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Expression of a mammalian cytochrome P-450 in *Nicotiana tabacum* for bioremediation of PCB contaminated soils

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EXPRESSION OF A MAMMALIAN CYTOCHROME P450 IN *NICOTIANA TABACUM* FOR BIOREMEDIATION OF PCB CONTAMINATED SOILS

by

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STATEMENT BY AUTHOR

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ABSTRACT

EXPRESSION OF A MAMMALIAN PCB-METABOLIZING CYTOCHROME P450 IN *NICOTIANA TABACUM.* V.D. Wall, D. Galbraith, J.R. Halpert and D.P. Bourque

Polychlorinated biphenyls (PCBs) are resistant to metabolism in most animal species. The dog has the unique ability to metabolize and eliminate certain PCB congeners, as a result of the activity of the cytochrome P450 isozyme PBD-2. An expressible cDNA coding for PBD-2 has been introduced into the genome of tobacco plants. The PBD-2 cDNA coding sequence and a screenable marker gene coding for neomycin phosphotransferase II were introduced into tobacco leaf disks using a binary *Agrobacterium tumefaciens* vector system. Southern and Western blot analysis have confirmed chromosomal integration of the cDNA and expression of the PBD-2 polypeptide. Differential centrifugation and Western blot analyses have shown the PBD-2 protein to be associated with a membrane fraction in transgenic tobacco leaf homogenates. Measurements of marker enzymes from linear sucrose gradient fractions and Western blotting show the PBD-2 protein to be associated with the endoplasmic reticulum. Our goal is to develop transgenic plants in which the PBD-2 protein metabolizes PCBs, thus providing a novel method for bioremediation of PCB-contaminated soils.

CHAPTER 1

INTRODUCTION

A) History of Polychlorinated Biphenyl Production and Use

Commercial production of polychlorinated biphenyls (PCB's) began in the late 1920's when Monsanto marketed mixtures of PCB congeners under the trade-name of Aroclor. By the 1960's commercial production of PCB mixtures was occurring in most industrialized nations. It has been estimated that from 1929 and 1980, over 1 million tons of polychlorinated biphenyls have been manufactured by the United States, Western Europe and Japan, while estimates for the manufacture of PCB's in the U.S.S.R and Czechoslovakia are not available [1, p.100]. In 1977 the manufacture, commercial distribution and use of PCBs in non-closed systems was banned in the United States. By 1984 only France, Spain and possibly Eastern block countries were still manufacturing PCB's [1, p.100]. The method of production of PCB's involves batch chlorination of biphenyls for variable amounts of time depending upon the extent of chlorination desired, followed by separation and purification to yield the desired congener composition [2, p.12]. Theoretically, the chlorination reaction can synthesize 209 different congeners of which only about 100 are found in commercially available products [3, Figure 1]. The PCB production process often leads to contamination of commercial mixtures by polychlorinated dibenzofurans (PCDF's) and polychlorinated dibenzodioxins (PCDD's) [2, p. 15].

Polychlorinated biphenyls exhibit unique chemical and physical characteristics that are responsible for their widespread industrial use, including very low electrical conductivity, high thermal conductivity and high resistance to thermal and chemical breakdown. Industrial uses of PCBs can be classified as closed, open or nominally

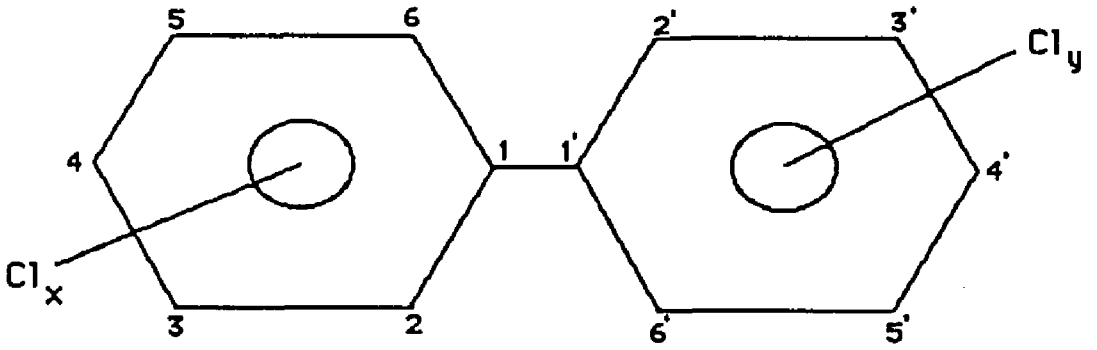

Figure 1: Generic structure of PCBs. There are 209 possible congener structures, where x and y represent the number (one to five) of the chlorines in each ring of the parent compound, biphenyl.

closed systems depending on the degree of exposure to, and potential of release into, the environment [1, p.104]. Nominally closed applications include hydraulic fluids, heat transfer fluids and lubricants, while open-ended applications include plasticizers, carbonless copy paper, adhesives, wax extenders, inks, dedusting agents, herbicides, pesticides and other uses [2, p.32]. The most significant use of PCB's from 1930-1980 among the United States, Western Europe and Japan was in closed systems, primarily transformers and capacitors. During the late 1960's and the early 1970's, the use of PCB's in the manufacture of plastics was the primary application in the United States.

B) Accidental Release of PCBs into the Environment

It is not difficult to imagine the potential for significant occupational exposure and global environmental contamination based upon the extensive industrial use of these compounds. The unique characteristics of PCB's that led to their wide-spread use also contribute to their environmental persistence and bioaccumulation. Significant incidents of PCB release into the environment continue to occur as a result of accidents and improper waste management procedures [1, p.17, Figure 2]. The two largest single incidents of accidental PCB release that have occured to date are Yusho and Yu-Cheng. The Yusho, ("oil disease" in Japanese), accident occured in October 1968 in Fukuoka, Japan. Over 1800 people were exposed to PCB mixtures by their consumption of contaminated cooking oils. The commercial mixture of PCB, Kanchlor 400, was being used to heat the cooking oil under reduced pressure to remove contaminants in the final step of production. Small holes were found in the metal casing separating the PCBs from the cooking oil [4, p.384]. The oil contained PCB levels of almost 1000 ppm [4, p.384], and it was estimated that, on average, the victims ingested 633 mg PCBs, consuming 157 μg PCBs/kg body weight/day [4, p.386]. The Yu-Cheng, (oil disease in

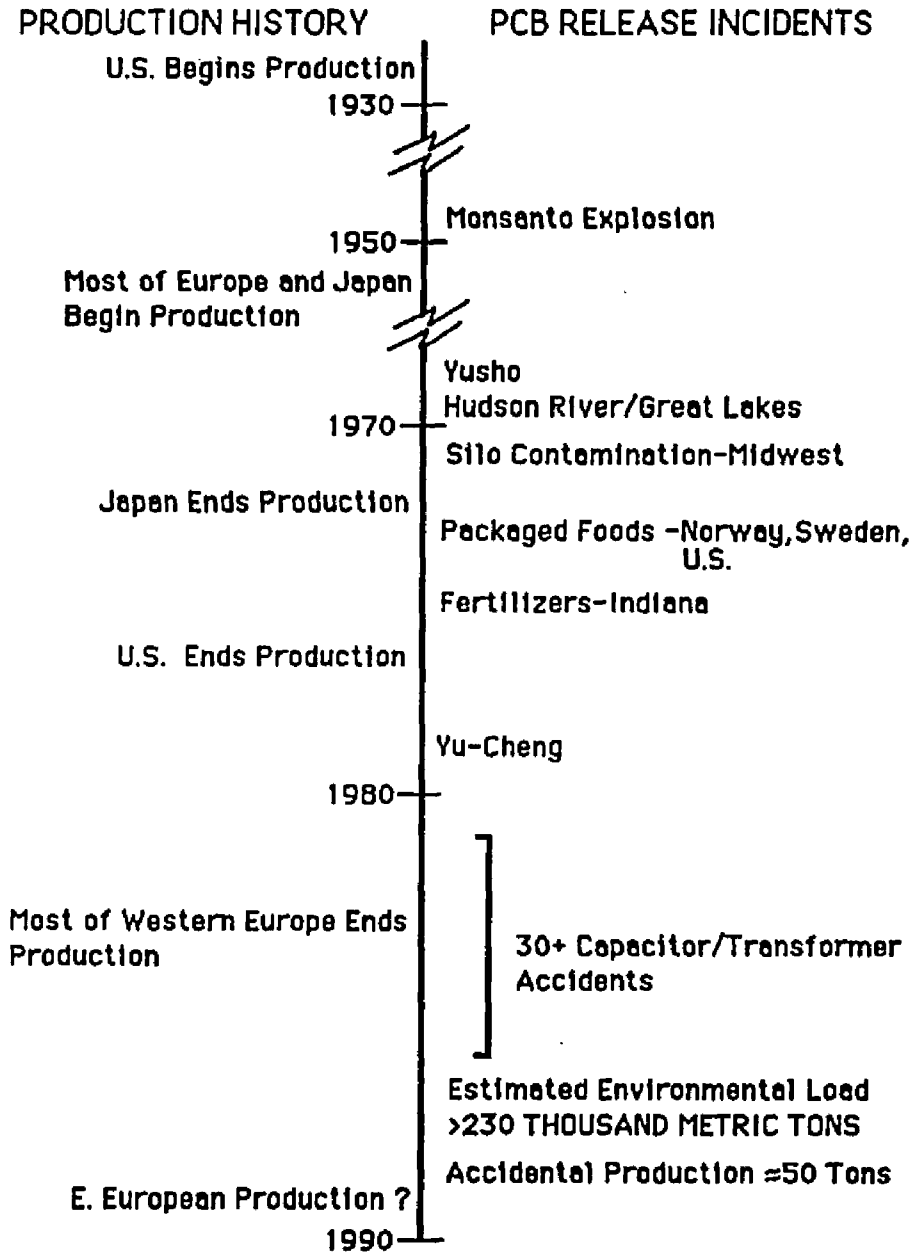

Figure 2 : Brief history of PCB production and major incidents of PCB release into the environment.

Chinese), incident occured in early 1979 in Taiwan and affected 2000 people. The accident was very similar to the Yusho poisoning, but the cause of the contamination has never been established. The concentrations of PCB found in the oil were considerably lower, but the rates of consumption were high enough to yield comparable exposures [4, p.386].

Previously unrecognized accidental production and release of PCBs into the environment have recently been discovered. It is estimated that, in the United States, 50 tons of PCBs have reached the environment as by-products of industrial processes. The USEPA has listed 80 compounds with the potential to form PCBs as contaminants during their production process [2, p.20]. Table 1 from O'Keefe et al.1989, [5, p.421] is a review of some of the recent transformer/capacitor (the primary closed application of PCB's) accidents that have occured in the United States, Scandinavia and France. There are many differing estimates of the environmental load of PCB's, but it is clear that hundreds of thousands of tons of these highly persistent compounds are present in various environmental matrices throughout the world [1, p.173].

C) Toxicity and Metabolism

After the possibility of global PCB contamination became a reality and the toxic effects of accidental PCB poisoning became apparent, there were, and continue to be, research efforts made to determine the effects of these compounds on various life forms. There are differences in the signs of toxicity dependent upon the dose, the duration of exposure, congener composition, chemical contaminants and animal species. Although a great deal of data concerning PCB metabolism and toxicity in animals has been collected and numerous effects have been claimed, there still remain many unanswered questions [6,161-162].

Table 1: Review of some of the recent transformer/capacitor accidents

Background information on PCB capacitor/transformer accidents

Location	Date of incident	Building type	Electrical equipment	PCDFs and related compounds	Description of accident	Reference
Nortalje, Sweden	Sept. 25, 1978	outdoors	capacitors	+	fire	Jansson and Sundstrom 1982
Binghamton, NY, U.S.A.	Feb. 5, 1981	multistory office	transformer	+	fire and explosion with release of 180 gals Askarel (65% Arocior 1254 and 35% chlorobenzenes); soot spread through building	O'Keefe et al., 1985
Stockholm, Sweden	Aug. 25, 1981	power station	capacitor	+	explosion and fire	Rappe et al., 1983, 1985a,b
Skovde, Sweden	March 19, 1982	metal treatment factory	capacitors	+	fire involving mineral oil and PCB capacitors	
Imatra, Finland	Aug. 2, 1982	paper mill	capacitors	+	explosion and fire with release of 26 gals Clophen A30	Rappe et al., 1985a,b
Maplewood, MN, U.S.A.	June 22, 1982	private school	transformer	-	pressurized release of 50 gals 45% Arocior 1260, 45% chlorobenzenes	Hryborczuk et al., 1986
Surhammar, Sweden	Sept. 23, 1982	steel mill	capacitors	+	explosion of an oven followed by molten steel igniting capacitors	Rappe et al., 1985a,b
Hallstahammar, Sweden	Nov. 8, 1982	foundry	capacitors	+	explosion in capacitors beside oven	Rappe et al., 1985a,b

Background information on PCB capacitor/transformer accidents

Location	Date of incident	Building type	Electrical equipment	PCDFs and related compounds	Description of accident	Reference
Southern, Sweden	Winter, 1982	locomotive	capacitor	+	explosion	Rappe et al., 1985a,b
Kisa, Sweden	April 25, 1983	saw mill	capacitor	+	explosion associated with external arcing and fuming	Rappe et al., 1985a,b
San Francisco, CA, U.S.A.	May 15, 1983	multi-story office	transformer	+	fire in PCB transformer vault beside building; fire generated soot while smouldering for 8 h.	Stephens, 1986
Chicago, IL, U.S.A.	Sept. 23, 1983	multi-story office	transformer	+	fire in a bus bar between the transformer and a switchgear	Orris et al., 1986
Syracuse, NY, U.S.A.	Dec. 1983	multi-story office	transformer	trace	an overheated transformer released 12 gals PCB through a pressure-relief valve	Stark et al., 1986
Reims, France	Jan. 14, 1985	six-story residence	transformer	+	an overloaded transformer exploded, paper contaminated with the dielectric fluid (60% PCBs, 40% chlorobenzenes) caught fire	Rappe, 1986
Santa Fe, NM, U.S.A.	June 17, 1985	two-story office	transformer	+	transformer overheated and released fluid (87% Arocior 1260 and 13% chlorobenzenes) for 65 min through a pressure-relief valve	NIOSH, 1986

1) Toxicity

a) Animal Studies

Pathological symptoms in most animals exposed to acute doses of PCB mixtures consisted of a decrease in body weight, edema, lymphoid atrophy, hepatomegaly, porphyria and others as summarized in Table 2. Clinical signs of toxicity with chronic doses of PCBs are similar to symptoms in animals given acute doses but include chloracne and reproductive dysfunction [6, p.165]. Chronic exposure of mice and rats to PCB mixtures has also been shown to cause neoplastic nodules and hepatocellular carcinomas at a frequency greater than controls [as reviewed in 7]. The 2,2'4,4'5,5'-hexachlorobiphenyl(2,4,5-HCB) congener is almost completely unmetabolized in the rat and is thought to be carcinogenic, while the readily metabolized 2,2',3,3',6,6'-hexachlorobiphenyl has not been shown to be carcinogenic [8, 9]. There are significant differences in susceptibility to intoxication between species, but generally female animals, young animals, poultry, guinea pigs and non-human primates are most susceptible [6, p.164].

b) Human Studies

Unfortunately, the occurrence of numerous accidental poisonings of human populations, Yusho and Yu-Cheng in particular, have necessitated epidemiological research into the toxicological effects of PCB's on humans. The aforementioned contaminants (PCDF's) and (PCDD's), the numerous congener mixtures created during the production of PCB's, differing research goals and differences in sensitivity of analytical techniques have made interpretation and comparison of many human epidemiological study results difficult. Table 3 is an overview of some of these results.

Acute exposure of humans to PCB mixtures has resulted initially in temporary immunological suppression as well as skin, respiratory and eye irritation. Over a

Table 2: Overview of toxicological responses of various animals to polychlorinated biphenyl exposure.

PCB congener or mixture	Pathological symptom	PCB Exposure Acute	Chronic	Animal species	Reference
Aroclor 1248	Learning Deficits		✓	Rhesus Monkeys	Bowman et al. 1978 [10].
Kanechlor 400	Pulmonary Edema	✓	✓	Rat	Shigamatsu et al. 1978 [11].
3,4-TCB	Immono-suppression, Hepatmegaly	✓		Mice	Silkworth et al. 1982 [12].
Aroclor 1260	Liver Tumors		✓	♀ Rats	Kimbrough et al. 1975 [13].
1-CB,6-CB	↑ Intestinal and Serum Marker Enzymes		✓	Rat Neonates	Walden et al. 1982 [14].
245-HCB	Keratinosis of Epidermis		✓	Rabbits	Vos et al. 1972 [15].
Aroclor 1254	Reproductive Dysfunction		✓	Mink	Aulerich et al. 1977 [16].

Table 3: Overview of toxicological responses of humans exposed to polychlorinated biphenyls.

PCB congener or mixture	Pathological Symptom	Exposure Incident	Reference
Kanechlor 400	Immunosuppresion	Yu-Cheng	Chang et al.1982 [17].
Kanechlor 400	Hyperpigmentation in Exposed Women and their Newborn Children	Yusho/Yu-Cheng	Rogan 1982 [18].
Fenclor 54	Miscarriage	Italy	Leoni et al. 1989 [19].
Kanechlor 400	Pulmonary Edema	Yusho	Shigematsu et al. 1978 [12].
Kanechlor 400	Hepatic Porphyria in Transplacentally Exposed Children	Yu-Cheng	Gladen et al. 1988 [21].
Kanechlor 300 and Kanechlor 500	Chloracne	Chronic Occupational Exposure	Hara 1985 [22].
Clophen A30	Elevated S-ASAT and S-ALAT Activities	Capacitor Explosion in Imatra, Finland	Elo et al. 1984 as reviewed in [1, p. 164].

period of months after acute exposure to high concentrations of PCB's in soot (16-20 mg/m^3 air), abnormal pigmentation, fingernail ridges and in some cases chloracne have resulted. Other effects of high level short-term exposure include headache, nausea, irritability, sleep disturbances, fatigue, nervousness and impotence [1, p.163]. One of the most important targets of PCB exposure in humans is the liver. Even acute exposure has been implicated in causing temporary changes in liver morphology, hormonal status, lipid metabolism and elevated serum levels of liver enzyme activities. Recovery may take several weeks or months [1, p.163].

Chronic exposure to PCB's has been found to cause a high incidence of chloracne, the severity of which has been correlated in a dose-response manner with the blood levels of PCB's, particularly with the highly chlorinated species [1, p.165, 21]. It is important to mention here that some researchers have attributed these symptoms to the contaminating PCDD and PCDF species [4, p.387-388]. The evidence concerning liver toxicity due to chronic exposure to PCBs is similar to the toxicity associated with acute exposures, except symptoms are usually more severe. Dose response correlations were made between serum PCB levels and increased liver enzyme activities in plasma, indirectly indicating liver damage [1, p.166], but morphological changes in liver cells are evidenced [7, p.249-250] as well as a suspected connection between liver cancer and PCB exposure [1, p.167, 22]. Although there appears to be a link between PCB exposure and cancer in humans, the few epidemiological results available generally suffer from an insufficiently- sized study group, lack of long term studies and mixed chemical exposure. Thus it is difficult to make conclusive statements concerning the carcinogenicity of PCBs [1, p.167].

2) Metabolism

a) General Aspects of PCB Metabolism

An outline of pathways of known PCB metabolism that have been documented for many species of animals (including humans) and bacteria is shown in Figure 3 [23, p.147]. Specific details of metabolic pathways are obviously complicated by the many different PCB congeners, and the effects of chlorine position have yet to be completely and systematically studied. However, the following generalizations have been made in an attempt to simplify the issue [as reviewed in 7, p.257-258].

a) "Unsubstituted adjacent carbon atoms facilitate metabolism (particularly carbons 3 and 4 or 4 and 5 of the biphenyl nucleus).
b) Hydroxylation is favored at the para-position in the least chlorinated ring unless sterically hindered (conversely, substituted para-positions hinder metabolism).
c) Unsubstituted meta-positions facilitate elimination (particularly in the dog).
d) As the degree of chlorination increases on both phenyl rings, the rate of elimination decreases.
e) Differences in the disposition of PCBs exist among animal species".
f) The most toxic congeners have low (0-1) ortho-chloro substitutions [23, p. 138].

