L&L Agricultural Services

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To: "Jennifer Thomson" <Jennifer.Thomson@uct.ac.za>
Cc: "Rozetta Ferguson" <Rozetta.Ferguson@uct.ac.za>
Sent: 08 April 2008 12:13 PM
Attach: L&L Map to Offices.doc
Subject: Maize Research Project on Drought Tolerance

Dear Jennifer

I refer to our telephone conversation earlier today and wish to confirm the following:
1. The Maize Trustees have requested you to address them with regard to the drought tolerant maize project at their next meeting in Pretoria;
2. The meeting is to be held at our offices at 1187 Cobham Road, Queenswood in Pretoria on 21 April 2008 (map attached for your convenience).
3. Your presentation is scheduled from 11:30 to approximately 13:00 and you are cordially invited to have lunch with the Trustees thereafter.
4. Your travelling and related costs in making the presentation will be borne by the Trust. The University can invoice us for these costs as soon as it is convenient.
5. The Trust will consider making a further payment with regard to the project after your presentation.

Please let me know if you should require any further information.

Kind regards
Leon du Plessis

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The Maize Trust Report – February 2008

As stated in my December 2006 report PhD student Richard Okoth produced a number of seeds from transgenic maize plants transformed with the various promoter constructs driving two *Xerophyta viscosa* genes, *Prx2* (encoding an antioxidant) and *Sap* (encoding a membrane signalling protein) as well as a reporter gene, *lux* (encoding luciferase). During the course of 2007 we took delivery of a new plant growth chamber (costing about R1 million including installation). We are now finally in a position to grow maize plants to maturity and set seed! Richard's plants are currently growing in this chamber, they will be allowed to set seed and the resulting plants will be tested for dehydration tolerance. This is a major milestone. In the meantime Richard has been testing the promoter constructs in other plant systems as outlined below.

1 Bioinformatics

Bioinformatics analyses of XvPsap1 (~2kb) promoter and its truncated fragments XvPsap2 (~1.5kb) and XvPsap3 (~1kb) were performed. The promoter sequences were blasted in the NCBI database to probe for sequence homology with existing plant promoters. No complete similarity was observed suggesting the novelty of the promoter sequences. Identification of sequence motifs in comparison with other plant promoters was conducted using the PLACE and PLANTCARE software. Various *cis*-acting elements including those involved in drought stress-inducibility, heat stress, abscisic acid, MeJA, light and low temperature responsiveness were identified. Additionally, common *cis*-acting element in promoter and enhancer regions, meristem specific activation, endosperm expression, auxin-responsive and circadian control elements were observed. These motifs serve as necessary guidelines on the possible stress experiments to be performed on the putative transgenics transformed with gene constructs harbouring XvPsap promoters driving reporter genes.

2 Evaluation of XvPsap promoter activity in *Nicotiana tabacum*

To evaluate the activity of XvPsap1 promoter and its truncated fragments in a dicot, *Nicotiana tabacum* was transformed with gene constructs containing the *luc* gene cloned downstream of XvPsap promoters and upstream of *nos* terminator. *Agrobacterium tumefaciens* strain EHA101 containing the expression vector pTF101.1 was used to transform tobacco leaf dics. Bialaphos was used for selection of putative transformants.

T_0, T_1 and T_2 putative tobacco transformants were screened by first applying 0.75 % basta on the leaves. Basta tolerant transgenics were further screened by PCR using *luc* gene specific primers. Positive transformants were then subjected to dehydration and salt stress treatments. For dehydration treatments, water was withdrawn for 9 days and leaf samples were collected at time points 0, 3, 6 and 9 days. Non-transformed plants and positive transformants regularly watered for the same period were used as negative and positive controls respectively. The activity of the XvPsap1 promoter was assessed by conducting a luciferase assay reaction. Protein was extracted from the corresponding dehydrated samples and luciferase assay was performed using a luciferase assay kit (Whitehead Scientific (Pty) Ltd) according to the manufacturer's instruction. Preliminary results indicate a gradual correlative increase in luciferase activity under XvPsap1 promoter with the elongation of the dehydration period. The highest relative light unit was recorded in leaf samples collected after 9 days of dehydration when the relative water content (RWC) was 41.1 %. Luciferase assay reactions on transgenics containing XvPsap2 and XvPsap3 promoter fragments are yet to be performed. For salt stress treatments, transformants were watered with 60ml of 200mM NaCl and leaf samples collected at time points 0, 6, 12, 24 and 48hrs. Similarly, non-transformed plants and positive transformants watered with 60ml of distilled water were used as negative and positive controls respectively. Luciferase assays on leaf samples collected under salt stress are still pending.

