Transformation of *Digitaria sanguinalis* with *XvPer1*, a novel antioxidant.

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Abstract

Digitaria sanguinalis, a widespread African grass, was micropropagated from immature inflorescences. Embryogenic calli generated was transformed with *XvPer1* using the Biolistic gene gun. *XvPer1* shares high similarity to a recently discovered group of antioxidants, the 1-Cys PRX's. A homologue was shown to be absent in *D. sanguinalis* by northern, Southern and western Blots.

Introduction

Plant water deficit affects every aspect of plant growth and hence productivity. Plant losses experienced due to water deficit stress such as drought, are the major limiting factors for plant growth and distribution worldwide (Boyer, 1982). The fact that world food production has been outpaced since 1984 by population growth, readily attracts attention (Dyson, 1999) and has caused a considerable amount of research into plant responses to water deficit. Water deficit or drought induces various biochemical and physiological responses within plants (Bray, 1997 and Ingram and Bartels, 1996). One consequence of water deficit is the generation of free radicals (Scandalios, 1993). Free radicals are known to be one of the factors responsible for ageing in humans and are the major cause of viability loss in desiccation-sensitive plant tissues (Smirnoff, 1993; Hendry, 1993; Krammer and Grill, 1997). These radicals are known as reactive oxygen species (ROS) and include anion radicals such as the superoxide ion $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). ROS causes protein oxidation, lipid peroxidation, polysaccharide depolymerisation and DNA modifications/ strand breaks etc. (Jacobson, 1996). As dehydration occurs in plant tissue, there is a marked increase in ROS. Mechanisms have evolved in plant tissues

to counteract the deleterious effects of ROS. These involve the up-regulation of biochemicals, both enzymatic and non-enzymatic. Non-enzymic compounds include vitamins C and E, flavonoids and some alkaloids amongst others, which can react directly with ROS (Larson, 1988 and Ames, 1983). It has been reported that there is an accumulation of anthocyanins, which also have antioxidant capabilities (Sherwin and Farrant, 1998).

Some of the antioxidant enzymes include ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD) and catalase. They are involved in the regeneration of glutathione and ascorbate that are essential in the detoxification of ROS (Allen, 1995). It has been shown that there is an upregulation of genes encoding enzymatic antioxidants, which are capable of removing, neutralising or scavenging free radicals and oxyintermediates during dehydration (Scandalios, 1993). APX and GR scavenge hydrogen peroxides in chloroplasts and mitochondria respectively (Foyer and Halliwell, 1976), and catalases also eliminate peroxides whilst SODs catalyse the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Scandalios, 1993). Recently discovered families of antioxidant enzymes, the peroxiredoxins, (PRX's), previously known as thiol-specific antioxidant (TSA) proteins, first identified in yeast (Kim *et al.,* 1988) have been identified in organisms from all kingdoms (Chae *et al.,* 1994a). The PRX's represent an emerging family of multifunctional enzymes most of which reduce peroxides using thioredoxin as the immediate electron donor (Chae *et al.,* 1994a, 1994b). PRX's show considerable amino acid sequence identity (Chae et al., 1994c), which indicates high biological significance. The PRX's are divided into two main types, the 1-Cysteine (1-Cys) and

2-Cysteine (2-Cys) PRX's, which, on the basis of immunological and amino acid sequence analysis, can be divided into six distinct groups PRX I-VI (Lee *et al.*, 2000). An N-terminal cysteine residue is conserved in all PRX family members (in the vicinity of the 47th position of the polypeptide sequence), which forms part of the peroxidatic centre of PRX's. PRX's that contain only this cysteine residue constitute the first group, 1-Cys PRX's. The majority of PRX's which have been studied more extensively, fall into the other group, (2-Cys), which have a second conserved cysteine residue in the C-terminal region, which is separated by roughly 120 amino acids from the first (Chae *et al.*, 1994b).

It has been suggested that the members of the 1-Cys and 2-Cys groups play different antioxidant roles, with the latter being general protectants and the former variant performing more specific tasks such as protecting DNA. 2-Cys PRX's, which include the subgroups of thioredoxin peroxidase and alkyl peroxidase-like enzymes, catalyse the reduction of hydrogen peroxide (H_2O_2) to water and alkyl hydroperoxides to their corresponding alcohols (Baier and Deitz, 1999a, Haslekas *et al.*, 1998). PRX's do not require cofactors such as metals or prosthetic like other peroxides (Henkle-Durhrsen and Kampkotter, 2001). The thioredoxin peroxidases reduce H_2O_2 with the use of electrons from the thioredoxin system (thioredoxin, thioredoxin reductase and NADPH). The many different isotypes of 2-Cys PRX's have very diverse cellular functions, such as antioxidants, endogenous regulators of apoptosis, and as intracellular signaling molecules (Lim *et al.*, 1998). The 2-Cys PRX proteins in plants contain an N-terminal transit peptide that was shown to function as a radical

scavenger coupled to the photosynthetic machinery in chloroplasts (Baier and Deitz, 1999a).