Generally, the greater the degree of chlorination of a particular PCB congener, the greater its concentration in animal tissue relative to its concentration in blood. For instance, in rats, 90% of the 4-chloro, 4,4'-dichloro (4-DCB) and 2,2',4,5,5'-pentachloro biphenyls were eliminated over 2, 4 and 10 day periods respectively [24, p.203; 25, p.377]. It was also demonstrated that only 5.5% of the 2,2',4,4',5,5'-hexachlorobiphenyl (2,4,5-HCB) was eliminated by rats in 7 days [as reviewed in 7,

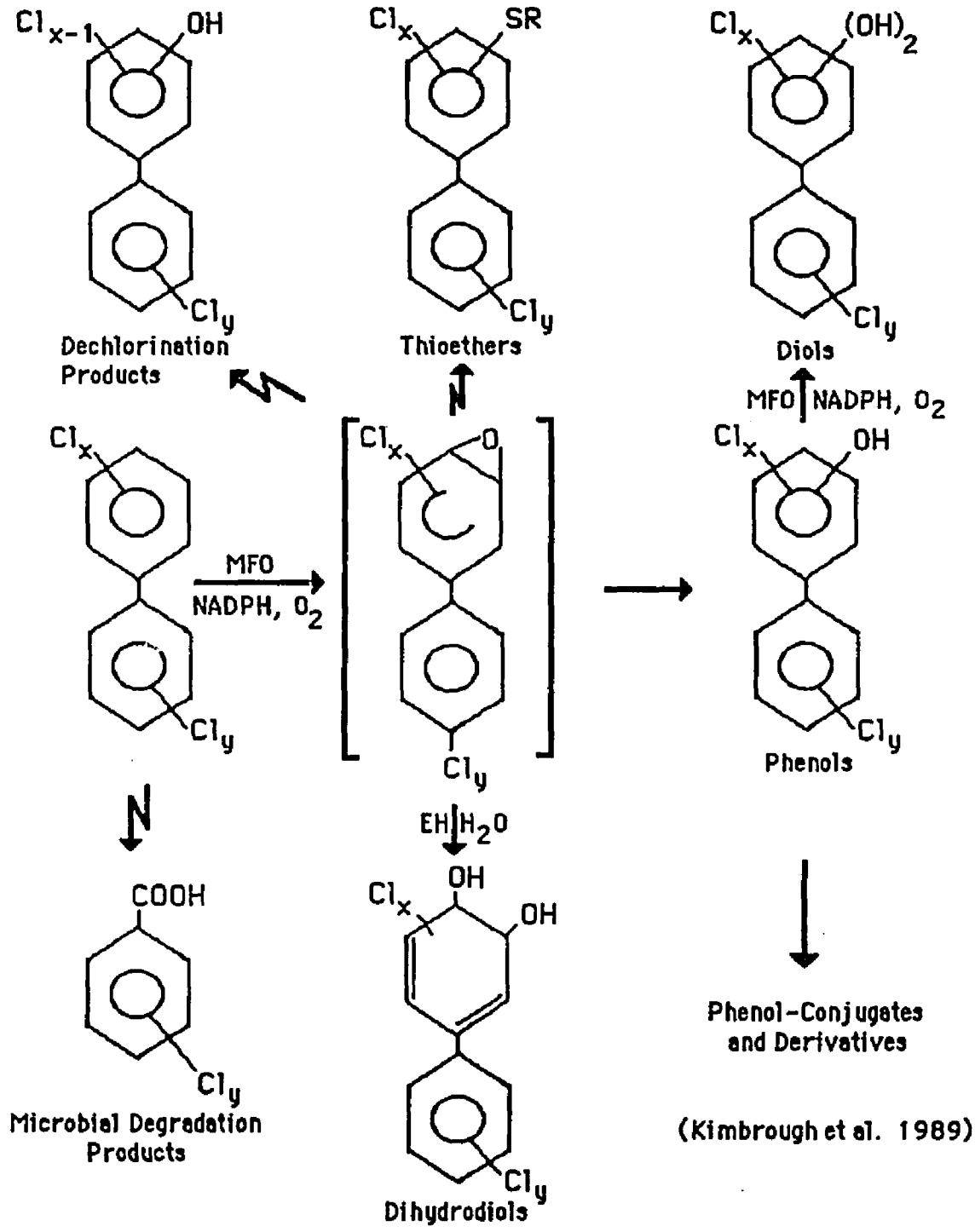

Figure 3: Overview of some of the known metabolic pathways for PCBs. Broken arrows indicate bacterial pathways.

p.257] Among animals studied, the dog appears to have the greatest ability to metabolize and eliminate certain highly chlorinated biphenyls. The studies supporting this have shown that the dog can eliminate 50% of an intravenously administered dose of 2,4,5-HCB while the rat and monkey eliminated only 5.5% and 18% respectively, of the administered dose in 8 days. It is interesting to note that the monkey and the rat also show significant differences in the elimination of 4-DCB, with the rat eliminating 50% of the dose in 1 day, and the monkey requiring 20 days [25, 26]. These results illustrate the very high lipophilicity and stability of PCBs that make biological elimination of certain congeners nearly impossible in some animal species and lead to bioaccumulation and biomagnification of PCB's in secondary and tertiary consumers.

b) Cytochrome P-450 Function and PBD-2 Catalyzed PCB Metabolism

The enzyme responsible for the metabolism of 2,4,5-HCB in the dog has recently been characterized [26-29] and a cDNA coding for the polypeptide has been sequenced [30]. The gene product, PBD-2, is a member of a membrane-bound family of enzymes called cytochromes P-450 that have evolved as proteins which can metabolize xenobiotics to which an organism may be exposed. The cytochrome P-450 system involves a flavoprotein, NADPH-cytochrome P-450 oxidoreductase and cytochrome P-450 hemoprotein (Figure 4) [31, p.67]. The substrate specificity of cytochromes P-450 varies greatly, but the final result is a family of proteins that can metabolize many classes of chemical substances.

In a general sense the reactions that are catalyzed by these proteins are "oxidative and reductive dehalogenation; N-hydroxylation and N-oxidation; oxidative deamination; S-, N- and O-dealkylation; and aliphatic and aromatic hydroxylation" [32, p.244]. The compounds that are acted upon by cytochrome P450s are generally converted from hydrophobic to hydrophilic compounds, which facilitates their

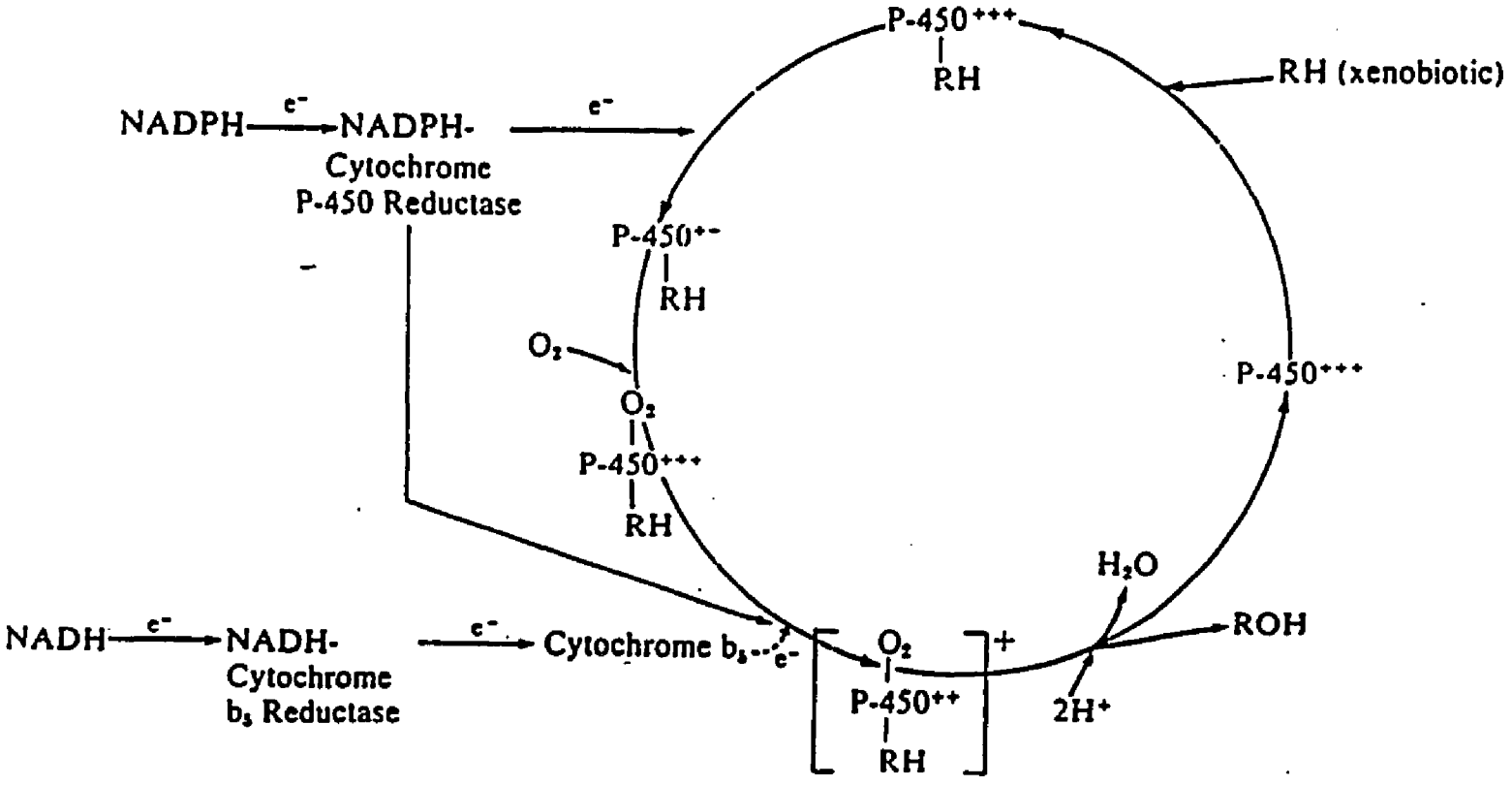

Figure 4 Cytochrome P-450 electron transport systems and oxidation of a xenobiotic.

(As reviewed by Sipes et al. 1980 [31])

elimination from the body. Further chemical modification, such as glucuronidation or sulfation, is also enhanced resulting in other more readily eliminated hydrophilic compounds. The cytochrome P-450 gene family also plays an important role in the metabolism of important biologically active endogenous compounds such as steroids, prostaglandins and fatty acids. Occasionally cytochrome P450-mediated reactions yield very reactive, and hence difficult to isolate, epoxide intermediates that may react with subcellular constituents causing cellular damage and toxicity [32].

In 1987 Duignan et al. [27] performed antibody inhibition studies which showed that the cytochrome P-450 protein, PBD-2, was responsible for >90% of the hepatic metabolism of 2,4,5-HCB in dog microsomes. Phenobarbital treatment increased the amount of PBD-2 protein in dog microsomes almost six-fold and increased the rate of metabolism of 2,4,5-HCB five-fold. Reconstituted systems containing purified PBD-2 protein showed a 10-fold greater rate of metabolism of 2,4,5-HCB than a reconstituted system containing purified PB-B from the rat. Both PBD-2 (dog) and PB-B (rat) are the major phenobarbital inducible isozymes of these animals [27]. Significantly, 95% of the ^{14}C labeled 2,4,5-HCB dog excreta were water soluble [26]. These data have been interpreted to show that the differences in the rates of metabolism of 245-HCB in the dog, relative to the rat, are due to the activity of the PBD-2 isozyme.

Sequencing of a 2.6 kbp cDNA that encodes for PBD-2 allowed deduction of a coding sequence for a 494 amino acid polypeptide. The deduced amino acid sequence of PBD-2 has been classified as a member of the cytochrome P450IIB subfamily. The numerous P-450 isozymes are classified based upon their amino acid sequence similarity. A particular family of cytochromes P-450 has ≤36% identity to P-450s in other families. Related subfamilies have about 40-65% identity and members of a given subfamily generally have greater identity [32, p.246, 251]. The dog cytochrome

IIB subfamily was determined, by Southern blot analysis, to contain at least 2 closely related genes. Northern analysis revealed two mRNA species which may result from use of an an alternative polyadenylation site or from expression of two genes [30].

D) Environmental Clean-up Methods for PCB's

1) Traditional Methods

The widespread contamination, environmental persistence and public health risk of PCB's has necessitated the need for preventive maintenance of existing PCB containing systems and adequate clean-up methods in the event of accidental or illegal discharge. The rehabilitation of land contaminated with levels of PCBs high enough to pose an immediate health hazard ($\geq \approx 100$ $\mu g/m^2$ [1, p.224]) may be dealt with by surface stripping and replacement with clean soil. If there is a large quantity of contaminated soil, it may be transported to landfills designed for long term storage. This method contains the PCB problem but is plagued with many flaws including; i) public opposition to selected disposal site ii) the expense and scale of the operation iii) special equipment and specially trained personnel to handle and transport hazardous materials iv) potential transit liabilities and v) extensive restoration and landscaping [1, p.50-51].

If the transport of contaminated soil cannot be facilitated, the affected soil may be immobilized *in situ*. Depending on the nature of the contamination, the area may be sealed to varying degrees of effectiveness. Surface sealing, as the name implies, consists of covering the area with an impermeable barrier (clay, concrete etc.). To prevent lateral spread of the contamination the area may be surrounded, to the necessary depth, with an inert material (steel sheeting, asphalt or granite). If the potential exists for significant groundwater contamination, total encapsulation by the addition of bottom sealing may be necessary. Total encapsulation is the most expensive and difficult of the

procedures. The area becomes a permanent disposal site and will require constant monitoring with the potential of becoming a problem to future generations [1, p.50].

Another *in situ* treatment of contaminated soil involves detoxication through chemical dechlorination using a sodium-polyethylene glycol-oxygen mixture which is sprayed on the contaminated soil. The method is less expensive than excavation and extraction methods, but the effects of the release of this solution into the environment have yet to be determined [1, p.67; 33].

The soil may also be subjected to on site treatment (such as thermal degradation, extraction or neutralization) and then redeposited. Due to the chemical nature of PCBs, on site thermal degradation of these compounds is difficult. Legal requirements state that the chemicals be exposed to a temperature of 1200°C for 2.0 seconds or more to achieve at least 99.9% combustion efficiency [33, p.134]. As the temperature is lowered, the time of exposure must be increased. The mobile treatment unit must be able to contain any volatilized compounds produced during the initial temperature increase and operate at an efficiency high enough to prevent the release of toxic by-products [1, p.60-62]. If the oxygen levels are too low during incineration, the potential exists for the emission of PCDDs and PCDFs [1, p.60; 33 p.135]. The use of mobile incinerators reduces the risks inherent to the transport of hazardous waste, but is generally only applicable in situations of limited contamination. At present there are a "few" units in operation in the United States [1, p.62].

Extraction of PCB's from soil has met with limited success. The solvents found to be most effective for extraction are generally low-molecular weight alcohols. Extracting a clay soil containing 2000 µg/g of Aroclor 1242 with isopropanol showed a >88% extraction efficiency [34]. The choice of solvent must be evaluated for each area of contamination due to differing soil characteristics. The extracted soil must then be

treated with a stream of warm air or an actively growing culture of *Pseudomonas* to remove any excess alcohol before it can be replaced. Depending on the extent of contamination, the cost of solvent and the risk of transport of solvent may make *in situ* immobilization a more attractive alternative [35].

2) Bioremediation

a) Bacterial

Recently there have been numerous discoveries of different strains of bacteria capable of "metabolizing" PCB's [36, 37, 38, 39]. These bacteria were isolated from various locations including PCB-laden slurries at contaminated sites. Rapid dechlorination has been observed in laboratory situations using growing cultures of bacteria, but currently there are no reports of time-efficient detoxification of contaminated sites. The reasons for the inability of the bacteria to function efficiently in field tests are complicated and not completely understood. The ability of a bacterium to metabolize certain compounds is often a fortuitous reaction in the metabolism of its primary energy source. This phenomenon is called cometabolism [40]. *Acinetobacter* strain P6 has been shown to mineralize a broad spectrum of PCB congeners when in log growth phase but only in the presence of a non-chlorinated biphenyl energy source [38]. Often the amounts of energy source required to facilitate the cometabolism of the "target" compound are financially inhibitory or else they posses toxic properties that limit their use [40].

In addition there are limitations to the spectrum of PCB congeners that a particular strain of bacteria can metabolize, possibly necessitating the use of several strains of bacteria to detoxify a particular area. Recent shifts in the PCB congener composition of contaminated stretches of the Hudson River have been observed. The cause of the shift was a controversial issue until the recently-demonstrated

biologically-mediated dechlorination of tri- and higher chlorinated congeners by anaerobic microorganisms isolated from the Hudson River [37]. The particular strain(s) of bacteria responsible for these actions was not isolated at the time the paper was published (1988), but the authors hypothesized that the initial dechlorination step(s) by the anaerobic bacteria facilitate further biodegradation by aerobic organisms [37].

In addition to the potential toxic effects of PCBs on the growth of the bacteria there are other difficulties encountered by this type of bioremediation. These difficulties include meeting nutritional, moisture, pH, temperature and oxygen tension requirements to enable optimal or near-optimal bacterial growth.

There are numerous methods for the remediation of PCB contaminated land, but none without shortcomings. Further research and development of alternative clean-up methods is necessary. Recombinant DNA technologies have recently been used to enhance or engineer organisms for numerous applications including toxic waste disposal. Studies of genetically engineered organisms capable of participating in the restoration of contaminated lands have primarily focused on bacteria [41].

b) Plant Accumulation and Metabolism of PCBs

Several authors have demonstrated PCB uptake by plants [42-47] and in some cases, metabolism of PCBs and biphenyl by endogenous plant activities [48-50]. The studies used a variety of different plants to analyze the extent of PCB uptake. There were significant differences in the ability to translocate PCB congeners, with purple loosestrife accumulating up to 210 ng PCB/gram plant tissue [42] and soybeans maintaining a 10-fold lower concentration [43]. The soil concentrations of PCBs at the experimental sites were similar for these studies (≈120-145 ng/g). Purple loosestrife exhibited a steady increase in PCB concentration in leaf tissue over time,

whereas soybeans maintained plateau levels and corn plants were actually able to decrease their PCB load over time [42, 43]. There is general agreement that the less chlorinated, more water soluble, more volatile congeners were preferentially translocated form soil to plants [42, 43, 45, 47]. The routes of PCB uptake (roots or vapor sorption) are dependent upon the water content of the soil, the concentration of the PCBs, the solubility of the individual congeners and the plant species [47, 51].

It has been reported that certain plant species are capable of metabolizing PCBs with endogenous proteins. Several plant species have demonstrated the ability to hydroxylate certain di- and tri-chlorinated PCB congeners [48, 49]. A recent investigation compared the rates of cytochrome P-450 dependent metabolism of xenobiotics using rat and artichoke tuber microsomes [50]. The results suggest that the artichoke was unable to hydroxylate halosubstituted biphenyls to a measurable degree, but was able to hydroxylate non-halogenated biphenyl at 1/25 the rate of the rat. It is apparent from these studies that plants may posses differing metabolic activities towards PCBs.

c) Potential for Remediation of PCB-Contaminated Areas with Transgenic Plants

Our hypothesis that transgenic plants can be used to decontaminate PCB affected sites is a relatively novel concept that has some advantages over other methods. In particular, transgenic plants can be designed for male sterility [51, 52] to prevent unchecked, ecologically deleterious spread throughout the environment. Furthermore recombinant DNA techniques allow many types of plants to be genetically manipulated. This allows one to choose a plant species capable of rapid growth in a particular environment. If a suitable plant(s) is chosen (ie. compatible pH, water, nutritional and climatic requirements) for a particular area, the amount of labor and expense involved in site restoration could be minimal in comparison to some existing methods. In

addition, the aesthetic value of this type of bioremediation could be very important to individuals living in the vicinity of the contaminated area.

E) Objectives and Rationale for this Research

We propose that the PBD-2 gene, incorporated in an appropriate plant genome, can be expressed and that the encoded PBD-2 protein might function as it does in the dog to metabolize PCBs to water soluble metabolites. The choice of plant for this research is *Nicotiana tabacum* , because it is a well established model for the introduction of expressible foreign DNA into its genome. The metabolic activity PBD-2 in the plant may initiate the *in situ* "decontamination" of certain persistent PCB congeners thus providing an alternative to existing reclamation methods.

F) Specific Aims

Initial experiments were designed to construct a plasmid capable of being used for transient expression assays in tobacco protoplasts to determine if the PBD-2 protein is expressed. Expression was confirmed by Western blot analysis. Next, a plasmid was constructed to function in an *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum*. Successful transformation of *N. tabacum* was confirmed using Western and Southern blot analysis. The subcellular localization of PBD-2 in the transgenic plants was determined by fractionating homogenized plant tissue on a linear sucrose gradient followed by analysis of the individual fractions for the presence of the PBD-2 protein and for activity of appropriate marker enzymes. PBD-2-containing fractions were then analyzed for enzyme activity using an androstenedione hydroxylase assay.

