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The use of carbon nanodots as a new method of genetic modification and gene editing in crop plants

The use of carbon nanodots as a new method of genetic modification and gene editing in crop plants.

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Current trends in food production estimate we will need to massively increase agricultural yields by 2050 to meet demands. Attempts are being made to increase crop productivity via genetic modification (GM) and gene editing (GE), although GM has proved challenging as many species are difficult, if not impossible, to GM with current methods.
To improve GM and GE alternative methods are being sought, and an area of interest is nanoparticles. Carbon-based nanoparticles, including carbon nanotubes, appeared to be effective in delivering genetic material, but could also result in tissue damage.
This work examines carbon nanodots (CNDs) as a method for GM and GE. This cost-effective, simple method was applied in various ways to the model plant species, *Arabidopsis thaliana*, and to a crop species, *Triticum aestivum*, to determine how effective for GM and GE this method is in a common crop plant.
CNDs were readily transported into plant tissues and appeared to travel via vascular tissues throughout the plant. CNDs conjugated with DNA based on electrostatic interactions and transported DNA it into cells, resulting in transient transformation. Foliar sprays were found to be the most effective method of application, with a higher average percentage of transformed cells in both plant species. GE did occur from CND treatment with a Spo11/GFP plasmid, with the CND GE methods being the first instance of spray on gene editing in *Triticum aestivum*. Stable transformation was not achieved, although future work may overcome this obstacle.

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Contents

1.Introduction

Aims

The aims of this chapter are to define what nanoparticles are, their sources, how they are manufactured and their applications. This chapter will examine the development of nanotechnology, including carbon-based nanoparticles, and then discuss genetic modification and how carbon-based nanoparticles might be applied to this field.

Abstract

Nanoparticles are defined as aggregates between 1-100nm and occur naturally and synthetically. Engineering nanoparticles have been utilised in many fields, from engineering to medicine, being used in tire production and drug delivery systems. Genetic modification is an area which has only recently had nanoparticles applied, with carbon-based nanoparticles being used to modify animal and plants cells. The use of nanoparticles in genetic modification is a relatively new practice, especially regarding living plant cells, and the production of carbon nanodots shows great potential in this field.

1.1 Nanotechnology development and application

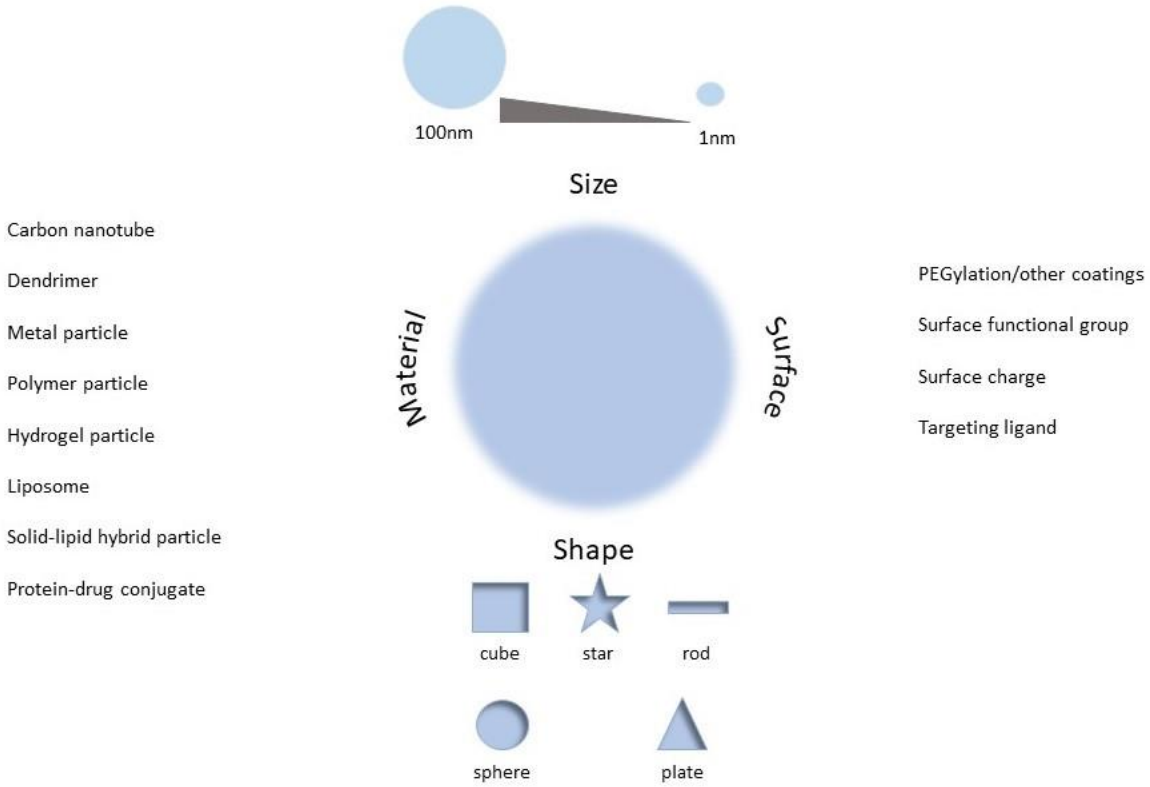


Figure 1 – The versatility of nanoparticles, including various sizes, materials, shapes and surfaces (figure personally produced).

Nanoparticles, defined as nanoscale aggregates between 1-100nm (fig.1), have been present throughout human history, occurring naturally from volcanic eruptions and as waste products from industrial processes (Monica & Cremonini, 2009). There has been an interest in nanotechnology since the 1970s (Tans, Verschueren & Dekker, 1998), but they have not been widely applied until the last decade. Nanoscale science is a rapidly developing field which aims to advance society and productivity, with global investment for the research and development of nanotechnology increasing six-fold between 1997 and 2003 (Roco, 2003). More recently nanoparticles have been engineered for use in multiple fields, from energy to medicine, and thus have become more diverse and specialised (Monica & Cremonini, 2009). There are currently four types of engineered nanoparticle, carbon-based, metal-based, dendrimers and composites, all of which have had an impact on the economy and society worldwide (Nel *et al.*,2006). There are also naturally occurring nanoparticles, such as lipoproteins and nanoclays (Sekhon, 2014), and natural nanostructures, including those involved in iridescence (Glover & Whitney, 2010), animal movement and camouflage (Sekhon, 2014).

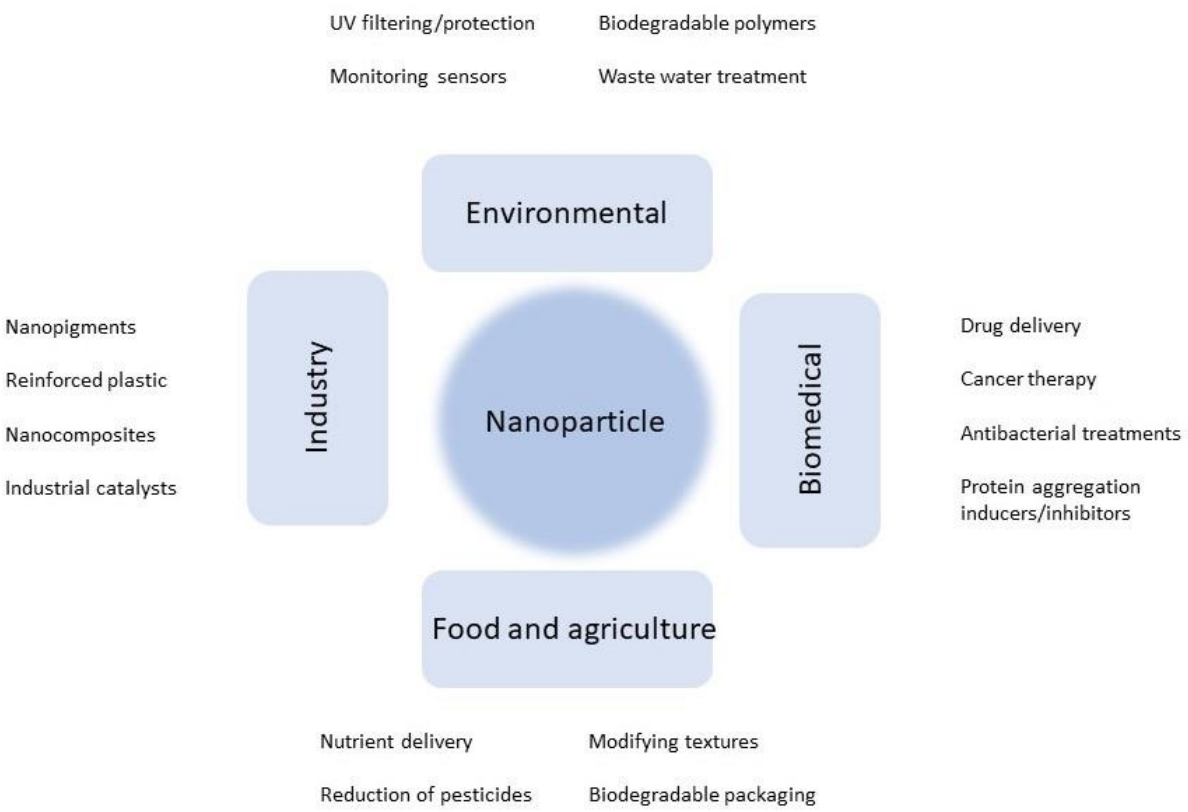


Figure 2 – Various applications of nanoparticles in a wide range of fields, including agriculture, industry, biomedical and environmental (figure personally produced).

Initially engineered nanomaterials were included in the production of tires, sunscreen and electronics, and are being increasingly used in medicine as part of drug delivery systems (fig.2; Table.1) due to their unique physio-chemical properties, including versatility of surface modification and biocompatibility (Nel *et al.*,

2006). The *in vitro* application of various nanomaterials in medicine have generally been highly successful and work to modify the properties of nanomaterials has led to the development of non-toxic, highly soluble and extremely versatile products (Brunner *et al.*, 2006; Nair *et al.*, 2010). These factors suggest that nanomaterials could be used to support or replace processes in cells, shown using nanotubules to arrange the mitotic spindle within a cell (Ball, 2002).

Nanoparticles have been applied to industries, with construction and energy storage companies investing in their development (Buentello *et al.,* 2005). Other areas which have harnessed the versatility of nanotechnology include environmental services, such as air and water remediation, and pest control (Table.1) (Buentello *et al.,* 2005).

Table 1 – Applications of nanotechnology and examples of the types used.

Applications	Example
Energy storage	Nanocatalysts (Hydrogen generation)
Agriculture	Nanosensors (soil quality)
Water treatment and remediation	Nanomembranes (desalination)
Construction	Nanomolecular structures (more robust concrete)
Drug delivery	Nanocapsules
Pest control (in agriculture)	Nanoparticles for pesticides
Air pollution and remediation	Gas separation nanodevices

These developments have led researchers to turn to agriculture as the logical step forward in the application of nanomaterials to increase productivity and solve major issues in the industry. While there are several types of nanoparticles, such as those made from heavy metals, the most appropriate type of nanoparticle for use in agriculture would be carbon-based nanoparticles, due to higher biocompatibility and low toxicity (Brunner *et al.*, 2006; Nair *et al.*, 2010).

1.2 Carbon-based nanoparticles

There are currently two main types of carbon-based nanoparticles: carbon nanotubes and carbon nanodots. Carbon nanotubes (CNTs) were originally developed for use in electronics as electrocatalysts (Zhao *et al*., 2013) and conductors in miniaturised devices (Tans, Verschueren & Dekker, 1998). They have the potential to be highly modified, with researchers developing single walled and multiwalled CNTs capable of penetrating cell walls of multiple plant species. Cañas *et al.* (2008) demonstrated the application of CNTs to multiple crop species, outlining the potential for CNTs to enhance or inhibit root elongation and how it appeared to be species dependent. They encountered the issue that the CNTs remained external to the plants, creating a film on the root surface, suggesting biocompatibility risks, problems with cell

penetration and potential damage to root physiology. To overcome these concerns water-soluble CNTs (wsCNTs) were developed and applied to crop species (Tripathi, Sonkar & Sarkar, 2011). This was not only highly successful, but they determined the nanotubes travelled via the xylem and increased the growth of both shoots and roots of crop species (Tripathi, Sonkar & Sarkar, 2011). As surface modifications and coatings applied to nanomaterials appeared to play a major part in their solubility and toxicity, they have been a major focus in the development of future materials for use in both medicine and agriculture (Brunner *et al.*, 2006).

As CNTs continue to exhibit problems with cell wall and cell membrane penetration researchers have begun to develop non-penetrating, highly soluble nanomaterials known as carbon nanodots (CNDs). These can pass through cell walls without causing significant damage and are highly biocompatible with low cytotoxicity (Wang *et al.*, 2014). Since their discovery in 2004 (Xu *et al.*, 2004), CNDs have been an area of interest for researchers, due to their photoluminescence and inexpensive production (Baker & Baker, 2010). The fabrication of CNDs has become increasingly simple, with one method taking a mere three minutes using microwave irradiation (Hill *et al.*, 2016).

There are several areas of interest for nanoparticles, with one major field being that of genetic modification (GM) (Liu *et al.,* 2010). Nanoparticles are an innovation which is thought to be able to overcome several of the current issues with GM and carbon-based nanodots, with their low cytotoxicity and high biocompatibility are strong contenders for the next main method (Liu *et al.,* 2010).

1.3 Genetic modification and gene editing

Genetic modification has been applied for over three decades, with *Agrobacterium*-mediated and particle bombardment-mediated transfer methods being two of the most widely used (Altpeter *et al.*, 2016).

The *Agrobacterium*-mediated method uses *Agrobacterium tumefaciens* and involves bacterial attachment and the use of the type IV secretory system (T4SS), which delivers the virulence effector proteins into the host cells (Altpeter *et al.*, 2016). T-DNA-protein complexes are trafficked through the cytoplasm and enter the nucleus where the proteins are removed, and T-strands of DNA are integrated (Altpeter *et al.*, 2016). This method allows large segments of DNA to be transferred relatively efficiently at a low cost (Wu *et al.,* 2003). It also appears to reduce the unnecessary DNA being transferred via segregation, which is beneficial under current guidelines (Wu *et al.,* 2003). However, to transfer the DNA plant embryos must be infected, resulting in necrosis of tissues and a reduction in transformation efficiency (Altpeter *et al.*, 2016). There are also limitations of which genotypes are successfully transformed using this method, with many monocots having very few transformable species (Wu *et al.,* 2003). This method is currently being modified to increase the efficiency of T-DNA delivery and transformation of plants, especially monocot crop species (Wu *et al.,* 2003).

Particle bombardment-mediated methods involve coating particles which are fired into the plant at high acceleration. One variation of this method involves the precipitation of DNA onto gold particles, which are

then launched at 7.6MPa (He *et al*., 1999). Other methods can apply various chemical coatings to particle and precipitate DNA into other metals (He *et al*., 1999). While usually immature embryos are bombarded, mature seedlings can also be transformed with this method (Becker, Brettschneider & Lörz, 1994). After the procedure treated plants are required to regenerate to determine if the procedure was successful (He *et al*., 1999). While this method has the advantage of being customisable in terms of particle coatings and metals used, it lacks accuracy and causes significant damage to tissues (Altpeter *et al.*, 2016).

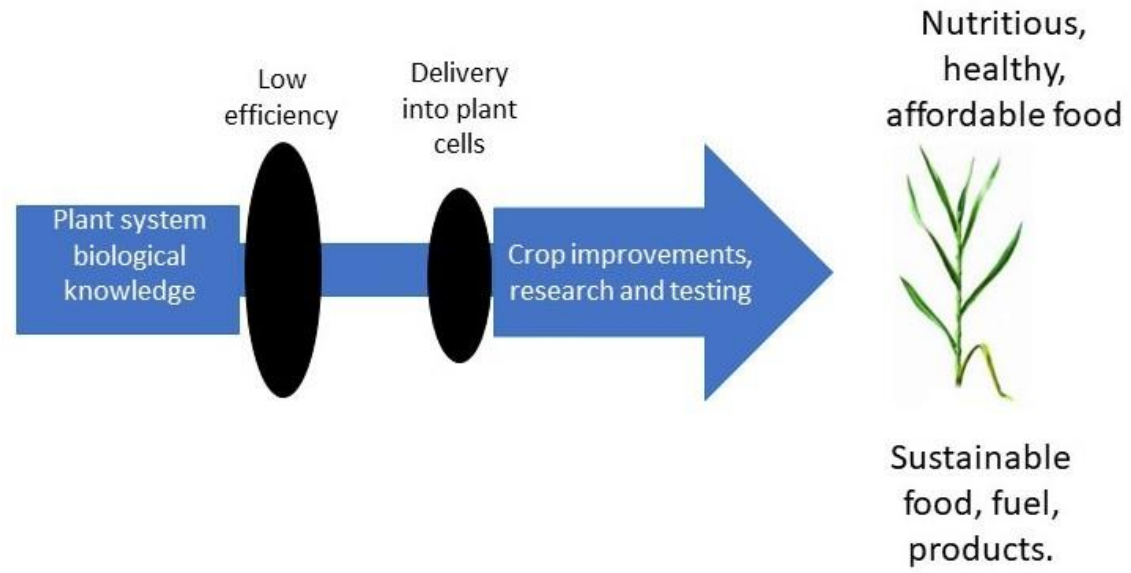


Figure 3 – The current bottlenecks and aims of genetic modification of plant cells. The two major barriers limit the ability to develop and provide a variety of products from food to fuel (modified from Altpeter *et al*., 2016).

Technology related to genetic modification has advanced rapidly in recent decades. There have been major revelations, including work to develop an optimised *Agrobacterium*-based method (Wu *et al.,* 2003), and the development of sequence-specific nucleases (SSNs), such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas system (Gaj, Gersbach & Barbas, 2013). SSNs target specific sequences in the genome for gene editing and are more efficient and programmable than previous methods (Humanes *et al.,* 2017). They introduce a single-stranded break in a specific locus which are then repaired using one of two methods: non-homologous end joining, which rejoins the ends of broken chromosomes and can result in mutations, or homologous recombination, which uses a template with a homology of the break site (Humanes *et al.,* 2017). These have been applied to important crop species, including *Zea mays* (Shukka *et al*., 2009), *Triticum* sp. (Shan *et al*., 2013), and *Oryza sativa* (Shan *et al*., 2013). These methods are more precise and efficient in transforming plant species, but still suffer from issues, including cell damage (Townsend *et al.,* 2009).

While these systems have been considered successful in achieving genetic modification and gene editing, they have several disadvantages, including cost, expertise required to produce them, and tissue damage

(Altpeter *et al.*, 2016; He *et al*., 1999; Becker, Brettschneider & Lörz, 1994). Due to this there is currently a lack of an efficient, universal genetic transformation and gene-targeting system for plants, which limits the ability to engineer species for food and fuel (Townsend *et al*., 2009). *Agrobacterium*-mediated and particle bombardment-mediated transformation, while being established methods, have issues related to cell damage, cost and low rates of success (Townsend *et al*., 2009; Nair *et al*., 2010; Sailaja, Tarakeswari & Sujitha, 2008). This is especially apparent with crop species such as wheat (*Triticum aestivum* L.) where not only is the rate of transformation dependant on genotype, but the process involves regeneration of the plants, resulting in many perishing during the process of transformation (Wu *et al.,* 2003).
The two major bottlenecks (fig.3) of this research are the low levels of efficiency, either due to DNA not being taken up successfully or plants perishing before expressing the proteins of interest (Altpeter *et al.*, 2016), and the struggle to deliver DNA into plant cells (Altpeter *et al.*, 2016). A biocompatible, easily modified, non-destructive, universal method is required to overcome these issues and create a system that can be applied across crop species. New technologies are emerging, such as nanodelivery systems. These have been used in medical practices and are now being examined with the potential for use in plant transformation system, especially in agriculture.

1.4 Genetic modification in agriculture

Agriculture is a major focus for genetic modification and transformation technology, as crops are required in far greater quantities and the industry has a huge environmental impact due to practices including pesticide, herbicide and agrochemical use (Sailaja, Tarakeswari & Sujitha, 2008).
While several genetic modification systems have been examined in relation to agriculture, one of the major focuses in recent years is the application of nanotechnology. With nanomaterials being successfully utilised in medicine, researchers have turned to other potential uses. The applications of nanotechnology in agriculture could include nanocapsulation of agrochemicals, surface modifications to allow nanoparticles to act like pesticides, and antimicrobial applications, such as silver nanoparticles. However, nanotechnology is still an area wrought with issues for researchers, as even carbon-based, non-cytotoxic nanomaterials cause damage to cells through punctures, and can represent a high cost due to materials required (Nair *et al*., 2010).
Recently there has been the genetic manipulation of Brassinosteroids in rice using *Agrobacterium*-mediated transformation, which achieved biomass and yield increases without fertilisation in field conditions (Sakamoto *et al*., 2006). Yield has not been the only factor manipulated through GM, but also taste and nutrition. Using *Agrobacterium*-mediated methods tomato plants were transformed, producing better tasting fruits with higher nutritional value (Davuluri *et al*., 2005). The GM of crop plants has been achieved in species including maize, rice, barley and legumes, with the promise of financial gains, environmental benefits and greater productivity (Stoger *et al*., 2005). However, there has been concerns regarding the use

of bacteria and particles of metal, such as those used in particle bombardment, in plants people would potentially consume.

1.5 Nanotechnology in agriculture

Nanotechnology has been applied to the agricultural field in multiple ways (Table.1), with phytonanotechnology expanding as a field. There has been an increasing demand for 'smart crops' involving nanoparticle (NP)-mediated delivery (Wang *et al*., 2016). Work has involved the application of NPs as a method of encapsulating pesticides, herbicides and other agrochemicals, to reduce the ecological impact of the industry. However, this has been expanded upon, with NPs being used to increase the durability and life of agrochemicals, as well as maintaining a more environmentally friendly approach. In such cases hollow silica NPs has been used to encapsulate pesticides, enhancing penetration into plants and protecting the chemicals from UV degradation (Li *et al*., 2006).

NPs have not only been used to deliver and improve agrochemicals but have been used as a method of gene delivery into crop plants. Starch NPs, with a positive charge and innate fluorescence, were conjugated with DNA and ultrasonic waves were induced to permit them to pass through the cell wall. It appeared NPs protected DNA from ultrasonic waves and, once inside the cell, DNA was released, and transformation occurred (Liu *et al*., 2008).

