 hours.

For post-hybridization washing, the membrane blots were removed from the hybridization buffer and washed three times at 27°C for 15 minutes each, once at the hybridization temperature for 60 minutes, and finally at 10°C above the hybridization temperature for 10 minutes. All washes were done using 6 x SSC, 0.05% sodium pyrophosphate. The membrane blots were air-dried, wrapped in a plastic sheet, and placed in a hypercassette (™ Amersham). Autoradiography was done at -70°C using

two intensifying screens and an X-ray film (Fuji) placed in the hypercassette. The period of exposure ranged from 12 hours to a week depending on the intensity of the probe.

After autoradiography results, the probe was removed from the membrane blots by washing the blots with boiling water. A minimum of five washes were done, each followed by monitoring the radioactivity with a β-Geiger counter. The blots were air-dried, wrapped in a plastic sheet, and stored at room temperature for future re-use.

2.4 PURIFICATION OF RECOMBINANT λ DNA (Verma 1989)

Ten to fifteen plaques were picked using a pasteur pipette and suspended in 300 µl of KW251 cells (see 2.1.1 for preparation). In some cases, 30–50 plaques were suspended in SM solution, and a solution equivalent to 10 plaques drawn from this stock to infect 300 µl of cells. The cell mix was incubated for 30 minutes at 37°C with occasional shaking, and then used to inoculate 20 ml of 2YT containing 10 mM $MgSO_4$ and 12.5 µg/ml tetracycline.

The cell mix was shaken at 37°C until a cell debris was visible, after which 300 µl of chloroform was added, and the solution shaken for another 30 minutes. The cells were spun at 12,000 g (10,000 rpm) for 10 minutes in a Beckman centrifuge (JA-20 rotor) and the supernatant was transferred to another tube.

An equal volume of saturated ammonium sulfate (saturated at 4°C) was added to the supernatant, mixed, and incubated on ice for 20 minutes. The solution was spun at 27,000 g (15,000 rpm) for 20 minutes at 4°C (Beckman, JA-20 rotor). The supernatant was discarded, the pellet was dissolved in 0.5 ml GSE (see appendix), and Proteinase K (Sigma) to a final concentration of 10 µg/ml was added. The solution was incubated at

52°C for 30 minutes. An equal volume of chloroform was added, the solution was vortexed and spun at 13,000 g (14,000 rpm) for 5 minutes (Eppendorf centrifuge).

The aqueous phase was transferred to a new Eppendorf tube and a one-tenth volume of 3 M sodium acetate pH 5.4, and 2 volumes of cold 100% ethanol were added. The phage DNA was spooled, washed in 75% and 100% ethanol respectively and dissolved in 50–100 µl of water. The quantity and quality of the phage DNA was determined by gel electrophoresis (see appendix) using 5 µl of sample DNA.

For digestion with *Eco*R I, 20 µl of λ DNA solution was mixed with 2 µl of 10 x multi-core buffer (Promega), and 4 units of *Eco*R I (Promega). Final volume was made up to 40 µl with water. Digestion was carried out for 3 hours at 37°C. 7–10 µl sample was analysed by gel electrophoresis. For Southern blotting (Southern 1975), agarose gel containing the DNA samples was inverted and placed on a pad of filter papers soaked in 0.4 M NaOH. A piece of nylon membrane was cut to the size of the gel and lowered onto it. A 3 MM Whatman filter paper and a stack of tissue papers were placed on it followed by two glass plates. DNA blotting was done for as short as 3–15 hours, after which the DNA blot was washed in 2 x SSC, air-dried and then subjected to hybridization carried out as per method 2.3.3.

2.5 SUBCLONING

2.5.1 Ligation

For subcloning into plasmid, DNA was digested with restriction enzymes, sample volume was made up to 50 µl, and the DNA purified by ethanol precipitation. All ligations were performed in a total volume of 20 µl where each reaction contained 2 µl of 10 x T_4 DNA Ligase buffer (Progen), 100–200 ng of insert DNA, 100–200 ng of plasmid vector (Promega), 2.5 units of T_4 DNA Ligase (Progen), and water added to 20

µl. The vector DNA and the insert DNA were heated separately at 65°C for 5 minutes to avoid self-annealing before mixing them together. Ligation was carried out for twelve hours at 12–15°C, or two hours at room temperature. Controls consisting of the vector DNA without ligase, and the vector DNA with ligase but without the insert DNA were set up to check the efficiency of ligation. The samples were ethanol precipitated and dissolved in 10 µl of water.

2.5.2 *Transformation by Electroporation* (Zabarovsky *et al* 1990)

Escherichia coli strain DH5α (Promega) was prepared for electro-transformation according to the protocol by Zabarovsky *et al* (1990), and stored at -70°C. The frozen cells were thawed at room temperature and placed on ice. 30–40 µl of the cells were transferred into a cold 1.5 ml Eppendorf tube and 2.5 µl of the sample DNA was added. The cell/DNA mixture was transferred to an ice-cold micro-cuvette (Biorad), and placed in position in the safety chamber of the BioRad Gene Pulser ™ (Biorad). The instrument was set to 25 µF capacitance, 2.5 KV, 200 ½ and pulsed once, with the resulting pulse of 12.5 KV/cm and a time constant of 4.5 msec. The cells were removed from the cuvette and mixed with 960 µl of SOC medium (see appendix), and incubated with shaking for 30 minutes at 37°C. 15–35 µl of the cells were plated on 6.5 cm LB agar plates containing 200 µg/ml of ampicillin together with 25 µl of a 2% solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). For plating on 9 cm plates, 75–100 µl of cells and 50 µl of 2% X-gal were used. The recombinants were identified either through plasmid prep digestion or through oligonucleotide screening. For the latter, ~100–200 recombinants were streaked on LB agar plates containing 200 µg/ml ampicillin. The colonies were incubated at 37°C overnight. The following day nylon membranes were placed on agar surface of these plates, and the plates incubated at 37°C for ~3–5 hours. Membrane blots and hybridization were carried out as previously described.

2.5.3 *Plasmid Miniprep*

Using a sterile toothpick, a white bacterial colony was randomly touched and the toothpick dropped into a tube containing 2.5 ml of SOB (see appendix) and 10 mM $MgCl_2$, or 2YT medium containing appropriate antibiotics. The cells were cultured ~24 hours with constant shaking at 37°C. 1.5 ml of the cell culture was poured into a sterile Eppendorf tube and the cells spun in a microfuge for 1 minute at 13,000 g (14000 rpm). The supernatant was discarded and the cell pellet resuspended by vortexing.

200 µl of 0.1 M NaOH/0.5% SDS was added to the cell suspension, mixed, and left to stand at room temperature for 5 minutes. 200 µl of 3 M sodium acetate pH 5.4 was added and the cell suspension placed on ice for 10 minutes followed by centrifugation at 13,000 g (14000 rpm) for 10 minutes. To the supernatant, 900 µl of 100% ethanol was added, and the solution spun at 13,000 g (14000 rpm) for 10 minutes. The resulting DNA pellet was washed with 500 µl of 75% ethanol, air-dried, and dissolved in 40 µl of sterile deionized water.

For restriction digestion, each reaction mix consisted of 1µl of 10 x multi-core buffer (Promega), 0.1µl of RNase A (Sigma) from a stock concentration of 6 mg/ml (see appendix), and 2 units of restriction enzyme (Promega), with the volume made up to 7.5 µl with water. 2.5 µl of DNA sample was added (~200 ng) and the digestion carried out for 3 hours at 37°C. Samples of 5 µl were analysed by gel electrophoresis.

2.5.4 *Deletion Subcloning*

In order to generate deletion subclones, the subclones in the pGEM 7Zf (+) plasmid vector (Promega), and the Bluescript SK (-) plasmid vector (Stratagene), were digested with restriction endonucleases present in the polylinker site and analysed by gel electrophoresis. Samples which after digestion produced inserts ranging from 400–2000

bp were incubated at 65°C for 10 minutes to inactivate the restriction enzyme. A ligase mix consisting of 3 µl of 10 x T_4 DNA Ligase buffer, and 2.5 µl of T_4 DNA Ligase was added to these samples. Total reaction was made up to 30 µl with water. Ligation was carried out at room temperature for 1–2 hours followed by ethanol precipitation. The DNA pellet was dissolved in 10 µl of water and electroporated into DH5α as previously described. In some cases, Southern analysis was carried out.

2.5.5 *Southern Hybridization Using cDNA Probes*

Bluescript SK (+) plasmids containing two *Arabidopsis* cDNA inserts viz: TOPP4 (1.3 kb), and KAPP (1.9 kb), were kindly provided by Dr J.C Walker (Division of Biological Sciences, University of Missouri- Columbia, Missouri). The cDNA inserts were homologous, in their translated amino acid sequences, to PP-1 and PP-2C respectively. These plasmids were electroporated into DH5α cells.

For making probes, pTOPP4 was digested with *Eco*R I while pKAPP was double digested with *Bam*H I and *Eco*R V to release the inserts from vector. cDNA was recovered from agarose gels by the method of Tautz and Renz (1983), and labelled according to the Megaprime ™ DNA Labelling Systems Kit protocol (Amersham Life Science). 150 ng of TOPP4 insert, or 200 ng of KAPP insert was used as DNA template. 2.5 µl of random oligonucleotide primers (Amersham) was added to the DNA template and the template denatured by heating for 5 minutes at 92°C To, prevent re-annealing of template DNA, the template was chilled on ice, for 2 minutes. 5 µl of dNTP/buffer mix was added to it followed by 1 unit of DNA Polymerase I Klenow fragment, 25 µCi of α ^{32}P-dATP (3000 Ci/mmole) (ICN), and water to bring the volume to 25 µl. DNA polymerization was carried out by incubating the sample at 37°C for 1 hour followed by purification in a G-50 sephadex column. The probe was first denatured at 92°C for 5 minutes, then chilled on ice for 2 minutes before mixing with the hybridization buffer.

The nylon membrane containing endonuclease digested DNA samples transferred from the agarose gels, was pre-hybridized for 4 hours followed by hybridization for 14 hours at 42°C using 25% formamide, 100 µg/ml sonicated salmon sperm testes DNA, 5 x Denhardt's solution, 50 mM sodium phosphate pH 6.5, 5 x SSC, and 0.2% SDS (Walker 1993). After hybridization, the membrane was washed in 25% formamide, 5 x SSC and 0.2% SDS three times at 24°C, two times at 37°C, and two times at 42°C for 15 minutes each. The membrane was dried and exposed, at -70°C, to an X-ray film using a hypercassette with two intensifying screens.

2.6 SEQUENCING

Sequencing was done using the dideoxy-chain-termination method of Sanger *et al* (1977). Sequencing reactions were run on a polyacrylamide sequencing gel runner (International Biotechnologies Inc.).

2.6.1 Preparation of Double-Stranded DNA Template

The bacterial cells were grown for 24 hours in 2YT medium containing 200 µg/ml ampicillin. The culture was poured into a 1.5 ml Eppendorf tube and spun at 13,000 g (14000 rpm) for 1 minute. The supernatant was discarded and the pellet resuspended by vortexing. 200 µl of 0.1 M NaOH/0.5% SDS was added, the solution mixed and left at room temperature for 5 minutes. 200 µl of 3 M sodium acetate pH 5.4 was added, the solution mixed and left on ice for 10 minutes followed by centrifugation at 13,000 g (14000 rpm) for 10 minutes.