CHAPTER 2

MATERIALS AND METHODS

A) Large Scale Plasmid Isolation

Ampicillin resistant E. coli, strain DH5α, cells containing a recombinant Bluescript KS(-) plasmid (Stratagene) with the entire 1.48 kbp PBD-2 coding sequence insert (Figure 5, Plasmid D-39, Graves, et al. [30]) were obtained from Drs. Penny Graves and James Halpert of the Pharmacology and Toxicology Department, University of Arizona. The cells were grown overnight at 37°C in 1 liter of LB medium (10 g bactotryptone, 5 g yeast extract, 86mM NaCl, 9mM NaOH and H_2O to 1 liter) supplemented with 50 µg/ml ampicillin. The cell-containing medium was transferred to 250 ml bottles and centrifuged at 8000 x g for 10 minutes in a Sorvall GSA rotor. The supernatant was discarded and the pellet resuspended in 18 ml of solution I (50mM glucose, 25mM Tris and 10 mM EDTA). Two ml of 10 mg/ml lysozyme from chicken egg white (Sigma) was added to the resuspended pellet, and the cells were incubated at room temperature for 10 minutes. To this mixture, 40 ml of solution II (0.2 M NaOH and 1.0% SDS) was added and it was incubated on ice for 10 minutes. Twenty ml of ice cold solution III (3M potassium acetate pH 5.5) was added and the mixture was incubated on ice for 15 minutes. After adding 2.5 ml H_2O, the mixture was centrifuged at 8000 x g for 10 minutes. The supernatant was filtered through 2 layers of sterile cheesecloth into a clean Sorvall bottle and mixed with 0.6 volumes of ice cold isopropanol. The precipitated DNA was centrifuged at 16,000 x g for 10 minutes. The DNA pellet was drained and briefly dried under a vacuum.

In order to separate the D-39 plasmid DNA from remaining contaminants (RNA, genomic DNA, protein) the DNA pellet was dissolved in 16.5 ml of H_2O and transferred to

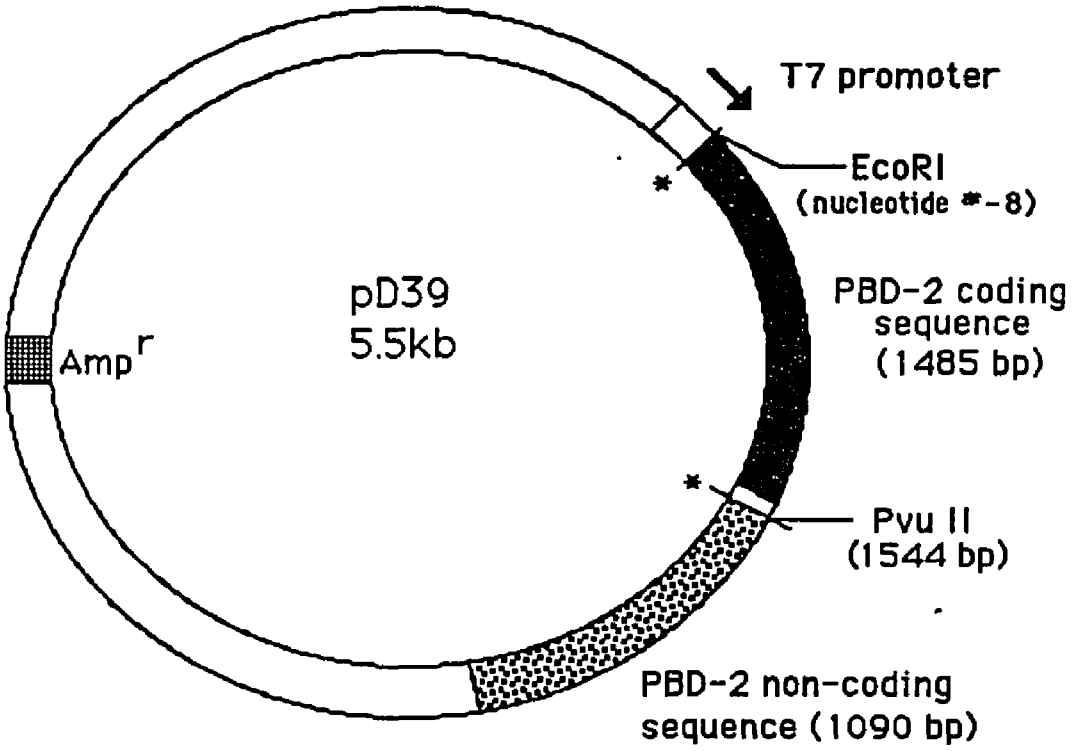

Figure 5: Diagram of D39 plasmid containing the PBD-2 coding sequence. Asterisks denote the location of restriction enzyme sites used to excise the PBD-2 coding sequence for future cloning (Graves et al. 1990, [30]).

a 30 ml Corex tube containing 17.5 g CsCl. When the CsCl was completely dissolved, 1 ml of 10 mg/ml ethidium bromide was added and the solution was centrifuged at 12,000 x g in a Sorvall SS-34 rotor for 10 minutes. The supernatant was transferred to a Beckman TLA 100.3 rotor in 13 x 51mm polycarbonate tubes and centrifuged in a Beckman TL-100 tabletop ultracentrifuge for 4 hours at 100,000 rpm. The plasmid DNA band was removed with a needle and syringe, diluted to an appropriate volume (i.e. evenly fill the least possible number of centrifuge tubes) with the CsCl solution (16.5 µl H_2O and 17.5 g CsCl). The solution is then subjected to another 4 hour centrifugation under identical circumstances. Again the plasmid DNA band was removed from the centrifuge tube and extracted 8-10 times with 1 volume TE (10mM Tris, 1mM EDTA, pH 8.0)-saturated butanol to remove the ethidium bromide. The DNA solution was placed in a 15 ml Corex tube, mixed with 4 volumes of 70% ethanol and incubated at -20^{o}C for 1 hour. The DNA was pelleted by centrifuging at 12,000 x g for 10 minutes in a SS-34 rotor. The pellet was washed with 70% ethanol and drained for 5 minutes. After dissolving the DNA pellet in 500 µl TE, pH 8.0, the solution was transferred to a 1.5 ml microfuge tube. Sequential extractions with equal volumes of phenol/chloroform (1:1) and ether (three times) were performed to remove any residual proteins. Any remaining ether was evaporated by heating the microfuge tube to 65^{o}C for 5 minutes. The DNA was precipitated with 0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol on ice for 10 minutes. The DNA was pelleted by centrifuging at 16,000 x g for 5 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. After draining and drying the pellet in a Speed Vac it was dissolved in 500 µl TE. The quantity and purity of the isolated D-39 plasmid DNA was determined by spectrophotometric measurement of its absorbance at 260 and 280 nanometers [54, p.468]. Typically the DNA preparations had an A_{260} /A_{280} ratio of 1.8. In order to

isolate a PBD-2 coding fragment from D-39 plasmid DNA (40μg) was digested with 60 units of EcoRI (Promega) and 120 units of Pvu II (Promega) restriction enzymes, according to manufacturers suggestions. An aliquot of the restricted DNA (0.5 μg) was electrophoresed for 1-1.5 hours at 50 V in a 10 X 7 cm 0.8% agarose gel (Maniatis et al. [54]). The size of the DNA fragments was estimated using 1 kb ladder DNA marker (Bethesda Research Laboratories).

B) Small Scale Plasmid Isolation

Using the method of Maniatis et al. [54, p.368], five ml of LB media containing the appropriate antibiotic was inoculated with a single colony of bacteria and allowed to grow overnight (or 48 hours in the case of *Agrobacterium tumefaciens*) at the appropriate temperature (37°C for E. coli and 30°C for *A. tumefaciens*). One and a half ml of the cell-containing media was pipetted into a microfuge tube and centrifuged for 30 seconds at 16,000 x g. The supernatant was removed and discarded. The pellet was resuspended by vortexing in 100 μl of an ice-cold solution of 50mM glucose, 10mM EDTA and 25mM Tris (pH 8.0). This solution was allowed to incubate at room temperature for 5 minutes. To this mixture was added 200 μl of a freshly prepared solution of 0.2 N NaOH and 1% SDS. The tube was inverted 2-3 times rapidly and stored on ice for 5 minutes. Next was added 150 μl of an ice-cold solution of potassium (3M) acetate (5M), pH 4.8. Again the tube was mixed by inversion and stored on ice for 5 minutes. The solution was centrifuged for 5 minutes at 16,000 x g at 4°C. The supernatant was removed and transferred to a fresh microfuge tube where it was extracted with an equal volume of phenol/chloroform (1:1). Multiple phenol /chloroform extractions (3-5) were necessary when isolating plasmids from *A. tumefaciens*. The aqueous phase was removed and mixed with 2 volumes of ice-cold ethanol and allowed to stand at room temperature for 4 minutes. The solution was

centrifuged for 5 minutes at room temperature at 16,000 x g. The supernatant was removed and the pellet drained thoroughly. The plasmid pellet was washed with 1 ml of 70% ethanol. The pellet was drained and dissolved in 50µl TE (pH 8.0) containing DNase-free pancreatic RNase (20 mg/ml). Ten µl of the plasmid-containing TE was analyzed by restriction digest mapping (see large scale plasmid preparation). The DNA yields were significantly lower when isolating plasmids from *A. tumefaciens.* Better results were obtained if the washed DNA pellet was resuspended in a 20µl volume of TE containing RNase. Low yields necessitated using all of the *A. tumefaciens* DNA obtained from the 1.5 ml culture for a single lane on a gel for restriction enzyme mapping.

C) Construction of PBD-2 Recombinant DNA Vectors

1) Ligation of PBD-2 coding sequence into PDG7 and transformation of E. coli

To test the ability of tobacco protoplasts to express PBD-2, an appropriate vector construction was necessary. The D-39 plasmid (40 µg) was subjected to digestion by the restriction endonucleases EcoRI and Pvu II (Promega), 60 and 120 units respectively, for 1 hour at 37°C in a total volume of 150 µl. All fragments resulting from the digestion were subjected to a fill-in reaction to blunt the sticky ends that resulted from cleavage at the EcoRI restriction site. The restriction endonuclease reaction mixture was heated to 70°C for 10 minutes to denature the EcoRI and Pvu II enzymes. The 4 dNTP's (P L Biochemical) were added to a final concentration of 0.125 mM/nucleotide followed by the addition of 5 units of DNA Polymerase I Klenow fragment (Promega). The mixture was allowed to incubate for 1 hour at 37°C to complete the fill-in reaction.

Digestion of D-39 plasmid with EcoRI and Pvu II yields 6 fragments of varying lengths including a 1554 bp fragment corresponding to the PBD-2 coding sequence. The 1554 bp sequence contains 8 bases 5' to the initiation codon and 62 bases 3' to the

termination codon for the coding sequence (Figure 6). The PBD-2 coding sequence was isolated from the other fragments by electrophoresis. All 40 µg of the digest was run on a 7.5% polyacrylamide gel at 50 volts for 17 hours. The DNA bands were visualized by their pattern of refracted light and the polyacryalamide containing the coding sequence fragment was excised with a sterile scalpel blade. The excised polyacrylamide was placed in a dialysis bag with a minimal amount of 0.5X TBE (0.089 M Tris-Borate, 0.089 M boric acid) buffer and subjected to electrophoresis at 75 volts for 1.5 hours. The 0.5X TBE buffer in the dialysis bag was removed and mixed with 0.1 volume of 3M sodium acetate and 2 volumes of ethanol and kept at -20°C for 1 hour to precipitate the DNA. The solution was centrifuged for 10 minutes, the pellet washed in 70% ethanol, dried in a Speed Vac and resuspended in 25 µl TE. Previous experience isolating DNA fragments in this manner allowed us to approximate a final DNA concentration of 0.1 µg/µl assuming a 25% yield for the procedure.

The purified PBD-2 coding sequence fragment was ligated into the SmaI site of the vector pDG7 (Figure 7), provided by Dr. David Galbraith (Department of Plant Sciences, University of Arizona). The pDG7 plasmid was cleaved at the SmaI (Promega, blunt end forming) recognition sequence in the polycloning region of the vector using the manufacturer's recommended conditions. To prevent self-ligation and recircularization of the plasmid, the 5' ends of 3 µg of the digested vector were subjected to dephosphorylation by alkaline phosphatase treatment (3 units) for 1 hour at 50°C. Calf Intestinal Alkaline Phosphatase was purchased from Boehringer-Mannheim and used in accordance with the manufacturers' recommended protocol for blunt-end dephosphorylation. The resulting dephosphorylated DNA was dissolved in 15 µl TE and its concentration was estimated, assuming a 50% yield, to be 0.15 µg/µl.

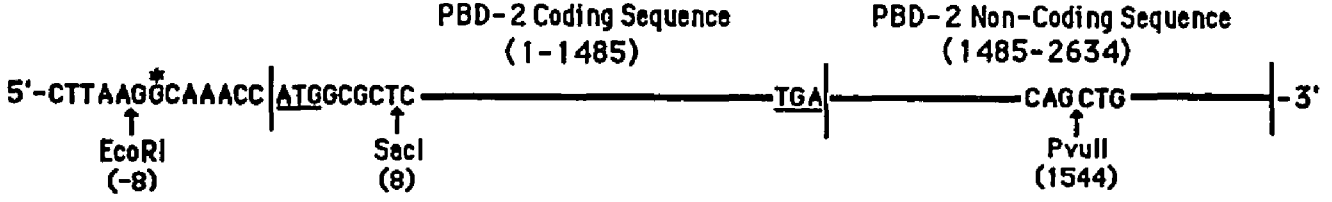

Figure 6: Schematic drawing of PBD-2 cDNA including initiation and termination codons and restriction enzyme sites used for construction and verification of pVPCB-1 and pBO-1 sequences (Graves et al. 1990, [30]). *5' end of cDNA, adjacent to an upstream EcoRI linker.

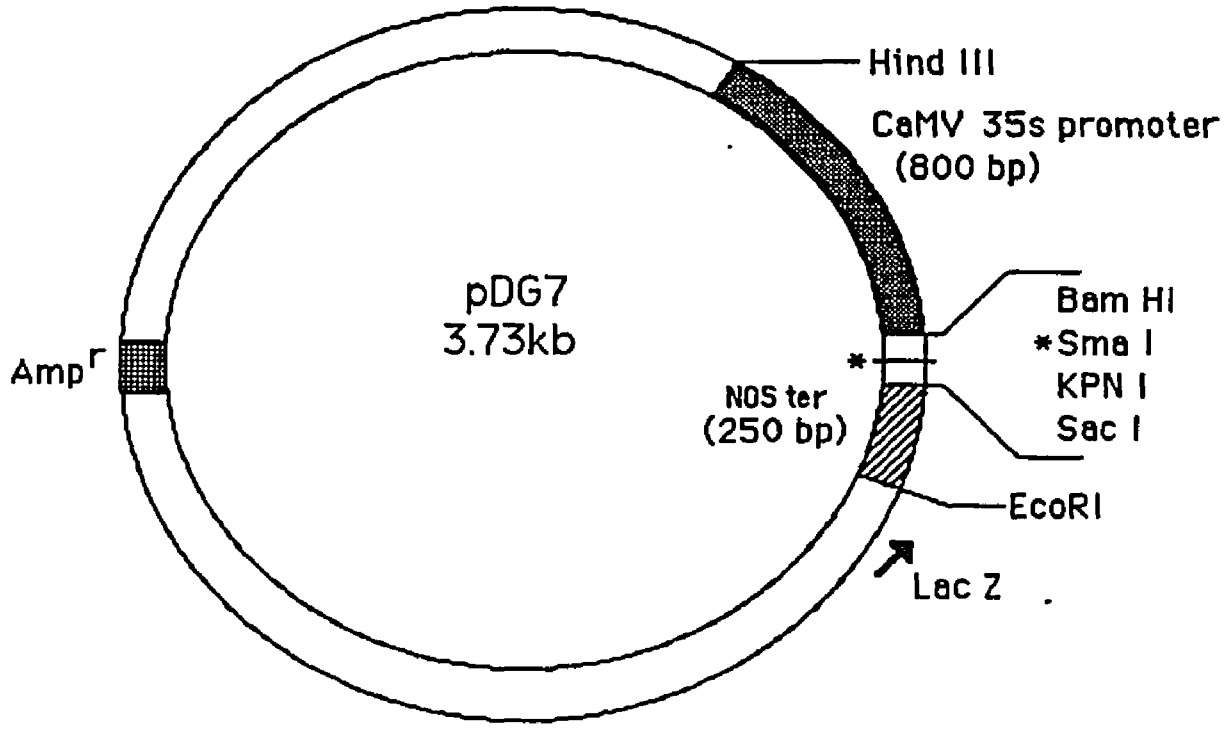

Figure 7: Illustration of pDG7 (pRKO2 subcloned into pUC8). Asterisk indicates the location of the SmaI site into which the PBD-2 sequence was cloned. The plasmid contains the CaMV 35s transcription promoter sequence and the nopaline synthase termination signal [75].

The ligation of the PBD-2 coding sequence fragment into the pDG7 plasmid (Figure 7) was carried out according to the recomendations of the manufacturer (International Biotechnologies Inc.) of the T4 ligase used for the reaction. Table 4 indicates the components used in the ligation reaction mixture (A) and the control mixtures (B, C).

The ligated DNA mixtures were used to transform competent E. coli DH5α cells. The preparation of competent cells began by growing 50 ml cultures of DH5α in LB to an optical density of 0.4 at 600nm. The cell-containing medium was centrifuged at 2600 x g for 4 minutes in a Sorvall GSA rotor. The supernatant was discarded and the pellet resuspended in 2 ml of 0.1 M $CaCl_2$ and placed on ice for 1 hour. Again the solution was centrifuged at 2600 x g for 4 minutes in a Sorvall GSA rotor and the supernatant discarded. The pellet was resuspended in 2 ml of 0.1M $CaCl_2$ and kept at 0-4°C overnight to increase competency. The transformation procedure required 200 µl of the competent cells plus 1 µl and 9 µl of each of the ligation mixtures listed in Table 4. These transformation mixtures were incubated on ice for 1 hour and then heat shocked in a 42°C water bath for 4 minutes. The transformed cells were then mixed with 700 µl of LB and incubated at 37°C for 1 hour. From each transformation solution, 20 µl and 200 µl were removed and spread-plated on LB plates containing 100 µg/ml ampicillin. Twenty individual ampicillin-resistant bacterial colonies were selected and subjected to a small scale plasmid isolation and restriction enzyme digestion analysis to verify the success of the ligation (see results A-1). After verification that the PBD-2 sequence had been ligated in the desired orientation, a large scale plasmid isolation was performed. The resulting plasmid was named pVPCB-1 (Figure 8). Figure 9 provides a summary of the strategy for construction of pVPCB-1 as discussed in this section.

		Controls	
	Complete	No Insert	No Ligase
Insert	2 µl (0.2 µg)	---	2 µl (0.2 µg)
Vector	3.5 µl (0.525 µg)	3.5 µl (0.525 µg)	3.5 µl (0.525 µg)
Ligase	1 µl (1 U/ml)	1 µl (1 U/ml)	---
Ligase Buffer	1 µl	1 µl	1 µl
H_2O	2.5 µl	4.5 µl	3.5 µl

Table 4: Ligation reaction mixtures and controls used for the construction of pVPCB-1.

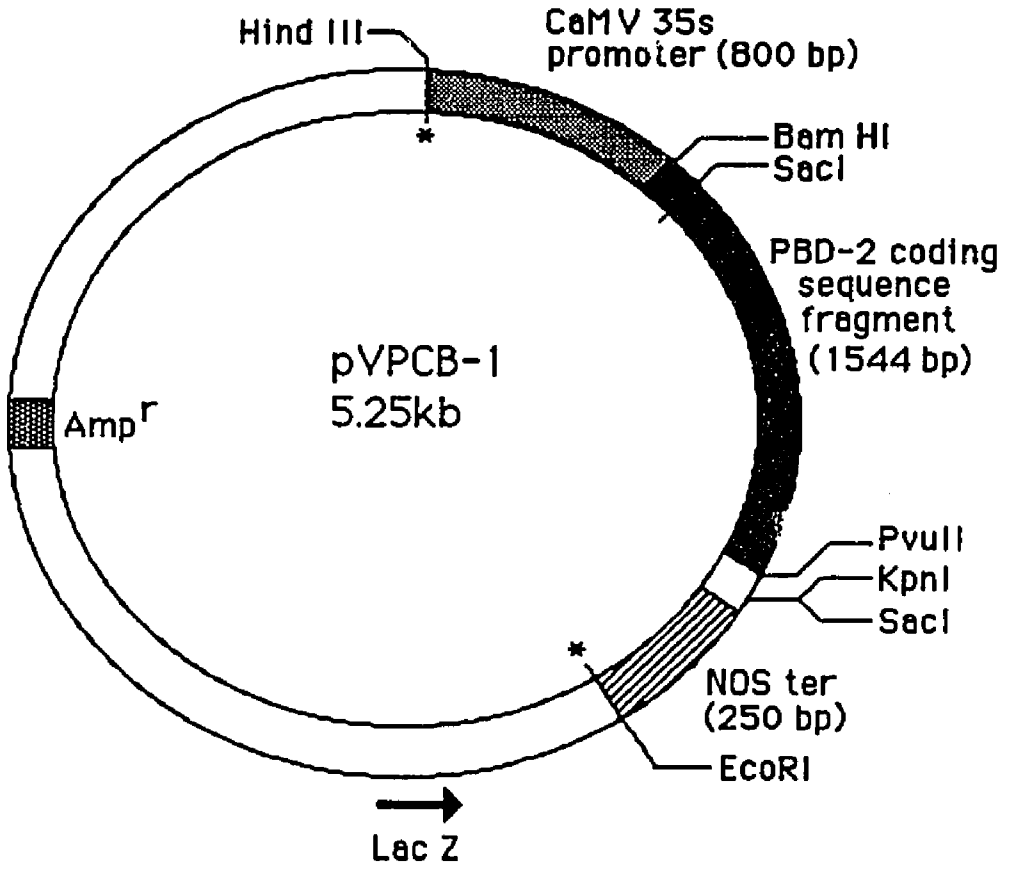

Figure 8: Illustration of pVPCB-1 plasmid, a construct with the PBD-2 coding sequence subcloned into pDG7.