The 1-Cys PRX's reduce peroxides in the presence of small thiols such as dithiothreitol (DTT) (Lim *et al.,* 1993) but the electron donor has not yet been identified (Henkle-Duhrsen and Kampkotter, 2001). In vitro mutagenesis in yeast showed that the N-terminal cysteine is the primary site for hydroperoxides (Chae *et al.,* 1994c).

PRX's exist as homodimers, with the monomers oriented in a head-to-tail fashion. Unusual structures have been observed from crystal structure in the human 2-Cys PRX isolated from erythrocytes. The structure consists of five dimers linked end-on end through predominantly hydrophobic interactions. This decamer structure is thought to form *in vivo* under conditions of oxidative stress (Baier and Deitz, 1999b). However, Western blot studies of rice 1-Cys PRX showed that the protein actually exist in the dimeric form (Lee *et al.,* 2000).

In plants 1-Cys PRX's are specifically expressed in the nucleus of immature embryos and the aleuronic layers of the seed. The expression level is significantly increased late in seed development and maintained in mature seeds during storage (Haskelas *et al.*, 1998). It had been thought that the role of 1-Cys PRX's in plants was in the maintenance of seed dormancy based on findings that in the imbibed non-dormant seed, the transcript level is reduced dramatically and disappears after seed germination (Stacy *et al.,* 1996). However this seems not to be the case as the transcript level of 1-Cys PRX did not correlate with the plant hormone abscisic acid

(ABA) levels, which is required for the induction of seed dormancy (Haskelas *et al.*, 1998). Recently it has been shown that rice 1-Cys PRX expressed constitutively in transgenic tobacco does not maintain dormancy but enhances antioxidant activity (Lee *et al.*, 2000).

The first plant 1-Cys PRX to be functionally described was from barley (*Hordeum vulgare* L.). It was isolated from an aleurone cDNA library and shown to belong to a group of *Balem* (*b*arley *al*eurone and *em*bryo expressed) transcripts (Aalen *et al.*, 1994). Further studies by the same group demonstrated that the protein PER1 protected DNA against cleavage by free radical attack and that *PER1* is a single copy gene downregulated by gibberellic acid (GA) (Stacy *et al.*, 1996).

A 1-Cys PRX was isolated from the resurrection plant *Xerophyta viscosa* using differential screening of a cDNA library (Ndima *et al.*, 2001). The library was constructed using mRNA isolated from dehydrated *X. viscosa* leaves (Mundree *et al.*, 2000). XvPer1 showed 74 % identity to pBS128 from *Bromus secalinus*, 73% identity to Per1 from *Hordeum vulgare* and 72% identity to AtPer1 from *Arabidopsis thaliana* amongst others. Southern blot analysis indicated that there is a single copy of *XvPer1* in the *X. viscosa* genome, with possible homologues. The transcript is induced strongly during dehydration, high light intensities, high and low temperatures as well as by ABA. The protein is thought to be localised in the nucleus, as there is a putative nuclear localisation signal (NLS) in the amino acid sequence.

The aim of this study was to authenticate the role of *XvPer1* as an important antioxidant enzyme. Using particle bombardment, we generated transgenic calli and plantlets constitutively expressing *XvPer1* in *D. sanguinalis,* a desiccation-sensitive monocotyledonous grass. Due to time constraints the plantlets could not be characterised.

Materials and Methods

Tissue Source

Embryogenic calli were initiated from immature inflorescences of *D. sanguinalis* plants (Chen *et al.,* 1998). The inflorescences were cut approximately into 2 cm segments and sterilised in $HgCl_2$ for 5 minutes then rinsed in sterile distilled water. Callus initiation media consisted of Murashige and Skoog (1962) medium (MS) containing 3% sucrose and 2.5 mg/l 2,-dichlorophenoxyacetic acid (2,4-D). Cultures were grown in the dark at room temperature. Type 1 Embryogenic calli was selected and subcultured every two weeks on a medium of the same composition.

Cloning of XvPer1 into pCAMBIA-UBQ.

A *Hind* III/ *Bam* HI fragment from pAHC25, which carries the maize ubiquitin promoter (Ubi) with its intron (Ubi-1) (Christensen & Quail, 1996), was cloned into the multiple cloning site of pCAMBIA 1201 which contains the hygromycin resistance gene (Hadjukiewicz *et al.,* 1994), creating the plasmid vector pCAMBIA-UBQ. *Escherichia coli* JM 109 competent cells were transformed with the plasmids pCAMBIA-UBQ and pGEM-T Easy-*XvPer1* to amplify the plasmids, which were then purified using the QIAGEN Plasmid Midi-Maxi prep kit (Qiagen, Germany).
The *Eco* RI fragment from the vector pGEM-T Easy-*XvPer1* (Mowla *et al.,* 2002) was cloned into *Eco* RI digested pCAMBIA-UBQ and the construct named construct #1 (Fig.1). High Pure PCR Product Purification Kit (Roche, USA) was used to isolate

specific restriction fragments from 1 % agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with Lambda DNA standards as well as spectrophotometrically.

