Preface

1. Problem Statement

Improvements in crop development, as in any discipline, rely on a combination of theoretical and practical advances. Both of these processes, in turn, require validation in the form of applicable experimental data. Improvements in experimental techniques are thus crucial for improvements in crops.

In the context of plant disease research, this requires elucidation and testing of a number of possible genetic components in the host organism. Narrowing focus towards maize foliar pathogen research, the relatively long life cycles of the host and its recalcitrance towards existing Agrobacterium-based systems make transient expression-based approaches attractive as an alternative to traditional stable integration via biolistic bombardment.

This project proposes the optimisation and further development – using a variety of approaches – of biolistic techniques as a platform for examining exogenous promoter and gene expression. It is hoped that this will lead to improved techniques for rapidly testing exogenous DNA constructs in maize, with an emphasis on analysing the effects of potential pathogen resistance genes in leaf tissue.

2. Aims

To optimise biolistic transient expression in maize leaf tissue.
To gain an improved understanding of biolistic transient transformation using microscopy and modelling the physical parameters.

3. Layout of Thesis

This thesis consists of an abstract, preface, literature review, materials and methods section, results and discussion.

Literature review

4. A brief history of plant transformation

In a series of retrospective articles, the noted biotechnologist Indra Vasil laid out an account of the history of plant biotechnology as defined by five broad 'foundations': the totipotency of plant cells, transgene delivery, integration into the host genome and expression, regeneration of transformed plants and Mendelian transmission of the transgene to any subsequent progeny (Vasil, 2008a; Vasil 2008b; Vasil, 2005). In as much as these concepts form a list of prerequisites for successful production of transformed plants, they are achieved through the use two broad technologies: the techniques of plant cell culture and regeneration and the use of some form of transformation system.

The history of both of these technologies gives an account both of the changing nature of science over the past century and the remarkable efforts that have gone into harnessing and improving the natural regenerative capacity of plant cells for the benefit of humanity. What is striking to note (besides the proliferation of multiple-author papers) is how often the conservative nature of funding comes into conflict with its supposed purpose of enabling 'new' science (Vasil, 2005). History, as the saying goes, does not repeat itself. But it does rhyme.

4.1 *Agrobacterium*-mediated transformation

Arguably the most common technique in use at present; the *Agrobacterium*-mediated transformation approach has been used to produce both transient expression and stable transformation in a number of plant species (Dai et al, 2001; Karami et al, 2009; Nadolska-Orczyk et al, 2000). As the first technique to demonstrate stable gene integration and recovery of transgenic plants, it rapidly became the standard by which all other transformation approaches were judged.

Initially used on dicots, it suffered from low efficiencies when confronted with certain plants (so-called 'recalcitrant' species, a term which broadly includes many monocots). As many of these species are cereal crops, and thus commercially important, a lot of work has been

done over time to develop improved *Agrobacterium* strains for transforming them (Cheng et al, 2004; Nadolska-Orczyk et al, 2000). The result has been a series of modified techniques that has extended the use of this eminently useful bacterium to ever more plant species.

Seventy-six years in the making

The history of *Agrobacterium* as a means of transformation can be traced back to the beginning of the 20th century, when Smith and Townsend (1907) identified the bacterium as the causal agent of crown gall disease. Here the matter rested, until the later discovery by Braun (1958) that the resulting tumor cells could continue to grow even when freed from bacteria. The implication of this finding was clear: some factor (quickly dubbed the Tumor Inducing Principle, or TIP) was responsible for permanently changing the host cells in a heritable manner. More evidence for this surfaced over a decade later, when the discovery of opines demonstrated that the change was strain-specific (Petit et al, 1970). This suggested that part of the bacterial genome was inserted directly into its host, although opinions on this were mixed (Vasil, 2008a). The discovery and isolation of the Ti megaplasmid virulent strains of *Agrobacterium* provided strong evidence that the bacterium was, in and of itself, a natural genetic engineer (Zaenen et al, 1974). With the discovery of the so-called T-DNA fraction of this plasmid in the nuclear DNA of transformed crown gall cells (Chilton et al, 1980; Willmitzer et al, 1980), the case was complete.

The next logical step was to harness the abilities of *Agrobacterium* for use as a gene insertion vector. The construction of 'disarmed' Ti plasmids (lacking auxin, cytokinin and opine/nopaline synthesis genes) gave researchers the ability to recover viable plantlets from cell cultures which had been transformed. This, along with the use of antibiotic resistance genes as selective markers for transformed cells, enabled three independent research groups to simultaneously produce transgenic plants. The results of these three groups (Bevan et al, 1983; Fraley et al, 1983; Herrera-Estrella et al, 1983), announced at the 1983 Miami winter symposium, have been argued as marking the beginning of the current age of biotechnology (Vasil, 2005; Vasil 2008a). Supporting this assessment is the fact that the lead researchers of each group (Marc Van Montagu, Mary-Dell Chilton and Robert Fraley) were jointly awarded the World Food Prize in October 2013.

With success in hand, researchers turned their gaze to the potential agricultural

applications of this new transformation system. Here, however, they were stymied by the fact that, in the wild, *Agrobacterium* simply does not infect the sorts of plants that feed the majority of the world's population. Cereals, and their recalcitrance to transformation by *Agrobacterium*, would be the next big challenge. This was eventually solved, albiet long after the first transformation of cereals using protoplasts (Rhodes et al, 1988), by the adoption of co-cultivation techniques. This approach; which uses a combination of co-cultivation of host cells, hyper-virulent *Agrobacterium* strains and acetosyringone; has now been extended to nearly all important cereal crops. Crucially, the high-virulence strains developed by Japan Tobacco (Komari et al, 1996) resulted in the company becoming a gatekeeper through which any group planning to perform *Agrobacterium*-mediated transformation of cereals had to pass. This tension between open research and free enterprise was played out in a similar fashion in biolistics – the result of which were a number of proprietary approaches and a few significant attempts to bypass them using self-consciously 'open' transformation systems.

4.2 Biolistic Approaches

Biolistic techniques, first developed in the late 1980's (Sanford, 1990; Sanford, 2000), have long been at the forefront of plant transformation. The robust, nature of this technique (resulting in broad applicability) has since kept biolistics a viable approach (Altpeter et al, 2005), though mostly confined to use in monocots where stable transformation is concerned.

