Summer 1 Laidlaw Report 2021:

Development of Tools and Methods to Investigate Crosstalk between Plant Transcriptional Responses to Stress

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1. Introduction

Transcriptional changes are a plant's major response to external stress. However, a defence response is taxing both in terms of nutrients and energy (Freeman *et al.*, 2011). The evolution of stress-inducible promoters allows the organism to adjust its transcriptome to produce protective proteins associated with that stress. A key feature of these are cis-acting motifs which bind co-activators or transcription factors to influence the transcriptional machinery at the core promoter according to their type, number, position and combination around the promoter (Hernandez-Garcia and Finer, 2014). Using transcriptional reporters linked to known promoters (e.g. luciferase) we can assess the plant's stress response to stress and where it takes place. Understanding these regulatory elements is vital to up/down regulating transcription rate or creating constitutive or inducible promotors (Mehrotra *et al.*, 2017). This knowledge can contribute to the development of new crop protection strategies, making them more robust to abiotic stresses or more efficient in the face of biotic stresses. Already, these reporters have helped elucidate phytohormone responses, calcium signaling and the mediator complex (Kaplan *et al.*, 2006; Whalley *et al.*, 2011; Lee *et al.*, 2021).

This project was limited to six weeks, therefore generating new transgenic plants lines was not an option. However, some pre-existing lines were already available in the lab such as wild-type *Arabidopsis* expressing a luciferase reporter for ABRE (abscisic-acid-responsive element) and DRE (drought-responsive element) responsive transcription. When a plant comes under multiple stresses, pathways interact and regulate their response through 'crosstalk'. For example, the abiotic stress hormone ABA (abscisic acid) has been shown to be influenced by the biotic stress hormone JA (jasmonic acid) (Aleman *et al.*, 2016). Also, since ABA is heavily involved in drought, cold and salinity response, we decided to test how exogenous treatment would affect the DRE motif. Likewise, Rabara *et al.* (2013) found that ABRE-enriched promoters were heavily influenced by JA. This provided a rationale for our crosstalk investigation.

Understanding how plants respond to stress can be aided by the ability to study their responses in mutant plants. *In planta* experiments are often limited by cost, time and scale so a new project appeared alongside the crosstalk investigation: to set up transient gene expression in protoplasts. These are stably expressing isolated mesophyll cells. This would allow us to study a variety of mutants without having to stably transform them. While they lack a cell wall they retain many of the same physiological characteristics (Yoo, Cho and Sheen, 2007) and have several advantages. Versatile and cheap, they can be used for high-throughput screening of many molecular pathways, such as promoter-reporter constructs used to describe phytohormone response (Lehmann *et al.*, 2020).

Two aims were set out for the six-week project: 1) to investigate DRE and ABRE motifs with JA and ABA; 2) to set up calcium-PEG protoplast transfection and optimise it for the lab.

2. Materials and Methods

General

Plant Growth

Seeds were planted on Murashige and Skoog 1964 agar medium, according to the method in Hemsley *et al. (*2014). The seedlings were moved to 44-mm peat plugs (LBS Horticulture, Colne, UK) and left for 4 weeks under the conditions stated: 20°C; 12 h : 12 h, light : dark; 150-200 µE m2 s 1 light. The plants were between 5 and 7 weeks old during the experiments.

Protoplast Isolation

Protoplasts were isolated using the 'Tape-Sandwich' method described in Wu *et al.* (2009). The same Time tape (Time Med, Burr Ridge, IL) was used to fix the dorsal side of the leaf, however, ordinary Sellotape was used to peel off the lower epidermis. Senescent leaves with more developed vasculature had their midribs flattened gently with a fingernail. Another deviation included cutting off the exposed edges of the leaves at the Time tape stage for easier peeling. Usually around 0.5g of leaf material could be digested easily with 8mL of enzyme solution in a 6-well tissue culture plate. After 1.5-2 hours at room temperature (~20°C) the free protoplasts were filtered through a 70µm cell strainer and washed in an equal volume of cold W5 solution. Using 3ml dropping pipettes the mixture was transferred to 50mL Falcon tubes and centrifuged at 400rpm for 2 minutes. When the pellet was carefully isolated and the washing process repeated, the cells were diluted to $2x10^5$/mL in MMG. Cells were made fresh and used the same day.