E. coli JM109 cells were transformed with the construct and colony PCR was performed as described in the following section.

Reverse Transcription –Polymerase Chain Reaction (RT-PCR).

The Omniscript RT kit (Qiagen, USA) was used for the synthesis of first strand cDNA. Protocol followed was according to manufacturers instructions. The primers used were the oligo-dT 18mer primer and the *XvPer1* reverse primer, CATTCACTCAGACGTTCGTAAAACG. PCR amplifications were carried out in 50μl reactions containing 1X reaction buffer, 1.5mM $MgCl_2$, 10 μm dNTP's, 10 pmols each forward and reverse primer, 100 ng template, 1.25 units Taq DNA polymerase (SR products, UK). Reactions were conducted using a GeneAmp PCR system 9700 at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and a 7 minute final extension step at 72°C.

XvPer1 forward primer, CCATGCCGGGGCTCACCATT and reverse primer produced *XvPer1* amplicons. A ubiquitin promoter specific forward primer, CTGCAGGTCGACTCTAGAGGATCC was synthesised to check orientation of the *XvPer1* insert in construct #1 with the *XvPer1* gene specific reverse primer under the same reaction composition and conditions above.

Bombardment and selection of transformed calli.

Twenty-four hours prior to microprojectile bombardment, embryogenic calli were transferred onto the centres of plates of high osmoticum MS medium containing 0.2 M mannitol and 2.5mg/l 2,4-D with an extra 50mg/l myoinositol.

A total of 2μg plasmid DNA was precipitated onto 1μm gold particles (Dunder *et al.*, 1995). A co-bombardment was performed for most of the calli where 1μg of the construct #1 was co precipitated with 1μg of the plasmid pUBIBAR 35S. Since the efficacy of the hygromycin gene under the 35S promoter had not yet been tested in *D. sanguinalis*, another vector was co-bombarded with the construct and calli selected on bialaphos (Meiji Seika Kaisha, Tokyo). The vector pUBIBAR 35S contains the bar gene that encodes phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin the active compound in the herbicide bialaphos (D'Halluin *et al.*, 1992;Wilmink and Dons, 1993). The rest of the calli were bombarded with 2 μg of the construct #1 and selected on 50 mg/l hygromycin (Sigma USA).

Bombardment was performed using the PDS-1000 Helium Biolistic particle bombardment delivery system from Biorad (USA). Each dish of calli was bombarded twice at 900 psi.

Bombarded calli were transferred back to initiation media with no selection marker for 14 days. The calli were then transferred to MS media that contained 3mg/l bialaphos or hygromycin as above and subcultured every two weeks. After 6 weeks of growth, the calli were transferred onto MS-Regeneration media containing 3% sucrose, 0.1mg/l naphthyleneacetic acid (NAA), 10 mg/l 6-benzylaminopurine (BA) and 3 mg/l bialaphos or hygromycin. The calli were kept in the dark for 5 days after

the transfer and under dim light (33.4- 42.72μmolm^{-2}s^{-1}) for 9 days. Subsequently the calli were kept under full light (54.8-74.5μmolm^{-2}s^{-1}) at 25°C.

DNA and RNA extraction

Genomic DNA was isolated from *D. sanguinalis* leaves according to the method of Dellaporta *et al.,* (1983). RNA was extracted using the TRIzol method (Life technologies, USA) from fully hydrated plants and plants that were not watered for 2 weeks and were showing dehydration stress. DNA samples were quantitated spectrophotometrically (Sambrook *et al.,* 1989) and by quantitative agarose gels using lambda DNA standards. RNA quality was assessed by formamide denaturing agarose gel electrophoresis (Sambrook *et al.,* 1989).

Southern and Northern blot analysis

Southern blot analysis was performed using the NaOH method modified from Reed and Mann, (1985). 10μg of DNA per lane was used after digestion of the DNA using the endonucleases *Eco* RV and *Hind* III (Roche, USA). Northern blots were performed on formamide denatured RNA run on 0.8% agarose gels at 80 V and transferred to Nylon membranes (Hybond-XL, Amersham Pharmacia Biotech, UK) using 0.4M NaOH.

DNA Labelling and hybridisation probes

Radioactive *XvPer1* hybridisation probes were synthesised using ^{32}P α-dCTP PCR labelling. Reaction compositions were as previously described and performed in a Hybaid thermocycler at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 10 minutes, and a 7 minute final extension step at 72°C. The probe was purified from unincorporated radioactive nucleotides using Sephadex G-50. Hybridisation of the probe was performed at 65°C.