Currently, the standard practice for biolistic transformation involves the use of high pressure helium gas to accelerate small, dense particles (gold or tungsten) coated with DNA up to transonic/low supersonic speeds (Sanford, 2000; Liu et al, 2006; Liu, 2007) into a live target. Post impact, a certain subset of these particles will lodge in cells in such a way as to leave behind usable DNA (Hunold et al, 1994). An even smaller subset of these cells will then express the DNA thus deposited for a short time (so-called transient expression). In time, a yet smaller subset of these cells (a microscopic fraction of the initially bombarded) will integrate the foreign genetic material into their own genomes, resulting in stable transformation. And of these stably transformed cells, only a few will integrate in such a way as to be useful to plant geneticists.

Sanford and the development of biolistics

Biolistics owes its existence primarily to the work of John Sanford, Edward Wolf and Theodore Klein, who conceived and developed the technique during the late 1980s. In a later retrospective article (Sanford, 2000), John Sanford laid out the genesis and development of biolistics during that time.

Initially, Sanford had been working on various other transgene insertion approaches, including the use microlasers to cut holes in pollen tubules that DNA could then be inserted into. In the course of this work (which bore no lasting fruits), he consulted with Dr Wolf on the feasibility of using one of the ion guns at the Cornell submicron facility to replace the microlaser. From here, the idea of simply shoving DNA in directly (a 'DNA beam') evolved into speculation on the use of a carrier particle to do the actual work of penetrating the cell.

With a rough idea of the speeds required to allow a 'bullet' of this sort to punch through living tissue, Sanford and company proceeded to the use of modified air rifles firing 4um particles of tungsten into onion epidermal cells. Having had some success with this approach, and with some fortuitous funding in hand, the team (now including Klein) proceeded to develop a gunpowder-driven device utilizing a particle-loaded macrocarrier (a polyethylene bullet) and a stopping plate (a thick sheet of lexan). This device then went on to produce the first biolistic transgenics (Klein et al, 1988a) and perform the first transformations of corn (Klein et al, 1988b). With the addition of a vacuum system, the final form of the gene gun began to take shape.

These initial experiments demonstrated the need for optimisation in a number of areas (particle size and type, DNA precipitation procedure and the physical parameters of the gun itself), each of which required a number of trials and experiments to hone. This process (which has continued into the present day) has slowly extended and broadened biolistics into a reliable technique for the insertion of foreign DNA into plant cells.

Biolistic Systems

Over the years, a number of approaches have been used in biolistics. Of these, only a few have continued in regular use. It is not entirely clear why this should be so, but cost, and technical difficulties in implementation (discussed below) all seem to have resulted in a

field in which new approaches are constantly being invented and re-invented before being subsumed by more established systems.

An early break-away from the use of expanding gasses to launch microparticles was made by using an electrical discharge to produce a usable firing impulse. This was first commercialised in the form of the ACCELL gene gun (McCabe and Christou, 1993), which worked by arcing through a water droplet to produce a vapour pulse. This pulse then propelled the macrocarrier and microparticles as normal.

Another approach to propelling microparticles is to use the macrocarrier itself. These devices (referred to as 'drum-head' gene guns) were tested very early on in the development of biolistics, but never really achieved much (Sanford, 2000). Recently, however, the use of high-intensity pulsed lasers has allowed the concept to re-emerge. This approach uses a focused pulse of light to ablate the back of the macrocarrier (made from a thin metal foil) and, in doing so, transmit a shock-wave through it to the microparticles coated on the front (Nakada et al, 2008). These are then flung off at hypersonic velocities (up to 1400m/s) into the target (Menezes et al, 2012). Although a recent development, this approach holds much promise for the future.

The most common systems, however, are still those using compressed helium to drive particles. The most common of these (arguably the most commonly-used biolistic device in the world) is descended more-or-less directly from the gunpowder-driven machines developed by John Sanford and company. This device (the PDS-1000/He), marketed by Bio-Rad Laboratories inc., uses a plastic macrocarrier to transport microcarriers down a short barrel (Kikkert, 1993). At the end of its flight the macrocarrier is arrested by a wire mesh stopping screen, freeing the particles to enter the vacuum chamber and impact their target. The stopping screen and vacuum limit damage to the target tissue somewhat, although the driving pulse of helium gas still causes problems.

An alternative approach, developed by John Finer et al (1992), is to do away entirely with the moving macrocarrier and inject microparticles directly into a high-speed gas stream. By simplifying the process down to the two most necessary components (the microcarriers and something to propel them), it was hoped that biolistic bombardment could be made more simple and affordable without sacrificing performance. The resulting device, the

Particle Inflow Gun (PIG), was conceived as a simple, low-cost, non-commercial alternative to existing biolistic systems which could be put together using common scientific and commercial components. In practice it has achieved a number of successful transformation events up to the present day, including a number of South African firsts (Biswas and Potrykus, 1997; O'Kennedy et al, 1998; Abumhadi et al, 2001; O'Kennedy et al, 2001; Girgi et al, 2002; O'Kennedy et al, 2004; Williams et al, 2004; Grootboom et al, 2010). As such, it has also taken up an important role for researchers who wish to avoid the extensive intellectual property concerns that come with patented commercial systems.

The use of a stationary carrier in biolistic bombardment also lead rapidly to the development of systems with what could be termed a 'cartridge-based' gene gun approach. In a similar fashion to the PIG, microcarriers are laid onto a macrocarrier and then subsequently stripped off into a stream of high-speed gas. Here, however, the focus is on cartridge preparation and storage, rapid reset and bombardment into ambient atmosphere. This culminated in the Helios device (also produced by Bio-Rad), which adds a revolver-style tube cartridge holder system and venturi nozzle barrel to help dissipate some of the gas blast caused on firing.

4.3 Other approaches to transformation

Use of protoplasts

The development of protoplast culture methods in the 1960s may be argued (in a number of ways) as setting the stage for biotechnology as a whole. In one interpretation it did so by allowing, for the first time, artificial introgression of genetic material across diverse species using protoplast fusion (Power et al, 1970). However, although this technique has had some successes in generating novel plant varieties, it has not had the revolutionary effect that it seemed to promise at the outset. Under another interpretation, however, the value of protoplasts in the development of biotechnology cannot be overstated. This is because the path leading from protoplasts, although filled with dead ends, has stimulated the development and improvement of other important technologies.