As phytonanotechnology has developed researchers have moved away from metal-based NPs and have begun to focus on more natural, carbon-based NPs. There are many current carbon-based NPs, including carbon nanoscrolls for antifungal treatments, and carbon nanotubes (CNTs) for gene delivery (Kim *et al*., 2017). Previous NPs were successful only in transforming protoplasts, while CNTs can penetrate and transform plant cells which have retained their cell walls (Liu *et al*., 2009). Multiwalled CNTs (MWNTs) were found to accumulate in plant tissues and transform cells without the need for protoplast extractions. It was determined that the most likely method of movement of MWNTs through the plant was via the xylem, and while smaller NPs were able to be absorbed and transported larger ones were unable to enter and move through the plant (Lin *et al*., 2009).

Despite the potential benefits there are concerns regarding the safety of NP use. Their behaviour in ecological systems is largely unknown and there is limited data on NP toxicity, or the results of them being released into the environment (Scheringer, 2008). Research is being done to establish what effects NPs may have on the environment, and relatively recent work has found that NPs, especially those which are carbon-based, have low toxicity when interacting with biological organisms (Liu *et al*., 2009).

1.6 Carbon nanodot development and application

This research builds on previous work to apply nanomaterials to crop plant species. While CNTs have been relatively successful there have been issues as they must orient in the correct way to enter the plant due to their tubular shape (Lin *et al*., 2009). Therefore, it was reasoned that a spherical shape would be more appropriate for entry and movement through plant tissues. This was found to be the case with carbon nanodots (CNDs).

Using the protocol outlined in work by Hill *et al.* (2016) CNDs were modified with poly(ethylene glycol) (PEG) diamine chains connected to carboxylic acids on the surface via amide conjugation. PEG was chosen as it has frequently been used in medicine, or "Polymer Therapeutics", due to its high aqueous solubility and biocompatibility (Banerjee *et al.*, 2012). The free amide on the PEG chain allowed the attachment of plasmid DNA through covalent conjugation. The CND-pDNA complexes were then applied to crop plant species in multiple ways, replicating previous methods of genetic modification (GM) and modifying methodologies to be more appropriate for the material.

While other forms of carbon-based nanomaterials have been produced and applied to plant species to genetically modify them, CNDs with PEG chains have not been tested before and offer the potential of a cost effective, simplistic, effective transformation method, without the risk of cell puncture, seen in CNTs, or the use of expensive or dangerous materials, seen in quantum nanomaterials. This research project aims to develop, test and optimise a transformation system for mature wheat (*Triticum aestivum* L.) and *Arabidopsis thaliana* plants, both transient and stable transformation, without the need for regeneration.

2.The production and modification of carbon nanodots

Aims

This chapter aims to define carbon nanodots and outline their production and applications. Initially it will examine where carbon nanodots originated, then how they are produced, and finally characterise them using DLS, Fluorometer and Zeta potential measurements and gel electrophoresis. Characterisation will include the CNDs alone and conjugating with plasmid DNA to illustrate any changes.

Abstract

Carbon nanodots (CNDs) are a type of carbon-based nanomaterial usually under 10nm in diameter. Previous nanomaterials had caused cell damage through punctures or toxicity, or were not suitable for the delivery of molecules, including drugs and genetic material, into live cells. This work aimed to modify CNDs to allow them to conjugate with plasmid DNA (pDNA) and transport it into mature plant cells. The CNDs were surface passivated with polyethylene glycol (PEG) of 600mW or 1000mW and the attributes were measured. It was found that PEG 1000mW had a more positive charge than 600mW, while the hydrodynamic radius was similar. This made PEG 1000mW more appropriate as a surface passivation agent due to the positive charge allowing a stronger electrostatic interaction with the negatively charged pDNA, and the resulting particle would not be significantly larger than if a lower molecular weight PEG was used.

2.1 Introduction

Nanotechnology has been of great interest over the course of the last century, with the first recorded size measurements of nanoparticles carried out in 1902 (Ochekpe, Olorunfemi & Ngwuluka, 2009). They have been constructed from a multitude of different sources, including silver as an antimicrobial agent (Sondi & Salopek-Sondi, 2004) and iron oxide for Magnetic Resonance Imaging (Na, Song & Hyeon, 2009).
Inorganic, engineered nanoparticles have been in use since the 1950s across several industries, including electronics and medicine (Lohse & Murphy, 2012). In medicine, nanoparticles have been of great interest as they have been shown to be capable of delivering drugs to animal cells and be applied as a diagnostic tool in vitro and in vivo (De Jong & Borm, 2008). These nanoparticles have been found to be able to bind and carry compounds such as drugs and proteins (De Jong & Borm, 2008). To apply nanoparticles to live cells a level of biocompatability is required, and so more biological sources were identified, including silica, lipids and carbon (De Jong & Borm, 2008).
Carbon nanodots (CNDs) have been an area of interest in cell biology, due to their photoluminescence properties, low toxicity, biocompatibility, and stability (Roy *et al.*, 2015). CNDs are spherical or quasi-spherical carbon particles under 10nm in size (Xu *et al.*, 2004) and have a multitude of functional groups, including carboxylic acids and amino acids (Bao *et al.*, 2011). Due to the variety of surface-functional groups CNDs are easily functionalised with organic and inorganic molecules, including polymers and biological materials (Kong *et al.*, 2012).

Previous work using carbon-based nanomaterials has involved the application of carbon nanotubes (CNTs) in drug delivery, as they can be functionalised to carry and deliver cargo to cells (Bianco, Kostarelos & Prato, 2005). However, these nanomaterials have been found to cause damage to cells, making them potentially unsuitable for drug or gene delivery in living tissues (Zhu *et al.*, 2007).

An alternative carbon-based nanomaterial known as carbon nanodots (CNDs) was discovered in 2004 (Xu *et al.*, 2004) and have become increasingly cheap and simple to produce, using methods such as microwave irradiation (Hill *et al.*, 2016).This investigation aimed to modify CNDs to allow them to act as transporters of genetic material into living plant cells. The CNDs were produced using microwave irradiation and were modified with polyethylene glycol diamine to create a positively charged linker that could join CNDs to DNA. Interactions were measured using established methods, including dynamic light scattering (DLS) to determine the size of the particles, and zeta potential to identify the electrostatic charge between particles.

2.1.1 Objectives of this chapter include:

1. To produce carbon nanodots using the synthesis by Hill *et al.* (2016).
2. To modify the CNDs with PEG diamine to allow the attachment of negatively charged genetic material.
3. To determine the size and charge of CNDs using DLS and zeta potential.
4. To extract plasmid DNA and attach it successfully to the PEG diamine-functionalised CNDs.

2.2 Methods

2.2.1 CND synthesis

The method for producing the CNDs was based on work done by Hill *et al.* (2016) using the microwave pyrolysis method. H_2O, Glucosamine HCl and TTDDA were added to a conical flask. Microwave pyrolysis of the solution was performed for three minutes using a domestic microwave. The solution was then filtered through a VIVASPIN 20 with a 10 kDa molecular weight cut off filter at 8000 RPM for 20 minutes.

Succinic Anhydride, Amide and ethanol were added to the CND solution and this was stirred constantly for 16 hours. This was then washed in Tetrahydroforan 3-5 times through filter paper until the liquid became clear.

2.2.2 Polyethylene glycol surface passivation

Polyethylene glycol 600mW or 1000mW, and EDC were added to the CND acid solution and shaken for 16 hours. The solution was then put on dialysis for 12-16 hours using snakeskin Dialysis tubing. The surface passivated CNDs were freeze dried for 12 hours and diluted in ethanol to a concentration of 50mg/ml.

2.2.3 Plasmid DNA extraction and quantification

E. coli containing the plasmid of interest was provided as stabs by Professor Keith Edwards, University of Bristol. These were grown in selective Luria-Bertani (LB) medium, which was standard LB medium with Kanamycin added at a 1:1000 ratio. Plasmid DNA was extracted as in ZymoPURE Midiprep kit protocol. The DNA was quantified using gel electrophoresis and a Nanodrop 1000.

2.2.4 Plasmid DNA conjugation

The plasmid DNA, surface passivated CNDs and TE buffer pH 8.0 were added in equal volumes to 1.5 mL microcentrifuge tubes and mixed by vortex for 5-10 seconds. The solution was then stored at 4^{o}C for 30-60 minutes until use.

2.2.5 Dynamic light scattering

Dynamic light scattering (DLS) measurements of CNDs in solution were taken on a Zetasizer Nano to determine the size of the nanoparticles. The CNDs were diluted in 100% ethanol and readings were taken three times.

2.2.6 Zeta potential

Measurements of the zeta potential and conductivity were taken on a Zetasizer Nano to determine the charge of the passivated CNDs. Samples were diluted in 100% ethanol for readings.

2.2.7 Fluorometer

A Fluorometer was used to determine the intensity and wavelength of excitation of the CNDs. To establish if concentration had an effect. CNDs in solution were measured at different concentrations, from 1ug/ml to 500ugml in increments of 50.

2.2.8 Nanodrop

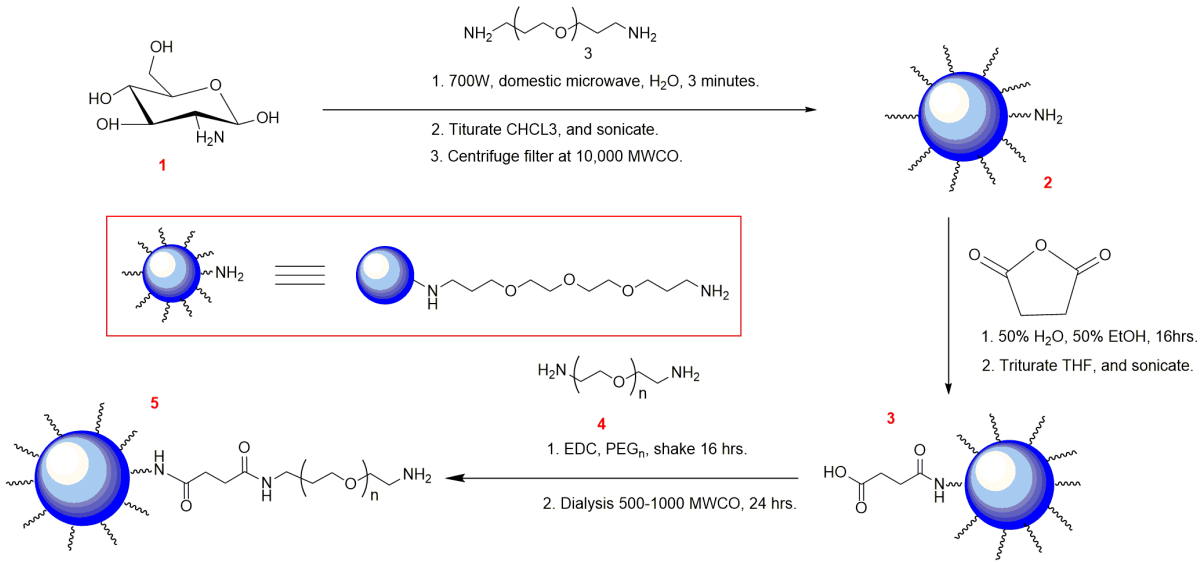
A Nanodrop ND 1000 was used to determine concentration and purity of pDNA samples. To calibrate the machine, 1.5ul of distilled water was applied and measured, then 1.5ul of each sample was applied and measured, with the machine being cleaned with distilled water in between each one.

2.2.9 Gel eletrophoresis

A 1% agarose-based gel electrophoresis was run at 70V for 2 hours with a standard 1kb DNA ladder. 1.5ul of Cas9-GFP pDNA and w127YFP pDNA, CNDs, CND-Cas9-GFP, and CND-w127YFP complex samples were mixed with 0.5ul of ethidium bromide and pipetted into the wells.

2.3 Results

2.3.1 CND structure



Scheme 1: 1 Core CND structure 2 Amine-functionalised CND structure 3 Surface passivated CND structure 4 Poly(ethylene) glycol diamine molecule 5 CND with PEG 1000 bound to the amine group. (Modified from Swift *et al.,* 2018).

The structure developed had a carbon core with a negatively charged outer shell (Scheme 1). The PEG diamine attached through ionic bonding due to its positive charge (Scheme 1.3) and created a chain which could then attach the CND to negatively charge genetic material, in this case pDNA.

2.3.2 Dynamic light scattering

Dynamic light scattering (DLS) was used to determine the hydrodynamic radius of the CND solution, providing information of the size distribution of the particles in solution based on the Brownian principle of motion (fig.1).

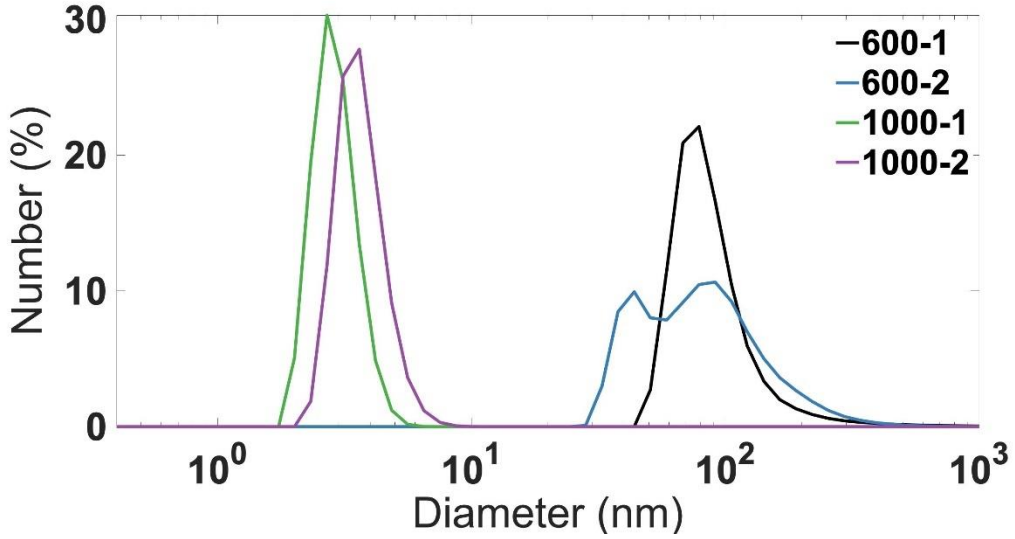


Figure 1: Dynamic light scattering results for surface passivated CNDs. Samples were passivated with PEG 1000mW and PEG 600mW. The average diameter and number of passivated CNDs were determined across five samples per passivation across 30 minutes.

Two PEG molecular weights, 600mW and 1000mW, that were used for surface passivation were compared when combined with CNDs. The measurements showed the particle size of those surface passivated with 1000mW PEG were similar, with sample 1000-1 (sample 3) having a larger percentage number and a smaller diameter than sample 1000-2 (sample 4) (fig.1) Sample 600-1 (sample 1) had a larger diameter and lower number percentage than both 1000 mW measurements, and differed from 600-2 (sample 2), which showed aggregations forming with an irregular number percentage and diameter plot (fig.1). The results suggested the diameter of the CNDs functionalised with 1000mW PEG was between 1 and 10 nm in solution, while those functionalised with 600mW PEG was between 10-1000nm.

2.3.3 Zeta potential and conductivity

The zeta potential of the CNDs was measured for four surface passivated samples, two with 600mW PEG and two with 1000mW. This was used to establish the electrostatic interaction between the particles to determine electrostatic bond formation. The samples 1 and 2, both with 600mW PEG, had a negative charge, while samples 3 and 4, with 1000mW PEG, were positive (fig. 2). The 600mW CNDs had a mean mV between -4.9 and -6.1, while the 1000mW CNDs have a mean mV between 2.9 and 4.3.

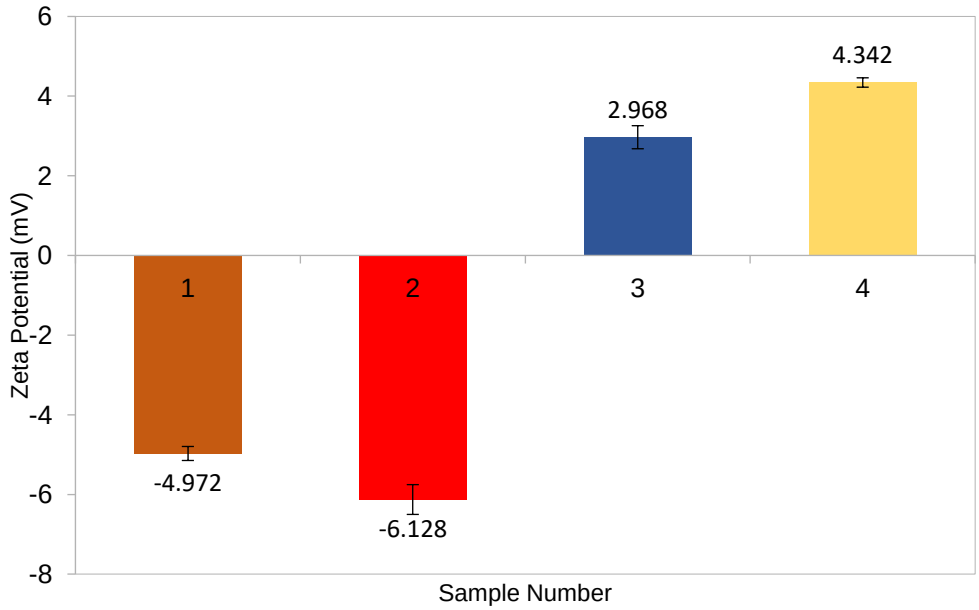


Figure 2 - The zeta potential (mV) of surface passivated CNDs. Samples 1 and 2 were passivated with PEG 600mW and samples 3 and 4 were passivated with PEG 1000mW.

The electrical conductivity was measured for the same samples used in the zeta potential measurements. The unit mS/cm was used based on the concentration of the solutions. Samples 2 ,3 and 4 had a conductivity below 0.6 mS/cm, while sample 1 had a conductivity over 4 mS/cm (fig.3). The error of sample 1 was also much larger than the other samples.

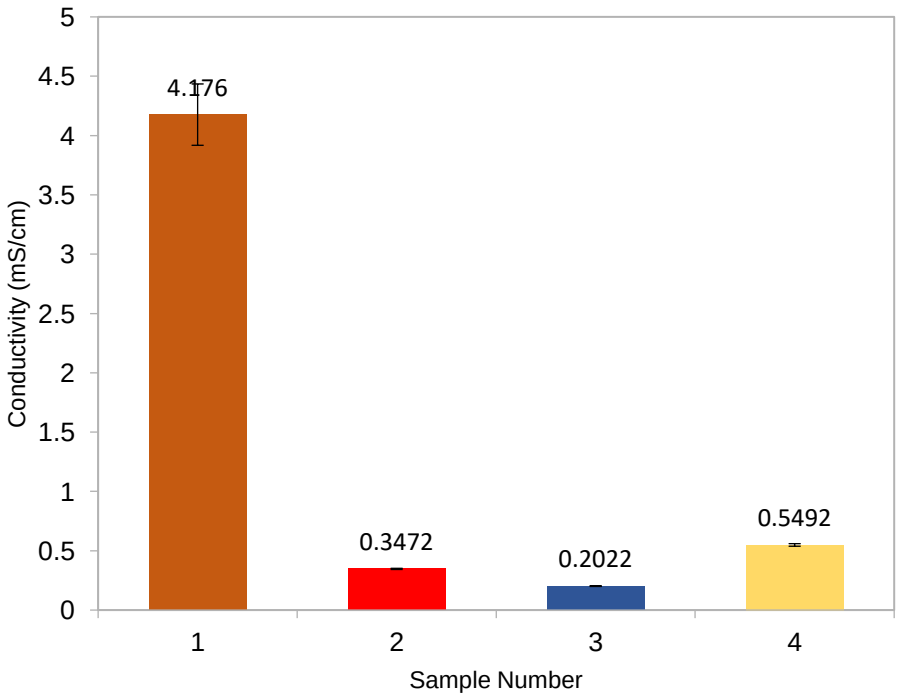


Figure 3 - The electrical conductivity of surface passivated CNDs at 25°C. Samples 1 and 2 were passivated with PEG 600mW and samples 3 and 4 were passivated with PEG 1000mW.

2.3.4 Plasmid DNA yield

The Nanodrop allowed quantification of the pDNA, providing the yield for each extraction (fig.4). This allowed the same range of DNA yields to be used in the conjugation with CNDs, ensuring that the results would be consistent.

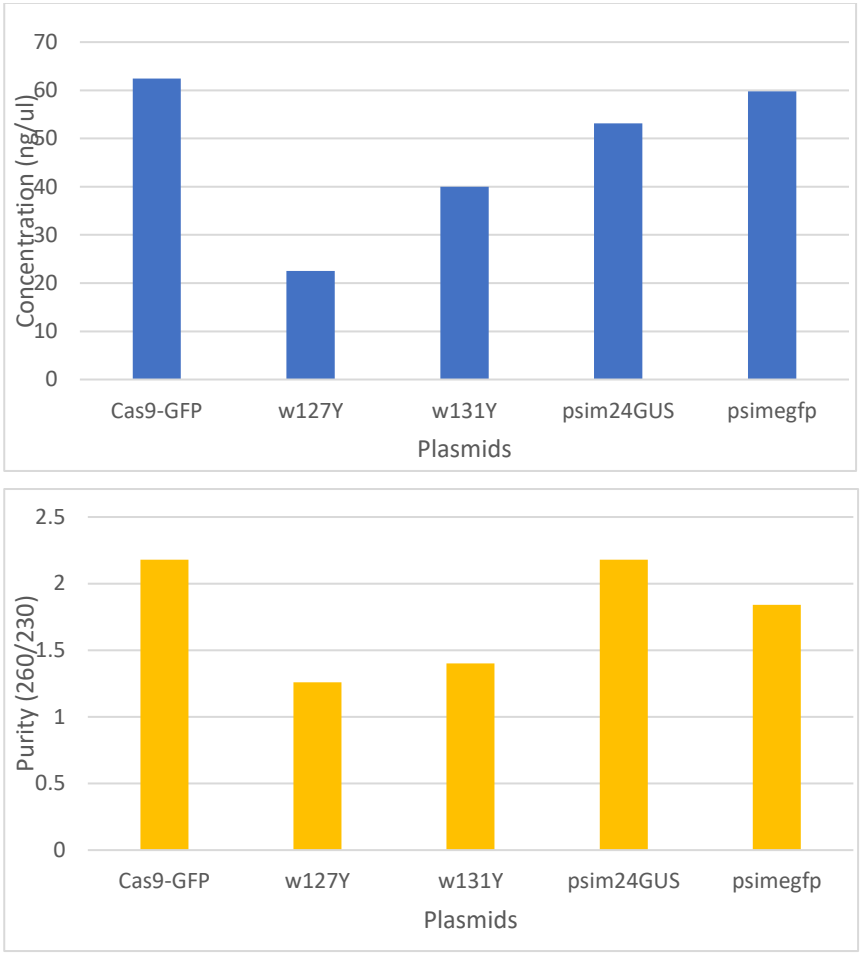


Figure 4: Nanodrop ND 1000 readings for 1.5ul pDNA samples. Concentration of the samples (top) and purity of samples (bottom) were taken as an average of three readings per sample.

