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Assessment of the Cell Specific Expression of the Cytochrome b_6f (Rieske) in the Guard Cells of *Arabidopsis thaliana* (L) Plant

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Abstract
The role of guard cell chloroplasts and the amount and importance of guard cell photosynthesis has been of great challenge due to the complex and functional plasticity of guard cell signalling pathways. Elucidating the role of the guard cell chloroplasts and guard cell photosynthesis presents a serious challenge for researchers in the field. Electron transport in guard cells has been proposed and current research efforts towards improvement of plant productivity have also focussed on improved photosynthetic carbon assimilation and manipulation of photosynthetic enzymes. In this study, electron transport chain enzyme, the Rieske FeS protein was manipulated by molecular tools using the specific guard cell promoters and polymerase chain reaction, PCR. Transgenic Arabidopsis (*Arabidopsis thaliana*) plants overexpressing in the guard cells were generated showing the localization of the enzyme specifically in the chloroplast of the guard cells. This provides tools to explore importantly the potentials for manipulating stomatal behaviour towards increased crop improvement and productivity. More opportunities await the exploitation of cell specificity in the guard cells metabolism.

Keywords: Guard cells, Yellow fluorescence protein, Cytochrome b_6f (Rieske), DNA Stomata

Introduction

Stomata are potential targets which determine the flux of gases between the inside of the leaf and the external atmosphere and therefore greatly influence photosynthetic carbon assimilation and water use. Sustainable agriculture will require a major reduction in plant water use in many areas as fresh water available for crops will decrease due to climate change and the predicted increase in global water usage due to increasing global population (IPCC 2013). Therefore, in order to maintain crop productivity to feed the growing population, it is necessary to identify potential targets for manipulating plants for improved water use and productivity (Jezek & Blatt, 2017; Lawson, von Caemmerer, & Baroli, 2011). Guard cells controls stomatal size by the opening and closing of the stomata. The figure 1 shows the guard cells where photosynthetic activity takes place and the stomata where exchange of gas occurs.

Stomata has therefore attracted the attention of scientists for almost three centuries (Meidner & Willmer, 1993), and a great deal of knowledge related to the structure, development, and physiology of stomata have so been acquired (Bergmann & Sack, 2007; Berry, Beerling, & Franks, 2010; Buckley, 2005) nevertheless, there are still many unanswered questions regarding signaling pathways, osmoregulation and the co-ordination between stomatal behaviours. For an optimal plant productivity and water use efficiency, stomata must open and close to environmental stimuli and internal signal to balance CO_2 uptake for photosynthesis (A) with water lost through transpiration (E) for optimal plant productivity and plant water use efficiency (WUE = A/E (Lawson & Blatt, 2014). Stomata therefore as a medium for gas exchange play a vital role in photosynthesis and improving photosynthesis has shown and can contribute toward greater food security in the coming decades as a result of climate change and world population increase. Lawson and Blatt (2014) highlighted the importance of having greater knowledge and understanding of the physiological and molecular mechanisms that mediates the speed of stomata and coordination with mesophyll demands for CO_2 towards achieving this crucial goal. Genetic mutants especially in Arabidopsis have already begun to reveal the

mechanisms that mediate regulation of stomatal conductance (Azoulay-Shemer et al., 2015; Kelly et al., 2013; S. W. Wang et al., 2014) but in the whole plant. Transgenic plants with guard cells specific manipulation are therefore important for the identification and characterisation of the signal transduction mechanisms that mediate such regulation of stomatal conductance. Guard cell photosynthesis therefore demands genetic analyses by guard cell-specific manipulation of photosynthesis. Single or multiple targets identified could be manipulated to aid more understanding to maximize crop production. Some of these targets are the SBPase and Rieske enzymes which have demonstrated their significance in controlling photosynthetic processes.