Protoplasts were first produced by Edward Cocking (Cocking, 1960) as a means to understand biochemical processes in plant cells (Cocking, 2000). Soon it was realised

that, lacking a cell wall, the protoplasts were amenable to taking up substances they normally wouldn't. Secondly, it was found that the protoplasts could be regenerated to produce adult plants (Takebe et al, 1971). Once these two processes were combined, the development of a useful plant transformation system seemed immanent. In the event, the first protoplast transformations were carried out using *Agrobacerium*-derived plasmids (Davey et al, 1980), producing non-regenerable tumor cells. . However, with attention shifting to crop plants, protoplasts were used to transform cereals, including the first transgenic maize (Rhodes et al, 1988).

The state of affairs was not to last: biolistics proved better at transforming cereals and *Agrobacterium* proved to be flexible enough to do the same. Even so, protoplasts have found a number of transformation-related uses: as a test system for targeted transformation approaches and novel vector systems (Puchta and Fauser, 2013), as well as being a useful culture/regeneration technique in its own right.

Whisker-mediated transformation

Aside from Biolistics and Agrobacterium, a whole constellation of putative transformation systems have been envisioned and tested (Rakoczy-Trojanowska, 2002; Songstad et al., 1995). So far, all of them have struggled to find broad acceptance due to a combination of technical (high cost, low efficiency) and socio-economic factors (opportunity costs associated with switching over to new systems, normal human resistance to new techniques over known ones and so on). The effect of the latter, especially, has limited the development of these techniques. Due to competition by more mature technologies for limited time and funds there is often no incentive to develop an otherwise unremarkable technology to the point at which it would become competitive.

The history of whisker-mediated transformation is illustrative of this process. First developed in the late 1980s, the technique saw initial success in transforming cultured monkey cell using conventional plasmid constructs (Appel et al, 1988). Thereafter, researchers applied themselves to extending the technique to other organisms. This resulted in the first successful transient transformations of plant cell cultures, a feat which was followed up with the transformation and recovery of stable clones in the early 90's (Kaeppler et al, 1990; Kaeppler et al, 1992). The approach was subsequently developed to the point where recoverable maize transgenics were produced, with routine development

of transgenics continuing until the present day (Frame et al, 1994; Asad et al, 2008).

Vaccines and viral approaches

Viral approaches, which have been used as vectors for trangene expression for almost as long as *Agrobacterium* and biolistics, suffer somewhat from categorisation issues in regards to constructing a coherent narrative of plant transformation. This is partly due to the difficult-to-classify nature of viruses themselves, which could plausibly be argued to be both a biotic and abiotic vector at the same time, and partly due to the nature of the expression which arises from viral interactions with host plants.

The use of RNA-based viruses – principally mosaic viruses – in plant transformation goes back almost as far as the other major approaches. In the early 1990s, Hamamoto et al (1993) succeeded in producing a modified form of Tobacco Mosaic Virus (TMV) expressing a recombinant protein in tobacco and tomato. This approach, which was subsequently expanded to include cowpea mosaic viruses, was recognised as a promising step towards the use of plants for producing novel chemicals (Goddijn and Pen, 1995). This plants-as-bioreactors idea had a number of things to recommend it, not least of which was the fact that the plants themselves were the ultimate source of a number of the medicinal compounds used in pharmacology. The concept of 'pharming', when allied with the continued development on viral expression systems, seemed to promise a future in which previously expensive medical treatments might actually grow on trees.

Among the first uses of viral expression in this field was the production of vaccine antigens for use against rabies (Modelska et al, 1998). These trials, which expressed the proteins in plant leaf material (for either direct consumption or further processing as the situation dictated) proved to be remarkably effective in vaccinating mice. Similarly, a viral surface protein – expressed, in this case, in stably-transformed plants produced using *Agrobacterium* – proved effective in combating hepatitis B (Kapusta et al, 1999). The confluence between transformation approaches lead, over time, to the realisation that seed-specific expression would be an ideal approach for stably packaging pharmacologically-useful proteins for subsequent shipping and processing. This was especially true for maize, which displays a number of useful characteristics for seed-based pharming (Ramessar et al, 2008).

Viral approaches have also been found to be useful in preventing expression rather than producing it. This Virus-Induced Gene Silencing (VIGS) approach uses a viral vector to to knock down gene expression after transcription. The effect was first noticed in the resistant phenotype generated by plants that had been previously exposed to a viral pathogen (Lindbo et al, 1993). It was was rapidly determined that this process was linked exogenous RNA sequences, with the effect being spread systematically from the point of origin (Baulcombe, 1996; Depicker and Montagu, 1997). From here, it was established that the process could be applied to non-viral genes, both endogenous and exogenous (transgenes), with the effects being similar to the previously-studied phenomenon of transgene silencing (Ratcliff et al, 1997; Ruiz et al, 1998). VIGS, it seemed, was simply one manifestation of a broader post-transcriptional gene silencing pathway.

With the publication by Fire et al (1998) of an article demonstrating the potent effect of double-stranded RNA in nematodes, the link between all of these seemingly disparate phenomena became clearer. This link was strengthened by research which both demonstrated the existence of complementary RNA strands in silenced plants (Hamilton and Baulcombe, 1999), as well as replicating the effect itself in *Arabidopsis* using double-stranded RNA (Chuang and Meyerowitz, 2000).
Since then, VIGS has come to be seen as a useful tool in studying gene function in plants. The processes' rapid ability to knock down expressed genes in a targeted fashion makes it attractive for a number of applications which require large amounts of genes to be assessed. This, combined with the flood of data generated by genomic studies, seems to offer the prospect of high-throughput elucidation of complex genetic systems for crop improvement (Becker and Lange, 2010).

Other influences on transgene expression
Alongside the physical process of putting a construct into a cell and the cell's innate ability to make use of it, the make-up of the construct itself is a key factor in determining transformation efficiency.. Minimal gene cassettes have shown, in some cases, orders-of-magnitude higher expression levels over whole-plasmid vectors (Beyene et al, 2011). Similarly, minimal vectors have been shown to be significantly more efficient for the purposes of transformation (Coutu et al, 2007).