2.3.5 Gel electrophoresis of CNDs and pDNA

Gel electrophoresis showed that most CNDs had a positive charge due to the PEG 1000mW, although some displayed a negative charge if not bound to PEG 1000mW (fig.5). The pDNA had a negative charge (fig.5). When CNDs were mixed with pDNA in solution the gel showed a small amount of the CNDs moving in the opposite direction to the pDNA, while most of the solution remained close to the well (fig.5). This suggested that, when mixed, CNDs and pDNA had an overall neutral charge.

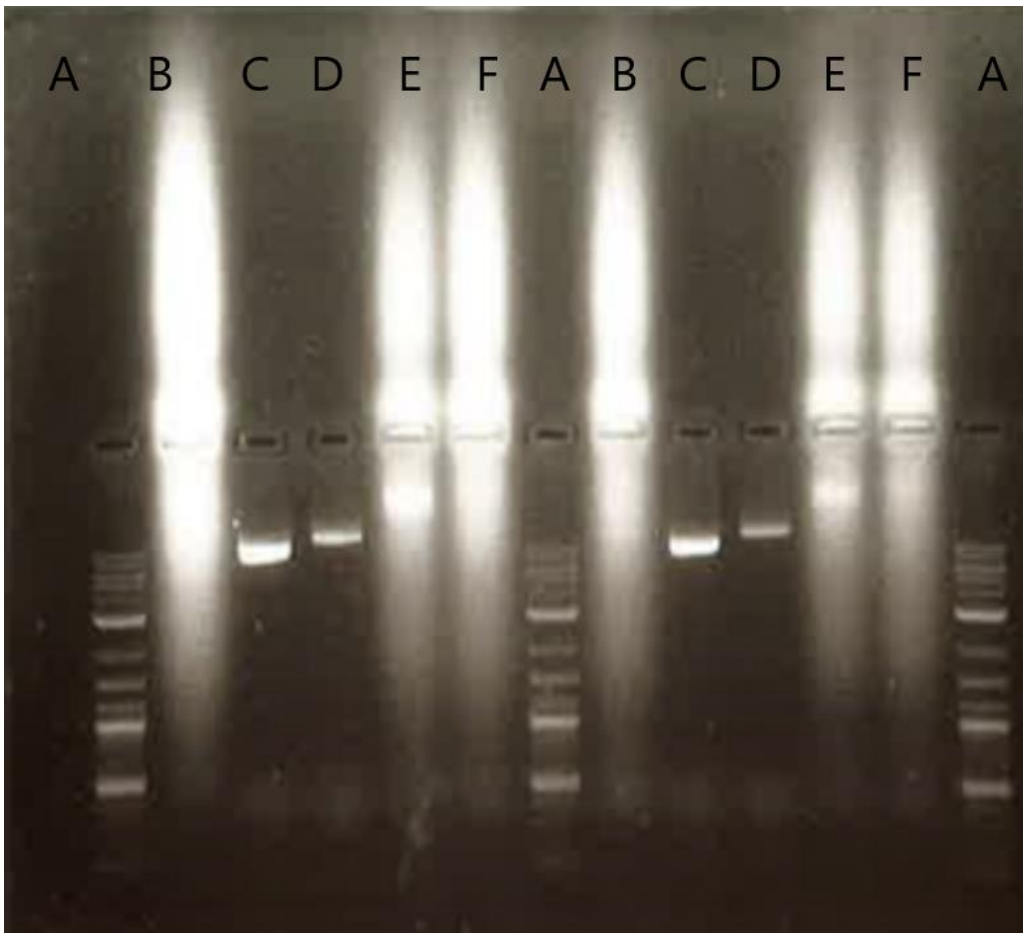


Figure 5 – All samples were mixed with ethidium bromide before loading, including autofluorescent CNDs to maintain consistency. Wells were central in the gel and the positive charge was located at the lower portion, where the ladder moved towards. A 1Kb Ladder B CNDs with PEG 1000mW C pDNA (Cas9-GFP plasmid) D pDNA (YFP plasmid) E Cas9-GFP pDNA conjugated with CNDs F YFP pDNA conjugated with CNDs.

2.4 Discussion

It was hypothesised that the small size of the CNDs and their hydrophilic nature would make them ideal for use in plants, as small particles, including viruses and bacteria, appear to be taken up and transported through the vascular system to plant tissues (Thorne *et al.,* 2006; Opalka *et al.,* 1998). The movement of CNDs is covered further in chapter 2. The modification of surface passivating the CNDs allowed negatively charged pDNA to attach through ionic bonds and be carried with the CNDs throughout the plant.

The surface passivated CNDs conjugated with genetic material due to the opposing charges, with 1000mW PEG possessing a positive charge over 2mV (fig.2) and the pDNA exhibiting a negative charge (fig.5). Using gel electrophoresis, the pDNA and CNDs conjugation was visualised, with free CNDs moving in the opposite direction to the free pDNA, and conjugated pDNA and CNDs being most concentrated around the well (fig.5). This suggested that, once conjugated, the particles formed a bond which would allow pDNA to be transported with the CNDs. Although some CNDs exhibited a negative charge, this can be explained due to not all CNDs binding to the PEG 1000mW and therefore not acquiring the positive charge.

The DLS data suggested a size of 1-1000nm for the CNDs in solution (fig.1). However, it had been reported that DLS data of nanomaterials overestimated the size and that usually they are much smaller. The overestimation has been found to be due to agglomeration of the CNDs in solution (Kaasalainen *et al.*, 2017) or because of conjugations with larger molecules, such as metals (Kang *et al.*, 2015). In this case it is likely the conjugation with 600mW PEG caused the estimation to be much larger, as PEG 600mW appeared to aggregate if any free, negatively charged CNDs were present (fig.1). Those CNDs with PEG 1000mW had a much smaller diameter and cleaner peaks, suggesting fewer aggregations and a higher number present in the solution (fig.1) and so future work was done with CNDs functionalised with PEG 1000mW.

Due to the opposing charges the pDNA was electrostatically bound to the surface passivated CNDs when mixed in solution. This was shown through gel electrophoresis, with the bound pDNA-CND nanoplexes remaining in the well and the unbound molecules moving to opposite ends of the gel (fig.5). In previous work it was found that DNA was condensed when in contact with surface-passivated CNDs, with the molecule being far smaller than would be expected (Liu *et al.*, 2012). This produced a greater advantage in that the DNA was not only bound to the CNDs but would be easier to transport through the cells as it would be condensed and so would be less likely to be obstructed by pore size or detection.

These results suggested that PEG 1000mW would be the most appropriate surface passivation agent for the CNDs, and not 600mW, as it have a much more positive charge and so could conjugate with the negatively charged pDNA. The DLS results showed that the hydrodynamic radius was not significantly larger than CNDs with PEG 600mW, and so it would be transported through the plant tissues just as easily, but that this functionalisation would aggregate more readily (fig.1). Therefore CNDs with PEG 1000mW would be more appropriate for carrying materials into plants.

As the CNDs were able to bond with pDNA in solution they could be applied to biological material, such as plant tissues, using multiple methods. This could be used as a versatile system for transporting negatively charged molecules, such as genetic material, into tissues without the risk of cytotoxicity or cell damage, as witnessed with carbon nanotubes (Zhu *et al.*, 2007).

3.The uptake of carbon nanodots in plants

Aims

This chapter aims to examine the current methods of genetic modification in plants and to determine the uptake of CNDs and the nanoplex of CNDs+pDNA.

Abstract

Current methods of genetic modification rely on the use of damaging systems, such as *Agrobacterium*-mediated and particle bombardment-mediated genetic modification. These methods, while transforming plant species, have several limitations including damaging the plants, requiring tissue culture and only being effective with select species. To test a new method of genetic modification using CNDs, the uptake and successful transformation were determined. Two main methods were used to determine if CNDs were taken up, the first being CND pellets placed at different levels in the soil, and the second being foliar sprays. It was found that CND pellets placed lower in the soil resulted in a higher concentration of cells containing CNDs, while those at the soil surface resulted in a much lower concentration. Foliar sprays were found to be successful, and it appeared CNDs would travel via the vascular tissue and between leaf cells. Further work will be required to determine the exact method of transport and how genetic material is released into plants, as well as how effective this method would be for genetic modification of various plant species.

3.1 Introduction

Current methods of GM include *Agrobacterium*-mediated and particle bombardment-mediated approaches. *Agrobacterium tumefaciens* is the cause of crow gall in dicot plant species, inserting a segment of bacterial DNA into the plant's genome (Zupan & Zambryski, 1995). During infection, T-DNA, or transferred DNA, is inserted into plant cells and carries the tumour-inducing (Ti) plasmid bordered by cis-elements to direct DNA processing (fig.1) (Zupan & Zambryski, 1995). While this is a popular method for gene insertion, there are several problems, including that infection causes damage to cells, with necrosis being an issue, and it is limited by the species which can be infected by the bacteria (Zupan & Zambryski, 1995).

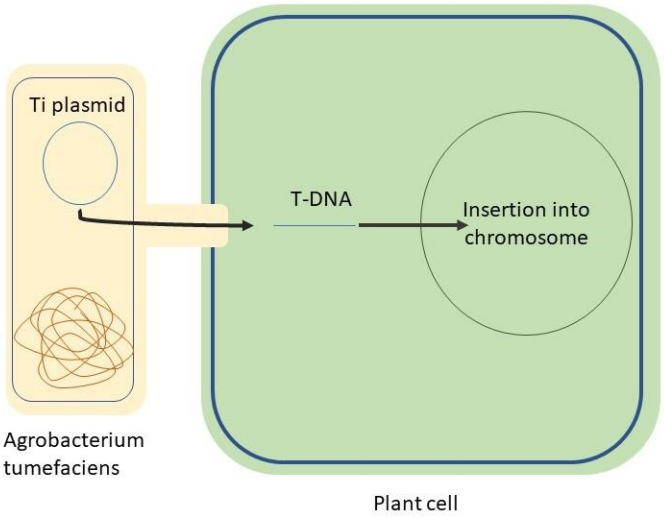


Figure 1 – The process of DNA insertion using *Agrobacterium tumefaciens*. The Ti plasmid is transported into the plant cell by T-DNA and Vir proteins, where it is processed and inserted into the chromosome.

While the *Agrobacterium*-mediated method relies on altering a natural infection process, particle bombardment, also known as biolistics, involves inserting foreign DNA through force. Particles coated with DNA are fired at high velocity into plant cells, allowing DNA to pass the cell wall and enter the cytoplasm where it can be processed (fig.2) (Ortigosa *et al.,* 2012). DNA is usually precipitated onto particles (fig.2), commonly made from gold, and helium pressure is used to fire them into the plant (Lorito *et al*., 1993). However, similarly to the *Agrobacterium*-mediated method, this causes cell damage, as particles are fired through cell walls, and this is generally limited to immature embryos (Ortigosa *et al.,* 2012).

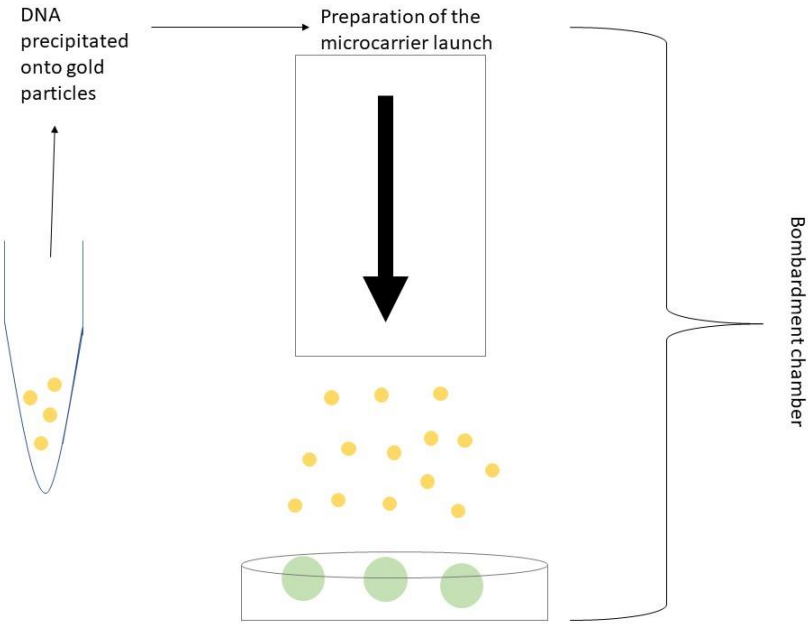


Figure 2 – The particle bombardment-mediated method of DNA transfer. DNA is precipitated into gold particles and a microcarrier launches the particles into young plant tissue.

These two commonplace methods of genetic modification have been applied to achieve both stable (germplasm) and transient (non-germline) transformation in various species (Kikkert *et al*., 2005; Russell, Roy & Sanford, 1992). While being considered successful, they have several major issues that negatively impact their use on a wider scale. These include cell damage, either through infection or puncturing cell walls, inaccuracy, and both methods require the use of immature embryos and tissue culture (Altpeter *et al*., 2016) which, is difficult to achieve in many species (Melnyk, 2016). Therefore, there is a demand for new methods to genetically modify plant species on a universal scale.

The use of carbon nanodots (CNDs) is a promising area of research, as they are highly biocompatible, and can be easily applied to adult plants without the destructive methods of infection or bombardment. The particle bombardment-mediated method involves the coating of microparticles (fig.2) with DNA being precipitated onto particles made from materials such as gold. Similarly, CNDs can be surface functionalised to allow them to carry different materials, including DNA. This method would also bypass the need for regeneration, which some plant species, especially monocots, are incapable of achieving for reasons which remain unknown (Melnyk, 2016). Foliar spray and CND pellets were examined to determine the uptake of CNDs into plants, and pDNA nanoplexed with CNDs were applied to plants to determine if CNDs could carry pDNA into plant cells and lead to transformation via different routes.

3.1.1 Objectives of this chapter include:

1) To determine if solid, soil-based CNDs can be taken up by the plant using a solid CND pellet.
2) To determine if aqueous CNDs applied to the leaves can be taken up by the plant via foliar spraying.
3) If CNDs conjugated with pDNA can lead to transient transformation of plant cells.

3.2 Methods

3.2.1 CND pellets

CND pellets were formed from liquid CNDs via lyophilization. Liquid CNDs were produced (chapter 1) and 20ml was placed in hard plastic moulds. The moulds were then placed into the lyophilizer for 12 hours. The pellets were cut into four quarters and placed in the soil of immature *Triticum aestivum* plants (scheme 1A). At seven weeks leaves from the plants were imaged via confocal laser scanning microscopy (CLSM) to determine uptake.

3.2.2 CND foliar spray

CND and TE buffer pH 8.0 nanoplex and control conditions were added to separate 100 mL spray bottles (Wilko Travel Spray Bottle 100ml, Bristol, UK). Then 2 mL dH_2O was added to each and they were inverted 3 times to mix.

19-day-old plants were separated into clean trays, to prevent contamination, before spraying (scheme 1B). The plants were kept separate during spraying and each plant was sprayed 3-4 times to coat the leaves.

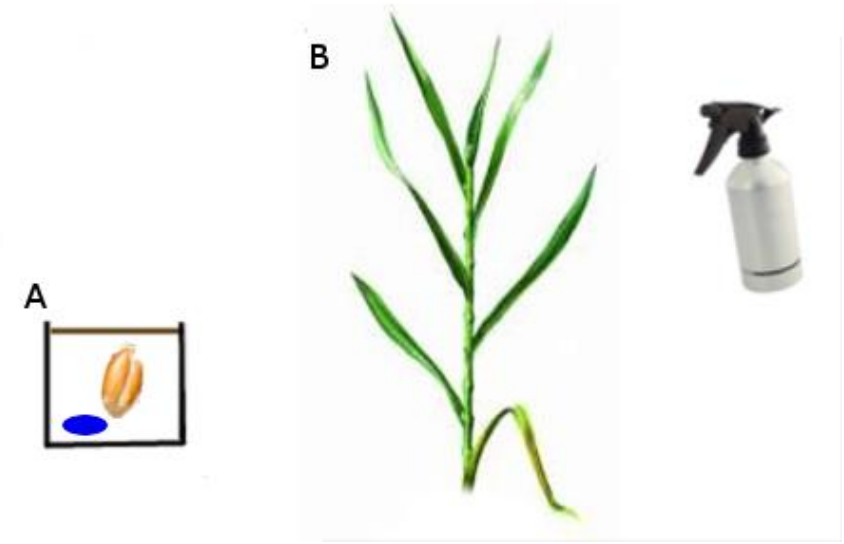
These were covered with lids to prevent contamination through evaporation and returned to their previous growth conditions.

The plants were sprayed weekly and once mature, leaves were taken for analysis.

3.2.3 CND and pDNA foliar spray

CND, DNA and TE buffer pH 8.0 nanoplex, and control conditions were carried out as with just the CND foliar spray (scheme 1B).

The plants were sprayed weekly and once mature, leaves were taken for analysis.



Scheme 1 – Applications of CNDs in the form of solid pellets and liquid spray. A CND pellets placed into soil as plants grow B foliar spray of CND solution onto immature plants.

3.3 Results

3.3.1 Uptake of CNDs from pellets

The application of CND pellets to the soil of immature *T. aestivum* plants showed uptake of the CNDs into the maturing leaves (fig.3 B, C, D). The CNDs accumulated around the vascular tissue of the leaves (fig.3 B, C, D) and stomata (fig.3 B).

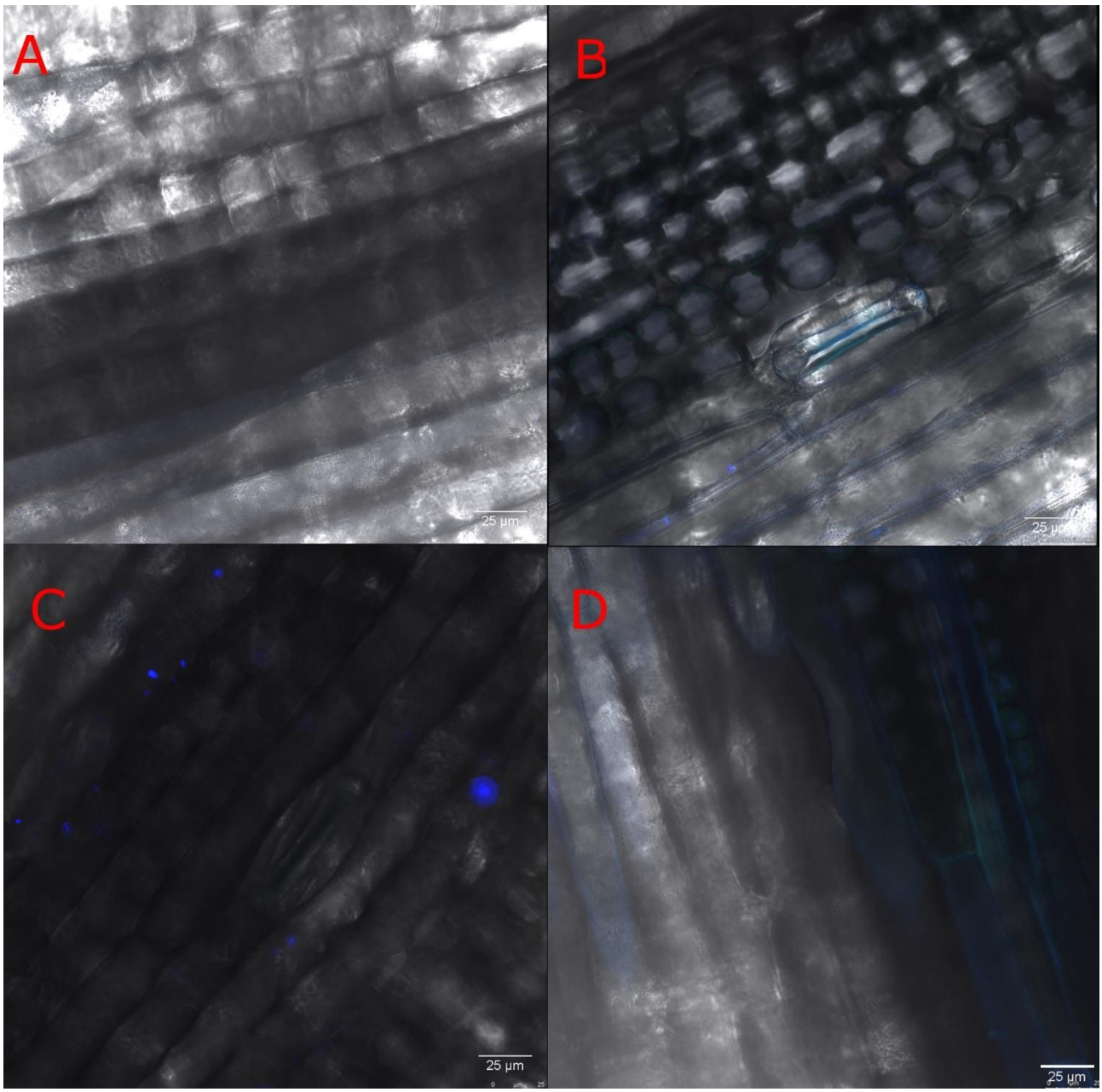


Figure 3 – Fluorescence depicting CNDs was artificially coloured blue and was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification. A WT leaf B-D leaves showing CND uptake from pellets. B had the pellet placed halfway down in the soil, C had the pellet placed at the bottom of the soil, and D had the pellet placed toward the surface of the soil.