The supernatant was transferred to another Eppendorf tube and a 0.7 volume of ambient-temperature isopropanol was added to it. The solution was mixed and incubated at room temperature for 5 minutes. Centrifugation was repeated at 13,000 g (14000 rpm) for 10 minutes. The supernatant was discarded and the pellet resuspended

in 90 µl of water. 10 µg of RNase A was added and the sample incubated at 37°C for 20 minutes. This was followed once by extraction with phenol, and once with chloroform:isoamylalcohol.

The upper aqueous phase was transferred to a fresh tube and a one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol were added, the mixture left at -20°C for 10 minutes and centrifuged at 13,000 g (14000 rpm) for 10 minutes. The supernatant was drained and the pellet washed in 70% ethanol, air-dried and dissolved in 10 µl of water. 1 µl of the sample DNA was analysed for quantity and quality by gel electrophoresis, where 100 ng/µl of pGEM 7Zf (+) linear vector was run as control.

2.6.2 *Sequencing Reaction*

All reactions were performed using the T^7 Sequencing ^{TM} Kit by (Pharmacia). All reagents were supplied by Pharmacia. Briefly, 8 µl of 2 M NaOH was added to the template DNA (1.5–2.0 µg), and the volume made up to 40 µl with water. The solution was kept at room temperature for 10 minutes followed by the addition of 7 µl of 3 M sodium acetate pH 4.8, 4 µl of water and 120 µl of absolute ethanol. The solution was kept on dry ice for 15 minutes followed by centrifugation at 13,000 g (14000 rpm) for 15 minutes. The DNA pellet was washed with ice-cold 70% ethanol, air-dried and dissolved in 10 µl of water.

The primer was annealed to the template in a reaction consisting of 5–10 picomole of primer, 10 µl of template DNA and 2 µl of annealing buffer. The denaturation was done at 65°C for 5 minutes, followed by annealing of the primer at 37°C for 10 minutes. The reaction was left at room temperature for 5 minutes. M_{13} universal primer/forward primer (Pharmacia), and M_{13} reverse primer (Promega) were used for annealing.

The labelling reaction mix consisted of 10 µl of the annealed reaction, 3 µl of labelling mix dATP, 10 µCi of α ^{32}P-dATP (3000 µCi/mmole), and 2 µl of diluted T^7 DNA Polymerase. The reaction mix was incubated at room temperature for 5 minutes. During this period 2.5 µl of Adenine, Guanine, Cytosine, and Thymine dideoxyribonucleotide 'mix-short' were aliquoted to separate Eppendorf tubes and warmed at 37°C for at least 1 minute. For termination, 4.5 µl of the labelling reaction was aliquoted to each Eppendorf tube containing the 'mix-short', and the reaction incubated at 37°C for 5 minutes after which, 5 µl of 'stop' solution was added to each tube.

4 µl of each 'stopped' reaction was aliquoted to a new Eppendorf tube and heated at 80–90°C for 2 minutes. 2 µl of each sample was loaded on a 6% polyacrylamide sequencing gel (see appendix) and the samples run at 45 Watts until the second dye, Xylene Cyanol, reached the end of the sequencing plate. The gel was lifted from the sequencing plate using a 3 MM Whatman filter paper and was wrapped in a plastic sheet. It was dried using the vacuum gel dryer (Biorad) and covered with an X-ray film. Exposure was overnight, at room temperature. The sequence was read visually and analysed using the Genetics Computer Group (GCG) Inc. sequence analysis software package, version 8, September 1994, Madison, Wisconsin.

Chapter 3: Cloning Protein Phosphatase Genes From Barley

3.1 SCREENING OF BARLEY GENOMIC LIBRARY

The initial round of screening of ~160,000 recombinants, using low stringency hybridization, yielded 21 positive clones with PPY1, 14 positive clones with PPS2C1, and 30 positive clones with PPS2A2, respectively (Table 3.1). Upon plaque purification and re-screening, 11 out of 21 clones, 3 out of 14 clones, and 24 out of 30 clones were found to hybridize strongly to the PPY1, PPS2C1, and PPS2A2 probes, respectively. A further round of screening, aimed at obtaining pure clones, did not yield any positive clones with PPY1, but purified 2 clones with PPS2C1. In the case of PPS2A2, 14 out of 24 clones were randomly selected for a final round of screening, which yielded 3 positive clones.

Primer	Recombinants Screened	Primary Screening	Secondary Screening	Tertiary Screening
PPY1	~160,000	21	11	0
PPS2C1	~160,000	14	3	2
PPS2A2	~160,000	30	24	3 (out of 14 clones screened)

Table 3.1: Putative Clones Identified Using Oligonucleotide Probes

Since the inserts were cloned in *Eco*R I site in the vector, they were digested with this enzyme and analysed on 1% agarose gels.

Figure 3.2 A shows the putative clones of PPS2C1 λ-DNA digested with *Eco*R I. The digestion of clone 1-C-2-2-1 yielded five fragments of 20 kb, 9 kb, ~5.5 kb, ~2 kb, and ~1 kb in size. The digestion of clone 3-C-1-3-1 yielded four fragments, two of which were 20 kb, one was 9 kb, and the other was ~2 kb. The digestion of clone 3-C-1-2-1 gave the same fragmentation pattern as that of 3-C-1-3-1, with the exception of the 2 kb band seen in 3-C-1-3-1.

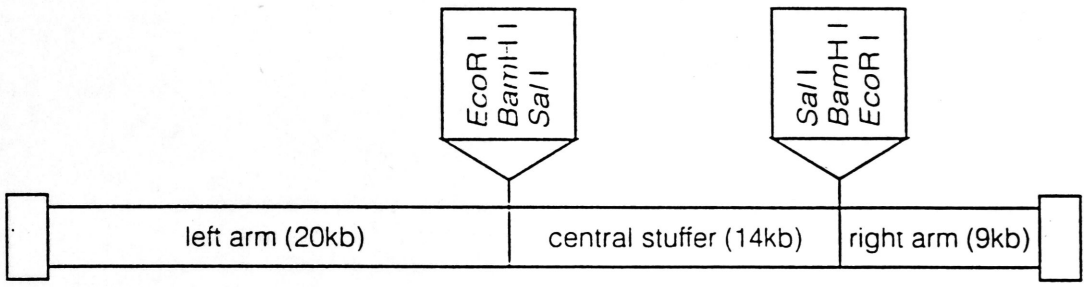


Figure 3.1: Schematic Diagram of the EMBL4 λ Cloning Vector Used for the Genomic Library of Barley (Promega). *The vector has a cloning capacity of 9–23 kb.*

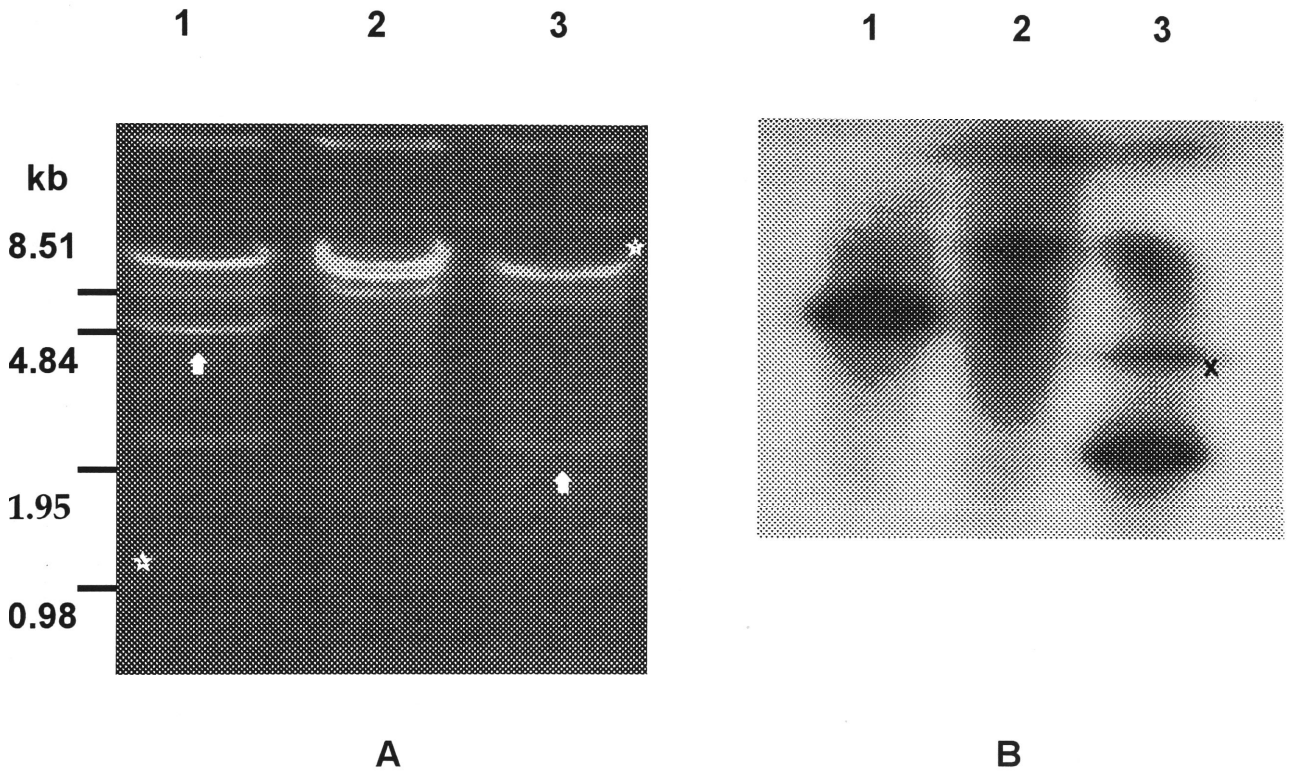


Figure 3.2: Southern Blot Analysis of the Putative λ PP-2C Clones. *Recombinant bacteriophage was digested with EcoR I and electrophoresed. The DNA was transferred onto membranes and probed with the 17 mer oligonucleotide PPS2C1. Hybridization and wash conditions were carried out under low stringency (37 °C, using 6xSSC, 0.05% sodium pyrophosphate).* ***A.*** *DNA fragments stained with EB after separation on 1% agarose gel.* ***B.*** *Radioautograph of a Southern blot of the electrophoresis gel (A) probed with oligo. Lane 1: 1-C-2-2-1, Lane 2: 3-C-1-2-1, Lane 3: 3-C-1-3-1. SPP-1 phage DNA/EcoR I was run as molecular size marker. The arrows indicate the fragments identified by Southern blotting. Asterisk indicates the position of the fragment not visible in this scan. (x) indicates an artifact that was verified through subsequent Southern blotting using the same sample.*

Figure 3.3 A shows the putative clones of PPS2A2 λ-DNA digested with *Eco*R I. The digestion of clone 11-A-c-1-1 yielded five fragments of 20 kb, 9 kb, ~3.2 kb, ~3 kb, and ~1.7 kb. The digestion of clone 14-A-b-1-3 produced seven fragments of 20 kb, 9 kb, ~7.2 kb, ~2.5 kb, ~1.7 kb, ~1.16 kb, ~0.8 kb, while the digestion of clone 10-A-a-2 gave three fragments of 20 kb, 9 kb, and ~7.2 kb.

EMBL4 genomic cloning vector has a cloning capacity of 9–23 kb. It has a 20 kb left arm, and a 9 kb right arm (Figure 3.1). The results from *Eco*R I digestion of the λ clones indicate that the first and the third largest bands in the case of 3-C-1-3-1 and 3-C-1-2-1 clones, the first and the second largest bands in the case of 1-C-2-2-1 clone, and the first and the second largest bands in the case of all PPS2A2 clones represent the two arms of the vector, while the remaining bands arise as a result of *Eco*R I sites in the insert.