Further advances in construct design have focused on mechanisms for targeted

integration, an approach which holds significant advantages when compared with the pseudo-random integration currently achieved by existing biolistic and *Agrobacterium*-mediated methods (Puchta and Fauser, 2013). These approaches tend to use the cell's existing repair systems to facilitate integration, with accuracy being achieved by the use of targeted strand breakage and homologous recognition/repair, or some combination thereof.

Homologous recombination-based systems have a long history, but until recently suffered from very low efficiencies in wild-type lines (Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999; D'Halluin et al, 2008; Xiao-Hui et al, 2008; Saika and Toki, 2009; Srivastava and Gidoni, 2010). This defect has subsequently been remedied to an extent by the use of engineered, site-specific nucleases such as Zinc-Finger Nucleases, or ZFNs (De Pater, 2009; Porteus, 2009 ; Townsend, 2009; Srivastava and Gidoni, 2010). Even more recently, the use of Tal nucleases (or TALENs) has provided a reliable tool for targeted genome modification in plants (Cermak et al, 2011), adding weight to the opinion that an age of reliable, precise transgene integration may rapidly be drawing near. The role that biolisic approaches will play in this new age is as yet unclear, but given the technique's inherent flexibility and broad applicability, it is almost certain to find applications at the vanguard of crop improvement research.

As with any host-vector relationship, the nature of the cells themselves in receiving, expressing and integrating the foreign constructs introduced by researchers is of high importance. It has long been known, for instance, that embryogenic, totipotent plant tissues are easier to transform with foreign DNA (Birch, 1997). With the early realisation of the importance of the cell wall as a barrier to entry, the development of wall-less protoplast culture systems provided another avenue for transformation research (Lörz et al, 1985; Hansen and Wright, 1999).

More recently, attempts have been made to use viral packaging proteins to slip constructs into the cell by stealth (Mäe et al, 2005; Chugh et al, 2010). This can be seen as an extension of the the approach used during *Agrobacterium*-mediated transformation, where a bacterium wielding a secretion apparatus (rather than a fragment of a virus' coat) is co-opted to act as the mediator between the researcher and his subject.

A rather more direct way of co-opting microbial vectors is the attempts to use them as components of the biolistic projectile itself (Rasmussen et al, 1994; Kikkert et al, 1999). This approach has some inherent advantages. Chief among these is the fact that the donor cell itself would carry the vector DNA, thus eliminating the need for its removal and purification prior to attachment to the micro-carrier. Unfortunately, achieving a sufficient dosage of DNA proved to be problematic and the technique was never developed further.

5. Cell culture systems

5.1 From cell to plant

Plant cell culture has an enormously long history, stretching back nearly two centuries to the cell theory of Schleiden and Schwann (Vasil, 2008a). This concept; made concrete by Virchow's aphorism “all cells arise from cells”; has lead over the intervening years to a number of attempts to culture plant cells in isolation from their parent plants.
Some of the first serious attempts at this were conducted in the early 1900s by Gottlieb Haberland (Krikorian and Berquam, 1969). Although not in any way successful (due to a combination of poor choice of starting material, very minimalist media and contamination by microorganisms), his experiments were useful in encouraging other attempts. These tended to concentrate on improvements in three aspects (improved nutrient solutions, better choice of plant material and more aseptic cultures) that became a recurring theme in each generation of research.

By the 1920s, this process had culminated in short-term cultures of embryo fragments and excised roots. With further refinement, the 1940s saw long-term cultures of cambial and root tissues become the norm (White, 1943). With the discovery of auxins, a large step was taken in controlling the growth and multiplication of cultured cells in media. This culminated in systems like White's media, which were then used until the mid-1960s.

With input generated by mineral requirement studies, the addition of greater amounts of mineral salts provided further improvement in culture growth and stability (Ozias-Akins and Vasil, 1985). This culminated in experiments using leaf extracts in culture media, the inorganic components of which were found to be principally important in maintaining cell

viability. This reconstituted leaf extract formula (with increased levels of minerals, chelated iron, four vitamins and myo-inositol) went on to become the foundation of Murashige and Skoog's nutrient solution (Murashige and Skoog, 1962) – a formula which is used to this day.

Further advances in enhancing the growth of cultures were obtained by the use of green coconut milk to fortify media. An investigation into why this should improve cell division moved in logical fashion from here to yeast extracts, the addition of DNA and finally the use of Herring sperm DNA extracts. At this point kinetin, which made up a component of the extract, was found to be the causal agent of the increased division (Skoog and Miller, 1957). The link between auxins, cytokinins and plant growth and differentiation had been made.

5.2 Somatic embryogenesis

With viable culture systems in hand, thoughts turned to their possible commercial uses. An immediate use was found for the use of plant hormones in the field of micropropagation, which then formed the basis for further advances in plant regeneration from cell cultures. A patent by Routier and Nickell (1956), by contrast, laid the groundwork for the concept of natural product production using liquid suspension cultures. Eventually, this gave birth to a number of commercial products (although ongoing technical and biological issues have hampered large-scale approaches). However, it was the development of somatic embryogenesis which had the greatest commercial impact by enabling the regeneration and breeding of transgenic plants.

First observed described in the late 1950s (Reinert, 1958; Steward et al, 1958), the phenomenon of somatic embryo development in culture pointed the way towards closing the loop between cells isolated from a parent plant and the production of new plantlets from them. This, it was immediately apparent, would allow changes to a single cell to propagate into a plant with heritable characteristics. Progress, however, was not easy: cereal crops proved recalcitrant and required further discoveries and developments before regeneration could be obtained (Vasil, 2005). Key among these was the use of the herbicidal Auxin 2,4-dichlorophenoxyacetic acid (2,4-D) to halt differentiation. This allowed

cell lines to be maintained and cultured for extended periods of time and enabled the development of viable embryos. Once formed, the embryos could differentiate and grow into plantlets by simply lowering the 2,4-D concentration in media. As an added bonus, the resulting plantlets tended to be genetically and physiologically normal – a result of selective advantage by meristematic cells in the formation of embryos.