Western Blot Analysis

Proteins were extracted from *D. sanguinalis* leaves using the TRIzol method as per manufacturers instructions (Life technologies, USA) and separated on a 10% denaturing polyacrylamide gel (Sambrook *et al.,* 1989). The amount of protein loaded was calibrated using a comassie blue stained identical gel. Electrophoretic transfer of proteins from SDS-polyacrylamide gels to a 0.45 micron nitrocellulose membrane (Osmonics, UK) using Biorad (USA) transfer apparatus was carried out as described by Rybicki and von Wechmar (1982). The membrane was probed with *X. viscosa* peroxiredoxin antiserum (1:2000) overnight at 4°C. The secondary antibody (anti-rabbit IgG, peroxidase linked whole antibody from goat, Sigma, Germany) was diluted 1:5000. Detection was done using the ECL detection system. Molecular weight was determined by comparison to low-MW electrophoresis calibration kit protein mixture (Amersham life sciences, USA).

Results

Cloning of XvPer1 into plant expression vectors

XvPer1 cDNA was obtained by endonuclease excisions from the cDNA clone in pGEM-T Easy (Mowla *et al.,* 2002). Initial attempts to clone the cDNA into pAHC25 (Christensen & Quail, 1996), proved futile. Successful cloning was performed into pCAMBIA 1201-UBQ. Sequence analysis of the clone confirmed the correct orientation of *XvPer1* cDNA with respect to the ubiquitin promoter as well as absence of mutations.

Expression of a peroxiredoxin homologue in D. Sanguinalis

To determine whether a peroxiredoxin homologue exists in *D. sanguinalis*, possible expression of it was examined in wild type plants. RNA was extracted from leaves of dehydrated and hydrated plants described in materials and methods section. The nucleic acids were separated by gel electrophoresis, blotted onto nylon membranes and hybridised with a radioactive PCR labelled DNA probe. No signal was detected in both samples as shown in Fig. 2.

Presence of a peroxiredoxin homologue in Digitaria sanguinalis genome

Genomic DNA was extracted from *D. sanguinalis* and *X. viscosa* leaves. *Eco* RV digests of *D. sanguinalis* DNA and *X. viscosa* were performed as well as a *Hind* III digest of *D. sanguinalis* DNA. *Eco* RV *X. viscosa* cleaved genomic DNA hybridised with the PCR radioactively labelled ^{32}P DNA probe, revealing one hybridising fragment at a previously reported size (Mowla *et al.*, 2002). No signal was detected for the *D. sanguinalis* DNA (data not shown).

PCR of genomic DNA and RT-PCR of Digitaria sanguinalis RNA

Since PCR is a very sensitive method of detection of minimal DNA quantities, we investigated the presence of a peroxiredoxin homologue in *D. sanguinalis*. No previous studies have been done in this regard and it was necessary to understand the genetic background of *D. sanguinalis*. PCR products were obtained from PCR of *D. sanguinalis* genomic DNA as well as from RT-PCR of dehydrated and hydrated *D. sanguinalis* RNA samples (Fig 3). Primers used were gene specific for *XvPer1*, and yielded products of the published size of *XvPer1* (Mowla *et al.*, 2002). PCR and RT-PCR products were transferred onto a nylon membrane and probed using a radioactive probe in order to authenticate the identity of the products. Signals were obtained from both PCR and RT-PCR products (Fig 4). This result validates the presence of a peroxiredoxin homologue being expressed, hence the positive RT-PCR, which would allow detection of minimal RNA amounts that might be present or non-peroxiredoxin specific binding of the primers.

Protein levels of a peroxiredoxin in D. sanguinalis.

In order to conclusively ascertain the presence or absence of a peroxiredoxin homologue in *D. sanguinalis*, total protein was extracted from wild type *D. sanguinalis* without any treatment and from wild type *D. sanguinalis* and *X. viscosa* that were treated under high light intensity for 5, 10 and 15 days. The protein was electophoretically fractionated on 10% SDS-PAGE denaturing gels (Fig 5A). No signal was detected from Western blot analysis of the same gel, except for the positive control (Fig 5B). This suggests that the gene is either not present in the genome hence no protein being detected, or that it was not transcribed and translated at the times represented by the samples. A signal was detected for the positive control, which rules out the possibility of technical problems such as problems with the antibodies.

Tissue Culture of D. sanguinalis

Type 1 embryogenic calli with a compact, pearly white appearance was initiated from immature inflorescence after 2-3 weeks on MS media (Figs. 6 and 7). The most embryogenic calli were obtained from the youngest inflorescence. This calli were regenerated on MS media for bulking up.

Co-bombardment of the vector pUBIBAR 35S and clone #1 containing *XvPer1* was performed on calli that were twenty-eight weeks old. The bombarded calli were maintained on MS plates. Five plates on the calli were put on MS regeneration media containing 10mg/l BA and 0.1 mg/l NAA after 3 weeks. Green growth was noted after 4 weeks from less than 1% of the calli. In two weeks the green calli had formed

plantlets 9mm-15 mm tall. These plants were then transferred onto MS rooting media without any plant growth regulators.