The placement of the CND pellets resulted in different uptake levels of the CNDs (fig.3). Plants displayed more CND fluorescence when the pellets were placed at the bottom of the pot than placed at the middle or the top (fig.4).

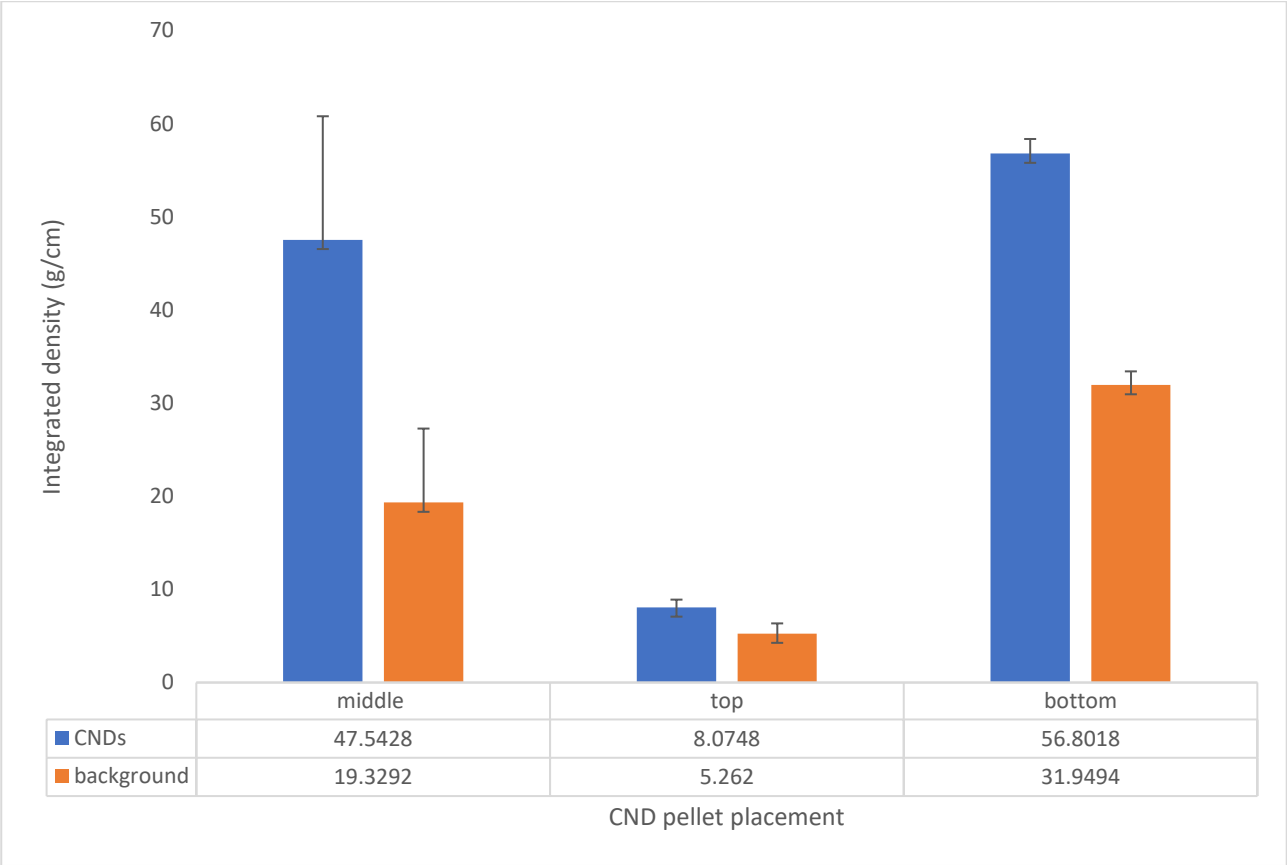


Figure 4 – Average fluorescence intensity of cells containing CNDs from pellets (CNDs) and background cells containing no CNDs (background).

3.3.2 Uptake of CNDs from foliar spray

Spraying plants weekly resulted in CNDs entering the cells, especially in the cytoplasm (fig.4). While the regions around the cells were clear of CNDs (fig.5).

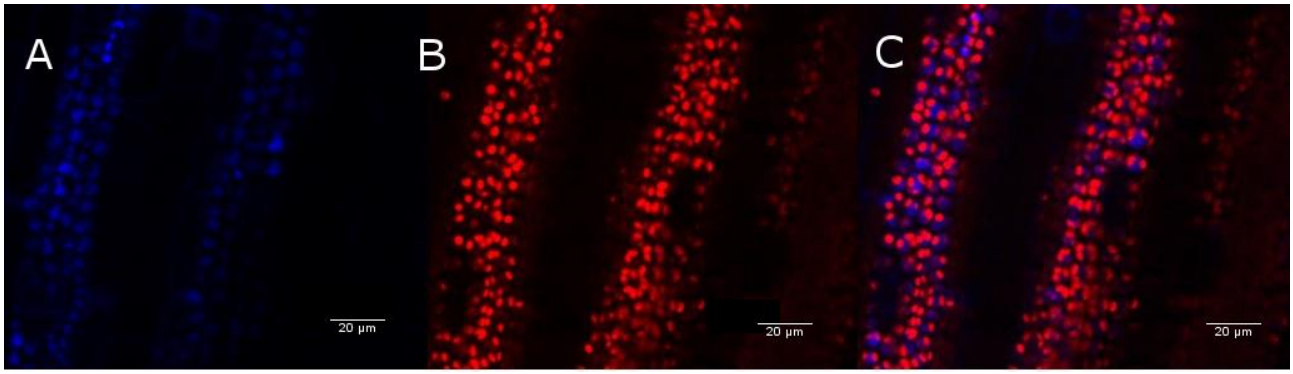


Figure 5 – Uptake of CNDs via weekly foliar sprays. Fluorescence depicting CNDs was artificially coloured blue and was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification. A CNDs only B chlorophyll only C CNDs and chlorophyll together.

The CNDs, when polyplexed with pDNA, continued to enter cells and allowed pDNA to enter the cytoplasm, where it was moved into the nuclei and proteins of interest were produced (fig.6)

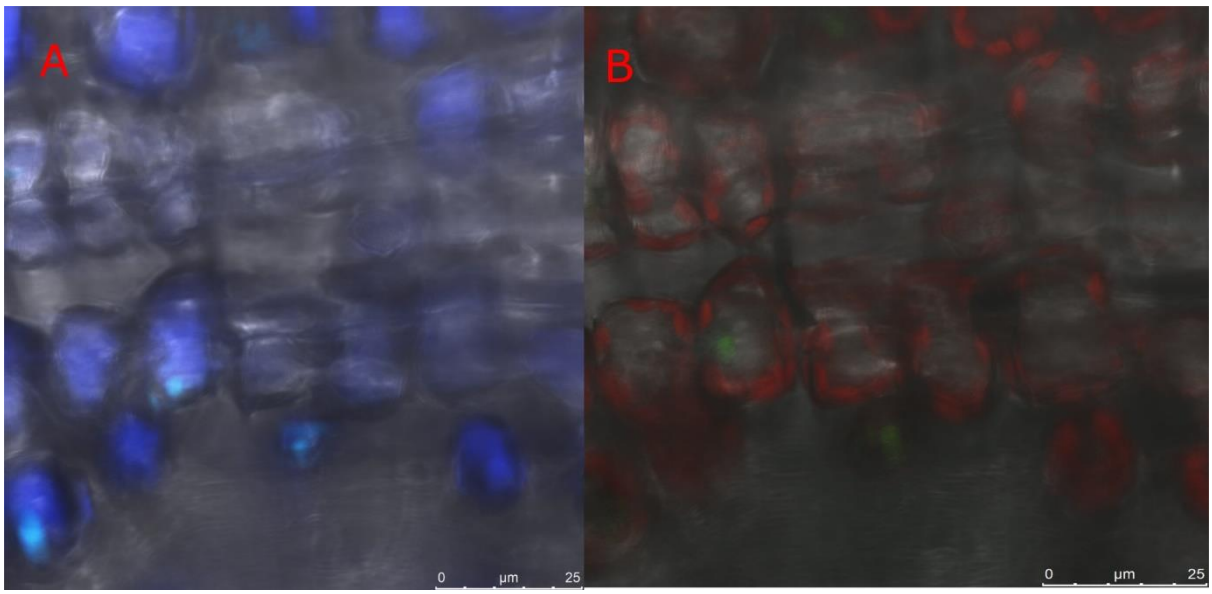


Figure 6 – CNDs and nuclear-targeted GFP in *T. aestivum* cells. Fluorescence depicting CNDs was artificially coloured blue and was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification. A CNDs in cells close to vascular tissue B Nuclear-targeted GFP present in cells.

3.4 Discussion

The uptake of CNDs into plants using these two methods of application had some success. CND pellets were placed at the top, middle and bottom of the soil in which *T. aestivum* was planted. The integrated density, measuring for fluorescence intensity in plant cells, was significantly higher in those which had the pellets places in the middle or at the bottom of the soil when compare to those placed at the top (fig.4). This suggests that CNDs were absorbed readily through the roots, but not so readily through the plant stem or seed coat in development. This was due to deeper roots having a more efficient uptake of water and nutrients than roots located closer to the surface (Lai & Katulab, 2000). As the pellets located at the bottom were closer to the source of water, as the plants were watered from the bottom, more water would have passed over those pellets than those located closer to the surface and so more of them would have been transported toward the roots. This opens the question of whether the amount of water passing over the pellets would have caused such a significant effect of their uptake, or if the efficiency of deeper roots was the cause.

When applying the CNDs to the foliage of the plants via foliar spray the uptake appeared to be highest in cells surrounding vascular tissue (fig. 5 & 6). This was due to plants taking up water via their foliage (fig.7), a mechanism that allows them to obtain water when there is low ground water or bypass it completely (Breshears *et al.*, 2008). Studies found that the application of artificial dew increased stomatal conductance (Boucher, Munson & Bernier, 1995). This appeared to be the main route of CND uptake from the foliar sprays, with stomata and vascular tissues showing higher levels of CNDs than surrounding cells (fig.5).

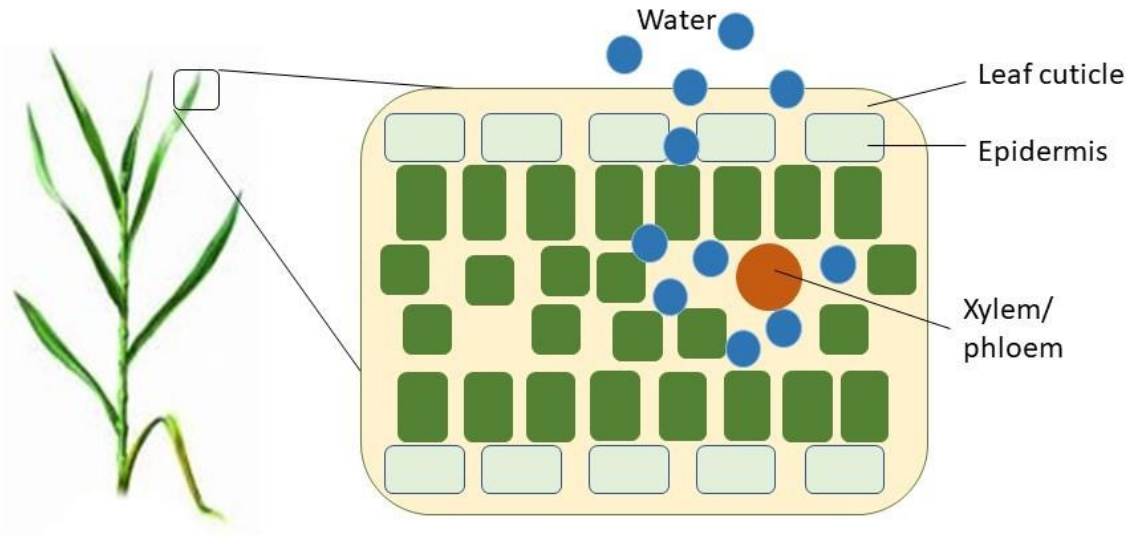


Figure 7 – Water being absorbed through the leaf's surface during precipitation or high humidity. Water, depicted by blue circles, moves through the leaf's surface and collects in the spaces surrounding cells and the vascular tissues, depicted as an orange circle.

As CNDs appeared to be primarily absorbed through the stomata, the CNDs were close to the vascular tissue of the plant (fig.3), supporting their movement pattern appearing to be similar to that of artificial dew (Boucher, Munson & Bernier, 1995) or sugar produced during photosynthesis in the chloroplasts (Damon *et al.,* 1988) (fig.8). The CNDs appeared to move from the leaf cells, where they entered via the stomata or leaf pores, moved into the vascular tissue, and from there to other areas of the plant following the route of transpiration (fig.8) (Boucher, Munson & Bernier, 1995). Cells containing CNDs showed high concentrations in the vacuole and cytoplasm (fig.6) where most sugars and fluids are moved and stored (Bush, 1993). This supports the hypothetical model (fig.7) that suggests movement being through the vasculature of the plant.

The movement of CNDs through plant cells would be affected by their positive charge. Electrogenic transport of materials in plant cells is affected by the charge of the particles (Bush, 1993) and CNDs are both acidic and charged (Swift *et al.,* 2019). This would explain how CNDs were able to move to and from the vascular tissue and to surrounding cells (fig.8), as plant cells utilise proton-sucrose symport for assimilate partitioning (Bush, 1993). This involves the movement of carbon and nitrogen, assimilates, between cells via active transport (Bush, 1992). As CNDs are carbon-based, nano-sized particles it is highly likely they would be transported between cells in this manner.

To determine if CNDs were capable of depositing material when travelling between cells pDNA was attached to CNDs using PEG diamine and ionic bonding. The cells displaying higher concentrations of CNDs also expressed the gene of interest, in this case nuclear-targeted GFP (fig.6). This suggests that high concentrations of CNDs passed through these cells and the ionic bonds were broken, allowing the pDNA to

be released into the cytoplasm of the cell and processed to allow transient gene expression. The percentage of transformed cells would need to be examined to determine how successful this method of gene delivery is, and if this method could prove successful in both monocot and dicot plant species.

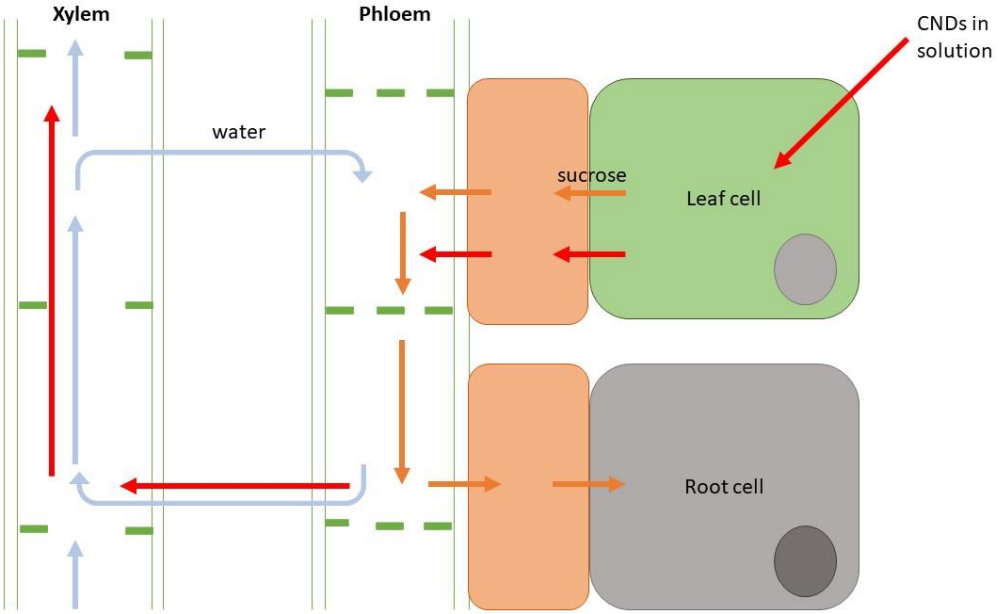


Figure 8 – The hypothetical route of CND movement through a plant. CNDs absorbed through the leaves move from leaf cells to the vascular tissue via companion cells. CNDs move through the plant vascular system similarly to sucrose and water (the movement of CNDs is discussed further in chapter 6, conclusions).

It was unclear if the CNDs moved via the xylem or the phloem, due to the vascular tissue association and both water and nutrients, such as sugars, being transported from chloroplasts and roots to other areas of the plant (fig.8). To determine the exact route of transport the CNDs would need to be modified to either 'drop' a different reporter gene in each cell they passed through, or carry a reporter dye that would map the route. However, it can be determined that the transportation of CNDs into plant cells involves the vascular tissue, where the fluorescence emitted was at its greatest (fig.5 and fig.6).
Another aspect of the transport of CNDs that requires investigation is how the pH affects their movement. Water transport is affected by pH, as it relies on pH-dependent signalling processes (Roux *et al.,* 2003) and so the acidic pH of the CNDs (Swift *et al.,* 2019) could have an impact on water transport mechanisms into and between cells. The pH may have affected the ionic bonding of the CNDs functionalised with PEG and the pDNA, causing the release of the genetic material into the cell. Further investigations should be made to determine what the cause of release of genetic material into the cells is and how it might be manipulated to release only at specific locations. If this was due to the pH, then pH manipulation of the CNDs could allow genetic material to be carried to specific locations in the plant and only then release their cargo.

To examine how successful the uptake of CNDs could be and the route of travel and release of their cargo pDNA was attached using PEG diamine chains and CNDs were applied to both dicot and monocot plant species using various methods.

4.The use of carbon nanodots in the genetic modification of *Arabidopsis thaliana*

Aims

This chapter aims to outline how CNDs can be applied to genetically modify the model plant species, *Arabidopsis thaliana*. The use of *A. thaliana* will be justified, then the background of *A. thaliana* and various methods of genetic modification will be covered. Nanoparticle use will be examined, and the applications of CNDs will be discussed. The methods of plant growth and the applications of CNDs complexed with pDNA will be outlined, including seed transformations, floral dipping, vacuum infiltration and foliar sprays. The results of these applications will be outlined and discussed, then the impacts of this work will be examined.

Abstract

Arabidopsis thaliana is the model species of plant for dicots and is used in genetic research. Current methods of GM include *Agrobacterium*-mediated and particle bombardment-mediated transformation, as well as gene editing using ZFNs and TALENs. However, these methods have several issues, including high cost and high cytotoxicity. There is the need for a cheap, effective and biocompatible method in GM of *A. thaliana*, and CNDs may be appropriate. To test an alternative method of GM using CNDs several methods of application were performed, including foliar sprays, vacuum infiltration and seed transformations. It was found that while most methods were successful in transforming plant cells, with multiple foliar sprays resulting on the higher percentage of transformed cells, seed transformations appeared to be unsuccessful.

4.1 Introduction

Arabidopsis thaliana is considered a model plant species, and therefore has been extensively studied across the plant science field. The ease of growing, fast generation time, and ability to be kept in laboratory conditions made it suitable for a wide range of investigations, including genetic studies. As the first plant species to have its genome completely sequenced it has been a key species in genetic modification (GM) experiments, as there is a greater understanding of its gene function and gene regulatory networks (Bevan & Walsh, 2005).

Several methodologies are currently used in the GM of Arabidopsis. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been utilised to cleave non-specific DNA domains. They have been found to be highly programmable and sequence-specific, giving a broad GM range (Gaj, Gersbach & Barbas III, 2013). There are, however, issues with ZFN and TALEN-based transformation. ZFNs require specialist design and construction, resulting in a high cost. The success rate of this method is uncertain and there have been issues with ZFNs having high cytotoxicity, limiting their use in live cells (Jiang

et al., 2013). TALENs are much simpler to construct and theoretically could target any sequence in an organism. They have lower levels of cytotoxicity than ZFNs, but there are still concerns overusing them in multiple living cells, and their success rates, although greater than ZFNs, are still not optimal (Jiant *et al*., 2013).

More recent techniques include *Agrobacterium*-mediated transformation and the CRISPR/Cas system. *Agrobacterium*-mediated methods, using a strain of *Agrobacterium tumefaciens*, are frequently used in numerous GM experiments due to its reliability, lower cost, and ease of use. There are multiple application methods possible, including floral dipping and injections (Zhang *et al.*, 2006; Jiang *et al.*, 2013), which make the method more versatile and accessible than previous techniques. The success rate is still low, at 1-2% in most cases (Zhang *et al.*, 2006) and there are concerns over the use of bacterial infection of cells causing damage either in the application or removal stages. The CRISPR/Cas system is more recent and, while still not fully developed to its fully potential, has shown promising results. The technique allows gene editing to occur with a single guide RNA (sgRNA) guiding the protein to the site of interest. It is considered simpler than ZFNs and TALENs and has been shown to be successful at gene disruption, gene activation and repression, and gene editing in *Arabidopsis* plants (Jiang *et al.*, 2013). The system has not only succeeded in transient transformation, but has been used to genetically edit the germline, with T2 plants being shown to be 22%± homogenous for the gene modification in the parent plants (Feng *et al.*, 2014). However, CRISPR/Cas relies on the entry and use of the Cas protein into cells, therefore relying on cell uptake. As plant cells have cell walls this is a limiting factor to the system and its ability to transform species.

Work has been done to overcome these issues using nanotechnology, which has been found to be extremely versatile in medical research. Initially work focused on toxicity to determine the effects of nanoparticles in plants. It was found that, while certain nanoparticles, especially those made with heavy metals, could inhibit plant growth, others were found to enhance growth and development (Lee *et al*., 2009). As heavy metals are known to be toxic, work was done to examine the effects of carbon-based nanoparticles on plants. Carbon nanotubes were applied to protoplasts via injection and it was determined that these could lead to cell aggregations, the accumulation of hydrogen peroxide and cell death when applied at medium to high concentrations. Low concentrations were found to have no effect (Shen *et al.,* 2010) or to enhance growth (Yuan *et al.,* 2010).