Luciferase Assay

The protocol published by Lehmann *et al.* (2020) followed with modifications suggested by the authors (Dominguez-Ferreras A) and was adjusted according to the experiment being conducted. All LUC detection was conducted in a 96-well white plate.

Reagents

Enzyme solution: 1.5% cellulase 'Onozuka' R10 (Yakult, Tokyo, Japan), 0.4% macerozyme 'Onozuka' R10 (Yakult), 0.4M mannitol, 10mM $CaCl_2$, 20mM KCl, 0.1% BSA and 20mM MES pH 5.7

W5: 2mM MES pH 5.7, 154mM NaCl, 125mM $CaCl_2$, 5mM KCl

W1: 4 mM MES pH 5.7, 0.5 M mannitol, 20 mM KCl

MMG: 4mM MES pH 5.7, 0.4M mannitol, 15mM $MgCl_2$

Luciferin buffer: 1mM Luciferin (Promega, Potassium salt 50 mg E1602), 3mM ATP, 15mM $MgSO_4$, 30mM HEPES 7.8

PEG solution: 40% w/v PEG4000, 0.2M mannitol, 100mM CaCl2

2.1 - Stable Lines

a) ABRE/DRE response

Protoplasts were extracted from plants stably expressing the constructs C6, DRE and ABRE (referred to from now on as ABRE protoplasts etc.) underwent a 'mock transformation' first

(described later). Three repeats were conducted for each hormone treatment plus a control. Samples were measured for 3.5 hours.

b) LUC buffer investigation – adaption of protocol for stable protoplasts

30µL ABRE protoplasts ($2.5x10^5$) were pipetted into a white plate along with 12 buffer and 6 hormone. HEPES buffer had 2 repeats whereas the MMG samples had none. JA response was also measured but is not relevant to the investigation and did not respond.

c) Protoplast concentration adjustment

Modified[1] p1000 tips were used to dilute the protoplasts to the correct concentrations in each well, an adjustment to the previous experimental method. ABRE PPs = $2.4x10^5$; C6 PPs = $2.1x10^5$ therefore 24µL ABRE + 76 W1 and 21ul C6 PPs + 79µL W1 results in ~10,000 PPs per well. The starting photon count was low and as the luciferin must be in excess an extra 10µL was added to each well.

d) Hormone pre-treatment

600µL ABRE protoplasts ($2.1x10^5$) were transferred to a 2mL tube. Instead of adding the hormone individually to each well, the protoplasts were pre-treated with 72µL JA/ABA/ethanol control for more precision. 20µL LUC buffer was added to each well along with 100µL pre-treated cells.

e) W1 wash steps for MMG

MMG previously caused issues with the protoplast's response to luciferin and previously this was the default solution for the stable protoplasts. Therefore the wash steps described in Yoo, Cho and Sheen (2007) for their protoplast transfection were carried out. 1200µL ABRE protoplasts ($2x10^5$/mL) were centrifuged at 100g for 2 minutes. The MMG supernatant was removed and protoplasts were resuspended in 1200µL W1. This wash was repeated then 144µL hormone treatment was added to the total volume for pre-treatment. 100µL PPs and 20µL LUC buffer were added to each well and expression measured.

2.2 - PEG-calcium transfection

Plasmid Construct

The vector contained a minimal 35S promoter sequence with four tandem repeats of the DRE or ABRE motif cloned into the Ncol site, upstream of the LUC+ reporter. The control (C6) lacked this concatamer element. See Pietrzak *et al.* (1986) and Whalley *et al.* (2011) for more detail.

DNA Extraction

AEQ plasmid pMAQ2 was extracted using the Invitrogen™ PureLink™ HiPure Plasmid Filter Maxiprep Kit (Knight *et al.*, 1991).

Aequorin Assay

Aequorin was an ideal marker of transformation as it is expressed under the same 35S promoter, meaning the levels do not change. Also, the measurement technique is well established in this lab in terms of equipment and expertise. In low light, transformed

[1] Aperture enlarged by removing the tip with scissors.

protoplasts were first reconstituted with coelenterazine for 2-3 hours to produce functional aequorin from the precursor. Samples were transferred to a 12mm Röhren cuvette (Sarstedt) and the luminometer cooled to 4°C to reduce 'thermal noise' (Knight and Knight, 1995). To measure, a cuvette was loaded into the luminometer adjacent to the photomultiplier tube and a 1mL syringe was filled with 500µL of 2M $CaCl_2$ with 20% ethanol. Before insertion, 10 seconds of background was taken and the needle wiped clean. Luminescence was measured and data extracted. A background reading was taken and subtracted from the final values, including the control (mock-transformed WT protoplasts under the same conditions). The full protocol is in Knight *et al.* (1991).

