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THE RECOMBINANT EXPRESSION AND POTENTIAL APPLICATIONS OF BACTERIAL ORGANOPHOSPHATE HYDROLASE IN *Zea mays L.*

A Dissertation

by

TERRENCE SCOTT PINKERTON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Biochemistry

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Approved as to style and content by:

James R. Wild (Chair of Committee)	John A. Howard (Member)
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May 2004

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ABSTRACT

The Recombinant Expression and Potential Applications of Bacterial Organophosphate Hydrolase in *Zea mays L.* (May 2004)

Terrence Scott Pinkerton, B.S., University of Nebraska-Lincoln

Chair of Advisory Committee: Dr. James R. Wild

Organophosphate hydrolase (OPH, EC 3.1.8.1) is a bacterial enzyme with a broad spectrum of potential substrates that include organophosphorus pesticides, herbicides, and chemical warfare agents. OPH has been expressed successfully in bacterial, fungal, and insect cell culture systems; however, none of these systems produces amounts of enzyme suitable for applications outside of the research laboratory. Therefore, a transgenic *Zea mays L.* (maize) system was developed to express OPH as an alternate to the current OPH expression systems. The bacterial gene encoding the OPH protein was optimized for transcriptional and translational expression in maize. The optimized gene was inserted into the maize genome under the control of embryo specific, endosperm specific, and constitutive plant promoters. Select transformants were analyzed for the expression of OPH. Expression was observed in the seeds of plants transformed with each of the three constructs with the highest expression observed with the embryo specific and constitutive promoter constructs. The highest OPH expressing lines of transgenic maize had expression levels higher than those reported for the *E. coli* expression system. OPH was purified from transgenic maize seed and analyzed for post-translational modification and kinetic properties. OPH was observed to undergo a

glycosylation event when expressed in maize that yielded at least two forms of OPH homogolous dimer. The glycosylated form of OPH bound tightly to the Concanavalin A sepharose and remained active after months of storage at room temperature. OPH activity was checked against a number of organophosphate herbicides. Enzymatic activity was observed against the herbicide Amiprophos-methyl and kinetic properties were measured. Enzymatic activity was also tested against the organophosphate Haloxon. Transgenic maize callus, leaf, and seed tissue could be screened for the presence of the optimized *opd* gene by enzymatic activity. Comparison of the growth of transgenic and control callus on media containing organophosphates showed that the transgenic callus was resistant to the herbicidal effects of haloxon. Transgenic plants expressing OPH were also resistant to the herbicide bensulide when compared to control plants. This indicates that OPH can be used as a screenable marker in plant systems and may be a potential scorable marker system as well.

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I would like to thank my family for supporting and encouraging me in my endeavors. Finally I would like to thank my wife, Jennifer, for the years of support she has provided while I was trying to accomplish this goal.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Organophosphates are neurotoxic chemical agents widely used as pesticides and herbicides [1]. Organophosphates inhibit the enzymatic activity of cholinesterases, thereby inhibiting proper nerve function. The powerful effect that organophosphates have on the nervous system has been harnessed not only to control insect and plant pests, but also for use as chemical warfare agents [2,3]. Since the initial discovery of organophosphates over sixty years ago, their use has spread worldwide [2]. Organophosphates used commercially are synthetic compounds not found in nature [2,3]. The only example of a naturally occurring organophosphate neurotoxin is Anatoxin-A(S), which is isolated from certain strains of the cyanobacteria of genus *Anabaena* [4]. Concurrent with the development and use of these agents, several enzymes have been identified that can degrade organophosphates [5-21]. These include mammalian serum paraoxonases (EC 3.1.8.1), squid DFPase (EC 3.1.8.2), organophosphate acid anhydrase (OPAA, EC 3.1.8.1), and organophosphate hydrolase (OPH, EC 3.1.8.1) [8-11,15,17,22]. With the exception of OPH, the hydrolytic activity of all of these enzymes against organophosphates appears to be an ancillary function rather than their natural metabolic function. A natural metabolic function for OPH has yet to be identified [23]. It is also interesting that none of the proteins

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This dissertation follows the style and format of *Nature Biotechnology*.

share sequence homology, indicating that the ability to degrade organophosphates arose independently in each.

OPH has been identified by several names in the literature including Organphosphorus Hydrolase and Phosphotriesterase (PTE) [23,24]. OPH activity was first observed in bacteria isolated from rice paddies treated with the organophosphate herbicide diazinon [18-21]. The enzyme was identified in *Pseudomonas diminuta* and *Flavobacterium spp*.[17,20]. In both organisms the gene encoding the enzyme (*opd*) was found on large plasmids; however, outside of the *opd* gene the plasmids were not similar in sequence [11,25,26]. More recently a similar gene was identified in *Agrobacterium radiobacter* isolated in Australia [7]. Studies with the enzyme have shown that it had the ability to degrade a wide range of organophosphates by hydrolyzing P-O, P-S, P-F, and P-CN bonds [24]. This gives OPH the widest range of substrates among organophosphate degrading enzymes. OPH without a 29 amino acid leader sequence has also been shown to be a highly stable enzyme, with the highest reported conformational stability reported for a dimeric protein [27,28]. At the heart of OPH are two active sites that contain two metal binding sites each [23,29,30]. These metal-binding sites can bind several different divalent transition metals with different kinetic properties attributed to the enzyme dependent on which metal is bound [30,31]. The highest activity against paraoxon has been observed with the cobalt form of the enzyme (k_{cat}=12,000s^{-1}). The type of metal bound can also have an effect on the stability of the enzyme with the zinc form of the enzyme being the most stable [28]. Studies to determine the enzymatic mechanism of OPH indicate that the ability to degrade organophosphates is metal dependent and the enzymatic center of OPH

contains a carbamylated lysine residue that has been shown in crystal structure to be involved in metal binding [23,31,32]. The reconstitution of OPH from metal free apo-enzyme is accelerated by the presence of bicarbonate, which supports the importance of the modified lysine for enzyme function [33]. Crystal structures of the enzyme isolated from recombinant expression systems have shown two divalent metals per subunit of enzyme [23,34]. Questions have arisen, however, on the exact number of metals needed for enzymatic activity. Initial atomic absorbtion experiments measuring the metal content of the enzyme indicated that for enzyme produced without the addition of extra transition metals to the growth media the enzyme was binding 1.1 zinc atoms per subunit of the monomer [30]. Further mechanism work indicated that OPH showed full activity against paraoxon was achieved only when two metals were bound per sub-unit. Turnover of paraoxon is extremely rapid with diffusion limited rates, which made it possible to conduct substrate inhibition experiments using the P-S bonded substrate demeton-S which has a turnover rate that is nearly 1000-fold lower than that of paraoxon [25,35]. The substrate inhibition experiments showed that the inhibition of paraoxonase with demeton-S was mixed non-competitive inhibition [35]. This indicates that there is might be a more complex mechanism for the hydrolysis of organophosphates by OPH. Experiments detailing the metal requirement for demeton-S activity have resulted in mixed results, with *Shim et al.* reporting that two metals are required and *diSioudi et al.* reporting only one metal is required [33,36]. The one metal mechanism is also supported by several mutant studies done on OPH [29,36]. The C59S mutant of OPH has an altered metal state, with only 0.86 metals bound per subunit, but the enzyme retains 33% of its

paraoxonase activity [30]. The replacement of histidine residues involved in structural interactions that form the active site of the enzyme resulted in increased activity the P-S bonded substrate demeton-S and maximal activity for both paraoxon and demeton-S degrading activity at 1 metal bound per sub-unit of enzyme [24,36]. The exact mechanism or mechanisms of OPH degradation of organophosphates remains elusive.

Given the broad substrate specificity and stable thermodynamic properties of OPH, the enzyme has been the enzyme of choice for several practical applications. These include enzyme engineering to increase enzymatic activity against P-S bonded chemical warfare agents such as the chemical warfare agent VX, for the immobilization of the enzyme for easier handling during remediation of organophosphates, and the use in biosensors to detect environmental organophosphate contamination [15,24,36]. With the increased demand for OPH protein for use in experimental applications, new methods for expressing the enzyme are being explored. OPH has been expressed in several prokaryotic and eukaryotic expression systems [17,23,27,31,37,38,39]. The workhorse of OPH expression to date has been the recombinant *E. coli* expression system [27]. The current laboratory shake flask expression system typically yields around 10-20mg of OPH per liter of media. Attempts to adapt this system to large-scale fermentation have resulted in expression levels 100 fold below the shake flask expression rate (Dr. M. Wales, personal communication). Insect cell culture has also been used for OPH expression, as has expression in fungal systems [38,39]. Neither of these two systems is ideal for scale-up to large-scale production of enzyme. In searching for alternative systems of expression,

plant based expression systems offers the ability to express OPH successfully in large quantities.

Plant based expression of recombinant proteins is increasingly becoming commonplace. The first transgenic plants were produced by *Agrobacterium tumefaciens* mediated transformation in the early 1980's [40]. Stable transformation was later achieved by particle bombardment [41]. Transfer of recombinant DNA to plant cells can also be achieved by tissue electroporation and silicon carbide fibers [42]. One of the advantages of the plant expression systems is the ability to target protein expression to specialized protein storage organs and tissues [43]. Plant seed is an ideal protein expression target due to its low moisture and high protein content, which are similar to the environment encountered by lyophilized proteins. In addition, carbohydrates and protease inhibitors are present in most seeds [43,44]. These factors serve to stabilize proteins expressed in seed to allow for germination at a later date, but also serve to stabilize recombinant proteins. Another advantage of plant expression systems is ability to quickly scale production of transgenic material from small to large scale [43]. This coupled with the simple input requirements for protein production (sunlight and water) gives the plant expression system a great advantage over bacterial fermentation and animal cell culture systems.

Transgenic plants have been used for applications ranging basic plant research to the commercial production of recombinant proteins [45-54]. The DNA transfer technology for producing transgenic plants has also been adapted for use with almost all types of higher plants including monocot grasses such as *Zea mays L*. (maize). The *Agrobacterium* system of transformation is based on the plant pathogenic bacteria

Agrobacterium tumefaciens. *Agrobacterium* naturally infects dicotyledonous plants at wound sites, typically on the plant stem near the soil surface, and cause a condition known as crown gall. The bacterium carries a large tumor-inducing plasmid (T_i) that contains genes that can be transferred and integrated into the plant genome. The genes encoded on the plasmid are divided into two sets, those that are transferred to the plant host, and those that are not transferred but are necessary for the transfer. Upon the transfer of the genetic material to the host cell the plant cell begins the production of a set of compounds called opines, which only *Agrobacterium* can metabolize. The transferred genes also cause the plant cell to begin rapid division resulting in a mass of cells containing the transferred genes. This pathogenic system was modified for use in plant transformation by removing the transferred material from the T_i plasmid and replacing it with DNA sequences desired for transformation. Further advancement was made with the use of a binary vector system, where one vector carries the genes needed for transfer of the DNA, and another vector carries the gene targeted for transfer [55]. This allows the system to use two smaller plasmids, which are easier to manipulate than a single large Ti plasmid. DNA manipulation can be carried out in *E. coli* and then transferred to *Agrobacterium* prior to transformation. Since the initial use of *Agrobacterium* for dicot transformation the Agrobacterium system has been modified and used for the transformation of monocots including maize [56-58].

Maize transformation resulting in fertile plants was first accomplished by particle bombardment with tungsten particles coated with the DNA targeted for integration [55,59]. A major advancement in the ability to generate transgenic maize was the

adaptation of the *Agrobacterium* system to maize transformation [50,56,58]. The advantage of this system is the control of gene copy number that it introduces, reducing the number of multiple gene inserts that can result in gene silencing. Maize plants have been genetically engineered for resistance to insect pests and herbicides [51,57,60]. Maize has also been used to produce recombinant proteins. A wide range of recombinant proteins have been expressed in maize including proteins from both prokaryotic and eukaryotic systems. Expression levels have been reported as high as 26% in transgenic maize seed for the protein avidin [61].

Given the wide range of expression systems that have been tested for the expression of OPH and the limited usefulness of these systems for large-scale expression of OPH, the ability of a maize plant expression system to successfully express OPH was explored. To reach this goal, the DNA sequence of the native *opd* gene was reconstructed into a new sequence that encoded OPH using the codons most common used in the maize genome. This change in coding sequence eliminated potential problem causing mRNA sequences that would have had a negative impact on OPH expression. The elimination of rare codons by optimization with common codons has previously been shown to increase expression of the *Bacillus thurengensis* toxin protein and an insect anti-freeze protein in plant systems [60,62]. The optimized gene was placed into plant expression vectors under three different promoters with constitutive, embryo specific, and endosperm specific expression patterns. The OPH protein also was fused n-terminally to the barley α-amylase signal sequence (BAASS). This signal sequence causes the export of the fused protein outside of the cell to the cell wall and is removed

during the export process [63]. By exporting the recombinant protein outside of the cell the protein is isolated from degradation that may be caused by internal cellular components. It also places the targeted protein into an environment that is high in carbohydrate, which can help to stabilize protein. The effect that the plant expression system had on OPH quality was also investigated. Plant expression systems are capable of post-translational modifications that are not found in bacterial systems, which can effect the quality of bacterial proteins expressed in a plant system.

It is interesting to note that OPH has a wide range of possible substrates that include herbicides and compounds that can be visually detected. This raises the possibility that OPH could be used as a selectable and scorable marker gene in plant transgene expression systems. Currently no commonly used marker is used as both a selectable and scorable marker system. Scorable markers, like β-glucuronidase (GUS), firefly luciferase (ff-LUC), and green fluorescent protein (GFP) are used to determine if tissue has been successfully transformed and are detected visually by their enzymatic activity (GUS, ff-LUC) or fluorescent properties of the protein itself (GFP) [64-67]. None of these proteins can be used as a selectable marker. Selectable marker genes such as *PAT* give transgenic tissue resistance to a toxic compound resulting in the ability to select transformants [68]. However, the degradation of the selection compound does not yield an easily detected product limiting the ability to use the selectable marker gene as a scorable marker. The wide range of compounds that OPH can degrade may mean that OPH can act as a selectable or scorable marker depending on which compound is used. Organophosphates have previously been used to screen bacteria for the presence of OPH

activity. Parathion impregnated filters were used to screen bacterial colonies for the degradation of organophosphates and the pesticide coumaphos has been added to agar plates to detect bacterial degradation of organophosphates [69,70]. The addition of a compound like coumaphos to plant tissue culture media may allow for the screening of tissue during the process of plant regeneration from transformed tissue. It may also be possible to use OPH substrates to screen tissue from plants after regeneration.

Organophosphate compounds have also been identified with herbicidal activity [1]. Among these compounds are the herbicides amiprophos-methyl, butamiphos, bensulide, anilofos, and piperophos [1]. Amiprophos-methyl and butamiphos block microtubual polymerization [71-75]. Bensulide blocks lipid synthesis, while Anilofos and Piperophos block cell division by inhibiting very long chain fatty acid synthesis [1,76]. All of these herbicides act in a pre-emergence fashion and are active against C4 monocots like maize [1]. None of these compounds have been previously tested as OPH substrates. If OPH has the ability to degrade an organophosphate herbicide it raises the potential of OPH as a selectable marker. Currently selectable marker systems for plant transformation rely on either the ability to degrade a herbicide such as bialaphos, the ability to degrade a broad spectrum antibiotic, or the inclusion of an enzyme which is resistant to herbicides which are metabolic inhibitors [48,57]. While the current selectable marker systems are useful as a selection mechanism, they do not act as a scorable marker system. If OPH has the ability to act as a scorable and selectable marker, it would make OPH unique among plant marker systems.

CHAPTER II

OPTIMIZATION, CONSTRUCTION, AND EXPRESSION OF A BACTERIAL ORGANOPHOSPHATE HYDROLASE GENE IN *Zea mays L.*

Organophosphate hydrolase (OPH) from *P. diminuta* is a dimeric metallo-enzyme with a broad substrate spectrum including the ability to degrade organophosphorus neurotoxins. The gene (*opd*) encoding OPH has been cloned into several expression systems including bacteria, insect cell culture, bacculovirus, and fungi. The current system of choice for the laboratory expression of OPH is *E. coli*. However, attempts to scale up production into fermentors have resulted in significantly lower expression of the enzyme when compared to shake flask expression levels. The aim of this work in maize was to develop a plant expression system, *Zea mays L.*, as an alternative expression system using several different promoters. This has required the optimization of the *opd* gene sequence to avoid problem sequences within the encoding mRNA, integration into plant expression vectors and transfer into the maize genome by Agrobacterium mediated transformation.

Introduction

Since the discovery of bacteria with the ability to degrade organophosphates during the late 1960's, extensive work in both basic research on the enzymes that catalyze the degradation of organophosphates, as well as the possible applications of these enzyme has been preformed [15,19,23]. Organophosphate hydrolyzing enzymes (E.C.

3.1.8) have been identified from both prokaryotic and eukaryotic systems. These enzymes include organophosphate hydrolase (E.C. 3.1.8.1, OPH, alternatively phosphotriesterase, PTE), Organophosphate Acid Anhydrase (E.C. 3.1.8.2, OPAA), the PON family of serum paraoxonases (E.C. 3.1.8.2), and squid DFPase (E.C. 3.1.8.1) [8,9,15,22]. Of these enzymes, OPH shows the widest range of organophosphate substrate specificity with the ability to hydrolyze P-O, P-S, P-F, and P-CN bonds [24]. This gives OPH enzymatic activity against a broad range of organophosphate pesticides, as well as the chemical warfare agents Soman, Sarin, Cyclo-Sarin, Tabun,VR, and VX. Because of the wide substrate range and chemical variety of OPH catalysis, the enzyme has been considered an ideal candidate for practical applications involving the degradation of organophosphorus neurotoxins [15,24,37]. OPH is a homodimeric metalloprotein that requires the presence of divalent transition metals, such as Zn^{+2}, Cd^{+2}, or Co^{+2}, for enzymatic activity [29]. OPH shows a remarkable thermal stability, with highest conformational stability reported for any dimeric protein (ΔG = 40 kcal/mol) [27]. The gene encoding OPH (*opd*) was originally isolated from *Pseudomonas diminuta* and *Flavobacterium* that contained large dissimilar plasmids [77]. Subsequently, this gene was transferred into *E. coli*, insect cell culture, and fungal expression systems for the production of recombinant OPH [29,37,38]. Currently, the *E. coli* expression system can yield up to 10-20mg of OPH per liter of culture when growth is carried out in shake flasks. When the same strains are used for fermentor expression the yield per liter of growth media is often 100 times lower than in the shake flask incubation. Given that OPH has been successfully expressed in several prokaryotic and eukaryotic expression

systems, the ability of a plant expression system, *Zea mays L.* (maize), to express recombinant OPH was tested.

The ability to use plants as expression systems for foreign proteins became a reality with the advent of plant transformation techniques in the early 1980's [40]. The transformation technology has been used to improve nutritional traits, add resistance to herbicides, increase resistance to pests, and express recombinant proteins [46-54]. Plants provide a recombinant expression system that has several advantages over bacterial, fungal, and animal systems that include the ability to compartmentalize expression of the recombinant protein into organelles or protein storage organs [44,78]. Plant seeds are ideal protein storage structures with an environment that is low in moisture and contains high concentrations of protein and carbohydrate. Protease inhibitors are also found in high concentrations in seeds [44]. Seed expression has the added benefit of not needing cold storage or processing that plant green tissue, such as leaves, requires. Plant expression systems also have a high degree of scalability of production with minimal amount of required resources [49]. Increasing the scale of production of a plant system is not dependent on specialized facilities, such as fermentors, and is only limited by the area being planted with the transgenic plant.

Fertile transgenic maize was first produced in the early 1990's by physical bombardment of plant tissue by tungsten particle coated in DNA containing the transgene (biolistic transformation) [41,59]. More recently *Agrobacterium tumefaciens* has emerged as a viable maize transformation system [56,58,79]. Maize has been used to successfully express several proteins of both prokaryotic and eukaryotic origin that

include the commercialized expression of avidin, β-glucuronidase, trypsin, and aprotinin [46-54]. Given the wide range of proteins successfully expressed in maize, including metallo-proteins and proteins from bacterial sources, maize appears to be a capable system for the heterologous expression of OPH. Levels of expression of over 26% total soluble protein (%TSP) have been achieved for recombinant expression of avidin in maize [61]. The high level of heterologus protein expression achieved in maize with a diverse set of proteins, coupled with the advantages that a multifaceted plant expression system has over other expression systems, led to the inquiry of whether OPH could be successfully expressed in a maize expression system.

Results

Assembly and Transformation of the Optimized opd Gene

Previous studies have shown that optimization of a foreign DNA sequence can dramatically effect expression levels when that sequence is transformed into plant tissue [60,62,80]. In order to maximize the expression of OPH in maize tissue, the native *opd* gene was optimized by substituting for the existing codons with codons used most often in the maize genome. This changed the coding sequence of the gene, but left the amino acid sequence of the protein unchanged. The barley α-amylase signal sequence (BAASS) was incorporated into the design of the optimized gene. The signal sequence results in the transport of the targeted protein to the cell wall, and has previously been used to target recombinant proteins in maize [49,63]. In order to generate the maize optimized *opd* gene, the entire sequence was produced as 50 base-pair oligomers, which were then

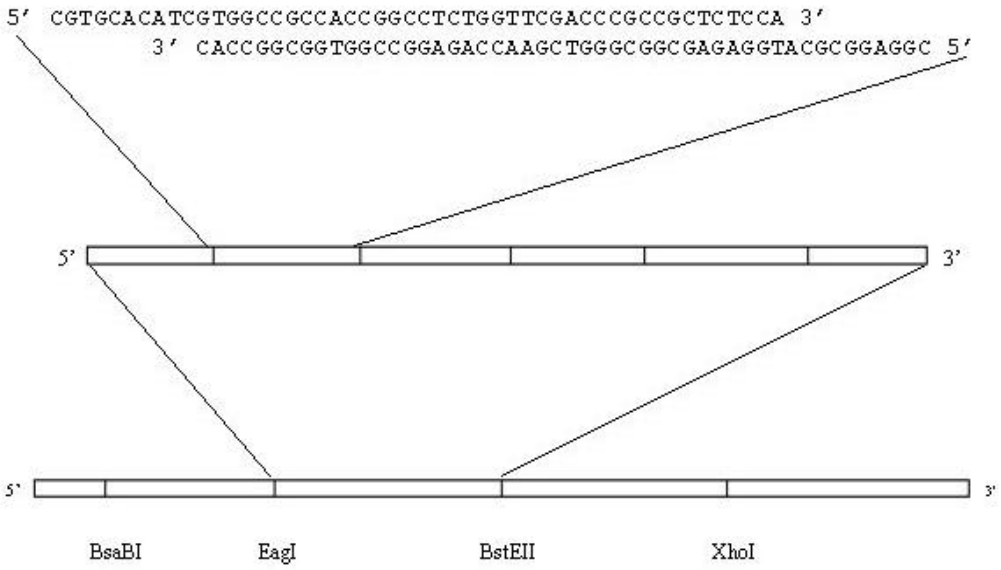


Figure 1. Assembly of the optimized *opd* gene. 50 base-pair oligos with 10 basepair ovelaps were used to assemble the optimized *opd* gene by PCR. Assembled fragments were trapped in Invitrogen PCR-TOPO 2.1 vectors and sequenced. Fragments were then sub-cloned using restriction sites indicated and the TOPO vector as a backbone. A summery of the changes made to the native *opd* gene and the mRNA problem sequences detected can be found in Appendix A.

```
          M  A  N  K  H  L  S  L  S  L  F  L  V  L  L  G  L  S  A  S  L  A  S
    1 CCATGGCCAA CAAGCACCTG AGCCTCTCCC TCTTCCTCGT GCTCCTCGGC CTCTCCGCCT CCCTCGCCAG
      GGTACCGGTT GTTCGTGGAC TCGGAGAGGG AGAAGGAGCA CGAGGAGCCG GAGAGGCGGA GGGAGCGGTC
        G  T  G  D  R  I  N  T  V  R  G  P  I  T  I  S  E  A  G  F  T  L  T
   71 CGGCACCGGC GACCGCATCA ACACCGTGCG CGGCCCGATC ACCATCTCCG AGGCCGGCTT CACCCTCACC
      GCCGTGGCCG CTGGCGTAGT TGTGGCACGC GCCGGGCTAG TGGTAGAGGC TCCGGCCGAA GTGGGAGTGG
       H  E  H  I  C  G  S  S  A  G  F  L  R  A  W  P  E  F  F  G  S  R  K  A
  141 CACGAGCACA TCTGCGGCTC CTCCGCCGGC TTCCTCCGCG CCTGGCCGGA GTTCTTCGGC TCCCGCAAGG
      GTGCTCGTGT AGACGCCGAG GAGGCGGCCG AAGGAGGCGC GGACCGGCCT CAAGAAGCCG AGGGCGTTCC
         L  A  E  K  A  V  R  G  L  R  R  A  R  A  A  G  V  R  T  I  V  D  V
  211 CCCTCGCCGA GAAGGCCGTG CGCGGCCTCC GCCGCGCCCG CGCCGCCGGC GTGCGCACCA TCGTGGACGT
      GGGAGCGGCT CTTCCGGCAC GCGCCGGAGG CGGCGCGGGC GCGGCGGCCG CACGCGTGGT AGCACCTGCA
       S  T  F  D  I  G  R  D  V  S  L  L  A  E  V  S  R  A  A  D  V  H  I
  281 GTCCACCTTC GACATCGGCC GCGACGTGTC CCTCCTCGCC GAGGTGTCCC GCGCCGCCGA CGTGCACATC
      CAGGTGGAAG CTGTAGCCGG CGCTGCACAG GGAGGAGCGG CTCCACAGGG CGCGGCGGCT GCACGTGTAG
       V  A  A  T  G  L  W  F  D  P  P  L  S  M  R  L  R  S  V  E  E  L  T  Q
  351 GTGGCCGCCA CCGGCCTCTG GTTCGACCCG CCGCTCTCCA TGCGCCTCCG CTCCGTGGAG GAGCTCACCC
      CACCGGCGGT GGCCGGAGAC CAAGCTGGGC GGCGAGAGGT ACGCGGAGGC GAGGCACCTC CTCGAGTGGG
         F  F  L  R  E  I  Q  Y  G  I  E  D  T  G  I  R  A  G  I  I  K  V  A
  421 AGTTCTTCCT CCGCGAGATC CAGTACGGCA TCGAGGACAC CGGCATCCGC GCCGGCATCA TCAAGGTGGC
      TCAAGAAGGA GGCGCTCTAG GTCATGCCGT AGCTCCTGTG GCCGTAGGCG CGGCCGTAGT AGTTCCACCG
        T  T  G  K  A  T  P  F  Q  E  L  V  L  K  A  A  A  R  A  S  L  A  T
  491 CACCACCGGC AAGGCCACCC CGTTCCAGGA GCTCGTGCTC AAGGCCGCCG CCCGCGCCTC CCTCGCCACC
      GTGGTGGCCG TTCCGGTGGG GCAAGGTCCT CGAGCACGAG TTCCGGCGGC GGGCGCGGAG GGAGCGGTGG
       G  V  P  V  T  T  H  T  A  A  S  Q  R  D  G  E  Q  Q  A  A  I  F  E  S
  561 GGCGTGCCGG TGACCACCCA CACCGCCGCC TCCCAGCGCG ACGGCGAGCA GCAGGCCGCC ATCTTCGAGT
      CCGCACGGCC ACTGGTGGGT GTGGCGGCGG AGGGTCGCGC TGCCGCTCGT CGTCCGGCGG TAGAAGCTCA
         E  G  L  S  P  S  R  V  C  I  G  H  S  D  D  T  D  D  L  S  Y  L  T
  631 CCGAGGGCCT CTCCCCGTCC CGCGTGTGCA TCGGCCACTC CGACGACACC GACGACCTCT CCTACCTCAC
      GGCTCCCGGA GAGGGGCAGG GCGCACACGT AGCCGGTGAG GCTGCTGTGG CTGCTGGAGA GGATGGAGTG
        A  L  A  A  R  G  Y  L  I  G  L  D  H  I  P  H  S  A  I  G  L  E  D
  701 CGCCCTCGCC GCCCGCGGCT ACCTCATCGG CCTCGACCAC ATCCCGCACT CCGCCATCGG CCTCGAGGAC
      GCGGGAGCGG CGGGCGCCGA TGGAGTAGCC GGAGCTGGTG TAGGGCGTGA GGCGGTAGCC GGAGCTCCTG
       N  A  S  A  S  A  L  L  G  I  R  S  W  Q  T  R  A  L  L  I  K  A  L  I
  771 AACGCCTCCG CGTCCGCCCT CCTCGGCATC CGCTCCTGGC AGACCCGCGC CCTCCTCATC AAGGCCCTCA
      TTGCGGAGGC GCAGGCGGGA GGAGCCGTAG GCGAGGACCG TCTGGGCGCG GGAGGAGTAG TTCCGGGAGT
         D  Q  G  Y  M  K  Q  I  L  V  S  N  D  W  L  F  G  F  S  S  Y  V  T
  841 TCGACCAGGG CTACATGAAG CAGATCCTCG TGTCCAACGA CTGGCTCTTC GGCTTCTCCT CCTACGTGAC
      AGCTGGTCCC GATGTACTTC GTCTAGGAGC ACAGGTTGCT GACCGAGAAG CCGAAGAGGA GGATGCACTG
        N  I  M  D  V  M  D  R  V  N  P  D  G  M  A  F  I  P  L  R  V  I  P
  911 CAACATCATG GACGTGATGG ACCGCGTGAA CCCGGACGGC ATGGCCTTCA TCCCGCTCCG CGTGATCCCG
      GTTGTAGTAC CTGCACTACC TGGCGCACTT GGGCCTGCCG TACCGGAAGT AGGGCGAGGC GCACTAGGGC
       F  L  R  E  K  G  V  P  Q  E  T  L  A  G  I  T  V  T  N  P  A  R  F  L
  981 TTCCTCCGCG AGAAGGGCGT GCCGCAGGAG ACCCTCGCCG GCATCACCGT GACCAACCCG GCCCGCTTCC
      AAGGAGGCGC TCTTCCCGCA CGGCGTCCTC TGGGAGCGGC CGTAGTGGCA CTGGTTGGGC CGGGCGAAGG
         S  P  T  L  R  A  S  *  V  N
 1051 TCTCCCCGAC CCTCCGCGCC TCCTGAGTTA AC
      AGAGGGGCTG GGAGGCGCGG AGGACTCAAT TG
```

Figure 2. Optimized *opd* gene sequence. Figure was generated with Vector NTI 5.0 (Informax, North Bethesda, MD). Translated protein sequence appears above the nucleotide sequence. Sequence has been added to Genbank accession # AX384799. Star indicates the end of the coding sequence.

assembled into the optimized gene by PCR. Assembly was carried out as visualized in Figure 1. The assembly process resulted in the direct change of 217 individual base pairs. This resulted in an overall change in the GC content of the gene from 57% to 70%. Analysis of the new mRNA sequence with the GCG pattern find program revealed a reduction in the number of potential mRNA problem sequences from 40 to 10 when compared to the native gene. Degradation of mRNA due to problematic sequences has been implicated in the low expression levels of the insecticidal protein encoded by the *Bacillus thurigiensis* gene *cryIA* in plant systems [60,80]. Of these 10 sequences identified, one was a 5' splice site, the other 9 were 3' slice sites caused by the glutamine codon CAG. None of the problem sequences could be removed without the inclusion of rare codons, which could also have an adverse effect on expression. The final optimized *opd* sequence with the added BAASS signal sequence is presented in Figure 2.

The assembled optimized gene was subcloned into plant expression vectors under the control of maize promoters with constitutive, embryo specific, and endosperm specific expression patterns [48,81,82]. The selectable marker gene *pat* under control of the CMV35s promoter was included for selection of transformants (Figure 3). The vectors were transferred to *Agrobacterium* and used to transform immature maize embryos.

A summary of the transformation results is presented in Table 1. OPA, OPB, and OPC transformations were carried out with maize line HiII. The OPA′ transformation was carried out with the same vector as the OPA transformation, but in HiII/SP122 cross embryos. Transformed embryos were selected using bialaphos [68]. Enzymatic activity

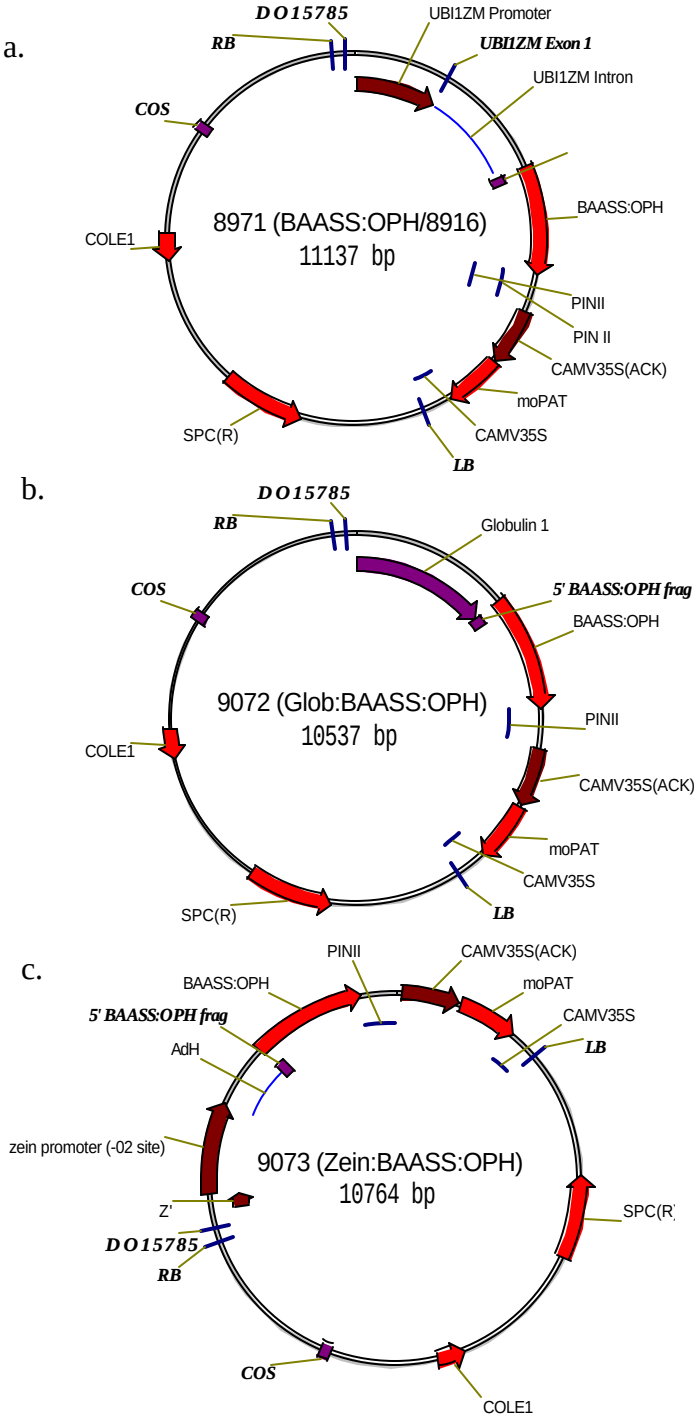


Figure 3. Assembled maize transformation vectors. **a.** OPA **b.** OPB **c.** OPC. The graphic maps of three maize transformation vectors used in this work were generated in Vector NTI.

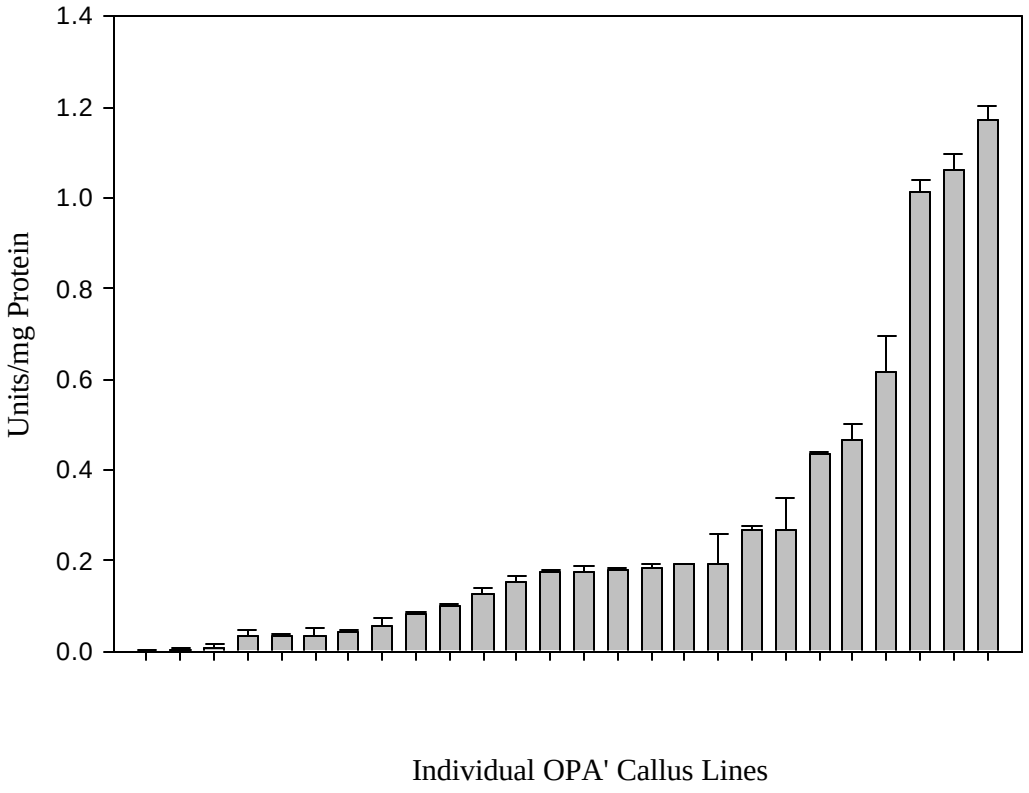


Figure 4. Paraoxonase activity of transgenic callus. A second transformation experiment was generated using the OPA transformation vector. Expression levels were calculated based on enzymatic activity against paraoxon. Each bar indicates the expression level observed in callus from a single transformation event with three replicate assays.

