Use of The Plant Accelerator to analyse salinity tolerance responses of transgenic barley expressing *AtAVP1*

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The project

The aim of the project was to study the effects of transgenic expression of *AtAVP1* (an Arabidopsis type I vacuolar H^+-translocating PPase) on the growth of barley, in particular whether it increased the salinity tolerance of the transformed plants. With high throughput, automated watering to weight and accurate and consistent measurements, The Plant Accelerator allowed the generation of high-quality time-course growth data for large numbers of replicates of multiple different transgenic barley lines.

It was established that transformed lines, despite displaying what would technically be considered reduced salinity tolerance, actually gained biomass at a higher relative rate under saline conditions than did their respective control lines. They also proved to have greater water use efficiency, growing more for a similar quantity of water usage under both salt stress and control conditions.

Crucially though, although *AtAVP1* expressing lines were larger, their relative growth rates were identical to control lines throughout the period of measurement in The Plant Accelerator. The only possible explanation for this is that the transgenic plants either started larger (because their seeds were larger) or they had a greater relative growth rate in their very early stages of growth, before being measured in The Plant Accelerator. This important result was only revealed because of the non-destructive measurements of growth of large numbers of plants made possible by The Plant Accelerator.

The project was carried out by Rhiannon Schilling, an Honours student at the University of Adelaide School of Agriculture, Food & Wine (2010), and detailed information about the protocols and varieties used and the background behind the projects is described in her thesis, "*Evaluating the salt tolerance of barley expressing the Arabidopsis vacuolar H^+-PPase (AtAVP1)*".

Background

Salinity tolerance is an issue of major importance to the Australian cereal industry, as well as in many other regions around the world. With up to 80% of Australian cereal-growing regions in Australia potentially at risk from salinity problems (1), and estimates of the salinity-caused production losses in the hundreds of millions of dollars annually (2-4) any improvements to the salinity tolerance of cereal crops has potentially huge benefits.

One possible route to the improvement of salinity tolerance in Australian cereal varieties is the development of varieties expressing higher levels of type I vacuolar H^+-PPase. Constitutive over-expression of the Arabidopsis type I vacuolar H^+-PPase *AtAVP1* has been shown to increase the salinity tolerance of a range of crop and model plant species (5-9). If similar findings could be replicated for commercial wheat and barley lines, it may lead to new opportunities for crop improvement, such as through the use of marker-assisted selection for more active alleles of the endogenous *AVP* genes or even the development of genetically modified crops.

In this project, barley strains over-expressing the *AtAVP1* gene were evaluated for their response to salt stress by measuring relative growth rates and water usage over time. Because of the complex, multi-component nature of salinity tolerance, reverse genetics experiments such as this require controlled conditions and accurate and reproducible measurements to effectively characterise the effects of this genetic manipulation. Large numbers of plants from several different transformed lines also needed to be screened to provide statistical confidence in the results, and to compensate for any insertional effects of the over-expression cassette. This required high throughput and accurate measurements - The Plant Accelerator is an ideal facility for projects of this nature.

Experimental design

A total of ten plant lines were used, consisting of two to three lines each from independent transformation events constitutively expressing *AtAVP1* under the CaMV 35S promoter, plus respective null controls. Eight replicates for the two test conditions (0mM and 150mM NaCl) were used for each line, giving a total of 160 test plants. Plants were grown in sealed pots, with salt applied by preparing the lower half of the soil to 300mM NaCl and the upper half with no added NaCl. The pots were then watered from above with reverse osmosis water to field capacity every two days, using the watering to weight capabilities of The Plant Accelerator, and over a period of days the salt redistributed throughout the pot to give a final overall concentration of 150mM.

Seeds were germinated on filter paper before potting. Seedlings were then grown for seven days, after which the plants were transferred to the Smarthouse and imaged every two days for a period of 34 days. Data was captured at all five of The Plant Accelerator's imaging stations, but detailed analysis was only performed on the data acquired from the visible light imaging camera, from which biomass can be accurately inferred using projected shoot area measurements (10).