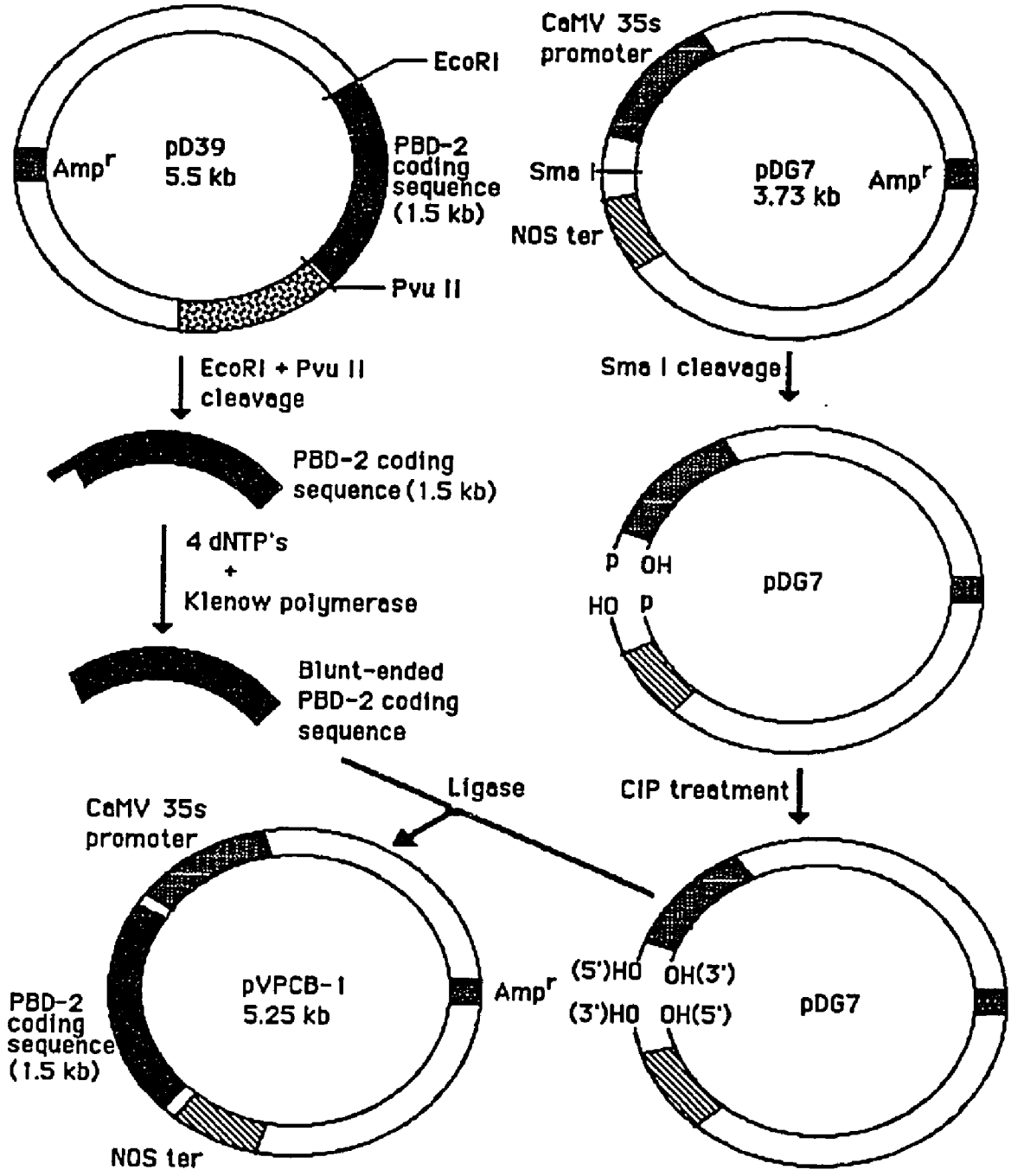

Figure 9: Strategy for construction of pVPCB-1

2) Cloning of pVPCB-1 into Bin 19

A plasmid was constructed for use in *Agrobacterium tumefaciens*-mediated introduction of the PBD-2 gene into tobacco. Five micrograms of Bin 19 (Figure 10 [55]) and 5.2 µg of pVPCB-1 were digested simultaneously with 36 units of EcoRI (Promega) and 72 units of Hind III (Promega) in a high salt EcoRI buffer (Promega). In addition to the digestion described above, 2 µg of Bin 19 (Figure 10) was cut with EcoRI and Hind III under similar conditions to be used as a negative control for the ligation. The ligation of the PBD-2 coding sequence into pDG7 was performed according to the suggestions provided by the ligase enzyme manufacturer (International Biotechnologies Inc.) for the unidirectional ligation of compatible 5' overhangs. Table 5 lists the components of the ligation reaction designed to create the recombinant Bin 19 vector (pBO-1, Figure 11) containing the PBD-2 coding sequence.

The ligated DNA mixtures and control mixtures were used to transform DH5α cells according to the procedure listed above. One-hundred and fifty microliters of the transformed cells and the positive (Bin 19 + no insert) and negative (no ligase) control cells were spread-plated on LB media supplemented with 50 µg/ml kanamycin and 40 µg/ml X-GAL. Bacterial kanamycin resistance conferred by Bin 19 (Kan^r) and the β-galactosidase gene (Lac Z) present in the polycloning region of Bin 19 were used to help identify bacterial colonies with recombinant Bin 19. Bin 19 without an intact LacZ gene can be distinguished from Bin 19 containing an insert that interrupts the Lac Z gene. Bacterial colonies growing on X-Gal supplemented media containing the PBD-2 insert are white in color whereas those colonies without an insert appear blue. Twelve individual white colonies were selected and subjected to a small-scale plasmid isolation and restriction digest analysis as described previously (see results A-2) to determine whether the PBD-2 insert had been successfully cloned.

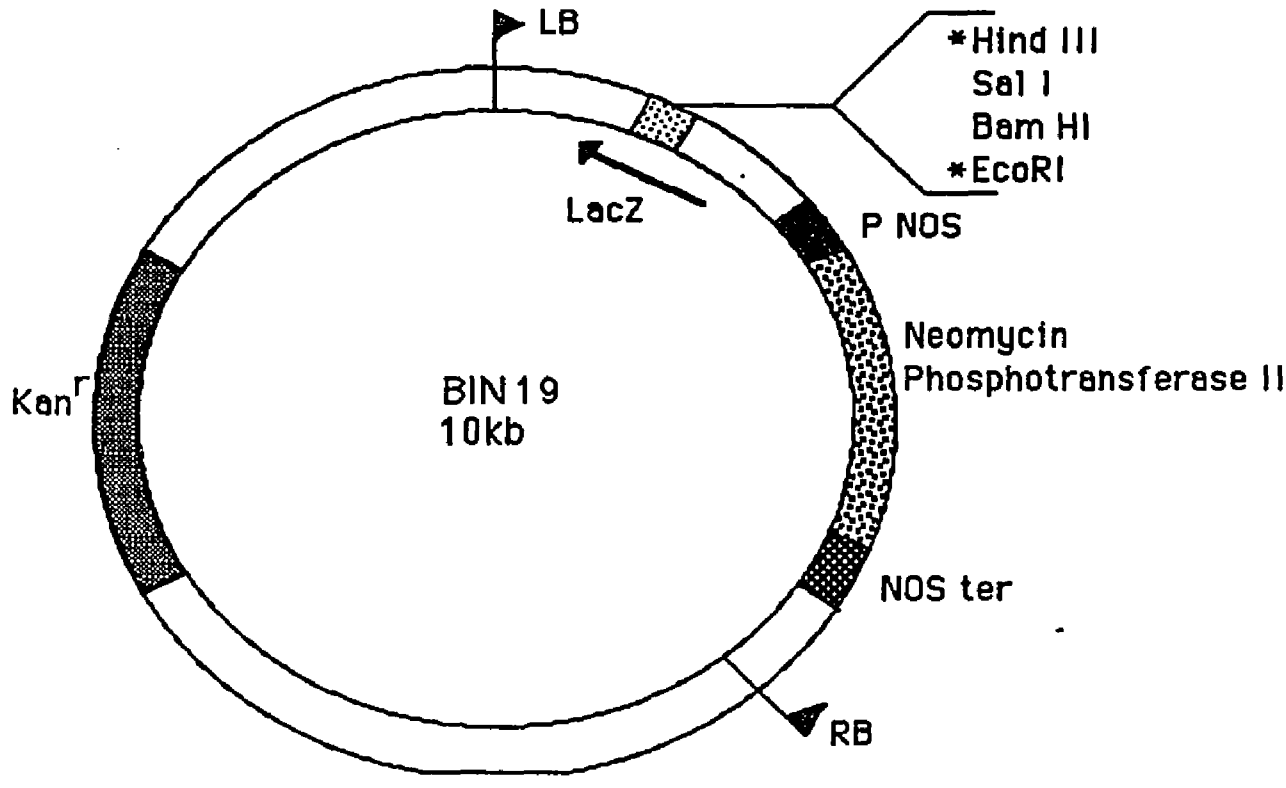

Figure 10: Diagram of an *Agrobacterium* compatible vector, Bin 19. Asterisks indicate restriction enzyme sites used for directional introduction of a PBD-2 coding sequence insert from pVPCB-1. These restriction sites are within the lac Z gene; recombinants are lac(-).

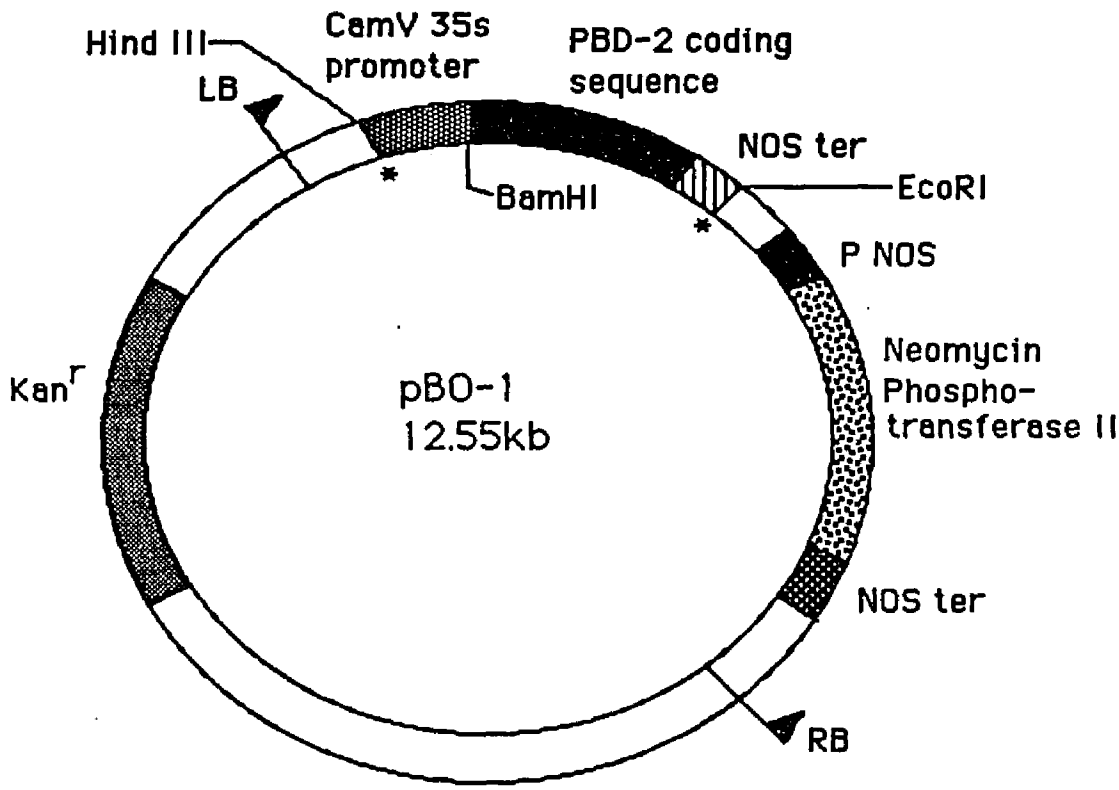

Figure 11: Diagram of Bin 19 containing the PBD-2 coding sequence and the appropriate promoter and terminator to facilitate expression in plants. The resulting plasmid is named pBO-1.

Table 5: Ligation reaction mixtures and controls used for the construction of pBO-1.

Component	Complete	Controls	
		no ligase	no insert
Insert	0.2 µg	0.2 µg	---
Vector	0.2 µg	0.2 µg	2.85 µl (2.0 µg)
Ligase	1 µl (1 U/ml)	---	1 µl (1 U/ml)
Ligase Buffer	2 µl	2 µl	2 µl
H_2O	16.1 µl	17.1 µl	15.1 µl

D) Mobilization of pBO-1 into *Agrobacterium tumefaciens*

A triparental mating, using the helper plasmid pRK2013 [56], was necessary to facilitate the mobilization of pBO-1 from DH5α cells into *Agrobacterium tumefaciens* [55]. Cultures of *Agrobacterium tumefaciens* LBA4404 (provided by Dr. David Galbraith, Plant Sciences Department, University of Arizona), DH5α containing pBO-1 and DH5α cells containing pRK2013 [56] (provided by Dr. Don Bourque, Biochemistry Department, University of Arizona) were incubated overnight in LB media (LBA4404 media contained 50 μg/ml kanamycin) The culture containing LBA4404 was incubated at 30°C and the others at 37°C. The tubes were centrifuged at 600 x g for 4 minutes to pellet the cells. The supernatant was removed and the pellet resuspended in 10 ml of LB broth. The following tubes were prepared using 200 μl aliquots of the relevant cultures:

1. DH5α containing pBO-1
2. DH5α containing pRK2013
3. LBA4404
4. DH5α containing pBO-1 + DH5α containing pRK2013
5. DH5α containing pBO-1 + LBA4404
6. DH5α containing pRK2013 + LBA4404
7. DH5α containing pRK2013 + LBA4404 + DH5α containing pBO-1

The cultures were vortexed briefly and 200 μl from each tube was spread-plated on LB media and incubated at 30°C overnight. Individual colonies were then streak plated on Minimal T (see Appendix A) media containing kanamycin (50 μg/ml) and incubated for three days at 30°C. Single colonies from plates exhibiting bacterial growth were restreaked two more times on identical media. Minimal T media will not support DH5α growth but will support LBA4404 growth. Therefore, the growing

colonies were LBA4404 bacteria containing pBO-1. Eight individual colonies were selected and subjected to a small scale plasmid isolation and restriction digestion analysis to verify mobilization of pBO-1 into *Agrobacterium tumefaciens* (see results B).

E) Transformation of *Nicotiana tabacum* with *Agrobacterium tumefaciens*

Fully expanded leaves (1-2 cm wide) removed from young *Nicotiana tabacum* axenic shoot cultures provided the tissue for the transformation events [55]. The shoot cultures were grown in Magenta boxes in 30 ml of Nutrient media consisting of 4.3 g of Gibco MS [57] salts, 30 g sucrose, 8 g agar and 1 ml vitamin stock. The pH was adjusted to 5.7, the volume was adjusted to 1 liter and the solution autoclaved. The vitamin stock consisted of 50 mg nicotinic acid, 10 mg thiamine-HCL, 50 mg pyridoxine-HCL, 200 mg glycine and enough H_2O to make 100 ml of solution. The leaves were cut with a sterile #4 core borer yielding 21 leaf disks. A 2 ml culture of *Agrobacterium tumefaciens* containing pBO-1 was grown overnight at 30°C in LB media. The leaf disks were placed in a 50 ml conical tube with the *Agrobacterium tumefaciens* culture, briefly (30 sec) mixed, removed and blotted on sterile Whatman 3mm paper.

The disks were then placed adaxial (top) side down onto tobacco cell feeder (see Appendix A) plates and incubated for 48 hours at 24-26°C in a lighted growth chamber. The disks were then transferred onto shooting media (see Appendix A) supplemented with 200 µg/ml kanamycin and 500 µg/ml carbenicillin, and subsequently transferred to fresh shooting media every 7 days. Within 3 weeks callus began to develop on the leaf disks. The calli were isolated individually from other calli developing on the same disk to insure selection of single transformation events. Within 6 weeks differentiated shoots began to appear on the callus. When the shoots reached a height of ≈ 1 cm they were removed with a sterile scalpel, under sterile conditions, and placed upright in a Magenta

box containing 30 ml of rooting media (see Appendix A) supplemented with 150 µg/ml kanamycin and 500 µg/ml carbenicillin. Plants that were transformed with, and expressing the neomycin phosphotransferase gene on pBO-1 were resistant to kanamycin. Within 10 weeks roots began to develop on the transformed plants. When the plants reached a height of approximately 10 cm, subcultures were started by removing the apical meristem with a sterile scalpel, under sterile conditions, and placing it upright in Nutrient medium (see materials and methods E). When there was a sufficient number (3-4) of subcultured plants, selected clones were transplanted to soil and gradually, over the period of a week, exposed to laboratory environmental conditions. The transplanted plants were then moved to a greenhouse and kept at 24-26°C while the axenic cultures were maintained in an incubator at 24-26°C under lighted conditions.

F) Transient Expression of pVPCB-1 in *N. tabacum* Protoplasts

Protoplasts were prepared according to Galbraith et al. [58] from fully expanded (1.5-2.0 cm) *N. tabacum* leaves selected from sterile axenic shoot cultures grown in Magenta boxes on the Nutrient medium described above. Under sterile conditions, 2-4 leaves were cut perpendicular to the midrib into 1 mm slices, in 15 x 100 mm petri dishes containing about 10 ml of sterile enzyme digestion medium (see Appendix A). The covered petri dish was sealed with parafilm and placed in a lighted incubator at 24-26°C for 16-18 hours. The digestion medium containing the leaf slices was removed with a pasteur pipette and filtered through 2 layers of cheesecloth into a 50 ml conical tube under sterile conditions. The protoplast-containing medium was diluted with an equal volume of W5 solution (see Appendix A) and incubated for 15 minutes at room temperature. The solution was centrifuged for 8 minutes at 600 rpm in a clinical centrifuge. The supernatant was discarded and the pellet was resuspended in 20 ml of

25% (w/w) sucrose solution (see Appendix A) using a sterile capillary pipette. Five milliliters of W5 solution was then gently pipetted over the 25% sucrose solution maintaining a sharp interface. The tube was centrifuged for 10 minutes at 600 rpm in a clinical centrifuge. The protoplasts at the interface were removed with a capillary pipette, placed in a 50 ml conical tube under sterile conditions and diluted with 1 volume of W5 solution. The number of viable protoplasts (approximately 5 x 10^5 protoplasts from 4 leaves) were then estimated with a hemocytometer. The criteria for viability were spherical shape and uniform distribution of organelles. The protoplasts were placed into sterile 15 ml conical tubes in aliquots of 7.5 ml or less and diluted with 1 volume of W5 solution. The tubes were allowed to incubate at room temperature for 15 minutes with periodic inversions. The tubes were centrifuged for 2 minutes at 600 rpm in a clinical centrifuge. The superantant was discarded and the protoplast pellet(s) was resuspended in W5 solution to give an approximate concentration of 1.6 x 10^6 protoplasts/ml.

Plasmid VPCB-1 DNA and sheared calf thymus carrier DNA were added to the protoplasts at a concentration of 20 µg/ml and 50 µg/ml, respectively. To this solution was added 1.5 volumes of a 40% polyethylene glycol(PEG) solution (see Appendix A) followed by gentle mixing and a 25 minute room temperature incubation. Then was added 8 volumes of NTTO + mannitol + glucose + ampicillin (see Appendix A). Using a sterile capillary pipette, the solution was transferred to a 15 x 100 mm petri dish and allowed to incubate overnight in the dark. The number of viable protoplasts surviving the transfection (approximately 20-50%) were estimated with a hemocytometer. The tube was centrifuged for 2 minutes at 600 rpm in a clinical centrifuge. The supernatant was discarded and the protoplasts resuspended in 1 ml of W5 solution which was then transferred to a microfuge tube and centrifuged for 10 minutes at 16,000 x g. The

supernatant was discarded and the pellet was either stored at -80°C or used immediately for SDS-PAGE and subsequent Western blot analysis of PBD-2 expression. Yields were variable but, generally, 3-4 leaves would result in 1 x 10^5 transfected protoplasts.