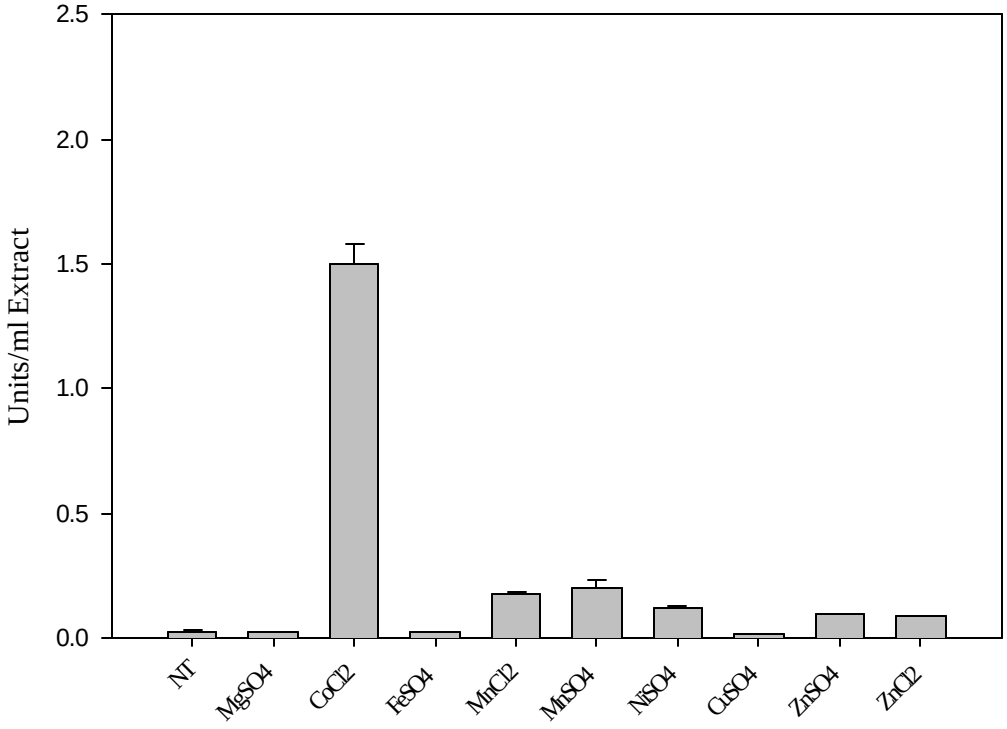


Figure 5. Increase of enzymatic activity in seed extract after incubation with transition metal salt. OPA0403 T2 extract was incubated with each salt at 10mM concentration for 30 minutes. Paraoxonase activity was measured by production of *p*-nitrophenol from paraoxon Each bar represents the average of three activity measurements of an extract treated with each salt. NT sample had no additional salt added.

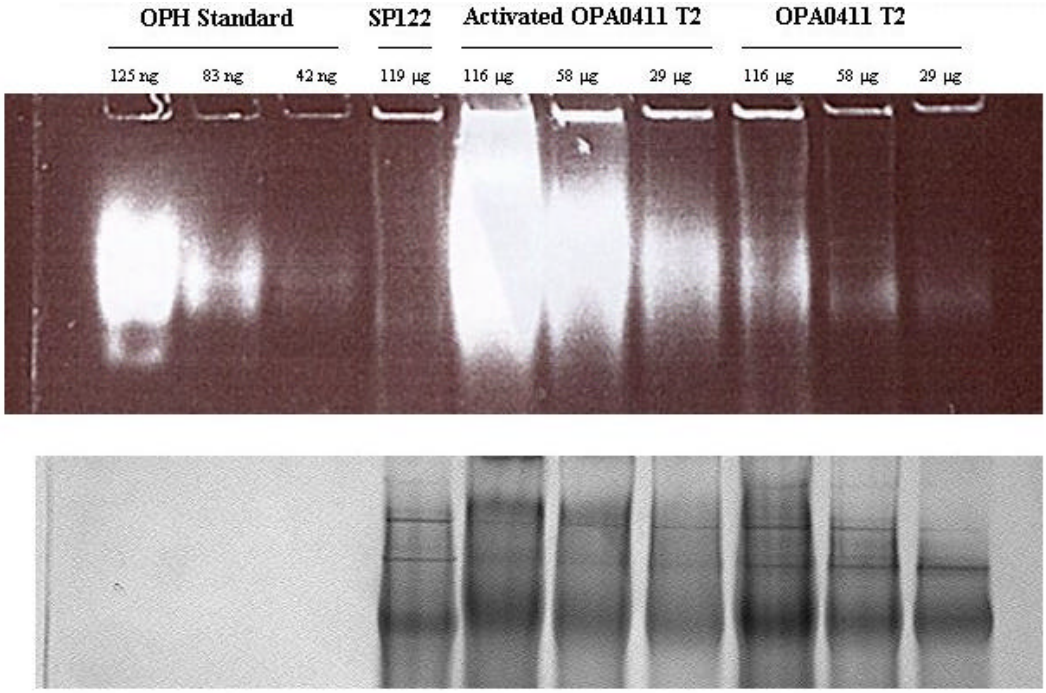


Figure 6. In gel hydrolysis of coumaphos by OPA seed extract. Control OPH was recombinant enzyme from an *E. coli* source. SP122 was an extract made from non-transgenic control seed. Extract protein levels as measure by Bradford protein assay are at the top of each well. Top panel shows in gel activity detected by UV fluorescence. Bottom panel shows the same gel after staining with Gel Code Blue (Pierce). OPA411 extract showed a specific activity of 0.42 u/mg paraoxonase activity before activation and 5.73 u/mg after activation.

was observed in different callus lines expressing OPH (OPA′) under control of the constitutive ubiquitin promoter (Figure 4).

Table 1. Transformation of Maize Embryos with OPA, OPB and OPC Vectors

Expression Tissue	Code	Z.E.'s Transformed	Stable Events	Transformation Frequency
Constitutive	OPA	819	23	2.81%
Embryo	OPB	2137	13	0.61%
Endosperm	OPC	2000	20	1.00%
Constitutive	OPA′	2287	52	2.27%

Enzyme Activation

Plants regenerated from the callus tissue were used to produce first generation transgenic seed (T1 seed). T1 seed was a product of the crossing of the initial transgenic line (T0) with an elite in-bred line. Initial analysis of T1 seed by enzymatic activity indicated expression levels under 0.001% TSP. It has previously been shown with fungal laccase expressed in corn seed that incubation with the appropriate metal co-factor could substantially increase the amount of observed activity [83]. Seed extracts were therefore incubated with a range of metals known to produce active forms of OPH (Figure 5).

The results indicated that the incubation extracts from OPH expressing lines of corn with various divalent transition metals resulted in an overall increase of up to 20 fold in the observed activity. The amount of activity observed closely follows the previously described pattern of activity for different metal substituted forms of OPH and was independent of the anion of the metal salt used to increase the observed activity [30]. Activity in callus extracts had a similar response to incubation with transition metal salts

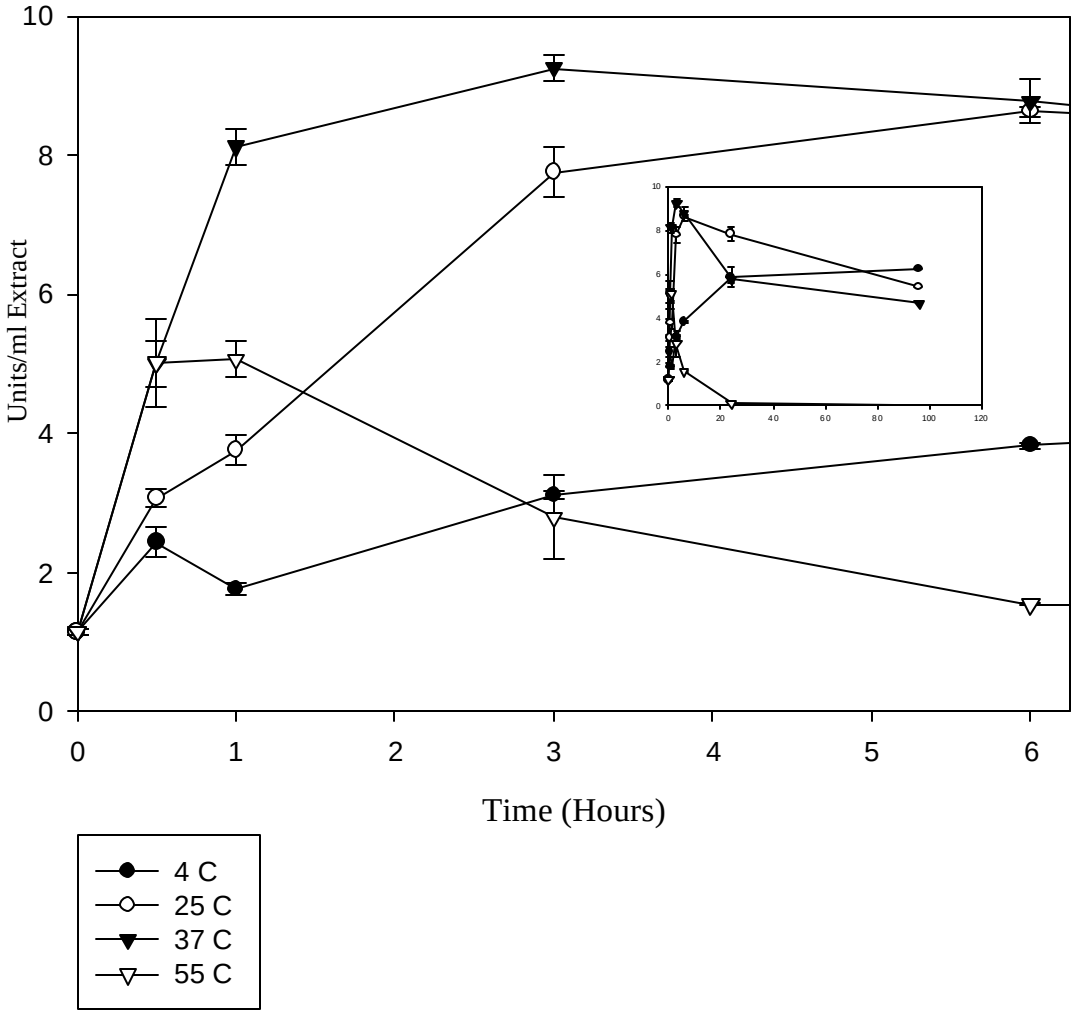


Figure 7. Temperature effect on the increase of activity detected in seed extract incubated with 10mM $CoCl_2$. T2 Seed Extract from line OPA0411 was incubated in the presence of 10mM Cobalt Chloride. Samples were assayed for paraoxonase activity and activity vs. time was plotted using sigma plot. Inset shows 0-96 hr. time frame.

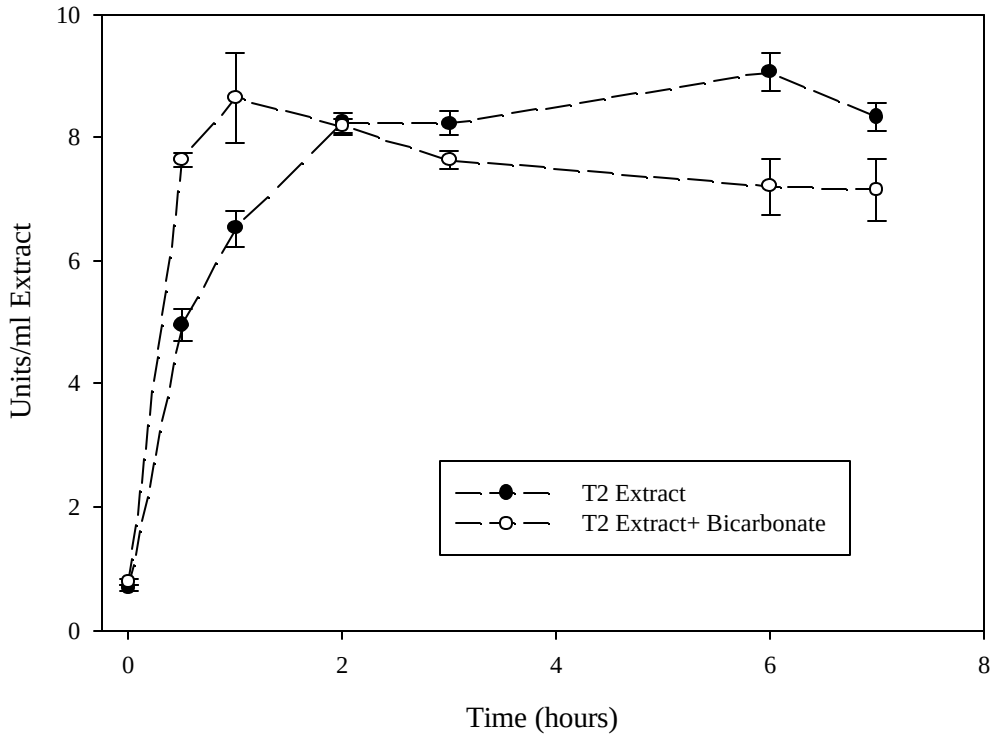


Figure 8. Effect of bicarbonate on the incubation of seed extract with 10mM $CoCl_2$. OPA0411 T2 seed extract was incubated in the presence and absence of 100mM sodium bicarbonate at 37°C. Paraoxonase activity was measured spectrophometrically and plotted vs. time with Sigmaplot 8.0.

(data not shown). Similar results were obtained for cobalt when the enzymatic activity was visualized after native gel electrophoresis using coumaphos as a substrate (Figure 6).

Incubation with cobalt chloride over a 100-hour time frame, and at four different temperatures, indicated that the increase in activity was a dynamic process that could be effected by temperature (Figure 7). Activity increased rapidly over time for extracts at 55, 37 and 25°C. The increase in activity was much slower at 4°C. The 55°C extract rapidly increased in activity over a 30-minute period and then rapidly lost activity until no OPH activity was detected. The 37 and 25°C extracts both increased in activity with the maximal activity reached in 3 and 6 hours respectively. At 100 hours the 4, 25, and 37°C extracts had similar final activity levels.

Inclusion of bicarbonate in buffer during the reconstitution of the apo-enzyme form of OPH has been shown to increase the rate at which activity is recovered, but not alter the final overall activity recovered [33]. The inclusion of bicarbonate in the buffer of seed extracts had the similar effect of increasing the rate of activity formation, but not the overall final activity observed (Figure 8).

T1 Seed Expression

T1 seed from all three constructs was analyzed for OPH enzymatic activity. Individual seeds were pulverized and extracted with buffer with added cobalt chloride and allowed to incubate in order to maximize the observed activity. Activity was determined by a micro-plate assay developed from the standard cuvette assay with a standard curve of purified recombinant OPH. Samples were compared to the standard

curve on an international enzymatic unit basis. The activity in each sample was then used to estimate the amount of OPH present in each sample. Expression levels of OPH in OPA and OPB T1 seed were similar to one another (Figure 9). Expression in OPC seed was below the level detectable with the plate assay system.

When OPC reactions with paraoxon were allowed to proceed overnight, *p*-nitrophenol was visually detected in half of the wells, which is the expected number of positive seeds generated by the initial cross.

Internal Localization of OPH Acitivity in Seed

To further determine the expression pattern of OPH in OPA and OPB seed, transgenic seed was manually separated into endosperm and germ fractions. The separated fractions were then extracted and extracts assayed for OPH activity. Results are presented in Table 2. The majority of activity in both constructs was isolated from the germ fraction indicating that most of the OPH activity in both constructs is located in the seed embryo.

Table 2. Separation of OPH Activity in Seed Fractions

Seed	**% Total Seed Mass**	**% Total OPH**
OPA0403 T2		
Embryos	14.4	91.0
Endosperm	85.6	9.0
OPB0301 T2		
Embryos	8.1	82.4
Endosperm	91.9	17.6

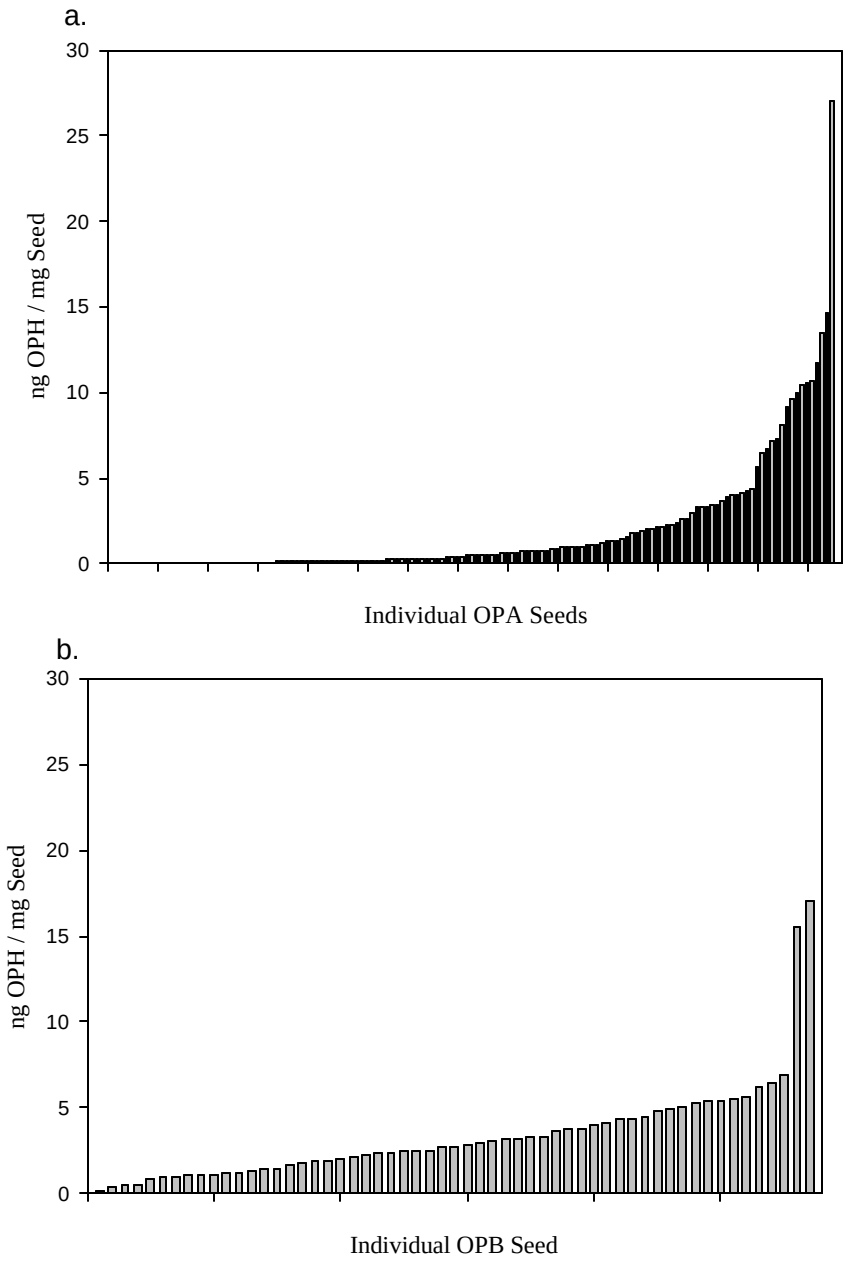


Figure 9. Expression of OPH in individual OPA and OPB T1 seeds. Enzymatic activity levels were measured across a global population of individual seeds from all OPA and OPB lines. Amount of OPH activity was used to estimate the total amount of OPH present per seed. All seed extracts were incubated with 1mM $CoCl_2$ overnight prior to the activity assay. The graphs show seeds that were positive for OPH expression by assay. Expression data can be found in Appendix B.

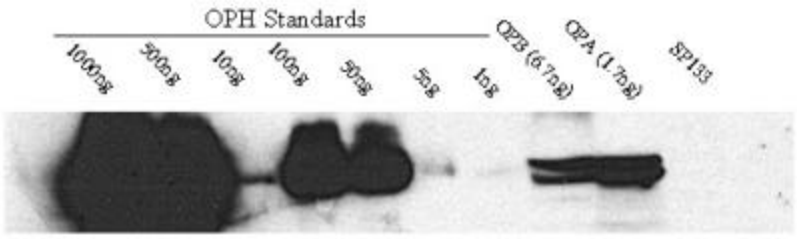


Figure 10. Western blot of OPA and OPB ammonium sulfate precipitates. 45% ammonium sulfate precipitated proteins were used for western bloting. Estimated OPH present by activity for OPA and OPB sample is shown in parentheses. SDS-PAGE samples of ammounim sulfate precipitated proteins were run out on a NOVEX 4-12% Tris-Glycine PAGE gel (Invitrogen). Gel was transferred to PVDF membrane and probed using purified anti-OPH antibodies with a horse radish peroxidase linked secondary antibody. Detection was carried out by chemiluminesence using an ECL-Plus western detection kit (Amersham Bioscience). SP133 is a no transgenic control line. Total protein loaded per well was 54µg for SP133, 80.5µg for OPA(OPA0411), and 52µg for OPB(OPB0310).

T2 and Field Expression

OPA and OPB lines were used to test expression in further generations as well as expression under field growing conditions. OPA0403 and OPA0411 were used to generate T2 seed, as well as Field grown T3 seed. T2 seed was generated by a self-cross of greenhouse grown OPA0403 and OPA0411 T1 plants. Bulk samples of 50 seeds from each line were analyzed for expression level by enzyme assay. The OPA0403 bulk sample had an enzyme level of 0.072%TSP and OPA0411 0.142%TSP. Both OPA and OPB were grown under field conditions. Lines from both constructs grew well under field conditions and seed was analyzed for OPH expression by enzyme assay. The expression level of OPH in field grown samples is presented in Table 3. OPA lines were grown in a 0.5-acre production field and generated over 1 metric ton of grain that contained OPH activity. The estimated amount of OPH enzyme present in the field grown OPA seed was 0.45 ng/mg of ground seed. This indicates that the total production of the OPA field was approximately 450 mg of OPH. OPB T2 field samples showed an overall higher expression level of OPH compared to the OPA field-grown seed. Average expression in positive bulk samples of OPB T2 seed was 0.22% TSP.

Immunological Detection

Extracts from OPA and OPB seed were used for immunological detection of OPH by western blot. Ammonium sulfate precipitates showed cross-reactivity with purified anti-OPH antibodies (Figure 10). A doublet band appeared in both the OPA and OPB samples, with molecular masses similar to that seen for standard recombinant OPH.

The doublet was identical for both the OPA and OPB sample and the lower band of the doublet was identical in mass to the bacterial OPH standard. A sample of ammonium sulfate precipitate from a control corn line showed no cross-reactivity with the antibody. It is interesting to note that the estimated OPH loaded for both transgenic samples was lower than the signal on the western blot compared to the OPH standards. This may indicates a loss in activity during the ammonium sulfate precipitation of OPH from seed extracts resulting in an under estimation of OPH present in the sample.

Discussion

Prior to this work, OPH has been produced in several eukaryotic and prokaryotic expression systems [17,23,28,32,39,40]. Organophosphate hydrolase from *Psudeomonas diminuta* has been successfully expressed in *Zea mays L*. Expression was successfully controlled by three different promoter systems. Under the control of the maize ubiquitin (constitutive) promoter and the maize globulin (embryo specific) promoter, highest expression level was nearly identical in the T1 seed generation. The performance of zein (endosperm specific) driven expression in seed was much lower then expression seen in the globulin and ubiquitin constructs, indicating that the ubiquitin and globulin constructs are superior for the production of OPH in corn seed when compared to the zein construct. This is similar to results obtained with the same promoters driving expression of several other proteins in maize [45,49,83]. Expression levels were measured for the constitutive construct at both the tissue culture (OPA′) and seed (OPA) levels. Expression levels varied between transformation events, which is consistent with the

random gene insertion achieved by *Agrobacterium* mediated transformation. Highest expression in OPA′ maize callus tissue was approximately 20 fold lower than expression in the *E. coli* expression system based on %TSP (Table 3). Expression for individual seeds in the OPA and OPB T1 generation were higher than that of the bacterial expression systems, but in OPA T2 bulks expression was lower than that of the *E. coli* system. Bulk samples from OPB T2 field grown plants were close in expression level to *E. coli*, with the best expressing sample two fold higher that the bacterial system and the average for all positive OPB T2 seed samples being nearly identical to the *E. coli* expression level. While in general the expression level in corn is lower than the expression in the bacterial expression system initially, higher levels of expression can be selected for in later generations [49]. Maize has advantages as a much more scalable system in terms of material that can be grown. This may give a maize expression system advantages over a bacterial system for the production of OPH for practical applications in the clean up of pesticides and defense against chemical weapons such as VX and Sarin. The bacterial system has also been shown not to respond well to growth in a fermentor, limiting the usefulness of the bacterial expression system for the large-scale production of OPH. OPH was expressed in a plant expression system without having an adverse effect on plant health and OPH expressing lines were able grow under field conditions. The OPH grown under the control of the constitutive promoter (OPA) and field conditions yielded an estimated 450mg of OPH enzyme per metric ton of grain produced. The same mass of the highest expressing line of the embryo specific (OPB) transgenic corn would yield an estimated 13kg of OPH. Both sets of field samples are

from early generations of transgenic plants (T2) and it has been observed that the expression of a transgenic protein can increase as much a 20 fold by proper selection of transgenic and inbred lines for further breeding [83]. It will also be possible to concentrate produced OPH by as much as 10 fold by separating the germ portion of the seed from the endosperm portion of the seed. With both promoter systems used in this study, the majority of recombinant protein activity was located in the embryo portion of the maize seed.

Table 3. Comparison of OPH Expression Levels in Corn Seed and Callus to Other Expression Systems. %TSP is estimated from the reported specific activity of extracts using a specific activity of 8000 units/mg protein for pure OPH.

Expression System	Expression Target/Location	Units/mg Protein	% Total Soluble Protein	Ref
P. diminuta	External	2	0.026	37
E. coli (DH5α)	Internal	18-25	0.225-0.312	37
Sf9 Cell Culture	Internal	15-20	0.188-0.250	37
S. lividans	External	1	0.012	84
OPA'08 Callus	External/Consitutive	1	0.013	-
OPA1606 (T1 Single Seed)	External/Consitutive	49	0.617	-
OPB0103 (T1 Single Seed)	External/Embryo	125	1.569	-
OPA0403 (T2 Bulk)	External/Consitutive	6	0.072	-
OPA0411 (T2 Bulk)	External/Consitutive	11	0.143	-
OPA0403 (T4 Bulk, Field Grown)	External/Consitutive	0.3	0.003	-
OPB0107 (T2 Bulk, Field Grown)	External/Embryo	48	0.604	-

Western blots of ammonium sulfate precipitates of OPA and OPB extracts resulted in the immunological detection of OPH expressed in maize. The protein detected on the western blot was detected as a doublet, indicating that at least two species of cross-reacting protein are present in the transgenic plants. There was no cross reactivity in the control sample. This shows that the two cross-reacting bands are specific to the transgenic lines. The origin of the two bands may be due to incorrect

processing of the n-terminal BAASS sequence added to the optimized gene. It is also possible that doublet is due to the possible post-translational modification of the OPH protein as it passes through the endo-membrane system during transport to the cell wall. N-terminal sequencing of OPH purified from maize should identify any differences in n-terminal processing that may result in the doublet band.

It is also interesting to note that OPH expressed in corn seed was "activated" when extracted in the presence of divalent transition metals that have previously been shown to form active OPH enzyme. This activation event had not been observed with enzyme produced in other systems. The most similar phenomena to that observed is apo-enzyme reconstitution with divalent transition metal salts [33]. The pattern of metal dependent reconstituted activity was similar to that seen with apo-enzyme reconstitution[30].

It was also observed that the addition of bicarbonate to extraction buffer increased the rate at which activity increased, but did not increase the overall final activity observed. It is possible that expression in maize or the transport of the OPH product through the endo-membrane system by the BAASS signal sequence may retard the inclusion of metals into the folded protein. The inclusion of metal into the extraction buffer remedies this lack of metal and results in the increase in activity observed over time.

Maize does serve as a successful expression system for OPH. While the bacterial system of expression remains adequate for the laboratory scale expression of OPH, the failure of laboratory strains to perform well in fermentors has limited the large-scale

production of OPH for practical applications. The maize-based expression system has advantages in scalability that makes lower expression levels less problematic compared to a bacterial or animal cell culture system. The maize-based expression system also has the advantage of long term storage of seed. This would mean that large amounts of OPH could be stored as whole seed and then processed into extracts or purified enzyme as needed.

Materials and Methods

Materials

All buffers were obtained from Sigma-Aldrich (St. Louis, MO). Protein A sepharose was obtained from Pharmacia. Paraoxon was obtained from Chemservice (West Chester, PA). Enhanced Chemiluminescence kit was obtained from Amersham Biosciences (Arlington Heights, IL). Gel Code Blue stain was obtained from Pierce. Anti-rabbit IgG labeled with horseradish peroxidase was obtained from Jackson Immunoresearch Laboratories. Bradford reagent was obtained from Bio-Rad. PCR TOPO vectors and NOVEX polyacrylamide gels were obtained from Invitrogen.

Methods

Optimization and Assembly of the Optimized *opd* Gene

Back-translating the OPH protein sequence using a maize-high codon usage table generated an optimized sequence of the bacterial *opd* gene. The sequence for the Barley α-amylase signal sequence was added to the 5' end of the optimized gene. The optimized sequence was analyzed for restriction sites and five were chosen for the assembly of the full-length gene. The entire gene sequence was then split into 50 bp

oligos with 10 bp over-lapping sequences. The oligos were synthesized commercially (Gibco-BRL). DNA manipulations were carried out using standard methods [85]. Oligos making up one of the restriction fragment were mixed with their matching pairs and annealed by cycling between 94°C and 25°C thirty times. Each annealed pair was then mixed and ligated using T4 DNA ligase. The ligated fragments were then amplified by PCR using primers to the end of each fragment. The amplified fragment was then trapped in a PCR TOPO 2.1 vector (Invitrogen). Insertions in the TOPO vector were analyzed by restriction digest and sequencing (Iowa State). When fragments were identified with the correct sequence, they were sub-cloned together using the restriction site previously identified and the TOPO vector as a backbone. Once the full-length gene had been assembled, the gene was transferred to a plant expression vectors under the control of the ubiquitin (OPA, OPA′, constitutive), globulin (OPB, embryo), and zein (OPC, endosperm) promoters [48,81,82].

Transformation in Maize Cells

The completed transformation vectors were transferred to *Agrobacterium tumefaciens* by triparental mating. Maize transformation was carried out as described in U.S. Patent 5,981,840 [58]. In short, immature maize embryos were isolated and incubated with *Agrobacterium* containing a binary transformation system with the desired construct. Embryos were then plated and regenerated into callus that was selected using the herbicide bialaphos. The first 15 stable transformation events for each construct (OPA, OPB, and OPC) were regenerated into plants and T1 seed was collected.

Analysis of Callus

Callus from the OPX line was placed into tubes with 500µl of 10mM HEPES pH 8.4 + 1mM $CoCl_2$. A ball bearing was placed in each tube and the tubes were shaken for 40 seconds in a Geno/Grinder 2000 (SPEX CentriPrep INC.). Extracts were then spun at 3000xg for 15 minute. Extracts were incubated at room temperature overnight. Assays were conducted the next day using 1ml of 1mM Paraoxon in 50mM CHES buffer pH=9.0 and 5µl of each extract. Each sample preparation was repeated in triplicate. Protein assays were preformed using a microplate Bradford assay (Bio-Rad).

Analysis of Seed

Five individual T1 seeds from each individual OPA plant and six seeds from each OPB and OPC T1 plants were analyzed for OPH activity. Single seeds were placed into a stainless steel vial and mechanically pulverized. Each seed was then extracted with 1000µl of 10mM HEPES buffer + 1mM $CoCl_2$ by shaking with a ball bearing in the dental vial for 20 seconds. Extracts were then spun for 15 minutes at 3000xg. Extracts were incubated at room temperature overnight and assayed for OPH activity. Assays were conducted using a microplate assay derived from the cuvette assay used to determine callus activity [28]. 200 µl of 1mM paraoxon in CHES buffer was added to 20µl of a 1/20 dilution of extract. Activity was measured over a 5-minute period and compared to a standard curve of recombinant OPH from bacteria on a unit/ml basis. The units/ml extract value was used to compute an estimate on the amount of OPH in a sample using a specific activity of pure bacterial OPH of 8000units/mg protein.

For T2 and higher generations, bulk samples of 50 seeds were used to generate expression data. Samples were ground in a conventional kitchen coffee grinder. The ground material (meal) was then extracted overnight with buffer (same as with T1 extractions) in a 3/1 or 5/1 ratio of buffer to meal. After extraction the extracts were spun in a Beckman J-25I centrifuge at 70,000xG for 30 minutes. Protein levels were determined by microplate Bradford protein assays.

OPA field grown corn was grown during the summer of 2002 in central Illinois. Bulk samples were analyzed as described above. A total of 0.5 acres of OPA corn was grown and yielded 1.005 metric tons of grain. OPB field grown corn was grown in Nebraska during the summer of 2002. Due to the large number of samples, 100mg samples of meal from each 50 seed pool were extracted with 1ml of buffer and in Corning clustar tubes with a ball bearing added to each tube. The tubes were then shaken using a Genogrinder 2000 tissue grinder/shaker and spun a 3000xg for 15 minutes. Samples were then transferred to a 96 well plate and assayed using the plate assay described above.

Activation of OPH Extracts

Corn seed from lines expressing OPH was ground into meal using a common electric coffee grinder. OPH was extracted from ground meal for 3 hours with 10mM HEPES buffer in a 3/1 ratio of buffer to meal. The extract was then aliquoted and brought up to 10mM concentration of various metal salts using 1M stocks. Enhanced extracts were then incubated and assayed for OPH activity using a cuvette paraoxon assay. In short, 5 µl of extract was added to a cuvette containing 1 ml of 1mM paraoxon

in CHES buffer. The mixture was inverted to mix and the production of p-nitrophenol was monitored at 400 nm on a Beckman 7400 spectrophotometer. All samples were measured in triplicate. Bicarbonate activation was carried out as above with the exception of the addition of 1M sodium bicarbonate to bring the final concentration to 100 mM. The control reaction included the addition of water to account for the dilution.

In Gel Hydrolysis

In gel hydrolysis was carried out by a method modified from Harcourt *et al*. [69]. Extracts of transgenic and control tissue were used for native gel electrophoresis on a Novex 12% Tris-Glycine gel as per the manufacturers instructions. After the completion of electrophoresis the gel was equilibrated for 5 minutes in 50mM Tris pH=8.0. After equilibration, the gel was moved to a reaction mixture of 8µM coumaphos in Tris buffer. The gel was left to react for ten minutes and then placed on a UV light box and recorded by digital camera. After recording the image, the gel was washed in distilled water and stained with Gel Code Blue stain (Pierce). The transgenic extract used for this experiment showed a measured specific activity of 0.42u/mg protein prior to incubation with 10mM $CoCl_2$ and 5.73u/mg after incubation.

Dissection and Extraction of Seed

Transgenic seed from both the OPA and OPB constructs was dissected to remove the embryo portion of the seed from the endosperm portion of the seed. 20g of seed from each line were imbibed for 3 hours with distilled water. After removal of the water the seed was manually de-germed by removing the embryo from the seed using a scalpel. The separated seed fractions were then dried for 3 hours in a 34°C oven. After drying the

material was ground in a consumer coffee grinder, massed, and extracted as noted above. The extracts were assayed for paraoxonase activity and the percentage of the total activity for each fraction was determined.

Immunological Detection and Western Blots

Rabbit serum containing antibodies raised against OPH was a gift from Dr. Janet Grimsley. Anti-OPH IgG was purified from the anti-sera by chromatography on Protein A CL-4B sepharose (Pharmacia). In short, the protein-A sepharose was hydrated in buffer A (20mM MOPS, 150mM NaCl pH=7.4). Serum was mixed in a 1:1 speharose:serum ratio and rotated for 90 minutes. After the incubation, the slurry was run onto a column and the sepharose was washed with ten column volumes of buffer A. Non-specific proteins were then eluted with five column volumes of buffer B (20mM MOPS, 1M NaCl pH=7.4). Antibodies were then eluted with buffer C (20mM Glycine, 50mM NaCl pH=2.5). Fractions were assayed for protein content by measurement of absorbance at 280nm. Protein containing fractions were then dialyzed overnight into phosphate buffered saline (PBS, 10mM Phosphate buffer 130mM NaCl pH=6.9). Purified antibodies were then checked again for protein content and used for western blots.

