Discussion

24. Interpretation of Results

Calculated optimal conditions for maize leaf transient transformation, used during subsequent experiments, were determined with the Taguchi method and by analysis of a Generalised Linear Model constructed from the experimental data. The GLM results were chosen as optimal conditions: a burst disc pressure of 1350PSI, a DNA loading of 5ug, microparticle (tungsten) loading of 0.75mg and selection of samples from the inner leaves of the non-emergent leaf whorl.

24.1 Development and testing of optimised conditions

Selection of optimal conditions

During the course of the optimisation experiment, it became clear that one aspect of the Taguchi approach (the verification of statistical differences among input factors via ANOVA) would not be applicable to the system being studied. The data which were produced proved to be too skewed and with too many outliers for statistical approaches based on normal distributions to be effective. It was at this point the the University of Pretoria department of statistics was consulted on the correct path forward, with the subsequent assistance by Prof. Steffens providing both an analysis of the data and an alternate optimisation approach.

Notably, the outputs of the Taguchi and GLM actually produced similar results from a statistical standpoint. This can be illustrated by the in-subset comparisons produced using Wilcoxon rank-sum testing (tables 6, 8, 10 and 12). Here, there was a clear convergence between factor levels that were higher-yielding and singular, and those that were better only in relation to a much inferior factor level (figure 19). Only in the case of sample location within the non-emergent leaf whorl was there a significant difference.

In the end, a decision was made to use the optimal conditions with better statistical support (those produced using the GLM), with the knowledge that the predicted effects would strongest in the cases where the two approaches converged (microparticle loading and burst disc pressure). Finally, it is interesting to note that, in the case of this experiment

at least, an accurate gauge of which condition to take as optimal would have been to simply count up the total number of spots generated by each factor (as illustrated by comparing the chosen (highlighted) optimal conditions to the total spot counts of tables 5, 7, 9 and 11).

Comparison of Baseline and optimised conditions

During the optima validation experiments, useful data was gathered on the differing responses between the baseline and optimal conditions. Here, the available statistical tools show that the difference between the two is not significant at high confidence levels (tables 18 and 19,figure 21). Intriguingly, there was shown to be a significant difference between the original baseline expression experiments and the baseline condition subset of the validation experiments. This, along with the clear difference between the baseline experiment and Taguchi optimisation experiment data, seems to indicate that a process of learning and improvement had occurred in regards to experimental technique. This underscores the need for good comparisons and controls when performing experiments of this nature.

These results are explicable when comparing the range of conditions tested - and the variations in results generated - in the optimisation experiments (figure 13) against both the baseline and optimal conditions used. Given the small differences both in input and output between the two conditions, it can be established that existing protocols are already well-optimised in terms of physical inputs.

24.2 Testing of an extra factor

The effect of distance along the leaf axis on expression

Comparison of transient expression level between the optimised and baseline protocol sample sets revealed statistically non-significant differences in expression. However, a comparison between sample sets isolated according to sample distance along the leaf axis from the ligule revealed highly significant differences in expression (fugure 20). The two data-sets, although drawn from subsets which mixed different input parameters, showed significant statistical differences in terms of mean, median, variance and distribution.

Comparison with published literature

The results found during the optimisation experiment are well correlated to those obtained by Kirienko et al (2012). This can be seen both in terms of the general differences in expression along the leaf axis, as well as factors such as cell size and the relative differences between expression level and cell density (figure 1). In this regard, the results presented in this study can be regarded as a validation of the work done by these researchers.

Declared optimal conditions

It is the opinion of the author that the results of the experiments conducted to optimise the input parameters of the transient biolistic assay system in maize leaf show that those factors are, in fact, as good as can be expected given the large variations typically seen in biolistic systems. The declared optima for this system, then, cover a broad range of physical input parameters. Optimal burst disc pressures should be sufficient both to project microparticles into the target tissue and break up any clumps or aggregates that may have formed. Here, it is recommended to use the highest gun pressures that can be obtained without the gas blast itself becoming a negative factor. Optimal particle loading parameters may vary, but should be above 0.2mg per bombardment.