While a new method of transformation is not necessary for *Arabidopsis thaliana*, as it is effectively transformed using current methods, such as floral dipping (Koornneef & Meinke, 2009), it is a useful species to use to compare the effectiveness to alternative methods. As it is a model species that is readily transformed using current methods it is ideal to test new methods of transformation on to compare those to current methods (Koornneef & Meinke, 2009). This is discussed further in chapter 6, conclusions.

This research examines the application of carbon-based nanoparticles, carbon nanodots (CNDs), to GM mature *Arabidopsis thaliana* plants without cell damage or invasive procedures, while retaining low costs and successful transformations. The CNDs were combined with plasmid DNA (pDNA) and several application methods were utilised to determine the most appropriate and successful method.

4.1.1 The objectives of this chapter include:

1. To determine if CNDs can be used to transform *A. thaliana* plant cells in mature plants by applying CNDs conjugated with pDNA.
2. To determine which application method is the most effective for transformation of cells by applying CNDs conjugated with pDNA in various ways, including foliar sprays and vacuum infiltration.
3. To determine how CND-transformed plants compare to waveline control plants, already expressing the gene of interest.
4. To monitor the movement of CNDs in the plant by identifying which cells express the gene of interest.

4.2 Methods

4.2.1 Plasmid DNA preparation

Waveline (127Y and 131Y) pDNA was selected from and supplied by the Arabidopsis Biological Resource Center (2011). The plasmid for use in GUS experiments were supplied by Dr Jill Harrison, University of Bristol. The plasmid for GFP/Cas9 experiments were supplied by Professor Keith Edwards, University of Bristol.

Plasmid DNA was grown as stabs in selective media, then grown overnight in LB with a selective antibiotic at a 1:1000 ratio. The pDNA was initially extracted using QIAGEN Miniprep kits, using the standard QIAGEN protocol. This extraction method was later substituted for the ZymoPURE midiprep kit method, as it produced higher yields of pDNA. The pDNA was eluted in autoclaved water rather than ethanol.

4.2.2 Carbon nanodot preparation

The Carbon nanodots (CNDs) were produced using a modified method based on the publication by Hill *et al.* (2016). Further information is provided in chapter 1.

4.2.3 Carbon nanodot and DNA incubation

85µl Carbon nanodots (CNDs), 85µl plasmid DNA and 85µl TE buffer pH 8.0 were added to a 2mL sterile Eppendorf tube, vortexed for 30 seconds and left in the dark at room temperature for 10 minutes to allow CNDs to bind the DNA. This created a CND and DNA complex in TE buffer pH 8.0.

4.2.4 Plant material

Soil

Soil was made at ¾ F2 compost (Levington F2 Seed and Modular Compost, Suffolk, UK) and ¼ horticultural sand (Melcourt Horticultural Silver Sand, Gloucestershire, UK). These were mixed, sieved to remove debris, and autoclaved for 15 minutes at 121°C or frozen between -15 to -30 °C for 24 hours to sterilise.

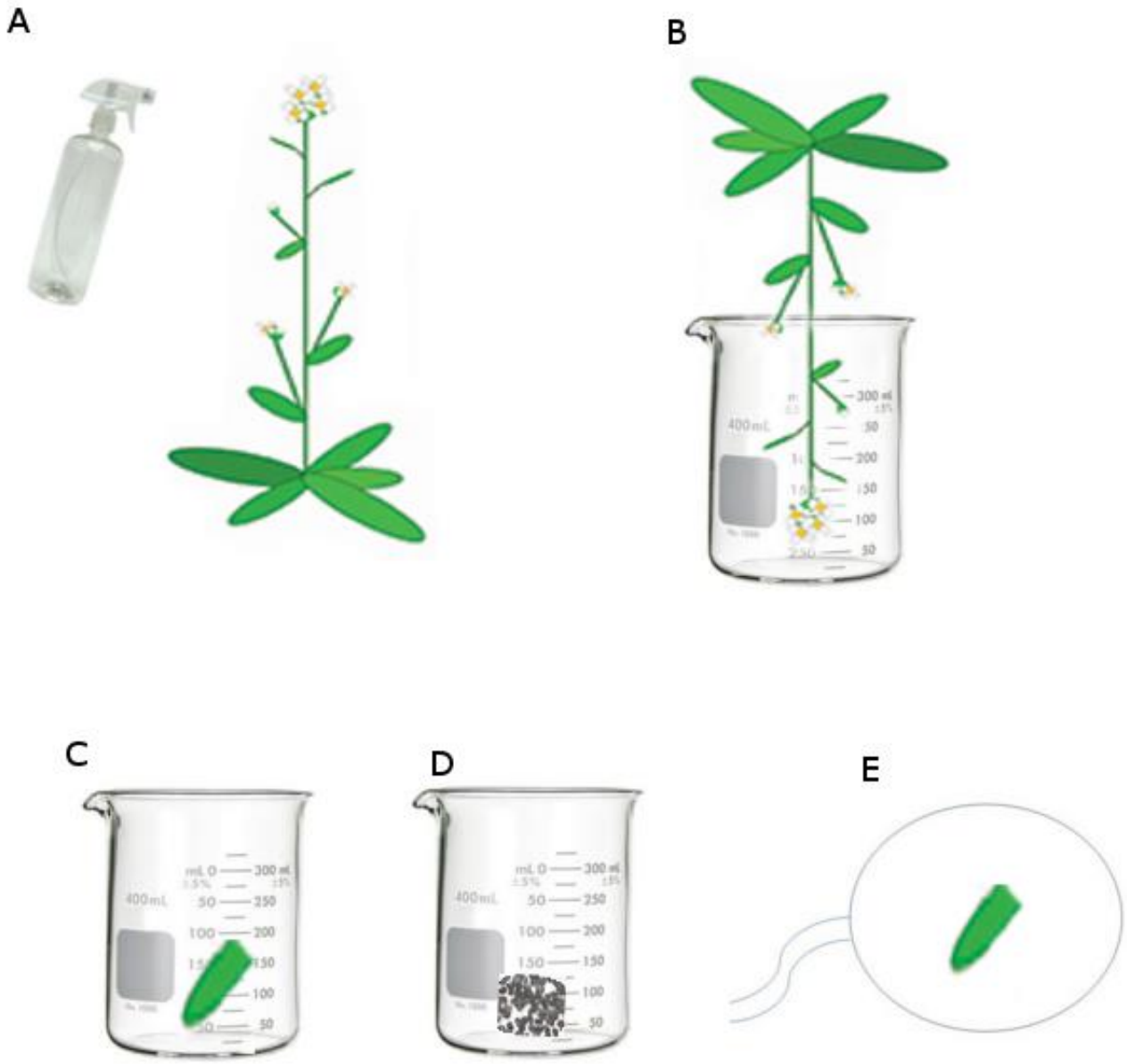
Plant growth conditions

The soil was transferred to pots (x24) in a tray containing water. The soil was left to absorb water for 5 minutes and sterilised seeds were pipetted onto the surface. The plants were grown in the University of Bristol's GroDome under 22°C, 16 hour day lengths

4.2.5 Seed Surface Sterilisation

Arabidopsis thaliana Columbia seeds were placed into sterilised 2mL Eppendorf tubes and washed in 70% EtOH. The tubes were closed and inverted 6 times to ensure all seeds were coated. The EtOH was removed and 20% bleach was added. The tubes were inverted 6 times and left to sit for 8 minutes. The bleach was removed and the seeds were washed three times with autoclaved, distilled water. The seeds underwent stratification at 4°C for 3 days.

4.2.6 Treatments of Arabidopsis thaliana *plants*



Scheme 1 – The application methods of the CND+DNA solution to *A. thaliana* **A** Foliar spray **B** Floral dip **C** Leaf dipping **D** Seed dipping **E** Vacuum infiltration.

A. thaliana plants were treated in a variety of ways in order to determine which method resulted in the highest level of transformed cells. Initial applications of CND-pDNA solution were on leaves either taken from or growing on mature plants, while later applications were on seeds.

4.2.6.1 Leaf transformation

Full basal leaves were cut from wild type plants. An equal number of leaves were placed into one of four conditions in sterile 2mL Eppendorf tubes. Condition 1 was CNDs and DNA in distilled water and TE buffer pH 8.0; Condition 2 was CNDs in distilled water and TE buffer pH 8.0; Condition 3 was DNA in distilled water and TE buffer pH 8.0; Condition 4 was distilled water and TE buffer pH 8.0.

The leaves were placed with the cut petiole in the solution. These were incubated at room temperature in the dark for 24 hours.

4.2.6.2 Seed transformation

Surface sterilised seeds were placed in 50 mL falcon tubes with 25 mL liquid MS 4.4 g/L as recommend by the manufacturer and incubated at 22^{o}C, shaking at 120 RPM with constant light for 24 hours.

The MS was removed, and the seeds were separated into four 50 mL falcon tubes. 25 mL liquid MS was added to each and the conditions in 4.1.2 with the solution prepared as in 4.1.1. However, the amounts of CNDs, DNA and TE buffer pH 8.0 were increased to 85µl to improve uptake in a more dilute end solution. The tubes were incubated in the same manner for another 24 hours. The seeds were washed with distilled water three times. This method was adapted from Feldmann and Marks, (1987).

Seeds were pipetted onto MS30 plates, made using 4.4 g/L MS, 30 g/L sucrose and 8 g/L agar pH 5.8. The plates were sealed with parafilm and incubated in a Micro Clima-series economic lux chamber (Snijders Labs, Tilburg, Netherlands) with day cycles of 25^{o}C for 16 hours and night cycles at 22^{o}C for 8 hours. The plates were placed upright to allow stem and root extraction from the surface of the agar.

4.2.6.3 Foliar spray

CND, DNA, TE buffer pH 8.0 complex and control conditions were added to separate 100 mL spray bottles (Wilko Travel Spray Bottle 100ml, Bristol, UK). Then 2 mL dH_2O was added to each and they were inverted 3 times to mix.

19-day-old plants were separated into separate trays, to prevent contamination, before spraying. The plants were kept separate during spraying and each plant was sprayed 3-4 times to coat the leaves. These were covered with lids to prevent contamination through evaporation and returned to their previous growth conditions.

The experimental condition was split, with half of the plants receiving the complex via spray once, while the other half was sprayed once weekly.

Plants leaves were taken from mature plants for analysis.

4.2.6.4 Multiple foliar sprays

CND, DNA, TE buffer pH 8.0 complex and control conditions were added to separate 100 mL spray bottles (Wilko Travel Spray Bottle 100ml, Bristol, UK). Then 2 mL dH_2O was added to each and they were inverted 3 times to mix.

19-day-old plants were separated into separate trays, to prevent contamination, before spraying. The plants were kept separate during spraying and each plant was sprayed each day for three days, left for a day and

then sprayed for another two days. Samples were taken one hour before spraying and one hour after each subsequent spray. The samples were imaged using CLSM as soon as they were taken and then frozen using liquid nitrogen They were stored at -80^{o}C for RT-PCR analysis.

4.2.6.5 Vacuum infiltration

The DNA/CND/TE solution was made with 85ul of each solution added and vortexed. This was mixed via inversion of the tube with 0.02% silwet in 20 mL dH_2O.

Young plants between 2-5 weeks old were removed from the soil and the roots were washed with dH2O. The aerial portion of the plant was placed into a square 90mm petri dish filled with the solution. This was place inside the Nalgene Vacuum Desiccator (Sigma Aldrich, UK) and was put under vacuum for 2 minutes and was left in the closed vacuum for 3 minutes before being removed.

The plants were rinsed with dH2O to remove the solution and replanted. These were allowed to grow, and leaves were taken for analysis. Once siliques had formed, they were taken and dried, then plated onto selective media or grown and imaged using CLSM.

4.2.6.6 Leaf injection

The CND/DNA/TE solution was added to a sterile 3 mL syringe and any air was removed through depressing the plunger with the syringe held upright. The barrel tip was placed firmly against the leaf and the plunger was depressed for 5 seconds until the area around the barrel tip turned a darker shade than the surrounding leaf. The area injected was marked with a permanent marker and the plant was allowed to grow for 3-4 days before imaging.

4.2.7 Confocal laser scanning microscopy

A Leica SP5 confocal laser scanning microscope was used to image treated and control plant leaves.

4.3 Results

Mature *A. thaliana* plants had CND+pDNA solution applied to them, with each treatment condition kept to separate groups of plants. If one group of plants was treated with a spray, they would not undergo a second, different treatment. The success of each treatment was measured by the integrated density and the percentage of transformed cells. Five leaves were taken from each plant that had undergone treatment and these were examined to determine if transformation had occurred.

4.3.1 Treatment efficiency

The treated, mature *A. thaliana* plants were examined using confocal laser scanning microscopy (CLSM) and fluorescence microscopy. Presence of the protein of interest, usually a fluorescent protein, and the percentage of transformed cells was calculated and compared between each treatment (Table 1). The level

of success of each treatment was determined as the highest number of cells expressing the protein of interest.

Table 1 – Treatment type and success of transformation in *Arabidopsis thaliana* plants.

Treatment Type	Presence of Fluorescent Protein (Yes/No)	Average percentage of transformed cells (%)
Leaf in solution	Yes	47
Seed transformation	No	N/A
Foliar spray (single)	Yes	11
Foliar spray (multiple)	Yes	23
Floral dipping	Yes	6
Vacuum infiltration	Yes	9

The spray method was found to be the most successful, with a high average percentage of transformed cells and a high frequency of transformed plants after treatment. This method was more successful when applied multiple times, with four sprays of the plants giving the highest average percentage of transformed cells (Table 1).

Other application methods were less successful. Injecting the solution into the leaves was the second most successful technique, followed by vacuum infiltration, and dipping being the least successful (Table 1).

Individual leaves were examined using confocal laser scanning microscopy (fig.1). The fluorescent protein expression was imaged and the level of fluorescence was measured in comparison to control cells. When compared to controls the level of fluorescence was far greater in plants which had the CND-pDNA solution applied to them.

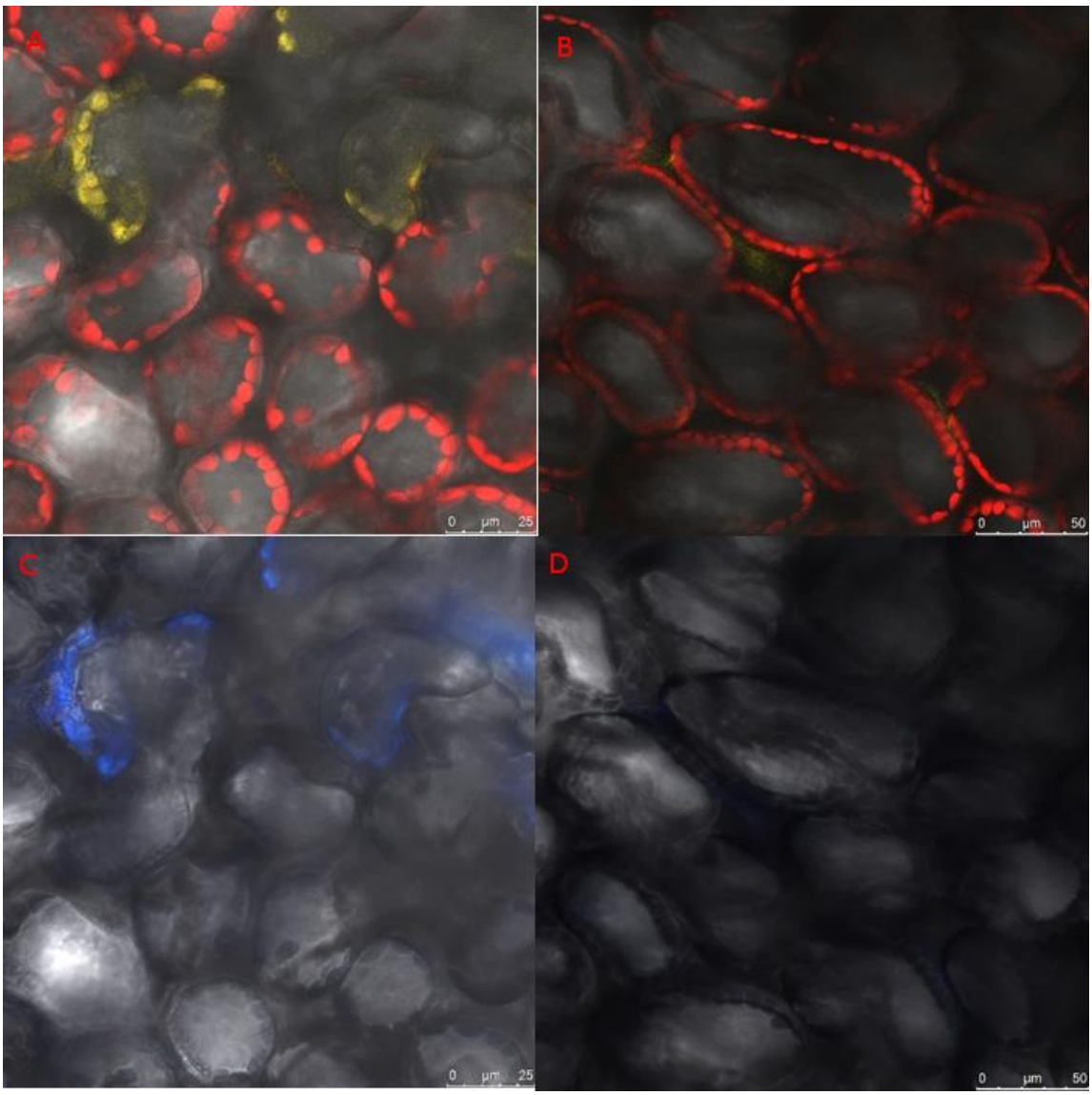


Figure 1 – The expression of waveline 127Y YFP in *A. thaliana* leaf cells. Fluorescence depicting CNDs was artificially coloured blue and was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification. **A** waveline 127Y transformed cells **B** WT chlorophyll **C** CND fluorescence detection where YFP was detected **D** WT fluorescence.

The fluorescence did vary between treatments, with the multiple spray method producing the highest levels of fluorescence (Table 1). Injections of the solution into the leaves of plants resulted in the second highest level of fluorescence, while vacuum infiltration and dipping of the leaves produced the lowest level (Table 1).

4.3.2 CND treatment compared to waveline controls

To compare with positive controls, the plants treated with waveline pDNA+CNDs were directly compared to waveline controls, known to produce the proteins of interest throughout the plants without treatment (fig.2a). The fluorescence of the transformed cells and the waveline controls were measured and compared

to determine the level of success. When compared, the level of fluorescence in CND-transformed plant cells were close or identical to those of the controls (fig.3), suggesting successful transformation.

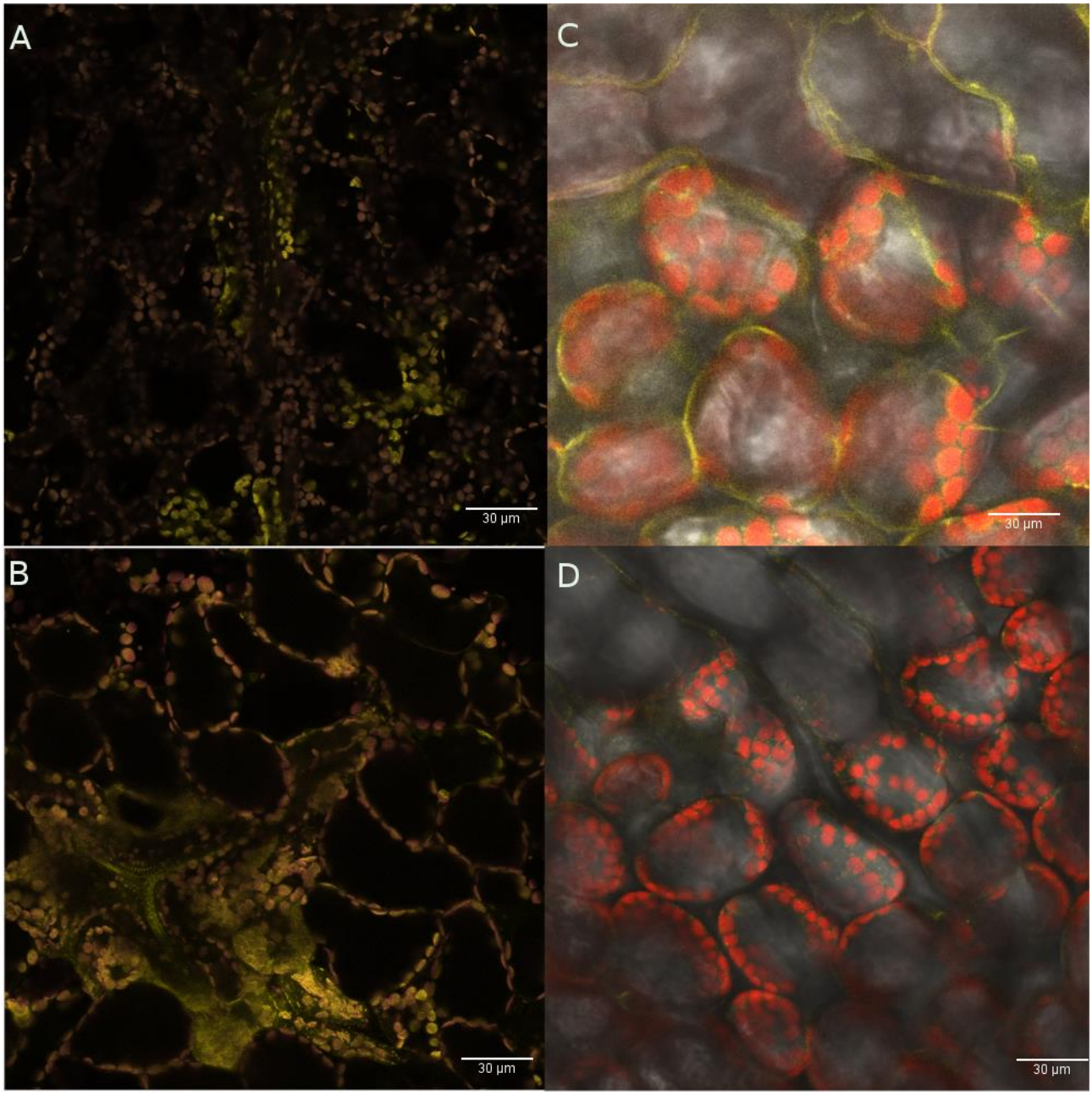


Figure 2a - CND-YFP-treated *A. thaliana* leaves compared to waveline control *A. thaliana* leaves, which express the proteins of interest throughout the plant without treatment. **A** 127Y+CND treated leaf **B** 131Y+CND treated leaf **C** 127Y waveline control leaf **D** 131Y waveline control leaf.