3 Evaluation of XvPsap promoter activity in BMS cells

The activity of XvPsap1 promoter and its truncated fragments was also tested in a monocot. Black mexican sweetcorn (BMS) cells were transformed using microprojectile bombardment with gene constructs containing the *gfp* gene cloned

downstream of the XvPsap promoters and upstream of the *nos* terminator in a pA53 plasmid. After 24hrs, the BMS cells were harvested and viewed through florescence microscopy for green florescence. The results show a low intensity of the green florescence compared to the level observed from positive control BMS cells, transformed with *gfp* regulated by the constitutive ubiquitin promoter. From bioinformatics analyses, the presence of various *cis*-acting in the XvPsap promoters suggests that the promoters could be stress inducible. Arguably, the low levels of green florescence observed under normal conditions could, therefore, be attributed to the basal activity of most promoters including stress-inducible promoters. Experiments assessing the levels of *gfp* expression under salt (200mM NaCl) and PEG treatments are yet to be conducted. BMS cells will also be transformed with XvPsap promoter-constructs containing the *luc* gene.

4 Cloning

Various stress tolerant genes such as *XvSap1, XvPrx2, XvAld1* have previously been isolated and characterised from *Xerophyta viscosa*. The ability of these genes to confer stress tolerance has been tested in Arabidopsis and tobacco. However, the present challenge is whether these novel genes can confer the same stress tolerance when transformed into economically important crops such as maize. As initial attempt towards the ultimate transformation of maize, *XvSap1, XvPrx2* and *XvAld1* were independently cloned downstream of a constitutive ubiquitin promoter and upstream of the *nos* terminator in a pA53 biolistics plasmid. Two of these gene constructs will be transformed into BMS cells through particle bombardment. Their ability to confer stress tolerance will be tested in BMS cells under salt and PEG treatments.

The aldose reductase gene has also been cloned downstream of the Psap promoters. These constructs were bombarded into BMS cells and conferred tolerance to 200 mM salt and 200 mM sorbitol. These results are extremely promising and transgenic maize plants will be generated with these constructs during 2008.

Following up on earlier work with the aldose reductase gene cloned downstream of a constitutive promoter, transgenic Arabidopsis plants with simple integration patterns

showed enhanced tolerance to salt, osmotic and dehydration stresses. Transgenic plants accumulated lower levels of lipid peroxidation-derived aldehydes than controls implying that XvAld1 protects against oxidative damage.

5. Proteomics

Proteomics involves the systematic study of proteins in order to provide a comprehensive view of the structure, function and regulation of biological systems. It is envisioned that qualitative and quantitative comparisons of the proteomes of X. viscosa and maize cell suspension cultures under different osmotic stress conditions using two-dimensional electrophoresis and mass spectrometry will help to identify key proteins or mechanisms involved in the responses.

PhD student, Mr Hlahane has nearly completed all the physiological experiments on maize cell suspension. He has optimized high throughput protein extraction from multiple cultures and has begun one-dimensional analyses. In the near future he will begin with two-dimensional gels of the maize proteomes.

PhD student Mr Abdalla has isolated nuclei from X. viscosa in both hydrated and dehydrated states followed by protein extraction and proteomic analysis. He has verified the protocols, established nuclei purity using DAPI staining and protein integrity on 2-D gels. He is about to begin analyses of the nuclear proteins using iTRAQ technology at the UCT Centre of Genomics and Proteomics.

The Maize Trust Report – March 31st 2007

a) Project Leader: Prof Jennifer A Thomson

b) Actions Taken, Progress Made and Results Achieved:

As stated in my December 2006 report 160 putative transgenic maize seeds were brought back to UCT by PhD student Richard Okoth who had done the maize transformations using 3 different promoter-gene combinations at Kenyatta University, Nairobi. Richard also brought back leaf material from the parental plants used to set seed. He has now tested these using Polymerase Chain Reaction (PCR) to determine which are indeed transgenic. The results are shown in Table 1.