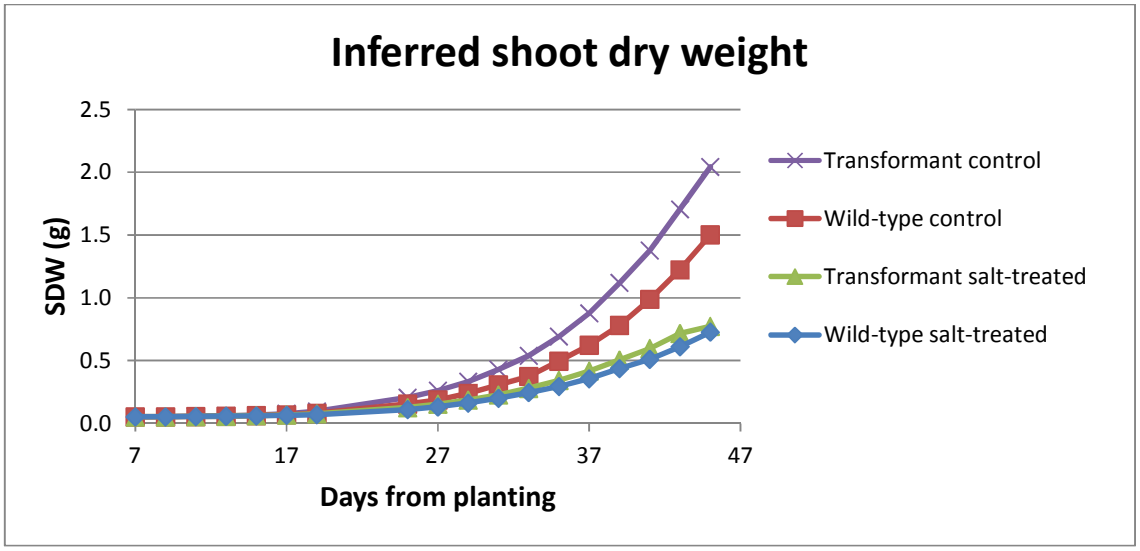
The project was carried out and grown under natural lighting between June and August 2010 with the greenhouse maintained in a temperature range of 16°C (night) to 22°C (day). This provided irradiance, day length and temperatures similar to those occurring during the commercial cropping season in southern Australia.

Outcome

Salinity tolerance is typically expressed as the biomass of salt-stressed plants as a percentage of the biomass of non-salt-stressed control plants. Using this relatively simple metric, the slightly surprising outcome of this project was that the transformed plants had a lower salinity tolerance than the wild-type lines; surprising since this is the opposite of what has been observed in many other species.

The nature of the data generated at The Plant Accelerator allows us to look in much more detail at the causes underlying this conclusion. For example, we can determine that the transformed lines still grew better in saline conditions than did the wild-type plants; it was just that they grew relatively even faster than the controls in control conditions (Figure 1).

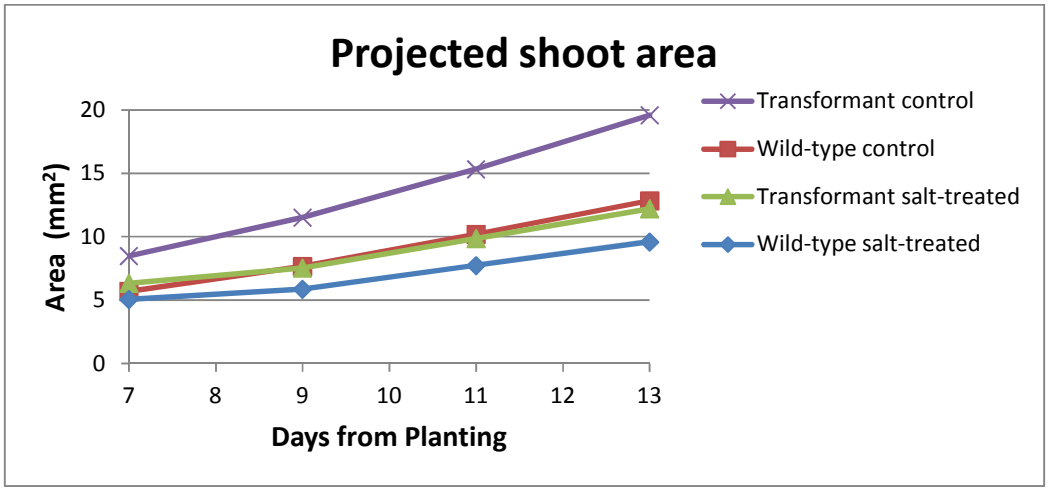
Figure 1. *Growth plots of inferred shoot dry mass, calculated using the equation of Golzarian* et al *(10). Controls grow substantially faster and bigger than salt-treated lines, and transformants grow faster than wild-types*



Yet even this does not show the whole story; further examination of the data reveals that from the very start of imaging, the transformed lines in control conditions were substantially larger than the wild-type control (Figure 2). In terms of size, the transformed controls were three to four days growth ahead of the other samples after just seven days of growth, a head start that they retained throughout the experiment. More detailed characterisation of seed size, embryo size and early seedling vigour is required.