6. Biolistics and Cereal Crops

Due to the substantial amount of literature generated by the research community on the application of biolistics to plants, a comprehensive survey on the subject would be in danger of becoming both unwieldy and opaque. This is true even when the scope of the subject is narrowed to cereal-specific applications.

However, the large amount of research also allows a very detailed snapshot of specific developments and technologies. This chapter will thus examine areas pertinent to this project: South African cereal (and sugarcane) biolistic transformation efforts (4.1), the use of biolistic approaches to transiently transform maize (4.2) and the use of biolistics to produce transient expression in leaf tissues (4.3).

6.1 The South African perspective

Biolistic monocot transformation in South Africa has, as with elsewhere in the world, been overwhelmingly dominated by projects related to crop improvement; often with a focus on disease-resistant traits. This can be seen in summary form in table 1, which collects all the available literature on the subject and sorts them by time and type of plant being studied.

In this context, the energetic work by researchers at the South African Sugarcane Research Institute (SASRI) can be seen to have produced a number of publications devoted to the transgenic improvement of sugarcane. Of these publications, the production of transgenic, herbicide-resistant plants from embryogenic calli of multiple commercial sugarcane varieties (Snyman et al, 1996) represents something of a milestone for South African biotechnology. The protocols developed during this project would subsequently be

used for a number of future sugarcane-related experiments. These included further refinements to sugarcane bombardment and selection (Snyman et al, 2000; Snyman et al, 2006), further research into the production of transgenic, herbicide-resistant plants (Liebbrandt and Snyman, 2003; van der Vyver et al, 2013) and the production of metabolically-altered transgenics for increased sucrose yield (Groenewald and Botha, 2001; Groenewald and Botha, 2008).

Moving to cereal crops, the production and recovery of transgenic maize (O'Kennedy et al, 1998), pearl millet (Girgi et al, 2002; O'Kennedy et al, 2004), sorghum (Grootboom et al, 2010) and wheat (Lacock and Botha, 2000) plants have all been undertaken by various workers. In the case of maize and pearl millet, this work was used as a foundation for studies into the expression of anti-fungal proteins for pathogen resistance (O'Kennedy et al, 2001; O'Kennedy et al, 2011).

Development of transgenics using positive selectable markers has been enthusiastically taken up by South African workers, projects in both pearl millet and sorghum using mannose-based selection (O'Kennedy et al, 2004; Grootboom et al, 2010). This is in line with a general trend towards improved biosafety and an awareness of the issues posed by transgene escape to wild relatives of African crop species such as sorghum and pearl millet (Stewart et al, 2003). In this context, the use of positive selection provides some clear advantages over more traditional negative selection techniques using herbicide or antibiotic-resistance genes by limiting the potential spread of genes which would provide selective advantages to plants exhibiting weedy characteristics (Miki and McHugh, 2004).

Table 1. Survey of South African articles: biolistic transformation of cereals and sugarcane

Article	Authors	Year	Plant
Stable transformation of Hi-II maize using the particle inflow gun	O'Kennedy et al.	1998	Maize
Transformation of elite white maize using the particle inflow gun and detailed analysis of a low-copy integration event	O'Kennedy et al.	2001	Maize
Maize streak virus resistant transgenic maize: a first for Africa	Shepherd et al.	2007	Maize
Transgenic and herbicide resistant pearl millet (Pennisetum glaucum L.) R.Br. via microprojectile bombardment of scutellar tissue	Girgi et al.	2002	Pearl millet
Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase	O'Kennedy et al.	2004	Pearl millet
Expression of a β-1, 3-glucanase from a biocontrol fungus in transgenic pearl millet	O'Kennedy et al.	2011	Pearl millet
Biolistic mediated sorghum (Sorghum bicolor L. Moench) transformation via mannose and bialaphos based selection systems	Grootboom et al.	2010	Sorghum
Establishment of embryogenic callus and transient gene expression in selected sugarcane varieties	Snyman et al.	1996	Sugarcane
Direct somatic embryogenesis for rapid, cost effective production of transgenic sugarcane (Saccharum spp. hybrids)	Snyman et al.	2000	Sugarcane
The introduction of an inverted repeat to the 5' untranslated leader sequence of a transgene strongly inhibits gene expression	Groenewald et al.	2000	Sugarcane
Manipulating sucrose metabolism with a single enzyme: pyrophosphate-dependent phosphofructokinase (PFP)	Groenewald and Botha	2001	Sugarcane
Stability of Gene Expression and Agronomic Performance of a Transgenic Herbicide-Resistant Sugarcane Line in South Africa	Liebbrandt and Snyman	2003	Sugarcane
Refining the application of direct embryogenesis in sugarcane: effect of the developmental phase of leaf disc explants and the timing of DNA transfer on transformation efficiency	Snyman et al.	2006	Sugarcane
Down-regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes	Groenewald and Botha	2008	Sugarcane
In vitro selection of transgenic sugarcane callus utilizing a plant gene encoding a mutant form of acetolactate synthase	van der Vyver et al	2013	Sugarcane
Genotype variation in regeneration and transient expression efficiencies of 14 South African wheat cultivars	Lacock and Botha	2000	Wheat
Optimisation of transient transformation of a South African spring wheat cultivar with particle bombardment	de Villiers and Laib	2000	Wheat

6.2 Transient transformation

Transient biolistic transformation of various maize tissues began to be investigated almost as soon as biolistic protocols themselves were established. In large measure this was because achieving transient expression was as a step along the pathway towards stable transformants. The result is that most research on biolistic transient expression in maize tissues is geared towards optimising the process for the production of stable transformants or assessing transient expression as part of the same process (table 2).

The study of transient transformation in cereals, as a phenomenon in and of itself, can thus be argued to have begun with the work of Oard et al (1990), who tested a biolistic device that they had constructed against a number of callus cultures of various crop species. This article was also an early example of the ongoing attempts to optimise the biolistic system generally through variation in physical input parameters. At around the same time, Reggiardo et al (1991) were busy testing a gunpowder-driven device against various maize tissues and culture types. Here, the focus was squarely on finding the optimal vector design and tissue type to achieve high expression.

