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Dr Leon du Plessis
Maize Trust
P O Box 12203
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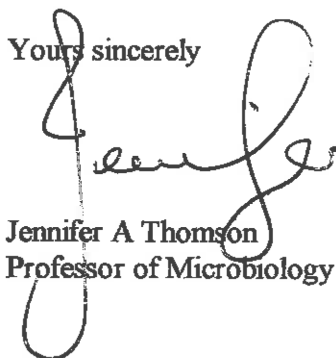
Dear Leon

Please find enclosed 20 copies of my final report, and application for new funding. I will courier the 20 copies of the financial report in the next few days. As the Trustees were satisfied with my 2004 report I have resubmitted it with new information highlighted in bold print. I have deleted the Future Work section of the 2004 report and incorporated that into the new application. I have included the funding agreed to by the Rockefeller Foundation for the period July 2005-June 2007. We will also be receiving funding from PlantBio some time in 2005 for a period of three years, but the amount has not yet been finalised.

Yours sincerely



Jennifer A Thomson
Professor of Microbiology

PROPOSAL FOR MAIZE TRUST – 2005-2008

DEVELOPMENT OF DROUGHT TOLERANT MAIZE BY GENETIC MODIFICATION

We are embarking on an extremely important new approach to maize transformation. While genetic bombardment has been the favoured method since it was developed in the 1980s, it has a number of drawbacks. The most important of these is the fact that bombardment often leads to the introduction of more than one copy of the gene. This can lead to problems in backcrossing as well as the phenomenon of gene silencing. This is the switching off of the expression of all copies of a gene if more than one copy is present in the genome. We have encountered this in our work on *Maize streak virus* resistance and wish to avoid it in our drought tolerance research. The first work on plant transformation used the bacterium *Agrobacterium tumefaciens* as the donor of foreign genes. However, initially this only worked in dicots. It has now been adapted for monocots and fairly recently scientists at Iowa State University made the method freely available to the public sector. We have started to use this system in our research, doing it in collaboration with Dr Rachel Chikwamba, of the CSIR/University of Pretoria who did her PhD in the Iowa State University lab. We have received all the necessary strains, vectors and protocols.

1. **Agrobacterium transformation of maize** (Dr Revel Iyer, post-doctoral fellow and Richard Okoth, new PhD student, technician)

We have already started working on this system using HiII and the results are extremely promising. However, HiII is such a poor maize variety that we will be using A188, one of the inbred parental lines of HiII. Rachel's lab is growing this variety and will send us immature embryos as soon as they are ready. These will be the perfect starting material for Agrobacterium transformation.

2. ***XvSAP1*** (Dr Revel Iyer, post-doctoral fellow and Richard Okoth, new PhD student, technician)

Due to the excellent protection this gene has given to Arabidopsis and tobacco (dehydration, heat and salt tolerance) this is an excellent candidate for maize. We wish to test it on its own and together with other genes, in a procedure called "stacking". The exciting thing is that no other group has found a similar gene to confer abiotic stress tolerance on transgenic plants. Moreover, the only proteins it has any remote identity to are Arabidopsis and wheat cold regulation proteins.

As we have bombarded this gene into HiII maize, we will carry on with the analysis of these transgenic plants, but it will be our first candidate for Agrobacterium transformation.

3. ***XvPer1*** (Dr Revel Iyer, post-doctoral fellow, new PhD student to be appointed, technician)

The results of *XvPer1* transgenic maize tolerant to high light intensity are very encouraging. We will repeat these experiments and make A188 transgenics using

Agrobacterium. Should this be successful we will stack this gene together with *XvSAP1*.

4. ***XvAR*** (Alice Maredza, PhD student, technician)

Initial results with Arabidopsis are very encouraging. These will be repeated. Transgenic HiII have been made and the plants will also be tested. We will repeat these experiments and make A188 transgenics using Agrobacterium. Should this be successful we will stack this gene together with *XvSAP1* and *XvPer1*.

5. **Promoters** (Dr Revel Iyer, post-doctoral fellow and Richard Okoth, new PhD student, technician)

The *XvSAP1* and *XVPer1,* together with the *AtPer1* (from Arabidopsis for comparative purposes) promoters, have been fused to the luciferase gene. They have been transformed into HiII maize and are currently being tested for stress inducibility. The promoter that gives the best results will be fused to the *XvSAP1*, *XvPer1* and *XvAR* genes for transformation into A188 using Agrobacterium.

