Molecular characterisation and expression profiles of the *CP4 EPSPS* gene in field-tested transgenic onions (*Allium cepa* L.)

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Abstract

The onion (*Allium cepa* L.*)* is the most agronomically important vegetable crop to New Zealand. In temperate climates, such as New Zealand, the most prominent problem associated with onion production is weed control. To address this problem, glyphosate-resistant onions were generated in a range of germplasm backgrounds via *Agrobacterium*-mediated transformation of immature embryos (Eady et al. 2003a). This thesis contains an analysis of these onions.

PCR and Southern analysis were used to create molecular profiles for individual transgenic events. Of the 17 putatively transgenic onion lines tested, 16 contained a single copy of the *CP4 EPSPS* gene integrated into their genomes, and one line had two copies of the gene. The spectinomycin resistance gene, located on the backbone of the vectors used in the transformation process, was detected in two of the glyphosate-resistant onion lines tested.

Ten different glyphosate-resistant onion lines were subjected to field trial evaluation over the 2004/2005 growing season in Canterbury. Glyphosate-resistant onion seedlings were transplanted into the field in a randomised block design and a subset of plants, chosen to represent selected lines, were sampled. Tissue was harvested from young leaves, old leaves, and bulbs.

Quantitative multiplex RT-PCR was used to assess expression of the *CP4 EPSPS* transcript, and protein levels were determined using an ELISA-based assay. The transgene appears to be highly transcribed in some lines, as expression of the *CP4 EPSPS* gene was in the same order of magnitude as the highly transcribed 18S internal control. The CP4 EPSPS protein levels of the glyphosate resistant onion lines presented in this thesis ranged between 0.36–7.44 µg CP4 g^{-1}FW in the leaf samples, which is a little lower than, but still similar to, values reported for glyphosate resistant corn and poplar.

This project reports the first in-depth analysis into the expression of the *CP4 EPSPS* gene in field-grown glyphosate-resistant onion lines.

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Abbreviations

ERMA	Environmental Risk Management Authority
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
CP4 EPSPS	Glyphosate-insensitive EPSPS gene from *Agrobacterium tumefaciens* strain CP4
MAF	Ministry of Agriculture and Forestry
PEP	Phosphoenol pyruvate
S3P	Shikimate-3-phosphate
EPSP	5-Enolpyruvylshikimate-3-phosphate
P_i	Inorganic phosphate
MurA	UDP-*N*-acetylglucosamine enolpyruvyl transferase
CaMV 35S	Cauliflower mosaic virus 35S promoter
P-Ract1	Rice actin promoter 1
gat	Glyphosate N-acetyl transferase gene
gox	Glyphosate oxidoreductase gene
AMPA	Aminomethylphosphonic acid
DNA	Deoxyribonucleic acid
T-DNA	Transferred DNA
GFP	Green fluorescent protein
FMV	Caulimovirus figwort mosaic virus promoter
HDGS	Homology-dependent gene silencing
aadA	Spectinomycin resistance selectable marker gene
PCR	Polymerase Chain Reaction
TAIL-PCR	Thermal Asymmetric Interlaced-PCR
GMO	Genetically modified organism
PLK	Pukekohe Long-Keeper
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
ELISA	Enzyme-linked immunosorbent assay

Chapter One

Introduction

1.1. Overview

Onions (*Allium cepa* L.*)* are the most agronomically important vegetable to New Zealand with exports contributing $92 million to the economy in 2004 (NZ Horticulture Facts & Figures 2004, http://www.hortresearch.co.nz/files/aboutus/factsandfigs/ff2004.pdf). Onions account for 42% of New Zealand's total vegetable exports and are exported to 44 countries. They are the world's second most economically important vegetable crop (FAO 2006, http://faostat.fao.org/). In part, the popularity of onions can be attributed to the unique sulphur metabolism pathways of the *Allium* species. These pathways are responsible for the distinctive odour and flavour characteristics, and are the basis for the numerous claims of health and nutritional benefits (Bloem et al. 2004).