DNA loading, by contrast, shows a mid-range optima independent of particle loading considerations, and should thus be around 5ug of plasmid DNA (7.94×10^{-7} umol in the case of pAHC25) per bombardment. Leaf sample selection should be on samples as close to the ligule as possible, with attention given to choosing samples which are as early as possible in development. Differentiation of leaf tissues has a drastic, negative effect on transgene expression and differentiated tissues should thus be avoided.

25. Theory Versus Practice

25.1 Choice of input factors

During the process of planning for the optimisation experiment, a decision had to be made on which factors to test. These would obviously have to be parameters which were

amenable to modification by experimenters, as well as being reasonably well-understood to have an effect on the levels of expression observed. In addition, the nature of the Taguchi approach (which has as one of it's core assumptions the independence of input variables) precluded combinations of factors which would be expected to have interacting effects. Finally, a decision was taken to limit species-specific biological factors (at least initially) in an attempt to maximise the broad applicability of the approach. From this available pool of testable factors, four (burst disc pressure, particle loading, DNA loading and sample location within the non-emergent leaf whorl) were then chosen for testing.

25.2 Numerical approaches to optimisation

One hypothesis regarding biolistic bombardment of tissues, formulated at the start of this project, was that there existed some mathematical relationship between particle loading and transformation efficiency. This is because, at a fundamental level, the nature of the bombardment system relies on random chance. As has been mentioned, it was determined by researchers relatively early on that only particles landing in or adjacent to the nucleus resulted in transgene expression (Hunold et al, 1994). Extrapolating, this implies that the area ratio of nucleus to cytoplasm (as seen from the origin of the bombarding particles) is important in determining bombardment efficiency. This is because, assuming particles are randomly distributed along the surface of the bombarded tissue, the the number of particles impacting the nucleus will be greater if the relative volume of cell it occupies is larger.

This model also implies that two approaches to improve efficiency: greater particle loading (thus increasing the density of particles impacting an area) and selection of tissues with high nuclear-to-cytoplasmic volumes (thus increasing the likelihood that particles impacting the cell will be deposited in the nucleus). Note that under this model the only important factors to consider are the relative areas of cell and nucleus facing the bombardment, the area-density of the particles and the total area being affected (figure 30).

A simple mathematical model can thus be proposed. Assuming that the size of the cell changes during development, while the size of the nucleus stays the same – a problematic assumption given the changeable nature of genetic material in plant tissues (Baluska,

1990; Bindloss E, 1938) – one can use average cell sizes for different tissues to determine the likelihood that a randomly-fired particle will land in a given area. Taking a mature piece of leaf tissue with a cell size of 1500um^2 (Kirienko et al, 2012) and using a conservative meristematic nucleus area of 20um^2 (Baluska, 1990); one can determine that there is a 1.3% chance for any given particle striking a cell to hit the nucleus.

Extrapolating further it can then be determined that, with a target size of around 13 cm^2 (the average combined areas of the three samples used per bombardment), the ~870000 cells being shot would require 87 million tungsten particles – around 1.2mg – to 'ensure' full transformation of the bombarded tissues. It is at this point it should be noted that this is already far in excess of standard protocols (which use around 0.2mg per bombardment). Adding in the amount of empty space that the bombardments actually hit (around 64cm^2, as the particles cover an entire 90mm petri dish once shot) means that the amounts needed to hit each nucleus would soar to around 5.5mg of tungsten. Here tungsten toxicity alone will almost certainly be enough to prevent the cells from properly expressing the gene construct (Russel et al, 1992; Adamakis et al, 2012).

However, to replicate the best results seen in the optimisation experiments, only tiny amounts of tungsten would theoretically be required. So why are these results not seen with smaller amounts of microparticles? From the nature of the results, at least, it seems as if it would indeed be possible to achieve reasonable transformation efficiencies with minute amounts of tungsten. There is certainly a visual trend towards linear improvements in transformation efficiency as particle load increases (see figure 15), but it is unsupported by statistical evidence (table 8).

An explanation for this disparity between theoretical and experimental results, then, can be found by examining the underlying assumptions of the model itself. Firstly, there is an assumption that particles are evenly scattered across the surface of the plate. This is obviously false – the pattern of bombardment will tend to mirror the pattern in which the particles are laid onto the macrocarrier, with varying areas of greater and lesser density determined by the vagaries of pipette motion and the surface tension of the carrier alcohol as it dries.