Samples for western blots were obtained by extracting corn meal from transgenic and non-transgenic lines with 10mM Tris pH=8.4 buffer with 1mM CoCl2 added in a 5:1 buffer:meal ratio. Extracts were then clarified by centrifugation and cut with 45% saturation ammonium sulfate for one hour at 4°C. Precipitate was collected by

centrifugation and resuspended in 10mM Tris buffer. Samples were then dialyzed against 1000 volumes of Tris buffer overnight to remove excess ammonium sulfate. Protein was estimated using microplate Bradford assay and enzymatic level estimated by activity against paraoxon. SDS-PAGE samples were then prepared and run on Novex 4-12% gradient gels (Invitrogen). Gels were transferred to PVDF membrane (Millipore) overnight using a NOVEX transfer apparatus. Blotted membrane was then blocked with 5% non-fat dry milk in PBS overnight. Blocked membrane was then incubated with purified anti-OPH antibody in PBS for 1 hour, washed, and then incubated with a goat anti-rabbit horseradish peroxidase linked secondary antibody (Jackson Immunoresearch) for 1 hour. Blot was then washed with tris buffered saline with tween (TBST, 100mM Tris 90mM NaCl 0.5% Tween pH=8.0) and detection carried out using an ECL plus chemiluminesence detection kit (Amersham Biosciences). Blot was recorded on Kodak OMAT-AR film.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF ORGANOPHOSPHATE HYDROLASE EXPRESSED RECOMBINANTLY IN *Zea mays L.*

Organophosphate hydrolase is a dimeric metallo-protein capable of degrading a wide range of organophosphate pesticides and chemical warfare agents. A synthetic sequence derived from the bacterial sequence encoding OPH has successfully been transformed into the C4 monocot *Zea mays L*. Western analysis of ammonium sulfate precipitates of transgenic seed extracts showed that two distinct bands cross-react with anti-OPH antibodies. Studies were also undertaken to determine if the doublet-banding pattern seen in western blots of maize derived material were due to possible mis-processing of the barley α-amylase signal sequence or were the result of post-translational modification. This work requires the purification of OPH from transgenic seed and the analysis of the physical and kinetic properties of the recombinant proteins.

Introduction

Organophosphate hydrolase (OPH) is a bacterially derived enzyme capable of degrading a wide range of organophosphate triesters [23]. OPH hydrolytic activity has been observed against organophosphates containing P-O, P-S, P-F, and P-CN bonds [24]. This gives OPH a broader spectrum of substrates compared to the PON family of serum paraoxonases, organophophate acid anhydrolase (OPAA), and squid DFPase [8,9,22,24].

OPH's wide range of substrates makes it an ideal target for use in practical applications, such as remediation. However, OPH use in practical applications is limited by the amount of enzyme produced by current laboratory expression systems. The gene for OPH, *opd*, was first isolated from soil bacteria living in areas treated with organophosphate pesticides [10,19]. The native sequence encoding the enzyme isolated from *P. diminuta* and *Flavobacterium sp.* contains a 29 amino acid leader sequence that facilitates the export of OPH outside the bacterial cell, where it is found in complexes associated with the bacterial cell membrane [37]. Initial attempts at recombinant expression in bacterial and insect cell culture systems resulted in production of enzyme, but the levels of expression were far below the maximum for the expression system and promoters being used [31,39]. Removal of the leader sequence resulted in a significant increase in the level of protein expression [37]. Recombinant expression of OPH has also been successfully attempted in a fungal system [38]. However, none of these systems has been adopted for large-scale production of OPH for application purposes due to low expression and scale-up problems. These problems include the loss of enzymatic activity in bacterial systems adapted to large-scale fermentation. Recently the ability of the plant expression *system Zea mays L.* (maize) to express OPH was investigated as a possible alternative to expression systems previously used OPH expression systems (Chapter II).

OPH was successfully expressed in the maize expression system under the control of three distinct promoters. Western blotting of ammonium sulfate precipitates of seed extracts generated from two different constructs revealed two bands in the sample that cross-reacted with anti-OPH antibodies. Non-transgenic seed extract does not cross-

react with anti-OPH antibodies leading to the conclusion that two forms of OPH monomer must be present in transgenic seed extracts. The optimized gene utilized for maize expression of OPH had the barley α-amylase signal sequence (BAASS) N-terminally fused in order to export the protein through the endo-membrane system to the plant cell wall. This raises the possibility that the two bands identified are the product of either mis-processing of the BAASS sequence or are the result of a post-translational modification that takes place during transit of the endo-membrane system. Post-translational modification was not observed in insect cell culture expression, however the protein did not pass through the endo-membrane system and was not exposed to the cell's glycosylation mechanism.

In this report, the physical effects on OPH when it is expressed in corn with an N-terminal BAASS signal sequence are determined. To validate the plant-based system of expression, it is necessary to determine what effect the plant expression has on the quality of protein being produced. These effects could range from the exclusion of co-factors needed for enzymatic activity to the post-translational modification of the protein resulting in altered enzyme kinetics or protein stability. Previously, it has been shown that the expression of OPH in maize seed resulted in a protein that could be activated by the inclusion of divalent transition metal salts during or after extraction and that immunological detection by western blot detected a doublet band of appropriate mass for OPH. The aim of this study was to isolate and characterize OPH expressed in maize seed. This included the identification of possible modifications and the kinetic properties of the seed expressed enzyme.

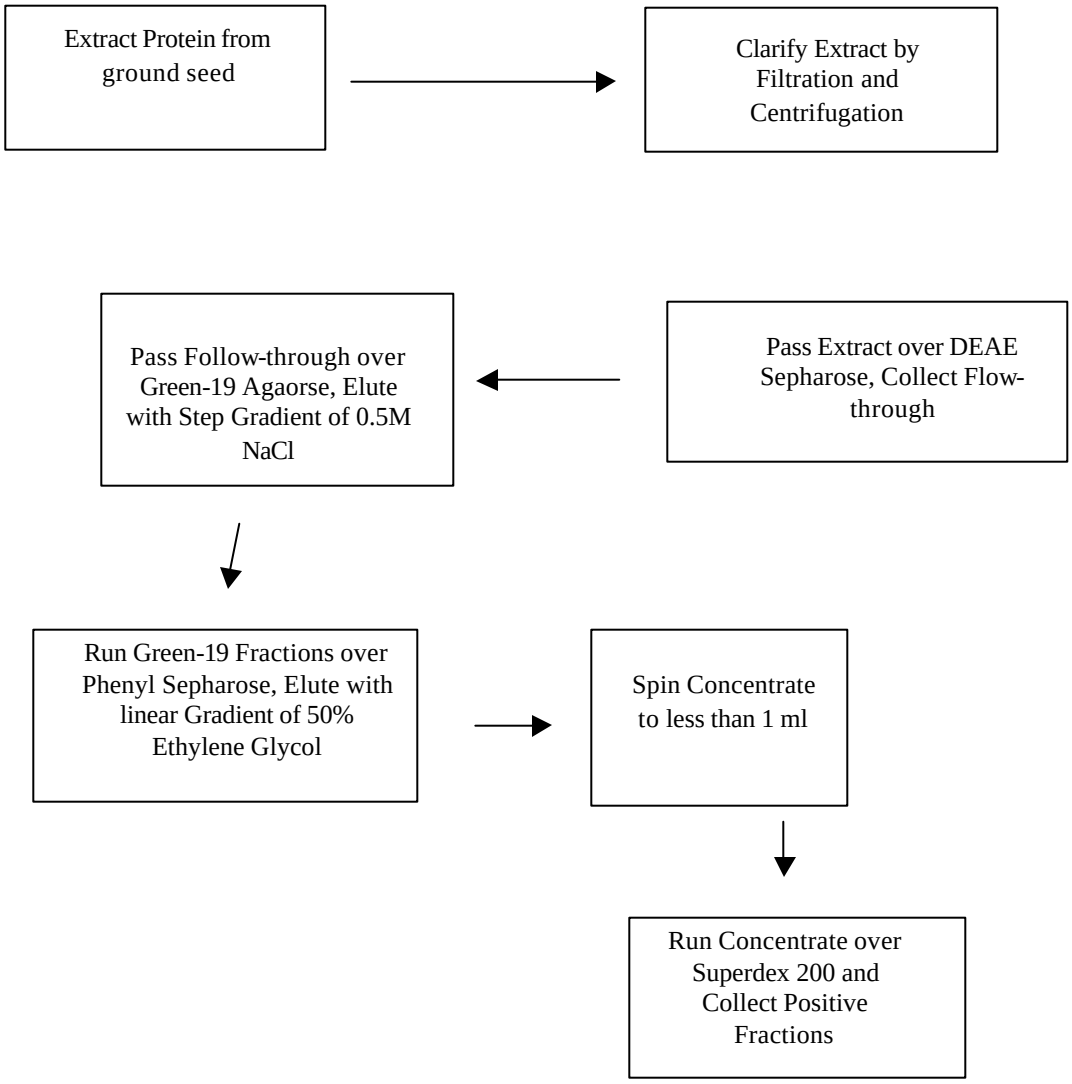


Figure 11. Purification of OPH from transgenic seed flow-chart. Steps of the purification process is indicated in each box. The purification procedure is a modification of a procedure used to isolate OPH from bacterial and insect cell culture expression systems [31,86].

Results

Purification

Table 4. Purification of OPH from transgenic maize seed. Purification flow chart can be found in Figure 11.

Step	Total Activity	Specific Activity	Purification	Recovery
	Units	units/mg	fold	%
Concentrated Extract	1891	0.38	1	100
Concentrated DEAE	596	0.75	2	32
Phenyl Sepharose	438	762.83	2007	23
Superdex 200	62	881.47	2322	4

Purification of OPH from seed was carried out using a procedure similar to that for the purification of OPH from insect cell culture and bacterial expression systems (Figure 11)[31,86]. Results of the purification procedure are presented in Table 4. The overall purification process resulted in over a 2000 fold increase in purity as measured by specific activity. The purification included an affinity chromatography step using green-19 agarose. It was noted during the development of the purification that the level of activity in the fractions from the green-19 elution degraded rapidly, resulting in the inability to accurately measure the results of the green-19 step. This instability was not present in steps prior to or after the green-19 elution. The nature of this instability was

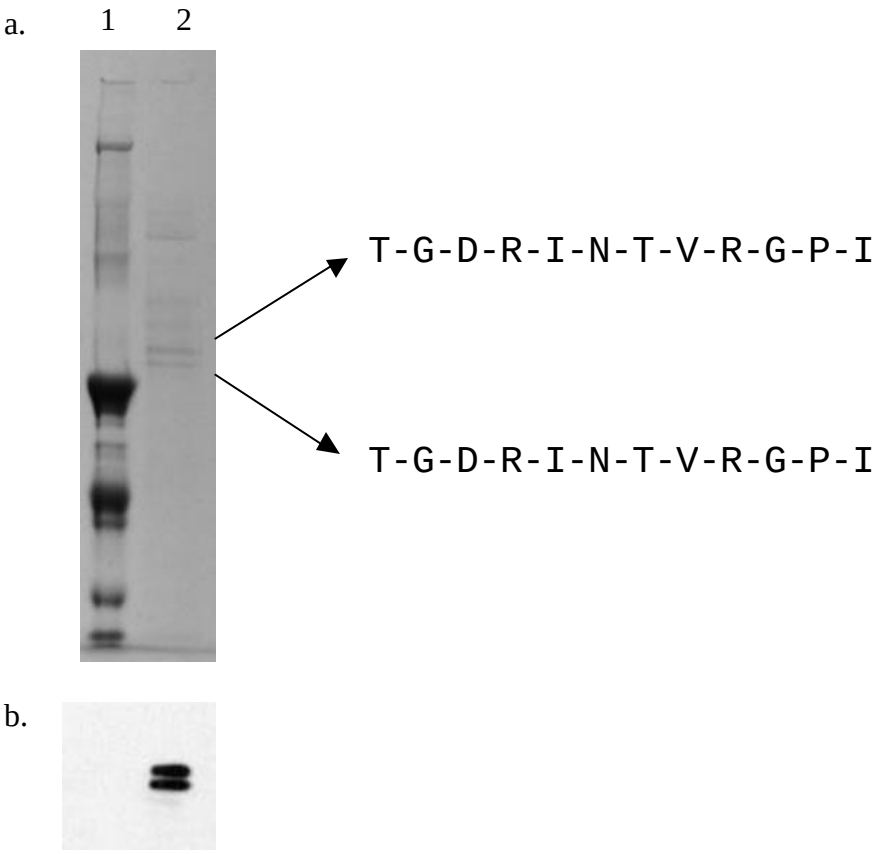
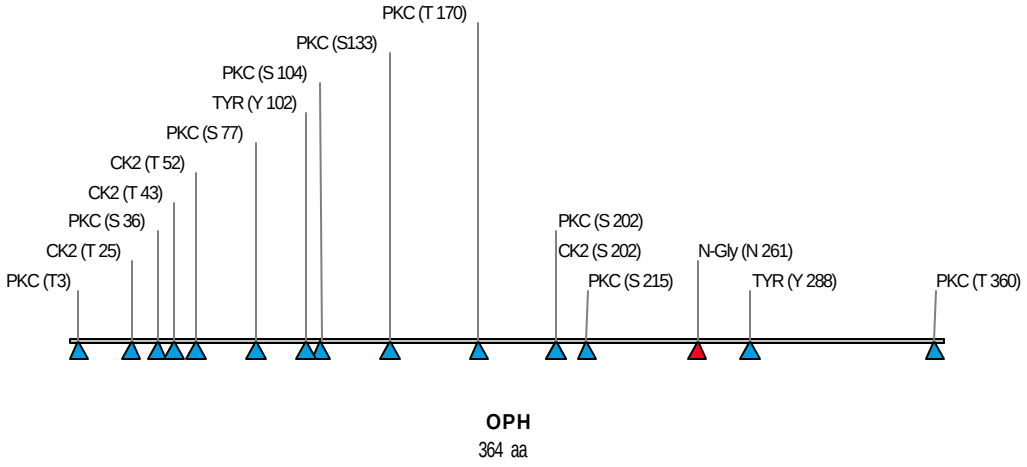


Figure 12. N-terminal sequencing of OPH expressed in maize. **a.** Lane 2 shows OPH purified from maize seed, lane 1 shows a molecular weight marker. Sequencing of both upper and lower bands that cross react with anti-OPH antibodies was carried out by the Protein Chemistry Laboratory at Texas A&M University. Predicted N-terminal sequence was generated using the web-based SignalP V1.1 program (http://www.cbs.dtu.dk/services/SignalP/). SDS-Page Gel shown is a Novex 12% Bis-Tris gel (Invitrogen) run with a MOPS buffering system and stained with Pierce Gel Code Blue stain. **b.** Western blot of purified OPH from transgenic maize.

not identified. To reduce the effects of the instability, the green-19 fractions were immediately run onto a phenyl sepharose column. Comparison of the superdex-200 elution profile of the purified maize recombinant protein to a molecular mass calibration kit indicates that the recombinant protein has a mass of approximately 85 kD. This is slightly more than the expected 72 kD of the recombinant protein from *E. coli* and is possibly due to the increased mass of the upper band observed in western blots. SDS-PAGE and western analysis of the purification product is presented in Figure 12. The two bands indicated in the figure also cross-reacted with anti-OPH antibodies in a western blot. Comparison of the two cross reacting bands with the molecular weight marker using Alpha Imager 2200 analysis software (Alpha Innotech, ver. 5.04) indicated that the size difference between the two bands was approximately 1.5 kD.

Sequencing

Protein purified from ground OPB meal was used for N-terminal sequencing of both bands observed in western blots (Figure 12). The results indicate that the n-terminal sequence for both bands is identical, and that both bands have an N-terminal sequence nearly identical to that predicted for the cleavage product of the BAASS:OPH peptide. The only difference is the predicted N-terminal residue was a glycine, while the observed is threonine. This difference in N-terminal residue may be because the natural cleavage site of BAASS is cleaved after a glycine residue.



N-Gly = N-Glycosylation Site
PKC = Protein kinase C
Phosphorylation Site
CK2 = Casein kinase II
Phosphorylation Site
TYR = Tyrosine kinase
Phosphorylation Site

Sequence analyzed with the PIR
Pattern Search program
(http://www-
nbrf.georgetown.edu/pirwww/search/
patmatch.html)

Figure 13. Possible OPH post-translational modification sites. The OPH peptide sequence was analyzed using the web based PIR pattern search program (http://www.nrbf.georgetown.edu/pirwww/search/patmatch.html)

Identification of Possible Post-Translational Modification Sites

The sequencing results indicate that the mass difference observed by SDS-PAGE and western of the two forms of OPH monomer is not due to the miss-cleavage of the BAASS sequence. Therefore, we used a web-based computer program to identify possible post-translational modification sites (Figure 13). Among the possible modification site predicted, there are several possible phosphorylation sites, as well as an N-glycosylation site at asparagine 261. The identification of a possible N-glycosylation site, along with the BAASS mediated export through the endo-membrane system and the difference in mass between the two forms of monomer lead us to investigate the possibility that the difference in mass was due to a N-glycosylation event.

Binding of Corn Derived OPH to Concanavalin A

Table 5. Binding and elution of maize expressed OPH to Concanavalin A sepharose.

Sample	Total Units OPH	% Recovery
Loaded	6.01	100.0
Flow Through	0.27	4.5
Elution	0.25	4.3

To identify if the modification observed by mass difference was a glycosylation, the ability of corn derived OPH to bind to Concanavalin A was tested. Partially purified protein (from phenyl sepharose fractions) was run onto a 1ml Concanavalin A sepharose

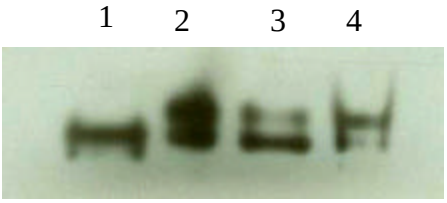


Figure 14. Western blot of Concanavalin A flow through and elution fractions. **1.** Recombinant OPH standard from E. coli (100ng). **2.**Loaded recombinant OPH from maize (90 ng). **3.** Concanavalin A flow through (113 ng). **4.** Concanavalin A elution (106 ng). Estimated OPH loaded is in parentheses. Partially purified OPH from OPB corn seed was passed through a 1ml Concanavalin A sepharose column. Samples were run out on a Novex 12% Bis-Tris Gel using MOPS buffering system. Gel was then blotted to PVDF and probed using anti-OPH antibodies using a HRP linked secondary antibody (Jackson Laboratories) and ECL Plus detection kit (Amersham Biosciences). Estimates of OPH loaded per well were determined by activity against 1mM paraoxon.

column. The column was then washed and protein eluted off using a single step gradient of 0.5M methyl α-D-glucopyranoside. Elution results are presented in Table 5. The vast majority of activity run onto the column bound to the Concanvalin A. Step-gradient elution only resulted in the recovery of a small amount of the loaded activity. A western blot was used to determine any difference between protein that flowed through the Concanavalin A column and material eluted from the column (Figure 14). The western blot revealed that there was an enrichment of the lower OPH band in the Concanavalin A flow through and an enrichment of the upper OPH band in the material eluted. This indicates that the upper band is bound preferentially over the lower band, providing stronger evidence that the upper band is glycosylated. It is also of note that greater than 90% of the activity run onto the column did not run through the column or elute with methyl α-D-glucanopyranoside. Of the 6 units loaded onto the column, only 0.27 units flowed through the column and 0.25 units were eluted. To test if activity remained bound to the column, 1mM paraoxon in buffer was loaded onto the column. Within 10 seconds, a noticeable yellow color was detected consistent with the production of *p*-nitrophenol, the product that is produced when OPH degrades paraoxon. Three months after the initial loading of protein onto the column, measurement of the absorbance of a paraoxon reaction mixture after exposure for 10 seconds indicated that an estimated 27% of the OPH loaded onto the column remained bound and active despite the elution step.

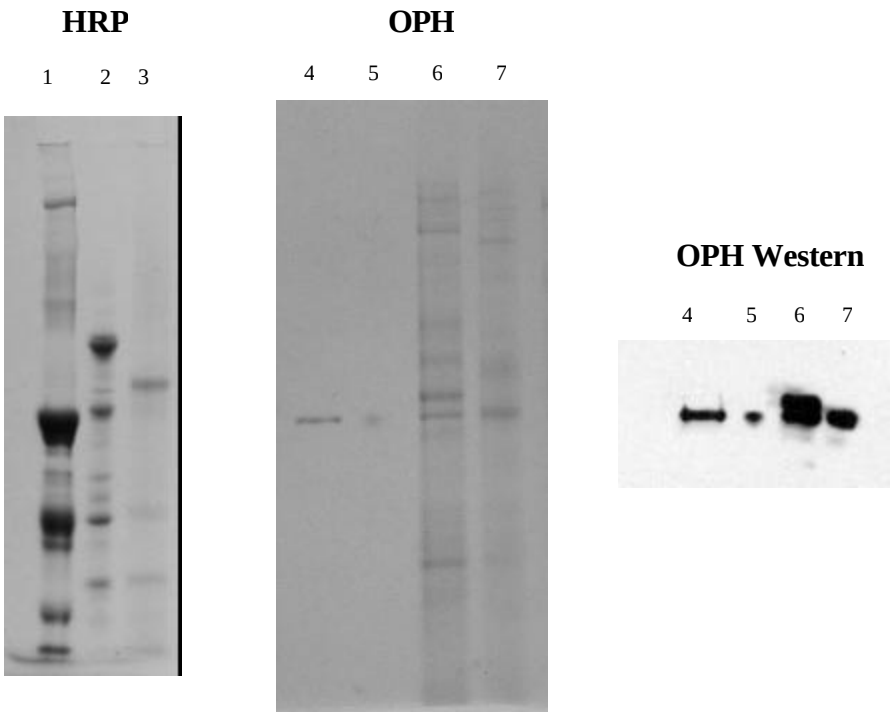


Figure 15. Chemical deglcosylation of maize expressed OPH. **1.** MW markers**. 2.** Horse Radish Peroxidase (HRP) no treatment. **3.** Deglycosylated HRP. **4.** Recombinant OPH from *E. coli*, no treatment. **5.** Deglycosylated *E.coli* OPH. **6.** Recombinant OPH from maize, no treatment. **7.** Recombinant OPH from maize deglycosylated. Partially purified OPH from OPB seed was subjected to chemical deglycosylation using a chemical deglycosylation kit (Glyko). Horseradish peroxidase was used as a positive control. Purified recombinant OPH from *E.coli* was used as a negative control. Treated samples were then run on a Novex 12% Bis-Tris SDS-PAGE gel and blotted onto PVDF. The lane numbers on the stained gel are the same lane numbers at the top of the western. The amount of OPH loaded per well was estimated by paraoxonase activity.

Chemical Deglycosylation

Removal of carbohydrate structures from glycoproteins is possible by enzymatic and chemical methods [87-89]. To further determine if the observed upper band was glycosylated, chemical deglycosylation was used to remove any carbohydrate structures. A sample of purified corn derived OPH was lyophilized into a reaction vessel. Chemical deglycosylation was carried out with a chemical deglycosylation kit (Glyco) with horseradish peroxidase used as a positive control. Products of the deglycosylation were run out on SDS-PAGE gels and stained, as well as used for western blot analysis (Figure 15). Results indicated the chemical deglycosylation resulted in the shifting of the upper band to the lower band position, which is indicative of the upper band being glycosylated.

Enzyme Kinetics

Kinetics of paraoxon hydrolysis for corn derived OPH were determined (Table 6). Overall kinetics for the corn-produced enzyme are similar to those of recombinant enzyme from *E. coli*. K_m values for enzyme passed through the Concanavalin A sepharose column were also determined. K_m values for the upper and lower band enriched fractions were very similar with values in the range of those reported for OPH K_m in the literature. The specific activity of the protein that passed through the Concanavalin A column without binding was 3 fold higher than the specific activity of the loaded sample, while the specific activity of the eluted sample remained at nearly the

same value. It is important to note that the sample loaded onto the Concanavalin A column had been through the phenyl sepharose step of the purification and concentrated prior to loading, but had not been run over the superdex 200 column.

Table 6. Paraoxon kinetics of OPH isolated from transgenic maize seed.

Recombinant OPH	K_m	Specific Activity	Reference
	μM	Units/mg Protein	
Purified *E. coli*	58-200	1760-8120	31
Partially Purified Maize	45 ± 8	882	-
ConA Upper Band	227 ± 87	1205	-
ConA Lower Band	85 ± 27	427	-

Material loaded onto the Concanavalin A column was a concentrated fraction taken from the phenyl sepharose step of the purification and had a specific activity of 445 units/mg protein.

Discussion

OPH has been expressed in several recombinant expression systems. The aim of this work was to evaluate the protein produced by a plant expression system, *Zea mays*. Previously it was shown that corn transformed with expression vectors containing an optimized OPH gene sequence resulted in the production of a protein with paraoxonase activity that cross reacted with anti-OPH antibodies. The western blot showed two bands that reacted with the antibodies, while no reaction was detected in non-transgenic seed samples. To determine the cause of the two-band phenomena we n-terminally sequenced both bands after purification. N-terminal sequencing showed that both bands had the same N-terminal sequence and that the sequence was identical to that predicted for the cleavage of the BAASS sequence with the exception of a missing N-terminal glycine

residue. This eliminated the possibility that the size difference observed for the two bands in western blots was due to incorrect processing of the BAASS sequence. While the experimentally determined sequence differed from the predicted sequence, it was only by a single glycine residue, not a large sequence of amino acids that would be needed to cause the significant size difference observed in the SDS-PAGE and western analysis.

Analysis of the protein sequence of OPH revealed that the sequence contains an N-glycosylation signal sequence. Post-translational modification of OPH expressed in eukaryotic systems has not previously been observed. Both expression in insect cell culture, as well as expression in filamentous fungi was cytoplasmically target, preventing the recombinant protein from coming into contact with the glycosylation machinery in the endo-membrane system [37,38]. The BAASS signal sequence used in corn expression causes the peptide to be exported to the cell wall via the endo-membrane system. During this transit, the peptide is exposed to the glycosylation apparatus. The idea that glycosylation was responsible for the doublet was re-enforced by the binding of the recombinant protein to Concanavalin A sepharose and the enrichment of the lower band in the flow through and upper band in the eluted fractions. It is also interesting that greater than 90% of the enzymatic activity loaded onto the column did not appear in either the flow through or the elution fraction. Loading paraoxon directly onto the column resulted in paraoxon hydrolysis, indicating that the activity remained bound to the column even after elution. Three months after the initial binding event, 14.5µg of enzyme remained bound and active on the Concanavalin A column. Results from the

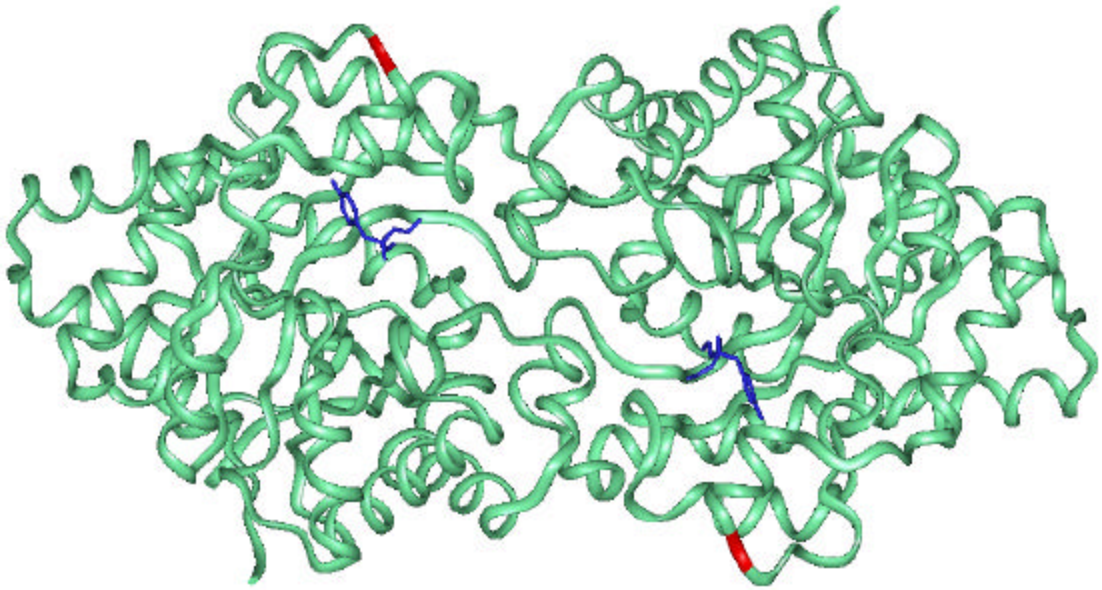


Figure 16. Potential glycosylation site on the structure of OPH. The previously identified residues (Figure 13) that make up the potential glycosylation sequence were mapped onto the crystal structure (1DPM, Vanhooke *et al*., *Biochemistry* 35 *pp*. 6020 (1996)) of OPH taken from the Protein Data Bank (www.rbsc.org). The red residue indicates the possibly modified asparagine residue. The blue wire-frame structure is a bound OPH inhibitor. The figure was generated using Insight II software.

Concanavalin A column also indicates that OPH may exist in three forms in transgenic seed. The fact that protein that flows through the column is enriched for the lower band and protein eluted from the column is enriched for the upper band, but not purified entirely from the lower band, indicates that the dimeric protein may exist as heterodimers of upper and lower band proteins. Western blots of the partially purified OPH from corn show that the abundance of both bands in the sample are nearly equal, therefore the 90% of activity that remains bound to the Concanavalin A column is not due to an overabundance of the upper band protein. This does not exclude the existence of upper band and lower band homodimers.

To experimentally determine if a glycosylation event is the reason for the doublet, chemical deglycosylation was used to remove any carbohydrate structures from partially purified OPH. The results indicate that the chemical deglycosylation caused the upper band to shift to the position of the lower band. This is consistent with the removal of a carbohydrate modification. The glycosylation of OPH did not seem to have an effect on the binding of paraoxon to the enzyme (Km). The effects of a modification on the overall stability of the protein, as well as on folding are still undetermined, but would be of interest. The site of possible modification lies on an exposed loop on the surface of the enzyme (Figure 16). This modification site may be ideal for further chemical modification. The carbohydrate structure may also serve as a new site for potential modification or cross-linking for application purposes. Also of interest, is the strong binding of the modified OPH to the Concanavalin A sepharose column. Immobilized enzyme has been proposed as a possible remediation technology. The use of corn

produced OPH bound to lectin or other column materials could provide a new source of immobilized enzyme for the detoxification of liquid organophosphate waste, or the cleaning of contaminated water.

Materials and Methods

Materials

Buffers, salts and other chemicals were obtained from Sigma-Aldrich. Column resins were obtained from Sigma-Aldrich. Pre-packed phenyl-sepharose and Concanavalin A sepharose columns were obtained from Pharmacia. Paraoxon was obtained from Chemservice. Purified recombinant OPH was produced as described by *Grimsley et al.*[27].

Methods

Purification

Corn expressed OPH was purified from ground seed from construct OPB. Material used for the purification was field grown in Nebraska during the summer of 2002. Pools of 50 seeds from each transformation event were analyzed for expression level by enzyme assay. Material with expression levels estimated over 0.1% TSP were saved. Those under 0.1% TSP were pooled and used for purification. The pooled seed was ground twice in a Waring blender and then separated by size using a Fischer 75-500 micron sieve set and Ro-Tap sieve shaker (Laval Labs Inc., Laval, Quebec). Material was split into two pools, a fine (flour) with particles between 75 and 500 microns, and a coarse (meal) with particles over 500 microns. The amount of enzymatic activity per

gram of material was approximately two fold higher in flour compared to meal. Flour material was used and exhausted pioneering the purification process. Actual purification of enzyme was done using the meal material. Purification started with the extraction of OPH from meal material. Meal was mixed overnight with 50mM Tris pH=8.4 with 1mM $CoCl_2$ (extraction buffer) in a 1:5 meal/buffer ratio. Stirring was accomplished with a magnetic stirrer for volumes under 2 liters and with a motor driven impeller for larger volumes. After extraction, the slurry was passed through cheesecloth or a metal sieve to remove large particles. The extract was then adjusted back to pH 8.4 and spun at 16000xg for 30 minutes. For large volumes (<1L) the extract was then concentrated using a prep-scale 5kd cut-off membrane cartridge (Millipore). The pH of the extract was monitored at each step and kept at 8.4. The extract was then passed over a 30ml DEAE sepharose column. At pH 8.4 OPH passes through the column. The flow through was collected and the DEAE was then cleaned with extraction buffer + 1M NaCl. The DEAE flow through was then run onto a 20ml Green-19 agarose column using an AKTA Explorer chromatography system (Pharmacia). Green-19 agarose has previously been used to purify OPH from insect cell culture and bacterial sources. OPH bound to the green-19 and was eluted using a step gradient of 0.5M NaCl in extraction buffer. Identification of OPH positive fractions was by placing 5µl of each fraction in 100µl of 1mM paraoxon in 50mM CHES buffer pH=9.0 in individual wells of a 96-well plate and observing the generation of *p*-nitrophenol by visual observation of yellow color. Due to instability, positive fractions eluted from the green-19 column were immediately run onto a pre-packed 5ml phenyl sepharose fast flow column (Pharamacia). OPH bound to

the phenyl sepharose was then eluted by a gradient of 0-50% ethylene glycol in extraction buffer over a volume of 20 columns. Positive fractions were pooled and concentrated to a volume of less than 1ml using centricon-3 and centriprep-3 centrifuge concentrators (Millipore). The concentrated sample was then passed over an 83ml Superdex 200 size exclusion column. The superdex column was calibrated prior to sample application using a Sigma molecular weight marker kit for gel filtration chromatography (Sigma-Aldrich). Protein eluted with a molecular mass of approximately 85kD.

Protein Assays

Protein assays were carried out on 96 well plates using the Bradford dye binding method. Bradford reagent was obtained through Bio-Rad and diluted according to the manufacturer directions. The assay used 200µl of diluted reagent per well. Samples (1-5µl) were added in triplicate and absorbance values read using a SpectramaxPlus 384 plate reader (Molecular Devices). Absorbance values were used to determine protein levels by comparison to a standard curve made with Sigma bovine serum albumin standard (Sigma-Aldrich).

Enzyme Assays

Paraoxonase activity was measured by spectrophotometric determination of *p*-nitrophenol production. In short 1-5µl of sample was added to the side of a 1ml plastic cuvette containing 1ml of 1mM Paraoxon in 50mM CHES buffer pH=9.0. The cuvette was then inverted 5 times and absorbance at 400nm followed on a Beckman DU-7400 spectrophotometer. Amount of enzyme present was calculated using the extinction co-

efficient of p-nitrophenol (17000 $M^{-1}cm^{-1}$) and an estimate of the specific activity of purified OPH of 8000 units/mg protein.

Concanavalin A Sepharose Chromatography

Partially purified OPH fractions from phenyl sepharose chromatography were used for ConA chromatography. The sample (300µl) was added by syringe to a pre-packed 1ml ConA sepharose column (Pharmacia). The column was then washed with 10 volumes of ConA binding buffer. The protein on the column was then eluted using the binding buffer with 0.5M methyl α-D-glucopyranoside added.

CHAPTER IV

ENZYMATIC DEGRADATION OF AN ORGANOPHOSPHATE HERBICIDE BY ORGANOPHOSPHATE HYDROLASE

Studies on the bacterial Organophosphate hydrolase (OPH) have focused on the ability of OPH to degrade neurotoxic organophosphate compounds that inhibit acetylcholinesterase. In this study, the ability of OPH to degrade several different organophosphorus herbicides, which effect life processes in plants other than cholinesterase activity, was explored. OPH was able to degrade the phosphoramidate herbicide Amiprophos-methyl. HPLC analysis with the organophosphothioate herbicides anilofos, bensulide, and piperophos showed that OPH failed to degrade these compounds. Amiprophos-methyl had K_m values similar to those of paraoxon, but k_{cat} numbers closer to that of demeton-s. The type of substrate competition that was observed for OPH activity against a P-O ester substrate was dependent on the type of phosphoester bond in the competitor. Substrate inhibition of the demeton-s degrading activity of OPH was competitive, regardless of the type of phosphoester bond in the competitor. The results of these experiments indicate that OPH can degrade an organophosphate herbicide, and inhibition patterns of paraoxon and demeton-S hydrolysis by these herbicides may indicate that a complex mechanism or mode of binding is at work during OPH catalysis.

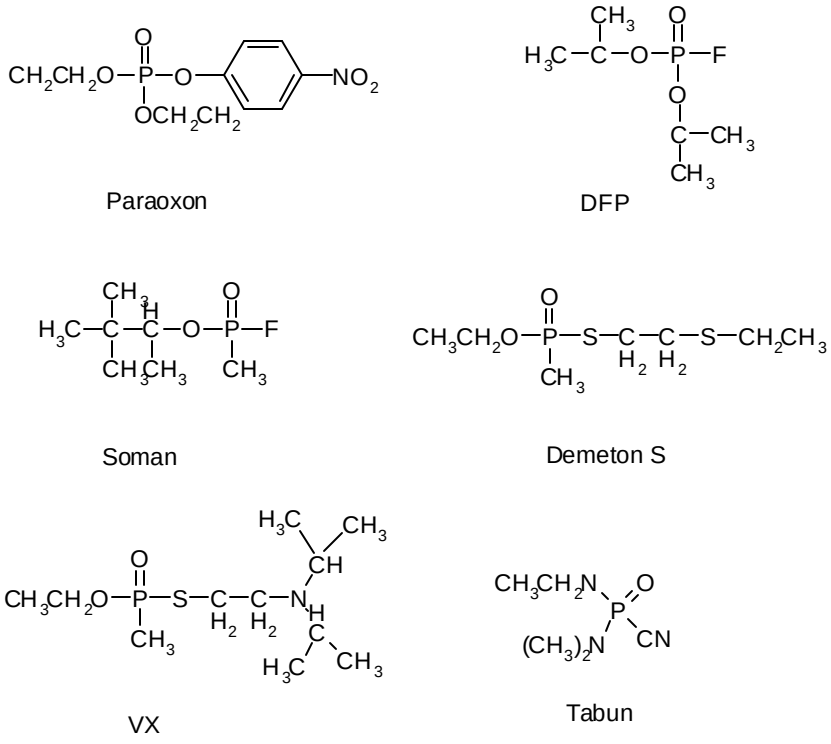


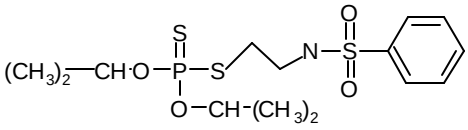
Figure 17. Representative OPH substrates I. Structures were drawn using ISIS Draw 2.4 (MDL Information Systems).