To identify the *Eco*R I fragment containing the nucleotide sequence homologous to the PPS2C1 or the PPS2A2 oligonucleotide probes, DNA restricted with *Eco*R I was transferred from agarose gels to nylon membranes and the membranes hybridized with PPS2C1 and PPS2A2 probes respectively.

Under the same conditions of hybridization as for screening the genomic library, the PPS2C1 probe hybridized with the ~5.5 kb fragment of the clone 1-C-2-2-1 and the ~2 kb fragment of the clone 3-C-1-3-1 (Figure 3.2 B). Hybridization was also seen in lane 2 (clone 3-C-1-2-1) but for a bigger fragment. Since the clones 3-C-1-2-1 and 3-C-1-3-1 originated from the same parent clone and would therefore have identical DNA, and since the ~2 kb fragment was not observed for 3-C-1-2-1, the hybridization result suggests that there was incomplete digestion for the clone 3-C-1-2-1. In addition to the ~2 kb fragment the probe PPS2C1 seems to have hybridized to an additional fragment (lane 3, marked x). No fragment of this size can be seen after gel electrophoresis. A second Southern blot using the same sample did not indicate hybridization in this region suggesting that the binding noticed is an artifact (result not shown).

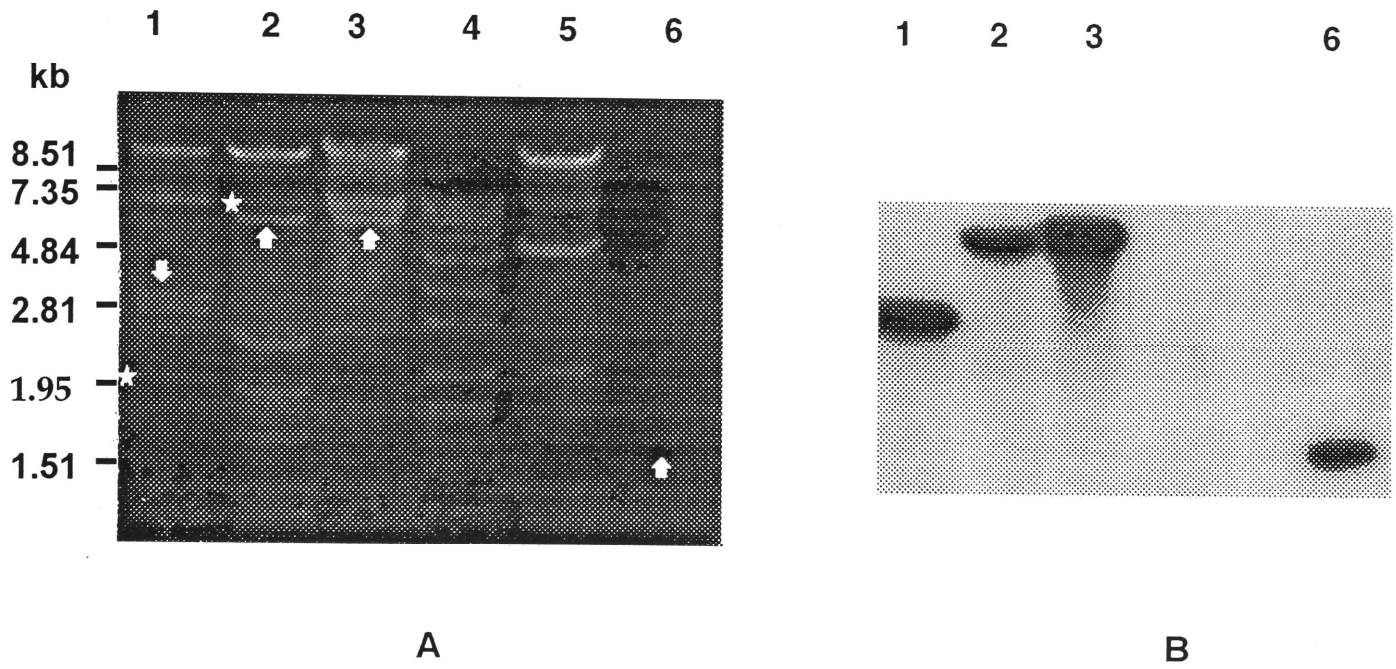


Figure 3.3: Southern Blot Analysis of the Putative λ PP-1/PP-2A Clones. *Recombinant bacteriophage was digested with EcoR I and electrophoresed. The DNA was transferred onto membranes and probed with the 20 mer oligonucleotide PPS2A2. Hybridization and wash conditions were carried out under low stringency (37 °C, using 6xSSC, 0.05% sodium pyrophosphate).* ***A.*** *DNA fragments stained with EB after separation on 1% agarose gel.* ***B.*** *Radioautograph of a Southern blot of the electrophoresis gel (A) probed with oligo. Lane 1: 11-A-c-1-1, Lane 2: 14-A-b-1-3, Lane 3: 10-A-a-2, Lane 4: SPP-1 phage DNA/EcoR I, Lane 5: λ 1-C-2-2-1 (as a negative control), Lane 6: cDNA TOPP4 (as a positive control). The arrows indicate the fragments identified by Southern blotting. Asterisks indicate the position of the fragment not visible in this scan.*

The experiment also revealed hybridization of the PPS2A2 probe with the ~3.2 kb fragment of the clone 11-A-c-1-1 and the ~7.2 kb fragments of the clones 14-A-b-1-3 and 10-A-a-2 respectively (Figure 3.3 B). The difference in the probe intensity (lane 2, 3) corresponds to a difference in the DNA concentration of the sample loaded. A positive control consisting of cDNA TOPP4, a PP-1 from *Arabidopsis* (Smith and Walker 1993), was used to check the efficiency of hybridization of the oligonucleotide PPS2A2. The oligonucleotide hybridized to the cDNA (lane 6).

3.2 PCR SUBCLONING OF λ PP-2C USING OLIGONUCLEOTIDE PPS2C1

In an attempt to subclone the core of the phosphatase catalytic domain directly from λ clones, PCR was carried out using the 17 mer oligonucleotide PPS2C1 and an oligo (dT_{20}) as primers. Amplification was carried out directly on the undigested λ-DNA samples of the clones 3-C-1-3-1, and 1-C-2-2-1, as well as on the *Eco*R I digested samples.

Initially, the λ-DNA was amplified unidirectionally using only the degenerate oligonucleotide PPS2C1. The second step involved adding a polynucleotide (A) tail to the amplified single strands, followed by amplification using both oligonucleotide PPS2C1 and oligo (dT_{20}) primers.

It was expected that the amplified product would contain the PPS2C1 sequence, as well as additional phosphatase sequences which could further verify the identity of the genomic clone. However, no amplification products were observed. Instead, only unbound primers could be seen (result not shown).

A number of parameters were altered to see their effect on the PCR products. These included increasing the concentration of the template, and decreasing the annealing temperature by 10°C in the second PCR. However, no amplification products were noted. Due to time constraints, the effect of altering polymerization time, annealing temperature, and concentration of primers could not be studied.