The second bombardment was performed on calli that were ten weeks old. The calli were maintained on MS media and transferred to MS regeneration media after eight weeks. Green growth was noticed for calli co-bombarded with the vector pUBIBAR 35S and clone #1 grown on selection media containing bialaphos. Calli bombarded with only clone #1 grown on selective media containing hygromycin did not show any green growth after four weeks on the media.

Discussion

Absence of a peroxiredoxin homologue in D. sanguinalis.

Resurrection plants such as *X. viscosa* have the ability to revive from an air-dried state (Gaff, 1971,1989). Their desiccation tolerance mechanisms have thus been a subject of much interest to researchers. One of the mechanisms they employ to overcome the challenges they face during dehydration involves the minimisation of toxin accumulation and free radical damage that occurs, as metabolism is impaired (Sherwin and Farrant, 1996; Smirnoff 1993). Studies at the molecular biology level as well as physiologically, have revealed that antioxidant enzyme transcripts are up-regulated during dehydration in desiccation tolerant plant species (Smirnoff, 1993; Krammer and Grill, 1997 and Sherwin and Farrant, 1998) and also in sensitive species (Seki *et al.,* 2001). Peroxiredoxin expression levels in *Arabidopsis* have been found to increase using DNA microarray technology during dehydration (Seki *et al.,* 2001) as well as in *X. viscosa* (Ndima *et al.,* 2001).

Western, Southern and northern blots done during this study in order to identify a peroxiredoxin homologue in *D. sanguinalis* seemed to indicate its absence. The absence of such a powerful antioxidant system in *D. sanguinalis* corresponds with its desiccation sensitivity, as it may not have sufficient mechanisms to ameliorate ROS generated during dehydration. These results were inconsistent with PCR and RT-PCR data obtained in the current study. However this may be as a result of the extremely sensitive nature of PCR that may allow the amplification of unrelated genes, resulting in false "positive" identifications.

Previous studies done by our group showed expression of *XvPer1* in *X. viscosa* plants treated under high light (Mowla *et al.*, 2002). However no protein was ever identified in that or the current study. This is possibly due to the expression noted being transient or instability of the protein in the plant or during processing for western blotting.

It is highly unlikely that there is a less homologous peroxiredoxin that was undetected. PRX's identified thus far show considerable sequence identity (Chae *et al.*, 1994c), a characteristic that enforces its biological significance, and if present would have been consequently detected.

Tissue Culture of D. sanguinalis.

The micropropagation of *D. sanguinalis* was successfully done with embryogenic calli being initiated after 2 weeks. Growth of bombarded calli on selective media as well as plantlets indicates the presence of some transformed calli and possibly plants. However, due to time constraints the plants regenerated from the bombarded calli could not be characterised in time for the submission of this paper.

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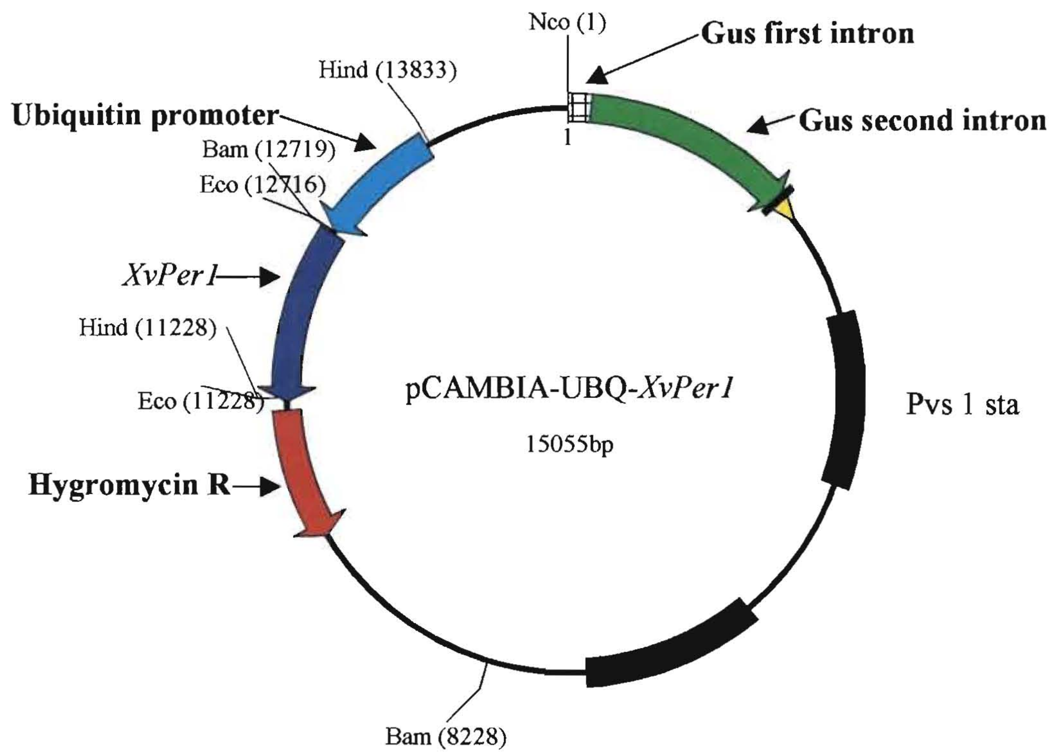
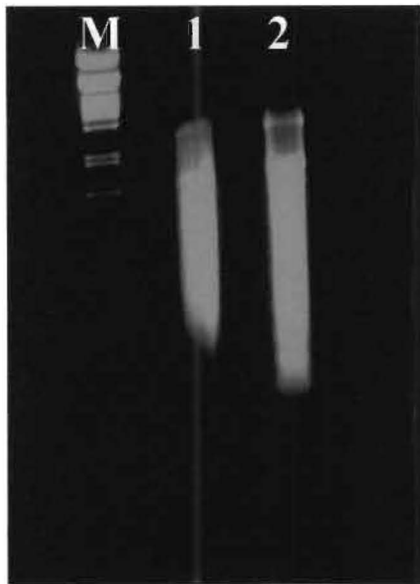