Introduction

Organophosphate hydrolase (OPH, E.C. 3.1.8.1, Aryldialkylphosphatase) is a dimeric metallo-enzyme capable of degrading a wide range of organophosphate insecticides and chemical warfare agents (Figure 17) [36]. The enzyme was first identified in *Flavobacterium* isolated from diazanon treated rice paddies in the Philippines and a *Pseudomonas diminuta* strain isolated in the United States [11]. The gene encoding OPH, *opd*, was found on dissimilar large plasmids in both species [77]. Other enzymes that can degrade organophosphates (DFPase, OPAA, and Serum Paraoxonase) show no homology to OPH and natural substrates have yet to be identified [23]. OPH has the ability to degrade a wide range of phosphate ester bonds including P-O, P-CN, P-F and P-S bonds [36]. The substrates that OPH has been shown to degrade are not naturally occurring, however a naturally occurring organophosphate di-ester, Anatoxin-A(S), is produced by some cyanobacteria of genus *Anabaena* [4].

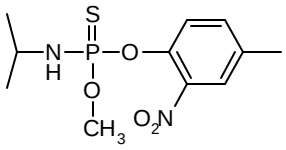
Most work to date has focused on the ability of OPH to degrade pesticides and chemical warfare agents. There are, however, organophosphate herbicides which effect plant life-processes other that cholinesterase activity (Figure 18). Amiprophos-methyl and a related herbicide, Butamiphos, are inhibitors of plant microtubual assembly [1,72-74]. Amiprophos-methyl has also been shown to effect plant calcium channels at high concentrations and is used in the study of plant microtubuals [72,74]. Piperophos and Anilofos are both inhibitors of cell division primarily used on transplanted and direct sown paddy rice for weed control, and are grouped with herbicides which inhibit very

Lipid Synthesis Inhibitors (N)

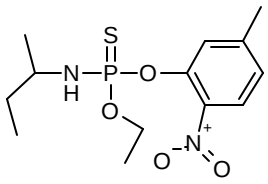


Bensulide (Prefar, Betasan)

Microtubule Assembly Inhibitors (K1)

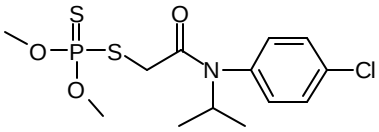


Amiprophos-methyl

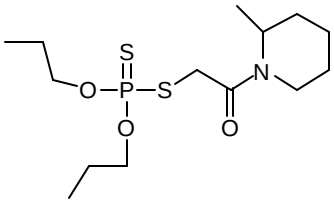


Butamiphos (Cremart)

Cell Division Inhibitors (K3)



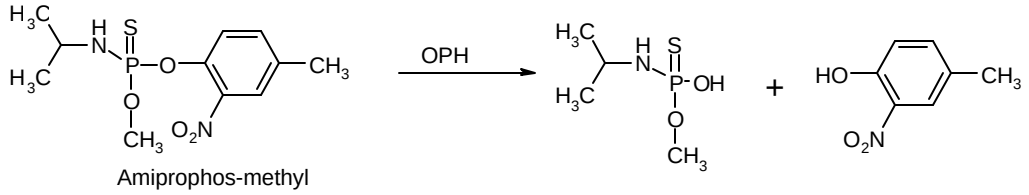
Anilofos (Aniloguard, Arozin)



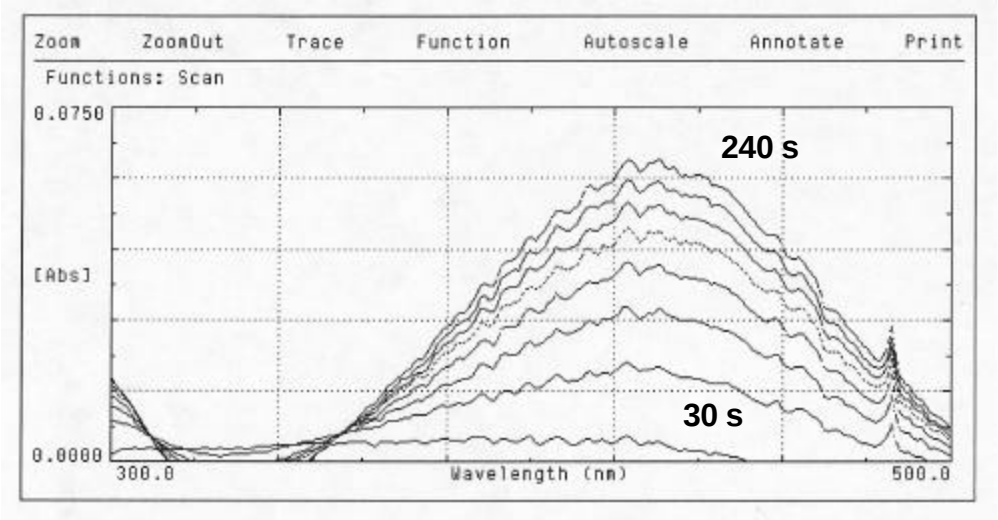
Piperophos (Rilof)

Figure 18. Organophosphate herbicides. Structures drawn with ISIS Draw 2.4. Pesticide structures and classifications obtained from the Weed Science Society of America HRAC web site: http://www.weedscience.org/summary/ChemFamilySum.asp

a.



Amiprophos-methyl



b.

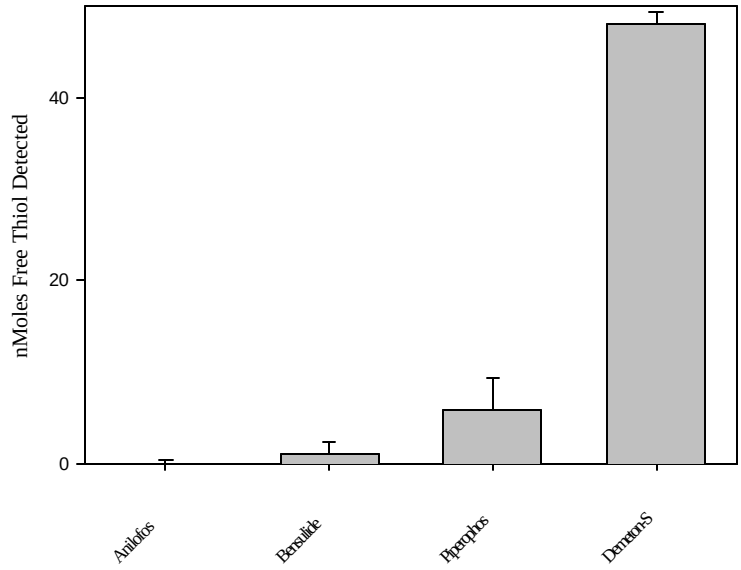


Figure 19. Degradation of organophosphate herbicides by OPH. **a.** The Degradation of Amiprophos-methyl by OPH. Each line represents a wavelength scan taken every 30 seconds. Degradation results in 4-methyl-2-nitrophenol, a colored product **b.** Degradation of P-S bonded organophosphate herbicides by OPH

long chain fatty acid synthesis [1]. Piperophos also has been shown to control rice blast, a fungal rice disease [94]. Bensulide is an inhibitor of lipid synthesis, which is commonly used on turf grass to control crabgrass [1]. Amiprophos-methyl, Butamiphos, and Bensulide are used as pre-emergence herbicides, while Anilofos and Piperophos can be used in both a pre and post emergence fashion [1]. Given the structures of these herbicides and their similarity to established OPH substrates, the ability of OPH to degrade these compounds was investigated. In this study, four organophosphate herbicides (amiprophos-methyl, anilofos, bensulide, and piperophos) were evaluated as substrates of OPH.

Results

Degradation of OP Herbicides

The ability of OPH to degrade the P-O ester organophosphorus herbicide amiprophos-methyl was explored (Figure 19). A reaction mixture of buffered amiprophos-methyl treated with OPH turned yellow in color over a period of 30 minutes. This is consistent with the breakdown of amiprophos-methyl into1-methylethyl phosphoramidic acid monomethyl ester and 4-methyl-2-nitrophenol. Pure 4-methyl-2-nitrophenol was used to determine an extinction coefficient and absorbance maximum. 4-methyl-2-nitrophenol in solution at pH 9 is yellow with an absorbance maximum of 435nm with a measured extinction coefficient of 1,800 $M^{-1}cm^{-1}$. Spectrophotometric monitoring of a 0.5 mM solution of amiprophos treated with 2µg of OPH showed an

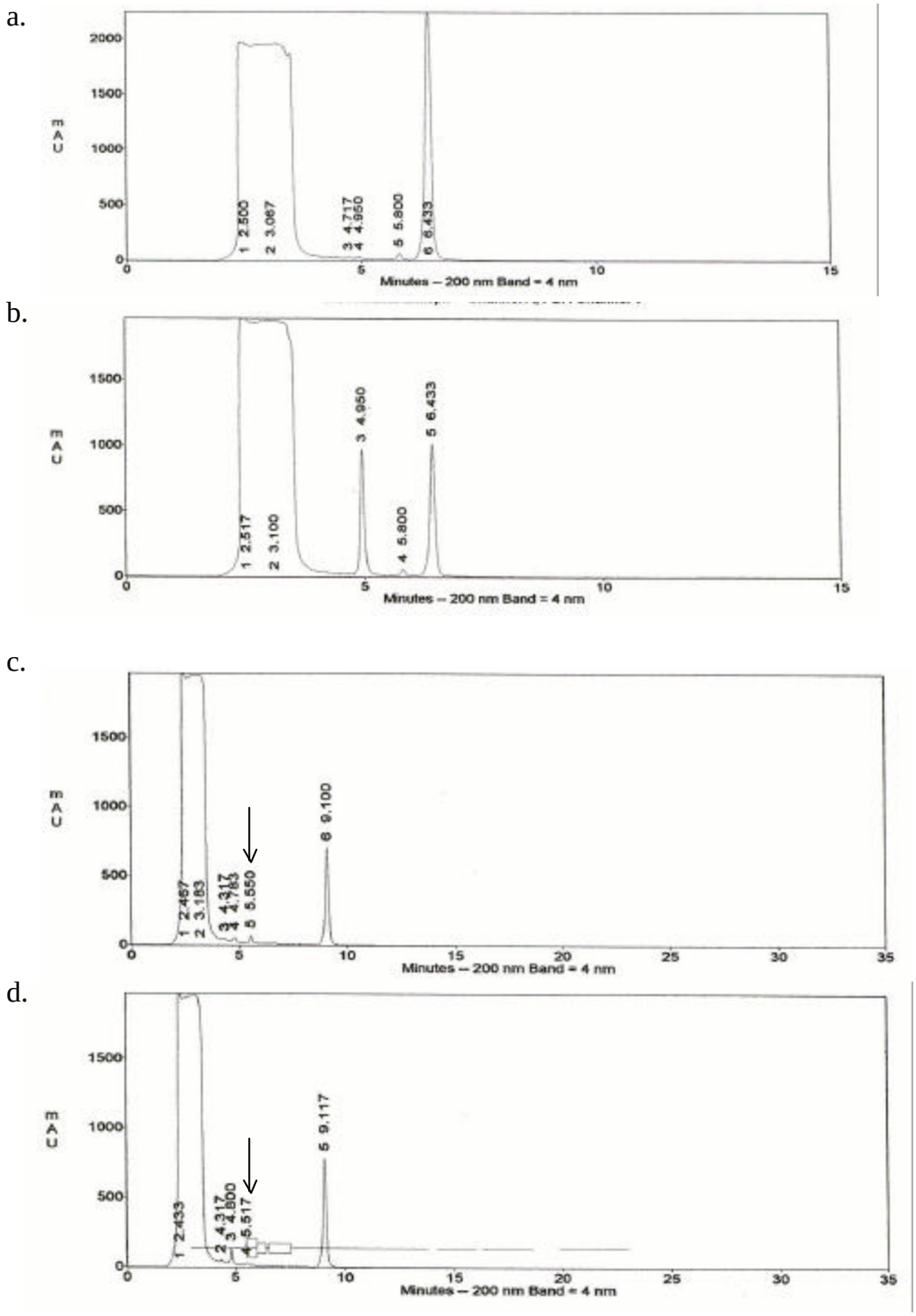


Figure 20. HPLC analysis of herbicide degradation by OPH **a.** 0.5mM Amiprophos-methyl **b.** 0.5mM Amiprophos-methyl + OPH **c**. 0.5 mM Piperophos **d**. 0.5mM Piperophos + OPH. Arrows denote the minor constituent degraded by OPH in piperophos reaction.

increase in absorbance as time increased at an absorbance maximum around 435nm (Figure 19a).

The ability of OPH to degrade P-S bonded herbicides was evaluated after a 20-hour incubation period. Reaction mixtures containing 1mM solutions of anilofos, piperophos, and bensulide in HEPES buffer were treated with 5 µg of OPH. After 20 hours of treatment, DTNB was added to detect free thiols using a modified method from *Lai et al*. [35]. Absorbance readings were taken at 412nm and compared to the control thioate-ester demeton-S. Of the P-S herbicides tested Piperophos showed a slight reaction at 20 hours (Figure 19b).

HPLC Analysis

HPLC analysis was carried out to confirm whether the herbicides were being degraded during treatment with OPH. Analysis of amiprophos-methyl reactions showed that the peak corresponding to amiprophos-methyl was diminished, while a peak with a retention time identical to that of 4-methyl-2-nitrophenol appeared (Figure 20 a&b). HPLC analysis of piperophos reactions showed little effect on the piperophos peak, but a minor peak was diminished, suggesting that the signal seen by Ellman's reagent is due to the degradation of a contaminant rather than piperophos itself (Figure 20 c&d). We were unable to detect any change in the bensulide and anilofos reactions (data not shown).

Kinetics of Amiprophos-methyl Degradation

Further investigation with Amiprophos and Piperophos hydrolysis yielded information on the kinetics of their degradation by OPH. Enzymatic activity was measured at various concentrations of amiprophos-methyl. Results were plotted on Sigma Plot and fitted to the Michaelis-Menten equation modified to take into account substrate inhibition (Figure 21). Results indicate that amiprophos-methyl has a K_m similar to that of paraoxon, but enzymatic turnover is much lower than that of paraoxon (Table 7). The K_m for amiprophos-methyl indicates a tightly bound substrate.

Table 7. Kinetics of amiprophos-methyl degradation.

Compound	$k_{cat}(s)^{-1}$	K_m (mM)	k_{cat}/Km ($M^{-1}s^{-1}$)	K_i (mM)	Bond	Ref
Paraoxon	15000 ± 300	0.12 ± 0.01	$1.3x10^8$	17 ± 1	P-O	24
Demeton-S	4.2 ± 0.1	4.8 ± 0.2	$8.7x10^2$	-	P-S	24
DFP	75 ± 6	0.96 ± 0.1	$7.8x10^4$	23 ± 8	P-F	24
Amiprophos -methyl	7.04 ± 0.15	0.071 ± 0.003	$9.9x10^4$	0.848 ± 0.150	P-O	-

Inhibition of OPH Activity by OP Herbicides

The ability of organophosphate herbicides to inhibit the activity of OPH against the P-O bonded substrate paraoxon was investigated. Anilofos, Piperophos, and

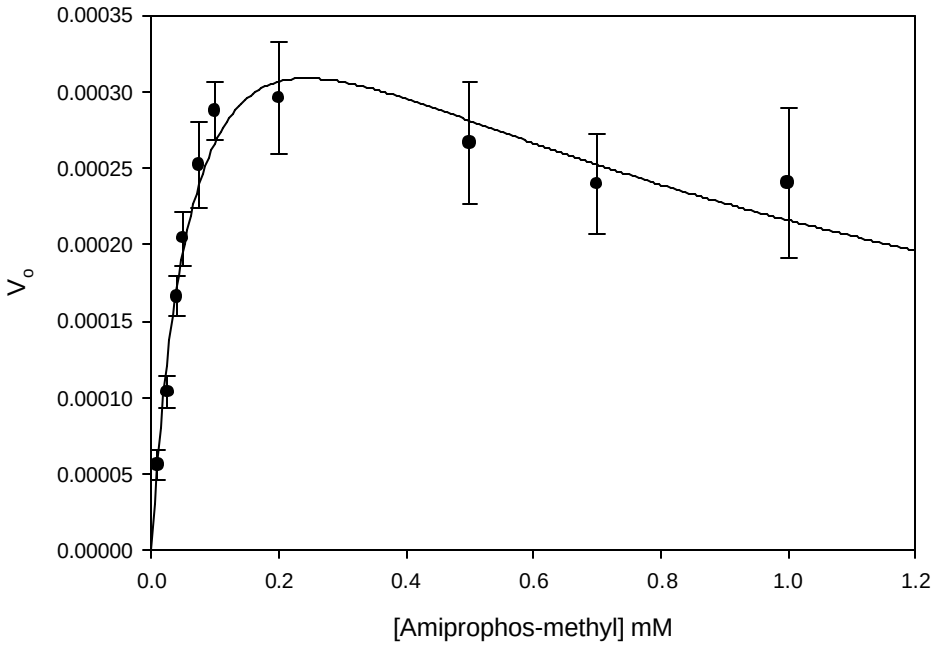


Figure 21. Michaelis-Menten Plot of the kinetics of amiprophos-methyl degradation by OPH. Enzymatic activity was measured by the production of 4-methyl-2-nitrophenol. Each point is the result of nine replicates. Data plotted in SigmaPlot 8.0.

Bensulide, all organophosphorthioates, were able to inhibit the Paraoxonase activity of OPH in a mixed non-competitive fashion (Figure 22 b,c, & d). This is similar to results obtained for the inhibition of paraoxon degradation by the P-S OPH substrate demeton-S [35]. Amiprophos-methyl, a P-O organophosphate, showed the ability to inhibit paraoxonase activity in a competitive fashion (Figure 22a).

Similar experiments were carried out with the P-S substrate demeton-s in place of paraoxon. The organophosphorthioate bensulide was able to inhibit the degradation of demeton-S in a competitive fashion (Figure 23b). Amiprophos-methyl, a P-O ester substrate, also inhibited demeton-S in a competitive fashion (Figure 23a).

Discussion

This work shows that organophosphate hydrolase is capable of degrading the herbicide amiprophos-methyl. This herbicide inhibits microtubual polymerization, and the ability of OPH to degrade it may make OPH a possible selectable marker gene for use in plant transformation. The data does not rule out the ability of OPH to degrade anilofos, bensulide, or piperophos. The competition studies indicate that they are able to influence OPH activity against paraoxon. These compounds may be similar to the organophosphothioate pesticide malathion, which showed less than 2% hydrolysis after an 18-hour incubation period with OPH [35]. It is also interesting to note that a minor constituent of the piperophos reaction was degraded by OPH. This compound does react with DTNB and its appearance is enzyme dependent. It is possible

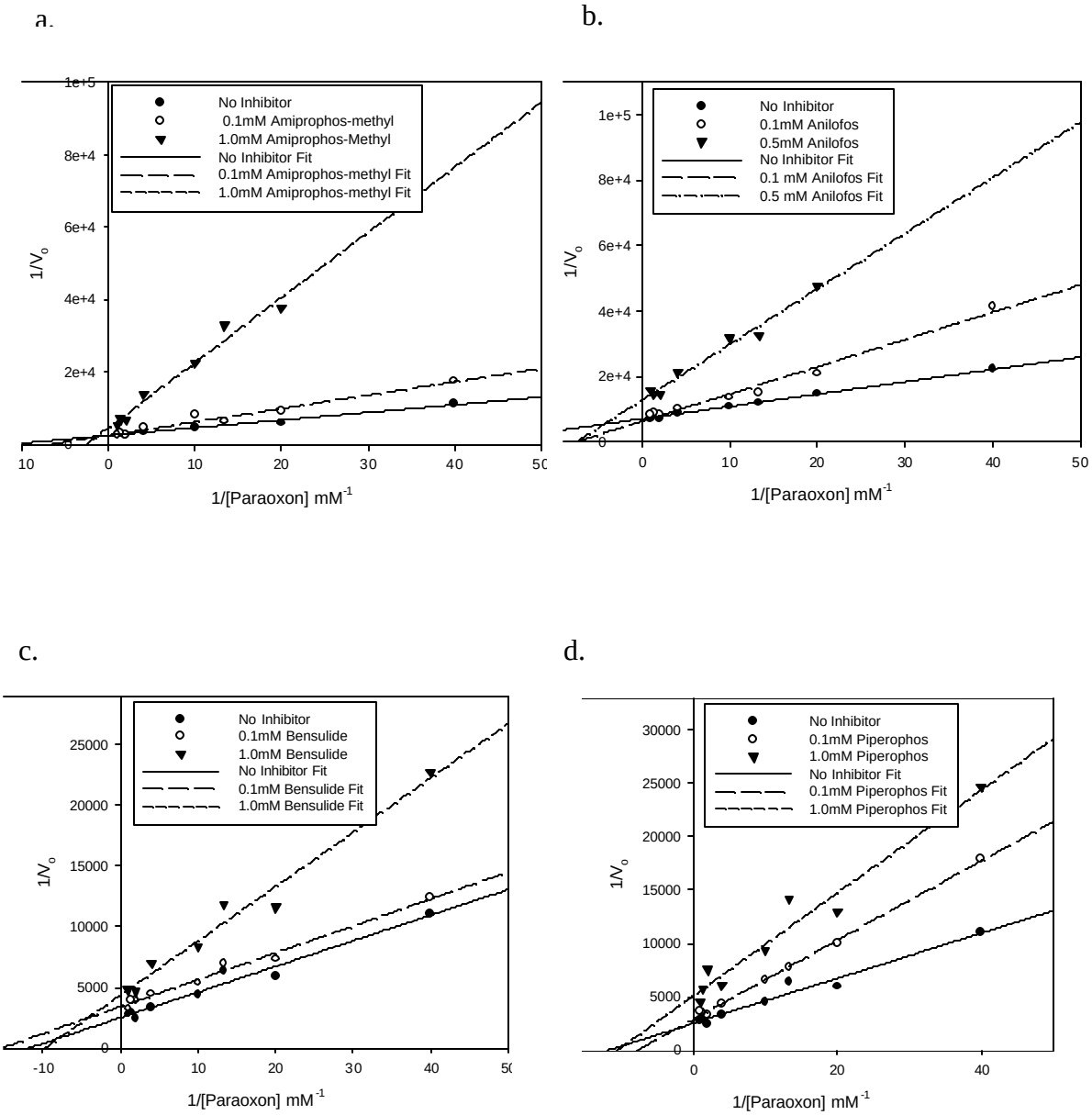
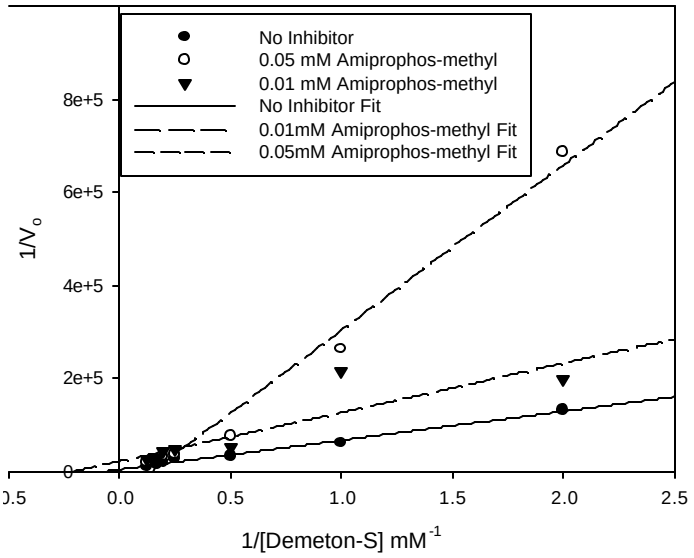


Figure 22. Substrate inhibition of the paraoxonase activity of OPH by organophosphate herbicides **a.** Amiprophos-methyl **b.** Anilofos **c.** Bensulide **d.** Piperophos

a.



b.

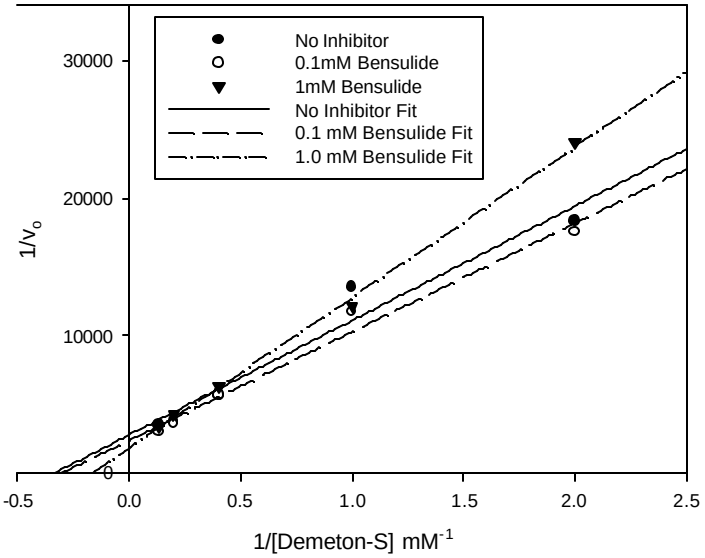


Figure 23. Substrate inhibition of demeton-Sase activity of OPH by organophosphate herbicides. **a.** Amiprophos-methyl **b.** Bensulide

that this compound is related to piperophos and its identification would be of great interest.

Kinetic studies show that amiprophos-methyl is a substrate that tightly binds to the OPH active site, but has a turnover number approximately 2000 fold lower than that of paraoxon. It is also interesting to note that amiprophos-methyl kinetics are subject to substrate inhibition much like those of paraoxon and DFP [36]. While substrate inhibition has been observed in P-O as well as P-F ester substrates, substrate inhibition with a P-S ester substrate has not been reported for wild type enzyme. Substrate inhibition indicates that P-O and P-F ester substrates can bind the OPH active site in more than one manner, resulting in substrate inhibition.

The ability of these herbicides to inhibit or compete with known OPH substrates was also investigated. When the P-O ester paraoxon was used, the P-O ester herbicide amiprophos-methyl was able to inhibit paraoxonase activity in a competitive manner. P-S ester herbicides bensulide, anilofos, and piperophos were able to inhibit paraoxonase in a mixed non-competitive fashion. When reciprocal experiments were carried out with the P-S ester demeton-S, the P-S ester bensulide was able to inhibit in a competitive manner at 1mM concentration. Amiprophos-methyl, a P-O ester, also inhibited demeton-S degradation in a competitive fashion. This difference in the ability of P-O and P-S esters to inhibit one another taken together with the substrate inhibition seen in P-O esters may indicate that there are at least two modes of binding for P-O substrate and possibly two different enzymatic mechanism at work. Work on the mechanism of OPH enzyme function has yielded two possible mechanisms involving one or two metals

respectively. Work with reconstituted enzyme appears to favor a two metal mechanism for the hydrolysis of paraoxon and demeton-S. As apoenzyme is reconstituted with metal it has been reported that the activity reaches a maximum near a molar ratio of 2 metals per sub-unit of enzyme for demeton-S and paraoxon [33,36]. It has also been shown that the rate at which reconstitution occurs can be increased by the addition of bicarbonate, which is thought to coordinate the two metals during the reconstitution process [36]. Crystallography has shown that the crystal structure of wild type OPH contains two divalent metals, and that the metal site can contain two different, as well as identical metals [34]. In support of a two mechanisms model, mutant studies by *diSioudi et al.* show that mutations to histidine residues near the active site can produce enzymes that work optimally with only one metal bound per sub-unit of the enzyme [36]. This is congruent with the mutants studies of *Lai et al.* which showed that mutant C59S, which contained only 0.86 metals per sub-unit when isolated, was able to degrade paraoxon with one third the activity of wild-type enzyme [29]. Both mutant studies show that mutants with reduced metal contents have reduced activities, but do not lose enzymatic activity all together, which may indicate that more than one mechanism is involved in substrate turnover. *diSioudi et al.* also reported that when reconstituted, the activity of wild-type OPH against demeton-S required only one metal for full activity while the same enzyme required two metals per sub-unit for full activity against paraoxon, which is in disagreement with the results of *Shim et al.* [33]. One important factor that has not been fully explored, is that enzyme produced in bacteria, without the addition of exogenous metals to the growth medium, contain only 1.1 zinc atoms per sub-unit when isolated

without additional metal added the purification buffers [30]. This would seem to indicate that the *in vivo* natural state of OPH produced in a recombinant bacterial system is that of an enzyme whose metal binding sites are only partially filled. Previous studies using demeton-S to compete with paraoxon showed a mixed non-competitive inhibition pattern, which is unexpected if only one mechanism is at work [34]. The competition/inhibition presented here support the results of *Lai et al.* that first showed the difference between P-O and P-S inhibition of paraoxon degradation by OPH [35]. The ability of a P-S ester to inhibit paraoxonase activity in a mixed non-competitive fashion would indicate that P-S ester substrates and inhibitors affect both the binding of paraoxon as well as actual enzymatic activity against paraoxon. Results with P-S vs. P-S and P-O vs. P-O ester substrates indicate that they directly compete for the same binding site. The most curious result from the present work is that the degradation of Demeton-S by OPH is inhibited in a competitive fashion by the P-O ester amiprophos-methyl. This would indicate that amiprophos-methyl is capable of interfering directly with the binding of demeton-s. The fact that demeton-s has been shown to interfere in a mixed non-competitive fashion with other P-O substrates, along with the competitive inhibition of demeton-s by amiprophos-methyl, may indicate that a complex set of mechanisms is at work. If P-O ester substrates are capable of being degraded by both a one metal as well as a two metal mechanism, while P-S ester substrates are only degraded by a one metal mechanism, this could possibly explain the inhibition results. The inhibition results taken together with the mutant studies, offer further evidence that the mechanism of organophosphate hydrolysis by OPH may be complex.

The addition of these herbicides to the spectrum of OPH substrates broadens the potential uses of the enzyme in both environmental detoxification, as well as potential use as a marker in plant biotechnology applications. The competition studies indicate that the mechanism of OPH action may be more complex than a single mechanism for all substrates. This, taken together with mutant studies and the substrate inhibition information, show that multiple mechanism are at work in OPH, and that the mechanism used is dependent on the type of substrate being degraded.

Materials and Methods

Materials

Amiprophos-methyl(98%) and Bensulide(99.2%) were obtained from Fluka. Anilofos(99.5%) and Piperophos(93.5%) were obtained from Cresent Chemical. Demeton-S(98.5%) and Paraoxon(98.5%) were obtained from Chemservice. 100mg/ml stocks of all herbicides were made up in dimethyl-sulfoxide (DMSO). Ellman's reagent (DTNB), 4-methyl-2-nitrophenol and all buffers were obtained from Sigma-Aldrich. Purified recombinant OPH was obtained as described in Chapter III.

Methods

Determination of 4-methyl-2nitrophenol Extinction Coefficient and Absorbance Maximum

Pure 4-methyl-2-nitrophenol was used to make up a 1mM solution in 50 mM CHES pH=9.0. This solution was then diluted with buffer to make 0.5mM and 0.1mM

solutions. One ml of each solution was placed in a 1ml disposable cuvette and the spectrum of absorbance was measured on a Beckman DU-7400 spectrophotometer. The absorbance maximum was used to determine the extinction co-efficient using Beer's law.

Enzyme Assays

Assays were carried out with purified recombinant OPH from *E. coli* isolated in the presence of cobalt [27]. Paraoxon based assays were carried out in 50 mM CHES pH=9.0. Demeton-S based assays were done in a 50mM HEPES pH=7.2 (13). Kinetic studies were done in CHES buffer. Degradation of Amiprophos-methyl was measured by the appearance of 4-methyl-2-nitrophenol (E_c=1800 $M^{-1}cm^{-1}$). Free thiols from the degradation of P-S substrates was detected with Ellman's reagent (DTNB) (E_c=13,600 $M^{-1}cm^{-1}$)

Kinetic results for amiprophos-methyl were fit with the Michaelis-Menten equation modified to account for substrate inhibition. All plots and curve fits were done with Sigma Plot.

Substrate competition was carried out in CHES buffer for paraoxon and HEPES for demeton-S. 5 ng of pure recombinant OPH from E. coli was used in each paraoxon reaction. 5 µg of pure enzyme was used for demeton-s reactions. Inhibition of demeton-s degradation with amiprophos-methyl was carried out using a reaction mixture without added DTNB as a blank to subtract out the formation of 4-methyl-2-nitrophenol from the degradation of amiprophos-methyl. All spectrophotometric readings were made on a

Beckman DU-7400 spectrophotometer. All results are the average of three runs. Results were plotted on double reciprocal plots.

HPLC Analysis

0.5 mM solutions of each of the herbicides in 50mM CHES pH=9.0 were made up from the herbicide stock solutions and treated with 5µg of pure OPH. The reactions were then analyzed after 24 hours by HPLC. HPLC was carried out using a Phenomix C-18 column with an 80/20% acetonitrile/water mobile phase. Standards for each of the herbicide reactions were run without added enzyme. For amiprophos-methyl a 0.5mM 4-methyl-2-nitrophenol solution was also used as a standard.

CHAPTER V

ORGANOPHOSPHATE HYDROLASE AS A MARKER SYSTEM FOR TRANSGENIC MAIZE

Organophosphate hydrolase (OPH) was evaluated as a scorable marker system for use in both plant cell culture and regenerated plant tissue. OPH has a wide range of substrates that create a colored or visually detectable compound when turned over by enzyme. The ability Organophosphate hydrolase activity expressed in the tissue of *Zea mays* to distinguish between transgenic and non-transgenic callus and plant tissue was investigated. In order to generate a wider range of screening compounds, haloxon was tested as an OPH substrate. Callus tissue was screened using the organophosphates coumaphos, coroxon, and haloxon. The coumaphos derivative coroxon was used to screen plant leaf tissue. Paraoxon was used to score seed. The use of OPH as a possible selectable marker system in cell culture and greenhouse settings was also investigated.

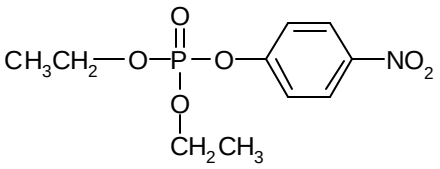
Introduction

The ability to transfer genetic material into plant tissue has first appeared in the 1980s [40]. With this ability came need for marker enzymes and proteins that could be used to evaluate the performance of promoters and distinguish between transgenic and non-transgenic materials. Among the genes that have been used as scorable markers are β-glucuronidase (GUS), green fluorescent protein (GFP), and firefly luciferase (ff-LUC)[65]. GUS is a bacterial enzyme that can be used to score tissue by enzymatic

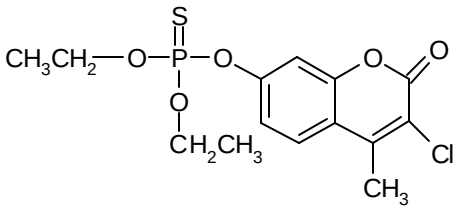
activity. The disadvantage of GUS is that the tissue used for the assay is generally not viable after the assay has been conducted. GFP is a protein isolated from jellyfish with a native fluorescence that is widely used as a scorable marker for biological systems. GFP is a relatively simple protein that is easy to detect visually due to its fluorescent nature, but its fluorescent signal can be masked by the fluorescence of naturally occurring compounds. GFP also lacks enzymatic activity that can be used as a selectable marker. LUC is an enzyme involved in the generation of bioluminescence in fireflies. The light generated during its enzymatic activity can score for the presence of the LUC marker, however this enzymatic activity cannot be used as a selectable marker. Commonly used plant selectable marker systems, such as glyphosate, rely on a mutation to a plant protein or the insertion of a foreign protein with resistance to the herbicide rather than the actual degradation of the chemical [57,68]. This limits the usefulness of these systems as scorable markers.

Organophosphate hydrolase (OPH) is a dimeric metalloprotein that acts against a wide range of organophosphorus compounds that include pesticides, chemical warfare agents, and herbicides (Figure 24) [23,24]. The gene encoding OPH was first isolated from *Peusdomonas diminuta* and *Flavobacterium spp.* and was present on large dissimilar plasmids in each organism [77]. The enzyme has been shown to have a high affinity and

Insecticides

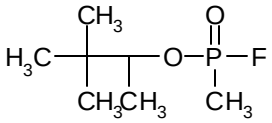


Paraoxon

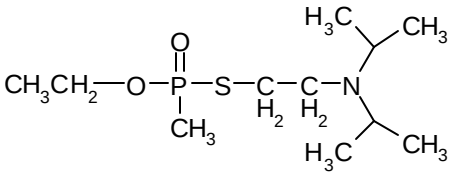


Coumaphos

Chemical Warfare Agents

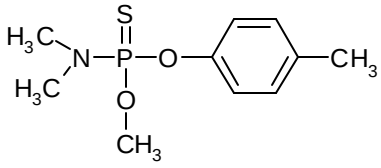


Soman



VX

Herbicides



Amiprophos-methyl

Figure 24. Representative OPH substrates II. Structures drawn using ISIS Draw 2.4.

turnover rate for the organophosphate paraoxon, with turnover reaching a rate that is diffusion limited [25]. One of the breakdown products of paraoxon is *p*-nitrophenol that is yellow in solution at basic pH. Coumaphos and a related compound, coroxon, break down to yield a coumarin derivative that fluoresces under UV illumination. The power of these compounds has previously been used to score bacteria for the presence of OPH or OPH like activity [69,70].