Table 1. Efficiency of maize transformation

Gene constucts	No. of transformants	No. of transformants that set seed	No. of seeds
P1-*Prx2-nos*	4	1	7
P3-*Prx2-nos*	2	0	0
P4-*Prx2-nos*	7	3	32
P1-*Sap-nos*	13	8	60
P3-*Sap-nos*	6	2	7
P4-*Sap-nos*	6	4	35
P1-*Luc-nos*	3	0	0
P3-*Luc-nos*	4	3	249
P4-*Luc-nos*	1	0	0
Total	**46**	**21**	**390**

Our conviron growth chamber arrived earlier this year. It is now running satisfactorily and the above maize plants are being grown in it to enable us to subject them to abiotic stresses and perform physiological analyses. We will also (finally) be able to set seed in our own lab!

As also stated in the December 2006 report Richard also brought back many seeds of putative transgenic tobacco. These have also been tested by PCR and the results are that we have thousands of transformed seeds to test.

Our post-doctoralscientist, Dr Revel Iyer, together with my colleague Dr Suhail Rafudeen, has spent a considerable amount of time investigating the nature of the XvSAP1 protein. All we knew was that it has limited homology to any known proteins in the data banks and that it is a membrane binding protein. It now appears as if it is a novel member of a class of membrane proteins, mainly found in animals, that transmit signals to other proteins within the cell. Recently a similar but unrelated protein in a dicotyledenous plant was shown to be involved in drought tolerance. If

we can determine which proteins XvSAP1 could be sending signals to it could help us determine which other gene to introduce into maize together with it. This process is called "stacking" and the best way to achieve this is by transforming maize with each gene singly and then breeding them together. We would really be lucky if one of the genes we have/are transforming into maize is the right one to stack with XvSAP1!

The promoter patent forms have been completed and forwarded to Spoor and Fisher for analysis and lodging. As this could take some weeks, Richard Okoth will not present the promoter work at the Rockefeller Foundation meeting in Maputo 26-29 March 2007.

PhD student Alice Maredza continues her work with the expression of XvAld1 in transgenic Arabidopsis. The plants continue to show good resistance to salt, osmotic stress and dehydration. Dr Thokozile Lewanika has joined our team and is working on cloning *XvAld1* into a maize vector for transformation into maize later this year. The conviron growth chamber will really be in full use by then.

Regarding the concerns expressed by the Trustees regarding Monsanto's work on dehydration, the conviron will certainly speed up our work. In addition Dr Lewanika is already proving to be an extremely useful member of our team. Finally I recently received a grant from the International Centre for Genetic Engineering and Biotechnology based in Trieste, Italy for two more PhD students to join our group. One is from Zimbabwe and one from Kenya. We are in the process of choosing these students.

c) Problems Encountered:
None during the months January – March 2007-03-20

d) Milestones that have not been achieved:
None

e) Adequacy of funding:
Adequate – refer to financial report that was submitted in January 2007

f) Duration of the project:
Three years

The Maize Trust Report – December 2006

a) Project Leader: Prof Jennifer A Thomson

b) Actions Taken, Progress Made and Results Achieved:

Table 1 shows the number of putative maize transformants and the number of seeds set. These will be brought back to UCT in January 2007 for molecular genetic analysis. This will enable us to determine which are true transformants and which are escapes.

Table 1 Number of putative maize transformants

Gene constructs	**No. of putative transformants**	**No. of putative transformants that set seeds**	**No. of seeds**
P1- *Prx2*-nos	4	1	7
P3- *Prx2*-nos	2	0	0
P4- *Prx2*-nos	7	3	32
P1- *Sap*-nos	13	8	60
P3-*Sap*-nos	6	2	7
P4-*Sap*-nos	6	4	35
P1-*Luc*-nos	3	0	0
P3-*Luc*-nos	3 (A188)	2	19
	1 (KAT * A188)	1	Still maturing
P4-*Luc*-nos	1	0	0
Total	**46**	**21**	**160**

Note: Some transformants could not set seeds probably due to somaclomal variation. (KAT*A188) means that KAT (Kenyan maize genotype) was cross-pollinated with pollen from transformed A188.