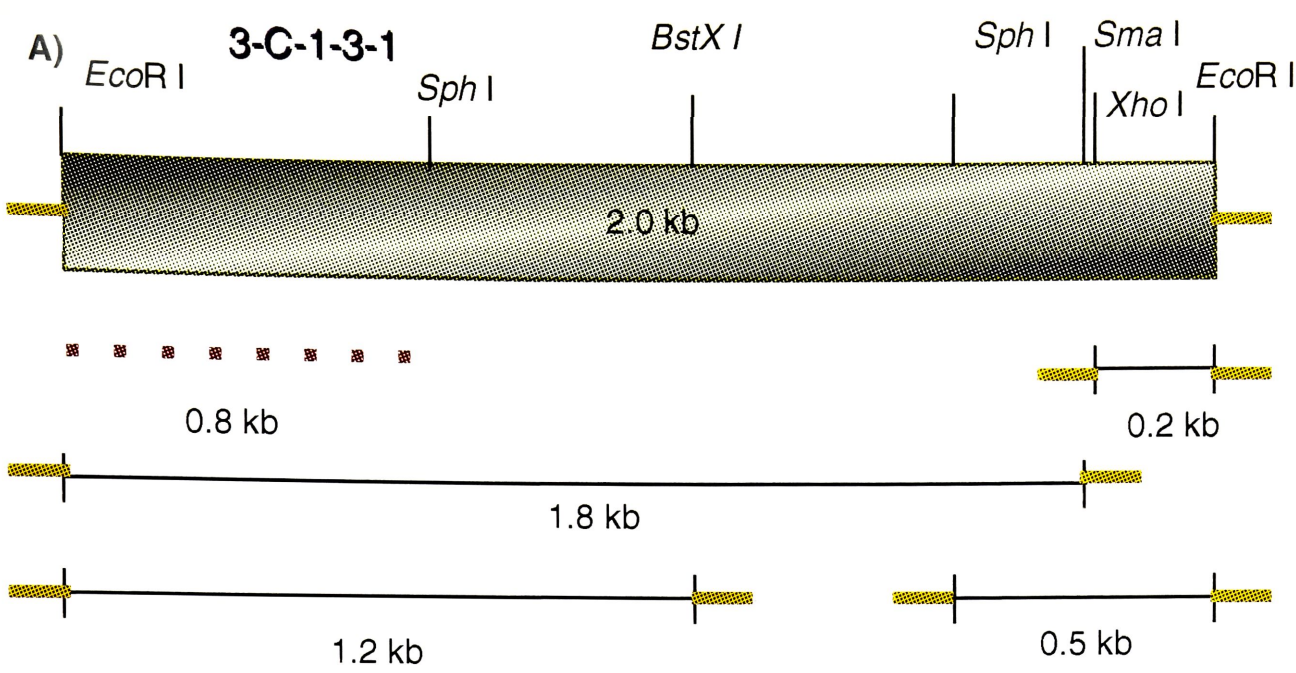
3.3 PLASMID SUBCLONING OF PPS2C1 AND PPS2A2 CLONED FRAGMENTS

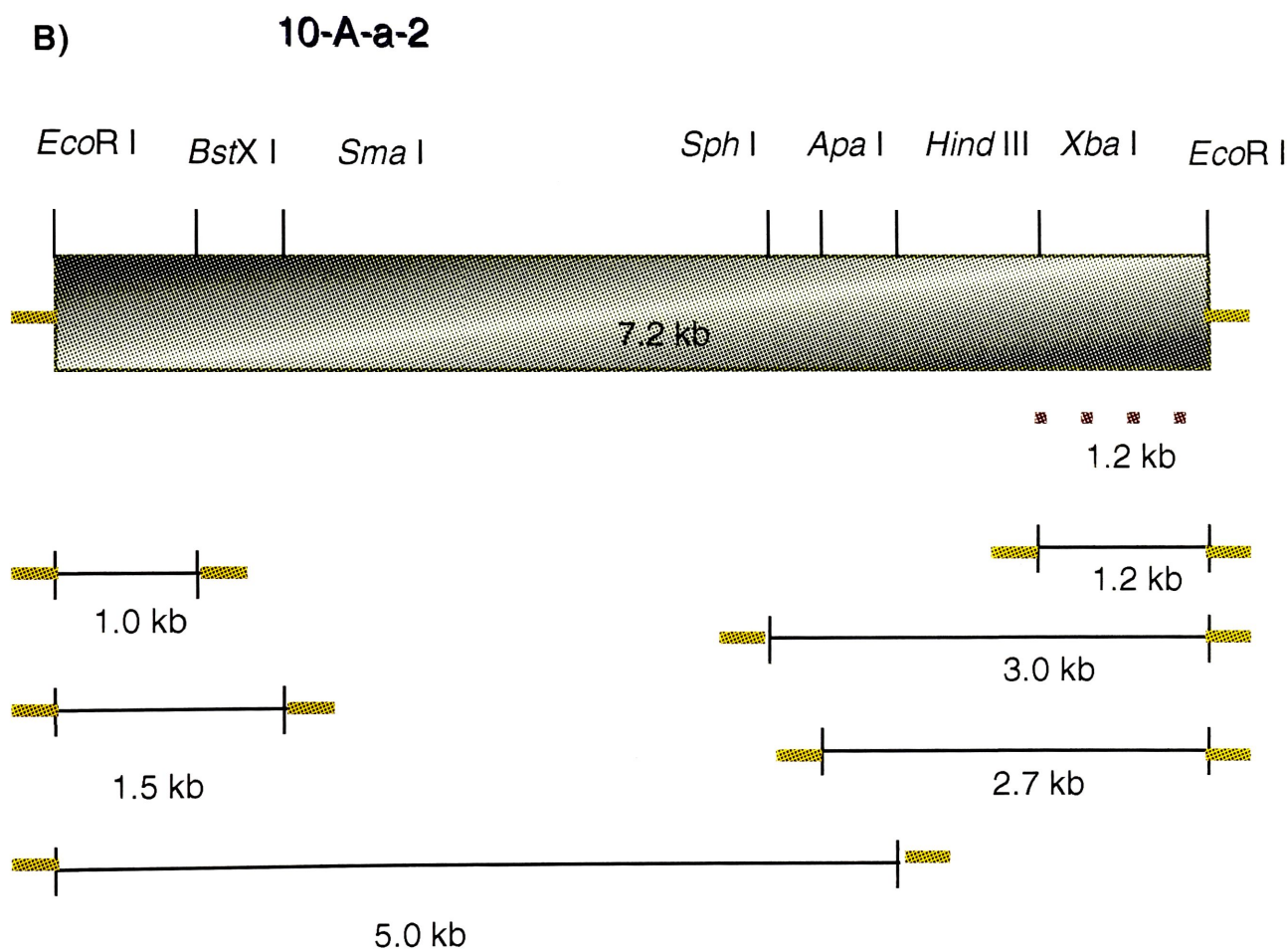
Since PCR amplification of genomic clones was not successful, the λ-*Eco*R I fragments hybridizing to the probes PPS2C1 and PPS2A2, respectively, were purified, and ligated into the *Eco*R I site of the pGEM 7Zf (+) vector. They were electroporated into the *Escherichia coli* strain DH5α, and the recombinants selected on plates containing ampicillin and X-gal.

The initial screening of a number of subclones using colony PCR and plasmid restriction methods failed to identify the desired subclones. Eventually, ~200 colonies of PP-2C and ~120 colonies of PP-1/PP-2A were subjected to colony hybridization. The positive subclones identified were digested with *Eco*R I and their DNA hybridized with the probes. *Eco*R I digested λ clones were used as controls to check the efficiency of oligonucleotide binding. This method succeeded in identifying the subclones with the target fragments.

3.4 RESTRICTION MAPPING AND SEQUENCING STRATEGIES FOR PPS2C1 AND PPS2A2 SUBCLONES

In order to sequence the putative phosphatase clones so that the amino acid sequence motifs corresponding to the conserved domains could be identified, thus confirming the identity of these clones, first, restriction maps of these clones was constructed


A)
3-C-1-3-1
EcoR I
Sph I
BstX I
Sph I
Sma I
Xho I
EcoR I
2.0 kb
0.8 kb
0.2 kb
1.8 kb
1.2 kb
0.5 kb


B)
10-A-a-2
EcoR I
BstX I
Sma I
Sph I
Apa I
Hind III
Xba I
EcoR I
7.2 kb
1.2 kb
1.2 kb
1.0 kb
3.0 kb
1.5 kb
2.7 kb
5.0 kb

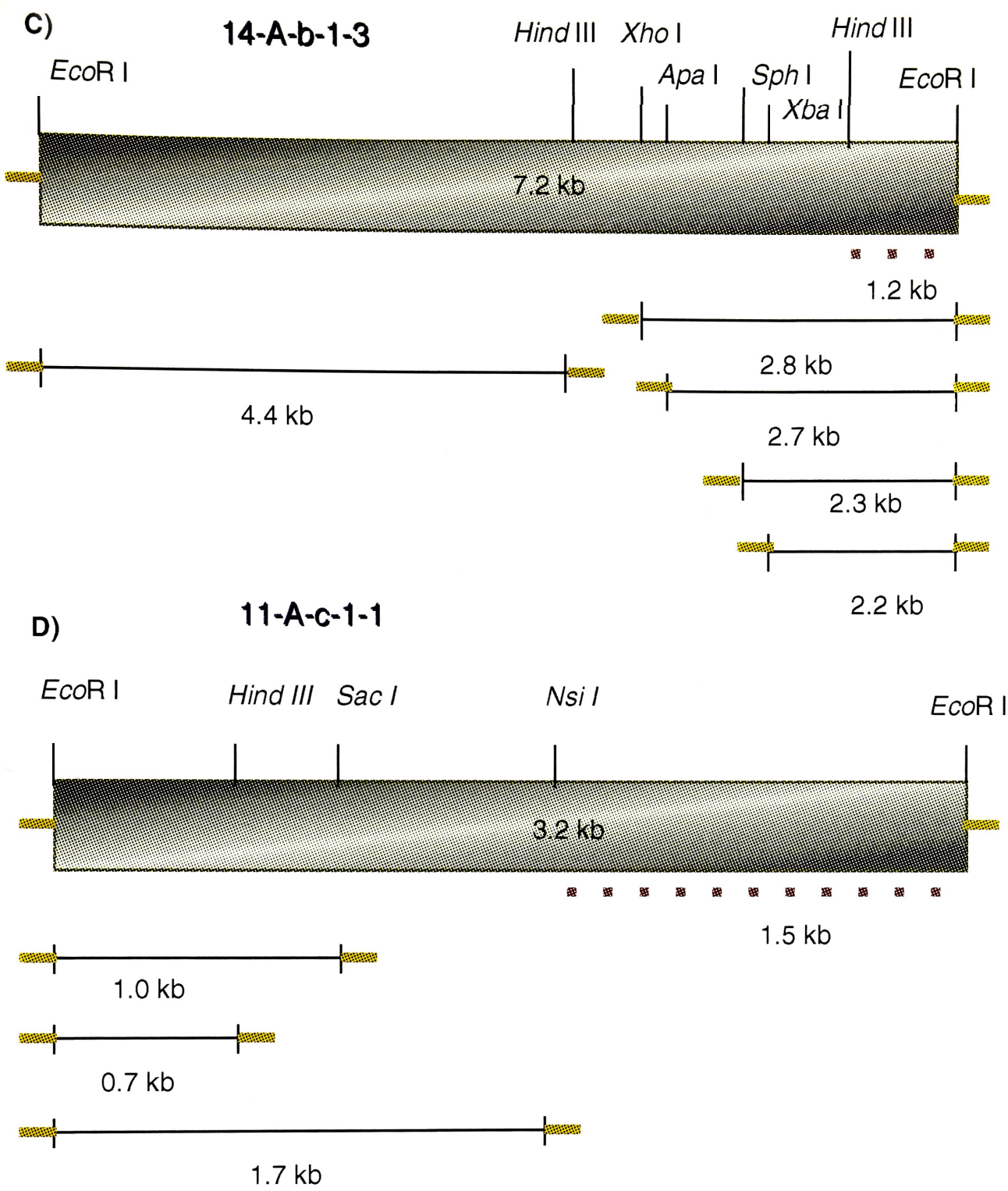


Figure 3.4: Restriction Mapping and Sequencing Strategies for Clones 3-C-1-3-1 (A), 10-A-a-2 (B), 14-A-b-1-3 (C), and 11-A-c-1-1 (D).
Restriction sites relevant for subcloning and sequencing are indicated. Red line represents the area to which the probe hybridized, yellow lines indicate vector sequences flanking the insert.

followed by screening with the oligonucleotide probe to identify the region to be sequenced.

The restriction mapping of clone 3-C-1-3-1 and its subsequent screening revealed a 0.8 kb *Eco*R I/*Sph* I fragment, that hybridized strongly to the oligonucleotide PPS2C1 (Figure 3.4 A). Similarly, a 1.2 kb *Xba* I/*Eco*R I fragment of the clone 10-A-a-2, a 1.2 kb *Hind* III/*Eco*R I fragment of the clone 14-A-b-1-3, and a 1.5 kb *Nsi* I /*Eco*R I fragment of the clone 11-A-c-1-1 hybridized strongly to the oligonucleotide PPS2A2 (Figure 3.4 B, C,D). These fragments were targets for subcloning before sequencing with M_{13} reverse, and forward primers. Restriction mapping was not done for clone 1-C-2-2-1.

3.5 HYBRIDIZATION OF THE PUTATIVE CLONES TO HETEROLOGOUS cDNA PROBES FROM ARABIDOPSIS

To determine the degree of binding of the subclones, identified initially using the oligonucleotide probes, to heterologous cDNA probes, the positive subclones of 3-C-1-3-1, 11-A-c-1-1, 14-A-b-1-3 and 10-A-a-2 were screened with two serine-threonine phosphatase cDNAs, KAPP (PP-2C) and TOPP4 (PP-1), respectively. Screening was done under low stringency. These essentially gave the same results as oligonucleotide screening. However, the screening results of their DNA digested with various endonucleases present in the polylinker region, were not as consistent.

Two anomalies were noted for screening the PPS2C1 subclones with the KAPP cDNA probe. Oligonucleotide PPS2C1 did not bind to the KAPP cDNA that had been used as a control (Figure 3.5 B). However, previous experiments (results not shown) of the same type had been successful. It is possible that the low ratio of probe to template DNA may have prevented its binding to the cDNA. In addition, the oligonucleotide bound to a 0.8 kb *Sph* I fragment of 3-C-1-3-1, whereas the KAPP cDNA bound to a 3.5 kb *Sph* I fragment of the same subclone (Figure 3.5 ; lane 1, A, C).