Figure 1. Map of the vector construct pCAMBIA-UBQ-*XvPER1*

XvPer1 was cloned into the Eco R1 site of the vector pCAMBIA-UBQ as described in the materials and methods

A



B

Figure 2. Per1 transcript levels in hydrated and dehydrated *D. sanguinalis.*

A :The RNA samples that were transferred onto a nylon membrane. M is a molecular weight marker and Lane 1 shows the hydrated RNA sample and lane 2 the dehydrated sample.

B: The hybridised membrane showing no signal.

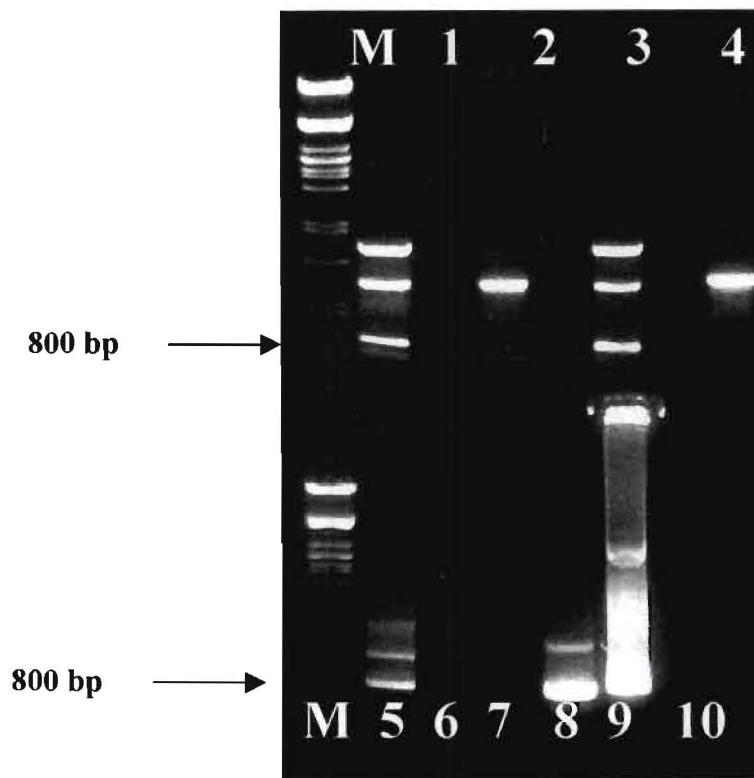


Figure 3. Primer extension by Polymerase Chain Reaction (PCR) on dehydrated and hydrated *D. sanguinalis* and *X. viscosa* cDNA and genomic DNA.

Lane1: PCR of cDNA synthesised using oligo dT primer during RT of RNA from dehydrated *D. sanguinalis* RNA using *XvPer 1* gene specific primers.