Table 2 shows the maize transformation efficiency. This is as good or better than other labs working in maize transformation. Unfortunately a consignment of Petri dishes to Kenyatta University was contaminated. Richard Okoth, the PhD student working on this project, only discovered this when maize transformants became overgrown with fungi. By then they had to be discarded. However, he has enough lines to analyse.

Table 2. Summary of maize transformation

Gene Constructs	No. of Explants Infected (x)	No. of Contam-inatecd explants (y)	Actual Explants (x-y)	No. of Calli that Survived Selection (z)	Transfor -mation Frequency % of z/x-y	No. of Putative Transformants (r)	Transformation efficiency % of r/x-y
P1-*Sap*-nos	2000	400	1600	50	3.125	13	0.8125
P3-*Sap*-nos	1300	200	1100	40	3.636	6	0.545
P4-*Sap*-nos	1300	150	1150	35	3.043	6	0.522
P1-*Prx2*-nos	850	100	750	25	3.333	4	0.533
P3-*Prx2*-nos	900	150	750	30	4	2	0.267
P4-*Prx2*-nos	1600	300	1300	50	3.846	7	0.538
P1-*Luc*-nos	700	150	550	30	5.454	3	0.545
P3-*Luc*-nos	300	20	280	13	4.643	3	1.071
P4-*Luc*-nos	250	40	210	8	3.8	1	0.476

As tobacco is relatively easy to transform he also introduced the three promoters linked to the marker luciferase gene (Table 3). It will be interesting to determine whether the expression patterns in a monocot and a dicot are the same.

Table 3. Tobacco transformation

Gene Constructs	Transformation Frequency	Transformation Efficiency	No. of transformed seeds
P1-Luc-nos	74	66	The tobacco seeds are too tiny to be counted. However, for each constructs there are thousands of seeds.
P3-Luc-nos	80	65	
P4-Luc-nos	77	60	

All the transgenic plants will be subjected to abiotic stresses and physiological analyses performed. The third *Xerophyta viscosa* gene to be tested, *XvAld1*, has not yet been cloned downstream of the promoters. Unexpected difficulties were encountered while this was attempted and we decided to leave that for another student to enable Richard to return to Kenyatta University in order to make the transgenic plants. This cloning will be done by a new post-doctoral fellow, Dr Thokozile Lewanika, who will be joining our team in January 2007. As mentioned in my July 2006 report I have bought a plant growth chamber that has sufficient light intensity to allow us to work with maize, including setting seed, in our own building. The chamber will be installed during December 2006. Thus Thokozile will also transform maize with her constructs.

A first draft of the promoter patent has been prepared and is under scrutiny by Mr Piet Barnard and colleagues in UCT Innovation. We need to complete this and lodge it early in 2007 as Richard Okoth wishes to present the work at a Rockefeller Foundation meeting in Maputo in March 2007. He can only do this if the patent has been lodged.

Our post-doctoral fellow, Dr Revel Iyer, together with my colleague Dr Suhail Rafudeen, has spent a considerable amount of time investigating the nature of the XvSAP1 protein. All we knew was that it has very little homology to any known proteins in the data banks and that it is a membrane binding protein. It now looks as if it is a novel member of a class of membrane proteins, mainly found in animals, that transmit signals to other proteins within the cell. If we can determine which proteins XvSAP1 could be sending signals to it could help us determine which other gene to introduce into maize together with it. This process is called "stacking" and the best way to achieve this is by transforming maize with each gene singly and then breeding them together. We would really be lucky if one of the genes we have/are transforming into maize is the right one to stack with XvSAP1!

PhD student Alice Maredza has continued research into XvAld1 expression in transgenic Arabidopsis. Plants showed increased root growth on 50, 75 and 100 mM salt and on 14% polyethylene glycol, an osmotic stress. Dehydration of the plants led to 50% survival compared to 20% of non-transgenic plants.

c) Problems Encountered:
Only the contamination mentioned earlier.
d) Milestones that have not been Achieved:
The cloning of the XvALD1 gene as mentioned above.

e) Adequacy of the Funding:
Please see the finance report which will be sent separately in January 2007.

f) Duration of the Project: Three years