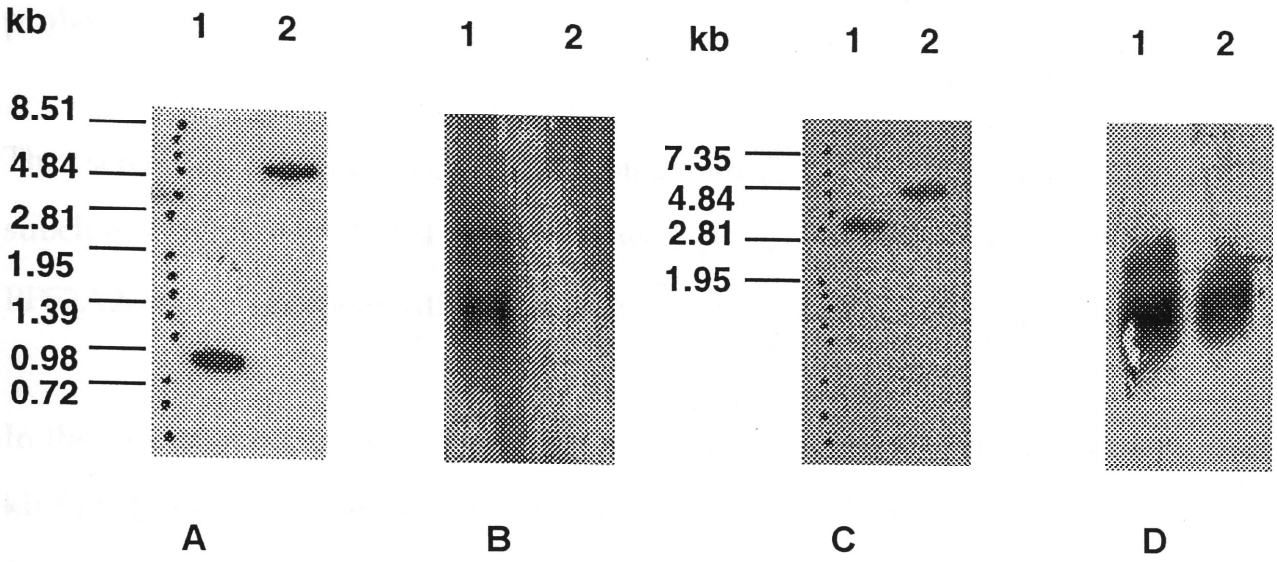


Figure 3.5: Southern Blot Analysis of EcoR I Subclone of 3-C-1-3-1 Using KAPP cDNA and PPS2C1 Oligo Probes. ***A.*** *Oligo screening of 3-C-1-3-1 digested with Sph I (lane 1), Cla I (lane 2).* ***B.*** *Oligo screening of uncut plasmid containing EcoR I insert of 3-C-1-3-1 (lane 1), and cDNA KAPP (Lane 2).* ***C.*** *cDNA screening of 3-C-1-3-1 digested with Sph I (lane 1), Cla I (lane 2).* ***D.*** *cDNA screening of uncut plasmid containing EcoR I insert of 3-C-1-3-1 (lane 1), cDNA KAPP (lane 2). DNA blots were first probed with oligonucleotide PPS2C1 (A, B). The same blots were probed with a 1.9 kb cDNA corresponding to PP-2C from Arabidopsis (KAPP) (C, D). Positive controls consisted of uncut plasmid containing EcoR I insert of 3-C-1-3-1 (lane 1; B, D), and the 1.9 kb cDNA (lane 2; B, D).*

KAPP also failed to bind to the λ PP-2C clones. This result may be explained by considering that the membrane blot in question had been re-screened several times as a 'control', and that frequent membrane stripping may have resulted in a loss of DNA samples. This would only be verified by re-screening the blot with the oligonucleotide probe.

The screening of endonuclease digested samples, of the three positive PPS2A2 subclones, with the TOPP4 cDNA showed positive binding as with oligonucleotide PPS2A2, except for some differences in fragment size (Figure 3.6 A).

In the case of subclone 11-A-c-1-1, PPS2A2 bound to a 0.6 kb *Hind* III (lane 1), and a 2.0 kb *Sac* I (lane 2) fragments, while TOPP4 bound to a 4.84 kb *Hind* III, and a 5.0 kb *Sac* I fragments. In the case of subclone 10-A-a-2, the oligonucleotide bound to the uncut (partially digested) *Apa* I band (lane 8), while the cDNA bound to the 5.0 kb band.

Similarly, the oligonucleotide bound to a 1.3 kb *Hind* III (lane 10) and a 2.0 kb *Sph* I (lane 11) fragments of the subclone 14-A-b-1-3, while the cDNA bound to the uncut *Hind* III sample and a 3.4 kb *Sph* I fragment respectively.

It is apparent that the oligonucleotide PPS2A2 has bound to shorter fragments except for lane 8. This suggests that the 20 mer sequence may be located on the shorter fragment but raises the question of why the cDNA probe does not bind to these fragments. Maybe the degeneracy has produced an oligonucleotide with a sequence not present in its heterologous counterpart? This contradicts previous experiments that had used the oligonucleotide probe to screen TOPP4 cDNA, and which resulted in a positive hybridization (Figure 3.3, lane 6). It is possible that the cDNA is more homologous to the insert at a region different from the one containing the oligonucleotide sequence. This may have caused the cDNA to hybridize strongly to larger fragments.

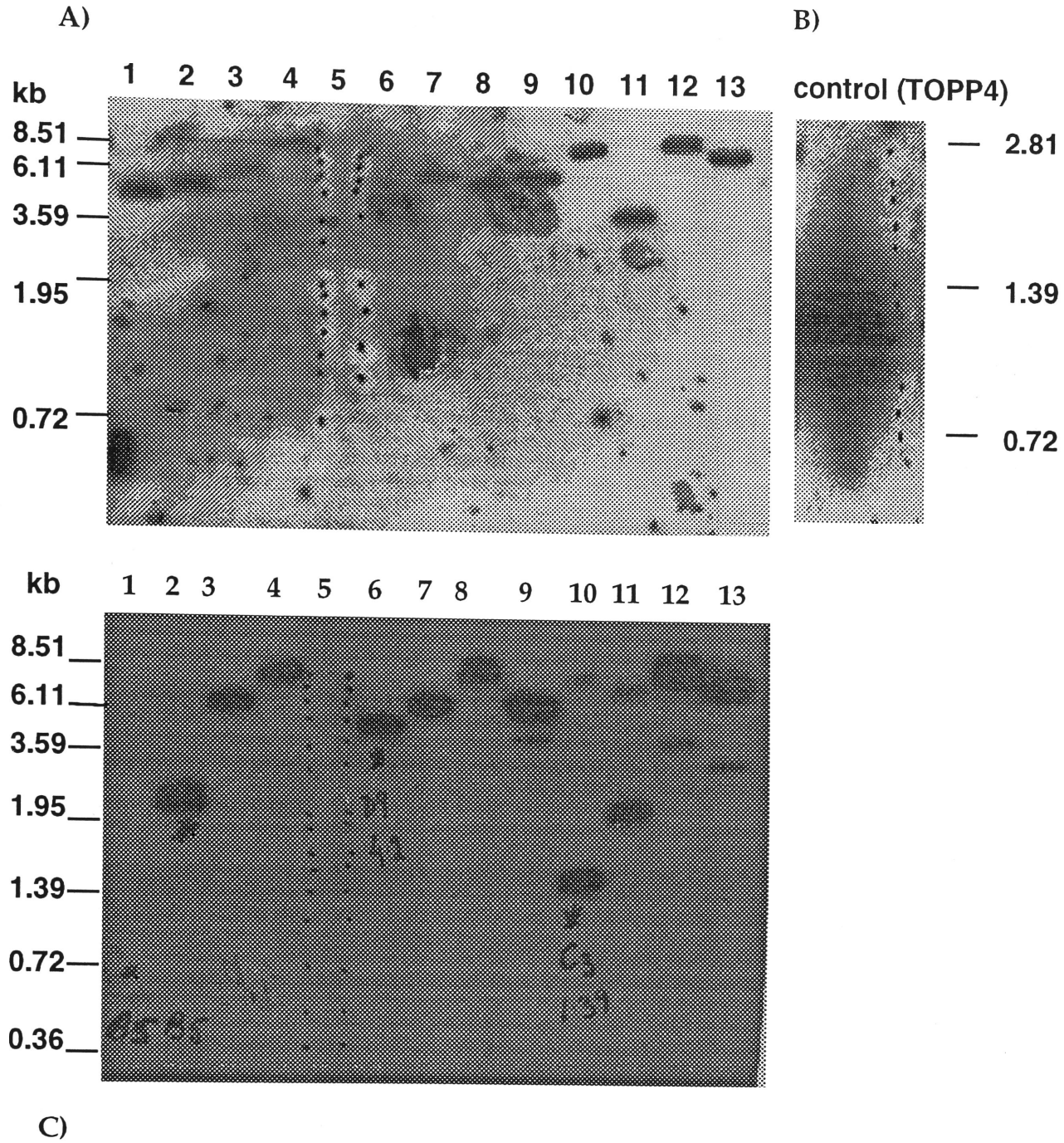


Figure 3.6: Southern Blot Analysis of EcoR I Subclones of PP-2A (pGEM vector) Digested with Various Restriction Enzymes. A. *DNA blots were first probed with 1.3 kb cDNA (TOPP4) corresponding to PP-1 from Arabidopsis.* ***B.*** *The cDNA was used as a positive control when screening with cDNA.* ***C.*** *The same blots were probed with oligonucleotide PPS2A2. cDNA was not used as a positive control when screening with oligonucleotide as it was previously shown to bind to the cDNA (see figure 3.3). Screening was at low stringency. 11-A-c-1-1 (lanes 1, 2; A, C), 14-A-b-1-3 (lanes 9-13; A, C), 10-A-a-2 (lanes 3, 4, 6-8; A, C). Lane 1: Hind III, Lane 2: Sac I, Lane 3: BstX I, Lane 4: Hind III, Lane 5: molecular size marker SPP-1 phage DNA/EcoR I, Lane 6: Xba I, Lane 7: Sma I, Lane 8: Apa I, Lane 9: Xba I, Lane 10: Hind III, Lane 11: Sph I, Lane 12: Apa I, Lane 13: Xho I.*

No binding of TOPP4 to the smaller bands in the above mentioned lanes was observed even after exposing X-ray film for a month. TOPP4 also failed to hybridize to λ PP-2A clones. A remote possibility is that the treatment of membrane blots to remove oligonucleotide PPS2A2 probe may have resulted in a loss of DNA from the membrane.

An alternative explanation for the different results obtained using the cDNAs may be suggested. It is possible that the cDNA probes were contaminated by the vector pBluescript sequences when they were isolated, and the bands observed in the Southern blot analysis against the plasmid subclones are due to cross-hybridization between the vector DNAs. This could also explain why both the cDNAs, TOPP4 and KAPP, failed to hybridize to the λ PP-2A, λ PP-2C clones, where no vector pGEM sequence is present. Similarly, the binding of TOPP4 to larger fragments as compared to the oligonucleotide PPS2A2 (Figure 3.6) may be explained to be the result of cross-hybridization between vector pBluescript sequence and the pGEM sequence of the plasmid subclones, where the binding of pBluescript to larger fragments is due to the production of larger fragments as a result of digestion of pGEM by the enzymes present in the polylinker.

3.6 SEQUENCING OF GENOMIC CLONES

An attempt was made to sequence the *Xba* I/*Eco*R I deletion subclones 14-A-b-1-3 and 10-A-a-2 using the M_{13} forward and reverse primers. No sequence could be obtained. Problems encountered with sequencing these templates included a failure to anneal the primers, possibly due to protein contamination. When conditions were altered to allow primer annealing, the resulting sequence contained band compressions. Because of time constraints alternative methods could not be used.

Sequencing of the original *Eco*R I subclones 3-C-1-3-1 and 11-A-c-1-1 from either end, using both M_{13} forward and reverse primers, gave a sequence of 201 bp and 276 bp

(forward primer), and a sequence of 195 bp and 312 bp (reverse primer) respectively (Figure 3.5). These only covered small portions of the two clones and the translation in all six reading frames could not identify the characteristic sequences of serine-threonine phosphatases.

In order to determine the proteins other than phosphatases that may be encoded by the sequenced regions, all four sequences were analysed using Blastx. For this, the nucleotide sequence was translated in all six reading frames and aligned with the combined GenBank/Swissprot protein database, at the National Centre for Biotechnology Information (NCBI), using the BLAST network service (February 28, 1996). The end sequences of 11-A-c-1-1 and 3-C-1-3-1 did not match significantly to any known protein sequences.