Lane 2: PCR of cDNA synthesised using a gene specific primer during RT of RNA from dehydrated *D. sanguinalis* RNA

Lane 3: PCR of cDNA synthesised using oligo dT primer during RT of RNA from hydrated *D. sanguinalis* RNA

Lane 4: PCR of cDNA synthesised using a gene specific primer during RT of RNA from hydrated *D. sanguinalis* RNA

Lane5 :PCR of *D. sanguinalis* genomic DNA (sample1)

Lane6: PCR of *D. sanguinalis* genomic DNA (sample2)

Lane 7: PCR of *X. viscosa* genomic DNA

Lane8:PCR of gel purified *XvPer1*

Lane 9:PCR of the clone pGEM-T Easy-*XvPer1* (Positive control)

Lane 10:PCR without template DNA (Negative control)

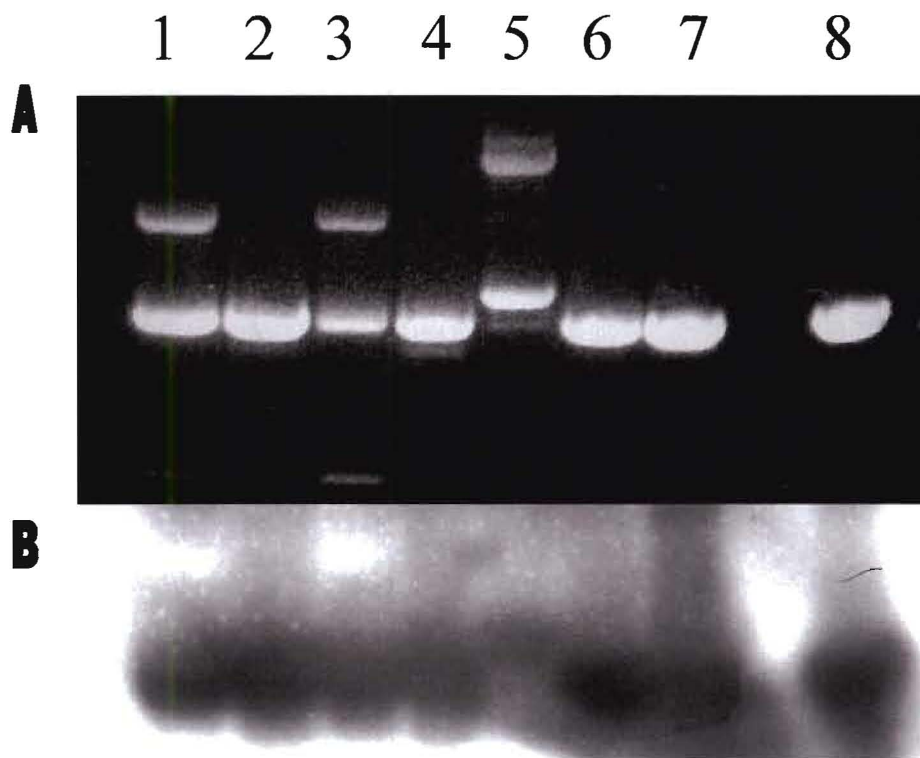


Figure 4. PCR of *D. sanguinalis* cDNA's using *XvPer1* gene specific primers and southern blot

A: PCR of different cDNA samples, B: Southern Blot of the products using a radioactively labeled *XvPer1* probe.

Lane1: PCR of cDNA generated from oligo dT primed RT of RNA from dehydrated *D. sanguinalis*

Lane2: PCR of cDNA generated from *XvPer1* reverse primer, primed RT of RNA from dehydrated *D. sanguinalis*

Lane 3: PCR of cDNA generated from oligo dT primed RT of RNA from hydrated *D. sanguinalis*

Lane 4: PCR of cDNA generated from XvPer1 reverse primer, primed RT of RNA from hydrated *D. sanguinalis*

Lane 5:PCR of *X. viscosa* genomic DNA using *XvPer1* primers

Lane 6: PCR of *D. sanguinalis* genomic DNA using *XvPer1* primers

Lane 7 and 8. PCR of *XvPer1* cDNA.