Method Development

Several investigations were designed to optimise the transformation method for the lab conditions and materials. Amongst those consulted were:

- 'Warwick method' (from correspondence with Dominguez-Ferreras A of Lehmann *et al.* (2020))

  This 'tube setup' involved suspending protoplasts in MMG at 200,000/mL for a single plasmid transformation. Protoplasts should be kept on ice until use. For a small scale transformation: 100µL of protoplasts (~20,000) are added to 4µg of plasmid (at 1 µg/µL) in a 2mL tube. This is mixed by gentle flicks before 200µL + plasmid volume PEG solution is added. Mixture is flicked again until homogenous and incubated for 20 minutes at RT. 500µL W5 stops the transformation. This is centrifuged for 2 minutes at 100g and the supernatant removed. 500µL W1 is added and the tubes are covered in Parafilm then incubated in the growth chamber overnight (16 hours).

- Yoo, Cho and Sheen (2007)

  Suggests higher initial plasmid concentration (10-20µg) and 1mL W1 for a well from a 6-well tissue culture plate. This is to prevent hypoxic conditions for the protoplasts. Also, culture plates are coated with 5% vol/vol sterile calf serum for 1-2 seconds to prevent sticking. Otherwise there is minimal deviation from above.

- Negrutiu *et al.* (1987)

  This method uses a much higher protoplast concentration ($2x10^6$), carrier DNA (herring sperm DNA) and a much longer transfection time. After the PEG addition stage was identified as a 'bottleneck' for protoplast survival (see later 2a), we took tips from this old method which was more careful with the cell's osmoticum. After 30 minutes in 20% PEG, W5 is added stepwise over a 20-minute period. This was also recommended in Abel and Theologis (1994).

- Development of Improved Techniques for the Genetic Transformation of Citrus Protoplasts (ufl.edu) - date accessed 27/09/2021

  This large report was not specific to Arabidopsis but contains a guide for the optimisation process of PEG-calcium transfection.

2.2 - Investigations

a) Cell mortality during transformation

A 'mock transformation' was conducted for stable protoplasts to help understand how many cells made it through the process and which stages were damaging. The 'Warwick method's' 'tube setup' was used: 300µL ABRE protoplasts mixed with equal vol. PEG and left for 15

minutes. 500µL W5 added to stop the transformation and the mixture centrifuged for 1 minute at 100g. Supernatant was removed and 250µL W1 added to get to original volume. Luciferase activity after 0.5 hours (time for fluorescence to stop) was compared to 'untransformed' protoplasts and relative cell mortality was calculated.

b) Transformation method

Alternate transformation methods were compared with the AEQ plasmid and tested for aequorin. Yoo, Cho and Sheen (2007) described a short transformation of 5 minutes before the W5 addition. The 'Warwick method's' protocol suggested 20 minutes. Older methods advised a 30 minute transformation followed by the gradual addition of W5 over another 30 minutes (Negrutiu *et al.*, 1987). Samples were centrifuged for 2 minutes at 100g and supernatant was removed before 500µL cold W1 was added. Tubes were incubated overnight under tissue paper in Sanyo MLR350 temperature- and light-controlled chamber with 12h light 12h dark cycles, same light level as in the stated above and temperature set to 20 +/- 1 degree. Samples were tested for aequorin as already described.

c) Plasmid/PEG concentration optimisation

WT protoplasts were made and diluted to 2.2x105/mL MMG. 9 2mL tubes were used for the transformation. The control had TE buffer in the same volume as the plasmid. Row A had 8µL plasmid, B 12µL, C 16µL while column 1 had 20% PEG, 2 had 15%, 3 had 10%. 200µL of protoplasts were added to each tube before the PEG. Cells were transformed for 15 minutes and underwent the same wash steps as above. Half the volume of each sample was removed after 2 hours incubation and tested for aequorin (as Yoo, Cho and Sheen's minimum incubation time (2007)). The rest was incubated overnight and tested the next day.

3. Results

<u>Aim 1 – Crosstalk Investigation</u>

a) DRE/ABRE response

DRE protoplasts did not respond to either ABA or JA treatment. The control plasmid (C6) also only showed background fluorescence (no luciferin luminescence) and was not used for further experiments. ABRE showed increased expression in response to ABA but further tests were conducted to confirm this.