A)

```
  1 AAATTTGTGG ACTGAAAATT TCAGAATTTT GTTCATAGGA AAAAGATGAT
 51 TTTCCTTAGG AAGTTTGCTT CGAGGAAGAA CTCCAAGGGG GTTATTGGGG
101 AGTCAtCTGA CAATGAtCTC ATACCCGGAG TTGGCAGAaC TACGgTCGTG
151 CGAGTGgCGC ATACGCGTCA TGTAGGgCTG ATCTCAGAGT CACACATATC
201 TGCTATGCGC TCACGACTCA TACGTGAACC GATAGGCGAT ACGATAGCTA
251 GGACTGGCcT ACGAACCTGC TAGAACCGCA GCTAACTGAC TGCTgCGACT
301 CAGTGACTAG CT
```

B)

```
  1 CGCCAACTGT GCTGGTTTTG TCACGGCAGA TGTCTCGTGA AAGGACTTAG
 51 TACTAGAACC ATCGCACCTT GGTGGCGACT CAAAGGGGTT AAGCGGGAGA
101 ACACGCGTTA CCAGGTTCGC ccTCGAGAGA GTATAGCTAC GtCTGCTTGG
151 GTTATGATAT GATCGATACA GAGCTACTGC TACTAGCACT GATGCATCTG
201 CTGCCTATCT CTGACTGCTA CTAGCTAGTA CGACTCTCTC AATGACATGG
251 CTAGCATCAG cGGATCAaGT CGACTG
```

C)

```
  1 TCGTTCTCCT TATTCCTCCT TGACAGCAAG GGTCTTTGTT CACAATGGAC
 51 ACAGGCCTGA TAACCTGatC TGTGATCCTC CACACAGAGG GTTCCAGCTG
101 ATCGGGAAAT CATGGATGgA CcATATGATG CAGCACGTAG ATGACTTTCT
151 TCGACCTGAG CATGCATGTA GgATCACTAG TCAGGATCGG AGACG
```

D)

```
  1 GCGATGGCAA GCTTGTGGGG CCCATGTGGC TCTGCGCCCC CAATCTCAGC
 51 TCTATAAATT CACTTTTGTC CAGAAAAAAT AAAAAGAGAG ATttCGTCGC
101 GTttGCGATA CGGAGGCGCG CACATCTGTT CTTCATCTGA GGCAGATCTG
151 AGTCGTtGGC TATGGAGAGG ATCGTCGCAT CGTCATCATC ACTTCTTCAT
201 C
```

Figure 3.7: Nucleotide Sequence of Genomic Clones. ***A.*** *11-A-c-1-1 (reverse),* ***B.*** *11-A-c-1-1 (forward),* ***C.*** *3-C-1-3-1 (reverse),* ***D.*** *3-C-1-3-1 (forward). Nucleotide numbers are indicated on the left. Nucleotides in the lower-case need to be reconfirmed through sequencing.*

Chapter 4:

Characterization Of A Putative Protein Kinase cDNA Clone From Maize

A maize cDNA library was screened previously with a PCR amplified 0.5 kb probe (Zhang and Pryor; unpublished results) corresponding to the catalytic subdomain of a known protein kinase gene *Pto* from tomato (Martin *et al* 1993). The heterologous screening identified a 0.8 kb fragment (McPk2.1) which was subsequently cloned in the *Eco*R I/*Xho* I restriction endonuclease sites on the Bluescript SK$^-$ plasmid vector.

At least 194 base pairs from the 5' end of the clone had been sequenced using the M_{13} reverse primer (Zhang and Pryor; unpublished results), and the resulting amino acid sequence showed similarity to *Pto*. This comparison indicated that McPk2.1 was probably from a serine-threonine kinase gene in maize.

4.1 RESTRICTION MAPPING OF McPk2.1

In order to sequence McPk2.1 further, deletion clones were constructed that would enable the sequencing of short stretches of the McPk2.1 clone. The clone was digested with restriction enzymes corresponding to the restriction sites in the polylinker region of the Bluescript SK$^-$ plasmid vector, and a restriction map prepared (Figure 4.1). Three fragments were chosen for further sequencing: an *Eco*R I/*Xho* I fragment for sequencing with M_{13} reverse primer, an *Eco*R I/*Kpn* I fragment for sequencing with M_{13} forward primer, and a *Pst* I/*Xho* I fragment for sequencing with both M_{13} reverse and M_{13} forward primers.

4.2 SEQUENCING OF McPk2.1

An additional 638 bp were sequenced. The complete sequence of 832 bp is shown in figure 4.2. The first in-frame translation initiation codon ATG is located 19 nucleotides from the start of the sequence. Since no untranslated region can be detected at this end, the cDNA may be truncated. The first stop codon TAG occurs after 612 nucleotides. This is followed by a 220 bp 3' untranslated region. However, no polyadenylation

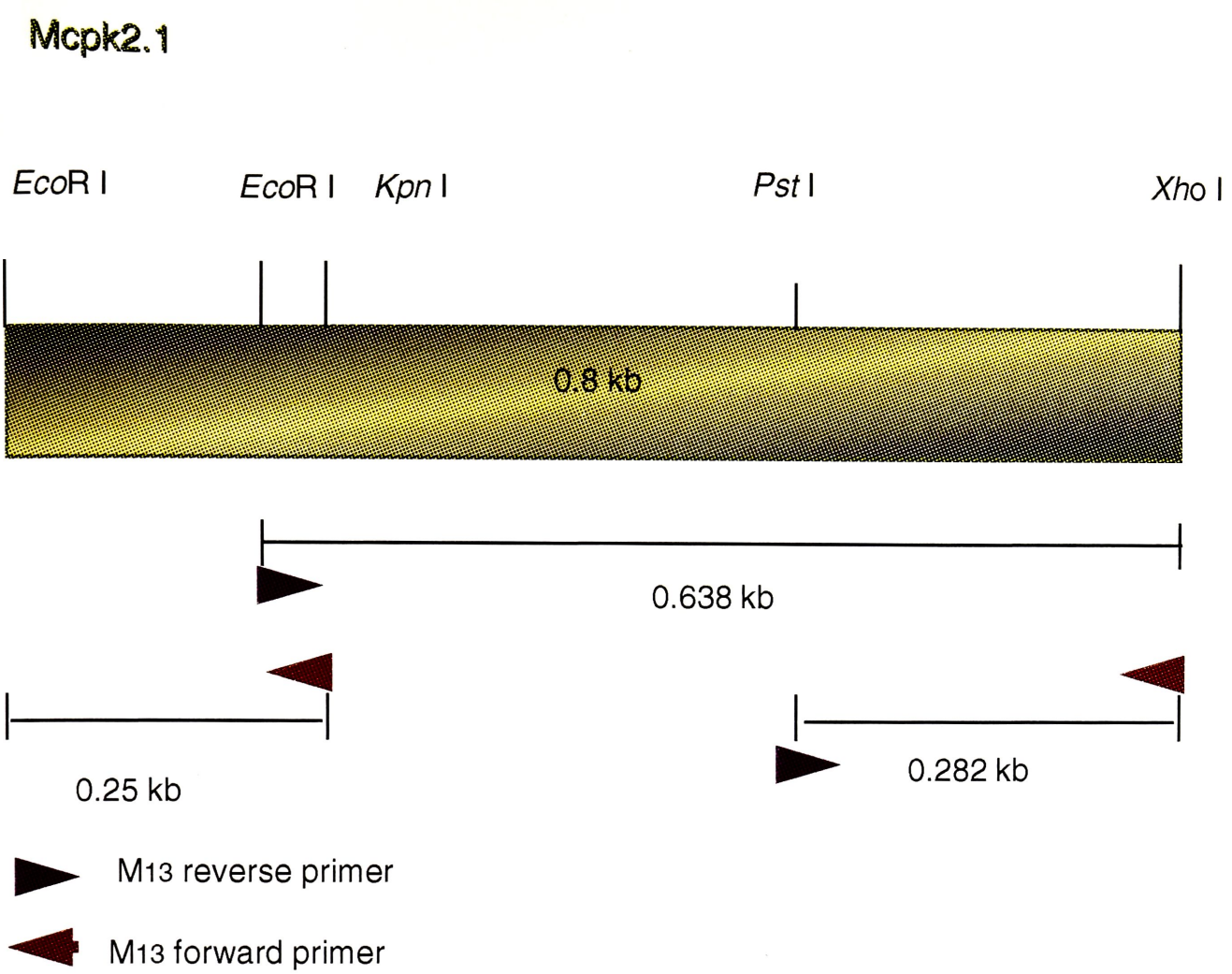


Figure 4.1: Restriction Mapping and Sequencing Strategy for Clone McPk2.1. *Restriction sites relevant for subcloning and sequencing are indicated.*

signal is found indicating that the cDNA transcript is incomplete from the 3' end. McPk2.1 was not sequenced completely from its 3' end; the sequence is ~13 bp short of *Xho* I cloning site in Bluescript SK⁻ plasmid vector.

4.2.1 Characterization of McPk2.1 Protein

The sequence analysis of McPk2.1 protein was performed using the BLAST network service (the National Centre for Biotechnology Information NCBI). Primarily, the service used was of BLASTx (combined GenBank/Swissprot protein database, February 1996).

The 832 bp DNA sequence of McPk2.1 insert was determined and a 612 bp open reading frame was found near the T_3 promoter in the Bluescript SK⁻ plasmid. The ORF encodes a 204 amino acid protein. Comparison of the deduced amino acid sequence of McPk2.1 with the protein sequences – using blastx (combined GenBank/Swissprot protein database), showed identity with the catalytic domains of several plant serine-threonine kinases (Table 4.1).

The ORF contains seven subdomains characteristic of protein kinases. The absence of subdomains I to IV indicates an incomplete cDNA. The remaining transcript contains all the invariant amino acid residues characteristic of protein kinases. In addition, the transcript contains sequences indicative of serine-threonine specificity that lie in subdomains VIb (DLKPEN), and VIII (G[T/S]XX[Y/F]XAPE). McPk2.1 protein contains 49.75% hydrophobic residues and 50.24% hydrophilic (charged and uncharged groups) residues. The comparison of the predicted McPk2.1 amino acid sequence with the deduced *Pto* amino acid sequence from tomato reveals that they share a highly conserved core region of about 113 residues (residues 5-118 of McPk2.1).