Weed control is the single most substantial cost involved in onion production (Rabinowich and Brewster 1990), particularly in temperate climates such as New Zealand. The short, upright stature and thin foliage of onion plants does little to diminish the amount of light available for competing weeds, and their relatively slow growth means that they can be easily overgrown. Also, they are shallow-rooting plants and have a low density of roots per unit of soil volume, so are easily out-competed for water and soil nutrients (Ghosheh 2004).

Herbicide-resistant lines of onion were produced in a range of germplasm backgrounds via *Agrobacterium*-mediated transformation of immature embryos at Crop & Food Research between the years 2000 – 2002 (Eady et al. 2003a). These onion lines were maintained within a containment glasshouse, where functionality of the transgene was evaluated by glyphosate spray tests, until enough glyphosate-tolerant lines were developed to warrant field assessment. However, in order to carry out field trials of genetically modified organisms in New Zealand, the national regulatory body, the

Environmental Risk Management Authority (ERMA), require an exhaustive application process. For the herbicide-resistant onions, this spanned many months and consisted of public hearings where scientific experts were consulted, and members of the general public were given the opportunity to relate their opinions and concerns. During this process, an overall evaluation of risks, costs and benefits were considered and weighed by ERMA. The principal benefit was considered to be that of scientific and other knowledge generated by the field trial and this was found to outweigh the potential risks of field testing the genetically modified onions in containment (ERMA Decision: Application GMF03001 and associated evaluation and review report http://www.ermanz.govt.nz/search/registers.html?aid=GMF03001).

This project is an investigation into the expression of the *CP4 EPSPS* gene in a number of these glyphosate-resistant onion lines grown under field conditions and treated with a glyphosate-based spray regime. Some lines tested in this project have been selected for further development. Due to the legal framework surrounding deregulation, data from these lines have been omitted from this thesis.

1.2. Weed control in onions

Severe reductions in growth, quality and yield result from the presence of weed competition during onion production (Wicks et al. 1973, Rabinowitch and Brewster 1990). As onions require a long growing period, and establishment of weeds can occur at any point in crop growth, successive weed control measures are required (Ghosheh 2004). Currently, onion production utilises complex weed control programmes, involving extensive use of herbicides applied at defined stages of crop growth, to reduce yield loss associated with weeds (Rabinowitch and Brewster 1990).

Initially, pre-sowing applications are applied to remove weeds already present and those that will emerge before the crop. Subsequently, pre-emergence applications are applied which free the crop from early competition. The timing of these applications is crucial, as contact with the herbicide will damage or destroy any newly emerged crop plants.

Finally, post-emergence applications of selective herbicides are required to maintain weed control throughout the growth of the crop. Mature onion plants have an increased herbicide tolerance compared with young seedlings due to increases in leaf surface wax and rooting depth (Brewster 1994). Therefore, this treatment is employed once the onions have reached the more herbicide-tolerant post-crook stage of growth. The selection of appropriate herbicides, and the timing of usage, is crucial in ensuring minimal crop damage.

Onion crops can receive up to 13 herbicide applications throughout a season, with most of the chemicals classified as poisonous or toxic, and persistent in the environment (Eady 2001). Also, the fuel and equipment required to administer these numerous herbicide applications increases the cost of onion production. In 1999, the Ministry of Agriculture and Forestry (MAF) reviewed the usage of agricultural pesticides in New Zealand. Onions were found to receive the most intensive spray programme of all vegetable crops studied, and concerns were raised over the sustainability of the current plant protection strategies (MAF Policy Technical Paper 99/11, http://www.maf.govt.nz/mafnet/publications/techpap.html).

The use of non-chemical alternatives has also been explored for weed control in onion production, but is problematic. Combinations of mechanical weed control techniques fail to provide complete weed control (Rabinowich and Currah 2002) and also risk inflicting damage to the crop because the crop plants are usually small at the time when weeds are most vulnerable to these methods. Manual weeding is the only reliable alternative, but this is expensive as it is time-consuming and labour intensive (Melander and Rasmussen 2001). Also, total weed seed numbers in the soil seed bank have been shown to increase significantly after changing from conventional chemical weed control to non-chemical means (Bond and Grundy 2001). Hence, the levels of input required using non-chemical methods are not sustainable or economically viable.