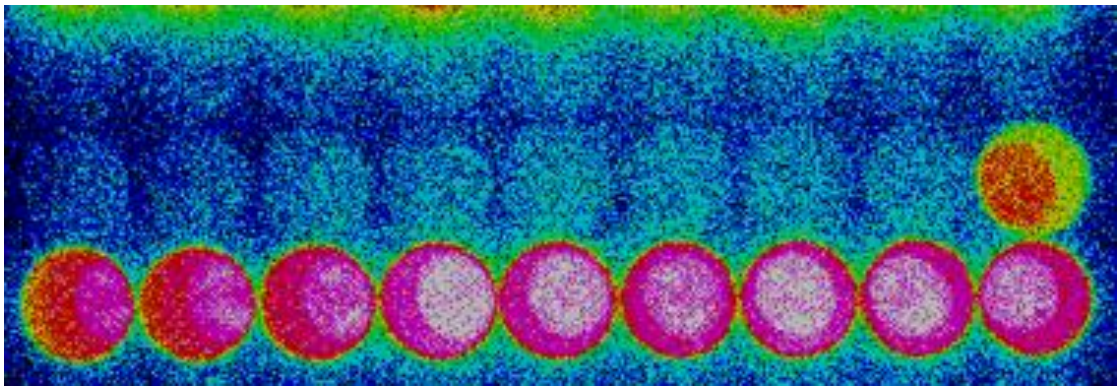


Image 1: Left three control, middle ABA, right JA; top row C6, middle DRE, bottom ABRE. See end of report for colour scale (and for following images).

b) Buffer analysis

The results show luciferase activity in the HEPES buffer but not for the MMG buffer. JA response was measured but is not shown for simplicity. The response becomes clear after 2 hours imaging and is shown below in Fig. 1.

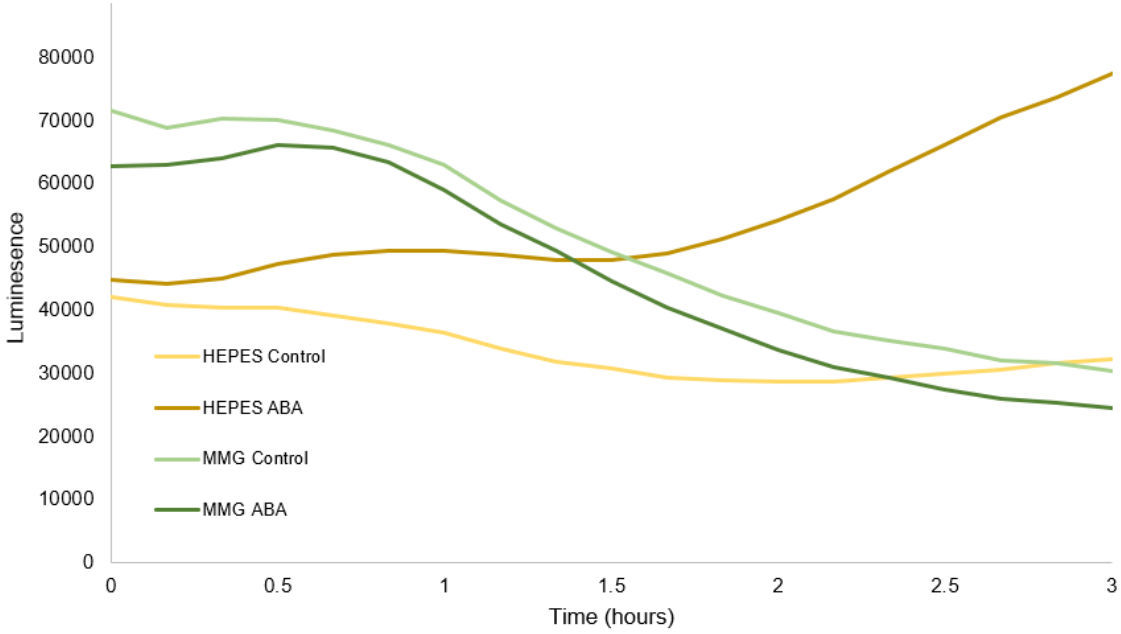
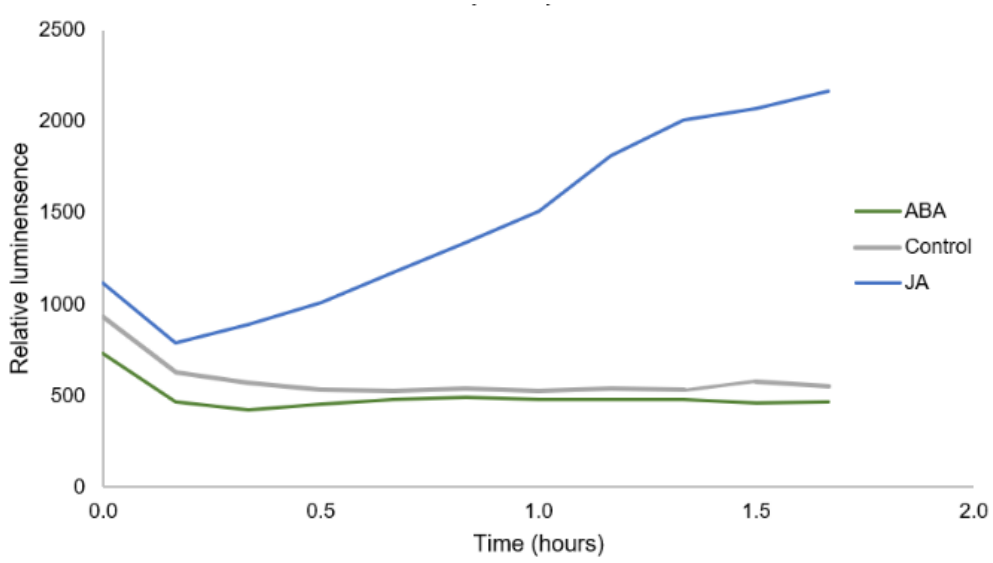