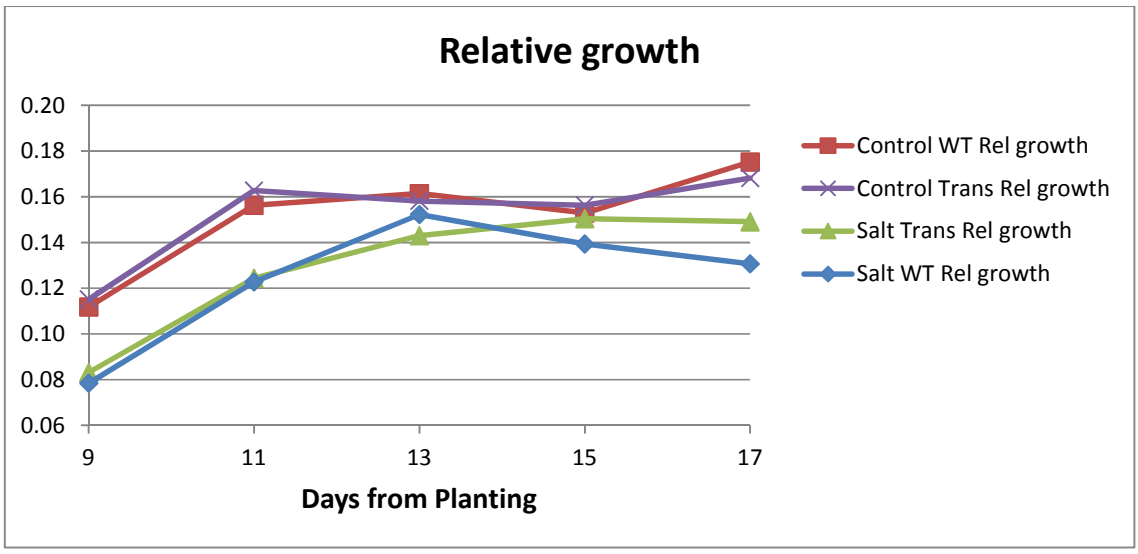
This observation by no means suggests that the subsequent apparent faster growth of the transformed lines under control conditions is simply an artefact or otherwise unimportant; rather it indicates that a different interpretation or angle of investigation is needed to characterise the difference. That the wild-type plants show no signs of converging growth with their transformed siblings is in itself an interesting and potentially valuable discovery; what is it about the transformed plants that generates and allows them to maintain this early vigour, and does it correspond to an eventual increase in yield or productivity?

Figure 2. *Shoot area increases over the first six days of imaging. The larger size of the control-condition transformants is clear even at this early stage.*