BUDGET

Item	2005/6	2006/7	2007/8	Total	Rockefeller Foundation funding
Running expenses	200 000	220 000	242 000	662 000	
Bursaries	100 000	110 000	121 000	331 000	
Technical support	80 000	88 000	96 800	264 800	
Small equipment	50 000	55 000	60 500	165 500	
Space and facilities	20 000	22 000	24 000	66 000	
Total	450 000	495 000	544 300	1 489 3000	700 000

Note: We expect additional funding from PlantBio some time in 2005 for a period of three years.

C:/reports/Maize Trust 2005

REPORT TO THE MAIZE TRUST:OCTOBER 2005

DEVELOPMENT OF DROUGHT TOLERANT MAIZE BY GENETIC MODIFICATION

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As the report submitted to the Maize Trust in October 2004 was accepted as satisfactory, I am re-submitting this final report with progress since then shown in bold print. In addition a new application for funding is submitted together with this report. In the budget for this new application I indicate that we have support from the Rockefeller Foundation for the period July 2005 – June 2007, and potential support from PlantBio (funded by the National Department of Science and Technology) starting sometime in 2005 for a period of three years.

In our project proposal to the Maize Trust in 2001, we reported that we had isolated a number of genes from the resurrection plant, *Xerophyta viscosa*, with the potential for protecting transgenic maize against drought and other abiotic stresses. As the project progressed we isolated more genes and became increasingly interested in stress-inducible promoters.

Our approach has been as follows:

- Determine the expression, at the level of transcripts, of each gene in response to a variety of abiotic stresses in *X. viscosa* leaves.
- Do the same for expression at the level of proteins. This often required the production of specific antibodies.
- Determine the mode of action of the protein during abiotic stresses.
- Introduce the gene into our model monocot grass, *Digitaria sanguinalis*. We use this as an intermediate step as transformation of maize is a long and costly process and we only wish to transform those genes which show significant abiotic stress protection in transgenic *D. sanguinalis*.
- Introduce the genes into maize and test for abiotic stress tolerance in greenhouse trials.

We will discuss our results under the following headings:

1. **Antioxidants**
2. **Osmoprotectants**
3. **Other proteins**
4. **Promoters**

1. Antioxidants

a) <u>1Cys-peroxiredoxin (*XvPer1*)</u> (Shaheen Mowla, PhD student; Tendai Rukanda, BSc (Hons) student)

This antioxidant is unique in that all 1Cys-peroxiredoxins described to date are seed specific, while this one is found in *X. viscosa* vegetative tissue. It has a nuclear localization signal and has been localized to the nucleus of dehydrated *X. viscosa* leaf cells. Northern and western blots show that the transcripts and proteins are absent in fully hydrated *X. viscosa* tissue but levels increased in tissues subjected to dehydration, heat (42°C), high light intensity (1500 $m^{-2}s^{-1}$) and when treated with sodium chloride (100 mM). *XvPer*1 was transformed into both *D. sanguinalis* and maize HiII under the control of the ubiquitin promoter. Transgenic lines of *D. sanguinalis* have been identified and will be tested for dehydration tolerance and resistance to high light intensity (see below).

Transgenic maize plants were sent to the African Centre for Crop Improvement (ACCI) at the University of KwaZulu-Natal for self-fertilization. However, the plants were all male sterile (previously encountered, especially when the HiII callus is not very fresh – for logistical reasons we have difficulty in obtaining a regular supply of fresh HiII callus). They were therefore crossed with an ACCI line of maize and the seeds sent to us. We grew six *XvPer1* PCR positive plants in our growth rooms, subjected them to dehydration, and no difference was found between them and control maize plants. Before testing more transgenic plants we will determine gene expression via western blot analysis. However, we have also decided to subject transgenic plants to high light intensity. This we need to perform in the phytotrons in the Botany Department and are therefore applying to the National Dept. of Agriculture for biosafety clearance. At present we only have biosafety clearance for the facilities in the Molecular Biology building.