G) Sample Preparation for Western Blot Analysis of PBD-2 Expression

1) Protoplast Preparation for SDS-Page

a) SDS-PAGE Analysis of Total Protoplast Proteins

The pelleted protoplasts (1.5-2.0 x 10^5) were mixed with 10 µl distilled H_2O and 10 µl 2X SDS sample treatment buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% ß-mercaptoethanol and bromophenol blue) to denature the proteins and boiled for 5 minutes [59]. The sample was cooled briefly and centrifuged at 11,000 x g at 4°C for 5 minutes. All of the supernatant was then loaded in one lane and subjected to SDS-PAGE and subsequent Western blot analysis to detect PBD-2.

b) Subcellular Localization of PBD-2 in Protoplasts

i) Soluble vs Particulate Proteins

In order to determine if the PBD-2 protein was associated with particulate proteins or with soluble proteins, protoplast lysates were fractionated by differential centrifugation. Approximately 2-3 x 10^5 protoplasts were resuspended in 0.5 ml of HBS (50 mM Tris-HCL pH 8.0, 1mM Na_2EDTA, 1 mM phenylmethyl sulphonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], 100 mg/ml butylated hydroxytoluene [BHT] and 0.3 M sucrose) solution [60]. The protoplasts were sheared by passing them through a 15 µm nylon mesh 3 times with a syringe. The protoplast lysates were transferred to a TLA100.3 rotor and centrifuged at 100,000 x g using a Beckman TL-100 tabletop ultracentrifuge. The soluble proteins contained in the supernatant were precipitated according to the procedure outlined by Schuster and Davies [61]. The supernatant was removed and mixed with 1 volume of phenol then vortexed for 10 minutes. The solution

was centrifuged at 11,000 x g for 10 minutes. The phenol layer was removed and transferred to a 15 ml Corex tube with 5 volumes of 0.1 M ammonium acetate in methanol. The tube was placed at -20°C overnight. The solution was centrifuged at 16,000 x g for 10 minutes. The pellet containing the precipitated proteins from the original supernatant and the original 100,000 x g particulate-containing pellet were both resuspended in 20 µl of 1X treatment buffer (see Appendix A), transferred to a microfuge tube, boiled for 5 minutes and centrifuged at 16,000 x g at 4°C for 5 minutes. All of the supernatant was subjected to SDS-PAGE and subsequent Western blotting to detect PBD-2.

ii) Subcellular Fractionation by Differential Centrifugation

Approximately 2-3 x 10^5 protoplasts were resuspended in 0.5 ml of HBS solution. The protoplasts were sheared by passing them through a 15 µm nylon mesh 3 times and the solution was then fractionated according to the differential centrifugations scheme in Figure 12. All of the samples were boiled for 5 minutes and centrifuged at 16,000 x g at 4°C for 5 minutes, then subjected to SDS-PAGE.

2) Whole Leaf Protein Extraction for SDS-Page

a) Analysis of Total Leaf Proteins for PBD-2

Fully expanded leaves were chosen from sterile axenic shoot cultures of transgenic and non-transgenic plants. The plant tissue was thoroughly homogenized in a 5 ml glass tissue grinder containing approximately 100 mg of glass beads (0.1 mm) and 500 µl of 1X treatment buffer/120 mg tissue (fresh weight). The homogenized tissue (50-250 mg) was centrifuged at 100 x g for 3 minutes in a clinical centrifuge. The supernatant was removed, boiled and electrophoresed as described above. Generally 20 µl of the supernatant was subjected to electrophoresis. The amount of total protein loaded per

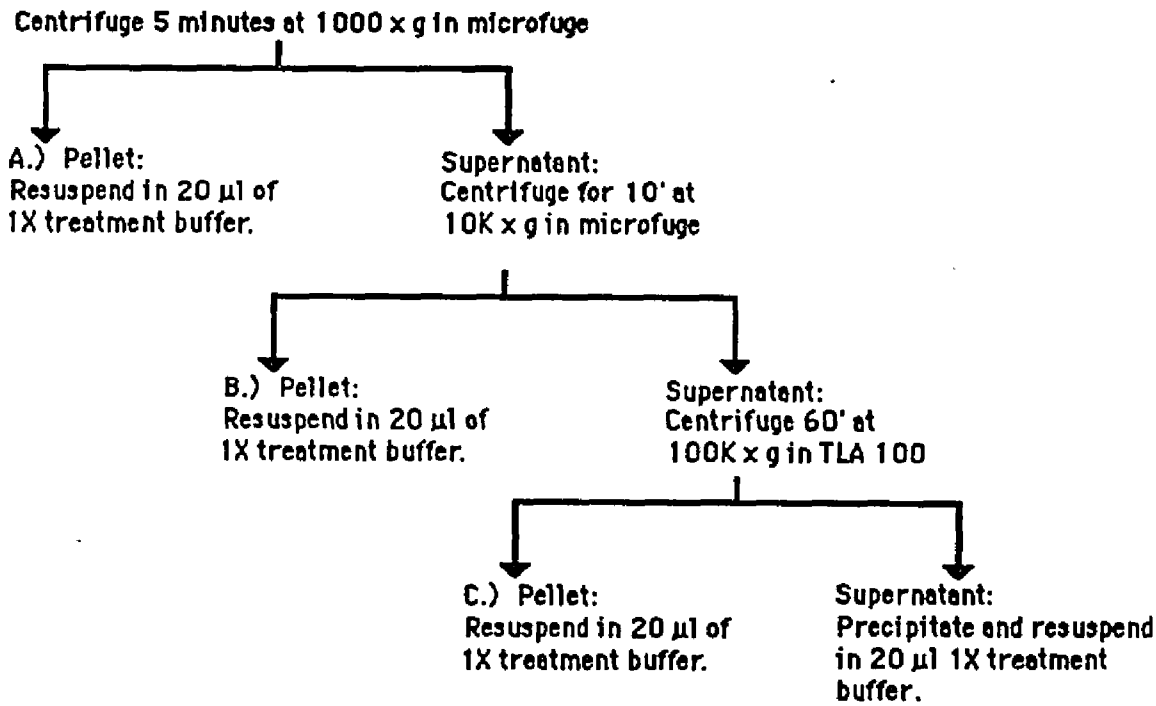

Figure 12: Centrifugation scheme for subcellular fractionation of transfected protoplasts and plant tissue with pellet (A) conating nuclei, cell walls and cell debris, chloroplasts and some microsomes and pellet (C) containing primarily microsomes, Golgi and some chloroplasts and mitochondria.

sample was approximately equal (100-150 µg) as determined by the "dot" method described below (G-3-a).

b) Subcellular Localization of PBD-2 in Transgenic Leaf Tissue

i) Soluble vs. Particulate Proteins

Transgenic leaf tissue was homogenized as above in HBS medium (120 mg tissue, fresh weight/500 ml HBS). The supernatant was subjected to the same centrifugation and precipitation procedures as described for the protoplast samples (section G-1-b-i) to obtain proteins from the 100,000 x g pellet and supernatant.

ii) Subcellular Fractionation by Differential Centrifugation

Transgenic leaf tissue was homogenized as above (G-2-a) in HBS medium (120 mg tissue, fresh weight/500 ml HBS). The supernatant was subjected to the same centrifugations and precipitation procedures (Figure 12) as the samples in the subcellular localization of protoplast proteins section above (section G-1-b-i).

iii) Association of PBD-2 with Marker Enzymes Using a Sucrose Gradient

Leaves from greenhouse-grown transgenic and non-transgenic plants (2 grams each) were homogenized separately using a chilled mortar and pestle in 4 volumes of a 50mM, Tris pH 8.0, 1mM EDTA and 0.3 M sucrose buffer (HBS) supplemented with 0.4% 2-mercaptoethanol, 0.1% bovine serum albumin, 0.5% insoluble polyvinyl-polypyrrolidone (PVPP, Sigma P-6755) and 1mM phenylmethylsulphonylfluoride (PMSF) for 2 minutes. The homogenization was facilitated by adding approximately 100 mg of glass beads (0.1 mm). The homogenate was filtered through 8 layers of pre-moistened cheesecloth and centrifuged at 400 x g for 10 minutes. It was necessary to remove secondary plant products (ie. phenolics, quinones), and the chemical supplements were added to the HBS buffer to inactivate these secondary products, since it was found that they interfered with the cytochrome c reductase and oxidase assays

described below [62]. To accomplish this, the homogenates (2ml) were layered over 50% sucrose in TE (w/w, 3ml), 15% sucrose in TE (w/w, 7ml) step gradients in 12 ml (Beckman 14 x 89 mm) SW-41 tubes. The SW-41 rotor was then centrifuged at 150,000 x g for 1 hour. The 15/50% sucrose interfaces (which contained the membranes) were removed and 5 µl aliquots diluted with 95 µl HBS buffer for analysis of chlorophyll fluoresence using a Fluorescan fluorimeter with excitation and emission wavelengths of 644 and 684, respectively [63].

One milliliter containing 1.4 x 10^6 chlorophyll fluoresence units of homogenate were layered over two 10 ml, 15-50% (w/w) linear sucrose gradients in 12 ml SW-41 tubes. The gradients were centrifuged to band membrane fractions to their isopycnic densities (overnight 8 hours) at 150,000 x g at 4°C [64]. The gradients were fractionated mechanically into 20 fractions of 0.6 ml using an Isco (model 640) fractionator. All steps of this procedure required that the samples be kept on ice or under refrigeration. The gradient fractions were analyzed for protein content using the modified Lowry microassay described below in Section 3-b and chlorophyll fluoresence as described above. Western blot analysis for the presence of PBD-2 was conducted after diluting 175 µl of each fraction to 1 ml with chilled TE, pH 8.0. The membranes in each sample were centrifuged at 100,000 x g for 20 minutes using a Beckman TLA 100.3 rotor with 13x51 mm polycarbonate tubes in a Beckman TL-100 tabletop ultracentrifuge. The pellet was resuspended in 20 µl SDS-treatment buffer, boiled for 5 minutes and subjected to SDS-PAGE and immunoblotting for the presence of PBD-2 (see sections H and I). The sucrose concentration (w/w) was determined for each fraction using a hand-held refractometer and the membrane marker enzyme activities for cytochrome c reductase and cytochrome c oxidase were determined according to the procedure of Briskin et al. [65].

Location of endoplasmic reticulum in gradient fractions was determined by an antimycin A-insensitive NADH cytochrome c reductase assay, performed immediately after fractionation of the gradient to avoid loss of activity. One hundred microliters of each fraction was mixed with 0.85 ml of a 1.67 mM KCN, 1mM antimycin A (Sigma A-2006), 0.3 mM cytochrome c (Sigma C-7752) and 43 mM KPO_4, pH 7.4 solution (see Appendix A for stock solutions). The reaction was initiated with 50 µl of 2.0 mM NADH (Sigma N-8129, see Appendix A) and the increase in absorbance at 550 nm was measured over a 2 minute time interval. Using Beer's law and an extinction coefficient for cytochrome c of 18.5 $mM^{-1}cm^{-1}$, the nmoles of cytochrome c reduced per minute per ml of enzyme can be determined by subtracting the activity measured in the presence of NADH from the activity in the absence of NADH (see Appendix A)[65].

For localization of mitochondrial membranes, the cytochrome c oxidase assay requires the addition of 75 µl of a 0.6 mM cytochrome c (Sigma C-7752) solution to an 0.825 ml solution containing 0.3% Triton-X 100, 44 mM KPO_4, pH 7.4 and 50 µl of the enzyme solution (see Appendix A for the stock solutions). The 0.6 mM cytochrome c solution was reduced with sodium dithionite prior to use. One milligram of sodium dithionite was enough to reduce 3 ml of a 0.6 mM cytochrome c solution. The reaction solutions were mixed and the decrease in absorbance over a 3 minute interval at 550 nm was measured. Calculations for enzymatic activity were performed as described above (see Appendix A for calculations).

3) Protein Concentration Determination

a) Dot Method

A standard curve was made using one microliter aliquots of dilutions of a 1 mg/ml stock of bovine serum albumin (BSA) protein in 1x treatment buffer spotted on 3 mm Whatman paper. The concentration of BSA in each 1 µl aliquot ranged from 0.2

µg/µl-1.0 µg/µl in 0.2 µg/µl increments. On the same paper were spotted 1 µl of 1:5 and 1:10 dilutions as well as 1 µl of an undiluted sample containing the proteins extracted from protoplasts and plant tissue. The paper was dried for 15 minutes under a stream of warm air. The paper was then stained for five minutes with Coomassie blue (0.25% Coomassie blue R250 in methanol: ddH_2O: acetic acid [4.5:4.5:1]) and then destained, until background levels were low, with Coomassie blue destain (methanol: ddH_2O (double distilled water): acetic acid [4.5:4.5:1]). The intensity of the dots from the BSA standards were visually compared to the unknown samples to estimate protein concentrations [66].

b) Modified Lowry Microassay

A standard curve was made using 200 µl duplicate samples containing 0, 1, 3, 5, 7, 15 and 20 µg BSA protein, respectively. The samples were diluted from a 100 µg protein/ml stock solution in water containing 200 mg/liter sodium azide. Duplicate 200 µl aliquots of the fractions from sucrose gradients were diluted 1:10 and 1:5. Twenty microliters of 0.15% sodium deoxycholate was added to each sample, mixed and allowed to stand at room temperature for 10 minutes. This was followed by the addition of 20 µl of 72% trichloroacetic acid and centrifugation at 3000 x g for 15 minutes to precipitate the proteins. The supernatant was removed and 200 µl water was added to the pelleted proteins and 200 µl reagent A (see Appendix A) was added to all samples, including the standard curve samples. The samples were mixed and allowed to incubate at room temperature for 10 minutes. Then 100 µl of reagent B (see Appendix A) was mixed with the samples, which were incubated for 30 minutes at room temperature. The absorbance of each sample was measured at 750 nm and compared to the BSA protein standards to determine approximate protein concentrations [67].

H) SDS-PAGE of Protein Samples

1) Gel Preparation and Electrophoresis

A 12% polyacrylamide gel in 0.375 M Tris pH 8.8, 0.1% SDS and 5.0% glycerol prepared in a Bio-Rad Mini-Protean II electrophoresis cell was used to separate the proteins. The running gel was composed of 3.75 ml 4X running buffer, 6.25 ml Bis: Acrylamide (29.2%:0.8%) solution, 4.2 ml H_2O and 0.75 ml glycerol (see Appendix A). The solution was degassed for 15 minutes under a mild vacuum before the addition of 75 µl of 10% ammonium persulfate and 7.5 µl of TEMED (Bio-Rad). After the solution was poured into the vertical Mini-Protean gel apparatus, it was overlayed with 30 µl of H_2O-saturated isobutanol. The gel was allowed to polymerize for 15 minutes before the isobutanol was removed and replaced with 1x running buffer (see Appendix A). The gel was allowed to polymerize for 1 hour. The buffer was removed and a 4.5% polyacryalamide stacking gel in 0.125 M Tris pH 6.8 and 0.1% SDS was poured. The stacking gel was composed of 1.5 ml 4 X stacking buffer, 0.9 ml Bis: Acrylamide stock solution and 3.6 ml ddH_2O (see Appendix A). Again the solution was degassed before the addition of 30 µl of 10% ammonium persulphate and 6 µl of TEMED. The solution was gently mixed and pipetted over the running gel and a 1mm thick 10 lane comb was inserted. After 30 minutes of polymerization, the gel was placed in the electrophoresis tank with SDS tank buffer (see Appendix A). The samples were loaded and electrophoresed for 35 minutes at 200 V [59]. The protein size markers (high and low molecular weight standards) were purchased from Bio-Rad and boiled for 5 minutes according to manufacturer's directions using 5 µl of each per gel.

I) Western Blotting

1) Electroblotting

The polyacrylamide gel containing the electrophoresed proteins was placed in a Bio-Rad Mini Transblot electrophoretic transfer cell according to the manufacturers specifications. The transfer cell was filled with Matsudaira's recommended transfer buffer (see Appendix A) and electrophoresed for 60 minutes at 100V. The gel was placed adjacent to the polyvinylidene difluoride membrane (PVDF, Millipore) and sandwiched between 4 pieces of Whatman 3mm paper. The proteins were electrophoretically transfered to the PVDF membrane [68] and then probed with PBD-2 antibody.

2.) Antibody Probing of Proteins to Detect PBD-2 Protein

The PVDF membrane containing electrophoretically transferred proteins was incubated for 1 hour in 50 ml of TSW (see Appendix A) followed by a 30 minute incubation in fresh TSW at room temperature. The TSW was replaced by 24 ml of a 1: 600 dilution of the IgG fraction of anti-PBD-2 (courtesy of Dr. Jim Halpert, of the Department of Pharmacology and Toxicology, University of Arizona) in TSW and incubated for 1 hour. The membrane was washed twice for 15 minutes in TSW followed by a 30 minute incubation with 24 ml of a 1:2000 dilution of an alkaline phosphatate-conjugated goat anti-rabbit antibody (Bio-Rad) in TSW. Again the membrane was washed twice for 15 minutes in TSW. The membrane was rinsed briefly in Color Developing Solution (CDS) then developed in a nitro-blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate system (BCIP, see Appendix A) [69].

J) Tobacco Genomic DNA Isolation

To demonstrate successful transformation, genomic DNA, isolated from our transgenic plants, was necessary for Southern blot analysis. Using the method of Rogers et al. [70], approximately 500 mg of leaf tissue was ground in a mortar and pestle with

dry ice. The powdered tissue and dry ice were placed in a microfuge tube and gently mixed with 1 volume of 65°C 2X cetyltrimethylammonium bromide solution (CTAB, Aldrich, see Appendix A). The mixture was incubated at 65°C for 5 minutes and extracted with 1 volume of chloroform/isoamyl alcohol (24:1). The chloroform/isoamyl alcohol-containing solution was mixed thoroughly and centrifuged at 11,000 x g for 30 seconds. The aqueous phase was removed and mixed with 0.1 volumes of 10% CTAB (see Appendix A) and mixed. Another chloroform/isoamyl alcohol extraction was performed and the aqueous phase removed. The aqueous (upper) phase was added to 1 volume of CTAB precipitation buffer (see Appendix A) and gently mixed. The solution was centrifuged for 1 minute at 11,000 x g to pellet the DNA. The supernatant was discarded and the DNA pellet was dissoloved in the necessary (<500 µl) volume of high salt TE buffer (see Appendix A). The DNA was precipitated again by the addition of 100% ethanol and centrifuged for 15 minutes at 11,000 x g. Again the supernatant was discarded and the pellet was resuspended in the appropriate (enough to dissolve the DNA pellet) volume of 0.1 x TE buffer. The DNA was treated with RNase (100 µg/ml RNase A for 1 hour at 37°C) and the concentration determined spectrophotmetrically. An O.D. $_{260}$ of 1 corresponds to approximately 50 µg/ml of double-stranded DNA [53].

K) Southern Blot Analysis

DNA samples were digested for 3 hours using 2 units of EcoRI/µg genomic DNA and electrophoresed at 80 V for 3.0 hours in a 1% agarose gel in TAE buffer (0.04 M Tris-acetate and 8 mM EDTA). The gel was stained with ethidium bromide and photographed. After DNA transfer by the Southern method the gel was incubated twice with shaking for 15 minutes in denaturing solution (see Appendix A) [71]. The gel was then incubated with shaking twice for 15 minutes in neutralizing solution (see Appendix

A). The transfer apparatus was assembled according to Figure 13 and the DNA was allowed to transfer overnight in 20 x SSC (3M NaCl, 0.3M sodium citrate, pH 7.0).

The Nytran (Schleicher and Schuell) membrane was removed, rinsed in 5x SSC and the DNA linked to it with ultraviolet light (254 nm) in a Stratalinker using 1200 microjoules. The membrane was prehybridized in a sealed plastic bag for 4 hours at 50°C in a prehybridization mixture listed in Appendix A. The prehybridization solution was removed and replaced with the hybridization solution (see Appendix A) containing the α-^{32}P dCTP (ICN Biomedicals) labeled probe. The membrane was incubated for 24 hours at 50°C. The probe (PvuII/EcoRI fragment of PBD-2 cDNA) provided courtesy of Betty Glinsmann-Gibson was labled according to the manufacturer's specifications for the Random Primed DNA Labeling kit (Boehringer-Mannheim) using the Klenow fragment of DNA polymerase I [72]. Labeled DNA generally had a specific activity >1 x 10^8 cpm/µg and was used in the hybridization mixture at a concentration of 8 x 10^5 cpm/ml. After hybridization, the membrane was washed twice for 15 minutes at room temperature in 2X SSC and 0.1% SDS. The membrane was then subjected to two high stringency washes fof 15 minutes each at 50°C in 0.1X SSC and 0.1% SDS followed by a 15 minute rinse at room temperature in 2X SSC and 0.1% SDS. The membrane was exposed to X-ray film at -80°C for 2-14 days.