1.3. Glyphosate tolerance

The advent of biotechnology offers an alternative to the weed control methods presently used in onions. The process of weed control in onions may be modified through the manipulation of a single enzyme within the onion plants to confer tolerance to the herbicide glyphosate.

Glyphosate (*N*-phosphonomethylglycine) is a systemic, non-selective herbicide which causes inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS is a nuclear-encoded, chloroplast-localised enzyme crucial for the manufacture of aromatic amino acids via the shikimate pathway (Amrhein et al. 1980, Bradshaw et al. 1997). As this biosynthetic process is unique to plants, microorganisms and fungi, glyphosate is relatively non-toxic to humans and animals (Kishore and Shah 1988). Glyphosate binds tightly to soil and therefore shows no pre-emergence or residual soil activity. It is also short-lived in the soil as it is readily degraded by microorganisms to produce phosphoric acid, ammonia and carbon dioxide (Franz et al. 1997).

The use of glyphosate-resistant onion lines, in combination with a glyphosate-based weed management strategy, has the potential to provide efficient and effective weed control in onion crops. In addition to reducing the complexity of the weed control regime, this technology offers a significant monetary saving estimated to be as much as $500/ha not including fuel costs (Eady 2001).

1.4. The shikimate pathway and the EPSPS enzyme

1.4.1. The shikimate pathway

The plastid-localised shikimate pathway (Figure 1.1) is crucial for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan) and many secondary metabolites (Haslam 1993). It has been estimated that aromatic molecules derived from the shikimate pathway represent around 35% of total plant mass in dry weight (Franz et al. 1997). The formation of the important branch-point intermediate, chorismate, in the shikimate pathway is the last common step towards the synthesis of the aromatic amino

acids. There are at least three pathways that branch off from chorismate. One pathway facilitates the formation of quinones, a second produces tryptophan, and a third leads towards the production of tyrosine and phenylalanine (Gorlach et al. 1993). A major portion of chorismate is invested into phenylalanine and subsequently the phenylpropanoid pathway. This pathway is responsible for the synthesis of numerous secondary metabolic compounds, some of which are lignans, phytoalexins, pigments, UV light protectants and antioxidants (Dixon and Paiva 1995). Tyrosine is the precursor for the formation of tocopherols and tocotrienols, collectively known as vitamin E (Rippert et al. 2004), and tryptophan is the major precursor towards the formation of the plant growth regulatory hormone indole-3 acetic acid (Wakasa et al. 2006). 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is the sixth enzyme of the shikimate pathway, catalysing a key reaction that directly precedes the formation of chorismate (Herrmann and Weaver 1999).

1.4.2. The reaction catalysed by EPSPS

EPSPS, a monomeric enzyme of between 44–48 kDa, catalyses the reversible transfer of the enolpyruvyl group of phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) with the liberation of inorganic phosphate (P_i) (Kishore and Shah 1988) (Figure 1.2). This reaction varies chemically from most others that utilise PEP in that C-O cleavage of PEP is undertaken instead of P-O bond cleavage (Bondinell et al. 1971, Eschenburg et al. 2003). The only other enzyme known to share this reaction mechanism, UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), is also the only known architectural homologue of EPSPS (Eschenburg et al. 2003). MurA is essential for bacterial cell wall formation and is the target of the antibiotic fosfomycin (Schonbrunn et al. 1996).