This investigative approach continued with the work of Hunold et al (1994), who attempted to piece together the series of events leading from bombardment to stable expression. In the process, they elucidated a number of basic, physical, attributes of the biolistic system - particle location, cell damage, the relative levels of transient expression versus stable transformation - that determine whether a given cell will express a transgene or not. Complementary to this research, the work of Kemper et al (1996) analysed the effects of various parameters using a factorial design of experiments. This resulted in a clear elucidation of the trade-off between high expression and cell damage – a problem which the researchers sought to mitigate by using osmotic preparation to 'harden' cells by lowering the cytoplasmic volume and decrease the tension of the cell membrane.

As research progressed, focus shifted from understanding the processes underpinning biolistics to its uses as a tool for understanding other processes. Schenk et al (1998) took a step in this direction via a series of experiments designed to overcome the intrinsic variation in expression levels seen during biolistic transformation. To do so an internal standard (Green Fluorescent Protein, or GFP, whose expression was driven by a 35S

promoter with known effects on expression) was used to normalise the data at the transient expression level. This was then tested on maize leaf tissues, as the use of phenotypically abnormal cultured cells was thought to skew the data. Kotlizky et al (2000) used a similar system as a means to analyse the role of maize geminivirus protein subunits in the movement of the virus from cell to cell. This research, geared as it was to the use of transient biolistic transformation as a means to understanding other systems, pointed the way forward for research in the new century.

Even as this trend has continued, the refinement of biolistic approaches has remained part of the process of applying them to new experimental questions. The work of Kirienko et al (2012), although principally concerned with testing fluorescent visual marker constructs intended for cell localization studies, nonetheless focused significant time and effort on developing an optimised protocol for transient biolistic bombardment on excised maize leaf segments. In the process, it was discovered that distance from the ligule (down the axis of the leaf blade) was one of the prime determinants of transformation efficiency for samples (figure 1). This was found to be the case even when correcting for cell size, demonstrating that one or more biological factors were affecting the ability of cells to take up and express foreign DNA deposited by microcarriers.

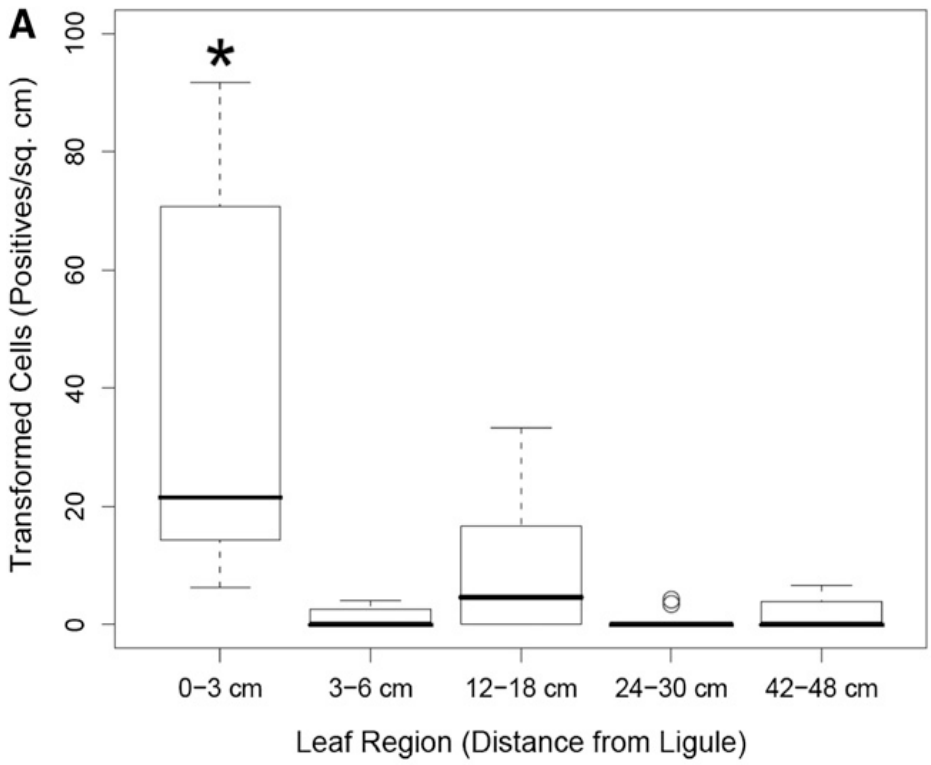

Figure 1. Results obtained in another biolistic transient expression assay incorporating a ligule-distance parameter (taken from Kirienko et al, 2012)

The transient expression approach has proven particularly appropriate for the study of plant-pathogen interactions, where rapid testing and visualisation approaches could greatly improve the development cycle for resistant crops (Panstruga, 2004; Carsono and Yoshida, 2008). Transient expression systems have been shown, however, to be limited in terms of their effect size (single cells or small clusters), expression duration and optimal tissue type (Panstuga, 2004; Kirienko et al, 2012; Kuriakose et al, 2012).