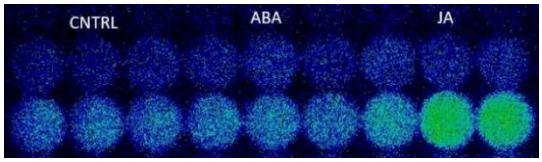
Figure 1: Buffer analysis for LUC detection of ABRE protoplasts treated with ABA. Counts at each time interval represent photon counts recorded over 600 second period (same for other graphs).

c) Protoplast concentration adjustment

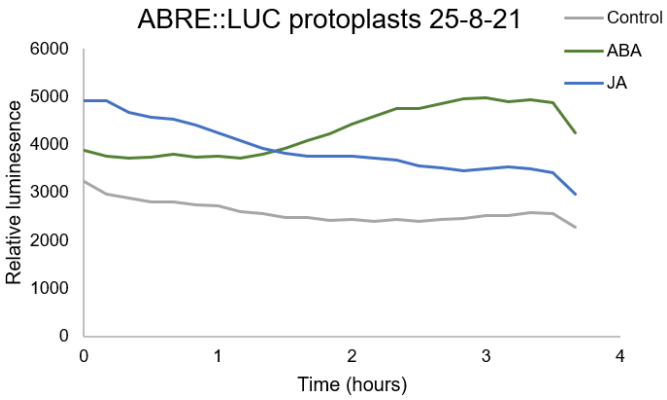
Results after just over 1.5 hours imaging indicate a response of ABRE protoplasts to JA but not to ABA. Luciferase activity in samples treated with JA seemed to increase after half an hour. Initial luminescence was significantly lower with the new setup.



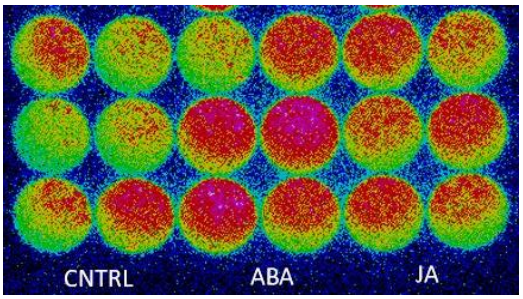
Above – Figure 2: ABRE protoplast reponse to ABA and JA treatment. Right – Image 2: protoplasts under imaging; top row contains C6, bottom row contains ABRE.



d) Hormone pre-treatment



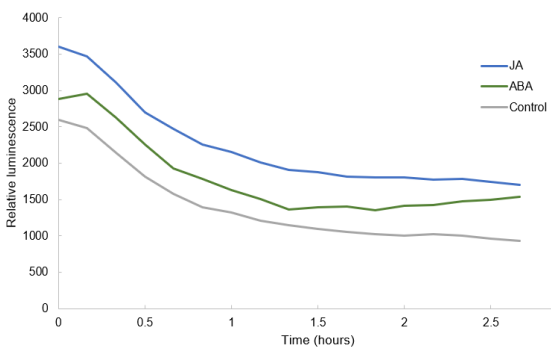
Left – Figure 3: ABRE protoplast reponse to ABA and JA treatment. Below – Image 3: protoplasts under imaging; ABRE protoplasts with 6 repeats.