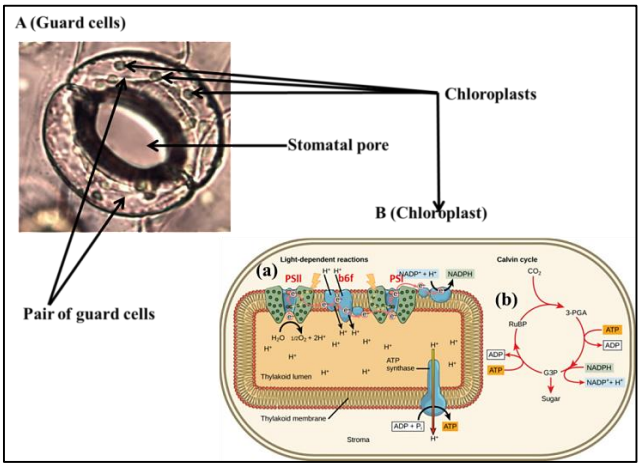

Figure 1. Guard cell as a potential tool for manipulation. (A) Guard cells from an epidermal peel showing chloroplasts which are the site of photosynthesis and the stomata through which exchange of gas takes place. The guard cells control the stomatal pore i.e the opening and closing of the stomata. (B) Schematic diagram of a single chloroplast illustrating (a) the thylakoid membrane where electron transport chain takes place. The cytochrome b_6f between the PSII and PSI shuttles electron which is used for the synthesis of NADPH and ATP as energy for fuelling the Calvin cycle. (b) Calvin Cycle found in the stroma of the chloroplast where sedoheptulose-1, 7-bisphosphatase (SBPase) a key component in the regeneration of RuBP in the Calvin cycle functions.

The Cytochrome b_6f (Rieske), a key component of the electron transport used towards ATP production were evaluated and assessed in the guard cells using specific promoters to drive the expressions in the guard cells. The KST1 and MYB60 promoters which are guard cell specific promoters developed by Müller-Röber et al. (1995) have been used to drive the expression of target genes specifically in guard cells. The use of a specific guard cell promoter has demonstrated an excellent use by Y. Wang et al. (2014) demonstrating an enhanced light-induced stomatal opening, greater photosynthesis and improved growth rate in Arabidopsis over expressing H^+-ATPase. Guard cell specific promoters allow the elucidation of the roles of specific transcripts involved in electron transport, ion channel function and carbohydrate biosynthesis to be evaluated on a cell by cell basis. This influence of manipulated electron transport and other enzymes on stomatal responses to CO_2 concentration and photon flux density can be determined physiologically in the guard cells specifically as well as how stomatal responses contribute to the capacity of guard cell function (Lawson, 2009; Lawson & Blatt, 2014; Lawson et al., 2014)

This study therefore describes the process of generation or production of guard cell specific enzyme of the transgenic homozygous Rieske (AT 4G03280) Arabidopsis plants. All homozygous lines were compared to the wild type (Col-0) in the Arabidopsis plants. Expression vectors from the cloning strategy 'Golden gate' where used to generate the construct with guard cell specific promotors. Constructs were designed to alter expression of the Rieske gene in a cell specific manner driven by the KST1 promoter. The yellow fluorescent protein, YFP tags were included in the construct to demonstrate cell specificity.

Materials and Methods

Seed sterilization

Arabidopsis seeds from the University of Essex were sterilized following a modified protocol based on those described by Aronsson and Jarvis (2002) and Aronsson and Jarvis (2011). Seeds were surface sterilized by a series of washes of which they were submerged in 70% ethanol for 3 min. Following this, they were rinsed with sterile water and then submerged in 4% bleach + Tween 20 (1 drop in 50 mL) for 15 m and agitated frequently. Finally the bleach was removed and the seeds were rinsed 3 to 5 times with sterile water. These seeds were placed on sterile filter paper inside the flow hood and allowed to air dry. Seeds were then sown on agar plates immediately or stored in the final rinse water at 4°C for stratification.

Generation of transgenic plants and plant growth conditions

The construct plasmids were introduced into wild-type Columbia (Col-0) Arabidopsis (*Arabidopsis thaliana*) by floral dipping using a strain of *Agrobacterium tumefaciens* GV3101. The Arabidopsis plant transformation was achieved using the floral dipping method described in Clough Steven and Bent Andrew (2008). Plants were grown in growth chambers approximately 7 weeks in short day length at which time they were flowering. The first bolts were clipped to encourage the proliferation of many secondary bolts and plants were dipped 10 days after this, 10 ml cultures of the strains carrying the genes of interest were grown overnight at 37°C in LB with antibiotics (rifampicin 50 µg mL-1, gentamicin 25 µg mL-1, kanamycin 50 µg/ml. These were used the next day to inoculate a 500 mL culture in a 1:100 proportion. These cultures were grown under the same conditions for approximately 24 hours until they reached stationary phase. The bacteria were then spun down (3000 g for 15–20 min) and resuspended in a 5% sucrose solution. Before dipping, 0.05% (500 µL/L) Silwet L-77 was added. Following the addition of silwet the whole inflorescences were dipped in the bacterial suspension for 2 to 3 seconds, with gentle agitation. The plants were then placed in large containers and covered with autoclave bags to maintain high humidity and left in the dark at 18°C for 16 to 24 hours. After this period, the plants were returned to the greenhouse and grown normally. The seed were harvested when dried and the transformants selected using antibiotics (50 µg ml-1 Kn or herbicide selectable marker (0.82 mM of glufosinate-ammonium). For antibiotic selection, plants were grown on soil watered with glufosinate-ammonium until selection were carried out.