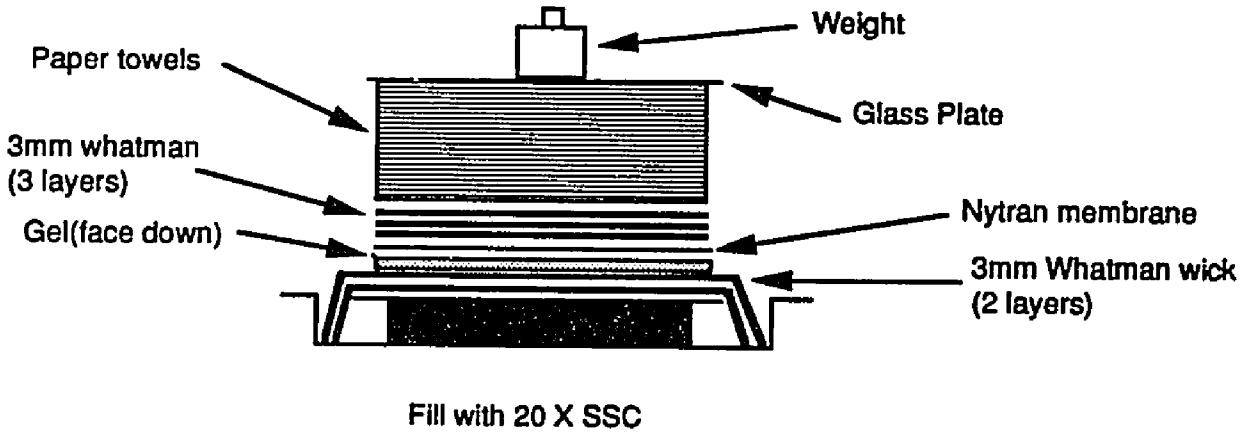

Figure 13: Diagram of Southern blot transfer apparatus.

CHAPTER 3

RESULTS

A) Construction of PBD-2 Recombinant Vectors

A plasmid vector (pVPCB-1) containing the PBD-2 coding sequence was constructed to demonstrate the feasibility of expression of PBD-2 in plants. Initial experimentation involved analysis of the transient expression of PBD-2 in protoplasts. This was followed by the construction of a Bin 19 vector (pBO-1) for *Agrobacterium tumefaciens*-mediated transformation of tobacco plants. Both plasmid constructions were verified by restriction digest analysis of the DNA from small-scale plasmid isolations [54] performed on the transformed cells.

1) Vector Construction for Transient Expression of PBD-2 in Protoplasts

The ligation of the PBD-2 coding sequence (EcoRI/PvuII fragment) from pD39 (Figure 5) into the SmaI site of pDG-7 (Figure 7) resulted in the plasmid VPCB-1 (Figure 8). Competent E. coli DH5α cells were transformed with pVPCB-1, which carries a gene conferring ampicillin resistance, and grown on ampicillin-supplemented media. Plasmids were isolated from several ampicillin resistant colonies to determine that a single copy of the PBD-2 coding sequence was ligated, in the correct orientation, into pDG-7. If the PBD-2 insert were ligated in the correct orientation, one would expect DNA fragments of 1.5 and 3.73 kb (Figure 8) following digestion of pVPCB-1 with SacI. If the insert were in the opposite orientation, the expected fragments would be 5Kb and 0.05Kb. To verify the presence of a single copy of the PBD-2 coding sequence into pDG7, the isolated plasmid [54] was digested simultaneously with BamHI and EcoRI. Resultant fragments of 1.75 and 3.48 kb are expected. The results shown in Figure 14

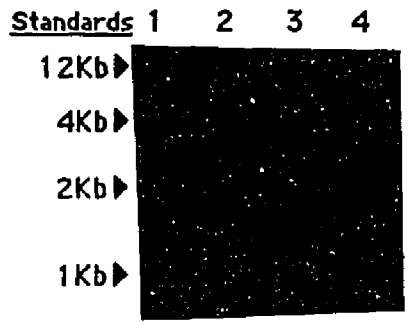

Figure 14: Photograph of an ethidium bromide stained 1% agarose gel containing digested pVPCB-1 DNA. The DNA is from a small-scale plasmid isolation derived from a single colony of DH5α cells that exhibited kanamycin resistance. The digestions were performed to verify the presence and orientation of the PBD-2 cDNA subcloned into pDG-7. Lane 1 contains plasmid DNA digested with both EcoRI and BamHI. Lanes 2 and 3 contain pVPCB-1 DNA digested with BamHI and EcoRI, respectively. Lane 4 contains plasmid DNA digested with SacI.

verify that a single copy of the PBD-2 sequence is ligated into pDG7 in the correct orientation.

2) Vector Construction for Transformation of Tobacco Plants

A 2.55Kb region of pVPCB-1 consisting of a CaMV 35S promoter and a nopaline synthetase poly A signal was ligated into Bin 19 (Figure 10) to construct a plasmid containing a PBD-2 cDNA which could be mobilized into *Agrobacterium tumefaciens.* This was constructed by a unidirectional ligation using Hind III and EcoRI restriction sites of both pVPCB (Figure 8) and Bin 19 (Figure 10), resulting in the plasmid BO-1 (Figure 11). The CaMV/PBD-2/Nos cassette was ligated into the lacZ gene. Transformed DH5α cells have a $lacZ^-$ phenotype, and are unable to metabolize X-galactose. Thus, on kanamycin and X-Gal supplemented media, DH5α cells having a Bin 19 plasmid with an uninterrupted lacZ gene give blue colonies. Colonies containing a recombinant Bin 19 plasmid were white in color. Twelve white colonies were selected for small-scale plasmid preparations [54], and restriction enzyme analysis were performed to verify that the CaMV/PBD-2/Nos cassette was correctly ligated into Bin 19 to yield pBO-1 (Figure 11). If the DNA cassette is correctly ligated into Bin 19 the isolated pB0-1 plasmids will, upon simultaneous digestion with with EcoRI, BamHI and Hind III, yield DNA fragments of 0.8, 1.75 and 10 kb. The results presented in Figure 15 show that 10 of the 12 colonies selected for restriction enzyme analysis with EcoRI, BamHI and Hind III yielded DNA fragments of the expected size with two colonies giving uninterpretable results. Colony 5 was chosen as a source of pBO-1 to be mobilized into *Agrobacterium tumefaciens.*

B) Mobilization of pBO into *Agrobacterium tumefaciens*

To obtain transformed tobacco plants it was necessary to mobilize pB0-1 from DH5α cells into the *Agrobacterium tumefaciens* strain LBA4404 [55] by conjugation

Figure 15: Photograph of an ethidium bromide stained, 1% agarose gel containing digested pBO-1 DNA. The DNA is from a small-scale plasmid isolation derived from 12 individual colonies of DH5α cells that exhibited kanamycin resistance and the inability to metabolize X-galactose. The digestions were performed to verify the presence of the EcoRI, Hind III fragment of pVPCB-1. The isolated plasmids were digested with EcoRI, BamHI and HindIII simultaneously to yield expected DNA fragments of 0.8,1.75 and 10 kb (see Figure 11). Lanes 1-12 are from 12 individual colonies. Colony (lane) 5 was chosen to mobilze pBO-1 into *Agrobacterium tumefaciens.*

(triparental mating, see Materials and Methods) using the pRK2013 helper plasmid [56]. Small-scale plasmid isolation [54] and restriction enzyme analysis were performed on 7 colonies of the transformed LBA4404 cells to verify the presence of pBO-1. The isolated plasmid was simultaneously digested with EcoRI and HindIII yielding 2.55 (PBD-2 cassette, Figure 11) and 10Kb (Bin 19) DNA fragments. In addition to restriction enzyme analysis following the triparental mating, the *Agrobacterium tumefaciens* strain LBA4404 was repeatedly streaked on minimal media supplemented with kanamycin to insure selection for *Agrobacterium tumefaciens* with pBO-1. The results of the restriction enzyme analysis of plasmid DNA from transformed LBA4404 cells (Figure 16) show that 6 colonies yielded the expected DNA fragments and 1 colony yielded uninterpretable results. Colony 3 was chosen for transformation of tobacco plants.

C) PBD-2 Expression in Transfected Protoplasts

The transient expression of PBD-2 in tobacco protoplasts provided preliminary information regarding the location and expression of the PBD-2 protein in tobacco cells. The pVPCB-1 vector was used for the polyethylene glycol (PEG)-mediated transfection of tobacco protoplasts and the expression of PBD-2 was determined by SDS-PAGE of the protoplast proteins and Western blotting for the presence of PBD-2. Preliminary information regarding the intracellular location of PBD-2 was obtained by differential centrifugation of protoplast proteins and subsequent Western blotting for the presence of PBD-2.

1) Western Blot Analysis

Figure 17 is a Western Blot of total protein from transfected and non-transfected protoplasts. The results show that the PBD-2 cDNA is capable of being

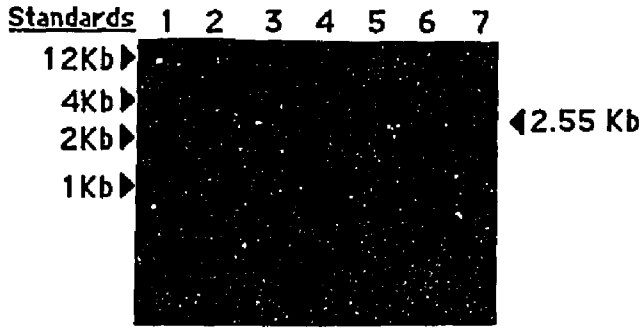

Figure 16: Photograph of an ethidium bromide stained, 1% agarose gel containing digested pBO-1 DNA. The DNA is from a small-scale plasmid isolation derived from 7 colonies of *Agrobacterium tumefaciens*, strain LBA4404 that were growing on minimal media supplemented with kanamycin. The digestions were performed to verify the presence of pBO-1 in LBA4404. Lanes 1-7 are plasmids digested with both EcoRI and HindIII. The resultant DNA fragments are expected to be 2.55 and 10 kb (see Figure 11). The colony in lane 3 was used for the transformation tobacco plants.

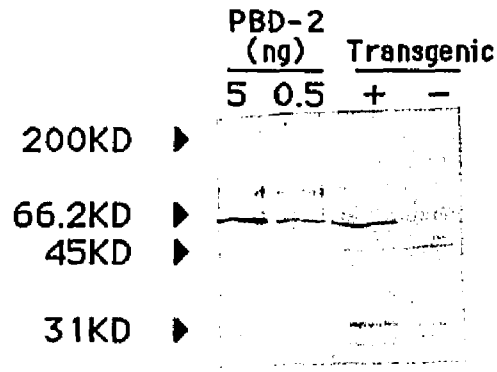

Figure 17: Western blot analysis of protoplasts transfected with pVPCB-1 to verify expression of the PBD-2 protein in plant cells. The lanes (L to R) contain purified PBD-2 protein, 5.0 and 0.5 ng, respectively, 2.1 x 10^5 protoplasts transfected with pVPCB-1 and 2.1 x 10^5 mock-transfected protoplasts.

expressed in tobacco protoplasts at levels of approximately 5 ng PBD-2/2.1 x 10^5 transfected protoplasts.

In addition to determining if PBD-2 would be expressed in detectable quantities, it was desirable to determine its subcellular location. Cytochrome P-450 proteins require the presence of NADPH oxidoreductase protein to provide electrons necessary for oxidation of the substrate (Figure 4). The oxidoreductases are membrane bound proteins, located primarily in the endoplasmic reticulum. Thus for enzymatic activity of PBD-2 in plant cells, it was assumed that PBD-2 would have to be present in the endoplasmic reticulum. Transfected protolasts were subjected to differential centrifugation and the different fractions analyzed by the Western blot method. Figure 18 compares proteins from the 100,000 x g pellet with precipitated soluble proteins from the supernatant of transfected protoplasts. The results indicate that PBD-2 is associated with the particulate protein (membrane) fraction of transfected protoplasts.

Further differential centrifugation was applied to the transfected protoplast lysates (Figure 12) and the various fractions were similarly analyzed by immunoblotting. Figure 19 shows the protein from the 100,000, 10,000 and 1,000 x g pellets and soluble proteins after being subjected to Western blotting. PBD-2 is present in both the 100,000 and 10,000 x g fractions which are the fractions that contain the endoplasmic reticulum [73]. The evidence was encouraging enough to warrant attempts to transform tobacco plants with a PBD-2 coding sequence that was likely to be expressed as it was in protoplasts.

D) Analysis of PBD-2 Expression in Transformed Plants

Agrobacterium tumefaciens cells strain LBA4404 containing pBO-1 (Figure 11) were placed in contact with tobacco leaf disks to allow transformation by insertion of the CaMV/PBD-2/Nos/neomycin phosphotransferase gene cassette into the tobacco

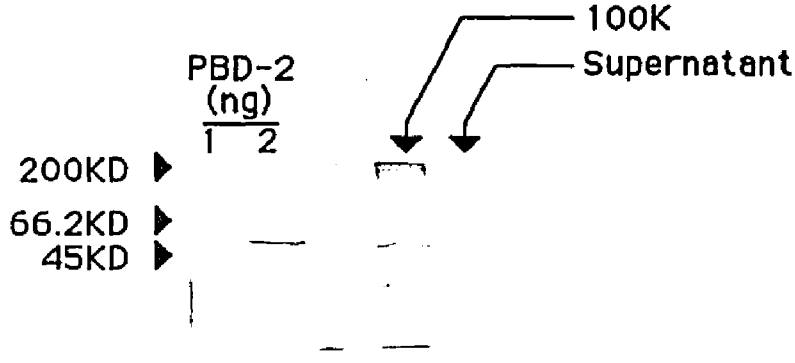

Figure 18: Western blot analysis of protoplasts transfected with pVPCB-1. The protoplasts were lysed and centrifuged at 100,000 x g to pellet the particulate proteins. The soluble proteins in the supernatant were precipitated and analyzed. From L. to R. the lanes contain 1 and 2 ng of purified PBD-2 protein respectively, and the 100k x g pellet and the supernatant.

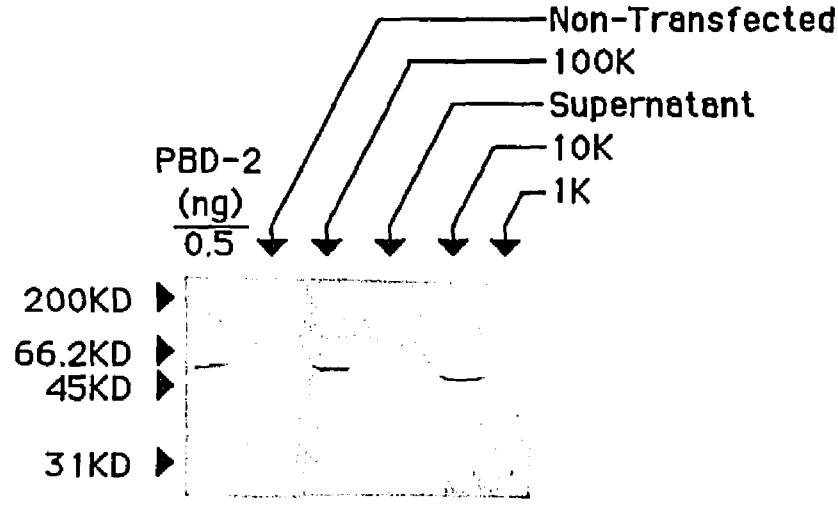

Figure 19: Western blot analysis of proteins from pVPCB-1 transfected protoplasts. The 6 x 10^5 protoplasts were lysed and subjected to centrifugation at 10k x g for 10 minutes. The supernatant was then centrifuged at 100k x g for 60 minutes. The precipitated proteins from the supernatant and from 6 x 10^5 mock-transfcted protoplasts were also analyzed.

genome. After about 3 months of incubation with appropriate media, the leaf disks were regenerated into plants transformed with PBD-2 (see materials and methods).

1) Western Blot Analysis

Over 50 individual plants were regenerated from callus cultures derived from transformed leaf disks. Of the 50 plants placed in rooting media supplemented with kanamycin (see Appendix A) 14 continued to exhibit resistance after several months of growth on the selective media. Of the 14 surviving plants, 9 showed detectable (0.5 ng PBD-2/150 µg total leaf protein) levels of PBD-2 expression, with 4 exhibiting relatively high (10 -50 ng PBD-2 /150 µg total leaf protein) levels of the protein based on visual inspection of Western blots. For more details see Appendix B.

a) Total Protein

Figure 20 shows the results of SDS-polyacrylamide electrophoresis and Western Blot analysis of the total protein from the homogenate of leaves from 10 transgenic plants. The amount of total leaf proteins loaded per lane are approximately equivalent. This assertion is based on the fresh weight of the leaf tissue and an estimation of 100-150 µg protein/lane using the "Dot" method [66]. The plants in Figure 20 that apparently did not express the protein were further probed by increasing the amount of total protein loaded on the gel to facilitate detection of PBD-2. After increasing the amount of total protein, the PBD-2 protein still was undetectable but plants 18 and 35 both contain the PBD-2 coding sequence as verified by Southern Blotting. Figure 21 is a Western Blot of the plant (#16) used in Figures 22 and 23.

b) Soluble vs Particulate Protein

In order to determine if the PBD-2 protein was associated with membranous or soluble proteins, fractionation of leaf homogenates by differential centrifugation was employed in a manner consistent with the previous analysis of the transfected

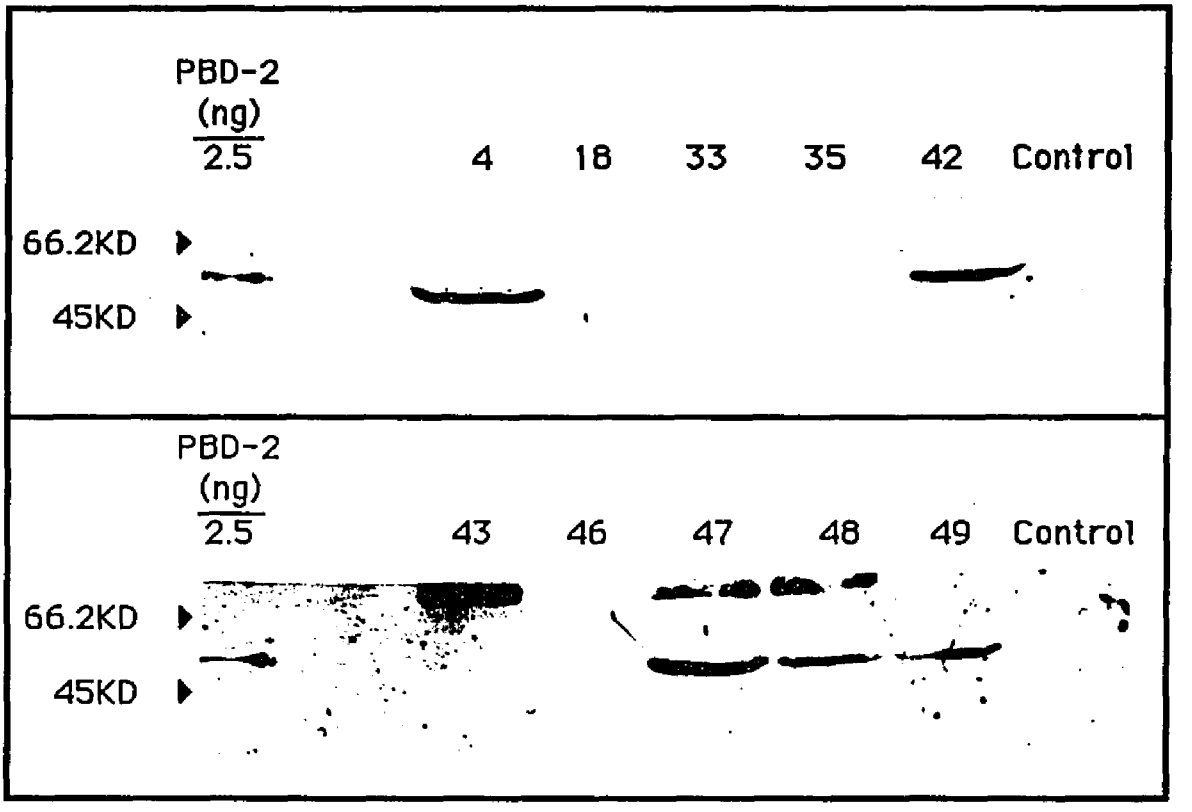

Figure 20: Western blot analysis of protein from 10 different transgenic plants and 2 non-transgenic (control) plants. The leaf tissue is from 1-2 cm wide leaves taken from axenic shoot cultures. Each lane contains approximately 100-130 µg total protein.