OPH has been expressed in the higher plant *Zea mays L.* (Chapter II). Expression was achieved using both constitutive and seed embryo specific promoters. Enzyme produced in plant tissue was shown to undergo a glycosylation event, which effected 50% of the monomer expressed and had little effect on the overall enzymatic activity of the enzyme. The enzyme was shown to have increased activity after the addition of divalent transition metal salts and incubation. Enzymatic activity was shown in both tissue culture callus tissue, as well as mature seed. Given the successful expression of OPH in plant tissue and the wide range substrates OPH will degrade, we set out to determine if OPH activity could be used to score plant tissue for the presence of the OPH gene.

Results

Screening of Transgenic Seed

Seed derived from plants expressing OPH was tested for the ability of intact seed

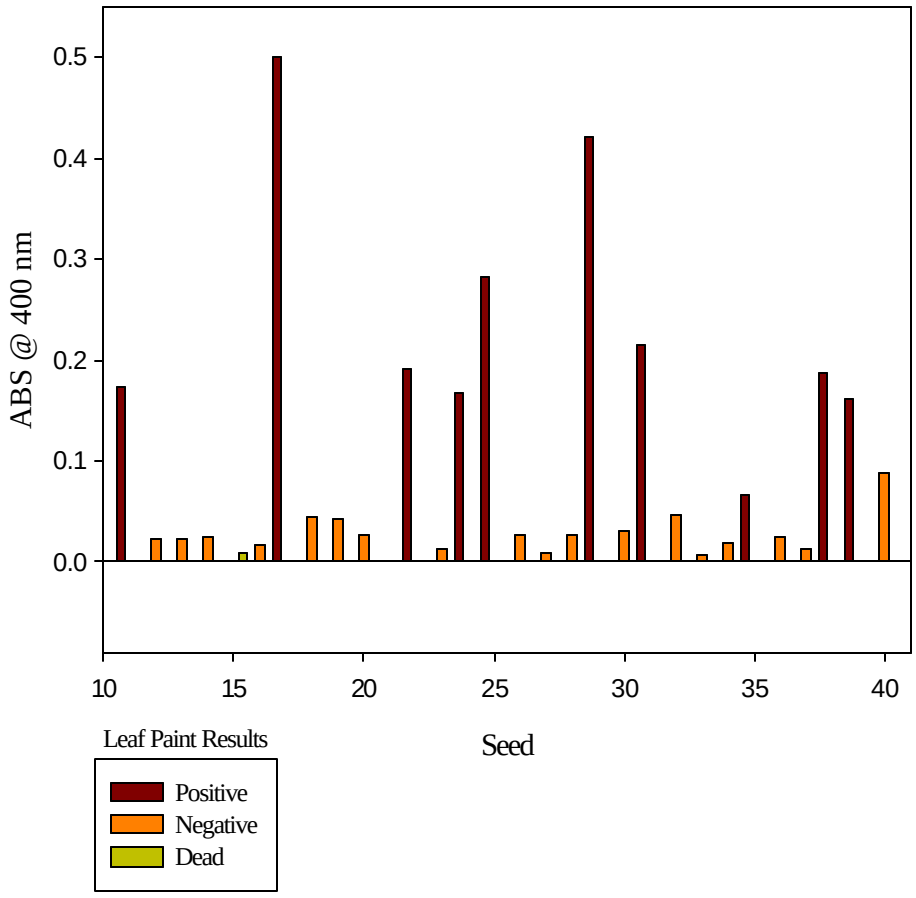


Figure 25. Intact seed assay with OPB seed. Whole OPB0301 seeds were placed individually in wells of a 24 well corning cluster plate with 1ml of 1mM Paraoxon in 50mMTris buffer pH=7.2. Absorbance readings were taken at 3 hours. Leaf painting results were obtained three weeks post-germination.

to turn over the OPH substrate paraoxon (Figure 25). Transgenic seed from both the constitutive (OPA) and embryo specific (OPB) construct were tested. The transgenic seed was the product of out-crossing to an elite in-bred and was expected to be 50% transgenic. The seed was incubated for 3 hours in 1mM paraoxon buffered at pH 7.4 in a 12 well cell culture plate. At the end of 3 hours, the paraoxon was removed and absorbance of the paraoxon solution at 400nm was measured spectrophotometerically. The seed was transferred to soil and allowed to germinate under greenhouse conditions. The plants were then leaf-painted with the herbicide Bialaphos to test for the presence of the *pat* gene, which was included in the transformation vector for selection purposes. As is shown in Figure 25, OPB seed showed a strong correlation between OPH activity measured by absorbance and the resistance to bialaphos, indicating that OPH activity can be used to differentiate between transgenic and non-transgenic seed. Subjecting seed to the buffered paraoxon did not seem to have a major effect on seed germination, with only one seed of the total 20 failing to germinate. Similar results were obtained with OPA seed (data not shown). The correlation of the whole seed activity, as measured by the intact seed assay and the amount of activity extracted from each individual seed, was also tested (Figure 26). In general, seeds that showed higher activity in the intact seed assay showed a higher enzymatic activity in extracts.

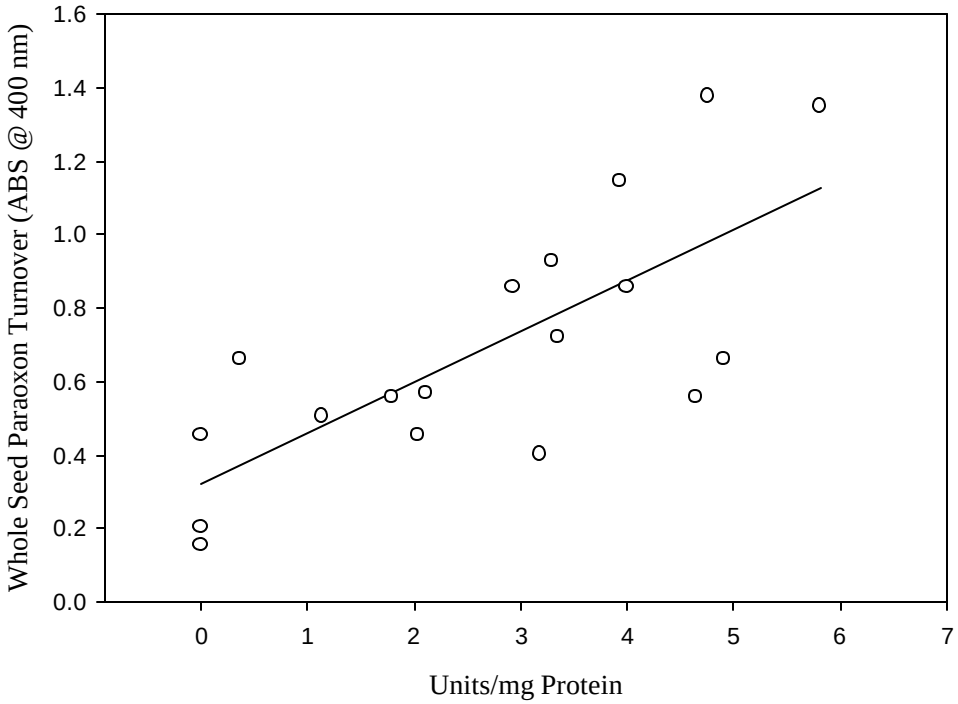


Figure 26. Whole seed turnover vs. extracted OPH activity. OPA0411 seed was treated as described in materials and methods. After intact seed assay, seeds were manually crushed with a hammer and extracted. Extracts were tested for paraoxonase activity and plotted against the intact seed data.

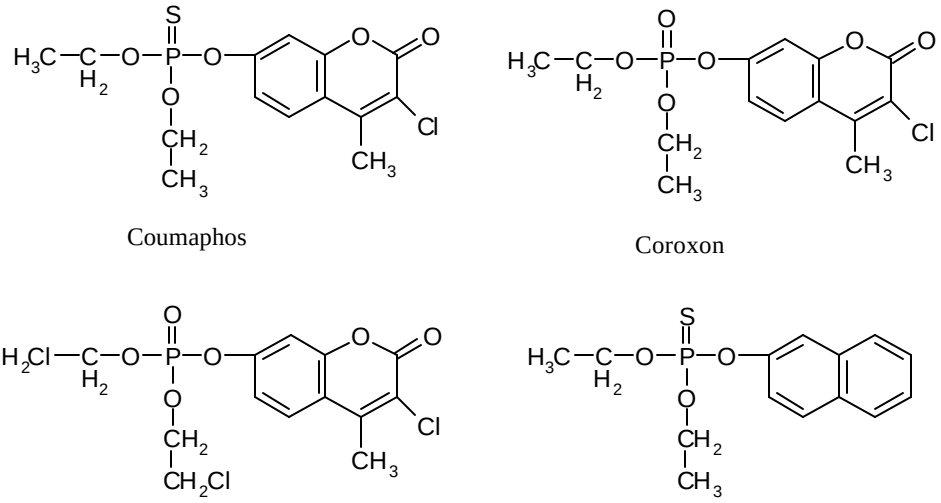


Figure 27. Coumaphos and Related Compounds. The compound from Patent 3,324,494 has been show to have herbicidal activity. Figures made with ISIS Draw 2.4. Structure for O-(2-Napthyl), O'O"-Diethyl Phosphorothionate taken from U.S. Patent 3,328,394 [94].

Enzymatic Activity of OPH against Haloxon

OPH has already been shown to have activity against the insecticide coumaphos [31]. This activity has been used to screen bacteria for the presence of OPH [69]. There are several other compounds with similar structures to coumaphos. These include coroxon, haloxon, and a compound patented as a herbicide (Figure 27). Haloxon is a chlorinated derivative of coroxon that is commonly used to treat intestinal parasites in livestock [90]. In order to broaden the range of compounds that can be used for screening purposes, haloxon was tested as a potential OPH substrate.

Initial qualitative results indicated that addition of OPH to reaction mixtures containing haloxon resulted in the production of a compound that fluoresces under UV illumination (Figure 28). This is consistent with the production of chlorferon seen when OPH degrades coumaphos and coroxon.

Kinetic results indicate that haloxon is kinetically is similar to coroxon. Haloxon had limited solubility above 0.25mM, limiting the utility of Michaelis-Menten analysis. Results were generated using the Lineweaver-Burke treatment over eight concentration points (Figure 29a). Coroxon was examined with an identical set of concentrations (Figure 29b). Comparison of results was done using the Lineweaver-Burke analysis for both compounds. Results of the kinetic analysis are presented in Table 8. Michaelis-Menten treatment of coroxon kinetics was also carried out and indicated that enzymatic degradation of coroxon undergoes substrate inhibition similar to paraoxon (Figure 30). Haloxon was not soluble at high enough concentrations to observe this effect.

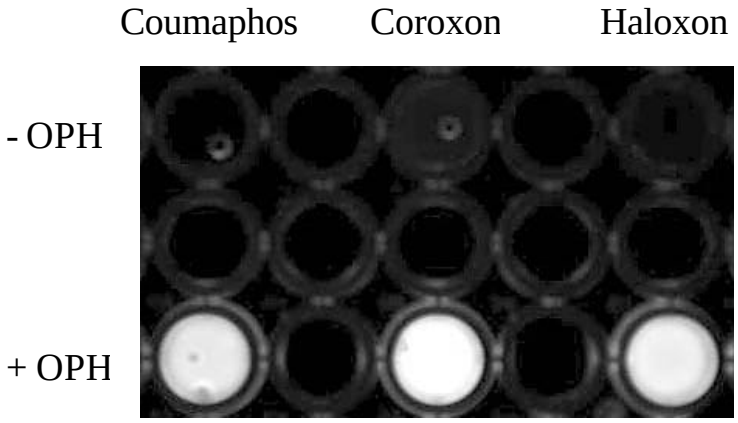
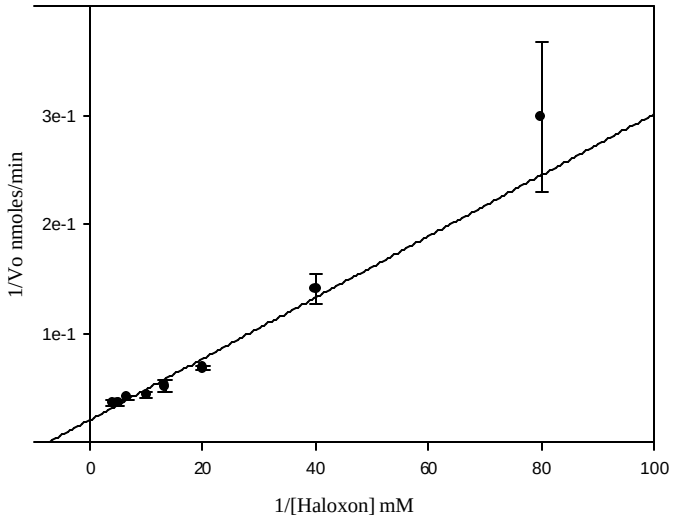


Figure 28. Enzymatic degradation of haloxon. Each +OPH well was treated with 0.87 μg of OPH

a.



b.

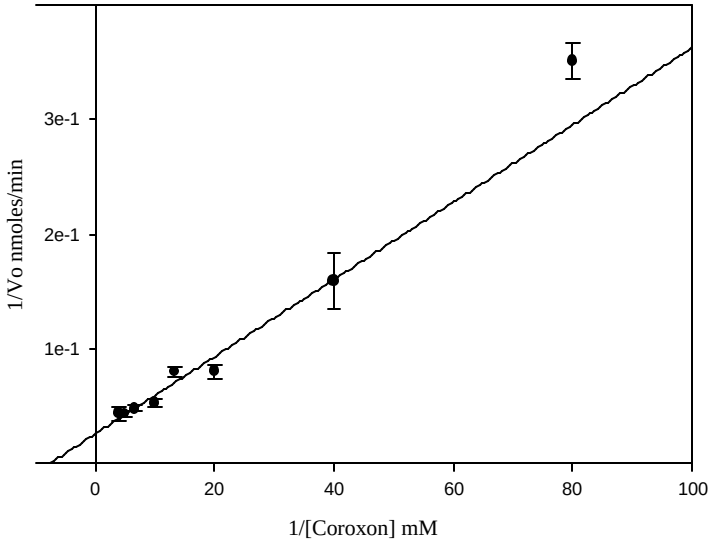


Figure 29. Lineweaver-Burke analysis of haloxon and coroxon kinetics **a.** Kinetics of Haloxon Hydrolysis **b.** Kinetics of Coroxon Hydrolysis

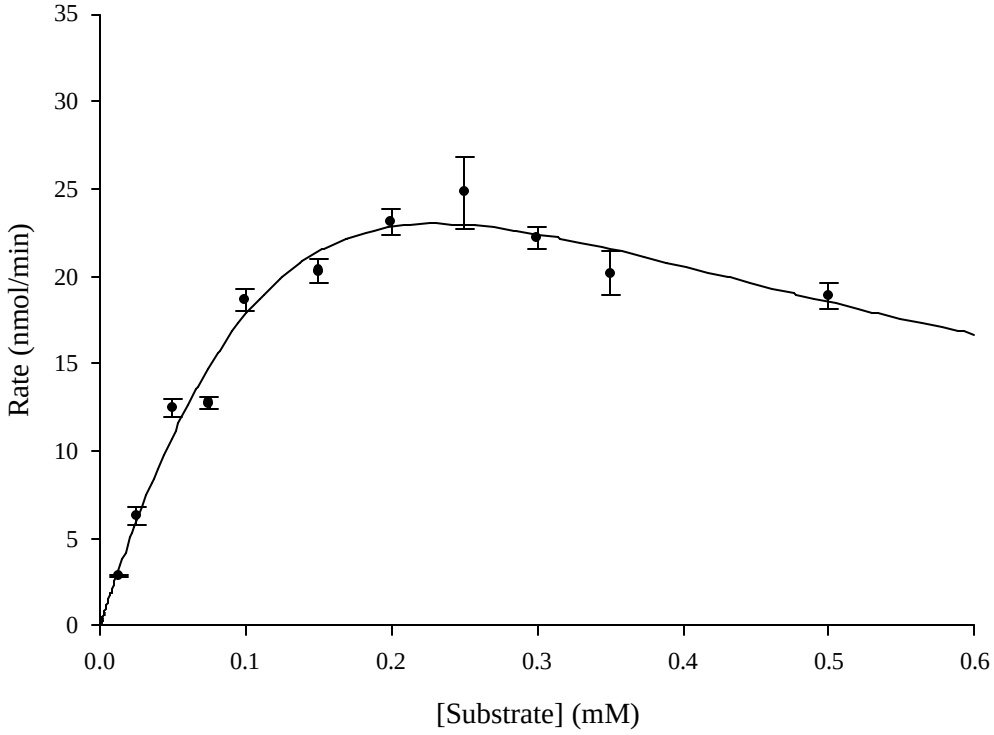


Figure 30. Michaelis-Menten analysis of coroxon kinetics. Kinetic data for the hydrolysis of Coroxon by OPH were plotted using SigmaPlot v.8.0 and fitted using the Michealis-Menten equation modified to account for substrate inhibition.

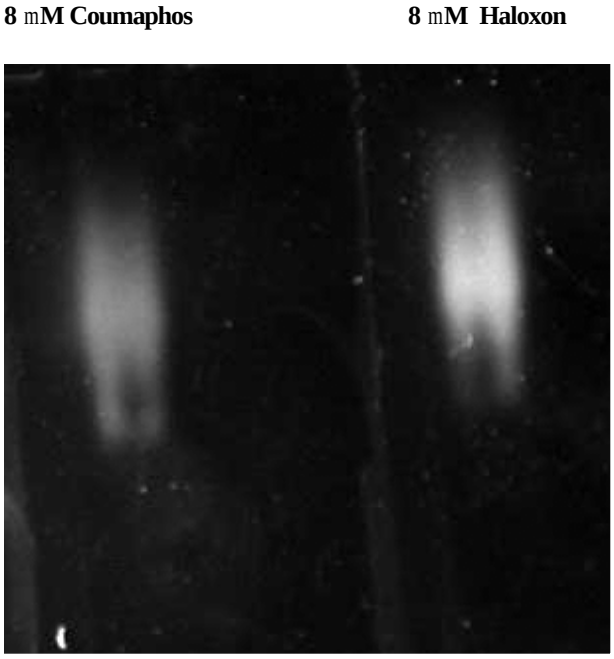


Figure 31. In gel hydrolysis of haloxon and coroxon by OPH

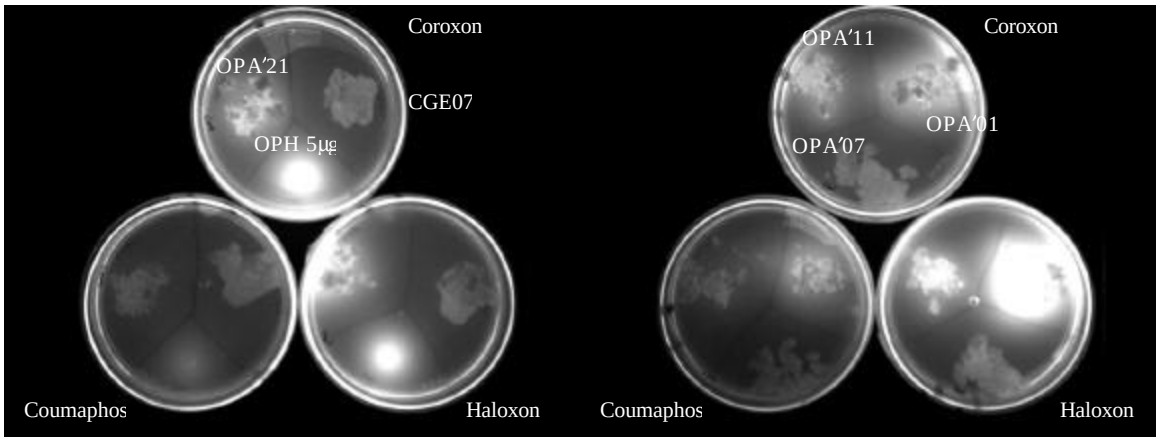
Table 8. Kinetics of OPH degradation of haloxon and coroxon.

Substrate	Fit	Km (mM)	Vmax (nmoles/min)	kcat (s^{-1})	kcat/Km ($M^{-1}s^{-1}$)
Coroxon	Lineweaver-Burke	0.132	38.9	537	4.07E+06
Haloxon	Lineweaver-Burke	0.134	47.9	660	4.93E+06

Haloxon was also evaluated as a substrate for in gel hydrolysis. 500 ng of recombinant OPH was run on a 4-12% Tris-Glycine native gel. After a five-minute incubation period in 8µM substrate, chlorferon production was detected by illumination on a UV light box (Figure 31).

Screening of Transgenic Callus

To prove the utility of OPH as a scorable marker in other phases of transgenic plant genesis, we tested the ability to score maize callus tissue. Coumaphos, coroxon, and haloxon were tested as potential screening compounds. OPH degradation of these compounds results in a product that fluoresces under UV illumination. Previously, coumaphos has been used to detect OPH activity in bacteria by plate assay [69]. Callus (OPA′) derived from a transformation with an optimized *opd* gene under the control of the maize ubiquitin promoter was used as positive test material. Callus was plated onto standard tissue culture media with 1mg/plate of the screening compound added. The plates were allowed to sit for 24 hours and then illuminated with UV light to detect any



Assayed Expression:
OPA′21- 0.27 u/mg

Assayed Expression:
OPA′11 – 0.15 u/mg
OPA′01 – 0.62 u/mg
OPA′07 – 0.001 u/mg

Figure 32. Detection of transgenic callus with coumaphos derivatives. Callus (OPA′) was plated onto tissue culture plates containing 1mg/plate coumaphos, coroxon, or haloxon. After 24 hours the plates were illuminated with UV light. CGE07 serves as a transgenic negative control, recombinant OPH from *E.coli* serves as a positive control. A total of 22 OPA′ lines were tested with similar results that can be found in Appendix D.

breakdown of the screening compound. Extracts of the tissue were then made and tested for OPH activity using paraoxon. Figure 32 shows the results of plate detection and enzyme assays. Transgenic callus was detected by all three compounds. The transgenic control callus, CGE07, showed no activity against any of the scoring compounds. Overall, a stronger reaction was seen to coroxon and haloxon when compared to coumaphos. In general, there was correlation between the amount of fluorescence observed on the plate and the amount of activity measured by paraoxonase activity in extracts. The coumaphos/coroxon/haloxon system for the detection of the presence of the OPH gene in callus tissue can be used to distinguish between transgenic and non-transgenic material, but cannot be reliably used to predict what the actual expression level is in the tissue being tested.

Scoring of Leaf Tissue

To further test the capability of OPH as a scorable marker for plant systems, the ability to score leaf tissue from a mixture of transgenic and non-transgenic plants was tested. Coroxon, the oxon derivative of coumaphos, was used to score leaf tissue from both plants with constitutive (OPA) and seed specific (OPB) expression. Leaf tissue was excised from 3-week old plants. A segment of tissue, just large enough to fill the well on a 24-well cluster plate, was placed in each well with 1ml of 0.1mM coroxon. The plate was then incubated for 20 hours and illuminated with UV light. Tissue from elite inbred SP133 was used as a negative control. Plants were also tested for the presence of the

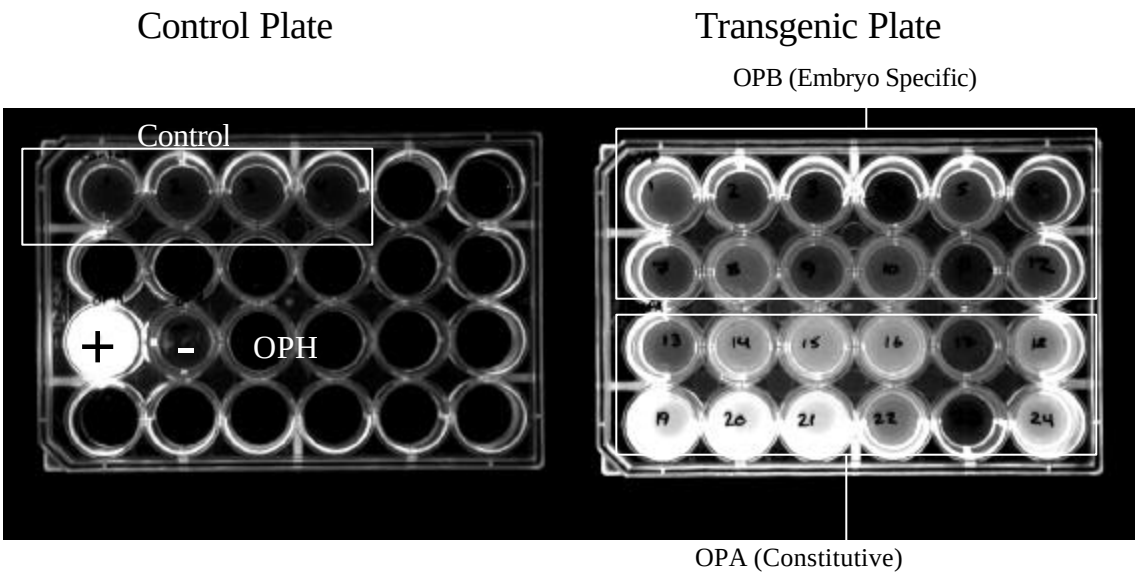


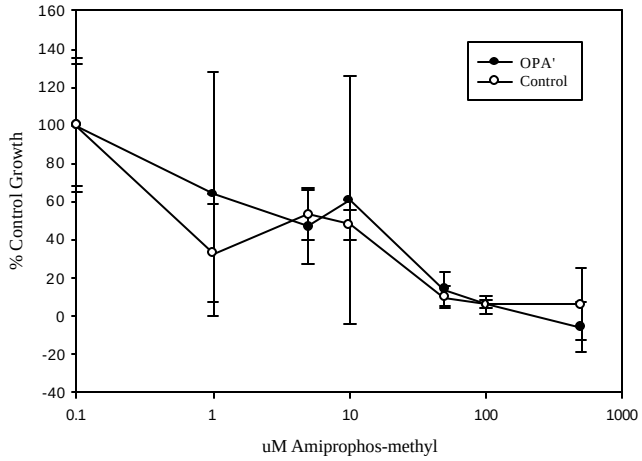
Figure 33. Detection of OPH activity in leaf tissue with coroxon. Leaf tissue was incubated for 20 hours in 1ml of 0.1mM coroxon in 50mM Tris pH=7.4. Tissue was removed prior to the taking of the images. Positive control was 0.87 µg of pure OPH. Well 11 of the transgenic plate had no tissue added and serves as a negative control. By leaf paint 2,3,4,9,17,22, and 23 were damaged by 1% Finale. Well 13 and 21 had questionable resistance to Finale. 1-12 are OPB0302 T2. 13-24 are OPA0403-1 T3 plants.

pat gene by leaf painting with 0.1% Finale (bialaphos). At 20 hours there was definite turnover of coroxon in some OPA wells. This is indicated by the glowing wells in the plate picture (Figure 33). The glowing wells correlate well with the results of the leaf painting that indicated plants 17, 22, and 23 were not transgenic. The results with OPB plants were not as clear, but that is expected due to the seed specific expression of the globulin promoter.

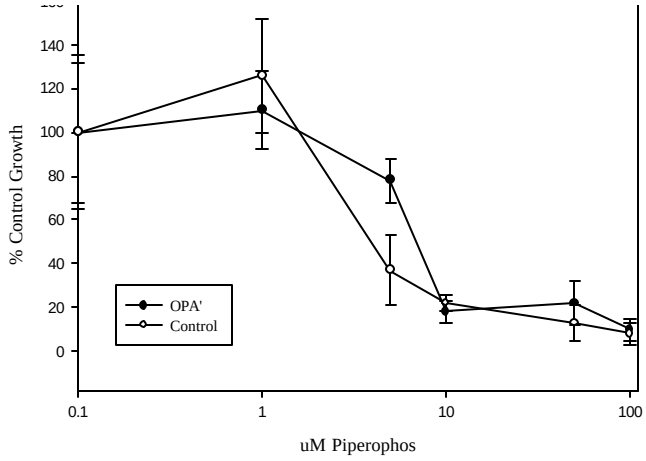
Selection of Transgenic Callus

The ability of OPH expressing callus lines to resist organophosphate herbicides was tested. Transgenic callus (OPA), expressing OPH under the control of a constitutive promoter, was plated onto cell culture plates containing a range of concentrations of herbicides. The experiment included the herbicides amiprophos-methyl and piperophos, as well as the anthelminitic drug haloxon, which has structural similarities to a known herbicide (Figure 26). Growth was measured by monitoring callus mass over a six week period and comparing the growth in treated plants to the growth of callus on plate without any added herbicide. Results for the 2 to 4 week period are presented in Figure 34. Results were similar when compared from week 4 to 6 and over the entire 6 week period. Overall, no difference was observed between OPH expressing callus and for treatment with amiprophos-methyl. OPA′ callus did appear to show some resistance to piperophos when compared to non-transgenic control callus. The most dramatic results were obtained with haloxon. OPA′ callus was able to grow well on concentrations of haloxon that greatly reduced the growth of control callus.

a.



b.



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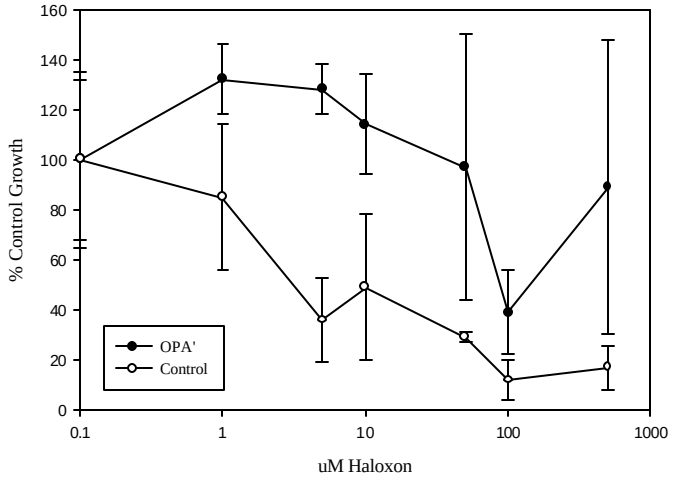


Figure 34. The effect of organophosphate herbicides on the Growth of transgenic and control callus tissue. **a.** Amiprophos-methyl **b.** Piperophos **c.** Haloxon. Error bars are from three replicates. Concentrations of 0µM for all herbicides plotted at 0.1 on the log scale.

Selection of Transgenic Plants

The ability to score transgenic tissue using OPH would be enhanced if a compound could be used to select for transgenic plants. There are a number of organophosphate herbicides that can be used against C4 monocot like *Zea mays*. The ability of transgenic plants expressing OPH (OPA) to resist the effects of the commercially available organophosphate herbicide Bensulmec-4LF (bensulide) was tested. Previously, it has been shown that recombinant OPH from bacteria does not show an activity against pure bensulide, but the bensulide was able to inhibit OPH activity against other OPH substrates (Chapter IV). Bensumec-4LF was added to trays of soil prior to planting, at both ten fold lower and ten fold higher than the recommended rate, as well as the recommended rate of application for the given area. Twenty seeds each of OPA and control were planted and allowed to germinate in the treated soil. Pictures of the trays at 3 weeks are shown in Figure 35. At the treatment rate of 0.35 ml/tray no effect was observed when compared to the control tray. At 3.5ml/plate, the recommended application rate, there was a noticeable difference between the OPA plants and the HiII/SP133 control plants. The control plants that did germinate at the 3.5ml/tray treatment level lacked a developed root system, which is consistent with the mode of action of besulide. At the highest application rate, both sets of plants seemed equally effected. Similar results were obtained with Prefar, another commercially available formulation of bensulide (data not shown).

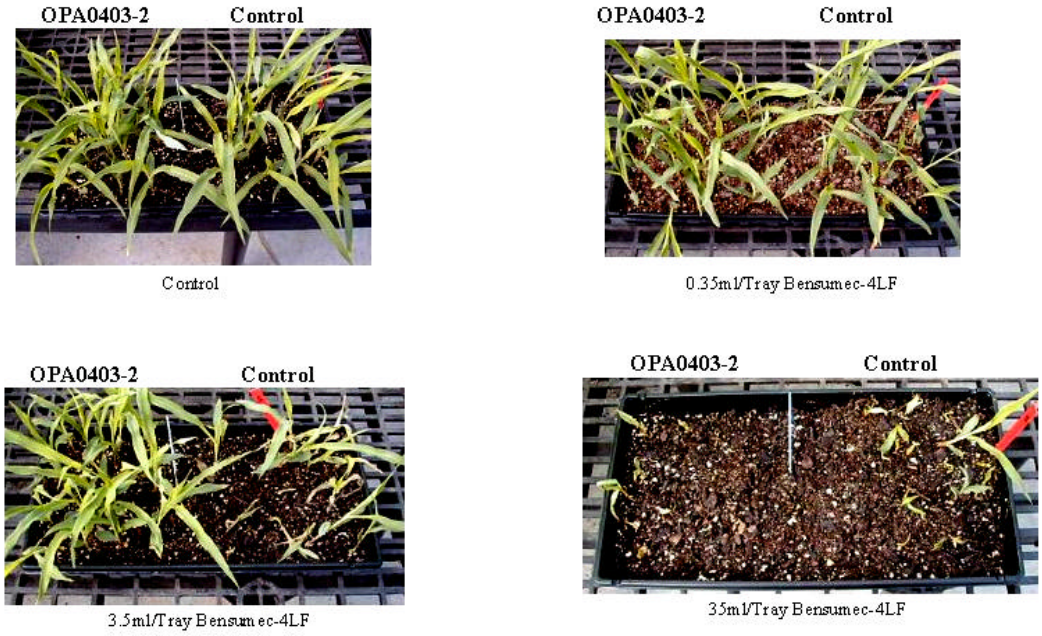


Figure 35. Resistance of OPA plants to Bensumec-4LF. OPA0403 T2 seed was used to determine resistance to besulide in the form of the formulated herbicide Bensumec-4LF. Control seed is of the same genetic background as the transgenic seed. Pictures were taken three weeks after germination. Shoots of control plants at 3.5ml/tray treatment level as well as all shoots at the 35ml/tray treatment level lacked a developed root system.

Discussion

Several selectable and scorable markers are available for use in plant biotechnology and research. To date none of these markers has been exploited as both a scorable, as well as selectable marker system. Organophosphate hydrolase has the unique ability to degrade a wide range of compounds that include chemicals used as herbicides. OPH has also been successfully expressed in plant tissue. Here we show that the wide substrate range of OPH can be harnessed to serve as a scorable marker system in seed, leaf, and callus tissue.

Intact seed from OPH producing plants was detected by the ability of that seed to degrade the compound paraoxon. Control tissue did not degrade paraoxon, and paraoxon turnover in non-transgenic seeds derived from a transgenic parent was significantly lower than transgenic seed. The act of subjecting the seed to the reaction mixture environment did not have an effect on plant germination and growth. This means that scoring with an OPH system results in the ability to screen out not transgenic seed, and that the seed that passes through the screening process remains viable. This is a great advantage in the generation of plants for the production of recombinant proteins. Out-crossing to elite in-bred lines results in seed pools where only half the seed contains the transgene. Use of this seed for production results in fields where only half the plants are transgenic. OPH could be used as a marker to pre-screen the seed prior to planting, resulting in fields that are uniformly transgenic and yielding product seed that has a higher concentration of product due to the elimination of the non-transgenic material.

Haloxon, a compound similar in structure to coumaphos, is a substrate for OPH. Kinetics results indicate that as a substrate for OPH haloxon is very similar to coroxon. The inclusion of chlorine on the ethyl groups of haloxon did not appear to significantly alter the binding of substrate to the enzyme when compared to coroxon. Catalytic turnover was also very similar between haloxon and coroxon. Kinetics of coroxon hydrolysis showed substrate inhibition at high concentrations, similar to the kinetics of paraoxon and DFP hydrolysis [24]. Coumaphos and Parathion have been used to screen bacteria as well as other biological materials for the presence of OP degrading activities [69,70]. Coumaphos has also been used to detect OPH activity in weakly denaturing SDS-PAGE gels [69]. Results with haloxon show that it can also be used to detect OPH activity in native page gels in the same manner as coumaphos.

Coumaphos, Coroxon, and Haloxon were used to screen plant leaf material and callus tissue. The use of these compounds expands the utility of OPH as a scorable marker by making it possible to screen callus material during the transformation and regeneration process. The screening of callus tissue could be of use in the production of transgenic plants. Typically, this is done by selection on media containing a herbicide. While this has the advantage of a selection system where non-transgenic material dies, it takes several weeks to manifest itself. The scoring system with OPH as a marker would yield results in as little as 24 hours and leave tissue viable for further propagation. This advantage would be magnified if the method were successfully adapted to freshly transformed embryos. Current methods for the screening of growing plant for a transgene involve painting the plants leaf with herbicide to which transgenic plants are

resistant. This system of scoring takes several days to yield results. The OPH system has the advantage over bialaphos leaf painting of yielding results in 24 hours, expediting the identification of transgenic plants.

OPA′ transgenic callus expressing OPH, challenged with the herbicide and OPH substrate amiprophos-methyl, showed no difference in growth inhibition when compared to control callus. The lack of resistance to amiprophos-methyl may be due to the toxicity of the breakdown products, or due to the slow rate at which OPH breaks down amiprophos-methyl. OPA′ callus did show some resistance to piperophos at concentrations that effected control callus growth. Piperophos has been shown not to be an OPH substrate in vitro, but it is possible that piperophos undergoes a biotransformation to another compound by the plant cell and that this new compound may be an OPH substrate. Plants have been shown to convert organophosphates from a thion to oxon form, so it is possible that piperophos could undergo a similar reaction [6]. OPH has been shown to have different activities against the thion and oxon forms of an organophosphate, such as parathion and paraoxon [31]. Haloxon showed the greatest promise as a selectable marker in tissue culture. The herbicidal activity of haloxon was not completely unexpected due to the structural similarity between it and the O-(2-napthyl) phosphorothionates that have been patented as herbicides. OPX callus showed the ability to continue to grow at concentrations of haloxon that inhibited the growth of control callus. Further experimentation with haloxon, as well as coumaphos and coroxon as compounds for selection in tissue culture are warranted.