Fluorescence microscopy to assess cell specific expression.

The constructs were designed with a yellow/green fluorescent protein, YFP cassette included to check cell specific (guard cell specific) expression of the construct in the leaf tissue of the T0/T1 generations. These were subjected to high resolution chlorophyll fluorescence and confocal microscopy to detect the presence of the yellow fluorescent protein YFP.

Small (about 1/2cm) leaf samples were cut from the leaf and mounted on a slide which were covered with a # 1.5 cover slip gently to avoid damaging the tissue. For live image acquisition, a high resolution microscopy and Nikon A1si inverted confocal microscope equipped with filters for YFP analysis were used. YFP images were acquired by exiting at 515 nm LEDs and emission collected with a band pass filter (530 ± 20 nm) for the high resolution microscope while YFP images for Nikon Alsi were acquired by exiting at 488nm with lasers and its emission collected at 530nm. Chlorophyll auto fluorescence was exited at 480 nm with emission collected at 685 nm which allowed to distinguish between the two signals. A 4X and 40X objective were used with a numerical aperture (N.A.) of 1.4.

Results

Selection of Arabidopsis transformants

Transformed floral plants in the Arabidopsis plants were allowed to mature to seed. Seeds were collected and planted for the screening of the T1 generation and selection of positive transformants achieved. The selection of positive transformants was identified by the application of BASTA watered on the soil in which the T1 germinated seedlings as transformants were resistant to the herbicide (Fig 2).

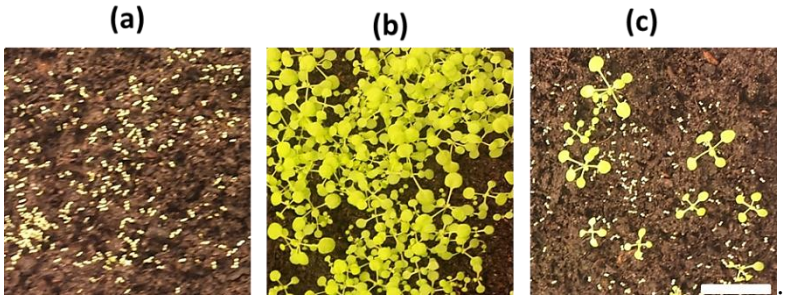

Figure 2. Hebicide (BASTA) selection of transformed Arabidopsis plants. Resistant transformants were selected by growing on soil and spraying with BASTA (presence of bar gene confers resistance to the glofusinate ammonium herbicide BASTA). **(a)** WT control showing complete death of cotyledons grown on BASTA, **(b)** WT control grown without BASTA and **(c)** selection of resistant transformants grown on soil watered with BASTA. Plants growth conditions maintained under controlled-environment growth room with (22°C, 8 h light, 16 h dark cycle). White scale bar represents 1cm. The resulting successful transgenic plants (T1 generation) were selected on the herbicide glofusinate ammonium (BASTA) and subsequent screening for homozygous lines began by PCR.

DNA analysis of transgenic plants.

The result (selected transformed T1 plants) from Fig 2 above and tissues from individual plants were checked for the presence of the transgene by PCR analysis. The result of the DNA analysis produced PCR fragment sizes exactly as the gene of interest in all the lines screened. The constructs pL2-BAR-(pKST1)-AtRieske-tHSP (512bp) with all ten lines selected positive for the presence of the transgene.