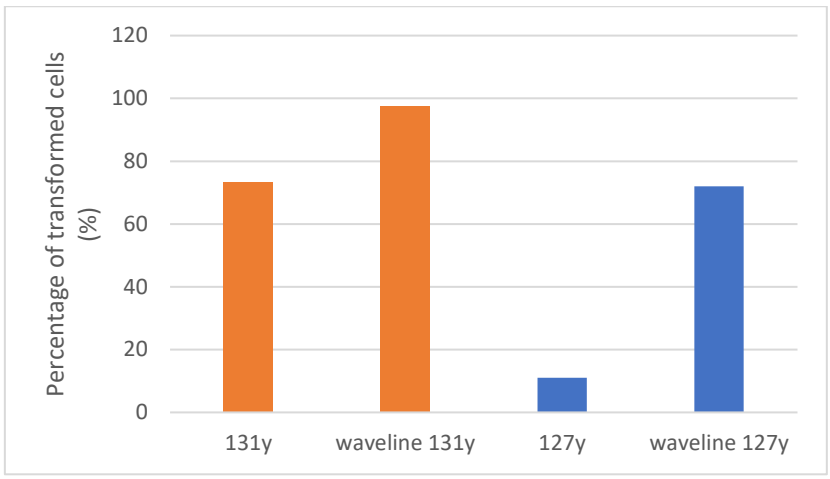
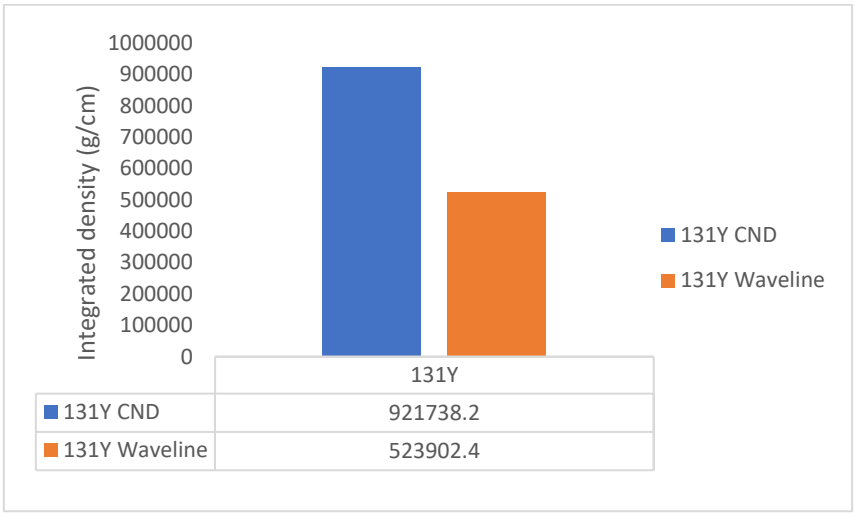


Figure 2b – Experimental treatment transformation percentage compared to waveline control plants.



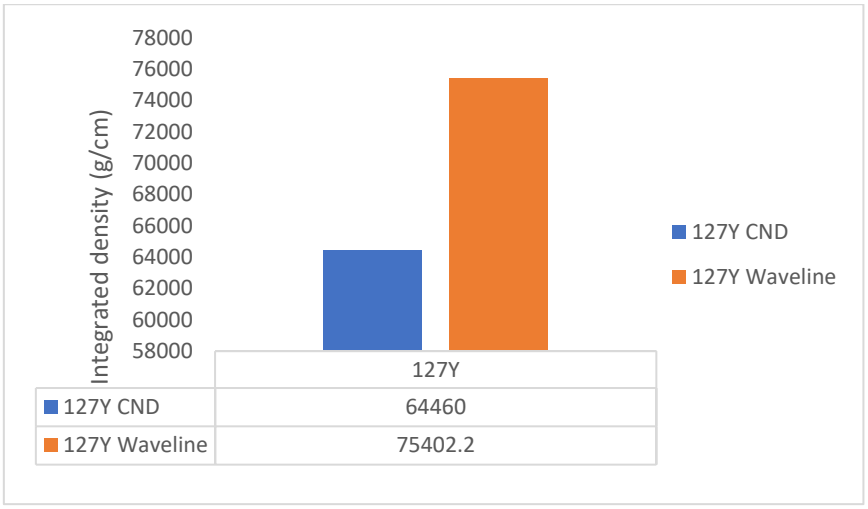


Figure 3 – Integrated density of CND+pDNA complex-treated *A. thaliana* plants compared to waveline control plants. 131Y+CND treated plants were directly compared to waveline positive 131Y control plants and 127Y+CND treated plants were directly compared to waveline positive 127Y control plants. Waveline controls expressed proteins of interest throughout the plant tissues.

4.4 Discussion

The presence of fluorescent proteins were found predominantly in the upper mesophyll cells towards the tips of the leaves. Genetic material targeting the plasma membrane resulted in higher fluorescence in the epidermal cells, as was the case with the 131Y plasmid, while others, including 127Y, were found only in mesophyll cells (fig.1).

There appeared to be colocalisation of the fluorescent proteins and the CNDs in the plant cells, with CND fluorescence detected in all cells (Table 1). The CNDs were also found between cells, especially shortly after the application of the solution (Table 1). Observing CNDs between cells, especially shortly after the application, suggested that CNDs travel through the xylem and are distributed into cells with water and small nutrient particles. This has been witnessed in previous work involving carbon-based nanoparticles, in which it was found that the nanoparticles travel through the xylem and vascular tissues, corticol cells and vascular bundles (Cifuentes *et al.*, 2010), which is what was found with the CNDs.

The method of application was found to affect the success of the treatment (Table 1). Multiple sprays of the solution were the most successful method due to repeated exposure over time, similarly to the *Agrobacterium* transformation method (Clough & Bent, 2008). Applying a single spray resulted in more transformed cells than other methods, but less than multiple sprays (Table 1). The injection method was similar in the number of transformants as the single spray method (Table 1). Dipping leaves and vacuum infiltration of leaves did result in transformed cells (Table 1), however there were fewer than the spray method or the injection method.

Not only was the spray method able to create the highest number of transformed cells, it was also the simplest to apply on a wide scale. However, it was less precise than other methods method, as some plants may not have been fully sprayed. The injection method was the most precise, as the CNDs would carry the pDNA to the tip of the leaf injected and nowhere else in the plant.

When comparing the fluorescence of CND-transformed cells to the waveline controls there was a significant difference (fig.3). While the waveline control for 127Y had a higher integrated density and more consistent in the distribution of the protein of interest, the CND+131Y transformed cells had a higher integrated density compared to the waveline control (fig.3). When comparing the percentage of transformed cells both waveline controls had a significantly higher percentage than the CND+pDNA treated plants (fig.2b). The transformation percentage of the plants treated with 131Y-CNDs was higher than those treated with 127Y-CNDs, which suggested a difference in uptake of genetic material varied based on the different factors of the pDNA such as size and electrostatic interactions. This comparison suggested that the CND-transformation method was able to transform cells with varied degrees of success, with the main difference appearing to be the pDNA conjugated with the CNDs. This is discussed further in chapter 6, conclusions.

The CND-transformation method resulted in transformants in the case of all but one of the application methods. As the different methods of application varied in their number of transformed cells and level of fluorescence it appears that this is a factor that will need to be considered when using the system. The multiple spray method produced the highest number of transformed cells and was easy to apply, therefore making it the most appropriate method to use for future work. Other methods may be improved through multiple applications or applying a different dosage of the CND-pDNA solution, as it may be that for other methods to be successful the solution may need to be more concentrated.

Conclusions

It was determined that the CND transformation method was successful when applied to the dicot model plant species *A. thaliana*. It was found to be effective for transient transformation when applied in numerous ways, including spraying and dipping mature plants. However, these application methods varied in their success and so future work would need to optimise the system to establish a transformation method which is reliably successful.

As the multiple spray method was found to be the most successful in producing mature plants with a high number of transiently transformed cells, it would be logical to establish exactly why this method was more successful in order to optimise the other application methods. This should be done by repeating the other application methods, such as dipping, multiple times to establish whether multiple applications were responsible for the increased success rate.

This transformation system was shown to be successful in a model dicot species. To establish whether this transformation system was viable for use on other plant species other plants had the system applied. The study examined the monocot and widely used cultivar, *Triticum aestivum* Apogee, to determine if this transformation system was applicable to monocot species.

5.The use of carbon nanodots in the genetic modification of *Triticum aestivum*

Aims

This chapter aims to examine the applications of CNDs on a common wheat species, *Triticum aestivum*. The use of *T. aestivum* will be justified, then the current methods of genetic modification (GM) and gene editing (GE) will be outlined, as well as issues present. The applications of nanotechnology will be examined, focusing on the use of CNDs and pDNA to GM and GE *T*. *aestivum*. The methods of pDNA extraction, plant growth and applications of CNDs complexed with pDNA will be outlined, including protoplast extraction and transformation, floral dipping, and foliar sprays. The results of these application will be presented and discussed, and the impacts of this work will be examined.

Abstract

Wheat is a major source of food produce worldwide and therefore there a high demand for increased production. To increase crop yields, combat disease and parasites, and allow continued growth in a changing climate there is the need for genetic modification of crop species to ensure demands are met. A common wheat species, *Triticum aestivum*, was used as a model for other monocot crop species. Plants were treated with pDNA and CNDs in a variety of ways to determine which was most effective. Gene editing was also examined using the Spo11 plasmid with Cas9. It was found that multiple foliar sprays resulted in the highest percentage of transformed cells, while vacuum infiltration, dipping and foliar injections were less effective, and seed treatments were unsuccessful. Gene editing was also found to have taken place in plants exposed to the Spo11-Cas9 pDNA.

5.1 Introduction

Wheat is one of the major staple grains for human and animal consumption, with over 17% of cultivatable land dedicated to its production worldwide (Xia *et al*., 2012; Ding *et al.,* 2009). As the human population is expanding at around 100 million per year, with an estimated 10 billion global population by 2050, sustainable food production is a major focus (Bhalla, 2006). To meet demands the agricultural industry needs to look to alternative methods to increase crop yield, production, and reduce environmental impact, as conventional breeding methods are unable to meet the projected targets (Vasil, 2007). GM has the potential to enhance agronomic performance through tailoring species to have increased yield, stress tolerance, increased vitamins, and disease resistance, to name a few of the areas of research being undertaken (Ding *et al*., 2009; Jones, 2005; Vasil, 2007; Xia *et al*., 2012). Despite socio-political concerns over GM the land dedicated to GM crops have increased by almost 90 million hectares in under 10 years,

and benefits have already been seen with a reduction in the use of pesticides and herbicides as plants are tailored to be resistant (Bhalla, 2006).

Currently several methods of GM have been applied to wheat plants, including electroporation, DNA application to pollen, PEG-mediated gene transfer and microbeam laser punctures (Ding *et al*., 2009). *Agrobacterium*-mediated methods and particle bombardment are the two dominant methods in GM of wheat and have efficiency rates of up to 10%. These methods are easier to apply, lower in cost, and allow larger amounts of DNA to be transferred (Wu *et al*., 2003; Xia *et al*., 2012; Ding *et al.,* 2009; Bhalla, 2006).

More recently CRISPR/Cas9 has been applied to wheat with relative success. Previously the system had been applied to *Arabidopsis*, but now studies have shown it is capable of transforming wheat protoplasts and embryos (Belhaj *et al.,* 2015). This system has shown promise in that it can target a large range of sites and allows engineering and multiplexing to take place, however it suffers from low efficiency due to guidance RNA targeting (Shan *et al*., 2014). The CRISPR/Cas system has been altered to attempt to increase efficiency and effectiveness. One alternative method involves the use of virus replicon systems, such as the wheat dwarf virus, to deliver Cas9 protein to a specific site and with a high level of efficiency (Gil-Humanes *et al.,* 2017).

Unfortunately, even with the advances made with CRISPR/Cas, *Agrobacterium*-mediated and particle bombardment-mediated GM, there is still a bottleneck present in GM and DNA delivery (Jones, 2005). GM of wheat is extremely complicated due to the complexity of the wheat genome and little progress has been made (Vasil, 2007). It is a very large hexaploid genome, over 16,000 Mb, consisting of a high number of transposable elements, repeated sequences, and the most agronomically important genes are not always triplicated (Bhalla, 2006). Due to the complexity genetic work is done to embryos, callus tissues and protoplasts, increasing success rates of initial transformation, but leading to the need to regenerate the plant entirely. This is challenging, with many transformed specimens failing to regenerate, and has a high financial cost (Wu *et al.,* 2003). To improve GM of wheat it has been suggested that a precise, marker-free, high-through put system is required, along with a greater understanding of gene function (Xia *et al*., 2012; Jones, 2005).

To resolve the issues with current GM methodologies, researchers have turned to nanotechnology as it has shown promise in work done in medical research. Work has previously been done using nanoparticles on wheat, but this has focused on toxicity, transport, cell interactions, and effects on plant growth and development (Lee *et al.,* 2008; Wild & Jones, 2009; Miralles *et al*., 2012; Wang *et al*., 2012).

This research examines the application of CND-pDNA complexes to mature wheat plants (*T. aestivum* Apogee) to demonstrate a novel method of transforming adult plants. The adult plants were transiently transformed using a variety of application systems, with many exhibiting precise and predictable transformation. This work expands on previous work with CND-pDNA complexes on *Arabidopsis thaliana* plants, demonstrating the transformation system can be applied to more complex, monocot species, while delivering similar levels of success.

5.1.1 Objectives of this chapter include:

1. To determine if the CND method is effective in *T. aestivum*, initially using protoplasts and then mature plants.
2. The determine the most effective method of application by comparing percentage of transformed cells for each treatment.
3. To determine if the CND method can result in gene editing using a plasmid targeting two of the Spo11 regions.

5.2 Methods

5.2.1 Plasmid DNA preparation

Waveline YFP pDNA, 127Y and 131Y, was supplied by the Arabidopsis Biological Resource Center (2011), explained in the previous chapter. The plasmid for GFP/Spo11 experiments was supplied by Professor Keith Edwards, University of Bristol.

Plasmid DNA was stored as stabs in selective plates, and was grown in LB with antibiotic selection at a 1:1000 ratio. pDNA was initially extracted using QIAGEN Miniprep kits, using the standard QIAGEN protocol. This extraction method was later substituted for the ZymoPURE midiprep kit method, as it produced higher yields of pDNA at ≤400ug. The pDNA was eluted in autoclaved water.

5.2.2 Carbon nanodot preparation

The Carbon nanodots (CNDs) were produced using a modified method based on the publication by Hill *et al.* (2016). Further information is provided in chapter 1.

5.2.3 Carbon nanodot and DNA incubation

85µl Carbon nanodots (CNDs), 85µl plasmid DNA and 85µl TE buffer pH 8.0 were added to a 2mL sterile Eppendorf tube, vortexed for 30 seconds and left in the dark at room temperature for 10 minutes to allow CNDs to bind the DNA. This created a CND and DNA complex in TE buffer pH 8.0.

5.2.4 Plant material

5.2.4.1 Seed Surface Sterilisation

T. aestivum cv. USU-*Apogee* seeds were placed into sterilised 50 mL Falcon tubes and washed in 70% EtOH. The tubes were closed and inverted 6 times to ensure all seeds were coated. The EtOH was removed and 20% bleach was added. The tubes were inverted 6 times and left to sit for 8 minutes. The bleach was removed, and the seeds were washed three times with autoclaved, distilled water. The seeds underwent stratification at 4°C for 3 days.

5.2.4.2 Hydroponics

400 mL ½ MS agar was prepared as instructed by the manufacturer by mixing 0.88g Mirashige and Skoog (MS), 3.2g Agar and 400 mL dH2O. This was autoclaved to sterilise and pipetted into hydroponics seed holders. These were allowed to set, and seeds were pipetted onto the agar, one per holder.

Plants were grown in hydroponics. *Triticum aestivum* cv. USU-*Apogee* were grown in high density compartments to allow full development. The water of the hydroponics was supplemented with autoclaved Hoagland's solution (0.5g/L) pH 5.8 and was refilled with standard water twice a week. Further Hoagland's solution of the same concentration was added as required, based on plant condition.

The plants were grown in the University of Bristol's GroDome under 20°C, 16 hour day lengths.

5.2.5 Soil

Soil was made at ¾ F2 compost (Levington F2 Seed and Modular Compost, Suffolk, UK) and ¼ horticultural sand (Melcourt Horticultural Silver Sand, Gloucestershire, UK). These were mixed, sieved to remove debris, and autoclaved 15 minutes at 121°C or frozen -15 to -30 °C for 24 hours to sterilise.

5.2.5.1 Plant growth conditions

The soil was transferred to pots (12x) in a tray containing water. The soil was left to absorb water for 5 minutes and sterilised seeds were pipetted onto the surface. The plants were grown in the University of Bristol's GroDome under 20°C, 16 hour day lengths.

5.2.6 Treatments of T. aestivum *plants*

5.2.6.1 Leaf transformation

Full leaves were cut from wild type plants. An equal number of leaves were placed into one of four conditions in sterile 50 mL Falcon tubes. Condition 1 was CNDs and DNA in distilled water and TE buffer pH 8.0; Condition 2 was CNDs in distilled water and TE buffer pH 8.0; Condition 3 was DNA in distilled water and TE buffer pH 8.0; Condition 4 was distilled water and TE buffer pH 8.0.

The leaves were placed with the cut petiole in the solution. These were incubated at room temperature in the dark for 24 hours.

5.2.6.2 Seed transformation

Surface sterilised seeds were placed in 50 mL falcon tubes (manufacturer) with 25 mL liquid MS 4.4 g/L as recommend by the manufacturer and incubated at 22°C, shaking at 120 RPM with constant light for 24 hours.

The MS was removed, and the seeds were separated into four 50 mL falcon tubes. 25 mL liquid MS was added to each and the conditions in 12.1.2 with the solutions prepared as in 12.1.1. However, the amounts of CNDs, DNA and TE buffer pH 8.0 were increased to 85µl to improve uptake in a more dilute end solution. The tubes were incubated in the same manner for another 24 hours. The seeds were washed with distilled water three times. This method was adapted from Feldmann and Marks, (1987).
Seeds were planted Care was taken to avoid any cross contamination from water run off by adding water only to the tray.

5.2.6.3 Hydroponics transformation

Seeds were sterilised and planted as described into hydroponics. Five separate hydroponics boxes were used to prevent contamination. They were grown under Grodome conditions until flower spikes emerged. CND and DNA and TE buffer pH 8.0 was added to two of the boxes. One box had the CND and DNA complex added once, and the other had the complex added once a week. The other three boxes were used as controls, one with DNA and TE buffer pH 8.0, one with CNDs and TE buffer pH 8.0 and one was left to grow as a wild type control.

5.2.6.4 Protoplast transformation

Seeds were planted into soil. These were then grown in continuous darkness at 25^{o}C for 3-5 weeks, until leaves were well developed.
Between 8 and 10 leaves were cut from the plants using scissors and placed in a 1 L beaker of dH_2O. They were swirled to wash and left to soak for 5 minutes.
5 mL of plasmolysis buffer was added to a sterile 9mm petri dish lid and the leaves were cut in 0.5-1.0 mm strips using a sterile razor blade. The cut leaves were transferred to a beaker containing 50 mL enzyme solution and put under vacuum for 10 minutes. The enzyme solution/leaf mix was incubated at 23^{o}C for 24 hours.
A nylon mesh was coated with plasmolysis buffer and the enzyme solution was carefully poured through into a 50 mL Falcon tube. The tube was kept at a 45^{o} angle to prevent damage to cells from falling. The plasmolysis buffer was added to the plant tissue in the beaker in 5 mL, swirled and poured through the nylon mesh twice more.
The leaf/plasmolysis buffer mix was centrifuged for 4 minutes at 1200 RPM and the resulting pellet was resuspended in 3 mL W5 solution on ice for 30 minutes. During this time an aliquot was taken, and protoplast density was estimated using a haemocytometer. The solution was centrifuged in the same manner again and the supernatant was removed. The pellet was resuspended in MaMg solution at $1x10^6$ cells per mL.
The protoplast solution was aliquoted into 2 mL Eppendorf tubes and a CND/DNA/TE buffer pH 8.0 complex, prepared as described in 2.1.1, was added to one, with the other acting as a control. This was

incubated for 1 hour at room temperature in the dark before 1 mL incubation medium was added to each tube. These were incubated for 12-16 hours at 28^{o}C in the dark.
The cells were imaged on a fluorescence microscope immediately after the incubation period to prevent protoplast deterioration.

5.2.6.5 Foliar spray

CND, DNA, TE buffer pH 8.0 complex and control conditions were added to separate 100 mL spray bottles (Wilko Travel Spray Bottle 100ml, UK). 25 mL dH_2O was added to each and they were inverted 3 times to mix.
19-day-old plants were separated into separate trays, to prevent contamination, before spraying. The plants were kept separate during spraying and each plant was sprayed 3-4 times to coat the leaves. These were covered with lids to prevent contamination through evaporation and returned to their previous growth conditions.
The experimental condition was split, with half of the plants receiving the complex via spray once, while the other half was sprayed once to five times weekly and left 2 days before imaging.
Plants leaves were taken from mature plants for analysis using confocal laser scanning microscopy and fluorescence microscopy.

5.2.6.6 Vacuum infiltration

The DNA/CND/TE solution was made as in chapter 2. This was mixed via inversion of the tube with 0.02% silwet in 20 mL dH2O.
Young plants of 2-5 weeks old were removed from the soil and the roots were washed with dH2O. The aerial portion of the plant was placed into a square 90mm petri dish filled with the solution. This was place inside the Nalgene Vacuum Desiccator and was put under vacuum for 2 minutes and was left in the closed vacuum for 3 minutes before being removed.
The plants were rinsed with dH2O to remove the solution and replanted. They were allowed to grow, and leaves were taken for analysis.