The ability of OPH to act as a selectable marker by challenging OPH producing plants with a commercial preparation of the herbicide bensulide was also tested. While the limited scope of our study does not definitively demonstrate that OPH is a viable selectable marker, it does suggest that OPH producing plants have some resistance to bensulide. Further work needs to be done with the wide range of organophosphate herbicides available, including those that are not commercially available but have been shown to effect plant growth. Organophosphates have been shown to have adverse reactions with some herbicides that result in damage of plants not targeted by the applied herbicide [91,92]. It is possible that this interaction coupled with OPH could be used as a selection system.

Overall, OPH has been shown to be a viable scorable marker for use in plant systems. OPH can be screened in tissue culture, leaf, and seed tissue with results that differentiate between transgenic and non-transgenic tissue. Screening of seed tissue resulted in the separation of transgenic from non-transgenic seed with no effect on the viability of the seed used in the screening. We also have shown initial results that OPH producing plants show some resistance to the OP herbicide bensulide and that OPH expressing callus shows resistance to piperophos and haloxon in tissue culture. This raises the prospect that OPH could be used as both a scorable and selectable marker, making it unique among the markers currently used in plant biotechnology. Organophosphates also have been shown to inhibit the growth of cyanobacteria and fungi, raising the possibility that OPH could be used as a selectable marker in those systems [93,94].

Materials and Methods

Materials

All buffers, haloxon, and Bovine Serum Albumin were obtained from Sigma-Aldrich (St. Louis, MO). Paraoxon, coumaphos, and coroxon were obtained from Chemservice (West Chester, PA). Bensumec-4LF was obtained from Hummert International. Prefar was obtained locally from Producer's CO-OP (Bryan, TX). Bradford reagent was obtained from Bio-Rad.

Methods

Transgenic Lines

OPA and OPA′ callus were generated by transformation with an *Agrobacterium* expression vector containing an optimized *opd* gene under the control of the maize ubiquitin promoter. OPB lines are the result of transformation with an optimized *opd* construct under the control of the maize globulin1 promoter, which is specific for embryo expression of the transgene.

Intact Seed Screening

Transgenic T2 seed expressing OPH were screened by incubation in a reaction mixture containing 1mM paraoxon in 10mM HEPES buffer pH 7.2. Individual seeds were placed in single wells of a corning tissue culture plate. 1ml of reaction mixture was added to each well and the plate was incubated with shaking for 3 hours at room temperature. After the incubation period the reaction mixture was removed from each well and absorbance at 400nm was measured on a Beckman DU-7400

spectrophotometer. The seeds were transferred to soil and allowed to germinate in the greenhouse. Three weeks post-germination, each individual plant was painted with 1% Liberty, a commercial formulation of bialaphos, to check for the presence of the selectable marker used during the transformation process. Leaf damage from the leaf painting was observed at 5 days. Data was plotted on Sigma Plot 8.0 (SPSS Inc.). Seed used for enzymatic analysis (Figure 25) was treated in the same way, with the exception that the seed was imbibed overnight in water prior to treatment with the reaction mixture. After the reaction mixture was removed, each seed was manually pulverized using a hammer. The seed material was then extracted with 1ml of 10mM HEPES 1mM $CoCl_2$ pH=8.3. The extract was allowed to sit overnight at room temperature and assayed the next day with 1mM Paraoxon in 50mM CHES buffer pH=9. The extracts were also assayed for total protein content using a microplate Bradford assay (Bio-rad) with a bovine serum albumin standard curve. The assay results were used to generate results in units/mg protein, which were plotted against the intact seed assay results.

Stock solutions of haloxon and coumaphos were made up to 100mg/ml in Dimethyl Sulfoxide (DMSO). A 100mg/ml stock of coroxon was made up in methanol. 0.1 mM solutions of each were made in 50mM TRIS pH=7.4 with 10% methanol added to aid in the solubility of the compounds. 100 µl of each reaction mixture was added to a well of a 96 well plate. 0.87 µg of purified recombinant OPH was added to each well and the reaction was allowed to proceed for 5 minutes. Control reactions with buffer added in place of the enzyme were also allowed to sit for 5 minutes. The plate was then

illuminated on an ultraviolet light box and a digital photograph taken using an Alpha Imager (Alpha Innotech).

Kinetics of Haloxon and Coroxon Degradation

Reaction mixtures containing various concentrations of coroxon and haloxon in 50mM TRIS pH=7.4 with 10% Methanol. 1ml of reaction mixture was added to a quartz cuvette and blanked at 348 nm on a Beckman 7400 spectrophotometer. 87 ng of OPH in 5 µl of 10mM Phosphate pH=6.8 was added to the top of the cuvette and mixed by inversion. Production of chlorferon was monitored at 348 nm. Each concentration was repeated in triplicate. Results were calculated using the extinction coefficient of chlorferon (9100 M^{-1} cm^{-1}). Results were plotted and fitted with Michaelis-Menten, Lineweaver-Burke, or Michaelis-Menten modified for substrate inhibition equations using SigmaPlot 8.0 (SPSS Inc.).

In Gel Hydrolysis of Coumaphos and Haloxon

In gel hydrolysis was run using a procedure similar to that of Harcourt et.al [46]. Native gels were run with Novex 4-12% PAGE gels (invitrogen) under the manufacturers conditions and instructions. 500ng of recombinant OPH was loaded per well. After electrophoresis the gel was cut into strips and soaked for 5 minutes in 100mM Tris pH 7.4. The gel strips were then transferred to Tris buffer containing either 8mM Haloxon or Coumaphos for five minutes and then visualized using a UV light box. Images were taken with an Alpha Imager.

Callus Scoring

Lines of maize callus expressing OPH under the control of the maize ubiquitin promoter were used to determine if OPH activity could be used to score transgenic callus directly on plates. Callus was plated on standard plant tissue culture media with the addition of 1mg per plate coumaphos, coroxon, or haloxon. The plates were stored in an opaque plastic container at 27°C for 24 hours. At 24 hours the plates were placed on UV light box and pictures were taken using an Alpha-Imager imaging system. 100mg tissue of each line was then extracted in 1ml of HEPES buffer and assayed for OPH activity. Protein was measured by microplate Bradford assays.

Scoring of Leaf Tissue

Plants of both of transgenic lines expressing OPH under the control of the maize ubiquitin (OPA) and maize globulin (OPB) promoters was used to test the ability to score transgenic leaf tissue by OPH activity. Plants were allowed to germinate and grow for three weeks prior to 25mm segment of leaf tissue being excised and place in a 24 well tissue culture plate. 1ml of 0.1mM coroxon in 50mM tris buffer pH=7.4 was placed in each well and the plate was incubated for 20 hours at room temperature. 0.87 mg of purified recombinant OPH was added to a well with coroxon as a positive control. Tissue from non-transgenic plants was added as negative controls. The tissue was removed from the plate after incubation and the plate was placed on a UV light-box and a digital image taken.

Selection of Transgenic Callus

Transgenic callus was selected using amiprophos-methyl, haloxon, and piperophos. Herbicides stock solutions were made up in DMSO. The stocks were then added to 30ml of plant tissue culture media in a 100x25 mm petri dish during pouring. The plates were massed prior to the addition of callus and then massed again after the addition of callus. Callus was transferred to fresh plates and massed every two weeks. Results were graphed on Sigma Plot 8.0. Three independent plates of each treatment were used for each data point.

Selection of Transgenic Plants

Transgenic plants expressing OPH under the maize ubiquitin promoter were tested for the ability to resist bensulide in commercially formulated forms Bensumec-4LF and Prefar. The recommended application rate for each herbicide was used to determine the amount need to treat a standard tray of soil. The experiment was carried out at the recommended rate (3.5ml per tray), ten fold below (0.35ml per tray), and 10 fold above (35ml per tray) the recommended rate. The formulated herbicide was diluted into 300ml of Acetone and mix by hand into the soil. The acetone was allowed to evaporate and twenty seeds each of control and transgenic lines were planted. The seed were allowed to germinate and were observed for three weeks. Pictures were taken with an Olympus digital camera after three weeks.

CHAPTER VI

CONCLUSIONS

Organophosphate hydrolase is an enzyme with a broad spectrum of substrates and potential applications. Previous experiments dealing with recombinant expression of the enzyme focused on the ability to generate enough enzyme for lab-scale use. Attempts to adapt these lab expression systems to larger scales of enzyme production, like bacterial fermentation, have been problematic in the unexpected fall off in enzyme expression observed. In order to provide an alternative expression system, the ability of a plant system, *Zea mays L*., to express OPH was explored. A synthetic gene construct was designed for maize expression and inserted by *Agrobacterium* mediated transformation into the maize genome under the control of constitutive, embryo specific, and endosperm specific promoters.

Analysis of the seed and callus tissue of transformants indicated that OPH was successfully expressed in tissue transformed with each of the three constructs. Highest expression was observed in tissue expressing OPH under the control of the constitutive and embryo specific promoters. The endosperm specific promoter did generate OPH positive tissue, but the expression levels were far below those observed for the other two constructs. Across plants generated from each construct, expression level varied between individual transformation events. This variation is consistent with the position effect caused by the random integration of *Agrobacterium* transformation. A comparison of the expression levels in transgenic maize to those of other expression systems indicated that on average maize expression levels in early generations were similar to that of the

original *Pseudomonas diminuta* strain that the gene was isolated from. Individual seed analysis indicated that the highest expressing individual seeds were expressing at a level above that of the *E. coli* expression system that is the laboratory workhorse of OPH expression. The highest expressing bulk sample of the embryo specific transgenic seed was also higher than *E. coli* expression. Manual separation seed tissues indicated for both the constitutive and embryo specific construct that the majority of activity in the seed was localized to the embryo. While maize will never replace *E. coli* for the laboratory expression of OPH, it may yield better results as an expression system for generating large amounts of enzyme for practical use in applications. The maize system, through breeding and selection of lines for out-crossing, has been shown to increase expression levels of a protein product from up to 20 times the initial expression level observed [83]. If a similar increase in expression is observed in latter generations OPH producing lines, and this increase is coupled with the inherent scalability of a plant system, maize could become an ideal expression system for expression of large quantities of OPH for application purposes. The protein stabilizing characteristics of the seed also means that seed containing OPH could be produced and stored as whole seed until need for purified OPH product.

Western analysis of ammonium sulfate precipitates of seed extracts showed that a doublet band cross-reacted with anti-OPH antibodies at a molecular weight consistent with monomeric OPH. No cross-reaction was observed for control samples. Close comparison of the corn derived OPH to the bacterial enzyme on Western blots showed that the lower band of the doublet migrated the distance in the gel as the standard,

indicating a similar molecular mass. The presence of two bands could have indicated an inappropriate processing of the BAASS signal sequence or a possible post-translational modification of the enzyme. N-terminal sequencing of both bands produced identical sequence that matched the expected N-terminal sequence of the processed OPH with the exception of an N-terminal glycine residue. The matching n-terminal sequences indicated that a post-translational modification event was most likely responsible for the doublet pattern on the western blot. Sequence analysis of the OPH protein sequence identified a possible N-glycosylation site that could be modified during transit through the endomembrane system. Passage of the corn derived OPH over a concanavalin-A sepharose column resulted in the partial binding of activity, with an elution of activity with 0.5M methyl α-D-glucopyranoside. This is consistent with the binding of a partially glycosylated population of protein molecules to the column. Western analysis confirmed that the material that passed through the column was enriched for the lower band of the doublet, and that material that was eluted from the column was enriched for the upper, modified band. It was also noted that a large portion of the activity loaded onto the column was not found in either the flow through or the elution fractions. Later experimentation indicated that a significant amount of activity remained bound to the column despite the elution step. The unexpected result that a majority of activity remained bound to the Concanavalin A column, even after elution, indicates that the modified form of OPH can be strongly immobilized on a column matrix without harsh chemical treatments that could effect enzyme function. The binding of corn produced OPH to concanavalin A sepharose may provide a new, unique method for the

immobilization of OPH onto solid matrices. In order to determine if a glycosylation event was the cause of the doublet band, corn expressed OPH was subjected to chemical deglycosylation. Western and SDS-PAGE results showed that the treated sample had been reduced from a doublet to a single band with a molecular mass identical to the lower band of the doublet, indicating the removal of carbohydrate from the protein structure. The glycosylation of OPH in the maize transformation system being used is unique. Other expression studies have not identified any post-translational modification of the recombinant product [37,38]. The addition of a carbohydrate moiety to the OPH protein may also provide new targets for modification and immobilization for practical applications.

Previous work with the copper dependent enzyme Laccase showed that the addition of copper to extracts could result in an increase in enzymatic activity [83]. The addition of divalent transition metal salts to extracts resulted in the increase of OPH activity of 4 to 10 fold, dependent on the sample being analyzed. The increase in activity was proportional to the different activity levels previously observed for enzyme constituted with different transition metals. Highest activity was observed for extracts incubated with cobalt. The increase in activity was time and temperature dependent, indicating a dynamic process. The addition of bicarbonate to the extracts during incubation resulted in an increase in the rate that activity rose, but not the final overall activity. This is consistent with previous results that indicated that the addition of bicarbonate to during the reconstitution of apoenzyme facilitated the formation of the metal center of the OPH active site [33]. This indicates that the incubation of extracts with

appropriate metals increases the OPH activity of the extract because of the formation of a metal center. The metal incubation results indicate that in order to achieve maximal activity when extracting OPH from transgenic maize added metal must be included in the extraction buffer.

The paraoxon kinetics of the maize produced enzyme was also explored. Enzyme was isolated from seed using a method originally used for the purification of enzyme from recombinant insect cells [31]. The purification resulted in a 2000 fold increase in enzyme purity. The purity was sufficient to obtain the N-terminal sequencing data and enzyme kinetic data. The enzyme showed a paraoxon K_m value similar to those published for enzyme derived from a bacterial expression system. K_m was also calculated for the flow through and elution fractions from the concanavalin-A column. The results indicated K_m values for both fractions within the range of those reported for enzyme expressed in bacteria. A direct comparison of the two fractions showed that the enzyme that was eluted from the Concanavalin A column had a significantly higher K_m value, indicating a weaker binding of substrate to the modified enzyme when compared to un-separated enzyme or the column flow through.

One of the unique aspects of OPH is the wide range of substrates that the enzyme can degrade. Organophosphates are primarily thought of as neurotoxic pesticides and chemical warfare agents. However, there is an entire class of organophosphate herbicides that have never been tested as OPH substrates. After analysis of the chemical structures of the organophosphate herbicides, four were chosen as potential OPH substrates. Both spectrophotometric and HPLC analysis were used to determine if the

organophosphate herbicides amiprophos-methyl, anilofos, bensulide, and piperophos were OPH substrates. Both spectrophotometric and HPLC analysis indicated that OPH had the ability to degrade the phosphoramidate herbicide amiprophos-methyl. The kinetics of amiprophos-methyl hydrolysis by OPH was determined. Amiprophos-methyl was shown to have a K_m value lower than that of paraoxon, but with a k_{cat} value that was similar to that of dementon-S. Spectrophotometric results with the phosphothioate herbicides indicated that anilofos and bensulide were not degraded appreciably by OPH, but piperophos showed a slight reaction. HPLC analysis showed that the spectrophotometric result was caused by a minor contaminant of piperophos and not by piperophos itself. The herbicides were also used as inhibitors of paraoxon activity. Amiprophos-methyl, a P-O ester organophosphate, showed competitive inhibition of paraoxonase activity. The other herbicides, all P-S ester herbicides, showed mixed inhibition of paraoxonase activity. These results are consistent with those observed for the P-S ester substrate demeton-S inhibition of paraoxonase activity. A reciprocal experiment was carried out using amiprophos-methyl and bensulide to inhibit OPH degradation of demeton-S. Both compounds inhibited demeton-S degradation in a competitive fashion, regardless if the inhibitor was a P-S or P-O ester organophosphate. These results support the theory that OPH has a complex enzymatic mechanism. If OPH had only a single mechanism, we would expect all inhibition of paraoxonase activity by another organophosphate to be competitive, independent of the type of ester bond found in the competitor substrate. The competitive inhibition of demeton-S degradation by amiprophos-methyl indicates that it can directly compete with demeton-S for catalysis.

The fact that the phosphothioate herbicides showed a mixed paraoxonase inhibition pattern indicates that they only were able to block part of the total enzyme activity against paraoxon. This would indicate that the degradation of P-O ester substrates might be more complex than a single mechanism or mode of binding.

OPH activity against coumaphos has previously been used to screen bacteria for OPH activity [69]. Coumaphos has several derivatives, one of which is the anthelmintic compound haloxon [90]. Haloxon was investigated as a possible OPH substrate. Enzymatic reactions with coumaphos, coroxon (the O-analog of coumaphos), and haloxon showed similar results. Kinetics of haloxon hydrolysis were similar to those of coroxon, indicating that the chlorinated side chains of haloxon had little effect on the ability of OPH to degrade the compound. Haloxon was also used in a native gel in gel hydrolysis reaction that has previously been tested with coumaphos. Haloxon has a higher solubility than coumaphos, which may be an advantage in some screening applications.

The ability of OPH to be used as a scorable marker for bacteria has already been established. The ability to degrade a herbicide the raises possibility that OPH could be used as a scorable and selectable marker system for plants. OPH was tested as a scorable marker for use in tissue culture by adding coumaphos, coroxon, and haloxon to tissue culture media plates and plating transgenic callus expressing OPH under control of a constitutive promoter. One day after plating, the plates were exposed to UV light and the scored by the appearance of fluorescence. Positive transgenic callus showed fluorescence in the plates with all three compounds. Control callus showed no reaction. Coumaphos and its derivatives were also used to screen leaf tissue. Leaf tissue from

transgenic plants expressing OPH was able to turn over substrate in a reaction mixture that could then be screened for fluorescence. Tissue from plants with OPH under the control of the constitutive and embryo specific promoters were tested with the best results obtained with the constitutive promoter plants. Control plants showed no reaction. Seed was screened using paraoxon. Paraoxon turnover was observed with both the constitutive and embryo specific constructs. The assay was able to distinguish between transgenic and non-transgenic seed. The screening of seed tissue had no apparent negative effects on the germination of scored seeds. This is an added advantage to an OPH scorable marker system, in comparison to the scorable marker β-gulcuronidase, which results in the destruction of the scored tissue. OPH expressing callus was resistant to the herbicidal effect of the organophosphate haloxon in tissue culture. This may indicate that an OPH/haloxon selection system can be developed for the selection of transgenic tissue. OPH can be used to score plant tissue for the presence of OPH in several different tissue types and stages in the production of transgenic plants. Plant expressing OPH, under the control of a constitutive promoter, were tested for resistance to the herbicide bensulide. At the recommended application rate for formulated bensulide, OPH expressing transgenic plants showed greater resistance when compared to non-transgenic control plants. In vitro bensulide has been shown not to act as a measurable substrate for OPH, however bensulide can inhibit OPH activity against other substrates such as paraoxon and demeton-S. It is possible, that like other organophosphates, bensulide is converted into another compound by other cellular enzymes. This converted compound may then be an OPH substrate. Differences in OPH

activity have been observed against the oxon and thion analogs of organophosphates such as coumaphos/coroxon and parathion/paraoxon. The development of OPH as a selectable marker would make it unique among marker genes for use in plants. It would be the first that is both readily scorable and also can be used to select for transgenic material by using a compound that is phytotoxic.

In closing, OPH expression in a plant system has yielded not only a new source of recombinant protein, but also a new modified version of the OPH protein with potential application uses and a series of potential uses for OPH in plant expression systems as a marker gene. Two new compounds, amiprophos-methyl and haloxon, have been identified as OPH substrates. The kinetic properties of these substrates have been determined and compared to other known substrates. The series of organophosphate herbicides have been used to determine inhibition patterns against both P-O and P-S bonded substrates and indicate that OPH uses more than one enzymatic mechanism. OPH has also been used to score plant callus, leaf, and seed tissue. The scoring process was able to distinguish between transgenic and non-transgenic tissue. There has also been a promising start to establishing OPH as a selectable marker system. Further refinement of the plant expression system, as well as the marker systems will yield not only new abundant source of enzyme, but also a new technological system for use in plant transformation research.

REFERENCES

1. Farm Chemicals Handbook. Meister Publishing, Willoughby, OH (2000).

2. Ehrich,M. *Encyclopedia of Toxicology*., pp. 467-470 (Academic Press, San Diego,1998).

3. Pope,C.N. Organophosphorus Pesticides: do they all have the same mechanism of toxicity. *Journal of Environmental Health, Part B* **2**, 161-181 (1999).

4. Hyde,E.G. & Carmicheal,W.W. Anatoxin-A(S), a naturally occuring organophosphate, is an irreversible active-site directed inhibitor of acetylcholinesterase (E.C.3.1.1.7). *Biochemical Toxicology* **6**, 195-201 (1991).

5. Chaudhry,G.R., Ali,A.N. & Wheeler,W.B. Isolation of a methyl parathion-degrading *Pseudomonas* sp. that possesses DNA homologous to the *opd* gene from a *Flavobacterium* sp. *Applied and Environmental Microbiology* **54**, 288-293 (1988).

6. Horne,I., Harcourt,R.L., Sutherland,T.D., Russell,R.J. & Oakeshott,J.G. Isolation of a *Pseudomonas montelli* strain with a novel phosphotriesterase. *FEMS Microbiology Letters* **206**, 51-55 (2002).

7. Horne,I., Qiu,X., Russell,R.J. & Oakeshott,J.G. The phosphotriesterase gene *opda* in *Agrobacterium radiobacter* p230 is transposable. *FEMS Microbiology Letters* **222**, 1-8 (2003).

8. Mackness,B., Durrington,P.N. & Mackness,M.I. Human serum paraoxonases. *General Pharmacology* **31**, 329-336 (1998).

9. McDaniel,C.S., Harper,L.L. & Wild,J.R. Cloning and sequencing of a plasmid-borne gene (*opd*) encoding a phosphotriesterase. *Journal of Bacteriology* **170**, 2306-2311 (1998).

10. Mulbry,W.W. Characterization of a novel organophosphorus hydrolase from *Nocardiodes simplex* NRRL B-24074. *Microbiological Research* **154**, 285-288 (2000).

11. Mulbry,W.W., Karns, J.S., Kearney, P.C., Nelson, J.O., McDaniel, C.S. *et al.* Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by southern hybridization with *opd* from *Pseudomonas diminuta. Applied and Environmental Microbiology* **51**, 926-930 (1986).

12. Munnecke,D.M. & Hsieh,D.P.H. Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. *Applied Microbiology* **28**, 212-217 (1974).

13. Munnecke,D.M. & Hsieh,D.P.H. pathways of microbial metabolism of parathion. *Applied and Environmental Microbiology* **31**, 63-69 (1976).

14. Munnecke,D.M. Enzymatic detoxification of waste organophosphate pesticides. *Journal of Agricultural and Food Chemistry* **28**, 105-111 (1980).

15. Raushel,F.M. Bacterial detoxification of organophospahte nerve agents. *Current Opinion in Microbiology* **5**, 288-295 (2002).

16. Rosenberg,A. & Alexander,M. Microbial cleavage of various organophosphorus insecticides. *Applied and Environmental Microbiology* **37**, 886-891 (1979).

17. Serdar,C.M. & Gibson,D.T. Enzymatic hydrolysis of organophosphates: cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta. Bio/Technology* **3**, 567-571 (1985).

18. Sethunathan,N. & MacRae,I.C. Some effects of diazinon on the microflora of submerged soils. *Plant and Soil* **30**, 109-112 (1969).

19. Sethunathan,N. & Pathak,M.D. Development of a diazinon-degrading bacterium in paddy water after repeated applications of diazinon. *Canadian Journal of Microbiology* **17** , 699-702 (1971).

20. Sethunathan,N. & Yoshida,T. A *Flavobacterium* sp. that degrade diazinon and parathion. *Canadian Journal of Microbiology* **19**, 873-875 (1973).

21. Sethunathan,N. Degradation of parathion in flooded acid soils. *Journal of Agricultural and Food Chemistry* **21**, 602-604 (1973).

22. Hoskin,F.G.G., Walker,J.E. & Mello,C.M. Organophosphorus acid anhydrolasein slime mold duckweed and mung bean: a continuing search for a physiological role and natural substrate. *Chemico-Biological Interactions* **119-120**, 399-404 (1999).

23. Raushel,F.M. & Holden,H.M. Phosphotriesterase: an enzyme in search of its natural substrate. *Advances in Enzymology and Related Areas of Molecular Biology* **74**, 51-93 (1998).

24. diSoudi,B., Miller,C.E., Lai,K., Grimsley,J.K. & Wild,J.R. Rational design of organophosphate hydrolase for altered substrate specificities. *Chemico-Biological Interactions* **199-120**, 211-223 (1999).

25. Caldwell,S.R., Newcomb,J.R., Schlecht,K.A. & Raushel,F.M. Limits of diffusion in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta. Biochemistry* **30**, 7438-7444 (1991).

26. Gunner,H.B. & Zuckerman,B.M. Degradation of 'diazinon' by synergistic microbial action. *Nature* **217**, 1183-1184 (1968).

27. Grimsley,J.K., Scholtz,J.M., Pace,C.N. & Wild,J.R. Organophosphorous hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. *Biochemistry* **36**, 14366-14374 (1997).

28. Kolakowski,J.E., Defrank, J.J., Harvey, S.P., Szafraniec, L.L., Beaudry, W.T. *et al.* Enzymatic hydrolysis of the chemical warfare agent vx and its neurotoxic analouges by organophosphate hydrolase. *Biocatalysis and Biotransformation* **15**, 297-312 (1997).

29. Lai,K., Dave,K.I. & Wild,J.R. Bimetallic binding motifs in organophosphate hydrolase are important for catalysis and structural organization. *The Journal of Biological Chemistry* **269**, 16579-16584 (1994).

30. Omburo,G.A., Kuo,J.M., Mullins,L.S. & Raushel,F.M. Characterization of the zinc binding site of bacterial phosphotriesterase. *The Journal of Biological Chemistry* **267**, 13278-13283 (1992).

31. Dumas,D.P., Caldwell,S.R., Wild,J.R. & Raushel,F.M. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta. The Journal of Biological Chemistry* **264**, 19659-19665 (1989).

32. Hong,S. & Raushel,F.M. Metal-substrate interactions facilitate the catalytic activity of the bacterial phosphotriesterase. *Biochemistry* **35**, 10904-10912 (1996).

33. Shim,H. & Raushel,F.M. Self-assembly of the binuclear metal center of phosphotriesterase. *Biochemistry* **39**, 7357-7364 (2000).

34. Benning,M.M., Shim,H., Raushel,F.M. & Holden,H.M. High resolution x-ray structures of different metal-substituted forms of phosphotriesterase from *Pseudomonas diminuta. Biochemistry* **40**, 2712-2722 (2001).

35. Lai,K., Stolowich,N.J. & Wild,J.R. Characterization of P-S bond hydrolysis in organophosphorothioate pesticides by organophosphate hydrolase. *Archive of Biochemistry and Biophysics* **318**, 59-64 (1995).

36. diSoudi,B., Grimsley J.K., Lai,K. & Wild,J.R. Modification of near active site residues in organophosphate hydrolase reduces metal stoichiometry and alters substrate specificity. *Biochemistry* **38**, 2866-2872 (1999).

37. Dave,K.I., Phillips,L., Luckow,V.A. & Wild,J.R. Expression and post-translational processing of a broad spectrum organophosphorus-neurotoxin degrading enzyme in insect cell culture. *Biotechnology and Applied Biochemistry* **19**, 271-284 (1994).

38. Dave,K.I., Lauriano,C., Xu,B., Wild,J.R. & Kenerley,C.M. Expression of organophosphate hydrolase in the filamentous fungus *Gliocladium virens*. *Applied Microbiology and Biotechnology* **41**, 352-358 (1994).

39. Phillips, J.P., Xin,J.H., Kirby,K., Milne,C.P., Krell,P. *et al.* Transfer and gene expression of an organophosphate insecticide-degrading gene from pseudomonas in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **87**, 8155-8159 (1990).

40. Fraley,R.T., Rogers,S.G., Horsch,R.B., Sanders,P.R., Flick,J.S. *et al.* Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Sciences* **80**, 4807 (1983).

41. Fromm,M.E., Morish,F., Armstrong,C.L., Williams,L., Thomas,J. *et al.* Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Bio/Technology* **8**, 833-839 (1990).

42. Songstad,D.D., Summers,D.A. & Griesbach,R.J. Advances in alternative DNA delivery techniques. *Plant Cell, Tissue, and Organ Culture* **40**, 1-15 (1995).

43. Jilka,J.M., Hood,E.E., Dose,R. & Howard,J.A. The benefits of proteins produced in transgenic plants. *AgBiotechNet* **1**, 1-4 (1999).

44. Valueva,T.A. & Mosolov,V.V. Protein inhibitors of proteinases in seeds: 1. classification, distribution, structure, and properties. *Fiziologiya Rastenii (Moscow)* **46**, 362-378 (1999).

45. Evangelista,R.L., Kusnadi,A.R., Howard,J.A. & Nikolov,Z.L. Process and economic evaluation of the extraction and purification of recombinant β-glucuronidase from transgenic corn. *Biotechnology Progress* **14**, 607-614 (1998).

46. Beyer,P., Al-Babili,S., Ye,X., Lucca,P., Schaub,P. *et al.* Golden Rice: Introducing the β-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin a deficiency. *Journal of Nutrition* **132**, 506S-510S (2002).

47. Christensen,A.H., Sharrock,R.A. & Quail,P.H. Maize polyubiquitin genes: structure, thermal pertubation of expression and transcript silencing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology* **18**, 675-689 (1992).

48. Christensen,A.H. & Quail,P.H. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Trangenic Research* **5**, 213-218 (1996).

49. Hood,E.E., Witcher,D.R., Maddock,S., Meyer, T., Baszczynski,C. *et al*. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction, and purification. *Molecular Breeding* **3**, 291-306 (1997).

50. Hood,E.E. From green plants to industrial enzymes. *Enzyme and Microbial Technology* **30**, 279-283 (2002).

51. Koziel,M.G., Beland,G.L., Bowman,C., Caarozzi,N.B., Crenshaw,R. *et al*. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Technology* **11**, 194-200 (1993).

52. Kusnadi,A.R., Hood,E.E., Witcher,D.R., Howard,J.A. & Nikolov,Z.L. Production and purification of two recomibnant proteins from transgenic corn. *Biotechnology Progress* **14**, 149-155 (1998).

53. Streatfield,S.J., Lane,J.R., Brooks,C.A., Barker,D.K., Mayor,J.M. *et al*. Corn as a production system for human and animal vaccines. *Vaccine* **21**, 812-815 (2003).

54. Witcher,D.R., Hood,E.E., Peterson,D., Bailey,M., Bond,D. *et al*. Commerical production of β-glucuronidase (GUS): a model system for the production of proteins in plants. *Molecular Breeding* **4**, 301-312 (1998).

55. Bevan,M. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12**, 8711-8721 (1984).

56. Frame,B.R., Shou,H., Chikwamba,R.K., Zhang,Z., Xiang,C. *et al*. *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiology* **129**, 13-22 (2002).

57. Howe,A.R., Glasser,C.S., Brown,S.M., Padgette,S.R., Hart,J. *et al*. Glyphosate as a selective agent for the production of fertile transgenic maize (*Zea mays L.*) plants. *Nature* **10**, 153-164 (2002).

58. Zhaou,Z.Y., Gu,W., Cai,T. & Pierce,D.A. Methods for *Agrobacterium* mediated transformation. (US Patent 5,981,840). 1999.

59. Gordon-Kamm,W.J., Spencer,T.M., Mangano,M.L., Adams,T.R., Daines,R.J. *et al.* Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* **2**, 603-618 (1990).

60. Diehn,S.H., De Rocher,E.J. & Green,P.J. Problems that can limit the expression of foreign genes in plants: lessons to be learned from *B.t.* toxin genes. *Genetic Engineering* **18**, 83-99 (1996).

61. Masarik,M., Kizek,R., Kramer,K.J., Billova,S., Bradzdova,M. *et al.* Application of avidin-biotin technology and absorptive transfer stripping square-wave voltammetry for detection of DNA hybridization and avidin in transgenic avidin maize. *Analytical Chemistry* **75**, 2663-2669 (2003).

62. Holmberg,N., Farres,J., Bailey,J.E. & Kallio,P.T. Targeted expression of a synthetic codon optimized gene, encoding the spruce budworm antifreeze protein, leads to accumulation of antifreeze activity in the apoplasts of transgenic tobacco. *Gene* **275**, 115-124 (2001).

63. Rogers,J.C. Two barley α-amylase gene families are regulated differently in aleurone cells. *The Journal of Biological Chemistry* **260**, 3731-3738 (1985).

64. Brandizzi,F., Fricker,M. & Hawes,C. A greener world: the revolution in plant bioimaging. *Nature Reviews: Molecular Cell Biology* **3**, 520-530 (2002).

65. de Ruijter,N.C.A., Verhees,J., van Leeuwen,W. & van der Krol,A.R. Evaluation and comparison of the GUS, LUC, and GFP reporter system for gene expression studies in plants. *Plant Biology* **5**, 103-115 (2003).

66. Escobar,M.A., Park,J., Polito,V.S., Leslie,C.S., Uratsu,S.L. *et al.* Using GFP as a scorable marker in walnut somatic embryo transformation. *Annals of Botany* **85**, 831-835 (2000).

67. Stewart Jr.,C.N. The utility of green fluorescent protein in transgenic plants. *Plant Cell Reports* **20**, 376-382 (2001).

68. Dennehey,B.K., Peterson,W.L., Ford-Santino,C., Pajeau,M. & Armstrong,C.L. Comparison of selective agents for use with the selectable marker gene *bar* in maize tranformation. *Plant Cell, Tissue, and Organ Culture* **36**, 1-7 (1994).

69. Harcourt,R.L., Horne,I., Sutherland,T.D., Hammock,B.D., Russel,R.J. *et al.* Development of a simple and sensitive fluorimetric method for isolation of

coumaphos-hydrolysing bacteria. *Letters in Applied Microbiology* **34**, 263-268 (2002).

70. McDaniel,C.S. & Wild,J.R. Detection of organophosphorus pesticide detoxifying bacterial colonies, using uv- photography of parathion-impregnated filters. *Archives of Environmental Contamination and Toxicology* **17**, 189-194 (1988).

71. Artus,N.N., Ryberg,M., Lindsten,A., Ryberg,H. & Sundqvist,C. The shibata shift and the transformation of etioplasts to chloroplast in wheat with clomazone (fmc 57020) and amiprophos-methyl (tokunol m). *Plant Physiology* **98**, 253-263 (1992).

72. Blume,Y.B., Strashnyuk,N.M., Smertenko,A.P., Solodushko,V.G., Sidorov,V.A. *et al.* Alteration of β-tubulin in *Nicotiana plumbaginifolia* confers resistance to amiprophos-methyl. *Theroretical and Applied Genetics* **97**, 464-472 (1998).

73. Katagi,T. Photochemistry of organophosphorus herbicide butamiphos. *Journal of Agricultural and Food Chemistry* **41**, 496-501 (1993).

74. Morejohn,L.C. & Fosket,D.E. Inhibition of plant microtubule *polymerization in vitro* by the phosphoric amide herbicide amiprophos-methyl. *Science* **224**, 874-876 (1984).

75. Ramulu,K.S., Dijkhuis,P., Famelaer,I., Cardi,T. & Verhoeven,H.A. Cremart: A new chemical for efficent induction of micronulceli in cells and protoplasts for partial genome transfer. *Plant Cell Reports* **13**, 687-691 (1994).

76. Menge,R.M. & Hubbard,J.L. Phytotoxicity of bensulide and trifluralin in several soils. *Weed Science* **18**, 244-247 (1970).

77. Harper,L.L., McDaniel,C.S., Miller,C.E. & Wild,J.R. Dissimilar plasmids isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) contain identical *opd* Genes. *Applied and Environmental Microbiology* **54**, 2586-2589 (1988).

78. Whitelam,G.C., Cockburn,B., Gandecha,A.R. & Owen,M.R.L. Heterologus protein production in transgenic plants. *Biotechnology and Genetic Engineering Reviews* **11**, 1-29 (1993).

79. Ishida,Y., Saito,H., Ohta,S., Hiei,Y., Komari,T. *et al.* High efficiency transformation of maize (*Zea mays L.*) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **14**, 745-750. (1996).

80. De Rocher,E.J., Vargo-Gogola,T.C., Diehn,S.H. & Green,P.J. Direct evidence for rapid degradation of *Bacillus thuringiensis* Toxin mRNA as a cause of poor expression in plants. *Plant Physiology* **117**, 1445-1461 (1998).

81. Belanger,F.C. & Kriz,A.L. Molecular basis for alleic polymorphism of the maize globulin-1 gene. *Genetics* **129**, 863-872 (1991).

82. Song,R., Llaca,V., Linton,E. & Messing,J. Sequence, regulation, and evolution of the maize 22-kd a-zein gene family. *Genome Research* **11**, 1817-1825 (2001).

83. Hood,E.E., Bailey,M., Beifuss,K., Magallanes-Lundback,M., Horn,M.E. *et al.* Criteria for high-level expression of a fungal laccase gene in transgenic maize. *Plant Biotechnology Journal* **1**, 129-140 (2003).

84. Steiert,J.G., Pogell,B.M., Speedie,M.K. & Laredo,J. A gene coding for a membrane bound hydrolase is expressed as a secreted, soluble enzyme in *Streptomyces lividans*. *Bio/Technology* **7**, 65-68 (1989).