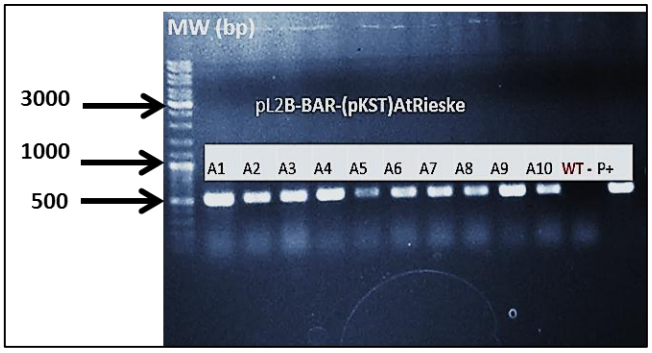

Figure 3. Genomic DNA PCR screening of transformants for presence of the transgene. The presence of transgenes were checked by PCR analysis of genomic DNA of T1 plants pL2-BAR-(pKST1)-AtRieske-tHSP(512bp). Ten lines were screened per construct. WT DNA (WT-red) and plasmid DNA containing the gene of interest (P+) were used as negative and positive controls respectively. PCR products were run alongside molecular weight marker (DNA generuler ladder mix from thermoscientific) in base pairs.

Fluorescence Microscopy to detect YFP expression in guard cells

Rieske YFP mutants T1 plants were rapidly screened for the presence and localization of the yellow fluorescence protein (YFP) specifically in guard cells using high resolution chlorophyll fluorescence microscope. Constructs fused to the yellow fluorescence protein confirmed that expressions driven by the cells specific promoters were confined to the guard cells. In total, all 83 plants analysed were found with detectable levels of YFP expression. Constructs tagged with the KST1 promoter revealed the guard cells with the YFP in them while wild type control had no signal (Fig 4.).

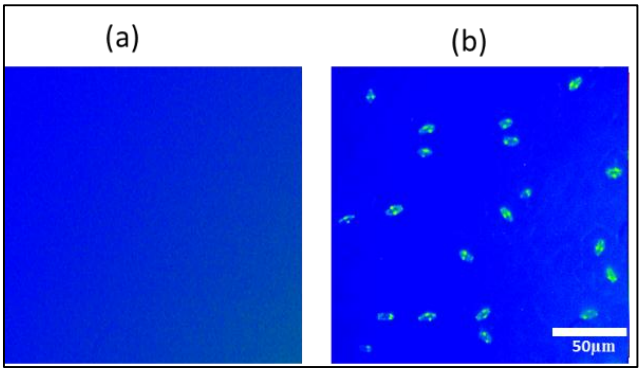

Figure 4. Specific YFP expression in the guard cell of T1 generation. Localization of the YFP in the chloroplasts of guard cells of Arabidopsis transformants**. (a)** Wild type (Col-0) tissue showing no signal while **(b)** Expression of the constructs tagged with YFP and driven by the KST1 promoter in pL2B-BAR-(pKST1)-AtSRieske-(pKST1)YFP-tHSP in leaf tissue were checked using the High resolution microscope. Images acquired by exiting with 515 nm LEDs and emission collected with a band pass filter 530 ± 20)

Furthermore, confocal microscope was also used to gain high resolution images of the cell specific expression in T2 generation plants. A Nikon A1si inverted confocal microscope was used to visualize cell specific expression. The expression of the YFP in the guard cells specifically can be seen in Figure 5.