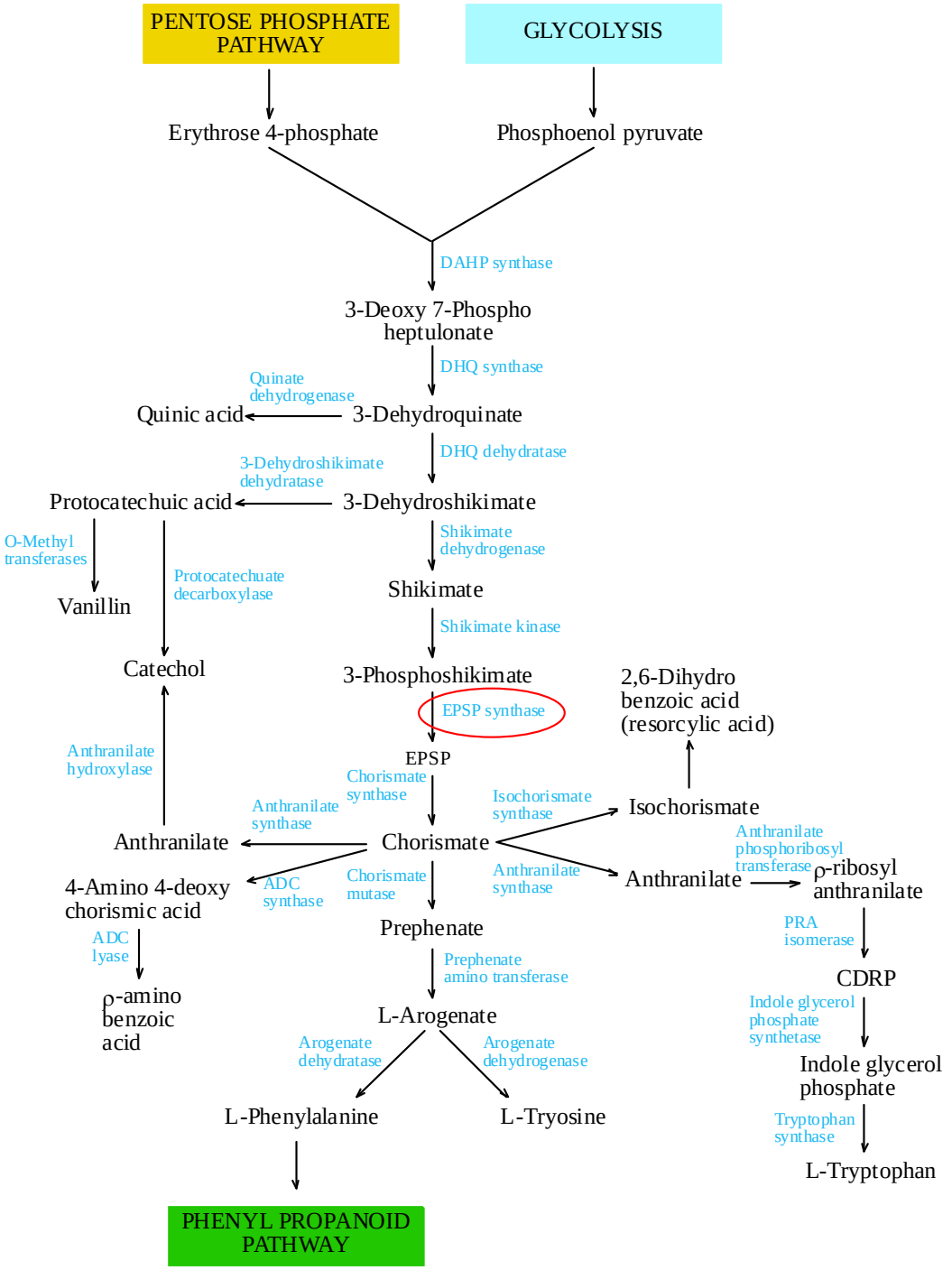

Figure 1.1. The shikimate pathway. 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS), circled in red, is the sixth enzyme in this pathway and is the target of the herbicide glyphosate (adapted from http://sites.huji.ac.il/malaria/maps/shikimatebiopath.html).

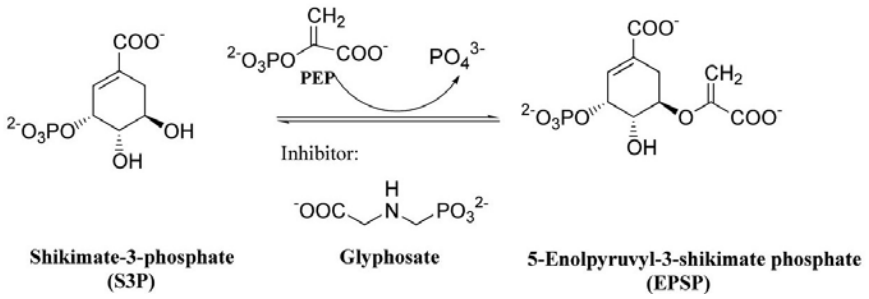

Figure 1.2. The reaction catalysed by EPSPS (Priestman et al. 2005).