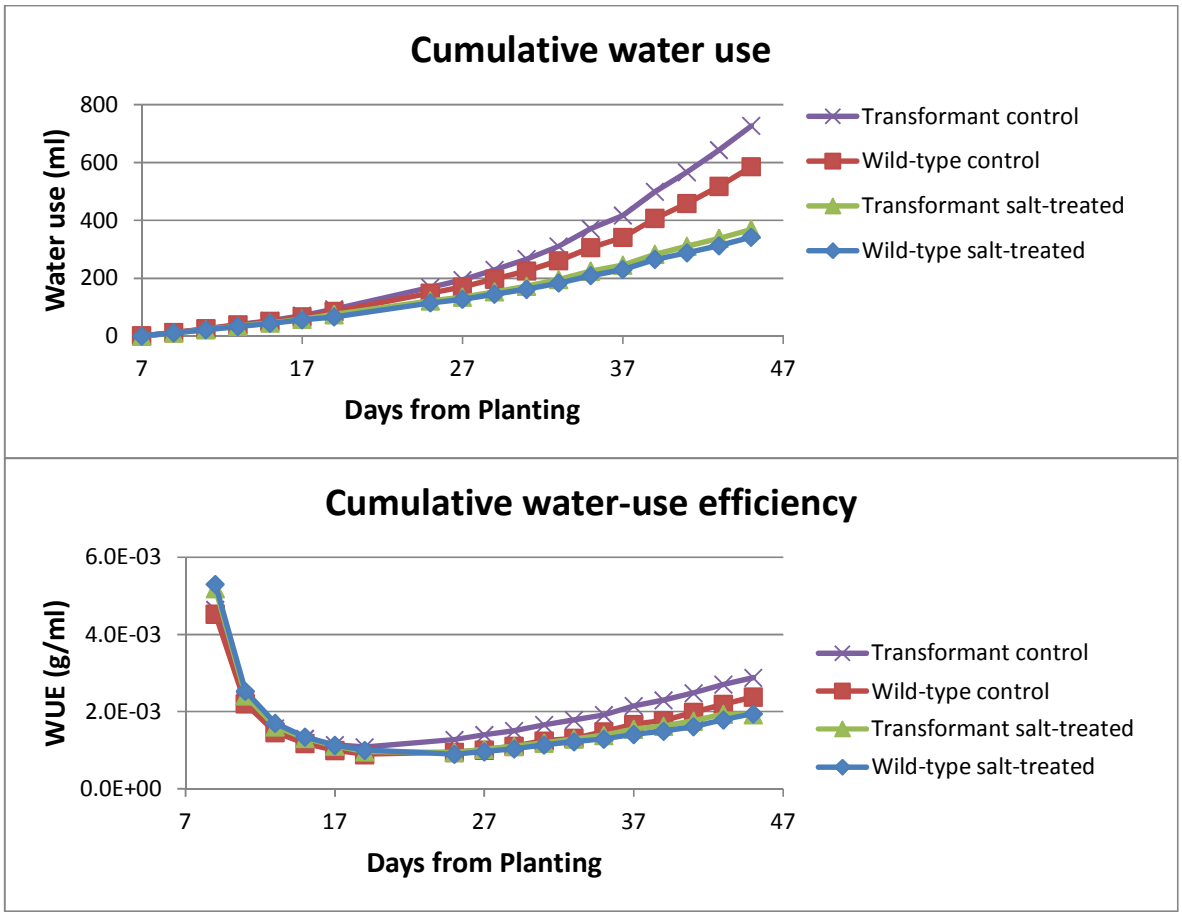
It was proposed in the methods description that the salt, which was applied only to the soil in the bottom of the pot, equilibrated throughout the soil during the experiment. In support of this assertion, clear differences can been seen between the relative increase in leaf area of the control and salt-treated plants from day two of imaging (Figure 3). This is the earliest time point by which relative growth differences can be determined, and corresponds to nine days after potting. This suggests that even by this relatively early stage, the salt had diffused up to the expanding root zone of these young seedlings and was having a rapid effect on growth rate. Quite possibly this is highlighting an osmotic response to the salt levels in the soil – the decreased osmotic potential caused by the salt causes reduced water uptake, and hence slower enlargement of cells in the salt-treated plants.

Figure 3. *Relative growth rate (proportional daily increase in shoot area) over the first ten days of imaging, showing the early inhibition of growth of the salt-treated plants.*



The project also identified differences in water usage and water use efficiency (amount of biomass accumulated for a given volume of water used) between the various lines and conditions. The cumulative water usage of controls was consistently higher than that for salt-treated samples, while the water usage of transformants was higher than for wild-type lines. Despite this, there was a strong positive relationship between water usage and water use efficiency, with the plants that used most water still being the most water efficient (Figure 4).

Figure 4. *Cumulative water usage (top) and cumulative water-use efficiency (biomass accumulated/total water used) (bottom). Despite their higher water use, the controls still produce more biomass per unit of water.*

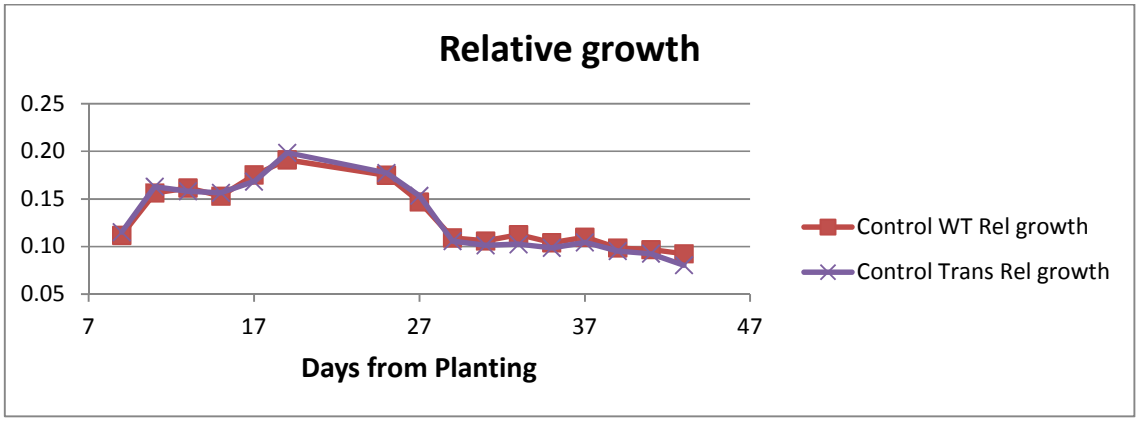


Benefits of The Plant Accelerator

The benefits of using The Plant Accelerator for this project are probably best summed up by Rhiannon's comment: "Without the Accelerator, I would never have been able to run such a large scale project with so many plants and replicates". The major focus of this experiment was the accumulation of biomass under salt stress conditions; to do this effectively requires the measurement of a time series of biomass data on a significant number of replicates. Furthermore, watering to weight is required to accurately maintain the intended salt concentration in the soil, which in itself is a time consuming and tedious task.