Table 2. Survey of articles: biolistic transient transformation of maize

Article	Authors	Year	Sample origin
Transfer of foreign genes into intact maize cells with high-velocity microprojectiles	Klein et al.	1988	Suspension culture
Transient gene expression in maize, rice, and wheat cells using an airgun apparatus	Oard et al.	1990	Callus culture
Transient transformation of maize tissues by microparticle bombardment	Reggiardo et al.	1991	Coleoptiles, embryogenic callus, leaf basal segments and suspension culture
Transient expression from cab-m1 and rbcS-m3 promoter sequences is different in mesophyll and bundle sheath cells in maize leaves.	Bansal et al.	1992	Leaves
Development of the particle inflow gun for DNA delivery to plant cells	Finer et al.	1992	Suspension culture
Dissection of a pollen-specific promoter from maize by transient transformation assays	Hamilton et al.	1992	Isolated pollen
Development of the particle inflow gun	Vain et al.	1993	Suspension culture
Early events in microprojectile bombardment - cell viability and particle location	Hunold et al.	1994	Leaves
Biolistic transformation of tobacco and maize suspension cells using bacterial cells as microprojectiles	Rasmussen et al.	1994	Suspension culture
Effect of microprojectile bombardment parameters and osmotic treatment on particle penetration and tissue damage in transiently transformed cultured immature maize (Zea mays L.) embryos	Kemper et al.	1996	Excised embryos and callus culture
Stable transformation of Hi-II maize using the particle inflow gun	O'Kennedy et al.	1998	Callus culture
Assessment of transient gene expression in plant tissues using the green fluorescent protein as a reference	Schenk et al.	1998	Leaves
Characterization of the regulatory elements of the maize P-rr gene by transient expression assays	Sidorenko et al.	1999	Suspension culture
Cis-elements and trans-factors that regulate expression of the maize Cat1 antioxidant gene in response to ABA and osmotic stress: H2O2 is the likely intermediary signaling molecule for the response	Guan et al.	2000	Excised embryos
Intracellular and intercellular movement of maize streak geminivirus V1 and V2 proteins transiently expressed as green fluorescent protein fusions	Kotlizky et al.	2000	Leaves
Estimation of biolistic transformation effect by transient expression of C1-R regulatory genes of anthocyanin biosynthesis	Shan et al.	2000	Callus culture
Inhibition of maize streak virus (MSV) replication by transient and transgenic expression of MSV replication-associated protein mutants	Shepherd et al.	2007	Suspension culture
A low-pressure gene gun for genetic transformation of maize (Zea mays L.)	Kao and Huang	2008	Excised embryos
Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study	Kirienko et al.	2012	Leaves

6.3 Transient expression in leaf tissues

Biolistic transient transformation of plant leaf tissues has been, as illustrated by table 3, a relatively unstudied area when compared with the massive amount of information available in the broader literature on plant transformation. Even so, there is enough literature available to draw together a timeline of developments within this field.

The biolistic transient transformation of plant leaf tissue was first described in the early 1990s in both monocots (Daniell et al, 1991; Reggiardo et al, 1991) and dicots (Seki et al, 1991). In both cases, the basal part of the leaf was targeted for expression. Within a very short space of time, this approach was used to study light-dependent promotion in maize (Bansal et al, 1992), with further work being driven mainly by a combination of disease-resistance and photosynthesis research. The use of GFP as a visual marker for expression was taken up early on (Schenk et al, 1998), although β-glucuronidase has remained popular throughout. The latter, while destroying assayed tissue, is both highly sensitive and easy to quantify. Fluorescent markers, on the other hand, are non-destructive but can be problematic to quantify in plants without treatments to reduce background fluorescence (Robic et al, 2009) in leaf tissues.

Table 3. Survey of articles: biolistic transient transformation of cereal leaf tissues

Article	Authors	Year	Plant
Cell-autonomous complementation of mlo resistance using a biolistic transient expression system	Shirasu et al.	1999	Barley
Transient transformation of maize tissues by microparticle bombardment	Reggiardo et al.	1991	Maize
Transient expression from cab-m1 and rbcS-m3 promoter sequences is different in mesophyll and bundle sheath cells in maize leaves.	Bansal et al.	1992	Maize
Assessment of transient gene expression in plant tissues using the green fluorescent protein as a reference	Schenk et al.	1998	Maize
Intracellular and intercellular movement of maize streak geminivirus V1 and V2 proteins transiently expressed as green fluorescent protein fusions	Kotlizky et al.	2000	Maize
Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study	Kirienko et al.	2012	Leaves
Transient expression of β-glucuronidase in different cellular compartments following biolistic delivery of foreign DNA into wheat leaves and calli	Daniell et al.	1991	Wheat
Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance	Schweizer et al.	1999	Wheat
A transient assay system for the functional assessment of defense-related genes in wheat	Schweizer et al.	1999	Wheat

7. Ballistics versus biology

7.1 Particle damage, toxicity and fate

When discussing biolistic bombardment, it should always be held in mind that the process itself is robustly physical. This central aspect of the technique (which, after all, results in cells being peppered with what amount to tiny bullets) is both the driving force for its wide applicability and the cause of the majority of its pitfalls. From the start, it was understood by researchers that the damage caused by the bombardment (along with the problems of gas blast) caused, as an unavoidable side-effect, the death of a large proportion of the cells that were being studied. This was dealt with even before the broad acceptance of the technique, with the addition of vacuum chambers to dissipate gas blast (Sanford, 2000). Further research on the phenomenon was carried out by Russel et al (1992), who investigated the effect of baffles and alternative launch systems (helium-driven, rather than gunpowder driven devices) on increasing the viability of bombarded cells. In addition, the role of the microcarriers themselves were investigated by comparison of tungsten and gold microparticles added to the culture medium. Here, large amounts of tungsten (over 100ug per ml of medium) were found to exert a toxic effect on plant cells.

7.2 Tissue morphology and development

As the research by Reggiardo et al (1991) and Kirienko et al (2012) has shown, the effect of tissue type, developmental state and morphology on transformation efficiency is profound. Often, this goes beyond physical features such as cell density, to the point that it becomes something like a factor in its own right – a sort of broad measure of 'receptiveness' to genetic manipulation. The underpinnings for this receptiveness are multifarious and often not clearly understood, but are occasionally traceable to particular causes. Tadesse et al (2003), for instance, found that the unwanted production of phenolic compounds from transgenic cultures of herbicide-resistant sorghum prevented regeneration of plantlets. Here, the problem of 'receptiveness' had manifested not at the level of transgene integration or expression, but at the point where the transgene product itself was preventing recovery.

8. Approaches to optimisation

Biolistic transformation is a process in which a multitude of physical, chemical and biological factors may become important in determining the final efficiency of the system (Sood et al, 2011). Added to this complicated picture is the fact that - in most cases - biolistic transformation systems also rely the use of sophisticated cell culture systems to recover transgenic plants from the small number of transformed cells thus produced; a process with it's own unique problems and pitfalls (Taylor and Fauquet, 2002). Improvements in biolistic transformation have thus gone hand in hand with improvements in cell culture techniques and methods for the selection of transgenic cells and plantlets (Moeller & Wang, 2008).