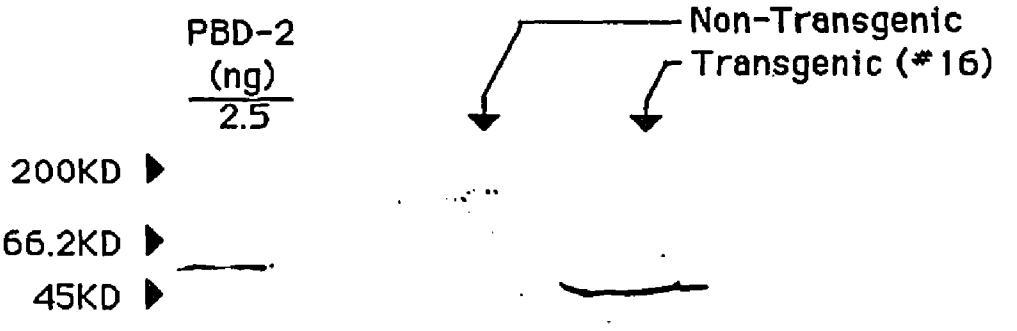

Figure 21: Western blot analysis of a transgenic tobacco plant transformed with the PBD-2-containing plasmid, pBO-1 compared to non-transgenic plant proteins. Lanes 1 and 2 contain 2.5 and 0.5 ng purified PBD-2 protein, respectively. Approximately 150 µg transgenic and non-transgenic total greenhouse-grown plant proteins were analyzed for the presence of the PBD-2 protein.

protoplasts. Leaf homogenates of the transgenic plants were subjected to sedimentation at 100,000 x g with the subsequent pellet constituting the particulate fraction and the proteins precipitated from the supernatant constituting the soluble fraction. Figure 22 illustrates the results of this experiment which show that the PBD-2 protein is associated with the particulate proteins of the fractionated transgenic leaf homogenate. This result is consistent with the results obtained using transgenic protoplasts.

c) Sucrose Gradient Fractionation and Marker Enzyme Analysis

The subcellular location of the PBD-2 protein was determined using leaf homogenate applied to an isopycnic linear (15%-50% (w/w)) sucrose gradient. The gradient was fractionated into 20, fractions of 0.6 ml and analyzed quantitatively for 5 characteristics: antimycin A-insensitive NADH cytochrome c reductase, cytochrome c oxidase, chlorophyll fluoresence, sucrose percentage (w/w), and presence of PBD-2. Chlorophyll fluoresence, cytochrome c reductase and cytochrome c oxidase enzyme activities were used to localize thylakoid, endoplasmic reticulum and mitochondrial membranes respectively [65]. SDS-PAGE and immunoblotting were performed on each fraction to locate the PBD-2 protein. Figure 23 illustrates the relationship between these parameters and the PBD-2 protein being expressed in transgenic plants. The distribution of marker enzymes and protein in non-transgenic control plants is shown in Figure 24. These results indicate that the PBD-2 protein is predominantly associated with the endoplasmic reticulum marker enzyme and to a lesser extent with the thylakoid membranes.

E) Southern Blot Analysis of DNA from Transformed Plants

Genomic DNA isolated from regenerated plants was analyzed by the Southern blot method to verify the successful integration of the PBD-2 cDNA into the genome of transgenic plants. Plants chosen for Southern analysis with radiolabled PBD-2 coding

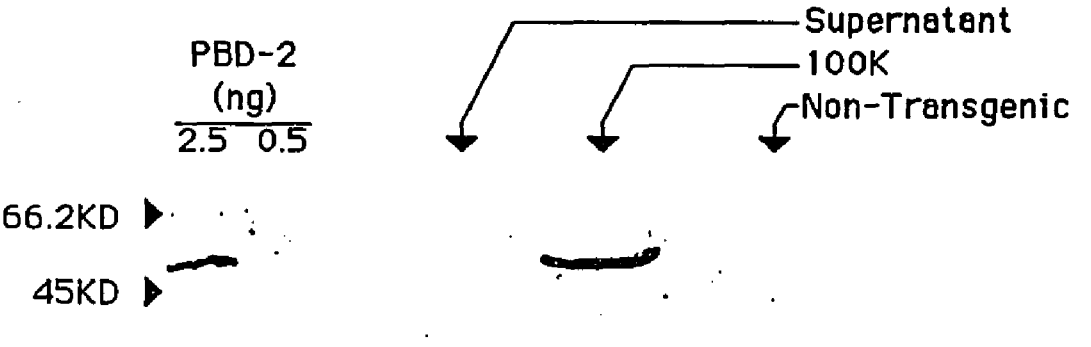

Figure 22: Western blot analysis of the particulate (100k) and non-particulate (Supernatant) fractions of a pBO-1 transformed tobacco plant. Approximately 300 µg of plant homogenate was centrifuged at 100,000 x g for 60 minutes. The resulting pellet and the precipitated proteins from the supernatant were then analyzed. Approximately 200 µg of tobacco plant proteins were used in the non-transgenic control. Both plants were grown in a greenhouse.

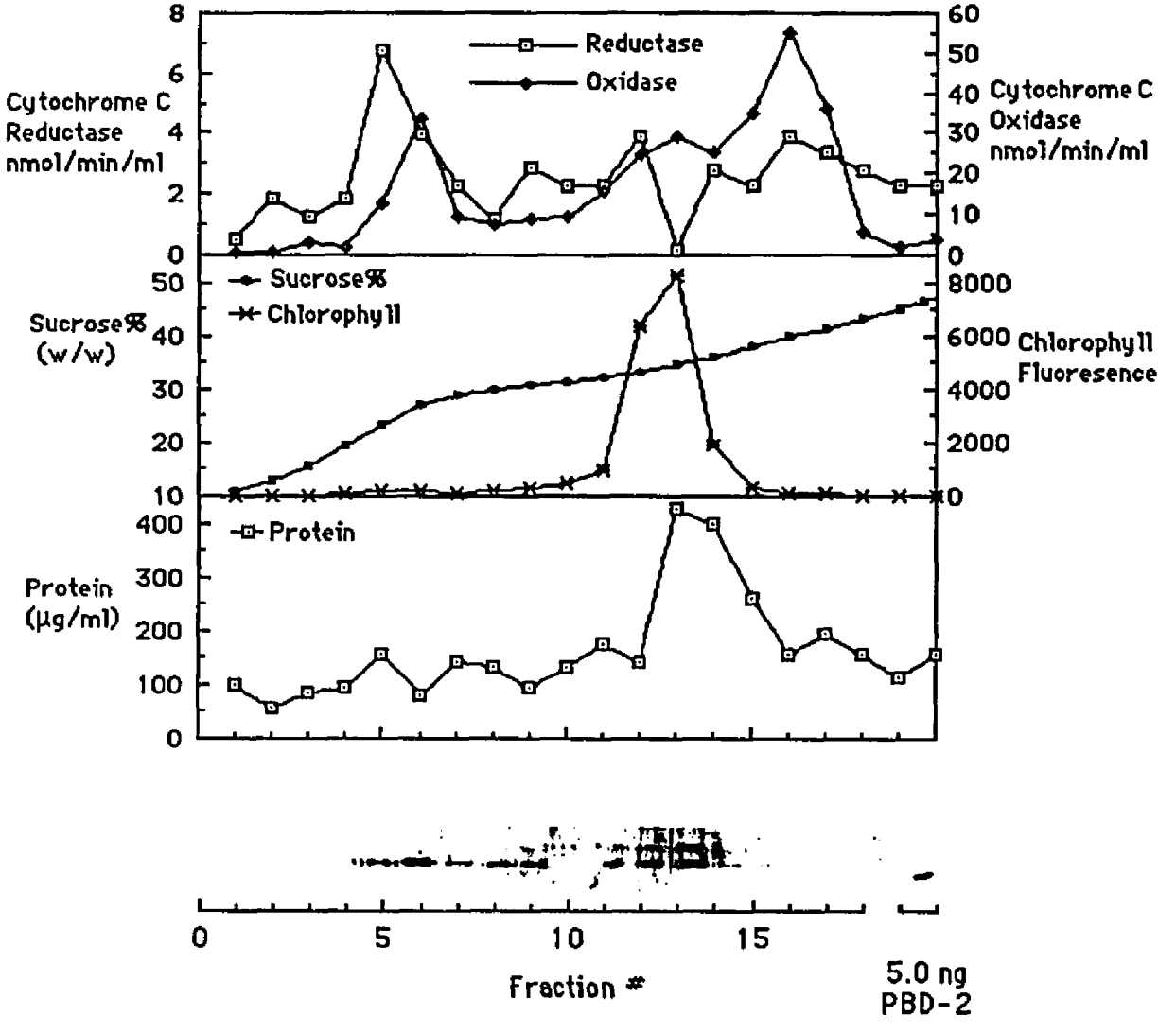

Figure 23: Analysis of fractions from a linear sucrose gradient to localize the PBD-2 protein. The top graph illustrates the relative quantities of 2 traditional marker enyme activities (Antimycin A-insensitive NADH-dependent cytochrome C reductase and cytochrome C oxidase) in the 20 aliquots of a linear sucrose gradient containing fractionated transgenic plant material. The middle graph shows the weight/weight sucrose concentration and chlorophyll fluoresence and the bottom graph shows the protein concentration of the same 20 fractions. The bottom portion of this figure is a Western Blot of 175 µl of each fraction for the presence PBD-2.

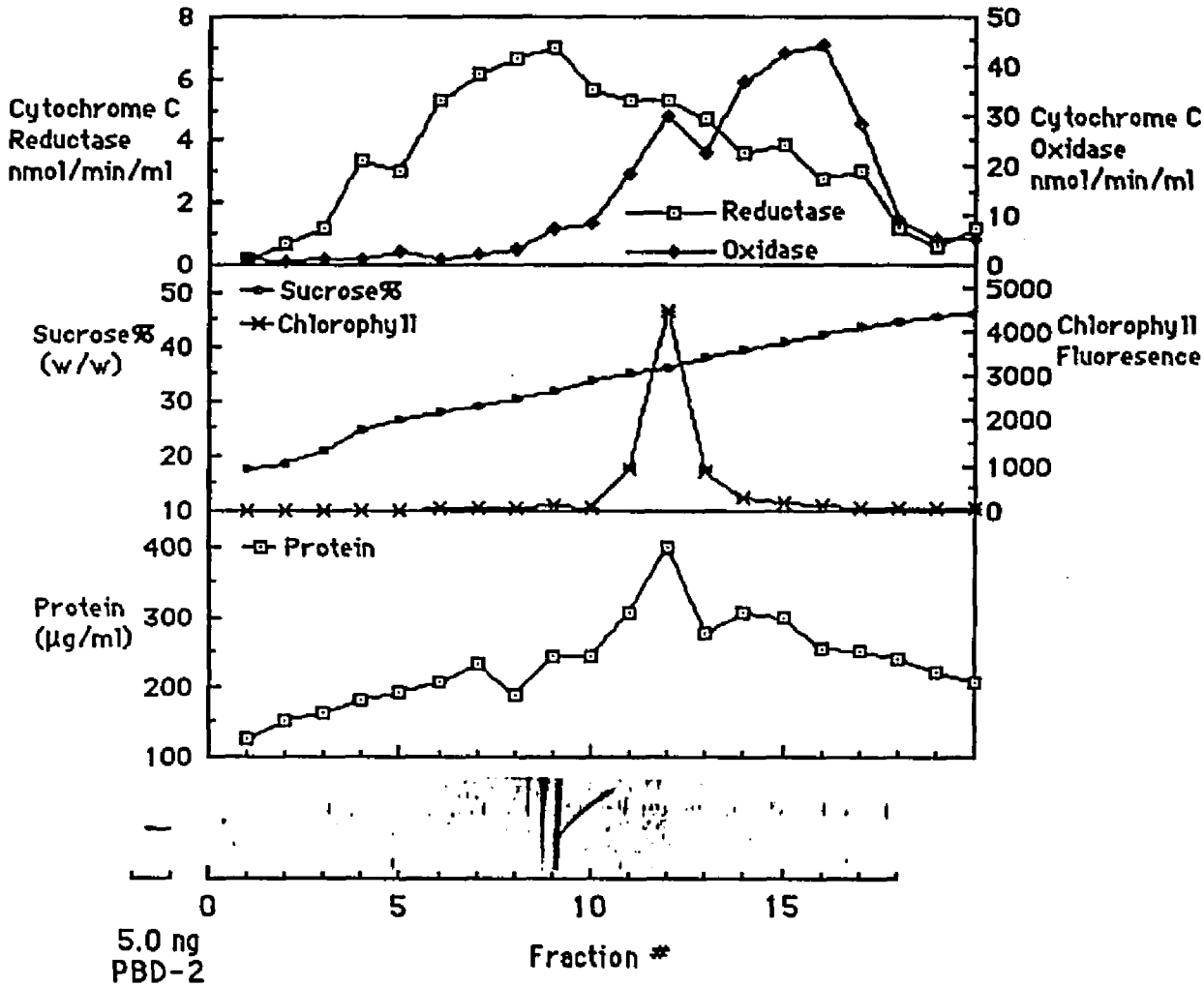

Figure 24: Analysis of fractions from a linear sucrose gradient containing non-transgenic leaf homogenate. The top graph illustrates the relative quantities of 2 traditional marker enyme activities (Antimycin A-insensitive NADH-dependent cytochrome C reductase and cytochrome C oxidase) in the 20 aliquots of a linear sucrose gradient containing fractionated non-transgenic plant material. The middle graph shows the weight/weight sucrose concentration and chlorophyll fluoresence and the bottom graph shows the protein concentration of the same 20 fractions. The bottom portion of this figure is a Western Blot of 175 µl of each fraction for the presence PBD-2.

sequence were those which expressed relatively high levels of PBD-2 as determined by visual inspection of Western blots. Figure 25 shows genomic DNA digested with EcoRI from clones of plants numbered 18, 47, 48, and 49 and DNA from non-transgenic plants. All plants tested positive for the the presence of the PBD-2 coding sequence and all plants, except plant 2, expressed detectable levels of PBD-2 protein.

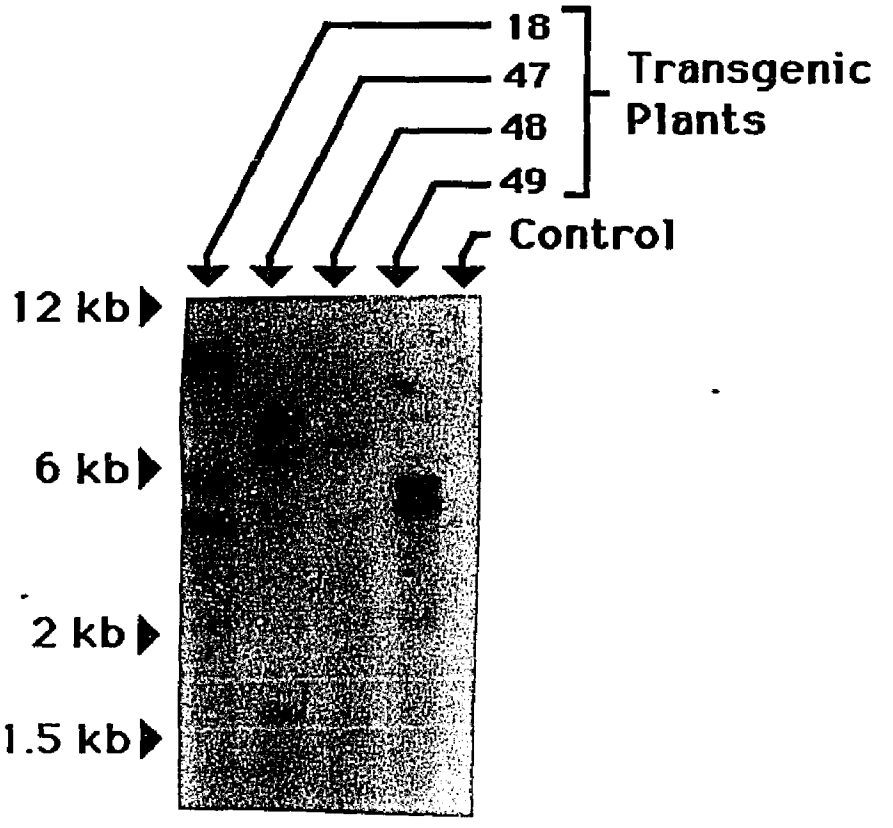

Figure 25: Southern blot analysis of genomic DNA of pB0-1-transformed plants. DNA was cut with EcoRI and probed with the PBD-2 coding sequence. Each lane contains 7µg of digested DNA.

CHAPTER 4

DISCUSSION

The PBD-2 enzyme functions to readily metabolize certain PCB congeners to water soluble metabolites in the dog [26, 29, 74]. A PBD-2 cDNA has been isolated from a dog liver cDNA library using a radiolabled rabbit P450IIB probe. The cDNA was sequenced and used as a probe to screen genomic dog DNA to determine the complexity of the PBD-2 gene family. It was determined through densitometric analysis of Southern blots that there are 2-5 copies of the PBD-2 gene in the dog genome. Northern blot analysis with the PBD-2 coding sequence revealed two mRNA species of 1.9 and 2.9 kb suggesting that either the two polyadenylation signals of a single transcript are used or two related PBD-2 genes are being transcribed [30].

Numerous researchers have demonstrated the ability of exogenous genes to function in transformed plants [55, 75, 76, 77] while there are few reports of functional mammalian genes [78] introduced into higher plants. Lefebvre et al. 1987 reported that a functional Chinese hamster metallothionein II gene was expressed in *Brassica campestris*. The metallothionein decreased the susceptibility of the plants to cadmium toxicity and was the first functional mammalian gene expressed in plants [78]. To our knowledge, no one has demonstrated a functional mammalian cytochrome P-450 in a transgenic plant, although Saito et al. 1990 [79] are attempting to create a transgenic plant containing a rabbit P-450 capable of modifying pharmaceutically useful secondary plant metabolites. The outcome of this research has yet to be published. The ability of the PBD-2 protein to function in a plant cell has yet to be determined, but

our research has provided information on the properties of a mammalian cytochrome P-450 as expressed in tobacco cells.

The catalytic activities of cytochrome P-450 isozymes are dependent on an oxidoreductase-flavoprotein component to provide reducing equivalents (Figure 4). With few exceptions, the cytochrome P-450 and the corresponding reductase are membrane bound proteins located in the endoplasmic reticulum [80]. It was therefore desirable to demonstrate the presence of PBD-2 in the endoplasmic reticulum where it may couple with the necessary oxidoreductase-flavoprotein.

Differential centrifugation schemes were used to determine the location of PBD-2 in leaf and protoplast homogenates. Protocols were designed according to the results of studies focusing on sedimentation rates of plant organelles. Nagahashi et al. (1982) reported approximately 85% recovery of mitochondrial marker enzyme activity (cytochrome c oxidase) from corn roots homogenized in 0.5 M sucrose when subjected to 10,000 x g for 10 minutes, with co-sedimentation of approximately 30% of the endoplasmic reticulum (ER) marker enzyme, antimycin A-insensitive NADH cytochrome c reductase. Centrifugation of the supernatant at 80,000 x g or greater for at least 35 minutes pelleted about 70% of the remaining ER marker enzyme [73]. In tobacco cell suspension cultures which were homogenized in 0.3 M sucrose and subjected to centrifugation at 10,000 x g for 10 minutes greater than 50% of the ER marker enzyme co-sedimented with the mitochondrial marker enzymes (Carol Zelher and David Galbraith-unpublished data). Based on these findings one could reasonably expect to find significant amounts of PBD-2 protein to co-sediment with mitochondria as well as being found in 100,000 x g pellets of transfected protoplasts and transgenic plants as illustrated in Figures 18, 19 and 22.

In a review article about plant cell fractionation, Quail (1979, [63]) reported that antimycin A-insensitive cytochrome c reductase activities have been shown to be predominantly (approximately 90%) located in the ER and have been used extensively in the identification of ER membranes. The other 10% of the antimycin A-insensitive cytochrome c reductase activity was associated with the outer membrane of mitochondria. Similarly, cytochrome c oxidase activity is a common mitochondrial marker associated with the inner membranes of mitochondria. Mitoplast (mitochondria inner membrane + matrix) densities have been reported to be 1.19 g/cm^3 (42% sucrose w/w). The proximity the cytochrome c oxidase activities in Figures 23 and 24 to the reported densities of mitoplasts suggest the mitochondria were ruptured during homogenization. If the mitochondria were intact their density would have probably been higher. The reported densities of thylakoid membranes (1.16-1.18 g/cm^3 = 36-40% sucrose w/w) and the location of maximum fluoresence on the sucrose gradient suggest the chloroplasts were also ruptured during homogenization [63]. Western blot analysis of the fractionated homogenate in Figure 23 shows the majority of the PBD-2 protein to be associated with cytochrome c reductase activity, and a small percentage associated with thylakoid membranes. More importantly, the amount of total protein in the thylakoid-containing fractions is at least 2-fold greater than the ER containing fractions. This, in conjunction with the relative intensities of the Western blot indicate that the ER fractions are more highly enriched in PBD-2 on a PBD-2/unit protein basis than the thylakoid membranes. The presence of PBD-2 with the thylakoid membranes was seen consistently in numerous gradients and may be due to aggregation of ER membranes with the thylakoid membranes [81].

All present data suggest that PBD-2 protein is localized in the ER but its functional status remains undetermined. Using the densities observed from numerous

sucrose gradients we used a sucrose step gradient to isolate ER membranes, as determined by antimycin A-insensitive NADH cytochrome c reductase, from homogenized plant leaf tissue. The gradient consisted of 7.5 ml of 28% sucrose (w/w in TE), 7.5 ml 26% sucrose (w/w in TE) and 15 ml of 15% sucrose (w/w in TE). Analysis of an ER enriched fraction of transgenic plant material showed no androstenedione hydroxylase activity [androstenedione hydroxylase assay details 82]. In fact, androstenedione hydroxylase activity of transgenic yeast microsomes containing PBD-2 was inhibited by the ER enriched tobacco samples (K. Kedzie-unpublished results). Every effort was taken to eliminate or inactivate phenolics and quinones in the tobacco leaf homogenate used for isolation of ER fractions. Even though there was significant cytochrome c reductase activities in the sucrose step gradient fractions used as samples, "secondary products" present in tobacco plants, may have negatively influenced the reaction. Hydroxyl groups from phenolics have been shown to form very strong hydrogen bonds to oxygen atoms of peptide bonds. Oxidation of phenolics to quinones and subsequent covalent binding of functional groups of peptides as well as ionic and hydrophobic interactions contribute to the damage of many cellular constituents [62]. While reports of microsomal preparations using plant tissue are readily available, tobacco leaf tissue being an exception, the difficulties presented during the disruption of plant cells as well as the relatively low protein content of plant leaf tissues suggest that alternative methods may be required to elucidate the functional status of PBD-2 in transgenic plants [50, 83, 84, 85].