Furthermore, the process of DNA binding causes noticeable clumping in tungsten particles

(although less so in the case of gold particles), presumably leading to local areas of high particle density even when the clumps are dispersed by the firing pulse. It is hypothesised that this improved scattering and clump-disaggregation was the cause of the higher transient transformation efficiencies seen when using high burst disc pressures (which did not, as was originally thought, result in increased penetration – more below).

It also bears mentioning that not all particles will be properly coated with DNA. The mechanism of DNA binding is known to be problematic and highly susceptible to small changes in reagent purity (Frame et al, 2000), so it follows that not all particles will have the correct amount needed for transgene expression once in a host cell. Even then, higher levels of DNA loading were correlated with lower expression levels (figure 16). DNA loading, it seems, is about getting 'enough' DNA onto particles over any other concerns.

The nucleus of a plant cell is not, as the model assumes, evenly placed around the cell. Instead, plant cells are dominated by their vacuoles, with the nucleus being distributed more towards the edges of the cytoplasm. Finally, the model ignores other biological factors - such as tissue 'suitability' for transformation and cell death caused by bombardment. This was touched on briefly when discussing the initial tungsten loading parameters, but requires elaboration due to the nature of the results obtained during the validation experiments. If, as the data has shown, the overwhelmingly important input variable is the distance along the leaf axis from the ligule, then biological factors - the combination and interaction of cell/nuclear ratio, developmental state, organelle arrangement, relative availability of enzymes and pathways and so on - rather than physical factors must be taken into account when attempting to optimise transient expression. This can be seen directly in the difference between cell size and expression levels seen during the validation experiment.

Comparing the published cell size measurement of the 0-3cm and 3-6cm sample groups, a close correlation can be found with the experimental data. Using this data and the same set of assumptions as the above discussion, it can be understood that, with an identical particle density and sample size, the smaller cells should have a roughly 2.3 times greater chance of being hit in the nucleus then the larger ones (with a corresponding increase in expression).

If the model proposed above was accurate, this could be expected to result in an equivalent improvement in expression. Instead, there is a massive difference in the number of transiently-expressing cells produced by the 0-3cm samples over their more distant sister groups (figure 22), both in the published and experimental data. Clearly, some other factor (or factors) are the part of the reason for such an enormous disparity. Numerical modelling, it seems, is simply too crude a tool to account for the real causes of transient expression seen in this system by itself.

25.3 Mathematical modelling and penetration limits

One assumption made at the start of the project was that there was a direct correlation between gas pressure particle penetration when firing into plant tissues. This assumption seems to have been fairly widely adopted, as even recent protocols (Ueki et al, 2013) recommend using higher pressures when bombarding thick or hardened tissues. However, when microscopic sections of bombarded samples were taken, none were seen showing expression beyond the second layer of cells (figure 25). Indeed, when assessing by depth of penetration rather than number of cell layers penetrated, the published literature itself seems to show similar results (Reggiardo et al, 1991). This is further corroborated by studies investigating depth-of-penetration in mammalian tissues, all of which show sub-100um penetration depths in these wall-less samples (Kendall et al, 2004; Mulholland, 2004; Soliman et al, 2011). From these results, it can be assumed that cell wall thickness (the major physical difference between plant and animal cells) plays an important role in altering the dynamics of particle penetration.

This is especially important in the case of leaf tissues, as thicker cuticles and cell walls would be expected to contribute to the low levels of expression seen during bombardments. Thus, an approach to understanding the actual mechanics of penetration would be important for future research into bombardment of leaf tissues.
The low penetration seen during microscopic examination of bombarded samples was, it should be emphasized, seen regardless of gas pressure (and thus particle velocity). Clearly, there existed a gap between the approach recommended by researchers and the behaviour of the system itself.

An initial explanation of the phenomenon was found in the application of the Newton's approximation for determining impact depth. This approximation (reputedly developed by Isaac Newton to estimate the depth of penetration achieved by cannonballs) uses momentum considerations only when determining depth of penetration. It is pleasingly simple to apply, as it requires no specific information on the velocity of the particle or the material properties of the target beyond its density. In addition, the assumptions of the approximation square reasonably well with the conditions seen during biolistic bombardment. Conversely, these same assumptions limit the validity of the approximation to scenarios where impact speeds are high, the projectile is rigid (ie: non-deforming), the target has no cohesion (ie: material properties such as tensile strength will not aid the target in resisting penetration) and there is no fluid flow of target material around the projectile.