Of particular interest in this experiment is the dissection of the higher growth rate of the transformant controls. Because of the ever-widening difference in biomass between the transformant and wild-type lines, it is easy to conclude that there is a difference in growth rate throughout the experiment. However, when one examines the time courses obtained from the Accelerator in more detail, the true situation becomes apparent – the real difference in growth occurs only within the first few days after germination. From then on, the transformants grow faster only because they have a greater photosynthetic area. The relative growth rates for the transformed and control lines (Figure 5) are almost identical, which is incompatible with a proposal of different growth rates, and leads to a rapid rejection of the hypothesis that the transformants have a higher intrinsic rate of growth. In other words, the transgenic plants are only larger because they start off larger, not because they have a higher relative growth rate.

Figure 5. *Relative growth plots of wild-type and transformant controls. The very similar profiles confirm that the transformants maintain their initial proportional size advantage over the wild-types for at least five weeks and that the transgenic plants are larger at the end of the experiment simply because of faster growth in the very early stages of growth, and not because of a sustained difference in rates of growth.*



This then begs the question of why the plants grow more rapidly in the very early stages of growth. One possible explanation is that the transgenic plants started larger (because their seeds were larger). Another possibility is that the transgenic plants had a greater relative growth rate in their very early stages of growth, before being measured in The Plant Accelerator. It is possible that AVP over-expression is stimulating growth not due to increased vacuolar sequestration, but due to higher PPi hydrolysis during the gluconeogenesis-intensive post-germination heterotophic growth (see Ferjani et al 2011 (11)).

Similarly, the ability to correlate growth data with water usage allows the analysis of results that could not readily have been interpreted with other approaches. In this case it is that the lines and conditions which lead to greatest water use, far from being the least water efficient, are in fact converting the consumed water into biomass more efficiently than others. Of course, in the Australian environment, it may not be desirable to have a barley variety which uses more water, even if that results in greater yield, since that water may simply not be available in the field. However, identifying these specific phenotypes allows us

to start looking into the mechanisms underlying them, and ultimately to start developing more commercially relevant alternatives.

Neither of these phenotypes could have been effectively identified using just endpoint destructive biomass testing, since the necessary time-course information would simply not have been measurable without the automation and non-destructive measurements provided by The Plant Accelerator. In fact, without the measurements made by The Plant Accelerator, data from a single end-point harvest would give misleading results likely to lead to significantly incorrect conclusions. Furthermore, even with the greatly reduced quality of results likely to be obtained using an endpoint-only measurement, the more basic project design required would still require approximately three hours of labour every other day just to carry out the watering to weight required to maintain salt levels accurately; labour that is not required when using The Plant Accelerator.

It is of course hypothetically possible to replicate the time-course capabilities of The Plant Accelerator by setting up many more plants at the outset of the experiment and destructively measuring one full set at each time point. Realistically though, this would not be viable; on top of the sixty or more work hours that it would take to prepare the 2,400 plants required to duplicate the experiment, it would then take in excess of twenty work hours every two days for harvesting and watering to weight. After all this, the data would still be less informative than that from the Accelerator, because of the error introduced in destructive testing by inter-plant variability, which is not present in time-courses measured on the same plants.

Other points to note

- Although this project was focused on a reverse genetics objective of characterising the effects of *AtAVP1* expression in barley, The Plant Accelerator could equally well be used for screening a large number of transformants for ones displaying a specific required phenotype. Two to three replicates each of a much larger number of transgenic lines could be grown under similar conditions to those described in this experiment and lines selected that displayed specific desirable characteristics such as high absolute growth rate, high salinity tolerance (c.f. the relatively low growth of transformants in salt in this project) and low water use. These lines could then be studied in further detail to identify what it was about the insertion site and other genetics that made them better than the other transformants.

- This project of 160 plants was followed immediately afterwards by a related one of 260 plants, consisting of 11 further lines of barley *AtAVP1* transformants. Carrying out detailed growth profiling in response to salt stress for 420 plants from 21 different transgenic lines in less than three months as part of an Honours project would not be feasible without the resources of a centre such as The Plant Accelerator.

Acknowledgements
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