1.4.3. Structure of the EPSPS enzyme

EPSPS consists of two distinct globular domains of equal size, each comprised of three sub-domains which are all replicates of a single folding unit (Figure 1.3). Each folding unit is made up of two parallel α-helices and a four-stranded β-sheet. The two domains are linked by two cross-over polypeptide segments creating an inter-domain cleft. The α-helices are oriented with their N-termini toward the cleft between the domains, forming a macrodipole. In each domain, three of the helices are buried and the surface of the molecule is formed from the three β-sheets and the solvent accessible faces of the other three helices. This structure has been referred to as a "mushroom button" or "inside/out αβ barrel" (Stallings et al. 1991).

The free EPSPS tertiary structure exists in an open conformation and undergoes a considerable conformational change upon substrate binding (Figure 1.4). The two domains approach each other in a screw-like movement with the active site forming in the cleft between the two domains (Schonbrunn et al. 2001). The α-helical macrodipole in this cleft creates a significant electropositive attraction for the binding of anionic ligands. X-ray structures and mutagenesis studies have shown that residues from both domains are required for ligand binding (Alibhai and Stallings 2001).

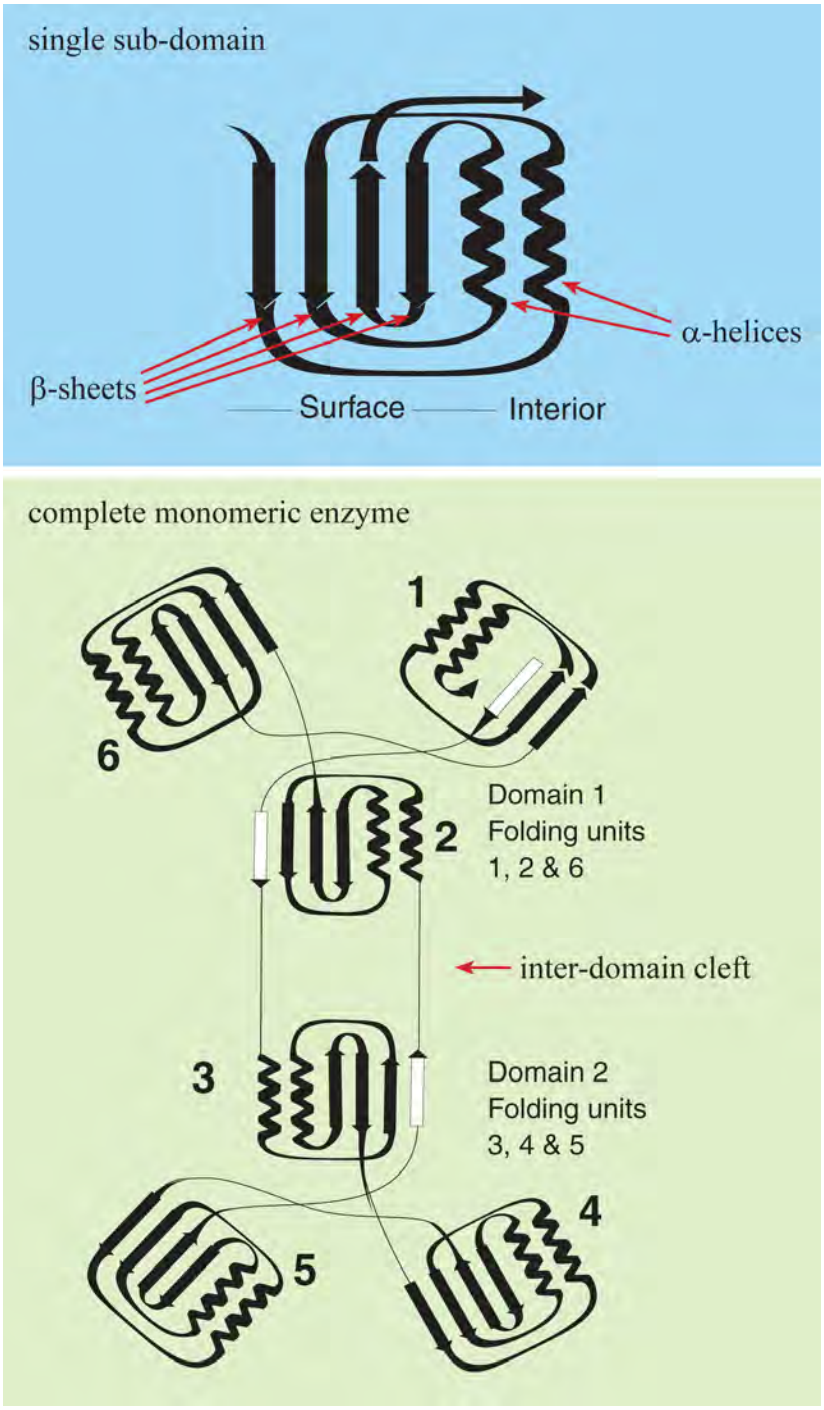

Figure 1.3. Folding and topological symmetry of *Escherichia coli* EPSPS (Alibhai and Stallings 2001).