Percent Amino Acid Identity	Source	Description	Accession Number	Database
64	*Lycopersicon*	disease resistance protein kinase *Pto*	A49332	PIR
62	*Lycopersicon*	Fen gene product	U13923	GenBank
58	*Nicotiana tabacum*	protein serine-threonine kinase	D31737	DDBJ
57	*Arabidopsis*	protein tyrosine-serine-threonine kinase	D10152	DDBJ
54	*Solanum lycopersicum*	*Pto* kinase interactor 1	U28007	GenBank
45	*Zea mays*	receptor-like protein kinase	P17801	SWISS-PROT

Table 4.1: Percent Amino Acid Identity between McPk2.1 and Some of the Plant Proteins, Obtained Using BLASTx (combined GenBank/Swissprot protein database)

```
CCATCTTTATGGCTTAATATGCCGCTACTCAGCTGGAAGAAAAGACTGGAAATCTGCG      58
   P  S  L  W  L  N  M  P  L  L  S  W  K  K  R  L  E  I  C

TAGGAGCAGCAAGAGGGCTCCACTACCTTCACACCGGTTTTCCCCAGTCAATCATACACC  118
                (VIa)                                    (VIb)
V  G  A  A  R  G  L  H  Y  L  H  T  G  F  P  Q  S  I  I  H

GTGACGTCAAGTCAGCAAACATACTACTCGATGAAAATCTCTTGGCCAAGGTTTCTGATT  178
                                                         (VII)
R  D  V  K  S  A  N  I  L  L  D  E  N  L  L  A  K  V  S  D

TTGGCCTCTCAAAGGTTGGGCCTGAATTCGACCAGACGCATGTGAGCACAGCAGTGAAAG  238
F  G  L  S  K  V  G  P  E  F  D  Q  T  H  V  S  T  A  V  K

GGAGCTTCGGGTACCTTGATCCTGAGTACTTCCGGAGACAGAAGCTGACTGACAAATCAG  298
                (VIII)
G  S  F  G  Y  L  D  P  E  Y  F  R  R  Q  K  L  T  D  K  S

ATGTGTACTCTTTTGGTGTGGTGCTGCTTGAGGTGATTTGTGCGAGAGCGCCGGTTATCG  358
                (IX)
D  V  Y  S  F  G  V  V  L  L  E  V  I  C  A  R  A  P  V  I

ACCCCGACTCTTCCAAGGGACATGATCAACCTCCGCGGAGTGGGCTATCAAGTGGCAAAA  418
                                                      (X)
D  P  D  S  S  K  G  H  D  Q  P  P  R  S  G  L  S  S  G  K

GAGAGGAGAGCTTGATCAGATTGTCGACCAGCGCAcTTGCTGGACAGTAGGCCTGAAGCAC 478
R  E  E  S  L  I  R  L  S  T  S  A  L  L  D  S  R  P  E  A

TGAGGAAGTTTGGAGAGCAGTGGAGAAATGCTTCGAGAGTATGGGTTTGTCGAGCTCTAC  538
                                                   (XI)
L  R  K  F  G  E  Q  W  R  N  A  S  R  V  W  V  C  R  A  L

TATCGAGTGTCTGTGACTGCAGTTTGTCGGCAGTGCAGAGCGGAGGTCCAGCAATGTCCA  598
L  S  S  V  C  D  C  S  L  S  A  V  Q  S  G  G  P  A  M  S

ACAATCAACAGTATTGAatacgatctctgaactcccttcaaatagaaggaaggtgaactg  658
N  N  Q  Q  Y  *

gatagaattggttccagtcactggaaaatgtttgttacaaactagagatgcctcttgtgt  718
gactacccagcctatcccttcctcacatataccaacaccaaggttggtgaatccttattg  778
aaattttgtttggccacttgtaactactatgtctagaaaggtagtagcttatgc        832
```

Figure 4.2: Nucleotide Sequence and Deduced Amino Acid Sequence of cDNA Clone McPk2.1 from Maize. *The deduced amino acid sequence is shown in single letter code. The region of McPk2.1 corresponding to consensus protein kinase sequence is underlined. The positions of subdomains characteristic of protein kinases are indicated in parenthesis above the sequence. Residues highly conserved in all protein kinases are shown in bold. Residues indicating possible serine-threonine specificity are italicized. Nucleotide 'c' at position 453 was deleted to produce a frameshift in order to maximize the identity of McPk2.1 with Pto. However this deletion needs to be confirmed through resequencing. Nucleotide numbers are indicated on the right.*

```
McPk2.1       5 LNMPLLSWKKRLEICVGAARGLHYLHTGFPQSIIHRDVKSANILLDENLL  54
                |    :||..|||||:||||||||||||   ..|||||||| |||||||::
Pto         131 LPTMSMSWEQRLEICIGAARGLHYLHT...RAIIHRDVKSINILLDENFV 177

McPk2.1      55 AKVSDFGLSKVGPEFDQTHVSTAVKGSFGYLDPEYFRRQKLTDKSDVYSF 104
                :|:.|||:|| |.|:||||:||.|||.:||:|||| :.:||:|||||||
Pto         178 PKITDFGISKKGTELDQTHLSTVVKGTLGYIDPEYFIKGRLTEKSDVYSF 227

McPk2.1     105 GVVLLEVICARAPV..............IDPDSSKGHDQPPRSGLSSGK 139
                ||||:||:|||.::               ::.... . :| . ..|..
Pto         228 GVVLFEVLCARSAIVQSLPREMVNLAEWAVESHNNGQLEQIVDPNLADKI 277

McPk2.1     140 REESLIRLSTSALL.........DSRPEALRKFGEQWR.NASRVWVCRAL 179
                |.||| ::::..|:          .|..:.|:|:: .:| ..| :
Pto         278 RPESLRKFGDTAVKCLALSSEDRPSMGDVLWKLEYALRLQESVI*      321

McPk2.1     180 LSSVCD............CSLSAVQSGGPAMSNNQQY*             204
```

Figure 4.3: Amino Acid Sequence Comparison of McPk2.1 from Maize with Pto from Tomato. *Identical residues are shown as dashes (-), similar residues as (:), variable residues as (.). Comparison was made using the program BestFit from the GCG sequence analysis package. Gaps have been introduced to maximize alignment. Amino acid numbers corresponding to each protein are indicated on the right.*

Chapter 5: Discussion

5.1 CLONING OF PROTEIN PHOSPHATASE GENES FROM BARLEY

Our approach consisted of using degenerate oligonucleotide probes to screen a genomic library of barley. This approach was based on the information that the catalytic domains of protein phosphatases have amino acid residues conserved within eukaryotes (Cohen *et al* 1990; Klumpp *et al* 1994). Since the serine-threonine phosphatase PP-1, and PP-2A are 50% identical in their conserved domains, PP-2B is 40% identical to PP-1 and PP-2A, while PP-2C is distinct (Cohen 1991), we designed two probes, one corresponding to a region of seven amino acids (YGFYDEC) conserved within the catalytic domains of PP-1, PP-2A and PP-2B, and the other corresponding to a region of six amino acids (NCGDSR) conserved within the catalytic domain of PP-2C. Similarly, we designed another probe corresponding to a region of seven amino acids (VHCSAGV) conserved within tyrosine phosphatases. Five putative clones have been isolated.

Within the serine-threonine phosphatases, several isoforms have been found for each class (Cohen and Cohen 1989). The use of genomic library would, therefore, facilitate the screening from a larger gene pool of protein phosphatases and potentially lead to the cloning of more novel protein phosphatases as compared to the use of a cDNA library, which would confine the gene pool to certain developmental stage or stimulus that permitted their transcription. In addition, the genomic library would enable the identification of controlling elements like promoters and enhancers that could be used in later studies.

We screened with degenerate oligonucleotide probes that were designed by adopting the frequently used codons in barley. The screening resulted in the isolation of three genomic clones which putatively encode catalytic subunits of PP-1/PP-2A/PP-2B, and two genomic clones which putatively encode catalytic subunits of PP-2C. No genomic clones were isolated for tyrosine phosphatases.

Positive and negative controls were used for each round of screening after the first screening. These consisted of plaque membranes, from previous screening, that contained clones showing positive hybridization, and plaque membranes that contained clones which showed no hybridization.

When screening for tyrosine phosphatase genes, the third round of screening eliminated all the positives that were identified from the previous screening. This indicated that those positives were background. No further attempt has been made to search for tyrosine phosphatase genes. It is probably not surprising that tyrosine phosphatase gene clones were not identified in our screening. Research indicates that no conventional tyrosine kinase or tyrosine phosphatase has been cloned, so far, from higher plants even though tyrosine phosphorylation plays a role in plant cellular regulation (Stone and Walker 1995). The absence or the rare occurrence of tyrosine kinases and tyrosine phosphatases in plants may be explained by the existence of dual-specificity kinases and phosphatases that can phosphorylate or dephosphorylate both serine-threonine and tyrosine residues, and, in some cases, histidine residues (Haring *et al* 1995; Kim *et al* 1993; Walker 1994). Our result seemed supportive to the above view.

Screening for serine-threonine phosphatase genes has resulted in five putative clones being identified. The DNA inserts of the putative clones for PP-1/PP-2A/PP-2B, and PP-2C were digested with *Eco*R I, in order to extract the cloned insert from the λ vector. *Eco*R I digestion cleaved each of the inserts into a few fragments. The restriction patterns indicated that they were five distinct clones. In order to identify fragments containing sequences homologous to the oligonucleotide probes, these fragments were screened with the oligonucleotide probes. *Eco*R I fragments of ~2 kb (3-C-1-3-1), ~5.5 kb (1-C-2-2-1), ~3.2 kb (11-A-c-1-1), ~7.2 kb (14-A-b-1-3), and ~7.2 kb (10-A-a-2) were identified. This approach led to restricting the region of the genomic inserts to facilitate its subcloning into the plasmid vector pGEM 7Zf (+).

In order to further locate the region within the subclone that contained the putative phosphatase gene domain, the subclones were treated with restriction endonucleases corresponding to the restriction sites on the polylinker in pGEM 7Zf (+), and the resulting fragments probed again with the degenerate oligonucleotides. Restriction maps were constructed for the clones 3-C-1-3-1, 11-A-c-1-1, 10-A-a-2, and 14-A-b-1-3. Screening indicated that the oligonucleotide PPS2C1 had hybridized to a region of ~800 bp in clone 3-C-1-3-1, while oligonucleotide PPS2A2 had hybridized to a region of ~1500 bp in clone 11-A-c-1-1, and ~1200 bp regions in clones 10-A-a-2, and 14-A-b-1-3.

The ~1200 bp region was located in the *Xba* I/*Eco*R I deletion subclones of 10-A-a-2, and 14-A-b-1-3. These subclones were sequenced subsequently using the M_{13} reverse and forward primers. However no sequencing data could be obtained. Two problems were encountered repeatedly — a failure to anneal the primers, and band compressions in the sequence. The former was possibly because the DNA was contaminated with proteins. The subsequent use of phenol extraction did not help. Because of time constraints, alternative methods including dimethyl sulfoxide in the annealing step, 7-deaza dGTP instead of dGTP, other sequencing primers such as T7 or PUC, or *Taq* DNA polymerase could not be used.

Due to time constraints deletion subclones were not made for 3-C-1-3-1 and 11-A-c-1-1, instead the original *EcoR* I subclones were sequenced from both ends using the M_{13} forward and reverse primers. The sequence data obtained only covered part of the two subclones and did not include regions coding for the amino acid sequence motifs corresponding to the oligonucleotide probes, or to any other conserved domains of serine-threonine phosphatases. It is possible, therefore, that the target domains lie further in the middle. Additional sequencing may lead into these domains.

The target domains (PPS2C1 and PPS2A2) are normally flanked by other domains (Figure 1.2). It is possible that the deduced sequences of the clones 3-C-1-3-1 and

11-A-c-1-1 have not identified these domains because the domains lie further upstream or downstream. Furthermore, the ~800 bp and the ~1500 bp regions, to which the oligonucleotide hybridized, are located within large inserts in the genomic DNA clones. It is possible that the flanking domains may be situated in the remaining stretch of the total insert.

Several alternatives were available to us for verifying the λ clones. These included PCR amplifying genomic inserts from λ vector using the oligonucleotide primers and sequencing the amplified product, or PCR amplifying from the plasmid subclones. The former approach was preferred since it would prevent subcloning. Furthermore, the inserts could be sequenced with degenerate primers. Our aim was to sequence the flanking region (s) and to obtain one of the conserved amino acid sequence motifs that would be sufficient to establish the identity of the putative clones. This would abolish the need for sequencing the entire stretch of the genomic insert or of producing deleted subclones for sequencing.