We have always been interested in comparing genes and proteins isolated from *X. viscosa* with orthologues in *Arabidopsis thaliana*. An opportunity arose to do this when Shaheen Mowla was funded by UCT to spend a year in the laboratory of Christine Foyer, at Rothamsted in the UK. Christine is an expert in elucidating the various antioxidant pathways that are present in plants and was recently ranked no. 8 in the world in citations in plant molecular biology. There Shaheen is comparing XvPer1, AC3 and AtPer1, a Lea-like antioxidant from *A. thaliana* and the XvPer1 orthologue, respectively. However, as this research is peripheral to our main aim, and was not funded by the Maize Trust or the Rockefeller Foundation, we only include this brief reference to it. Shaheen will be back in the lab in March 2005 and will continue her mainstream work on transgenic maize. However, our collaboration with Christine will continue. **Note: The Rockefeller Foundation is now interested in comparing *XvPer1* with its homologous gene from *A. thaliana* so they are funding Shaheen's return visit to Rothamsted until the end of 2005. Shaheen has submitted her PhD but we hope she will return as a post-doctoral fellow in 2006. The work she will do is part of our new application for funding.**

We made transgenic HiII maize carrying *XvPer1*. HiII was developed as a line amenable to transformation, but produces very few seeds. We therefore crossed it with a more robust variety and tested the progeny for the presence of the gene. We grew three transgenic and three control plants under conditions of high light intensity. The transgenic plants all showed greater tolerance to this treatment. We are repeating this experiment with larger numbers of plants.

b) Type II peroxiredoxin (*XvPrx2*) (Kershini Govender, PhD student)

This gene, which shows 77% identity with the *Oryza stativa* orthologue, was shown by northern blots to be induced by low temperature (4°C for 120 h), dehydration (8 days) and ABA. Kershini has also been funded by UCT to spend a year in the laboratory of Karl-Josef Dietz in Bielefeld, an expert in peroxiredoxins and antioxidant systems. While there she has purified the protein and raised antibodies to XvPrx2. Although she showed in vitro DNA protection by the purified protein at UCT, immunogold electron microscopy at Bieleveld showed that the protein was localized to the stroma of the chloroplast of *X. viscosa* plants, and the cytosol of *A. thaliana* plants. Therefore DNA protection might not be a physiological phenomenon. Although most type II Prxs possess two cysteins, at position 51 and 76, XvPrx2 only possesses the first. Interestingly enough, a point mutation in XvPrx2 introducing a second Cys at position 76 displayed lower in vitro activity. This shows yet another unique feature of *X. viscosa* genes and proteins. While in Bielefeld, Kershini made *A. thaliana* PrxC-antisense and knockout plants (PrxC is the orthologue of XvPrx2). She is currently making transgenic plants overexpressing XvPrx2 in both these *A. thaliana* genotypes. This could give us interesting insights into the role played by XvPrx2. Kershini will be back in the lab in October 2004 and should complete her PhD mid-2005. We need to decide whether to make transgenic maize expressing this gene.

Kershini is back in the lab. She is writing up her PhD thesis. We have decided that this gene is of theoretical interest only and will not use it to make transgenic maize.

2. **Osmoprotectants**

    a) Aldose reductase (*ALDRXV4*) (Alice Maredza, PhD student, Emily Davis, MSc student, in addition new PhD student, Priver Namanya)
    Note: Emily is writing up her MSc and will not continue and Priver will not be joining the lab.

This was the first gene we isolated from our *X. viscosa* gene bank and its nomenclature needs to be changed to *XvAR*. The protein converts glucose to sorbitol, a known osmoprotectant. Northern and western blots published by Mundree et al. (2000) showed that this gene was expressed only under dehydration conditions in *X. viscosa* leaves. Enzyme activity also increased as the leaf RWC decreased. Preliminary northern and western blots performed by Emily, show that the gene is not induced by ABA or NaCl.

Alice made transgenic *D. sanguinalis* and Arabidopsis plants carrying this gene under the control of the ubiquitin promoter. As expected, northern blots showed the gene to be constitutively expressed at varying levels in different lines of the transgenic plants and absent in controls (vector transformed and untransformed). Some transgenic *D. sanguinalis* lines showed twice the levels of expression than others. After production of an antibody, western blots showed similar results.

Stress experiments on transgenic Arabidopsis are underway. Preliminary experiments show increased tolerance to dehydration and salt stress. Dehydration experiments were performed on 3-week old plants grown in pots. All non-transgenics (vector

transformed and untransformed) dried after 7 days of dehydration, while the transgenic plants were still green and continued to flower. Salt stress was measured by comparing relative root growth (rrg) of plants growing in Petri dishes containing 50 and 100 mM NaCl. Under the latter conditions, wt seedlings had 30% rrg compared with normal growth media, and transgenic lines varied from 45% to 53%. These results need to be confirmed and expanded, including analysis of the transgenic *D. sanguinalis* plants which will be done by Priver.