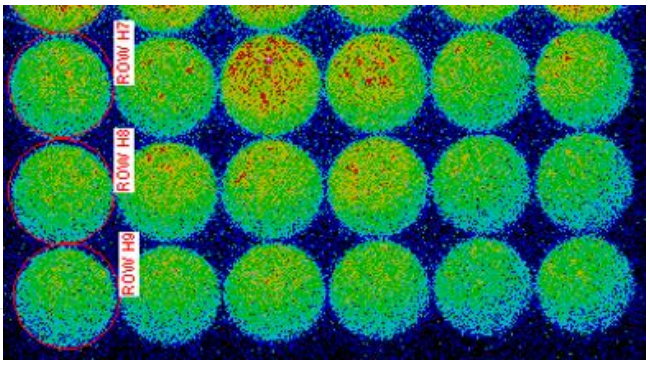


Protoplast response to ABA became visible after 2 hours. Samples did not respond to JA this time. Results shown in Fig. 3.

e) W1 wash steps

Luminescence was at in a similar range as the previous experiment. This time the ABA response was more subtle, even after 2 hours.





Left – Figure 4: Shows ABRE protoplast responses to JA and ABA treatment. Right – Image 4: Left 2 rows ABA, Middle 2 JA, Right 2 Control.

Aim 2 – Setting up transient protoplast expression of motif-reporter constructs

Up until this point we have used stably transformed lines to produce mutant protoplasts. Being able to express new constructs in wild-type cells would allow for much greater flexibility, even enabling them to be transformed with multiple constructs. Easily expressing and testing a variety of motifs could reveal much more about hormone pathways and potential crosstalk.

a) Mock transformation of stable lines

	Undamaged			Post mock transformation		
Time (hrs)	Control	ABA	JA	Control	ABA	JA
0.00	594	720	969.5	307.3333	393.5	400.5
0.17	320	408.5	697.5	157	202.5	222
0.33	321.5	423	685	135.3333	180.5	180
0.50	308.5	410.5	672	124	151	184

0.67	304	407	703.5	102	139	153.5
0.83	318	402	712	108.3333	142.5	165.5
1.00	324.5	400.5	701	108.3333	132.5	123
1.17	332	417	725	93	119	130
1.33	317.5	401	739.5	75.66667	117	132.5
1.50	308	386.5	715	92	97.5	129
1.67	304	386.5	747	68	114	120.5
1.83	220	301.5	512	56	81	72

Table 1: Comparison of stable protoplasts (left) and stable protoplasts that have undergone a mock transformation (right). Data shows their luminesence under different hormones.

The mock PEG-calcium transfection significantly reduced the luminescence of ABRE protoplasts. There was between a 70 and 82% reduction in light emission compared to the undamaged protoplasts. This was calculated using values from after 30 minutes to discount initial fluorescence.

b) Transformation method

Sample	Reading	Peak time (secs)
Control	425	1
Yoo, Cho and Sheen (2007)	2173	5
'Warwick method'	3255	5
Negrutiu *et al.* (1987)	1256	3

Table 2: shows luminometer values for varying methods of transfection for protoplasts.

The 15-minute transformation produced the highest reading on the luminometer.