Attempts were made to amplify the region flanking the oligonucleotide sequence (NCGDSR) from the putative PP-2C λ clones using the method of RACE-PCR (Innis *et al* 1990). Initially, the template was amplified unidirectionally with the degenerate oligonucleotide. After ten rounds of amplification, a polynucleotide-A tail was added. The next stage consisted of amplifying the PCR product using the degenerate oligonucleotide PPS2C1, and oligonucleotide dT, as primers. However, no desired PCR products were obtained.

Degenerate oligonucleotides ranging from 21 mer to 28 mer have been used in several studies as PCR primers for amplifying cDNA and genomic inserts (Arino *et al* 1993; Smith and Walker 1991). Our approach would have also been successful, given time. Only a few parameters like the template concentration and the annealing temperature were altered, without success. Because of time constraints we did not amplify plasmid

subclones. We did, however, sequence the PP-2C template with the degenerate oligonucleotide but obtained a poor annealing of the primer to the template.

Time has not allowed further characterization of clones 1-C-2-2-1, 10-A-a-2, and 14-A-b-1-3. The identity of these putative clones can only be speculated. They may either contain the same catalytic domain or belong to any one of the three catalytic domains of PP-1/PP-2A/PP-2B. Isoforms of serine-threonine phosphatases have been reported in plants and animals. In plants, reports have characterized at least five isoforms of PP-1 (Smith and Walker 1993), and at least four isoforms of PP-2A (Arino *et al* 1993). Therefore, the putative clones may be genes encoding isoforms of serine-threonine phosphatases. PP-X, PP-Y, and PP-Z are novel catalytic subunits occurring in plants and animals (Perez-Callejon *et al* 1993; Cohen and Cohen 1989), but they all contain the sequence of PPS2A2. It is probable that our screening with the oligonucleotide probe may identify genes coding for these phosphatases.

The sequencing of plant phosphatase genomic clones has in some instances revealed 2 to 11 introns (Arino *et al* 1993; Slabas *et al* 1994)). It is therefore possible that some of the five putative clones may contain introns. Furthermore, they may possess flanking sequences from adjacent genes that may have contributed to the difference in the size and the restriction pattern.

A wide array of techniques are available for cloning. In this study, homologous hybridization using degenerate oligonucleotide probes was employed. Oligonucleotide probes, by virtue of their short sequence, can bind to many more clones than cDNA probes. However, the degeneracy of the oligonucleotide as well as its short sequence may reduce its specificity. Heterologous cDNA probes could also be used. Two such cDNA clones were available only at a late stage and were used to re-screen the isolated clones.

Additional sequencing of the isolated clones is required to determine their identity. An efficient way of sequencing the subclones would be by making nested deletions. This involves digesting the DNA from a 5' protruding or blunt end using Exonuclease III and producing unidirectional deletions (Erase-a-Base system by Promega).

5.2 *CLONING OF SERINE-THREONINE KINASE GENES FROM MAIZE*

We have also isolated and partially characterized a cDNA clone McPk2.1 encoding a putative serine-threonine kinase from maize. The cDNA of 0.8 kb was sequenced mostly from both strands, however sequencing results indicate that it is partial. McPk2.1 encodes 204 amino acid residues that contains characteristic serine-threonine kinase domains. In order to obtain a full length cDNA, maize genomic library could be screened with the McPk2.1 insert.

The clone shows a good homology, in its amino acid sequence, to a disease resistance conferring serine-threonine kinase from tomato called Pto (Martin *et al* 1993). *Pto* is cytosolic, with a myristylation site at the amino terminal, necessary for membrane association. Since McPk2.1 is truncated at the amino terminal, a similar function can only be suggested.

Pto is one of several plant genes, cloned recently, involved in disease resistance. The others include *RPS2, N, Cf-9, Xa21* and L^6 (Jones *et al* 1994; Lawrence *et al* 1994; Mindrinos *et al* 1994; Song *et al* 1995; Whitham *et al* 1994). Except for the protein product of *Pto,* all have leucine-rich repeats thought to be involved in protein-protein interaction. *Xa21* is a transmembrane receptor-like protein kinase while most of the others are cytosolic with signal anchors at the amino terminal suggesting an interaction with transmembrane proteins. Among these proteins, *Pto* and *Xa21* contain serine-threonine kinase domains. All the proteins are thought to be involved in the initial stages of transduction of the elicitor signals that lead to a hypersensitive response.

Even though McPk2.1 was isolated using *Pto* as a probe, further studies would be required to ascertain its physiological role. One way would be to conduct complementation studies in mutants of *Pto*, using the full length McPk2.1.

The fact that the gene expression of *Pto* does not depend on infection and is observed in both susceptible and resistant plants suggests that *Pto* may be involved in other cellular activities as well (Martin *et al* 1993). Maize plants could be transformed with antisense RNA corresponding to McPk2.1 and the phenotypic effect observed. Furthermore, mapping studies of McPk2.1 in maize will serve to localize it at or near loci that may be carrying genes for important agronomic characteristics, allowing its use as a genetic marker.

Pto belongs to a gene cluster of five to eight members (Martin *et al* 1994). Other gene clusters common to plants include those encoding peroxidase isozymes, small subunits of ribulose bisphosphate carboxylase, and chlorophyll a/b binding proteins (Martin *et al* 1994 and references within). It is possible that McPk2.1 may belong to a similar gene cluster. This could be ascertained by Southern blot analysis of maize genomic DNA.

5.3 *FUTURE DIRECTIONS*

From the studies on cell-cycle regulation in animal cells, the serine-threonine phosphatases are known to regulate protein function positively and negatively. Their regulation by second messengers such as calcium for PP-2B, and cAMP-dependent PKA for PP-1, and their response to plant hormones like abscisic acid indicate that they may play key roles in signal transduction. However, the occurrence of the large gene pool as evidenced by the multigene families found for PP-1 and PP-2A in plants and the occurrence of isoforms for all classes of serine- threonine phosphatases suggest that they may either have a broad substrate specificity and contain functionally redundant members, or they may be highly specific. In the latter case their specificity may be

under the influence of several parameters such as their intracellular location, their coupling to regulatory subunits, and their isoforms. The specificity may also be developmentally regulated.

Plant serine-threonine phosphatases are suggested to be involved in cellular processes ranging from metabolic pathways to cell cycle to signal transduction. These roles have been assigned to phosphatases either through complementation studies (Nitschke *et al* 1992), structural studies (Stone *et al* 1994), or mutational analysis (Leung *et al* 1994). Many more isoforms have been identified in plants than the roles assigned to them. One of the long-term aims of the project is, therefore, to determine the functional significance of phosphatases in barley. The choice of the plant system barley was made on the fact that it is one of the important agronomic crops in the world, and that to date, phosphatases have not been cloned from it.

Although the amino acid sequence data would indicate the class to which the phosphatase belongs, its ability to phosphorylate serine-threonine residues or additional residues such as tyrosine or histidine would need to be demonstrated through phosphorylation assays. Questions like the sub-cellular location of the phosphatase, its developmental regulation, and the existence of multigene families would need to be addressed. Its physiological role could be assessed in two ways:

- expressing the antisense RNA in barley under normal or stressed conditions and studying its phenotypic effect in transgenic plants,
- conducting complementation studies in yeast or lower green plants such as algae.

Further elucidation of the signal transduction pathways in which both McPk2.1 and the putative phosphatases play a role will require the identification of proteins that act both upstream and downstream. One way of identifying the proteins involved is by the method of 'interactive cloning' (Skolnik *et al* 1991). This involves screening expression

libraries with polypeptides known to interact with other proteins. This method has been successful in cloning KAPP, that interacts with a receptor- like kinase RLK5 (Stone *et al* 1994), and a helix-loop-helix zipper protein that interacts with c-Fos (Blanar and Rutter 1992).

These results will pave the way for a better understanding of the regulation of phosphorylation in plants in normal plant function such as development, and in disease resistance, with useful implications for agriculture.

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Appendix

2.1.1

2YT media (For two litres):

Bacto-tryptone	32 g
Yeast extract	20 g
NaCl	10 g
pH 7.0	

2.1.2

SM Buffer (1 litre):

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g
Tris Cl (pH 7.5) (1 M)	50 ml
Gelatine (2%)	5 ml

NZCYM Top Agarose (For 400 ml):

Yeast extract	2 g
NZ amine	4 g
NaCl	2 g
$MgSO_4.7H_2O$	0.8 g
Casamino acids	0.4 g
0.7% Agar	0.7 g/100 ml

LB agar (1 Litre):

Bactotryptone	10 g
Yeast extract	5 g
NaCl	10 g
pH 7.5	
Agar	15 g

2.2.2

1 x STE buffer:

Tris Cl (pH 8.0)	10 mM
NaCl	100 mM
EDTA (pH 8.0)	1 mM

2.3.2

Denaturing Solution:

NaCl	1.5 M
NaOH	0.5 M

Neutralization Solution:

NaCl	1.5 M
Tris-HCl (pH 7.2)	0.5 M
EDTA	0.001 M

2.3.3

Prehybridization buffer for screening with oligonucleotide:

6 x SSC
1 x Denhardts
0.5 % SDS
100 µg/ml Salmon testes sperm DNA
0.05 % Sodium pyrophosphate

50 x Denhardt's Solution (500 ml):

Ficoll	5 g
Polyvinylpyrrolidone	5 g
Bovine Serum Albumin (Pentax Fraction V)	5 g

Stored at -20°C in aliquots of 25 ml

20 x SSC (For 1 Litre):

NaCl	175.3 g (3 M)
Sodium citrate	88.2 g (0.3 M)

pH 7.0

2.4

GSE Buffer:

Guanidine isothiocyanate	3 M
N- Lauroyl Sarcosine (Sarkosyl)	2.5 %
EDTA (pH 8.0)	10 mM

pH 7.5

Agarose Gel Electrophoresis

A Biorad Gel Electrophoresis apparatus was used to perform agarose gel electrophoresis. 1 % agarose gel was made in 1 x TAE buffer. After restriction digestion DNA samples were mixed with 1 µl of 10 x Ficoll dye and loaded into the wells. The gel was run in 1 x TAE buffer at 80–90 V and then stained in a solution containing ~3 µl ethidium bromide in ~100 ml 1 x TAE for 5 minutes. The gel was photographed over UV light, using 667 polaroid film in the polaroid land camera. SPP-1 bacteriophage DNA restricted with *Eco*R 1 was used as molecular weight marker

50 x TAE (1 Litre):

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (pH 8.0)	0.5 M

pH 7.0–7.5

Agarose Loading Dye (10 ml):

Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Ficoll	1.5 g

2.5.2

SOC Medium (100 ml):

SOB medium	100 ml
Glucose	20 mM

2.5.3

SOB Medium (1 Litre):

Tryptone	20 g
Yeast extract	5 g
NaCl (1 M)	10 ml
KCl (1 M)	2.5 ml
Autoclave and add $MgSO_4$	10 mM

RNase A (10 ml):

RNase A	100 mg
Tris.Cl (0.01 M)	100 µl
NaCl (0.015 M)	150 µl

Heat for 15 minutes at 100°C in a waterbath

2.6.2

6% Polyacrylamide Sequencing Gel (80 ml):

30% Acrylamide	16 ml
Urea	36.8 g (8M)
5xTBE	16 ml
H_2O to	80 ml
10% Ammonium persulfate	400 µl
TEMED	100 µl

30 % Acrylamide (300 ml):

Acrylamide	87 g
N, N' methylene bisacrylamide	3 g

pH 7.0 or less

30 x TBE (1 Litre):

Tris base	324 g
Boric acid	165 g
0.5 M EDTA (pH 8.0)	120 ml