85. Sambrook,J., Fritsch,E.F. & Maniatis,T. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

86. Rochu,D., Beaufet,N., Renault,F., Viguie,N. & Masson,P. The wild type bacterial co2+/co2+-phosphotriesterase shows a middle-range thermostability. *Biochimica et Biophysica Acta* **1954**, 207-218 (2002).

87. Sojar,H.T. & Bahl,O.P. Chemical deglycosylation of glycoproteins. *Methods in Enzymology* **138**, 341-350 (1987).

88. Tams,J.W. & Welinder,K.G. Mild chemical deglycosylation of horseradish peroxidase yields a fully active, homogenous enzyme. *Analytical Biochemistry* **228**, 48-55 (1995).

89. Edge,A.S.B., Faltynek,C.R., Hof,L., Reichert Jr.,L.E. & Weber,P. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Analytical Biochemistry* **118**, 131-137 (1981).

90. Martin,J.R. Modes of action of anthelmintic drugs. *The Veterinary Journal* **154**, 11-34 (1997).

91. Christopher,J.T., Preston,C. & Powles,S.B. Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. *Pesticide Biochemistry and Physiology* **49**, 172-182 (1994).

92. Kreuz,K. & Fonne-Pfister,R. Herbicide-insecticide interactions in maize: malathion inhibits cytochrome p450-dependent primisulfuron metabolism. *Pesticide Biochemistry and Physiology* **43**, 232-240 (2003).

93. El-Helaly,A.F., Abo-el-Dahab,M.K. & Zeitoun F.M. Effect of organic phosphorus pesticides on certain phytopathogenic bacteria. *Phytopathology* **53**, 762-764 (1963).

94. Buchner,B., Westfield & Jacoves,E. O-(2-Napthyl) Phosphorothinates. Continental Oil Company. (US Patent 3,328,494). 6-27-1967.

APPENDIX A

MAIZE HIGH CODON TABLE AND CHANGES TO THE NATIVE *opd* GENE

Maize codon usage table taken from the Codon Usage Database (www.kazusa.or.jp/codon)

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	11275.00	15.10	0.21
Gly	GGA	10838.00	14.51	0.20
Gly	GGT	11380.00	15.24	0.21
Gly	GGC	21059.00	28.20	0.39
Glu	GAG	29427.00	39.40	0.64
Glu	GAA	16250.00	21.76	0.36
Asp	GAT	17764.00	23.78	0.44
Asp	GAC	22752.00	30.46	0.56
Val	GTG	18314.00	24.52	0.36
Val	GTA	5370.00	7.19	0.11
Val	GTT	12238.00	16.39	0.24
Val	GTC	14896.00	19.94	0.29
Ala	GCG	15610.00	20.90	0.23
Ala	GCA	12429.00	16.64	0.19
Ala	GCT	16694.00	22.35	0.25
Ala	GCC	21904.00	29.33	0.33
Arg	AGG	10527.00	14.09	0.25
Arg	AGA	6855.00	9.18	0.16
Ser	AGT	6219.00	8.33	0.11
Ser	AGC	11381.00	15.24	0.21
Lys	AAG	29494.00	39.49	0.70
Lys	AAA	12567.00	16.83	0.30
Asn	AAT	10987.00	14.71	0.40
Asn	AAC	16252.00	21.76	0.60
Met	ATG	17706.00	23.71	1.00
Ile	ATA	7049.00	9.44	0.20
Ile	ATT	11943.00	15.99	0.33
Ile	ATC	17033.00	22.81	0.47
Thr	ACG	7650.00	10.24	0.21
Thr	ACA	7987.00	10.69	0.22
Thr	ACT	8958.00	11.99	0.24
Thr	ACC	12066.00	16.16	0.33
Trp	TGG	9747.00	13.05	1.00
End	TGA	828.00	1.11	0.45

Cys	TGT	4502.00	6.03	0.34
Cys	TGC	8650.00	11.58	0.66
End	TAG	568.00	0.76	0.31
End	TAA	447.00	0.60	0.24
Tyr	TAT	7989.00	10.70	0.37
Tyr	TAC	13868.00	18.57	0.63
Leu	TTG	10227.00	13.69	0.15
Leu	TTA	5819.00	7.79	0.08
Phe	TTT	10405.00	13.93	0.37
Phe	TTC	18021.00	24.13	0.63
Ser	TCG	7391.00	9.90	0.14
Ser	TCA	8270.00	11.07	0.15
Ser	TCT	9418.00	12.61	0.17
Ser	TCC	11779.00	15.77	0.22
Arg	CGG	6442.00	8.63	0.15
Arg	CGA	3662.00	4.90	0.09
Arg	CGT	4912.00	6.58	0.12
Arg	CGC	9761.00	13.07	0.23
Gln	CAG	17187.00	23.01	0.61
Gln	CAA	11189.00	14.98	0.39
His	CAT	7896.00	10.57	0.43
His	CAC	10355.00	13.86	0.57
Leu	CTG	17602.00	23.57	0.25
Leu	CTA	5856.00	7.84	0.08
Leu	CTT	12255.00	16.41	0.18
Leu	CTC	17598.00	23.56	0.25
Pro	CCG	10802.00	14.46	0.26
Pro	CCA	10609.00	14.20	0.26
Pro	CCT	10004.00	13.39	0.24
Pro	CCC	9946.00	13.32	0.24

Table of mRNA Problem Sequences Used to Scan the Native opd Gene

Name	Sequence
Killer	ATTA
mRNA Deg	AAAA
mRNA Deg	TTTT
mRNA Deg	TTTTRTY
AT String	W {5}
polyA	AANNAA
5' Splice	VRGTRANN
3' Splice	YAGV
mRNA Deg	ATTTA
mRNA Deg	AATAA
polyA	AATAAA
polyA	AATAAT
polyA	AACCAA
5' Splice	ATAGCAA

Summary of Changes to the Native *opd* Gene

	OPD	Maize Optimized OPD
# Base Changes	-	217
% GC	57%	70%
# of Potential Maize mRNA Processing Problems (by sequence analysis with GCG)	40	10

APPENDIX B

OPA AND OPB T1 SEED EXPRESSION DATA

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
OPA0301	0.9680	1.0100	1.0434	0.0130	0.1263	0.5050
	0.5060	0.1860	0.3676	0.0046	0.0233	0.0930
	1.2730	0.1820	0.1430	0.0018	0.0228	0.0910
	1.1490	0.3010	0.2620	0.0033	0.0376	0.1505
	0.4630	1.4850	3.2073	0.0401	0.1856	0.7425
OPA0304	2.1230	0.1230	0.0579	0.0007	0.0154	0.0615
	1.5750	0.0000	0.0000	0.0000	0.0000	0.0000
	1.8140	0.2130	0.1174	0.0015	0.0266	0.1065
	1.8590	0.0000	0.0000	0.0000	0.0000	0.0000
	1.9000	11.4190	6.0100	0.0751	1.4274	5.7095
OPA0305	1.4510	4.4290	3.0524	0.0382	0.5536	2.2145
	1.2570	0.1320	0.1050	0.0013	0.0165	0.0660
	0.0340	0.1880	5.5294	0.0691	0.0235	0.0940
	0.6770	0.4810	0.7105	0.0089	0.0601	0.2405
	0.7040	0.1070	0.1520	0.0019	0.0134	0.0535
OPA0306	6.7330	0.7310	0.1086	0.0014	0.0914	0.3655
	0.9300	0.0000	0.0000	0.0000	0.0000	0.0000
	1.8180	0.0000	0.0000	0.0000	0.0000	0.0000
	2.1220	0.1430	0.0674	0.0008	0.0179	0.0715
	1.7570	0.5470	0.3113	0.0039	0.0684	0.2735
OPA0307	1.4680	0.0000	0.0000	0.0000	0.0000	0.0000
	1.9820	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3020	1.4300	1.0983	0.0137	0.1788	0.7150
	0.9730	0.2390	0.2456	0.0031	0.0299	0.1195
	1.1150	1.0530	0.9444	0.0118	0.1316	0.5265
OPA0310	1.5060	3.9680	2.6348	0.0329	0.4960	1.9840
	1.8700	8.4480	4.5176	0.0565	1.0560	4.2240
	2.9820	0.1170	0.0392	0.0005	0.0146	0.0585
	1.6110	2.5630	1.5909	0.0199	0.3204	1.2815
	1.3460	3.7460	2.7831	0.0348	0.4683	1.8730
OPA0101	1.3650	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6140	0.0000	0.0000	0.0000	0.0000	0.0000
	1.6700	0.1800	0.1078	0.0013	0.0225	0.0900
	1.7890	0.2100	0.1174	0.0015	0.0263	0.1050
	1.4010	0.4060	0.2898	0.0036	0.0508	0.2030
OPA0102	2.2020	0.2400	0.1090	0.0014	0.0300	0.1200

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	1.3480	0.1830	0.1358	0.0017	0.0229	0.0915
	1.5360	0.4180	0.2721	0.0034	0.0523	0.2090
	1.1170	0.1480	0.1325	0.0017	0.0185	0.0740
	1.3390	0.1630	0.1217	0.0015	0.0204	0.0815
OPA0104	1.8360	0.2030	0.1106	0.0014	0.0254	0.1015
	1.8390	0.4050	0.2202	0.0028	0.0506	0.2025
	1.4850	1.3820	0.9306	0.0116	0.1728	0.6910
	0.9770	0.1530	0.1566	0.0020	0.0191	0.0765
	2.4420	0.0000	0.0000	0.0000	0.0000	0.0000
OPA0113	2.9090	0.2070	0.0712	0.0009	0.0259	0.1035
	2.3020	0.2360	0.1025	0.0013	0.0295	0.1180
	1.3020	0.1670	0.1283	0.0016	0.0209	0.0835
	0.8630	0.0000	0.0000	0.0000	0.0000	0.0000
	1.6630	0.4080	0.2453	0.0031	0.0510	0.2040
OPA0114	1.5140	0.1820	0.1202	0.0015	0.0228	0.0910
	1.6390	0.6540	0.3990	0.0050	0.0818	0.3270
	1.4280	0.4410	0.3088	0.0039	0.0551	0.2205
	2.0280	0.3770	0.1859	0.0023	0.0471	0.1885
	2.0960	1.1620	0.5544	0.0069	0.1453	0.5810
OPA0504	2.4730	0.3390	0.1371	0.0017	0.0424	0.1695
	1.3210	0.3930	0.2975	0.0037	0.0491	0.1965
	1.3810	0.1040	0.0753	0.0009	0.0130	0.0520
	2.2330	0.9950	0.4456	0.0056	0.1244	0.4975
	1.4810	0.0000	0.0000	0.0000	0.0000	0.0000
OPA0515	3.4460	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9880	0.0000	0.0000	0.0000	0.0000	0.0000
	2.1190	0.1530	0.0722	0.0009	0.0191	0.0765
	2.4690	0.2840	0.1150	0.0014	0.0355	0.1420
	1.8580	0.1860	0.1001	0.0013	0.0233	0.0930
OPA0402	2.1200	7.2960	3.4415	0.0430	0.9120	3.6480
	1.6770	0.4380	0.2612	0.0033	0.0548	0.2190
	0.1490	0.1330	0.8926	0.0112	0.0166	0.0665
	1.3420	0.1540	0.1148	0.0014	0.0193	0.0770
	1.4140	0.0000	0.0000	0.0000	0.0000	0.0000
OPA0403	1.9990	0.0000	0.0000	0.0000	0.0000	0.0000
	2.0620	0.1380	0.0669	0.0008	0.0173	0.0690
	1.9060	1.3160	0.6905	0.0086	0.1645	0.6580
	1.1510	0.0000	0.0000	0.0000	0.0000	0.0000
	2.0260	1.3240	0.6535	0.0082	0.1655	0.6620
OPA0406	2.9580	12.9930	4.3925	0.0549	1.6241	6.4965
	3.2290	8.2310	2.5491	0.0319	1.0289	4.1155

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	2.1840	18.3230	8.3897	0.1049	2.2904	9.1615
	3.1970	21.4130	6.6978	0.0837	2.6766	10.7065
	3.4270	7.8880	2.3017	0.0288	0.9860	3.9440
OPA0407	1.4500	0.0000	0.0000	0.0000	0.0000	0.0000
	1.6310	0.0000	0.0000	0.0000	0.0000	0.0000
	2.1280	0.0000	0.0000	0.0000	0.0000	0.0000
	1.6160	0.2360	0.1460	0.0018	0.0295	0.1180
	2.1030	0.1210	0.0575	0.0007	0.0151	0.0605
OPA0409	1.4050	0.5050	0.3594	0.0045	0.0631	0.2525
	1.3170	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8390	0.1070	0.1275	0.0016	0.0134	0.0535
	1.5340	0.4130	0.2692	0.0034	0.0516	0.2065
	1.5410	0.4790	0.3108	0.0039	0.0599	0.2395
OPA0410	1.9210	3.1340	1.6314	0.0204	0.3918	1.5670
	3.4450	4.7870	1.3896	0.0174	0.5984	2.3935
	2.3990	23.3640	9.7391	0.1217	2.9205	11.6820
	2.4340	7.9490	3.2658	0.0408	0.9936	3.9745
	2.7430	0.3170	0.1156	0.0014	0.0396	0.1585
OPA2102	0.0050	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0050	2.5840	2.5711	0.0321	0.3230	1.2920
	0.6560	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0170	2.5030	2.4612	0.0308	0.3129	1.2515
	0.3060	0.6600	2.1569	0.0270	0.0825	0.3300
OPA2103	0.4380	6.6140	15.1005	0.1888	0.8268	3.3070
	0.4930	2.1390	4.3387	0.0542	0.2674	1.0695
	0.6100	1.4940	2.4492	0.0306	0.1868	0.7470
	1.6810	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6000	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2104	0.3700	6.8410	18.4892	0.2311	0.8551	3.4205
	0.3860	5.1760	13.4093	0.1676	0.6470	2.5880
	0.0050	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9900	1.7440	1.7616	0.0220	0.2180	0.8720
	0.1470	0.2130	1.4490	0.0181	0.0266	0.1065
OPA2105	1.7360	0.0100	0.0058	0.0001	0.0013	0.0050
	0.1230	0.9460	7.6911	0.0961	0.1183	0.4730
	0.1790	3.6270	20.2626	0.2533	0.4534	1.8135
	0.9840	4.5060	4.5793	0.0572	0.5633	2.2530
	0.6650	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2110	0.0880	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8300	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1320	0.5210	3.9470	0.0493	0.0651	0.2605

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	0.0030	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7060	4.3700	6.1898	0.0774	0.5463	2.1850
OPA2111	0.4550	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4330	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6040	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8090	0.4260	0.5266	0.0066	0.0533	0.2130
	1.1320	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2113	0.5140	0.6620	1.2879	0.0161	0.0828	0.3310
	1.4980	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0480	0.6160	0.5878	0.0073	0.0770	0.3080
	2.0680	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1700	0.9120	5.3647	0.0671	0.1140	0.4560
OPA2114	0.6990	0.2720	0.3891	0.0049	0.0340	0.1360
	0.1780	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0680	0.4770	7.0147	0.0877	0.0596	0.2385
	0.3700	0.7400	2.0000	0.0250	0.0925	0.3700
	0.2950	1.1050	3.7458	0.0468	0.1381	0.5525
OPA1606	0.6750	19.8990	29.4800	0.3685	2.4874	9.9495
	0.6260	14.3360	22.9010	0.2863	1.7920	7.1680
	1.7000	29.2190	17.1876	0.2148	3.6524	14.6095
	0.8620	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2970	14.6560	49.3468	0.6168	1.8320	7.3280
OPA1609	1.5040	26.9730	17.9342	0.2242	3.3716	13.4865
	1.2770	54.1430	42.3986	0.5300	6.7679	27.0715
	0.7790	3.7100	4.7625	0.0595	0.4638	1.8550
	0.0340	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7310	4.1900	5.7319	0.0716	0.5238	2.0950
OPA1610	1.7480	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6800	0.0000	0.0000	0.0000	0.0000	0.0000
	0.5500	0.2630	0.4782	0.0060	0.0329	0.1315
	0.9150	13.5020	14.7563	0.1845	1.6878	6.7510
	1.1200	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2505	2.4700	0.0000	0.0000	0.0000	0.0000	0.0000
	2.0440	6.5970	3.2275	0.0403	0.8246	3.2985
	1.2510	5.2320	4.1823	0.0523	0.6540	2.6160
	0.2890	4.2810	14.8131	0.1852	0.5351	2.1405
	1.8000	20.9900	11.6611	0.1458	2.6238	10.4950
OPA2503	0.6520	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3320	0.5550	0.4167	0.0052	0.0694	0.2775
	1.1560	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3780	16.2310	42.9392	0.5367	2.0289	8.1155

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	1.0550	19.2870	18.2815	0.2285	2.4109	9.6435
OPA2506	0.7880	1.4500	1.8401	0.0230	0.1813	0.7250
	1.9730	0.0000	0.0000	0.0000	0.0000	0.0000
	1.2950	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9910	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7080	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2510	0.7700	1.9040	2.4727	0.0309	0.2380	0.9520
	0.6960	2.2590	3.2457	0.0406	0.2824	1.1295
	0.7930	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8460	0.9450	1.1170	0.0140	0.1181	0.4725
	0.3840	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2515	0.3480	0.0000	0.0000	0.0000	0.0000	0.0000
	1.2230	1.9800	1.6190	0.0202	0.2475	0.9900
	0.2840	1.0170	3.5810	0.0448	0.1271	0.5085
	0.8250	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0010	21.0790	21.0579	0.2632	2.6349	10.5395
OPA1203	1.1640	6.7550	5.8033	0.0725	0.8444	3.3775
	0.4940	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1920	2.0840	10.8542	0.1357	0.2605	1.0420
	0.0070	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9870	1.4170	1.4357	0.0179	0.1771	0.7085
OPA1205	0.6960	2.9070	4.1767	0.0522	0.3634	1.4535
	0.3990	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8450	0.0000	0.0000	0.0000	0.0000	0.0000
	1.2920	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3240	0.0000	0.0000	0.0000	0.0000	0.0000
OPA1207	0.4030	6.5660	16.2928	0.2037	0.8208	3.2830
	0.4450	2.1360	4.8000	0.0600	0.2670	1.0680
	0.7210	2.7060	3.7531	0.0469	0.3383	1.3530
	0.7750	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2180	6.0420	27.7156	0.3464	0.7553	3.0210
OPA1402	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2560	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3410	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4500	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3790	0.0000	0.0000	0.0000	0.0000	0.0000
OPA1210	0.6290	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4850	1.9960	4.1155	0.0514	0.2495	0.9980
	0.5910	1.4540	2.4602	0.0308	0.1818	0.7270
	0.4860	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8200	0.0000	0.0000	0.0000	0.0000	0.0000

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
OPA2602	0.4930	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2500	2.0510	8.2040	0.1026	0.2564	1.0255
	0.4260	0.6010	1.4108	0.0176	0.0751	0.3005
	0.5060	0.3890	0.7688	0.0096	0.0486	0.1945
	0.1690	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2603	0.3050	0.8420	2.7607	0.0345	0.1053	0.4210
	0.6130	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2260	8.1320	35.9823	0.4498	1.0165	4.0660
	0.6220	8.7160	14.0129	0.1752	1.0895	4.3580
	0.1490	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2604	0.2650	0.2680	1.0113	0.0126	0.0335	0.1340
	0.1630	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1820	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1060	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2760	0.0840	0.3043	0.0038	0.0105	0.0420
OPA2607	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4520	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1070	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4290	1.7600	4.1026	0.0513	0.2200	0.8800
	0.0950	0.0000	0.0000	0.0000	0.0000	0.0000
OPA2610	0.0720	0.0370	0.5139	0.0064	0.0046	0.0185
	0.0090	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1600	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0650	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1600	0.1620	1.0125	0.0127	0.0203	0.0810
OPA2611	0.4620	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4530	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1730	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1360	0.0720	0.5294	0.0066	0.0090	0.0360
OPA2613	0.2630	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3280	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1080	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1810	0.0000	0.0000	0.0000	0.0000	0.0000
	0.5330	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0102	0.0710	2.0610	29.0282	0.3629	0.2576	1.0305
	0.3550	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0620	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0740	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0680	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4290	8.2840	19.3100	0.2414	1.0355	4.1420

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
OPB0103	0.1680	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1030	12.9310	125.5437	1.5693	1.6164	6.4655
	0.3870	7.3100	18.8889	0.2361	0.9138	3.6550
	0.1220	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2350	7.9700	33.9149	0.4239	0.9963	3.9850
	0.1310	4.6270	35.3206	0.4415	0.5784	2.3135
OPB0104	0.2560	8.6840	33.9219	0.4240	1.0855	4.3420
	0.2150	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4460	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3110	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1390	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1190	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0202	0.0720	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1990	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1440	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1930	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0900	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0760	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0203	0.3760	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4610	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2740	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4200	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2280	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3080	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0204	0.1340	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2610	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0760	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0910	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3350	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0209	0.4860	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2970	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3900	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4140	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4440	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0210	0.4870	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3670	0.0000	0.0000	0.0000	0.0000	0.0000
	0.2140	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1100	0.0000	0.0000	0.0000	0.0000	0.0000

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	0.3070	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0303	1.4110	3.7830	2.6811	0.0335	0.4729	1.8915
	0.9440	0.0000	0.0000	0.0000	0.0000	0.0000
	1.4480	4.1650	2.8764	0.0360	0.5206	2.0825
	2.0220	3.5520	1.7567	0.0220	0.4440	1.7760
	1.4870	0.0000	0.0000	0.0000	0.0000	0.0000
	1.7780	4.0550	2.2807	0.0285	0.5069	2.0275
OPB0305	0.3460	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1970	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3250	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9630	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1490	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1240	3.3990	3.0240	0.0378	0.4249	1.6995
OPB0306	0.6890	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8890	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3520	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7860	2.0310	2.5840	0.0323	0.2539	1.0155
	0.7640	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0705	0.6940	0.2480	0.3573	0.0045	0.0310	0.1240
	1.3980	0.0000	0.0000	0.0000	0.0000	0.0000
	0.5040	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8350	0.7820	0.9365	0.0117	0.0978	0.3910
	1.1160	2.2630	2.0278	0.0253	0.2829	1.1315
	0.5310	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0706	1.2110	7.6430	6.3113	0.0789	0.9554	3.8215
	0.7660	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8220	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8100	5.0650	6.2531	0.0782	0.6331	2.5325
	1.9450	5.4810	2.8180	0.0352	0.6851	2.7405
	1.3480	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0709	0.3560	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7290	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8400	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7110	2.5390	3.5710	0.0446	0.3174	1.2695
	1.3120	0.9270	0.7066	0.0088	0.1159	0.4635
	1.0250	1.7490	1.7063	0.0213	0.2186	0.8745
OPB0712	1.1850	6.3610	5.3679	0.0671	0.7951	3.1805
	0.6510	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1180	2.8190	2.5215	0.0315	0.3524	1.4095
	1.2660	0.0000	0.0000	0.0000	0.0000	0.0000

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	0.8280	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0860	7.4120	6.8250	0.0853	0.9265	3.7060
OPB0714	0.9530	0.0000	0.0000	0.0000	0.0000	0.0000
	0.4860	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8270	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0050	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8830	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1640	2.4900	2.1392	0.0267	0.3113	1.2450
OPB0715	0.7540	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8770	0.0000	0.0000	0.0000	0.0000	0.0000
	0.1930	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7750	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6140	2.2710	3.6987	0.0462	0.2839	1.1355
	2.0950	5.7230	2.7317	0.0341	0.7154	2.8615
OPB0716	2.2330	10.8140	4.8428	0.0605	1.3518	5.4070
	0.9290	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1440	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3860	0.0000	0.0000	0.0000	0.0000	0.0000
	0.3250	6.5790	20.2431	0.2530	0.8224	3.2895
	1.0030	4.7100	4.6959	0.0587	0.5888	2.3550
OPB0803	1.3520	2.3240	1.7189	0.0215	0.2905	1.1620
	0.5220	3.7270	7.1398	0.0892	0.4659	1.8635
	1.3820	1.9530	1.4132	0.0177	0.2441	0.9765
	0.3940	0.9750	2.4746	0.0309	0.1219	0.4875
	0.5330	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0150	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0804	0.8700	6.4940	7.4644	0.0933	0.8118	3.2470
	0.6750	5.8420	8.6548	0.1082	0.7303	2.9210
	1.2830	8.8360	6.8870	0.0861	1.1045	4.4180
	1.7840	12.4470	6.9770	0.0872	1.5559	6.2235
	1.7440	2.8260	1.6204	0.0203	0.3533	1.4130
	1.3130	9.6100	7.3191	0.0915	1.2013	4.8050
OPB0805	1.1570	0.0000	0.0000	0.0000	0.0000	0.0000
	0.9000	10.6630	11.8478	0.1481	1.3329	5.3315
	1.6050	9.8340	6.1271	0.0766	1.2293	4.9170
	0.5660	0.0000	0.0000	0.0000	0.0000	0.0000
	4.7860	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3470	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0806	0.3990	4.9080	12.3008	0.1538	0.6135	2.4540
	0.8840	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7660	0.0000	0.0000	0.0000	0.0000	0.0000

Event	Protein	Units/ml	Units/mg	%TSP	mg OPH total	ng/mg Seed
	0.5800	5.3440	9.2138	0.1152	0.6680	2.6720
	1.3050	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7610	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0809	1.0440	11.2930	10.8170	0.1352	1.4116	5.6465
	0.7880	4.9960	6.3401	0.0793	0.6245	2.4980
	0.9650	0.0000	0.0000	0.0000	0.0000	0.0000
	0.6830	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7580	8.6670	11.4340	0.1429	1.0834	4.3335
	0.7260	10.0450	13.8361	0.1730	1.2556	5.0225
OPB0813	0.8580	6.2590	7.2949	0.0912	0.7824	3.1295
	0.5620	6.1230	10.8950	0.1362	0.7654	3.0615
	0.3210	0.0000	0.0000	0.0000	0.0000	0.0000
	0.8550	0.0000	0.0000	0.0000	0.0000	0.0000
	1.1800	4.6320	3.9254	0.0491	0.5790	2.3160
	1.8170	0.0000	0.0000	0.0000	0.0000	0.0000
OPB0901	1.7930	0.0000	0.0000	0.0000	0.0000	0.0000
	1.2660	0.0000	0.0000	0.0000	0.0000	0.0000
	1.0190	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7410	0.0000	0.0000	0.0000	0.0000	0.0000
	0.7940	13.8030	17.3841	0.2173	1.7254	6.9015
	1.0870	34.1080	31.3781	0.3922	4.2635	17.0540
OPB0905	1.0950	0.0000	0.0000	0.0000	0.0000	0.0000
	1.4910	0.0000	0.0000	0.0000	0.0000	0.0000
	1.8100	31.0580	17.1591	0.2145	3.8823	15.5290
	1.1660	0.0000	0.0000	0.0000	0.0000	0.0000
	1.3630	10.8400	7.9530	0.0994	1.3550	5.4200
	0.9100	11.0250	12.1154	0.1514	1.3781	5.5125

APPENDIX C

OPH EXPRESSION IN T2 AND HIGHER SEED

T2 Greenhouse Grown Expression

OPA0403-2

Sample	dABS/min	Volume	Protein	Units/ml	Total Units	Average	Total Units	Units/mg Protein	% TSP
T2 Extraction 1	0.3394	31	1.5820	4.0129	124.4001	129.3849	162.6492	2.5366	*0.0317*
	0.3565	31		4.2151	130.6677				
	0.3631	31		4.2931	133.0868				
T2 Extraction 2	0.0998	27	1.4280	1.1800	31.8597	33.2643		0.8263	*0.0103*
	0.1134	27		1.3408	36.2013				
	0.0994	27		1.1753	31.7320				

	Units/g Seed	ng OPH/mg Seed
T2 Total Extraction	10.1529	*1.2691*

T2 Bulk Sample of 16.02g (50 Seeds)
First extration was 1 Hour at Room Temp with 3ml buffer/g seed
Second Extraction was 30 min with same buffer/seed ratio
Protein measured by Bradford and expressed in mg/ml

OPA0411-2

Sample	dABS/min	Volume	Protein	Units/ml	Total Units	Average	Total Units	Units/mg Protein	% TSP
	1.1923	31	3.3380	14.0972	437.0130	410.6840	410.6840	3.9688	0.0496
	1.0936	31		12.9302	400.8366				
	1.0755	31		12.7162	394.2024				

Units/g Seed	ng OPH/mg Seed
27.4705	3.4338

T3 Greenhouse Grown Expression

OPA0411 Plant	Protein	Units/ml	Units/mg Protein	%TSP	Total mg OPH	ng/mg Seed
6	0.604	1.191	1.971	***0.025***	2.977	***0.475***
8	1.267	5.064	3.997	***0.050***	12.661	***2.022***
12	2.550	15.409	6.043	***0.076***	38.523	***6.153***
16	2.146	0.341	0.159	***0.002***	0.852	***0.136***
18	0.390	4.025	10.320	***0.129***	10.062	***1.607***
21	1.836	12.069	6.573	***0.082***	30.172	***4.819***
44	1.085	3.976	3.665	***0.046***	9.941	***1.588***
47	0.288	4.272	14.833	***0.185***	10.680	***1.706***
49	1.964	10.766	5.481	***0.069***	26.914	***4.299***
51	1.866	18.198	9.752	***0.122***	45.494	***7.266***
OPA0403 Plant						
1	1.133	1.479	1.305	***0.016***	3.698	***0.591***
4	1.112	2.265	2.037	***0.025***	5.663	***0.904***
5	1.046	0	0.000	***0.000***	0.000	***0.000***
8	0.692	0.382	0.552	***0.007***	0.955	***0.153***
10	0.957	1.909	1.995	***0.025***	4.773	***0.762***

T2 and T3 Field Expression

OPA Field
Illinois Summer 2002

Plant	Protein	dABS	Units/ml	Units/mg	Average (Units/mg)	%TSP	ng/mg protein	ng/mg Seed
OPA04-3079-IL02-06	2.7450	0.0444	0.5250	0.1912	0.1707	0.00213%	23.9055	0.3281
		0.0366	0.4327	0.1576				
		0.0379	0.4481	0.1632				
OPA04-3081-IL02-06	3.2660	0.0744	0.8797	0.2693	0.2528	0.00316%	33.6677	0.5498
		0.0713	0.8430	0.2581				
		0.0638	0.7543	0.2310				
OPA04-4030-IL02-06	3.0540	0.0632	0.7472	0.2447	0.2386	0.00298%	30.5848	0.4670
		0.0604	0.7141	0.2338				
		0.0613	0.7248	0.2373				

OPB Field
Nebraska Summer 2002

Extraction 1 Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
1	NE021334-3	OPB0109	0.480	2.975	6.198	0.077	1.488	0.186	1.859
2	NE021334)1	OPB0109	0.463	8.055	17.397	0.217	4.028	0.503	5.034
3	NE021333-A2	OPB0107	0.461	0.597	1.295	0.016	0.299	0.037	0.373
4	NE021333-3	OPB0107	0.587	0.000	0.000	0.000	0.000	0.000	0.000
5	NE021333)2	OPB0107	1.212	18.330	15.124	0.189	9.165	1.146	11.456
6	NE021333)1	OPB0107	0.543	4.288	7.897	0.099	2.144	0.268	2.680
7	NE021332-5	OPB0107	0.538	0.000	0.000	0.000	0.000	0.000	0.000
8	NE021332-4	OPB0107	0.588	1.351	2.298	0.029	0.676	0.084	0.844
9	NE021332-3	OPB0107	0.561	0.174	0.310	0.004	0.087	0.011	0.109
10	NE021332)2	OPB0107	0.321	0.000	0.000	0.000	0.000	0.000	0.000
11	NE021332)1	OPB0107	0.897	7.795	8.690	0.109	3.898	0.487	4.872
12	NE021331-5	OPB0107	0.481	0.000	0.000	0.000	0.000	0.000	0.000
13	NE021331-4	OPB0107	0.504	0.000	0.000	0.000	0.000	0.000	0.000
14	NE021331-3	OPB0107	0.762	0.316	0.415	0.005	0.158	0.020	0.198
15	NE021331)2	OPB0107	1.009	13.282	13.164	0.165	6.641	0.830	8.301
16	NE021331)1	OPB0107	0.497	6.072	12.217	0.153	3.036	0.380	3.795
17	NE021330-5	OPB0107	1.016	0.000	0.000	0.000	0.000	0.000	0.000
18	NE021330-4	OPB0107	0.746	0.000	0.000	0.000	0.000	0.000	0.000
19	NE021330-3	OPB0107	0.897	5.325	5.936	0.074	2.663	0.333	3.328
20	NE021330)2	OPB0107	1.182	0.753	0.637	0.008	0.377	0.047	0.471
21	NE021330)1	OPB0107	0.586	1.850	3.157	0.039	0.925	0.116	1.156
22	NE021328-6	OPB0107	0.433	20.858	48.171	0.602	10.429	1.304	13.036

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
23	NE021328-5	OPB0107	0.966	0.000	0.000	0.000	0.000	0.000	0.000
24	NE021328-4	OPB0107	0.862	0.000	0.000	0.000	0.000	0.000	0.000
25	NE021328-3	OPB0107	0.533	12.464	23.385	0.292	6.232	0.779	7.790
26	NE021360-5	OPB0302	0.908	4.822	5.311	0.066	2.411	0.301	3.014
27	NE021360-3	OPB0302	1.153	13.771	11.944	0.149	6.886	0.861	8.607
28	NE021358-6	OPB0116	0.430	1.332	3.098	0.039	0.666	0.083	0.833
29	NE021358-5	OPB0116	1.104	3.884	3.518	0.044	1.942	0.243	2.428
30	NE021358-A3	OPB0116	0.427	6.109	14.307	0.179	3.055	0.382	3.818
31	NE021358-A2	OPB0116	0.172	0.000	0.000	0.000	0.000	0.000	0.000
32	NE021358)1	OPB0116	0.301	6.408	21.289	0.266	3.204	0.401	4.005
33	NE021357-7	OPB0111	1.309	3.711	2.835	0.035	1.856	0.232	2.319
34	NE021357-5	OPB0111	0.160	3.892	24.325	0.304	1.946	0.243	2.433
35	NE021357-4	OPB0111	0.590	8.157	13.825	0.173	4.079	0.510	5.098
36	NE021357-3	OPB0111	0.285	4.461	15.653	0.196	2.231	0.279	2.788
37	NE021338-3	OPB0111	0.792	10.404	13.136	0.164	5.202	0.650	6.503
38	NE021338)2	OPB0111	0.780	14.775	18.942	0.237	7.388	0.923	9.234
39	NE021338)1	OPB0111	0.491	0.000	0.000	0.000	0.000	0.000	0.000
40	NE021337-6	OPB0111	0.609	6.213	10.202	0.128	3.107	0.388	3.883
41	NE021337-4	OPB0111	1.136	4.557	4.011	0.050	2.279	0.285	2.848
42	NE021337-3	OPB0111	0.822	9.207	11.201	0.140	4.604	0.575	5.754
43	NE021337)1	OPB0111	0.235	9.814	41.762	0.522	4.907	0.613	6.134
44	NE021336-6	OPB0111	0.672	7.736	11.512	0.144	3.868	0.484	4.835
45	NE021336-5	OPB0111	0.746	0.000	0.000	0.000	0.000	0.000	0.000
46	NE021336-4	OPB0111	0.360	4.333	12.036	0.150	2.167	0.271	2.708
47	NE021336)2	OPB0111	1.059	3.700	3.494	0.044	1.850	0.231	2.313
48	NE021336)1	OPB0111	0.551	0.608	1.103	0.014	0.304	0.038	0.380
49	NE021334-5	OPB0109	1.086	0.671	0.618	0.008	0.336	0.042	0.419
50	NE021334-4	OPB0109	0.560	3.803	6.791	0.085	1.902	0.238	2.377
51	NE021369-4	OPB0309	0.550	5.692	10.349	0.129	2.846	0.356	3.558

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
52	NE021369-3	OPB0309	1.371	7.268	5.301	0.066	3.634	0.454	4.543
53	NE021368-5	OPB0308	1.183	6.276	5.305	0.066	3.138	0.392	3.923
54	NE021367-3	OPB0307	0.956	0.000	0.000	0.000	0.000	0.000	0.000
55	NE021367)2	OPB0307	1.314	16.887	12.852	0.161	8.444	1.055	10.554
56	NE021364-7	OPB0306	0.736	17.813	24.202	0.303	8.907	1.113	11.133
57	NE021364-6	OPB0306	1.181	13.299	11.261	0.141	6.650	0.831	8.312
58	NE021364-5	OPB0306	0.053	0.000	0.000	0.000	0.000	0.000	0.000
59	NE021363-6	OPB0305	0.621	0.000	0.000	0.000	0.000	0.000	0.000
60	NE021363-5	OPB0305	0.602	9.782	16.249	0.203	4.891	0.611	6.114
61	NE021363)2	OPB0305	0.807	18.640	23.098	0.289	9.320	1.165	11.650
62	NE021363)1	OPB0305	1.556	12.948	8.321	0.104	6.474	0.809	8.093
63	NE021362-A0	OPB0304	0.683	16.302	23.868	0.298	8.151	1.019	10.189
64	NE021362-9	OPB0304	0.445	0.000	0.000	0.000	0.000	0.000	0.000
65	NE021362-3	OPB0304	1.189	12.886	10.838	0.135	6.443	0.805	8.054
66	NE021362)2	OPB0304	1.170	14.581	12.462	0.156	7.291	0.911	9.113
67	NE021362)1	OPB0304	0.889	7.077	7.961	0.100	3.539	0.442	4.423
68	NE021361-7	OPB0303	0.406	0.000	0.000	0.000	0.000	0.000	0.000
69	NE021361-5	OPB0303	0.566	2.193	3.875	0.048	1.097	0.137	1.371
70	NE021361-4	OPB0303	0.543	7.022	12.932	0.162	3.511	0.439	4.389
71	NE021361-3	OPB0303	1.381	8.738	6.327	0.079	4.369	0.546	5.461
72	NE021361)2	OPB0303	0.662	16.463	24.869	0.311	8.232	1.029	10.289
73	NE021360-8	OPB0302	0.581	8.073	13.895	0.174	4.037	0.505	5.046
74	NE021360-7	OPB0302	1.193	12.005	10.063	0.126	6.003	0.750	7.503
75	NE021360-6	OPB0302	1.008	15.159	15.039	0.188	7.580	0.947	9.474
76	NE021315-3	OPB0103	0.767	0.000	0.000	0.000	0.000	0.000	0.000
77	NE021314-3	OPB0103	0.946	14.547	15.377	0.192	7.274	0.909	9.092
78	NE021314-4	OPB0103	0.623	0.000	0.000	0.000	0.000	0.000	0.000
79	NE021314-5	OPB0103	0.813	0.000	0.000	0.000	0.000	0.000	0.000
79	NE021315)1	OPB0103	0.652	11.612	17.810	0.223	5.806	0.726	7.258