The approximation, as stated, uses momentum transfer of the projectile to the surrounding target (which is assumed to be pushed away from the projectile at the same speed as the projectile itself for simplicity, although this can also be accounted for) to calculate impact depth. Initially, the target will have no momentum (it is at rest), while the projectile will. Using conservation of momentum and assuming an elastic collision in one dimension, the projectile will then stop once the displaced mass of the target is equal to its own (Halliday et al, 2003 – pg 203):

Mass impactor (M) = mass of target displaced (m)

These masses can be determined using the volume and densities of the impactor and target:

Volume impactor (V) * density impactor (D_p) = volume hole in target (v) * density target (D_t)

As the hole produced by the displacement will have the same cross-sectional area (C) for both the impactor and target (the projectile will bore a hole equal to it's own cross section):

Volume = cross-sectional area * length
Therefore,

length impactor (L) * C * D_p = Depth of penetration (D) * C * D_t

This approximation thus becomes the equation:

$D \approx L \times (D_p/D_t)$

Where:

D = Impact depth in target

L = Length of projectile

D_p = Density of projectile

D_t = Density of target

To illustrate this, consider a macro-analogy for the process of biolistics: the bombardment of a wooden ship by cannon. The walls of the ship are hardened oak (0.75g/cm^3), while the cannon balls being shot at it are large cast iron 24-pounders (14.1cm diameter, with a density of 7.85g/ cm^3) from long guns. If the approximation is used, the iron shot (once fired quickly enough) will be able to penetrate around ten times its effective length in oak. Once the size of the ball is known, the depth of penetration can then be determined:

$D \approx 14.1 \times (7.85/0.75) \approx$ **147.5cm** penetration depth

This squares very well with figures published at the time (Manucy, 1994), where a long 24-pounder was supposed to be able to penetrate 4½ feet (~137cm) of “sound and hard oak”.

In the case of biolistics, the Newtonian approximation can be seen as supplying a theoretical maximum penetration path for particles due to the simplified nature of the model. As has been mentioned, the approximation ignores material factors which may affect particle penetration at low speeds - including the tensile strength of the target and viscous drag effects on the projectile - which would be highly important in retarding the movement of the particle. In addition, the small size of the microcarriers (which results in a very high surface area-to-volume ratio) would tend to exacerbate these factors.

Applied to the biolistic system tested in these experiments, the approximation resolves to:

$D \approx L \times (D_p/D_t)$

Where:

L = 1.1um for M17 particles

D_p = 19.25g/cm^3 for tungsten

D_t = 0.5-1g/cm^3 overall for plant tissue, depending on water content

Choosing a range of values for leaf tissue water content (a measured value of 0.75g/cm^3 was obtained for mature maize leaf tissue), this results in:

$D \approx 1.1 \times (19.25/D_t) \approx$ **21 – 42um** penetration depth (**28um** using the measured value)

Note that due to the similar densities of tungsten and gold, the difference in penetration between the two microparticles should be almost negligible.

These values - which correlate well with the observed and published results - are interesting for a number of reasons. Firstly, they imply that penetration will usually be limited to surface cell layers in the case of differentiated tissues, with multi-cell penetrations being limited to cases where cell layers are highly compact.

In addition, the approximation implies that surface layers (water films, cuticles etc.) encountered before penetration of the target can substantially affect biolistic expression by limiting the penetration of the projectile. This, along with one of the limits of the approximation - its assumption of uniform structure and density for both the projectile and target - argues that tissue microstructure may substantially affect the biolistic process. A target composed of a number of small, thin-walled cells (such as meristematic or embryogenic tissue) will thus naturally show greater levels of expression than one composed of large, thick, well-protected cells. This may, indeed, be true even if the macrostructural properties of the two tissues are identical to the naked eye.

Equally important, it should be noted that the assumptions of the approximation may not hold in all cases. Specifically, in cases where tissues are friable and lack cell walls the mode of penetration may more closely resemble one of an object undergoing hydrodynamic drag than anything else (with accordingly deeper penetration tracks). This can be seen in studies of mammalian tissues, which tend to show deeper penetrations both in absolute terms and relative to the type of tissue being penetrated (epidermal samples having shorter penetration tracks then neuronal tissue samples, for instance).