We have decided to discontinue the work on *D. sanguinalis* and to concentrate on maize transgenics. We will continue to use Arabidopsis as our model plant. Alice has shown excellent protection of Arabidposis against dehydration, salt and heat stress. She is currently making transgenic maize plants.

b) Galactinol synthase (*XvGolS*) (Shaun Peters, MSc student)

The GolS enzyme is the first committed to the raffinose family of oligosaccharides (RFO). It is unique to plants, is known to occur in large amounts in seeds and increases have been reported in plants exposed to dehydration and cold stress. The XvGolS shares >70% identity with a variety of other GolS proteins. It contains the conserved functional domain belonging to this class of galactosyl transferases. Northern and western blots showed that the expression of *XvGolS* was upregulated in response to water deficit and a -20°C cold shock for 1-3 hours, after which the plants freeze! Shaun also received UCT bursary to spend 6 months in the laboratory of Felix Keller at Zurich University, one of the leading groups working on carbohydrate metabolism. While there he showed an increase in raffinose in *X. viscosa* in response to low temperature (1°C) stress. In addition, recombinant XvGolS was functionally expressed in an in vitro reaction, thereby confirming the identity of the gene product as a bona fide GolS. As some variability was seen in the carbohydrate spectra of individual *X. viscosa* plants, some of which had been collected in the wild and some germinated from seed in the lab, studies are underway to determine if natural history plays a role in this variability. We need to decide whether to make transgenic maize expressing this gene.

Shaun has written up his MSc thesis and has a bursary from Felix Keller to return to Zurich to do a PhD on *XvGolS*. We are delighted by this and will continue to collaborate with Felix, one of the world leaders in this field, and Shaun. As his work develops we will decide whether to make transgenic maize with this gene.

3. Other proteins

a) *X. viscosa* stress associated protein, XvSAP1 (Dahlia Garwe, PhD student graduated December 2003, to be taken up by new PhD student, Priver Namanya in late 1994) **Note: Priver will not be joining the lab.**

This protein is a membrane protein with six transmembrane regions. It is induced in *X. viscosa* by dehydration, salt, low and high temperature and high light treatment. As Dahlia was seconded to us from the Zimbabwe Tobacco Research Board, she made transgenic tobacco and Arabidopsis plants which showed greater tolerance than vector transformed and untransformed control plants under the following conditions:

- Treatment at 42°C for 12 hours followed by growth at normal temperatures for 2 weeks
- Growth in media containing 50 mM and 100 mM NaCl (*A. thaliana*) and 200 mM NaCl (tobacco)
- Growth in media containing 150 mM mannitol

We have since shown that transgenic tobacco plants could withstand lack of water for two weeks by which time the control plants were dead. These results suggest that XVSAP1 may confer tolerance to a variety of abiotic stresses in transgenic maize. To this end Ms. Garwe has cloned the gene into a monocot vector and our tissue culture technician has bombarded it into fresh maize HiII callus obtained from colleagues in the USA. When Priver joins us towards the end of this year she will continue this research as part of her PhD.

Our technician has successfully obtained transgenic HiII maize carrying XvSAP1. Our new post-doctoral fellow is taking up this project.

b) Dehydration Response Element binding protein, DREB1A (Dr Sagadevan Mundree)

A truncated *Dreb1A* cDNA was isolated from the *X. viscosa* library but attempts to clone the complete gene were unsuccessful. More recently, Dr Mundree used a different library to screen for *Dreb1A* while spending time in a laboratory of colleagues in Seville, Spain. Although he identified a number of potential clones, sequencing showed that none were the required gene. We have decided to abandon this search.

c) Vacuolar H^+-ATPase subunit c (Saberi Marais, MSc student to graduate with distinction December 2004)

Vacuolar ATPases have been implicated in response of plants to salinity stress. Subunit c is integral to V-ATPase function, and its c-DNAs have been identified and characterised in a range of angiosperm species. Subunit c's steady-state transcript levels display a 2 to 4-fold increase in response to salinity stress. A gene for the c subunit, *XvVatp*, was isolated from the *X. viscosa* library and shown to be induced when *X. viscosa* leaves were subjected to 150 mM NaCl and dehydration. A yeast complementation system has been used to show that the XvVatp1 protein can supply the corresponding protein lacking in the yeast strain, *vma3*. Northern blot studies have shown that in *X. viscosa* the gene is upregulated in response to dehydration and NaCl stress, and remained at steady state in response to ABA application. We need to decide whether to make transgenic maize plants expressing this gene.