8.1 Optimisation and biolistics

Optimisation of biolistic procedures and parameters has (as mentioned above) long been of concern to workers in the field. This is speculated to be due, in part, to the fact that minor changes in transformation efficiency can greatly increase the number of recoverable transgenic plants being produced (Sanford, 1993; Frame et al., 2000; Carsono and Yoshida, 2008). This, in turn, has a knock-on effect when screening for suitable integration events; allowing faster development times overall. Given that transient transformation events lead to stably-expressing cells, it is reasonable to assume that maximising transient expression will thus have the effect of increasing the number of recovered plants overall by producing more stable transformants for selection. Optimisation parameters for transient expression and stable transformation may, however, differ due to their different operational requirements: maximising DNA transfer and expressing cell numbers in transient transformation versus transfer of complete constructs without multiple integration events in stable transformation. Furthermore, the tradeoff between expression and cell viability argues that the relationship between the two states is non-linear.

Due to these concerns, as well as existing uncertainties regarding the exact mechanisms of exogenous DNA expression and integration in plant tissues, optimisation has proceeded mainly on an experimental basis (Sanford et al, 1993; Oneto et al, 2010; Ueki et al, 2013). This has allowed experimenters to treat the cell as a classic black box system, altering

single inputs while holding other variables stable until a change in output occurs – all without having to build a reductive model of the underpinnings governing the process itself. Unfortunately, the use of such a factorial analysis can be both time consuming (if a number of factors are important in determining output) and does not allow a-priori optimisation of new techniques.

One variation of this single-input system which partly eliminates some of these disadvantages was developed by a Japanese industrialist named Genichi Taguchi in the 1940s (Ballantyne & van Oorschot, 2008; Kacker et al, 1991; Rao et al, 2008). Building on work done for the Electrical Communication Laboratory in designing telephone switching systems, he independently combined a multi-factor array-based system (the orthogonal array), a series of analysis tools and an overall design philosophy emphasizing quality control and loss reduction to create a complete experimental and industrial approach (Taguchi, 1986).

Taguchi's techniques, adopted widely during the late 1970's, formed an integral part of the robust design approach (so-called because it seeks to make the process 'robust' or insensitive to input variation) in industrial development. More recently, his approaches to optimisation have been taken up by biotechnology researchers to tackle problems where multiple input factors must be rapidly ranked, assessed and tuned to provide improved outputs (Khoudoli et al, 2004; Nava-Arenas et al, 2008).

In essence, the Taguchi methodology for experiments evenly tests a series of factors at different levels of intensity in a set sequence determined using an orthogonal array design for experiments. This allows a large, even sweep of the optimisation 'landscape' to be conducted while excluding factor interactions. The resulting data is then analysed statistically to determine which factors have the greatest effect on the desired outcome and isolate their signal-to-noise ratio in this context.

The major advantage of Taguchi optimisation when compared with standard single variable experiments (sequential testing of every variable with all other variables held constant) is that it greatly reduces the total number of experiments needed for optimisation, especially in systems with large numbers of input variables. As an example, a plant growth experiment testing ten separate parameters at three levels would require 3^{10},

or 59049 experiments if each factor and level was tested individually. For the equivalent Taguchi optimisation study, an L27 design array (which can be looked up using a suitable reference table) is recommended for the task, resulting on only 27 experiments to achieve the same end. This reduction in effort would, for obvious reasons, be very attractive to workers seeking to perform an optimisation on this system.

Taguchi's method, however, has not been universally embraced. Criticisms have been raised in regards to the novelty of the approach, the underlying philosophy of variation limitation, the statistical methods underpinning the analysis of the data and the lack of factor interaction testing (Pignatiello and Ramberg, 1991; Nair et al,1992; Smith, 1998 – pg 250).

8.2 Numerical modelling of the biolistic system

Of course, experimental optimisation is merely one approach to achieving better technique. With sufficient knowledge of the underlying processes, it is also possible to propose reductionist numerical models which can then be verified and refined using experimental outputs. This has the advantage of opening up the 'black box', allowing workers to determine the best inputs *a priori*.

One of the few papers currently available in the open literature on the physical modelling of biolistics is by Zhang et al (2007). This article uses a simulation-based approach to model the dynamics of particle acceleration, ballistic motion and penetration when using the Helios device on animal tissues. The core of the paper (at least from the perspective of biolistics modelling in plant tissues), is an equation for modelling penetration depth over time, using a simplified formula for drag in a viscous medium. This was then simulated *in silico* until maximum particle depth had been achieved. The results were claimed to show good agreement with experimental results, although as tissue became more 'resistant' (a term which was, unfortunately never quantified within the article itself) the penetration dropped significantly; to around 100um.

Another article (Liu, 2007) used a commercial computation fluid dynamics (CFD) software package to analyse the dynamics of a biolistic device intended for needle-free vaccination. Here a unified penetration theory, as proposed by Dehn (1987) is applied to the penetration phase of particle bombardment. This is then calibrated by comparison with experimental data obtained in bombardments of agar, before being extended to porcine epidermis (for which no experimental data is presented). The penetration model uses the inertial force required for penetration (which is density-dependent) and the yield strength of the target material to calculate the decelerating force acting on the projectile. In this case, skin was estimated to limit particle penetration to around 30um.

Soliman et al (2011) took a similar approach by using a semi-empirical formula based on inertial forces and the yield strength of the target material that they themselves derived. This formula was then applied on various numerical models of skin, each with different thicknesses and proportions of three component layers, to arrive at a penetration depth of 35-95um. These results were then compared with those obtained by Kendall et al (2004), who performed a series of experiments into penetration of various particle types, sizes and launch parameters in skin. The data they obtained, coupled with yet another inertial/yield force equation, showed that penetrations below 100um were expected, with high variability seen in tested samples.

These models, although confined to the physical process of particle firing and penetration into tissue (which is better understood than the complex biological process which governs expression of the cargo the particle carries) can nonetheless be seen as providing potential components for a larger theoretical framework of biolistic transformation.

In conclusion; it can be seen that in the case of transient transformation approaches which pertain to plant leaf tissues and cereal crops, there is a relative paucity of information. In this relatively narrow sub-field, the improvement of theoretical and practical understanding can best be achieved by working on optimising the delivery and uptake of exogenous DNA into intact tissues. To achieve this, improvements in the current best approach (biolistic bombardment) require an efficient means of optimisation. From here, additional factors can be investigated and elucidated using a combination of experimental research and, where appropriate, mathematical modelling.