5.2.6.7 Leaf injection

A solution of Cas9-GFP/CND/TE [85ul of each] was produced using methods from chapter 2. This was vortexed for 5-10 seconds and mixed with 20 mL dH2O.
The solution was added to a sterile 3 mL syringe and any air was removed through depressing the plunger with the syringe held upright. The barrel tip was placed firmly against the leaf and the plunger was depressed for 5 seconds until the area around the barrel tip turned a darker shade than the surrounding leaf. The area injected was marked with a permanent marker and the plant was allowed to grow for 3-4 days before imaging.

5.2.7 Confocal microscopy

A Leica SP5 confocal laser scanning microscope was used to image treated plant leaves.

5.2.8 RT-PCR

An RT-PCR was performed by Dr Chris Bellas and gene sequences was carried out by Professor Keith Edwards at the University of Bristol, UK. Leaf samples from the foliar spray experiment were provided to be prepared and analysed.

5.3 Results

5.3.1 Protoplast transformation

Initial work was done on *T. aestivum* protoplasts. Transformed protoplast cells expressed fluorescent proteins that appeared to be colocalised with the CNDs (fig.1). When compared to control protoplasts the level of fluorescence was greater and was expressed in multiple areas of the cell (fig.1). Once the treatment was found to be successful it was applied to mature plants. A more appropriate plasmid was used that was easier to identify with nuclear-targeted GFP and allowed Spo11 gene editing.

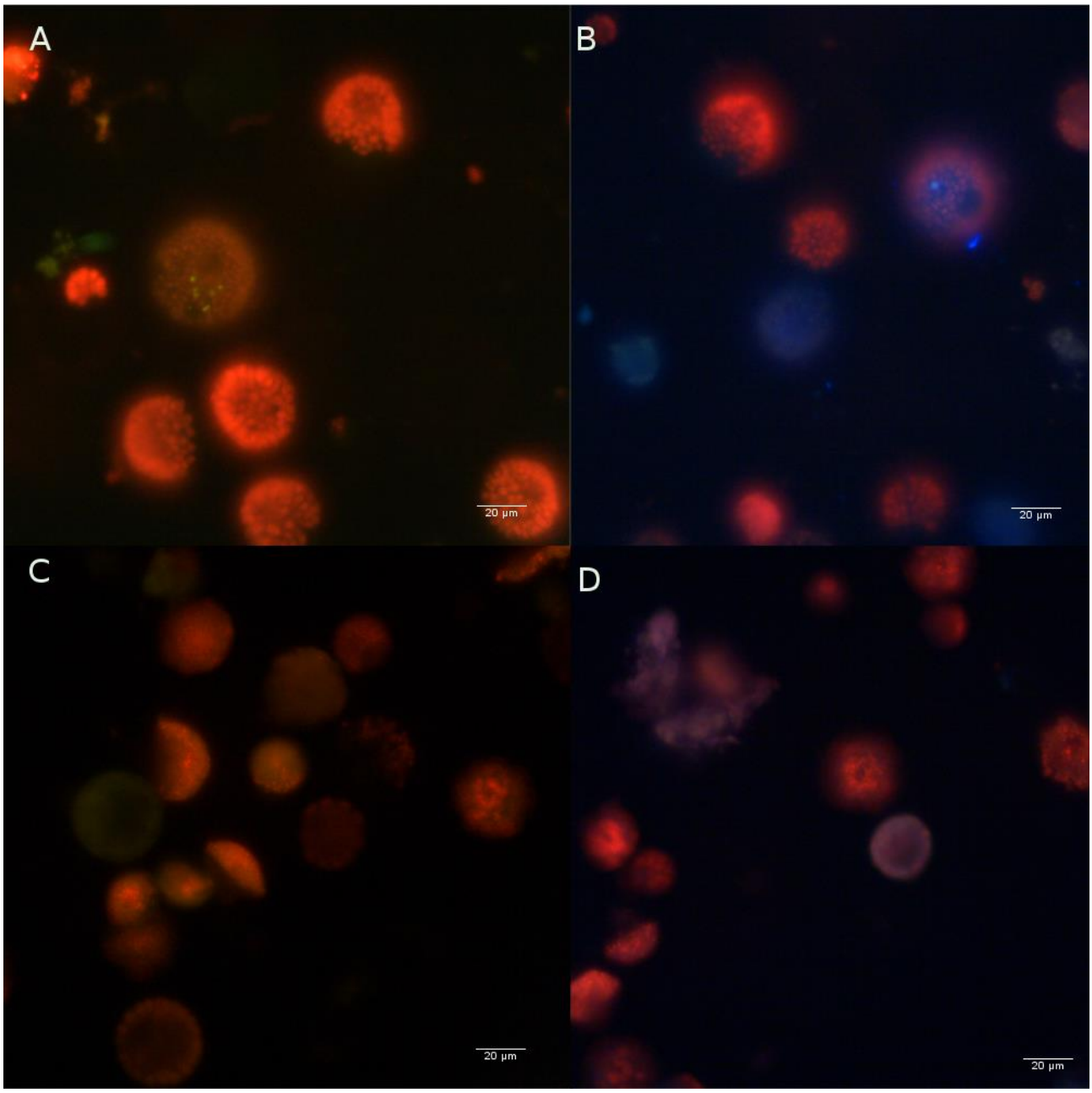


Figure 1: Fluorescence microscope images of protoplast transformations using CND-127Y conjugations. **A** YFP filter of 127Y-CND-treated protoplasts **B** Ultraviolet filter of 127Y-CND-treated protoplasts **C** YFP filter of untreated protoplasts **D** Ultraviolet filter of untreated protoplasts.

5.3.2 Treatment efficiency

The treated, mature *T. aestivum* leaves were examined with CLSM and fluorescence microscopy, and the number of cells expressing the protein of interest and the density were calculated for each treatment (Table 1). When compared there was a clear difference in transformation success depending on which method of application was used. The multiple spray method was the most effective, with a greater number of transformed cells than any other method (fig.2). The single spray method was the next most effective, followed by the injection, dipping and vacuum infiltration (fig.2).

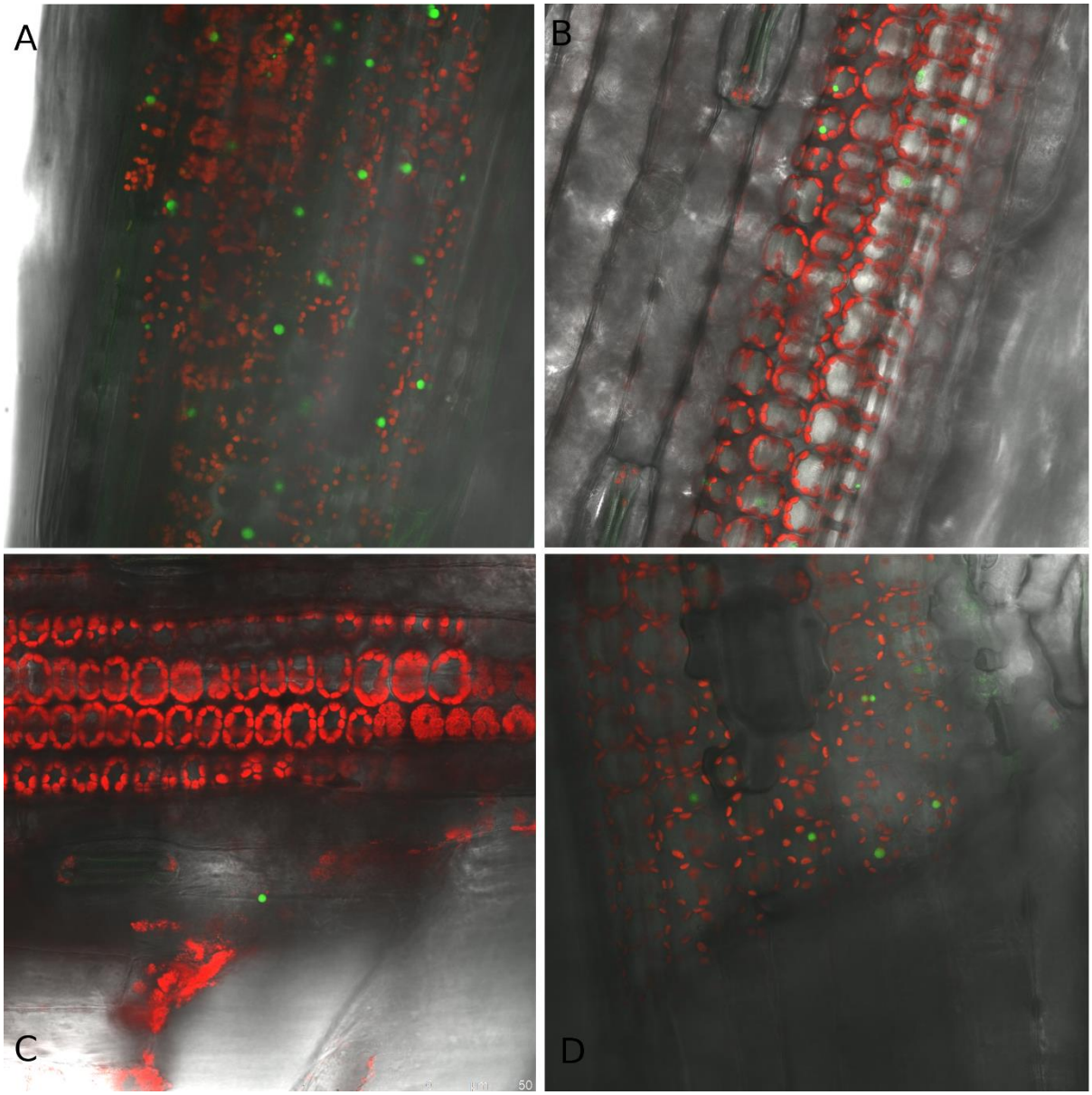


Figure 2: Treatment type comparison for CND+Cas9/GFP plasmid on *T. aestivum* leaves. Nuclear-targeted GFP was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification. **A** Foliar spray **B** Leaf injection **C** Dipping **D** Vacuum infiltration.

Table 1: Treatment type and fluorescence percentage of transformation in *T. aestivum* leaves.

Treatment type	Average fluorescence percentage (%)
Seed transformation	N/A
Foliar dipping	4
Foliar spray	21
Vacuum infiltration	13
Leaf injection	18

The expression of the protein of interest was found to be located predominantly in the mesophyll cells (fig.3). Plants treated with the CND-pDNA solution expressed fluorescent proteins at a greater level than control plants and the CNDs appeared to be colocalised with the fluorescent protein expression (fig.3).

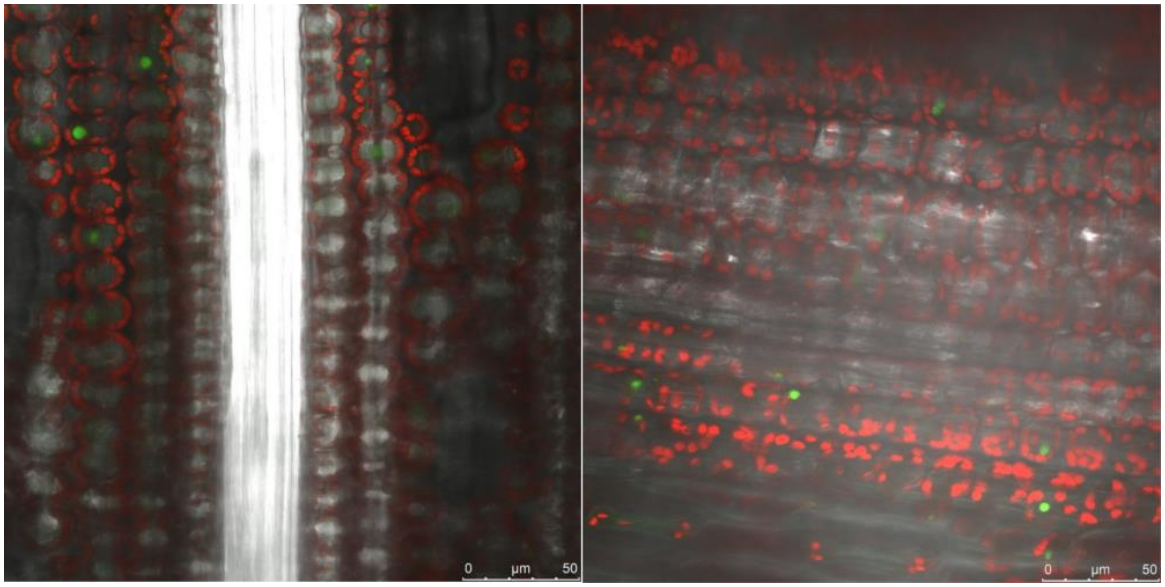


Figure 3 – The expression of GFP in CND+GFP/Cas9-treated *T. aestivum* leaf cells. Nuclear-targeted GFP was detected using a confocal laser scanning microscope (Leica SP5) at 63x magnification.

5.3.3 RT-PCR

Leaves from the foliar spray experiment were taken for analysis using RT-PCR. The analysis showed that gene editing was present in treated leaves, while it was not observed in the control leaves (fig.4). The Spo11 plasmid carried the Cas9 gene and guide RNAs (gRNAs) and targeted two regions of the Spo11 genes, approximately 250bp apart. If gene editing was successful, the sequence between these two points would be deleted.

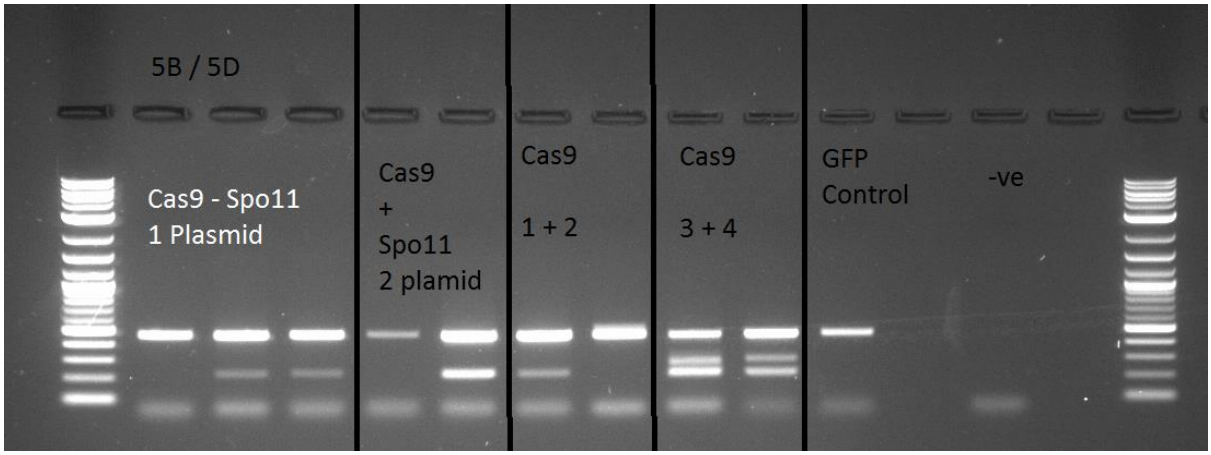


Figure 4 – RT-PCR of foliar sprayed *T. aestivum* leaves. Cas9 1+2 and Cas9 3+4 show the results of the leaves treated with Cas9/Spo11-CNDs. There were several controls: Cas9-Spo11, Cas9+Spo11, GFP control and a wild type (-ve). The Spo11 gene edits produced a PCR product approximately 230bp smaller than the unedited gene. This was sequenced and confirmed to have the correct gene editing by Professor Keith Edwards, University of Bristol.

5.4 Discussion

The CNDs were found to be present in the protoplasts in association with YFP fluorescence (fig.1). Previous studies had examined the use of electroporation of protoplasts (He *et al.,* 1994) which had been moderately successful. However, the use of CNDs to transform protoplasts allowed a non-invasive and non-damaging method to be applied, resulting in transformed protoplasts with few cells damaged or destroyed. As this transformation attempt was successful the treatment was then applied to unaltered, mature plants. Different methods were also found to affect the number of transformed cells. The most successful method was the multiple spray method, with a high concentration of transformed cells observed, while the vacuum infiltration and injection methods produced similar results (Table. 1/fig.2). The dipping method was the least successful but did produce several transformed cells (Table 1/fig.2). The multiple spray method being most successful suggested that multiple applications of the treatment allowed for a higher transformation rate, as all other factors remained consistent with the single spray method. The difference, therefore, in the success of the multiple spray method was due to multiple applications over times, which is likely because any cells missed in the initial application could become transformed in later applications. It has been found that floral sprays in *Arabidopsis thaliana* plants have produced results which are comparable to the vacuum infiltration method, similarly to what was observed in this experiment (Chung, Chen & Pan, 2000). The study applied to spray one to three times, which was similar to this experiment, with sprays applied one to four times, but across several days. However, the dipping method has been noted to be equally successful to vacuum infiltration in *A. thaliana* (Chung, Chen & Pan, 2000), which suggests that *T. aestivum* plants differ in their susceptibility to dipping. This may be due to a different composition of chemicals in the wax cuticle of the leaves (Wang *et al*., 2015), or that the wheat plants used for dipping should have been

covered prior to being submerged in solution, as this has been found to increase the likelihood of success (Clough & Bent, 2008).

It was found that, with each treatment, the number of transformed cells was highest in the mesophyll region of the leaves, with few cells transformed in the epidermal region unless targeted by specific plasmid sequences (fig.2). CNDs were observed in spaces between cells shortly after the application which suggested that CNDs travel through with water and small nutrient particles, which are transported via the lumen of the xylem, the middle lamella and between cell walls (Crowdy & Tanton, 1970). Previous work involving carbon-based nanoparticles has found that the nanoparticles travel through the xylem and vascular tissues, cortical cells and vascular bundles (Cifuentes *et al.*, 2010), Areas of free space, such as in the mesophyll, have been found to have a high level of water transport (Crowdy & Tanton, 1970), which allowed the transport of the CNDs and associated plasmids to travel more efficiently to cells in that region. The accumulation of CNDs around stomata and trichomes was likely due to the CNDs being carried out of the plant as water evaporated during gas exchange (Crowdy & Tanton, 1970).

The RT-PCR results showed that gene editing had taken place when *T. aestivum* had been treated with a plasmid containing GFP-Ca9/Spo11 (fig.4). This is one of the first instances of spray-on gene editing, transiently deleting a region of the Spo11 genes in somatic cells of the mature plants. While GFP expression had been observed in the plants previously, it had not been certain whether gene editing would be possible using the CND-transformation system. This result demonstrated it was possible to genetically edit mature *T. aestivum* plants using a simple spray-on application. This would allow a much simpler method of gene editing, without the need for protoplast extraction and plant regeneration as is currently the case (Nogué *et al.,* 2016). This method would permit the use of a gene-editing plasmid, such as the GFP/Spo11 plasmid, which could easily be applied to a mature plant, enter cells and genetically edit them in a relatively short time. Once this method has been optimised it would be logical to examine other plasmids and even siRNAs which could be applied to enhance desirable traits or removed undesirable attributes for the offspring of mature plants. The gene editing found in this study appeared to not enter the germline and so future work will need to focus on stable transformation and stable editing of mature plants in order to produce offspring which express the desired traits.

However, transient gene editing is sought in the production of recombinant protein production systems, such as pharmaceuticals, which have a lower cost and great potential to expand (Krenek *et al.*, 2015). This includes areas such as vaccinations and industrial enzymes (Yusibov, Streatfield & Kushnir, 2011). As this method is extremely versatile it could easily be applied on an industrial scale, such as via sprinklers, which would permit the method to be used easily in pharmaceutical and industrial protein production. This method has many benefits and could allow a greater level of research in the area of plant gene editing.

Conclusions

The CND transformation method has been shown to have a variety of application methods available, with others currently under investigation to establish germline transformation, including floral sprays and floral dips. This research has established a new transformation method with the potential to be applied to numerous species in a multitude of ways.

As observations have shown the transformation methods to vary, future work will focus on optimising the transformations systems which have produced the most successful transformant, such as the multiple spray application. This method will be altered in order to focus on germline transformation, with multiple sprays being directed into *T. aestivum* spikes or onto developing embryos, to create expression of the gene of interest in future generations.

6.Conclusions

6.1 Summary of findings

While the current methods of GM, including *Agrobacterium*-mediated and particle bombardment-mediated GM, have successfully transformed plant species they are limited in several areas. The need for a system that can be applied to mature plants, without the need for regeneration and with limited damage to cells (Zupan & Zambryski, 1995; Ortigosa *et al.,* 2012), would allow a much greater application in industry and reduce the resources required.

To improve on current methods and overcome these limitations the use of nanotechnology has been examined. While earlier forms of nanotechnology required costly materials and expert knowledge newer, carbon-based forms have proven to be more appropriate for use in areas such as medicine and GM. Carbon nanotubes (CNTs) have had various applications due to their properties, especially recently from wastewater treatment (Kavosi *et al.,* 2018) to breast cancer treatment (Zhang *et al.,* 2019). CNTs were previously found to be effective in transferring genetic material into plant cells but had the issue of causing cell damage and necrosis (Bianco, Kostarelos & Prato, 2005).

Another form of carbon-based nanomaterial was examined as an alternative, carbon nanodots (CNDs). These were simple and cheap to produce, while being less damaging to cells as they did not rely on puncturing cells to transfer genetic material. Core CNDs were modified with PEG diamine to attach genetic material via electrostatic bonds, and these were mixed with plasmid DNA and TE buffer. This mixture was applied to two plant species, *Arabidopsis thaliana* and *Triticum aestivum*, in various ways to determine the most successful method. *A. thaliana* was used to compare the effectiveness of the CND transformation system compared to those already being used, as it is a model plant species for GM (Koornneef & Meinke, 2009), while *T. aestivum* was used to test the effectiveness of the CND method of GM in a crop species that requires a new transformation system.

The movement of the CNDs with and without genetic material appeared to be via the vascular tissues, as the majority of the CND autofluorescence was found in cells surrounding that area. How the CNDs moved through the vascular tissue was not determined, as it was unclear if they travelled via the xylem or phloem. It was also not determined what caused CNDs to drop their cargo, such as genetic material.

It was found that while most methods were successful, resulting in transiently transformed plant cells, multiple foliar sprays resulted in the highest percentage of transformed cells. Based on this work it appears that CNDs have the potential to be used for GM on a large, industrial scale, as they are efficient, effective, cheap and can be applied to both monocot and dicot plant species. It was also found that CNDs could carry plasmids for gene editing, as shown in chapter 5. This new method could see spray-on gene editing become reality, simplifying the process and making it a more accessible area of research.