c) Plasmid/PEG optimization

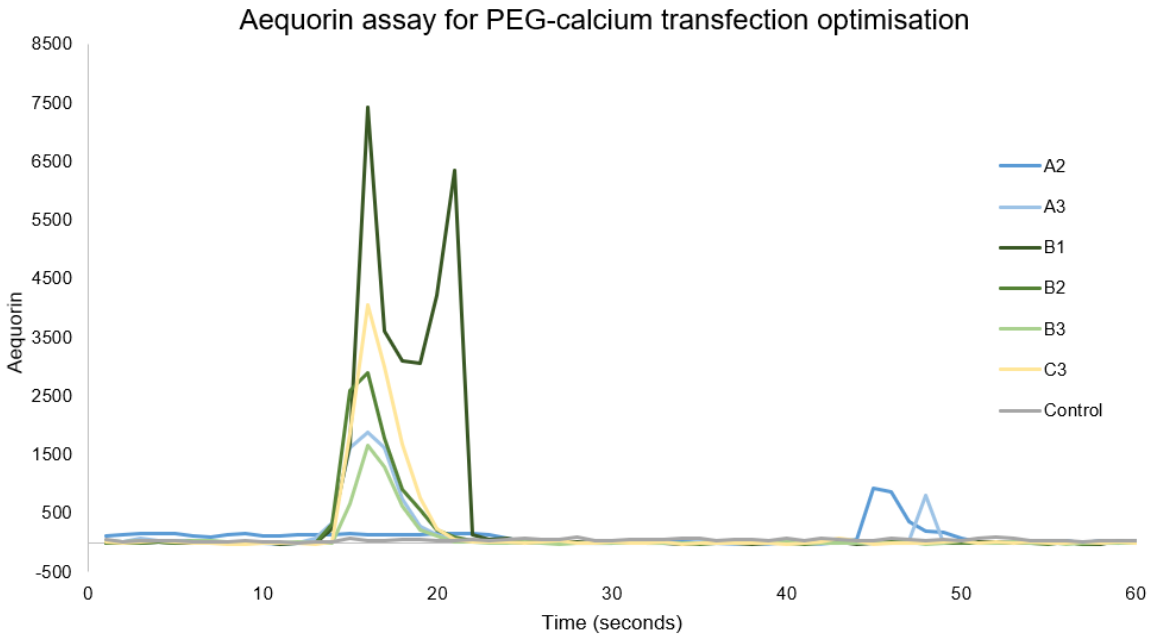


Figure 5: Dark to light = 20%, 15% & 10% PEG; blue = 8µL plasmid, green = 12µL, yellow 16µL. A1, C1 & C2 have been omitted.

B1 (20% PEG, 12µL plasmid) contained the highest amount of aequorin. A2 showed a slightly higher background reading and a late peak. A3 also had a late peak as well as an initial one at injection. C3 showed the second largest reading but the others from its plasmid bracket (as well as A1) were omitted as the reading was poor.

4. Discussion

Aim 1 – Crosstalk Investigation

Exogenous hormone treatment revealed the ABRE motif responded to ABA but not JA. The DRE motif was not activated by either ABA or JA. This second conclusion lacked a thorough repeatable experiment, but results were clear, so investigations were dropped quickly. As the protocol followed was designed for transient protoplasts, not all the conditions were the same. This meant the method had to be adapted for imaging stable protoplasts. The buffer analysis (1b) showed the solution luciferin is added to is vital for LUC imaging. MMG was the solution suggested for storing the protoplasts in so it was considered that it may make a better environment for protoplast imaging. Protoplasts are very fragile and sensitive to solute change too. However, the HEPES buffer allowed the response of ABRE to ABA. Possibly the MMG makes the luciferin inactive by denaturing it through the high solute concentration or affecting the hormone treatment.

The activation of the ABRE motif was reliably confirmed in experiment 1d. This is in accordance with previous findings, hence its name (Choi *et al.*, 2000). In experiment 1c ABRE protoplasts showed a response to JA but not ABA. This was consistent with Rabara *et al.*'s (2013) findings in which promoters enriched in ABRE were activated by JA. However, closer inspection of the data and later experiments reveal that conclusion to be misleading. In 1c, samples were measured for just over an hour. As seen later in 1d, the ABA response only becomes clear after around 2 hours. It is more likely that this JA response is background fluorescence from chlorophyll.

The use of wash steps with W1 emulated the post-transfection wash steps seen in the PEG-calcium method (Yoo, Cho and Sheen, 2007). This was not strictly necessary as responses had already been achieved in 1d. However, the effect of MMG on LUC imaging had been established and so it was hypothesized that removing residual MMG and placing the stable protoplasts in W1 would enable a stronger response. This was not the case, as seen in the results of 1e, where the ABA response is more discreet and luminescence lower. It should be noted that there were many uncontrolled variables between the experiments as they were carried out on different days and with different protoplast. For example, the protoplasts from 1e were left on ice for 1.5 hours instead of the recommended 30 minutes while being prepared for imaging. This may have contributed to slower luciferase production and action. Also, the two W1 wash steps, involving solute changes and centrifugation, may have been damaging to the fragile protoplasts, which need to be intact for LUC imaging. Despite the less obvious response we believe these wash steps should be carried out if conducting assays on stable protoplasts such as here, as these samples may have just required a longer imaging time to display their response. Furthermore, it ensures the conditions stated in Yoo, Cho and Sheen (2007) are replicated adequately.