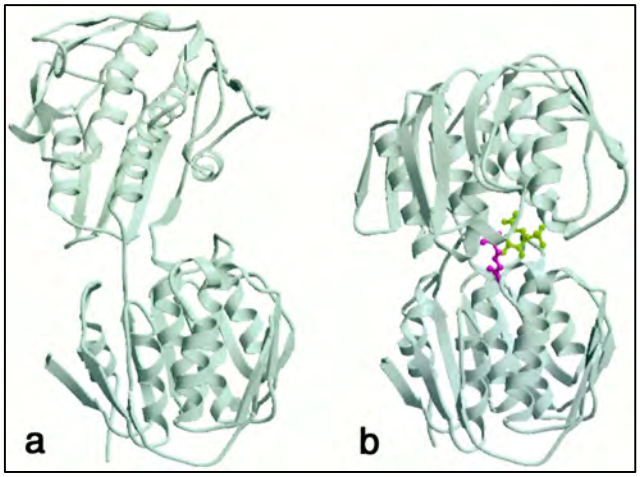

Figure 1.4. Diagram of EPSPS in the (a) open and (b) closed conformation (Schonbrunn et al. 2001).

Binding of substrates occurs in an ordered manner with S3P binding first and the second substrate, PEP, binding preferentially to the EPSPS-S3P binary complex (Kishore and Shah 1988).

1.5. Glyphosate inhibition of EPSPS

Inhibition of EPSPS is attained by glyphosate competing with PEP in binding to the enzyme in the presence of S3P (Steinrucken and Amrhein 1984). Glyphosate exhibits non-competitive behaviour with S3P, and is also non-competitive in the reverse reaction with EPSP (Kishore and Shah 1988).

There is substantial evidence that glyphosate occupies the PEP binding site in the EPSPS enzyme (Schonbrunn et al. 2001). However, glyphosate displays extreme specificity in its inhibition of EPSPS. EPSPS is its sole cellular target, and it does not inhibit any other PEP-utilising enzymes, including MurA (Steinrucken and Amrhein 1984). This has led to the conclusion that glyphosate does not directly mimic PEP.

Currently the most favoured hypothesis is that the bound ternary complex of EPSPS-S3P-glyphosate mimics the geometry of the enzyme-bound tetrahedral intermediate of S3P-PEP (Schonbrunn et al. 2001) (Figure 1.5). It has been suggested that the binding of S3P in conjunction with glyphosate stabilises the EPSPS enzyme in a closed conformation (Krekel et al. 1999).