6.2 Limitations of the CND method of GM

Due to the nature of CNDs being nanoscale it is difficult to monitor how they travel throughout the plant. While CNDs are fluorescent, this is on a low level and difficult to detect. The fluorescence is emitted from individual nanodots which are difficult to detect using a standard CLSM or fluorescence microscope, but instead 'clouds' of fluorescence can be seen, suggesting that larger groups of CNDs have formed. This would mean that CNDs may be present in other cells, but not be noticed due to their weak fluorescence and small scale.

It appeared they traversed the plant via the vascular tissue, but it was unclear if they moved through the xylem or phloem, and how they were able to move between cells. As most transformed cells appeared to be towards the tip of the leaf this suggests that CNDs move via the xylem, but the actual dispersion route of them throughout the plant is unknown.

It also appeared that while CNDs did move through plant tissues it was unpredictable which cells would be transformed. The cause of this was unclear but may be linked to cell pH, as this can vary across plant cell types and also in response to growth conditions, such as drought (Wilkinson, 1999). This would be an issue if specific cells needed to be targeted as the CND transformation method is difficult to control and direct. This lack of control meant the CND method of GM did not result in stable transformants or any pDNA being transferred into the germplasm. This may be due to CNDs releasing their pDNA cargo in the first cells they encounter and so they would not carry the genetic material far enough to reach the germplasm. This may be an issue if stable transformants were required, but also shows that CNDs will not deliver genetic material to the germplasm if applied to other areas of the plant. However, this may also be a benefit of this system. Transient transformations of plants have various applications, including testing the function of a construct before investing high amounts of time and money on producing stably transformed plants (Schillberg, Fischer & Emans, 2003). This could be used to test new vaccinations or other pharmaceuticals at a fraction of the price of stable transformations, and much quicker, usually within 2-3 days of the treatment (Schillberg, Fischer & Emans, 2003; Fischer *et al*., 2004). Not only could transient transformation be a huge benefit to biopharmaceuticals, it is much more convenient and cheaper for performing bioassays, such as with the FAST system in Arabidopsis (Li *et al*., 2009).

While the use of CNDs for transient GM was successful in most cases, the method did not work on seeds. This may have been due to CNDs being unable to penetrate the seed coat, as this is a barrier to protect the embryo from external factors. However, the seeds were soaked in a CND+pDNA solution without modification, and it may have been of benefit to puncture the seed coat to allow entry.

6.3 Future Work

The limitations of the CND method of GM suggest areas in which future work could be carried out. This method has its own limitations, including a lack of control, unclear routes of movement through the plant

and being unpredictable, which would need to be resolved before being applied in industry to ensure health and safety requirements were met.

The route the CNDs travel is still unclear and would be useful to determine as these areas would be exposed to more CNDs overall than the rest of the plant. The initial findings suggested that CNDs travel via the vascular tissue, but it was not clear if this was through the xylem or phloem. This could be investigated using time lapse fluorescence imaging or using a marker dye that would bond to the CNDs. To determine if the xylem or phloem was the primary route of movement one portion could be blocked or inhibited and CND travel could be imaged using methods in chapters three to five.

Once their movement was determined, it would be possible to attempt to alter the route and give the CNDs more direction, even targeting specific cell regions. It should also be examined what factors influence CNDs to drop their pDNA cargo in the cells and whether this could be manipulated to allow drops in specific locations.

There is also the question of the limitations of what CNDs can transport into and between cells. It should be examined what CNDs can carry, including smaller genetic material such as RNAs, or larger molecules such as proteins. It could also be tested whether CNDs could carry multiple cargos of different types, DNA and RNA for example, as this would permit fewer treatments to cause multiple transformations. It may be that CNDs act in a similar manner to histone proteins, interacting with genetic material and wrapping it around itself, making the material small enough to enter cells without active transport (Sims III, Nishioka & Reinberg, 2003). If this was the case then protein movement using CNDs would be unlikely, as the proteins would not wrap around the CNDs and so would remain too large to enter the cells. It may also be that CNDs, with their electrostatic charge, could affect the cell membrane, but this would need to be investigated using marker dyes.

As this technology would be of use in industry and research the effects of CNDs on a larger scale would need to be investigated. This would involve looking into optimising application methods so that CNDs could be applied to large numbers of plants, as well as improving the efficiency of transformation. Potential issues may be if CNDs have an impact on soil composition or function less optimally in specific soil types, affecting efficiency and success rates of transformation.

Due to the impact of CNDs on more biotic and abiotic factors being unknown it would be useful to test how CNDs may affect the environment. They may affect factors including pH of the soil and the soil microbiota among other areas, which would need to be considered before CNDs were applied to agricultural land.

6.4 Conclusions

This research has determined that not only can CNDs easily enter plant cells, seemingly via the vascular tissues, but can also carry genetic material and successfully transform cells of both monocot and dicot species. The multiple foliar spray method was found the be the most effective, but more research would be required, especially regarding large-scale applications such as in industry.

These finding present the option of a cheap and easy method for GM which can be applied to crop species. While more work is required to determine its limitations, this technology could be one of the first steps to overcoming current issues with GM, leading to a reduction in the bottleneck and expanding options available for industry and research.

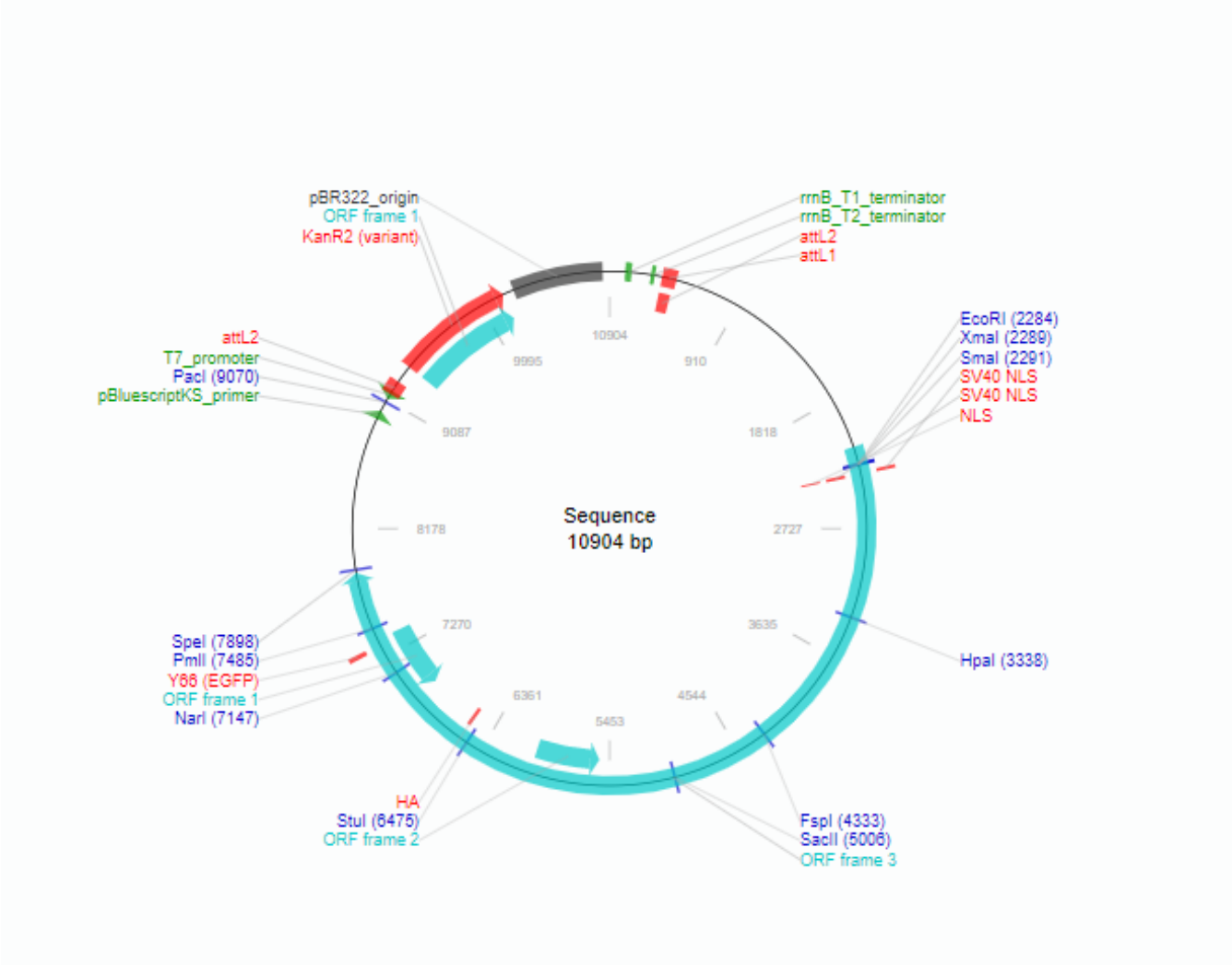
7. Supplementary material

7.1 Graphs and Statistics

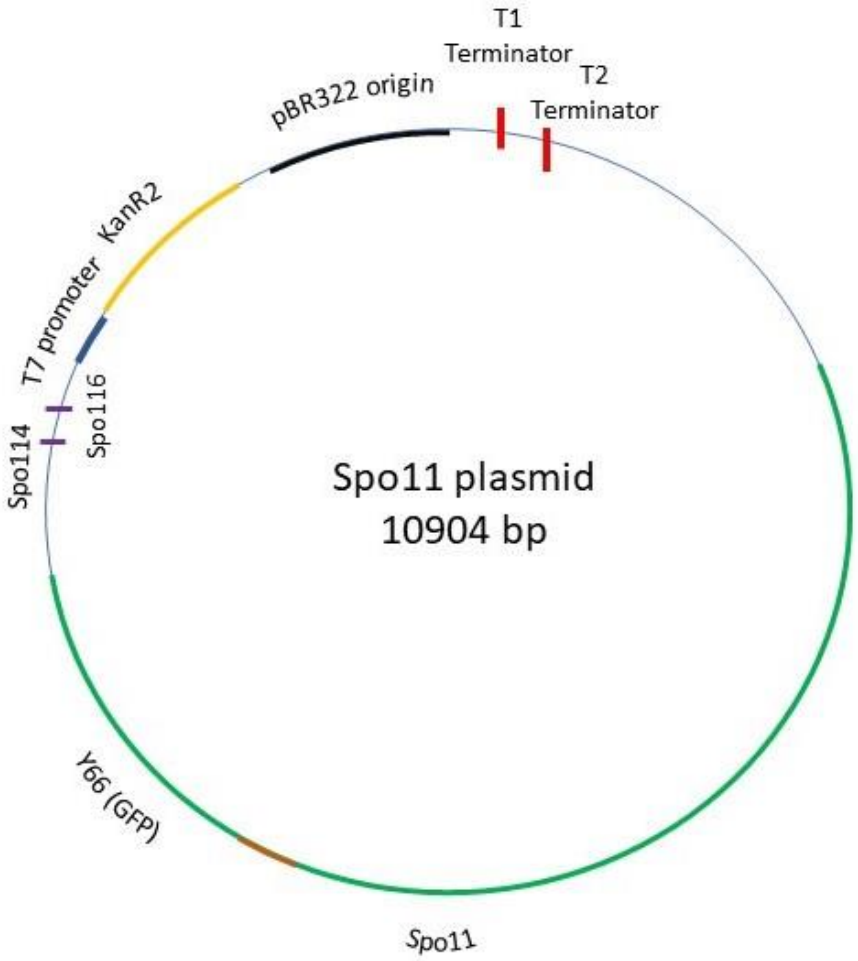
Plots were primarily produced using Microsoft Office Excel 16, while any others were made using MatLab R2018a.

7.2 Plasmid Maps and details

7.2.1 Spo11 Plasmid



Source: Addgene



Graphic created using Microsoft Office Powerpoint 16. Shows the locations of the Spo genes and GFP gene.

The cas9 construct was in pEntry 4 (Kan resistant)

Attl1 site 350-450 bp

Ubqu promoter 460-2280

Wheat optimised Live Cas 9 2280-6615

ATG at 2334

Porcine 2A 6620-6704

Histone 2A targeting sequence 6705-6941

Wheat optimised GFP 6942-7874

Nos terminator 7875- 8100

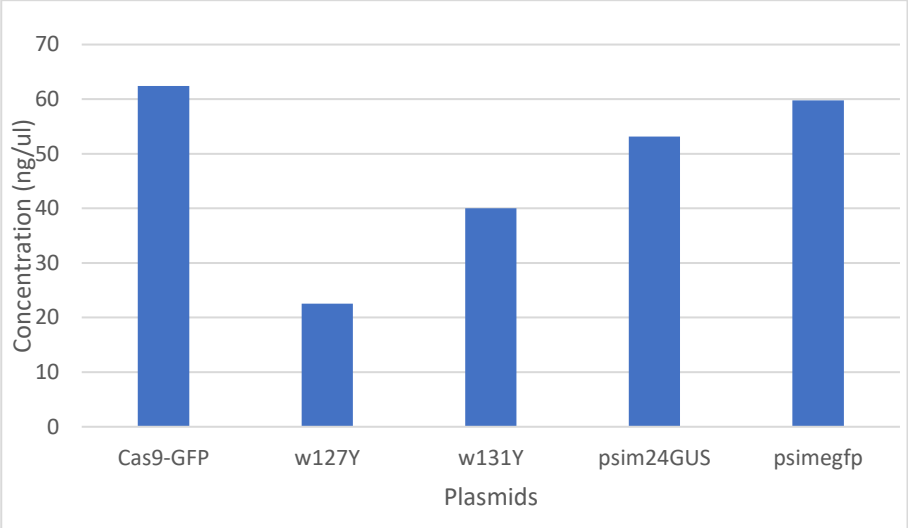
BssHII sites (used to clone in sgRNAs 8100 and 8124)

Attl2 8235-8330

Rest of vector 8,330 - 10,904 (courtesy of Professor Keith Edwards, University of Bristol).

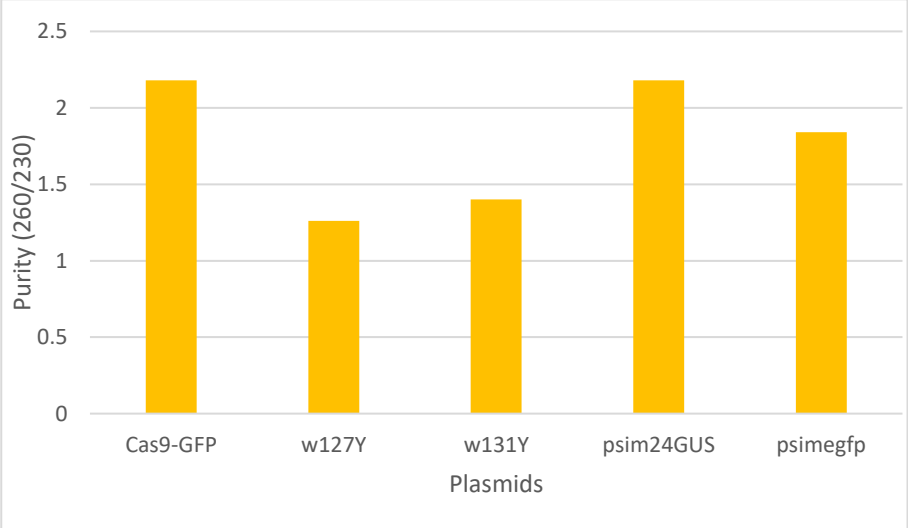
7.3 Nanodrop

7.3.1 Plasmid concentrations



Plasmid	Concentration ng/ul
Cas9-GFP (Spo11)	62.43
W127Y	22.55
W131Y	39.99
Psim24GUS	53.16
Psimegfp	59.8

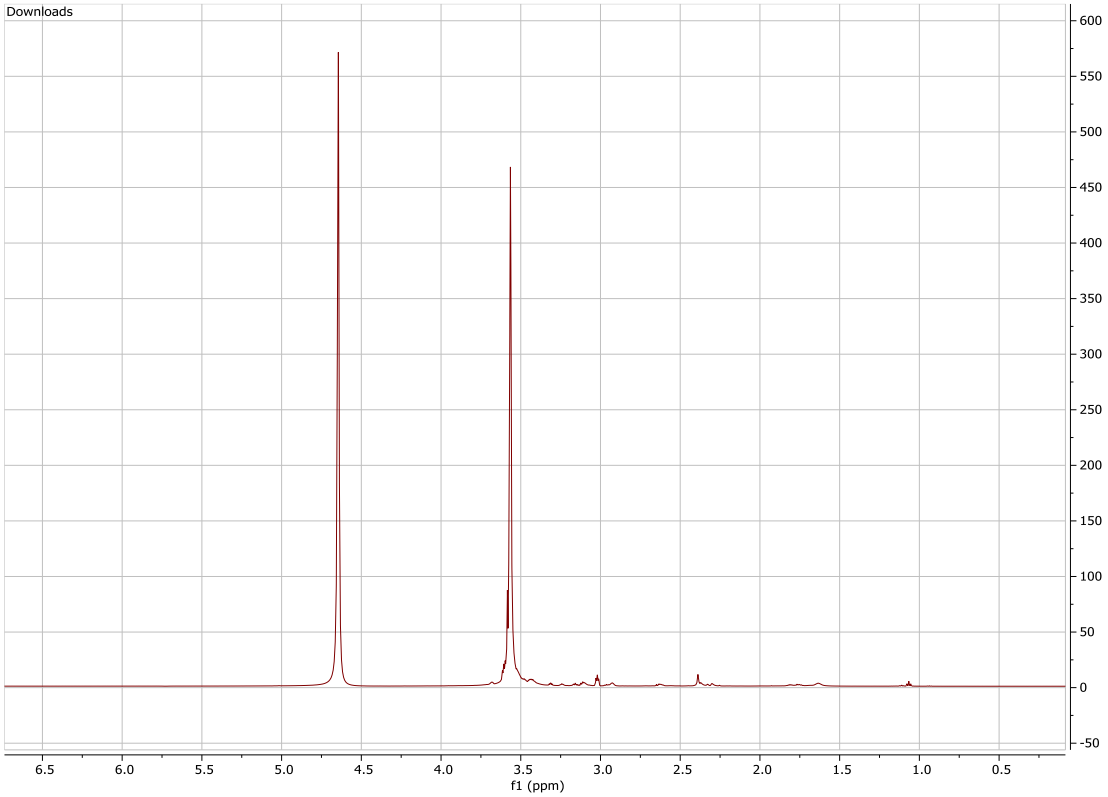
7.3.2 Plasmid purity



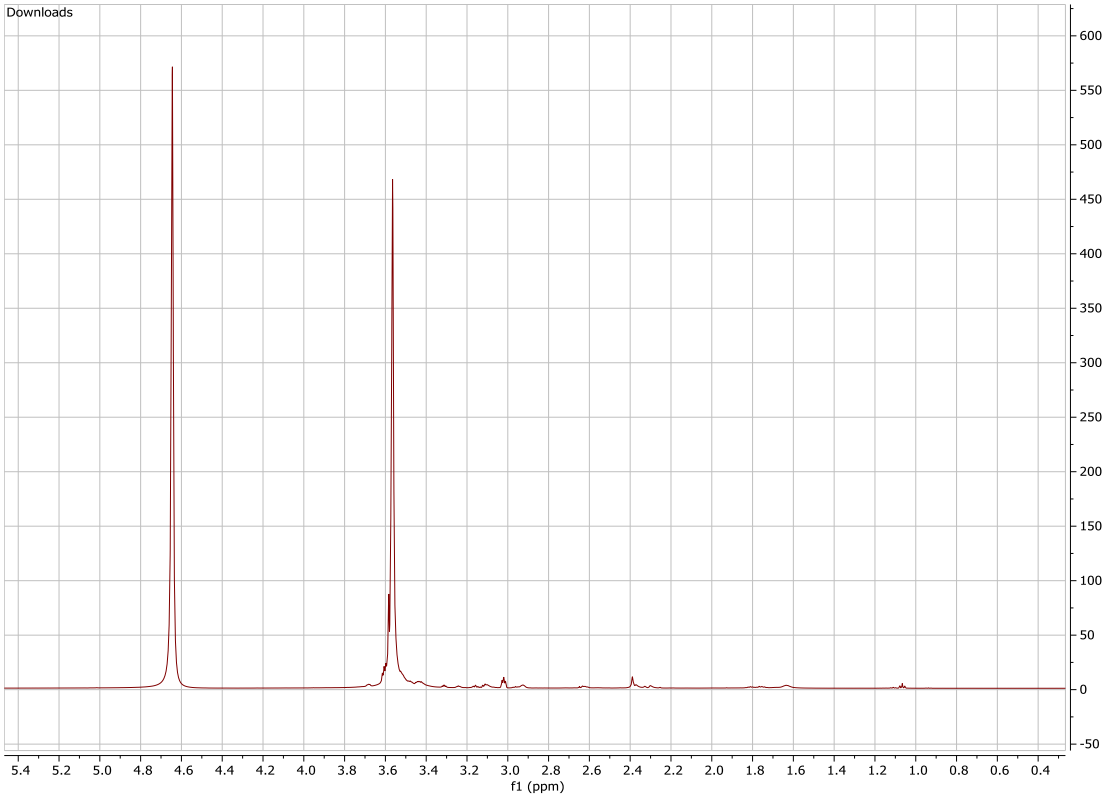
Plasmid	Purity (260/230)
Cas9-GFP (Spo11)	2.18
W127Y	1.26
W131Y	1.4
Psim24GUS	2.18
Psimegfp	1.84

7.4 NMR

7.4.1 CNDs with PEG 1000 Mw



7.4.2 CNDs with PEG 600 Mw



7.5 Confocal laser scanning microscopy

The CLSM used was a Leica TCS SP5 X. Magnification was 63x.

CLSM settings were as follows:

GFP – 488nm laser, emissions between 500-540nm, 20% power.

YFP – 514nm laser, emissions between 520-550nm, 20% power.

CNDs – UV laser (Fluo3), emissions between 415-470nm, 15% power.

Chlorophyll – 514nm laser, emissions between 644-713nm, 20% power.

8.References

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