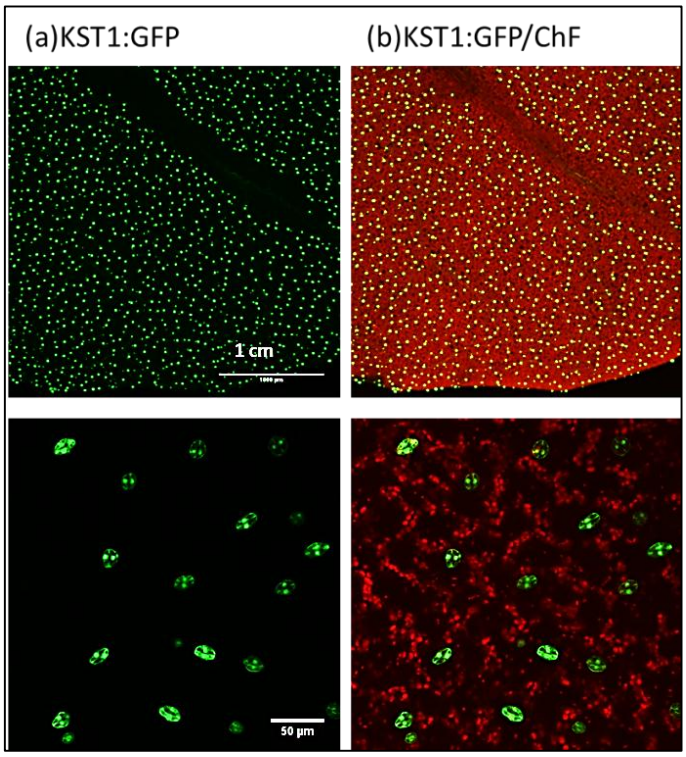

Figure 5: Specific YFP expression in the guard cell of T2 generation. Localization of the GFP/YFP in the chloroplasts of guard cells in T2 generation of Arabidopsis transformants (pL2B-BAR-(pKST1)-AtRieske-(pKST1)YFP **(a)** GFP/YFP flourescense, **(b)** GFP/YFP/Chlorophyll flourescense merged of the constructs tagged with YFP and driven by the KST1 promoter in leaf tissue were checked using Nikon A1si inverted confocal microscope. YFP images were acquired by exiting at 488nm with lasers and emission collected at 530nm. Chlorophyll auto fluorescence exited at 480 nm with emission collected at 685 nm

Discussion

Efforts to comprehend the involvement of guard cell photosynthesis in stomatal function requires manipulating photosynthesis specifically in guard cells (Lawson 2014). Therefore, the genetic manipulation of metabolites involved in metabolism and photosynthetic enzymes in the guard cells themselves could lead to changes in stomatal behaviour and potentially improve photosynthesis and water use efficiency in plants (Santelia & Lawson, 2016). Transgenic studies have provided numerous evidences that manipulation of certain enzymes are potential route for the improvement of plant productivity (Lawson, Lefebvre, Baker, Morison, & Raines, 2008; Lawson, Oxborough, Morison, & Baker, 2002; Lefebvre et al., 2005; Raines, 2011; Simkin et al., 2017; Simkin, McAusland, Headland, Lawson, & Raines, 2015; von Caemmerer & Furbank, 2016). The KST1 and MYB60 promoters, which are guard cell specific promoters developed by Müller-Röber et al. (1995) have been used to drive the expression of target genes specifically in guard cells. It was in line with this that Lawson (2009)Lawson (2009)Lawson (2009)this approach was employed and in this study, we generated transgenic Arabidopsis (*Arabidopsis thaliana*) plants overexpressing Rieske which has demonstrated the potential of generated transgenic plants with altered guard cell metabolism. We particularly have demonstrated specificity of the KST promotor and shown that expression was only in the guard cells. This has also shown the potential of this promotor for manipulating guard cell specific metabolism. This agrees with the earlier work of Muller-Rober et al., (1995) as well as the work by Kelly et al. (2013). The analysis carried out such as the DNA and herbicides screening plus the localization of the YFP have also revealed that these genes are present in the guard cells of the transgenic plants. The rationale for this study were two-fold. First, it is still not known if guard cell electron transport directly impacts on stomatal responses (Lawson et al., 2014); secondly, it has been hypothesized that guard cell photosynthesis could provide the mechanisms that co-ordinates stomatal responses with those of the underlying mesophyll (Lawson et al., 2002) as improving the rapidity of stomatal responses will greatly improve productivity and WUE but achieving this require greater knowledge of molecular mechanisms that determine the speed of stomata and coordination with mesophyll demands for CO_2 (Lawson and Blatt, 2014). The production therefore of these transgenic homozygous Arabidopsis plants and the cell specificity activities of this enzyme in the guard cells present the potential for the future advances exploiting guard cell functions. The following can further be exploited in order to elucidate mesophyll-stomatal interactions and subsequent impacts on WUE and plant productivity

- Exploitation of other single or individual enzymes in guard cell Calvin cycle transformations could lead to changes in stomatal behaviour by demonstrating the role of other enzymes in electron transport chain which could potentially improve photosynthesis and water use efficiency in plants.
- Quantification of the level of transcripts abundance in guard cells: Guard cell specific quantification in the transgenic and wild types from subsequent generations would be required to determine the impact of enzyme manipulation on functional processes and determine the mechanisms and pathways these contribute to stomatal behaviour..

Having said all these, guard cells metabolism is a fast-paced area of research and more opportunities still awaits its exploitation to bring to an unequivocal view of its metabolism. The findings highlighted using this approach can therefore be further exploited as researchers are continuously adopting multicity of approaches in order to shed more lights on plants WUE and productivity.

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