We will not be carrying on with this gene.

d) Calcium binding proteins (Nailah Conrad, MSc student)

Nailah is working on *XvCam*, encoding a calmodulin protein, and *XvEF*, encoding a calcium binding protein containing one EF-hand. Indications, using polysomal RNA in northern plots, are that the former is upregulated by dehydration stress. Our initial characterisation of XvEF also revealed that it is upregulated by dehydration stress.

Further northern and western blot analyses are currently underway. The significance of calcium binding proteins is to identify downstream targets during abiotic stress. Currently, we have no intention of producing transgenic maize plants until we have an indication of the role these proteins play in abiotic stress signalling.

Nailah is writing up her MSc thesis.

e) Microarray analysis (Bienyameen Baker, post-doctoral fellow; Rob Ingle, post-doctoral fellow) **Note: Rob's post-doctoral fellowship has come to an end. Bienyameen is carrying on this work.**

RNA extracted from 35% relative water content (RWC) leaves of *X. viscosa* was used to construct a full-length cDNA library. Individual clones (8736) were amplified and spotted onto microarray slides while Bienyameen was working for a year in the laboratory of Professor Malcolm Bennet at the Sutton Bonnington campus of the University of Nottingham, funded by UCT. The slides were screened with mRNA isolated from hydrated and dehydrated *X. viscosa* leaf tissue. 45 independent clones were identified as upregulated more than two-fold in response to dehydration. These were broadly categorized into genes coding for proteins involved in chlorophyll synthesis, translation machinery and other known stress responsive genes such as late embryonic abundant proteins (LEAs), dehydrins and other desiccation related proteins. Four cDNAs, not previously implicated in the dehydration response, included *Xvvtc2* with high similarity to the Arabidopsis *Atvtc2* (vitamin C) gene, which is thought to be involved in ascorbate synthesis or regulation. Ascorbate is involved in antioxidant systems that eliminate ROS. Northern blots confirmed that *Xvvtc2* transcription in *X. viscosa* is induced at low RWC which suggest it could be involved in drought tolerance. We need to decide whether to make transgenic maize plants expressing this gene or any of the other 41.

A more recent initiative has been instigated by Rob in collaboration with Dr Mel Oliver (USDA), who spent a month in our lab this year, and Dr Lee Sweetlove (University of Oxford), where Rob did his PhD. They are looking for RNA transcripts that are sequestered during dehydration and only translated into protein upon rehydration. Some of these proteins could play a role in the repair of dessication-induced damage and thus may be good candidates for developing drought tolerant maize. The project involves translational state microarray analyses and large scale proteomics. Results from these experiments are expected by the end of October 2004.

A number of interesting proteins have been identified. This work is more theoretical than practical and will not be part of an application for funding from the Maize Trust.

4. **Promoters** (Rob Ingle, post-doctoral fellow) **Note: This work is extremely important for the development of transgenic maize. It will be carried on by our new post-doctoral fellow, Revel Iyer.**

As the existing stress inducible promoters are stringently covered by patents we decided to clone our own. Rob has succeeded in cloning approximately 2 kb upstream of *XvPer1* and *XvSAP1*. Bioinformatic analyses of these sequences have

indicated the presence of multiple elements that may play a role in stress-induced expression of these two genes. In reality only some of these elements will play a functional role in gene regulation and to this end it is necessary to create a promoter deletion series. Reporter constructs have been made in which the promoter regions have been fused to the luciferase gene. Two systems have been chosen to test these constructs. The first is the moss, *Physcomitrella patens*. The plants were obtained from our collaborator Dr Mel Oliver at the USDA, and a culture system optimised. The second system is a black Mexican sweetcorn (BMS) tissue culture that has been extensively used in testing constructs for *Maize streak virus* resistance. The constructs have been made and we are now ready to test them. Rob will continue this when he returns from Oxford in November.

Rob was unable to introduce the promoter-luciferase constructs into *P. patens* and we decided not to waste time with BMS but to go directly into maize. We have transgenic HiII carrying the construct and Revel Iyer will be testing the inducibility of the promoters. We also have the Arabidopsis homologue of the *XvPer1* promoter to compare.

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