Aim 2 – Setting up transient protoplast expression of motif-reporter constructs

The results from the PEG cytotoxicity investigation suggested this was the major bottleneck for protoplast transfection. With over an 80% decrease in luminescence in some cases, we saw that this step needed optimisation. Yoo, Cho and Sheen (2007) expects around 50%

transfection efficiency, meaning that only an estimated 10% of protoplasts were healthy and transformed. Oxygen availability was also a major consideration when conducting the transformation in a 2ml tube. This anoxic environment during the overnight incubation stage could be another bottleneck, hence many other papers conducting the incubation in larger containers such as 15mL flasks (Abel and Theologis, 1994) and tissue culture plates (Yoo, Cho and Sheen, 2007).

	'Warwick' plate setup	'Warwick' tube setup	Yoo *et al.* 2007
DNA added (µg/µl)	1	4	10
Vol. of protoplasts (µl)	30	100	100
Conc. protoplasts added (mL^{-1})	330000	200000	200000
Volume PEG	33	100	120
Protoplast:PEG ratio	0.9	1.0	0.8
Vol. W5	170	500	420
Second dilution factor	0.73	0.71	0.66
Total vol.	233	700	640
Supernatant to remove	160	600	**stop**
Vol. left	73	100	
W1 to add	170	500	
New vol.	243	600	
Supernatant to remove (2)	160	**stop**	
Vol. left (2)	83		
W1 to add	40		
New vol.	123		
Overnight incubation	**stop**		

Table 3: transient protoplast production comparison (estimates have been entered where the protocol simply says remove "most").

Too treat the issues discovered in 2a, the transformation method was considered. First a protocol comparison was carried out to assess the amounts of protoplasts and concentrations at each stage. This helped with the design of the following experiments. The 5-minute transformation was suggested in Yoo, Cho and Sheen (2007). We hoped that less time in the PEG mixture would be less damaging to the protoplasts. This method produced the second-best results. This time factor may still be significant as the longest transformation method, suggested in Negrutiu *et al.* (1987) was the least effective. This method involved a slow dilution of the PEG-protoplast mixture so would have had less osmotic shock. However, we believe the prolonged time in the PEG mixture would have caused more damage over time on top of the initial PEG addition. The 15-minute transformation showed the highest level of aequorin, and therefore transfection efficiency, so was the preferred method.

The results from 2c suggest 12µL plasmid and 20% PEG are the optimal conditions for pDH51:AEQ. There is a decrease in aequorin levels as the PEG concentration is lowered B2 and B3. This suggests the cell membrane was less permeable to the DNA in these milder conditions so fewer cells were transformed. However, this pattern is not apparent for both the low plasmid samples (8µL) or the high plasmid samples (16µL). 10% PEG might cause less cytotoxicity therefore kills fewer cells. Conversely, 20% might have a higher success rate as the cell walls are more permeable to the plasmid vector. This may explain the difference between A3 and C3, where C3 could be limited by the permeability while A3 is limited by surviving cells. We estimated from investigation 2a that 20% PEG can kill up to

82% of protoplasts. Assuming fewer were killed by 10% this would leave more cells expressing the AEQ gene. It seems then there is a trade-off between cytotoxicity and transfection efficiency alongside the specific plasmid conditions. Both repeats and more work are needed to confirm the optimal method for pDH51, but we believe a plasmid amount of 12µL per 40,000 protoplasts is a good starting point (plasmid DNA was the most expensive material used but the results from using smaller amounts were much less effective). PEG concentration should be investigated further.

Tiwari *et al.* (2006) emphasises plant age as an important factor in protoplast transfection and we feel this could have affected our results as some were 2-3 weeks older than recommended. However, the Tape-Sandwich isolation method was effective throughout and we agree with Wu *et al.* (2009) in saying it should be widely adopted. This was in contrast with the transfection process which had more variable results. These experiments should be repeated before any firm conclusions have been made but we feel they offer some guidance for further research into crosstalk in the ABRE motif and for PEG-calcium transfection.

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