Finally, tests into cell-free media (such as agar or ballistic gelatine) show the deepest depth-of-penetration of all (Menezes et al, 2012). There is thus a continuum from cases which strictly fulfil the requirements for the Newtonian approximation, to cases where a hybrid model (such as inertial/yield strength approaches) is suitable, before transitioning to a fluid-like regime where a drag-based modelling approach is superior.

Finally, the approximation leads to some possible solutions for improving depth of penetration, should such an approach be required. The simplest option is to use larger projectiles: a doubling of microparticle diameter will, all things being equal, produce an equivalent doubling of penetration depth. However, the larger particles will also result in more damage to the cells. This can be seen to an extent in the effect of particle clumping on lowering cell viability at the impact site - a process which can, in extreme cases, result in visible holes being made in bombarded tissue.

Another approach would be to use silicon carbide whiskers as projectiles, as the penetration depth is directly proportional to the length of the projectile and whiskers have a more favourable length/projectile ratio. As an example, the Silar® SC-9M whiskers frequently used for whisker-mediated transformation have a diameter of 0.65um, a length of 10-12um and a density of 3.21g/cm^3. This would, if used in a biolistic bombardment, result in a penetration depth of 32 – 77um (a 50 – 80% increase).

If, on the other hand, tungsten whiskers grown using the vapour-solid-solid method are used (Wang et al, 2008), then penetration of 38-154um would be possible even in hardened tissues. This would potentially allow for either an increase in expression efficiency (more cell layers being penetrated would expose more nuclei to the carried DNA) or the targeting of deeper cells layers within a given tissue.

However, whiskers would also suffer from problems related to the fact that impact geometry (which had been unimportant when analysing the approximately spherical microparticles) will now play an important role in determining penetration efficiency. Indeed, if we assume that the orientation of particles is random, then only a small fraction of the bombarded whiskers will be in the correct orientation to penetrated to the maximum depth.

Conclusion

During the course of this project, a number of approaches were taken to optimise and improve biolistic transient expression in maize leaf tissues. This was done in order to develop an improved method for examining exogenous gene constructs in live tissue, as well as an understanding of the factors which may influence expression in this system. The results of this study have shown that, while theoretical improvements are feasible by modifying physical input parameters, the existing protocols for biolistic bombardment (developed from studies into improving stable transformation) are already very close to the broad optimal range that can be determined in such a statistically noisy and problematic system.

The efficiency of biolistic bombardment for testing gene constructs in maize leaf is, as demonstrated by the effect of the ligule-distance parameter, largely governed by the tissue being being targeted. This imposes limitations of the ability to study certain genes or interactions, as mature leaf tissues show much lower expression overall. Careful consideration should thus be taken of the nature of the construct, the type of interaction it will have with the target tissue and the overall aim of the experiment taking place.

Examination and modelling of the bombardment system emphasize the qualitative differences between thin-walled cells with high nucleur-to-cytoplasmic area ratios (such meristematic tissues and rapidly-dividing cell cultures) and the more thickly-walled cells found in mature leaf tissues. Even so, the difference in expression seen between the less developed cells closer to the leaf base and those further along the axis of the leaf indicate that another factor (or factors) besides gross morphology is principally responsible for the uptake and expression of gene constructs in maize leaf tissue.

The differences between biolistic transient expression in maize leaf tissues and other expression approaches in other plant systems also mean that different optimal parameters may exist. The broadly optimal nature of the physical input parameters tested during this study, along with the aforementioned contribution of sample location, indicates that other factors must be considered to achieve further improvements in transformation efficiency. Here, the generally applicable nature of the Taguchi methodology would allow large numbers of factors, once identified, to be tested using a relatively small number of

experiments.

In general, transient expression in maize leaf tissues via biolistic bombardment provides a rapid way of assessing certain gene constructs but requires both a visualisation system (to identify transformed cells) and an assay system of sufficient sensitivity and robustness to draw viable conclusions from variable data. Targeting of tissues deeper than a few cell layers from the surface would require modifications to be made to the microparticles being used. To achieve optimal results, the highest priority is to target areas very close to the base of the leaf.

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