Future experiments to determine the functional status of PBD-2 in plants could involve cell suspension cultures which are non-differentiated, non-green dividing cells suspended in a liquid medium. The use of cell suspension cultures for studying the ability of PBD-2 to function in plant cells has several advantages over isolation of ER-

enriched fractions. Androstenedione and 2,4,5-HCB would, due to their highly lipophilic nature, passively enter into growing suspended tobacco cells thus maintaining the structural integrity of the cell during exposure to these substrates. The tissue is easily grown under sterile conditions and large quantities of cells can be readily obtained and analyzed. Cell suspension cultures have been used to study primary and secondary metabolism in plants, but one should be aware that these cultures are sensitive to changes in their environment which may induce physiological responses [86, 87, 88]. Harms et al. (1990) addressed this issue directly by comparing the ability and rates of cell suspension cultures, root cultures and intact plants to metabolize a variety of xenobiotics including pentachlorophenol, 4-chloroanaline, anthracene and phenanthrene. The researchers report quantitative differences in the rates of metabolism, with the cell suspension cultures exhibiting greatest metabolite formation due to their ability to passively absorb lipophilic compounds. Among the three plant systems the relative orders of rates of metabolite formation were the same. Qualitatively, the metabolites are consistent among the three plant systems and they conclude plant cell suspension cultures are a useful method to study the behavior of xenobiotics in plants [89]. Researchers have also used plant cell suspension cultures to metabolize testosterone and certain PCB congeners [88, 90, 91, 92]. The methodologies used by these researchers could be directly applied to PBD-2 containing cell suspension cultures to determine the activity of PBD-2.

Additionally, similar procedures could be used to determine if PBD-2 metabolizes PCBs in transgenic tobacco cells with functioning chloroplasts (i.e. protoplasts) [58]. The incubation of tobacco protoplasts with the appropriate substrates would provide further evidence of the functional status of PBD-2 in transgenic plants.

Assuming that PBD-2 exhibits some metabolic activity, as determined by cell suspension and protoplast experiments, whole plant studies will need to be done. Fries et al. (1981) provide a detailed description of the type of planter that could be constructed to determine the ability of tobacco, or other plants, to translocate PCBs from soil [as reviewed in 53]. The planter would prevent the evaporation and volitalization of PCBs from the soil, allowing determination of the route of uptake by the plants and quantitative recoveries of radiolabled substrates. Further studies using these planters would allow determination of the plant(s) that are most able to translocate PCBs and most appropriate for field applications.

If the PBD-2 protein does not exhibit activity or exhibits very low activity in transgenic plant cells, it may be helpful to construct a fusion polypeptide consisting of a mammalian cytochrome P450 reductase gene adjacent to the PBD-2 cDNA. This composite gene would then be used to construct an *Agrobacterium* vector to transform tobacco plants. Murakami et al. (1987) reported a four-fold increase in activity of a rat cytochrome P-450c protein, as expressed in yeast, when fused with a rat NADPH-cytochrome P-450 reductase gene [93]. The fused enzyme is reportedly a "new self-catalytic P450 monooxygenase". A similar construction for plants may overcome incompatibilities or stoichiometric imbalances between PBD-2 and the endogenous cytochrome P450 reductase.

Additional studies to determine the metabolite profile of PCB congeners subjected to metabolism by PBD-2 in plants are necessary. The metabolites will subsequently need to be tested from a toxicological perspective. The probable hydroxylation of PCB substrates by PBD-2 will lead to an increase in their water solubility. These more hydrophilic compounds may decrease PCB persistence in biological tissues, thereby reducing toxicity. The metabolism of PCB congeners to water soluble compounds will

also increase their mobility in most environmental matrices. As hydrophobic compounds, PCBs are generally bound to soil particles and are less mobile than a compound which can dissolve in water. Hydroxylated species may therefore be dispersed more easily. This may not be desirable in certain situations and must be considered before application of a PBD-2 transfected plant to contaminated soils.

Field use of these transgenic plants in PCB contaminated soils would require special site management considerations. The proper water content, soil pH and soil compaction are all factors that would need careful monitoring during remediation to maintain healthy plants and to ensure PCB/plant contact. This idea of "land farming" has been conceptualized for microbial remediation [94] but could be readily applied to plants. The proper rotation of "crops" for adaptation to optimal weather conditions and possible soil supplementation with microorganisms that display PCB metabolizing activities are issues that also need to be explored.

The chemical nature of the compounds that are acted on and the broad substrate specificity demonstrated by the P450 gene superfamily offer much potential as bioremediating agents. In an even broader sense, demonstrating bioremediation potential with a P-450 gene may pave the road for further exploitation of any proteins that exhibit unique metabolic activities toward environmental toxicants. Similarly, it is hoped that the continuation of this research will encourage future studies of plants as important organisms for biological remediation.

APPENDIX A-SOLUTIONS

A. Transformation of *Agrobacterium tumefaciens*

1. Minimal T Media 400 ml

20 X Salts	20 ml
20 X Buffer	20 ml
20% Sucrose	10 ml
4.8 g Agar	
H_2O	350 ml

20 X Salts

NH_4Cl	20	g
$MgSO_4.7H_2O$	4	g
$MnCl_2$	0.04	g
$CaCl_2$	0.2	g
$FeSO_47H_2O$	0.1	g
H_2O	to 1 liter	

20 X Buffer

K_2HPO_4	210	g
KH_2PO_4	90	g
H_2O	to 1 liter	

B. Transformation of Tobacco Plants with *Agrobacterium tumefaciens*

1. Tobacco Feeder Plates

MS salts (Gibco)	4.3	g
sucrose	30	g
thiamine stock (0.4 mg/ml)	1	ml
myo-inositol	100	mg
kinetin stock (50 mg/ml)	2	ml
2,4-D stock (0.1 mg/ml)	30	ml

Adjust pH to 5.6 add 10 g agar and H_2O to 1 liter

Autoclave the medium and inoculate with 1 gram tobacco cells from tobacco cell suspension culture when the solution cools to 55°C. W38 tobacco cells were graciously provided by Randy Ryan of the Plant Tissue Culture lab at the University of Arizona.

2. Shooting Media

MS salts (Gibco)	4.3	g
sucrose	30	g
B-5 vitamin stock for 100 ml	1	ml
-myo-inositol	10	g
-thiamine HCL	1	g
-nicotinic acid	0.1	g
-pyridoxine	0.1	g

-H_2O to 100 ml
napthalene acetic acid stock (1 mM) 0.5 ml
benzyl adenine stock (4 mM) 1 ml
Adjust pH to 5.7 add 8 g agar
H_2O to 1 liter

3. Rooting Media
MS salts (Gibco) 2.15 g
sucrose 15 g
B-5 vitamin stock 1 ml
Adjust pH to 5.6 add 9 g agar
H_2O to 1 liter and autoclave.

C. Transfection of Tobacco Protoplasts

1. Enzyme Digestion Media

For 500 mls:
0.5 g cellulysin
0.5 g driselase
0.5 g macerase
0.515 g $CaCl_2$ $2H_2O$ (9mM)
0.293 g MES (Morphoethane Sulphonic acid)
45.55 g mannitol
After dissolving, adjust pH to 5.8 and adjust volume to 500ml H_2O.
Centrifuge the enzyme solution for 10 minutes at 10K rpm in GSA tubes.
After centrifugation, store 40 ml portions at - 20°C. Filter sterilize before use.

2. W5 Solution

component	concentration	g/l
NaCl	154 mM	9.0
$CaCl_2$	125 mM	18.36
KCl	5 mM	0.372
Glucose	5 mM	0.900

Adjust to pH 5.7 Add H_2O to 1 liter and autoclave.

3. NTTO 10X Stock

component	g/l	concentration
NH_4NO_3	8.25	0.1 M
KNO_3	9.50	0.1 M
$CaCl_2$ $2H_2O$	2.20	15 mM
$MgSO_4$ $7H_2O$	1.85	8 mM
KH_2PO_4	0.85	6.2 mM
$ZnSO_4$ 7 H_2O	0.0178	0.11 mM
H_3BO_3	0.010	0.16 mM
Inositol	1.00	5.6 mM
Calcium Pantothenate	0.010	2.1 µM
Niacin	0.01	- - - - -
Pyrodoxine HCL	0.010	- - - - -

Thiamine HCL	0.010	30 µM

As a 10X Stock (100 ml)- Add1ml to NTTO 10X Stock

$CuSO_4 5H_2O$	0.030	2 mM
$AlCl_3$	0.030	2.25 mM
$NiCl_2 6H_2O$	0.030	1.33 mM
KI	0.010	0.6 mM
$MNSO_4 H_2O$	0.0758	4.5 mM

Biotin Stock-Add 1 ml to NTTO 10X Stock
Add 10 mg of biotin to a few ml 0.1N NaOH
Add 80 ml of water and stir.
Bring to 100 ml and freeze.

4. 25% Sucrose Solution

50	ml	10X NTTO
125	g	sucrose
250	ml	dH_2O

Bring volume to 500 ml. Adjust the pH to 5.7. Sterile filter 40 ml into 50 ml conical tubes. Store at -20°C in 45 ml aliquots.

5. NTTO + Glucose + Mannitol + Ampicillin

50	ml	10 X NTTO
34	g	glucose
24	g	mannitol
250	ml	H_2O

Adjust pH to 5.7; and bring volume to 500 ml. Sterile filter into 50 ml sterile conical tubes, 40 ml/tube. Add ampicillin right before use at a final concentration of 0.075 mg/ml.

6. 40% Polyethylene Glycol (PEG)50 ml

Bakers 4000 PEG	20 g	
mannitol	3.64 g	
$CaNO_2$	1.1 g	0.284 M

Adjust pH to 7.0 and bring volume to 50 ml. Filter sterilize and aliquot. Store at -20°C.

D. Marker Enzyme Assays

1. Antimycin A-Insensitive NADH Cytochrome c Reductase

Component	[stock]	Vol. stock	[Final]
cytochrome c	0.6 mM in 50 mM KPi	50 µl	0.03mM
NADH	2.0 mM in 50 mM KPi	50 µl	0.1mM
KCN	33 mM in 50 mM KPi	50 µl	1.67mM
antimycin A	200 µM in ETOH	5.0µl	1µM
KPO_4 pH7.5	50 mM	745-845µl	50mM
	Made with;		
	-0.2 M K_2HPO_4	42ml	
	-0.2 M KH_2PO_4	8ml	
	-H_2O	150ml	

Calculations: Equation for NADH dependant Cytochrome c Reductase activity.

+NADH $(\Delta A/min + 18.5(\mu mol/ml \cdot cm)^{-1})x(1000\ nmol/1\ \mu mol)x(total\ vol/ml\ enzyme)$

-NADH $-(\Delta A/min + 18.5(\mu mol/ml \cdot cm)^{-1})x(1000\ nmol/1\ \mu mol)x(total\ vol/ml\ enzyme)$

= nmol/min/ml

2. Cytochrome c Oxidase

Component	[stock]	Vol. stock	[Final]
Triton-X 100	4% in 50 mM KPi	75 µl	0.3%
KPO_4 pH7.5	50 mM	825-875 µl	50 mM
	Made from		
	-0.2 M K_2HPO_4	42ml	
	-0.2 M KH_2PO_4	8ml	
	-water	150ml	
cytochrome c(reduced)	0.6 mM in 50 mM KPi	50 µl	0.03mM

Calculation; Equation for Cytochrome c Oxidase activity.

$(\Delta A/min + 18.5(\mu mol/ml \cdot cm)^{-1})x(1000\ nmol/1\ \mu mol)x(total\ vol/ml\ enzyme)$

= nmol/min/ml

E. Lowry Protein Assay

1. Reagent A

Component	[stock]	Vol. stock
CTC	00.1% $CuSO_4.5H_2O$	
	00.2% $K_2C_2H_4O_6.1/2\ H_2O$	1 Part
	10.0% Na_2CO_3 (add last)	
sodium dodecyl sulate	10.0%	1 Part
NaOH	0.5N	1 Part
distilled H_2O		1 Part

2. Reagent B

Component	[stock]	Vol. stock
Folin-Ciolcateu Phenol	2N	1 Part
distilled H_2O		1 Part

F. SDS-PAGE

1. 4X running buffer

Tris-Base	36.33 g
SDS	00.80 g

Adjust pH to 8.8 with HCL: Add H_2O to 200 ml: Filter through 0.2 µm filter.

2. Acrylamide:Bis stock solution

Acrylamide	29.2 g

Bis-acrylamide 00.8 g
Add H_2O to 100 ml: Filter through 0.2 µm filter. Store in the dark at 0-4°C

3. 4X Stacking gel buffer
Tris-base 12.0 g
SDS 00.8 g
Adjust pH to 6.8 with HCL: Add H_2O to 200 ml: Filter through 0.2 µm " filter.

G. Western Blot Analysis-Protein blotting
1. SDS Tank Buffer

component	g/l		concentration	
Tris-base	12.0	g	0.1	M
Glycine	57.6	g	0.77	M
SDS	4.0	g	0.4	M

Add H_2O to 1 liter and store at room temperature

2. Matsudaira Transfer Buffer
100 ml methanol
100 ml 100 mM CAPS, pH 10.5
800 ml ddH_2O

H. Western Blot Analysis-Antibody Probing
1.TSW
10 mM Tris-HCL pH 7.4 - 10 ml of 1M
0.9% NaCl - 9 g
0.25% Gelatin - 2.5 g
0.1% Triton X-100 - 1 ml
0.02% SDS - 0.2 g
Store at 0-4°C or -20°C. It may be necessary to warm the solution in the microwave to dissolve the gelatin.

2. CDS(color development solution)
100 mM Tris-HCL pH 9.5
100 mM NaCl
5 mM $MgCl_2$
Store at room temp.

3. NBT/BCIP System
66 µl of BCIP solution and 66µl of NBT solution in CDS comprise the
i. BCIP(5-Bromo-4-Chloro-3-Indolyl Phosphate: p-Toluidine salt)
25mg/ml in DMF(Dimethyl Formamide)
Store at 0-4°C in the dark. Shelf life is only 1 month.
ii. NBT(Nitro Blue Tetrazolium)
50 mg/ml in 70% DMF
Store at 0-4°C in the dark. Shelf life is only 1 month.

I. Genomic DNA Isolation

1. 2X CTAB(cetyltrimethylammonium bromide) Buffer

2% CTAB (w/v)
100 mM Tris (pH 8.0)
20 mM EDTA (pH 8.0)
1.4 M NaCl
1% PVP (polyvinylpyrrolidone) M_r 40,000

2. 10% CTAB

10% CTAB
0.7 M NaCl

3. CTAB Precipitation Buffer

1% CTAB
50 mM Tris (pH 8.0)
10 mM EDTA (pH 8.0)

4. High Salt TE

10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)
1 M NaCl

J. Southern Blot Analysis

1. Denaturing Solution

0.5 M NaOH
1.5 M NaCl

2. Neutralizing Solution

1.5 M NaCl
1.0 M Tris (pH 8.0)

3. Pre-hybridization Solution

50% Formamide
1% SDS
5X SSC (0.75M NaCl, 0.075M sodium citrate, pH 7.0)
2X Denhardts Solution (0.4% BSA, 0.4% Ficol, 0.4% PVP, 1.5% SDS)
0.05 mM NaPO4
2.5 mg Sheared Salmon Sperm DNA

4. Hybridization Solution

50% Formamide
1% SDS
5X SSC (0.75M NaCl, 0.075M sodium citrate, pH 7.0)
2X Denhardts Solution (0.4% BSA, 0.4% Ficol, 0.4% PVP, 1.5% SDS)
0.05 mM $NaPO_4$
10% Dextran Sulphate
2.5 mg Sheared Salmon Sperm DNA

APPENDIX B - SUMMARY DATA: HISTORY OF TRANSGENIC PLANTS AND PBD-2 EXPRESSION

Plant #	Placed In Rooting Media	Observations (6-29-90)	Survival (NO/YES)	Western Analysis	Level of PBD-2 Expression				Positive Southern Analysis	Seeds Collected	Sterile Plants Maintained
					H	M	L	NONE			
1	4/14/1990	Dying	N								
2	4/14/1990	Dying	N								
3	4/14/1990	Dying	N								
4	4/14/1990	Rooting/Infectio	Y	6/11/90	X				8/30/90	X	X
5	4/20/1990	Dying	N								
6	4/20/1990	Dying	N								
7	4/20/1990	Dying	N								
8	4/20/1990	Rooting/Sickly	N								
9	4/27/1990	Dying	N								
10	4/27/1990	Sickly	N								
11	4/27/1990	Dead	N								
12	5/3/1990	Sickly	N								
13	5/3/1990	Dead	N								
14	5/3/1990	Sickly	N								
15	5/3/1990	Sickly	N								
16	5/3/1990	Healthy/Rooting	Y	5/31/90	X				10/1/90	X	X
17	5/3/1990	Sickly	N								
18	5/3/1990	Healthy/Rooting	Y	6/11/90				X	7/13/90	X	
19	5/3/1990	Sickly	N								
20	5/3/1990	Sickly/Rooting	N								
21	5/3/1990	Sickly	N								
22	5/3/1990	Sickly	N								
23	5/3/1990	Sickly	N								
24	5/3/1990	Sickly/Rooting	N								
25	5/3/1990	Dying	N								
26	5/9/1990	Healthy/no roots	N								
27	5/9/1990	Healthy/no roots	N								

Plant #	Placed In Rooting Media	Observations (6-29-90)	Survival (NO/YES)	Western Analysis	Level of PBD-2 Expression				Positive Southern Analysis	Seeds Col-lected	Sterile Plants Maintained
					H	M	L	NONE			
28	5/9/1990	Sickly	N								
29	5/9/1990	Rooting	N								
30	5/9/1990	Healthy/no roots	N								
31	5/9/1990	Rooting	Y	7/18/90				X	8/30/90		
32	5/9/1990	Dying	N								
33	5/9/1990	Rooting	Y	6/11/90			X		Not Done		
34	5/17/1990	Healthy/no roots	N								
35	5/17/1990	Rooting	Y	7/2/90				X	8/30/90		
36	5/17/1990	Sickly	N								
37	5/17/1990	Sickly	N								
38	5/17/1990	Sickly	N								
39	5/17/1990	Sickly	N								
40	5/17/1990	Healthy/no roots	N								
41	5/17/1990	Healthy/no roots	N								
42	5/17/1990	Rooting/Bact. In	N	7/2/90			X		Not Done		
43	5/17/1990	Rooting/Bact. In	Y	7/5/90				X	Not Done		
44	5/30/1990	Rooting	Y	7/18/90	X				Not Done		X
45	5/30/1990	Sickly/rooting	Y	7/18/90		X			Not Done	X	
46	5/30/1990	Healthy/no roots	Y	7/5/90				X	Not Done		
47	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
48	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
49	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
50	5/30/1990	Healthy/no roots	Y	10/16/90				X	Not Done		
51	5/30/1990	Sickly	N								
52	5/30/1990	Healthy/no roots	N								
53	5/30/1990	Healthy/no roots	Y	10/16/90				X	Not Done		

APPENDIX B - SUMMARY DATA: HISTORY OF TRANSGENIC PLANTS AND PBD-2 EXPRESSION

Plant #	Placed In Rooting Media	Observations (6-29-90)	Survival (NO/YES)	Western Analysis	Level of PBD-2 Expression				Positive Southern Analysis	Seeds Collected	Sterile Plants Maintained
					H	M	L	NONE			
4	4/14/1990	Rooting/Infectio	Y	6/11/90	X				8/30/90	X	X
16	5/3/1990	Healthy/Rooting	Y	5/31/90	X				10/1/90	X	X
18	5/3/1990	Healthy/Rooting	Y	6/11/90				X	7/13/90	X	
31	5/9/1990	Rooting	Y	7/18/90				X	8/30/90		
33	5/9/1990	Rooting	Y	6/11/90			X		Not Done		
35	5/17/1990	Rooting	Y	7/2/90				X	8/30/90		
42	5/17/1990	Rooting/Bact. Int	N	7/2/90			X		Not Done		
43	5/17/1990	Rooting/Bact. Int	Y	7/5/90				X	Not Done		
44	5/30/1990	Rooting	Y	7/18/90	X				Not Done		X
45	5/30/1990	Sickly/rooting	Y	7/18/90		X			Not Done	X	
46	5/30/1990	Healthy/no roots	Y	7/5/90				X	Not Done		
47	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
48	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
49	5/30/1990	Rooting	Y	7/5/90	X				7/13/90	X	X
50	5/30/1990	Healthy/no roots	Y	10/16/90				X	Not Done		
53	5/30/1990	Healthy/no roots	Y	10/16/90				X	Not Done		

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