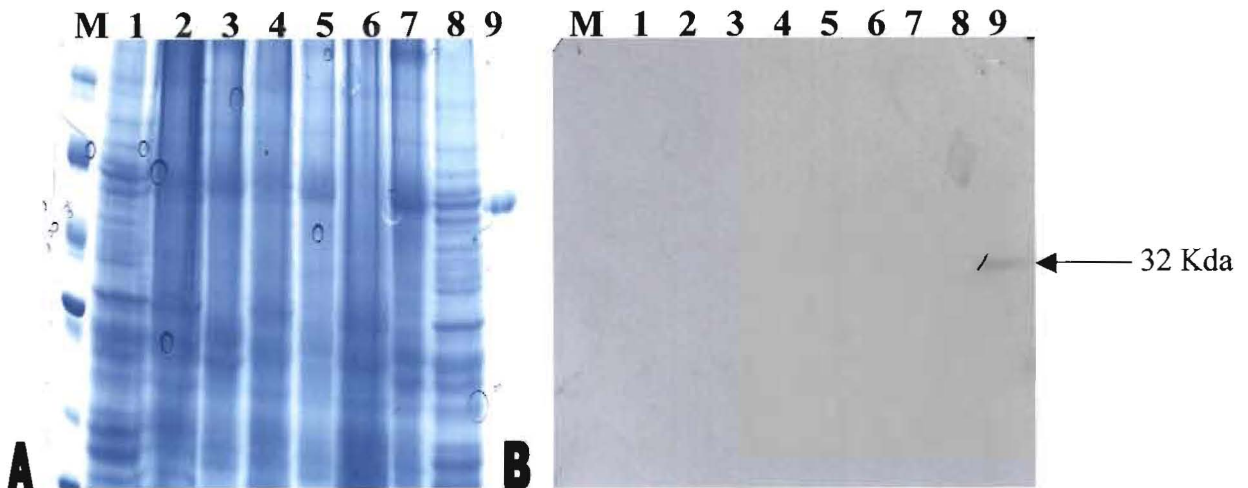


Figure 5. Peroxiredoxin protein levels in high light intensity (1500 $\mu molm^{-2}s^{-1}$) treated *X. viscosa* and *D. sanguinalis*.

A is a denaturing SDS-PAGE gel stained with Coomassie Blue and B is the Western Blot of those proteins.

Lane M:Molecular weight marker

Lane 1: Total *D. sanguinalis* protein from an untreated plant.

Lane 2: Total *D. sanguinalis* protein from 5 day high light treated plants

Lane 3:Total *X. viscosa* protein from 5 day high light treated plants

Lane 4:Total *D. sanguinalis* protein from 10 day high light treated plants

Lane 5:Total *X. viscosa* protein from 10 day high light treated plants

Lane 6:Total *D. sanguinalis* protein from 15 day high light treated plants

Lane 7: Total *X. viscosa* protein from 15 day high light treated plants

Lane 8: Total *D. sanguinalis* protein from an untreated plant.

Lane 9:Purified XvPER1 protein from heat treated X. viscosa

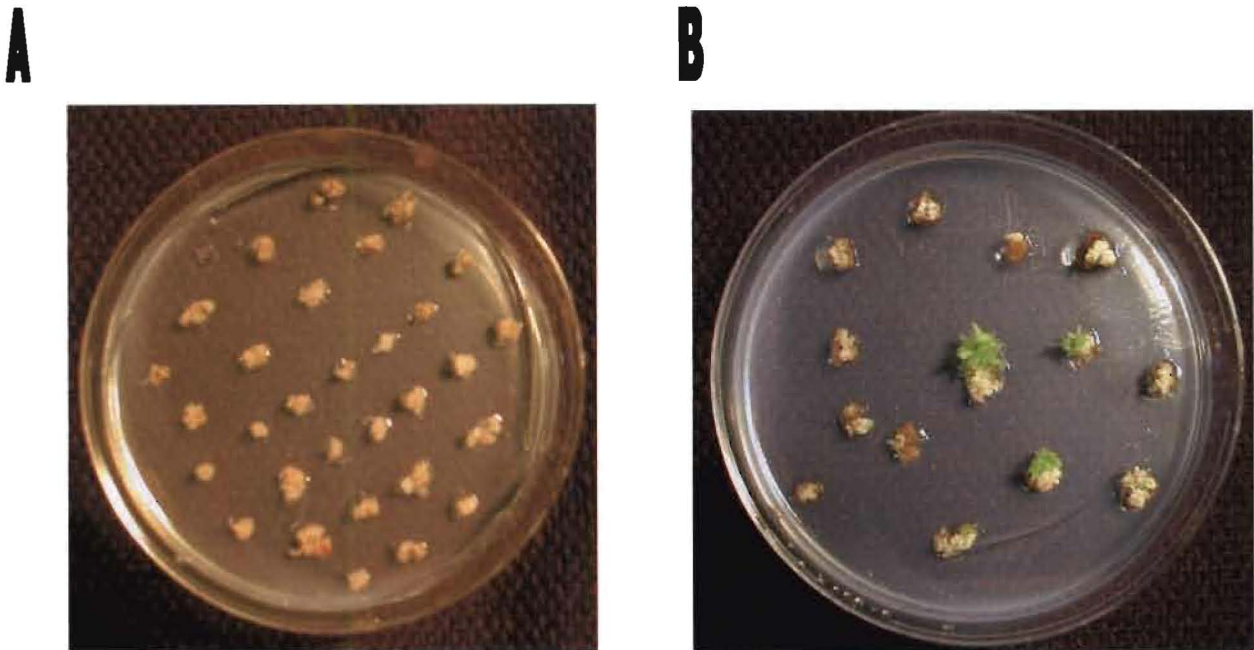


Figure 6. Regenerating bombarded *D. sanguinalis* calli on selective media.

A: Calli bombarded with the vector construct # 1, grown on MS-regeneration selection media containing Hygromycin

B: Calli cobombarded with the vector construct #1 and pUBIBAR 35S on MS regeneration media containing Biolophos showing green regenerating plantlets beginning to form

.

Figure 7.*D. sanguinalis* plantlet regenerated from co-bombarded calli growing on rooting media.