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
80	NE021315)2	OPB0103	0.774	0.000	0.000	0.000	0.000	0.000	0.000
81	NE021313-4	OPB0102	0.515	1.779	3.454	0.043	0.890	0.111	1.112
82	NE021313-5	OPB0102	1.458	3.411	2.340	0.029	1.706	0.213	2.132
83	NE021313-6	OPB0102	0.925	0.000	0.000	0.000	0.000	0.000	0.000
84	NE021314)1	OPB0103	0.553	6.060	10.958	0.137	3.030	0.379	3.788
85	NE021314)2	OPB0103	0.398	0.000	0.000	0.000	0.000	0.000	0.000
86	NE021312-3	OPB0102	1.171	0.000	0.000	0.000	0.000	0.000	0.000
87	NE021312-4	OPB0102	0.858	0.000	0.000	0.000	0.000	0.000	0.000
88	NE021312-5	OPB0102	0.917	0.000	0.000	0.000	0.000	0.000	0.000
89	NE021313)1	OPB0102	1.199	11.796	9.838	0.123	5.898	0.737	7.373
90	NE021313-3	OPB0102	1.157	13.755	11.889	0.149	6.878	0.860	8.597
91	NE021311)2	OPB0102	0.544	11.752	21.603	0.270	5.876	0.735	7.345
92	NE021311-3	OPB0102	1.040	0.654	0.629	0.008	0.327	0.041	0.409
93	NE021311-4	OPB0102	0.973	0.002	0.002	0.000	0.001	0.000	0.001
94	NE021311-5	OPB0102	2.124	8.693	4.093	0.051	4.347	0.543	5.433
95	NE021312)1	OPB0102	0.646	8.277	12.813	0.160	4.139	0.517	5.173
96	NE021320-4	OPB0104	0.720	2.578	3.581	0.045	1.289	0.161	1.611
97	NE021320-5	OPB0104	0.707	2.220	3.140	0.039	1.110	0.139	1.388
98	NE021319-3	OPB0104	0.306	0.000	0.000	0.000	0.000	0.000	0.000
99	NE021319-4	OPB0104	0.674	0.000	0.000	0.000	0.000	0.000	0.000
100	NE021320)1	OPB0104	1.557	17.166	11.025	0.138	8.583	1.073	10.729
101	NE021320)2	OPB0104	1.912	0.000	0.000	0.000	0.000	0.000	0.000
102	NE021320-3	OPB0104	1.263	0.000	0.000	0.000	0.000	0.000	0.000
103	NE021318-3	OPB0103	0.980	3.713	3.789	0.047	1.857	0.232	2.321
104	NE021318-4	OPB0103	0.700	0.000	0.000	0.000	0.000	0.000	0.000
105	NE021318-5	OPB0103	0.746	5.599	7.505	0.094	2.800	0.350	3.499
106	NE021319)1	OPB0104	1.714	4.812	2.807	0.035	2.406	0.301	3.008
107	NE021319)2	OPB0104	1.535	12.838	8.364	0.105	6.419	0.802	8.024
108	NE021317)1	OPB0103	0.778	17.094	21.972	0.275	8.547	1.068	10.684

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
109	NE021317)2	OPB0103	0.737	17.475	23.711	0.296	8.738	1.092	10.922
110	NE021317-3	OPB0103	0.878	10.284	11.713	0.146	5.142	0.643	6.428
111	NE021317-4	OPB0103	0.389	0.000	0.000	0.000	0.000	0.000	0.000
112	NE021318)1	OPB0103	1.147	9.998	8.717	0.109	4.999	0.625	6.249
113	NE021311)1	OPB0103	0.964	9.977	10.350	0.129	4.989	0.624	6.236
114	NE021371)2	OPB0411	0.545	7.090	13.009	0.163	3.545	0.443	4.431
115	NE021360-1	OPB0302	0.897	27.590	30.758	0.384	13.795	1.724	17.244
116	NE021376-4	OPB0420	0.582	2.719	4.672	0.058	1.360	0.170	1.699
117	NE021376-3	OPB0420	0.759	6.266	8.256	0.103	3.133	0.392	3.916
118	NE021376)2	OPB0420	0.778	1.533	1.970	0.025	0.767	0.096	0.958
119	NE021375)1	OPB0419	1.796	19.737	10.989	0.137	9.869	1.234	12.336
120	NE021371-4	OPB0411	0.679	5.322	7.838	0.098	2.661	0.333	3.326
121	NE021370-6	OPB0311	1.839	6.582	3.579	0.045	3.291	0.411	4.114
122	NE021370-5	OPB0311	1.670	11.247	6.735	0.084	5.624	0.703	7.029
123	NE021370-4	OPB0311	1.087	8.871	8.161	0.102	4.436	0.554	5.544
124	NE021370-3	OPB0311	2.415	6.896	2.855	0.036	3.448	0.431	4.310
125	NE021369-9	OPB0304	0.733	0.000	0.000	0.000	0.000	0.000	0.000
126	NE021369-5	OPB0304	0.759	12.038	15.860	0.198	6.019	0.752	7.524
127	NE021321)1	OPB0104	1.279	19.794	15.476	0.193	9.897	1.237	12.371
128	NE021321-3	OPB0104	1.324	3.164	2.390	0.030	1.582	0.198	1.978
129	NE021321-4	OPB0104	0.666	2.430	3.649	0.046	1.215	0.152	1.519
130	NE021324)1	OPB0104	0.783	13.307	16.995	0.212	6.654	0.832	8.317
131	NE021324)2	OPB0104	0.873	17.933	20.542	0.257	8.967	1.121	11.208
132	NE021324-6	OPB0104	0.661	0.000	0.000	0.000	0.000	0.000	0.000
133	NE021324-8	OPB0104	0.504	8.479	16.823	0.210	4.240	0.530	5.299
134	NE021324-A0	OPB0104	0.295	0.000	0.000	0.000	0.000	0.000	0.000
135	NE021325)1	OPB0104	1.136	22.322	19.650	0.246	11.161	1.395	13.951
136	NE021325)2	OPB0104	1.568	22.878	14.591	0.182	11.439	1.430	14.299
137	NE021325-3	OPB0104	0.736	0.000	0.000	0.000	0.000	0.000	0.000

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
138	NE021325-4	OPB0104	0.574	0.000	0.000	0.000	0.000	0.000	0.000
139	NE021326)1	OPB0106	0.781	22.764	29.147	0.364	11.382	1.423	14.228
140	NE021326)2	OPB0106	0.713	7.262	10.185	0.127	3.631	0.454	4.539
141	NE021326-3	OPB0106	2.827	13.326	4.714	0.059	6.663	0.833	8.329
142	NE021326-4	OPB0106	0.703	14.591	20.755	0.259	7.296	0.912	9.119
143	NE021326-5	OPB0106	0.747	11.377	15.230	0.190	5.689	0.711	7.111
144	NE021327)1	OPB0106	4.386	0.000	0.000	0.000	0.000	0.000	0.000
145	NE021327)2	OPB0106	6.734	12.872	1.911	0.024	6.436	0.805	8.045
146	NE021327-3	OPB0106	3.768	0.000	0.000	0.000	0.000	0.000	0.000
147	NE021327-4	OPB0106	4.583	6.213	1.356	0.017	3.107	0.388	3.883
148	NE021327-5	OPB0106	6.653	2.625	0.395	0.005	1.313	0.164	1.641
149	NE021327-6	OPB0106	4.374	0.000	0.000	0.000	0.000	0.000	0.000
150	NE021327-7	OPB0106	4.937	2.717	0.550	0.007	1.359	0.170	1.698
151	NE021328)2	OPB0107	4.484	25.425	5.670	0.071	12.713	1.589	15.891
152	OPA0411T2	OPA0411	3.429	17.142	4.999	0.062	8.571	1.071	10.714
153	OPB0310GH	OPB0310	6.997	0.769	0.110	0.001	0.385	0.048	0.481
154	LH244	LH244	3.639	0.000	0.000	0.000	0.000	0.000	0.000
155	LH244	LH244	3.463	0.000	0.000	0.000	0.000	0.000	0.000

Extraction 2 Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	ug OPH	ng/mg seed
1	NE021334-3	OPB0109	0.436	0.000	0.000	0.000	0.000	0.000	0.000
2	NE021334)1	OPB0109	0.666	5.637	8.464	0.106	2.819	0.352	3.523
3	NE021333-A2	OPB0107	0.262	0.000	0.000	0.000	0.000	0.000	0.000
4	NE021333-3	OPB0107	0.375	0.000	0.000	0.000	0.000	0.000	0.000

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
5	NE021333)2	OPB0107	0.974	15.367	15.777	0.197	7.684	0.960	9.604
6	NE021333)1	OPB0107	0.883	9.897	11.208	0.140	4.949	0.619	6.186
7	NE021332-5	OPB0107	0.527	0.000	0.000	0.000	0.000	0.000	0.000
8	NE021332-4	OPB0107	0.365	1.309	3.586	0.045	0.655	0.082	0.818
9	NE021332-3	OPB0107	0.924	3.168	3.429	0.043	1.584	0.198	1.980
10	NE021332)2	OPB0107	0.994	0.000	0.000	0.000	0.000	0.000	0.000
11	NE021332)1	OPB0107	0.891	8.163	9.162	0.115	4.082	0.510	5.102
12	NE021331-5	OPB0107	0.632	3.680	5.823	0.073	1.840	0.230	2.300
13	NE021331-4	OPB0107	0.399	0.000	0.000	0.000	0.000	0.000	0.000
14	NE021331-3	OPB0107	1.630	4.592	2.817	0.035	2.296	0.287	2.870
15	NE021331)2	OPB0107	0.973	11.254	11.566	0.145	5.627	0.703	7.034
16	NE021331)1	OPB0107	0.532	3.454	6.492	0.081	1.727	0.216	2.159
17	NE021330-5	OPB0107	0.736	0.598	0.813	0.010	0.299	0.037	0.374
18	NE021330-4	OPB0107	0.693	0.000	0.000	0.000	0.000	0.000	0.000
19	NE021330-3	OPB0107	0.286	0.376	1.315	0.016	0.188	0.024	0.235
20	NE021330)2	OPB0107	1.141	3.531	3.095	0.039	1.766	0.221	2.207
21	NE021330)1	OPB0107	0.828	3.537	4.272	0.053	1.769	0.221	2.211
22	NE021328-6	OPB0107	0.964	13.346	13.844	0.173	6.673	0.834	8.341
23	NE021328-5	OPB0107	0.647	0.000	0.000	0.000	0.000	0.000	0.000
24	NE021328-4	OPB0107	0.796	0.000	0.000	0.000	0.000	0.000	0.000
25	NE021328-3	OPB0107	0.476	8.478	17.811	0.223	4.239	0.530	5.299
26	NE021360-5	OPB0302	0.548	0.000	0.000	0.000	0.000	0.000	0.000
27	NE021360-3	OPB0302	0.409	10.786	26.372	0.330	5.393	0.674	6.741
28	NE021358-6	OPB0116	0.599	0.000	0.000	0.000	0.000	0.000	0.000
29	NE021358-5	OPB0116	1.055	3.681	3.489	0.044	1.841	0.230	2.301
30	NE021358-A3	OPB0116	0.237	8.207	34.629	0.433	4.104	0.513	5.129
31	NE021358-A2	OPB0116	0.213	0.000	0.000	0.000	0.000	0.000	0.000
32	NE021358)1	OPB0116	0.764	4.442	5.814	0.073	2.221	0.278	2.776
33	NE021357-7	OPB0111	0.858	0.000	0.000	0.000	0.000	0.000	0.000

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
34	NE021357-5	OPB0111	0.328	0.000	0.000	0.000	0.000	0.000	0.000
35	NE021357-4	OPB0111	0.309	0.000	0.000	0.000	0.000	0.000	0.000
36	NE021357-3	OPB0111	0.181	0.000	0.000	0.000	0.000	0.000	0.000
37	NE021338-3	OPB0111	0.803	5.920	7.372	0.092	2.960	0.370	3.700
38	NE021338)2	OPB0111	1.394	18.650	13.379	0.167	9.325	1.166	11.656
39	NE021338)1	OPB0111	0.382	0.000	0.000	0.000	0.000	0.000	0.000
40	NE021337-6	OPB0111	0.850	0.455	0.535	0.007	0.228	0.028	0.284
41	NE021337-4	OPB0111	1.280	4.593	3.588	0.045	2.297	0.287	2.871
42	NE021337-3	OPB0111	0.550	5.533	10.060	0.126	2.767	0.346	3.458
43	NE021337)1	OPB0111	0.462	12.842	27.797	0.347	6.421	0.803	8.026
44	NE021336-6	OPB0111	0.760	6.403	8.425	0.105	3.202	0.400	4.002
45	NE021336-5	OPB0111	0.712	0.000	0.000	0.000	0.000	0.000	0.000
46	NE021336-4	OPB0111	1.358	9.896	7.287	0.091	4.948	0.619	6.185
47	NE021336)2	OPB0111	0.267	0.000	0.000	0.000	0.000	0.000	0.000
48	NE021336)1	OPB0111	0.415	0.000	0.000	0.000	0.000	0.000	0.000
49	NE021334-5	OPB0109	0.417	0.000	0.000	0.000	0.000	0.000	0.000
50	NE021334-4	OPB0109	0.096	0.285	2.969	0.037	0.143	0.018	0.178
51	NE021369-4	OPB0309	0.441	9.398	21.311	0.266	4.699	0.587	5.874
52	NE021369-3	OPB0309	1.430	9.600	6.713	0.084	4.800	0.600	6.000
53	NE021368-5	OPB0308	0.418	3.683	8.811	0.110	1.842	0.230	2.302
54	NE021367-3	OPB0307	0.725	0.587	0.810	0.010	0.294	0.037	0.367
55	NE021367)2	OPB0307	1.197	14.903	12.450	0.156	7.452	0.931	9.314
56	NE021364-7	OPB0306	0.951	20.797	21.869	0.273	10.399	1.300	12.998
57	NE021364-6	OPB0306	1.399	17.493	12.504	0.156	8.747	1.093	10.933
58	NE021364-5	OPB0306	0.706	4.650	6.586	0.082	2.325	0.291	2.906
59	NE021363-6	OPB0305	0.569	3.809	6.694	0.084	1.905	0.238	2.381
60	NE021363-5	OPB0305	0.701	8.902	12.699	0.159	4.451	0.556	5.564
61	NE021363)2	OPB0305	0.661	15.447	23.369	0.292	7.724	0.965	9.654
62	NE021363)1	OPB0305	0.733	9.706	13.241	0.166	4.853	0.607	6.066

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
63	NE021362-A0	OPB0304	0.687	17.936	26.108	0.326	8.968	1.121	11.210
64	NE021362-9	OPB0304	0.976	2.530	2.592	0.032	1.265	0.158	1.581
65	NE021362-3	OPB0304	0.469	5.559	11.853	0.148	2.780	0.347	3.474
66	NE021362)2	OPB0304	1.058	12.205	11.536	0.144	6.103	0.763	7.628
67	NE021362)1	OPB0304	0.576	1.472	2.556	0.032	0.736	0.092	0.920
68	NE021361-7	OPB0303	0.473	0.000	0.000	0.000	0.000	0.000	0.000
69	NE021361-5	OPB0303	1.410	10.356	7.345	0.092	5.178	0.647	6.473
70	NE021361-4	OPB0303	1.036	11.636	11.232	0.140	5.818	0.727	7.273
71	NE021361-3	OPB0303	1.298	7.244	5.581	0.070	3.622	0.453	4.528
72	NE021361)2	OPB0303	1.259	19.134	15.198	0.190	9.567	1.196	11.959
73	NE021360-8	OPB0302	0.878	5.214	5.938	0.074	2.607	0.326	3.259
74	NE021360-7	OPB0302	1.420	10.329	7.274	0.091	5.165	0.646	6.456
75	NE021360-6	OPB0302	0.546	9.453	17.313	0.216	4.727	0.591	5.908
76	NE021315-3	OPB0103	0.416	0.000	0.000	0.000	0.000	0.000	0.000
77	NE021314-3	OPB0103	1.138	15.128	13.293	0.166	7.564	0.946	9.455
78	NE021314-4	OPB0103	0.913	1.857	2.034	0.025	0.929	0.116	1.161
79	NE021314-5	OPB0103	1.006	0.000	0.000	0.000	0.000	0.000	0.000
79	NE021315)1	OPB0103	0.793	10.892	13.735	0.172	5.446	0.681	6.808
80	NE021315)2	OPB0103	1.331	0.000	0.000	0.000	0.000	0.000	0.000
81	NE021313-4	OPB0102	1.390	1.415	1.018	0.013	0.708	0.088	0.884
82	NE021313-5	OPB0102	0.707	0.000	0.000	0.000	0.000	0.000	0.000
83	NE021313-6	OPB0102	0.796	0.000	0.000	0.000	0.000	0.000	0.000
84	NE021314)1	OPB0103	1.121	8.344	7.443	0.093	4.172	0.522	5.215
85	NE021314)2	OPB0103	0.466	0.000	0.000	0.000	0.000	0.000	0.000
86	NE021312-3	OPB0102	1.738	0.000	0.000	0.000	0.000	0.000	0.000
87	NE021312-4	OPB0102	1.856	0.000	0.000	0.000	0.000	0.000	0.000
88	NE021312-5	OPB0102	0.659	0.000	0.000	0.000	0.000	0.000	0.000
89	NE021313)1	OPB0102	1.345	6.623	4.924	0.062	3.312	0.414	4.139
90	NE021313-3	OPB0102	0.840	0.000	0.000	0.000	0.000	0.000	0.000

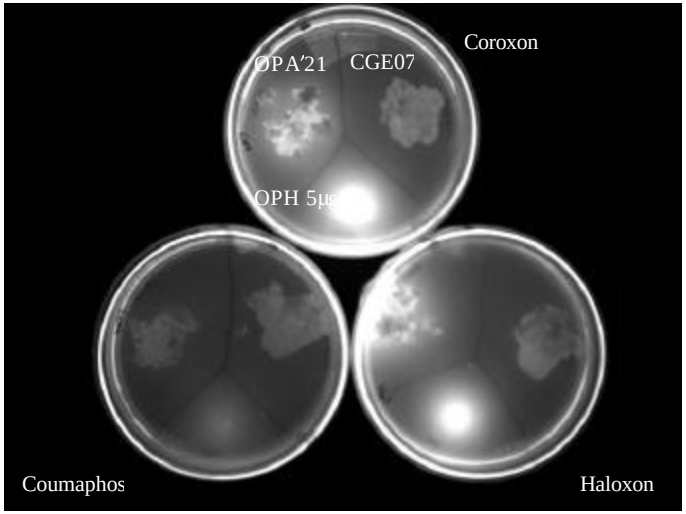
Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
91	NE021311)2	OPB0102	2.107	13.620	6.464	0.081	6.810	0.851	8.513
92	NE021311-3	OPB0102	1.037	2.297	2.215	0.028	1.149	0.144	1.436
93	NE021311-4	OPB0102	1.568	2.437	1.554	0.019	1.219	0.152	1.523
94	NE021311-5	OPB0102	1.941	8.606	4.434	0.055	4.303	0.538	5.379
95	NE021312)1	OPB0102	0.951	9.281	9.759	0.122	4.641	0.580	5.801
96	NE021320-4	OPB0104	0.102	0.000	0.000	0.000	0.000	0.000	0.000
97	NE021320-5	OPB0104	0.636	4.588	7.214	0.090	2.294	0.287	2.868
98	NE021319-3	OPB0104	0.000	0.000	#DIV/0 !	#DIV/0!	0.000	0.000	0.000
99	NE021319-4	OPB0104	0.332	0.000	0.000	0.000	0.000	0.000	0.000
100	NE021320)1	OPB0104	0.699	18.191	26.024	0.325	9.096	1.137	11.369
101	NE021320)2	OPB0104	0.604	14.522	24.043	0.301	7.261	0.908	9.076
102	NE021320-3	OPB0104	0.559	16.746	29.957	0.374	8.373	1.047	10.466
103	NE021318-3	OPB0103	0.686	4.851	7.071	0.088	2.426	0.303	3.032
104	NE021318-4	OPB0103	0.468	0.476	1.017	0.013	0.238	0.030	0.298
105	NE021318-5	OPB0103	0.570	4.229	7.419	0.093	2.115	0.264	2.643
106	NE021319)1	OPB0104	1.000	6.300	6.300	0.079	3.150	0.394	3.938
107	NE021319)2	OPB0104	1.503	17.263	11.486	0.144	8.632	1.079	10.789
108	NE021317)1	OPB0103	0.957	16.829	17.585	0.220	8.415	1.052	10.518
109	NE021317)2	OPB0103	0.556	10.362	18.637	0.233	5.181	0.648	6.476
110	NE021317-3	OPB0103	1.200	5.943	4.953	0.062	2.972	0.371	3.714
111	NE021317-4	OPB0103	0.262	0.000	0.000	0.000	0.000	0.000	0.000
112	NE021318)1	OPB0103	0.871	4.502	5.169	0.065	2.251	0.281	2.814
113	NE021311)1	OPB0103	0.723	11.086	15.333	0.192	5.543	0.693	6.929
114	NE021371)2	OPB0411	0.208	2.513	12.082	0.151	1.257	0.157	1.571
115	NE021360-1	OPB0302	0.981	19.607	19.987	0.250	9.804	1.225	12.254
116	NE021376-4	OPB0420	0.980	16.066	16.394	0.205	8.033	1.004	10.041
117	NE021376-3	OPB0420	1.661	11.691	7.039	0.088	5.846	0.731	7.307
118	NE021376)2	OPB0420	1.216	5.337	4.389	0.055	2.669	0.334	3.336

Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
119	NE021375)1	OPB0419	0.806	15.217	18.880	0.236	7.609	0.951	9.511
120	NE021371-4	OPB0411	0.720	6.226	8.647	0.108	3.113	0.389	3.891
121	NE021370-6	OPB0311	1.557	8.970	5.761	0.072	4.485	0.561	5.606
122	NE021370-5	OPB0311	1.877	14.001	7.459	0.093	7.001	0.875	8.751
123	NE021370-4	OPB0311	1.445	7.528	5.210	0.065	3.764	0.471	4.705
124	NE021370-3	OPB0311	2.032	0.000	0.000	0.000	0.000	0.000	0.000
125	NE021369-9	OPB0304	0.558	0.000	0.000	0.000	0.000	0.000	0.000
126	NE021369-5	OPB0304	0.349	3.858	11.054	0.138	1.929	0.241	2.411
127	NE021321)1	OPB0104	1.754	19.189	10.940	0.137	9.595	1.199	11.993
128	NE021321-3	OPB0104	1.148	7.804	6.798	0.085	3.902	0.488	4.878
129	NE021321-4	OPB0104	0.890	4.471	5.024	0.063	2.236	0.279	2.794
130	NE021324)1	OPB0104	1.157	21.812	18.852	0.236	10.906	1.363	13.633
131	NE021324)2	OPB0104	0.925	9.451	10.217	0.128	4.726	0.591	5.907
132	NE021324-6	OPB0104	0.998	3.414	3.421	0.043	1.707	0.213	2.134
133	NE021324-8	OPB0104	0.672	10.721	15.954	0.199	5.361	0.670	6.701
134	NE021324-A0	OPB0104	1.170	0.527	0.450	0.006	0.264	0.033	0.329
135	NE021325)1	OPB0104	0.804	21.449	26.678	0.333	10.725	1.341	13.406
136	NE021325)2	OPB0104	1.763	22.102	12.537	0.157	11.051	1.381	13.814
137	NE021325-3	OPB0104	0.333	0.000	0.000	0.000	0.000	0.000	0.000
138	NE021325-4	OPB0104	0.325	0.000	0.000	0.000	0.000	0.000	0.000
139	NE021326)1	OPB0106	1.303	21.206	16.275	0.203	10.603	1.325	13.254
140	NE021326)2	OPB0106	1.184	16.062	13.566	0.170	8.031	1.004	10.039
141	NE021326-3	OPB0106	1.343	18.600	13.850	0.173	9.300	1.163	11.625
142	NE021326-4	OPB0106	0.753	13.043	17.321	0.217	6.522	0.815	8.152
143	NE021326-5	OPB0106	0.847	10.719	12.655	0.158	5.360	0.670	6.699
144	NE021327)1	OPB0106	0.894	5.622	6.289	0.079	2.811	0.351	3.514
145	NE021327)2	OPB0106	0.693	2.799	4.039	0.050	1.400	0.175	1.749
146	NE021327-3	OPB0106	0.781	0.000	0.000	0.000	0.000	0.000	0.000
147	NE021327-4	OPB0106	0.770	7.363	9.562	0.120	3.682	0.460	4.602

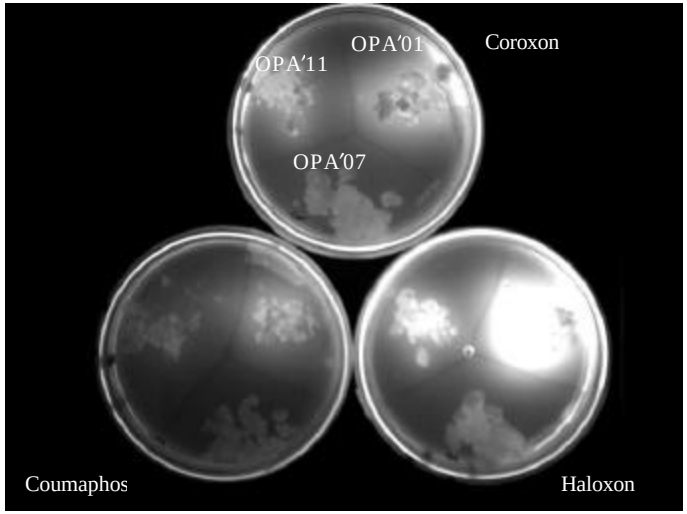
Plate Position	Ear	Event	Protein mg/ml	Units/ ml	Units/ mg	%TSP	Total Units	mg OPH	ng/mg seed
148	NE021327-5	OPB0106	0.925	0.000	0.000	0.000	0.000	0.000	0.000
149	NE021327-6	OPB0106	0.297	0.000	0.000	0.000	0.000	0.000	0.000
150	NE021327-7	OPB0106	0.379	0.000	0.000	0.000	0.000	0.000	0.000
151	NE021328)2	OPB0107	0.567	12.997	22.922	0.287	6.499	0.812	8.123
152	OPA0411T2	OPA0411	0.996	27.521	27.632	0.345	13.761	1.720	17.201
153	OPB0310GH	OPB0310	0.824	0.000	0.000	0.000	0.000	0.000	0.000
154	LH244	LH244	0.818	0.000	0.000	0.000	0.000	0.000	0.000
155	LH244	LH244	1.224	0.000	0.000	0.000	0.000	0.000	0.000

APPENDIX D

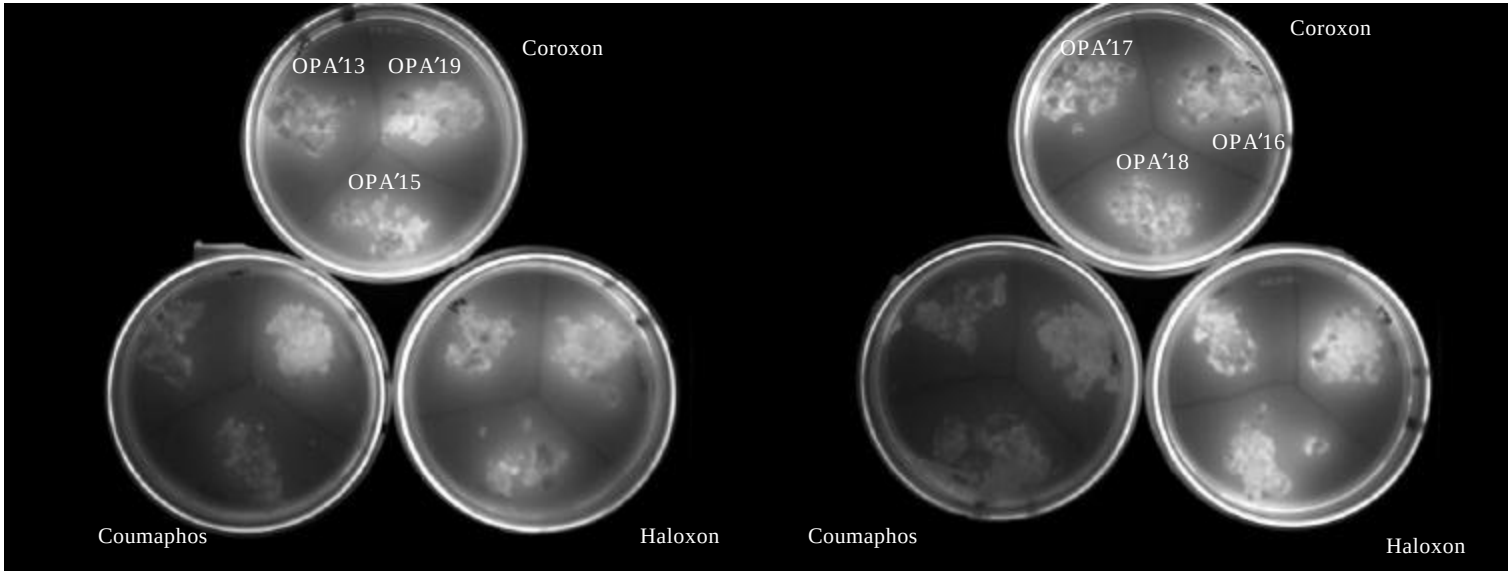
SCORING OF TRANSGENIC CALLUS



Assayed Expression:
OPA′21- 0.27 u/mg

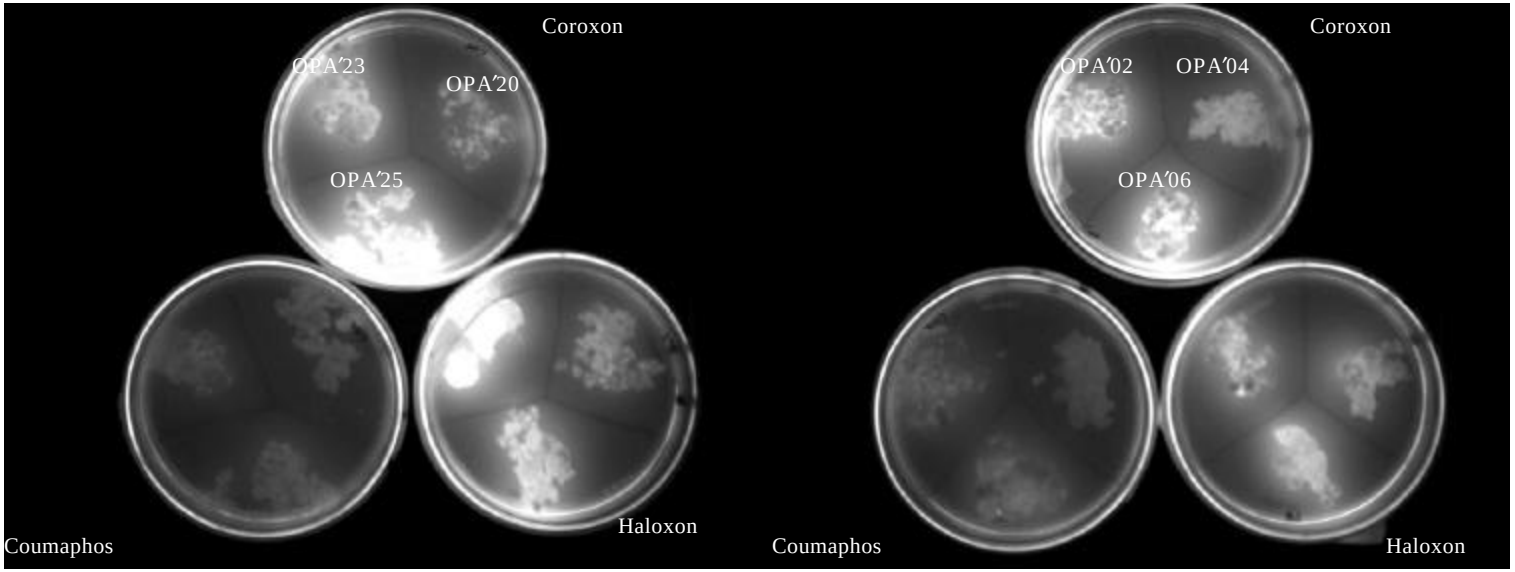


Assayed Expression:
OPA′11 – 0.15 u/mg
OPA′01 – 0.62 u/mg
OPA′07 – 0.001 u/mg



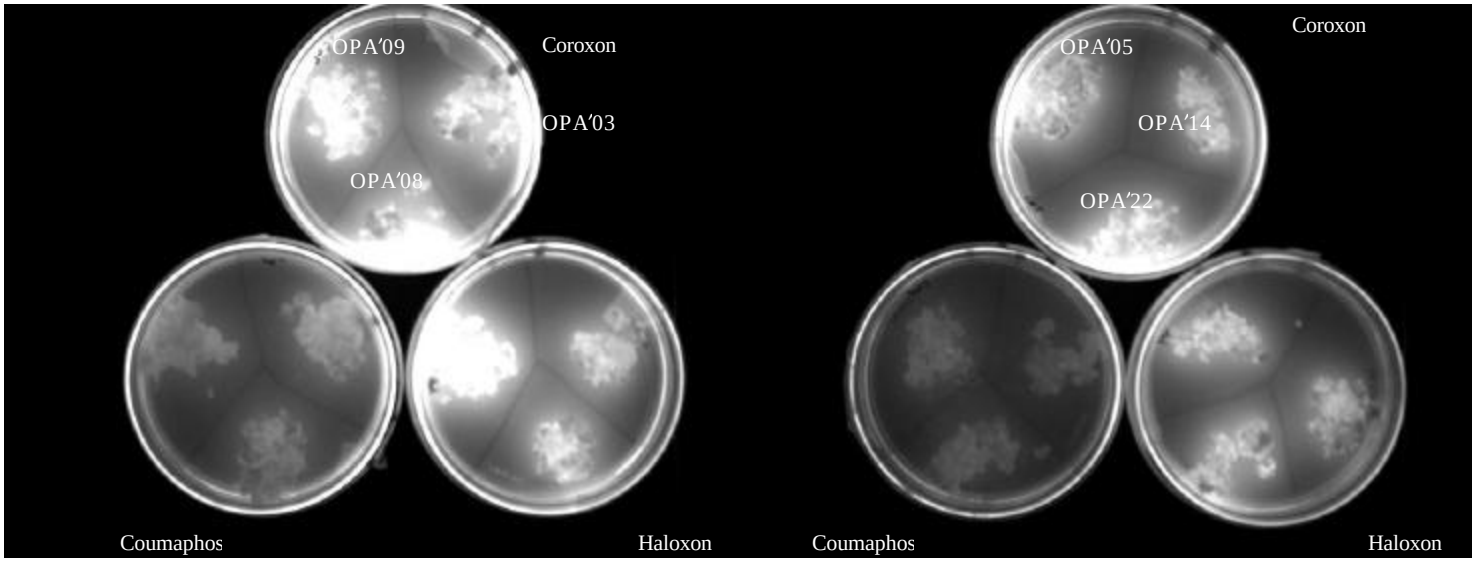
Assayed Expression:
OPA′13 – 0.19 u/mg
OPA′19 – 1.01 u/mg
OPA′15 – 0.27 u/mg

Assayed Expression:
OPA′17 – 0.03 u/mg
OPA′16 – 0.18 u/mg
OPA′18 – 0.04 u/mg



Assayed Expression:
OPA′23 – 0.10 u/mg
OPA′20 – 0.03 u/mg
OPA′25 – 0.06 u/mg

Assayed Expression:
OPA′02 – 0.01 u/mg
OPA′04 – 0.19 u/mg
OPA′06 – 0.44 u/mg



Assayed Expression:
OPA′09 – 0.18 u/mg
OPA′03 – 0.03 u/mg
OPA′08 – 1.06 u/mg

Assayed Expression:
OPA′05 – 0.47 u/mg
OPA′14 – 0.04 u/mg
OPA′22 – 0.18 u/mg

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Education

Doctor of Philosophy, Biochemistry, Texas A&M University, May 2004
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Publications and Abstracts

Relationship Between Nitrogen Nutrition and Photosynthetic Carbon Fractionation in Corn, S. Madhavan, S. Todd Swanson, T.M. Blackmer, T. Scott Pinkerton, and M.H. O'Leary. Supplement to Plant Physiology, June 1996, Volume 111 #2, pg. 97

Do Plant Cells Have the Receptor for Acetylcholine? Immunodetection of Acetylcholinesterase and Acetylcholine Receptor, S. Madhavan and T. Scott Pinkerton. Supplement to Plant Physiology, June 1997, Volume 114 #3, pg. 168

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