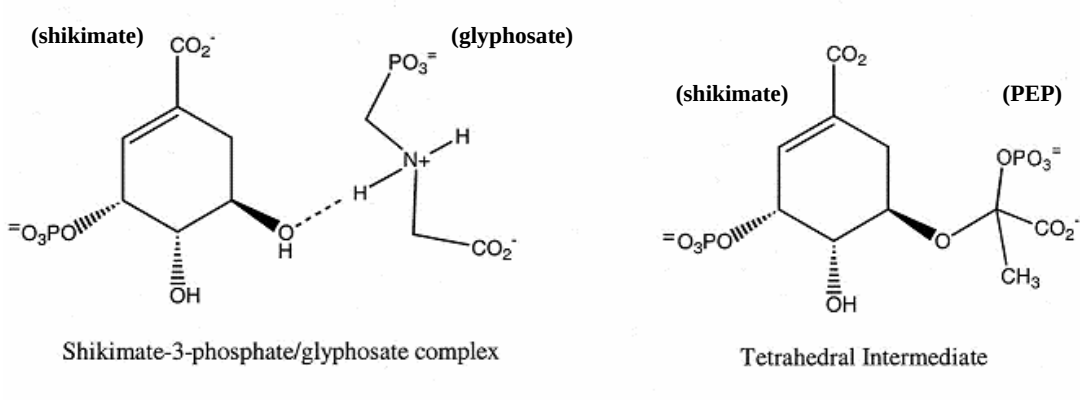

Figure 1.5. Comparison of the tetrahedral intermediate to the shikimate-3-phosphate/glyphosate complex (Alibhai and Stallings 2001).

Glyphosate has also been shown to inhibit import of the EPSPS pre-protein into chloroplasts (Della-Cioppa and Kishore 1988). The pre-protein is able to function catalytically as an enzyme and binds glyphosate with the same affinity as the mature enzyme (Della-Cioppa et al. 1986). The mode of inhibition by glyphosate is observed to be the same as that for the mature enzyme in that glyphosate binding requires the prior formation of an EPSPS-S3P binary complex.

Glyphosate is readily translocated through the phloem and accumulates in the meristematic regions of the plant (Franz et al. 1997). It has been estimated that in plants approximately 20% of all fixed carbon flows through the shikimate pathway (Haslam 1993). Glyphosate inhibition of EPSPS disrupts this carbon flow, resulting in the accumulation of large amounts of shikimate, and ultimately plant death (Sikorski and Gruys 1997).

1.6. The production of glyphosate-resistant plants

Plant biotechnology can be used to manipulate the EPSPS enzyme and introduce glyphosate tolerance by gene transfer and cell selection techniques. There are three methods to achieve this tolerance: overproduction of the native EPSPS enzyme, introduction of an insensitive form of the enzyme, and detoxification of glyphosate.

Overproduction of the EPSPS enzyme has been shown to confer resistance to glyphosate in plant cell cultures through stepwise selection on increasing concentrations of glyphosate in the growth medium. Amplification of the *EPSPS* gene was demonstrated as the molecular basis for the increase in EPSPS enzyme levels in tobacco (Goldsbrough et al. 1990). However, in *Corydalis sempervirens*, this increase was due to transcriptional and translational changes relating to the EPSPS sequence (Hollander-Czytko et al. 1988). Resistance via overexpression of EPSPS has also been observed in transgenic petunia containing the *EPSPS* gene under the transcriptional control of the highly expressed constitutive cauliflower mosaic virus 35S promoter (Shah et al. 1986). However, overproduction of the EPSPS enzyme has not been achieved at levels sufficient to provide adequate tolerance in the meristematic regions of the plants where glyphosate accumulates. As a result, herbicide treated plants over-expressing the EPSPS enzyme exhibit reduced plant growth compared with untreated plants (Kishore and Shah 1988). Alternatively, glyphosate resistance can be achieved by the introduction of a glyphosate-insensitive form of the EPSPS enzyme into plants using transformation techniques. Bacterial EPSPS enzymes have been isolated with point mutations that confer glyphosate resistance (Stalker et al. 1985), and a number of glyphosate-tolerant mutants of plant EPSPS have been produced by site-directed mutagenesis (Franz et al. 1997). However, whilst these enzymes show tolerance to glyphosate, they also demonstrate an increase in K_m for PEP, reducing their overall catabolic efficiency (Dill 2005). These EPSPS enzymes are known as Class I EPSPS enzymes, and their genes are required to be overexpressed in transgenic plants to compensate for their reduced efficiency. To date, only one Class I EPSPS enzyme has been used to generate commercial glyphosate resistance. The Roundup Ready corn line GA21 expresses a glyphosate tolerant, modified corn EPSPS that has 99.3% amino acid sequence homology with the wild-type

corn EPSPS enzyme (Sidhu et al. 2000). This glyphosate resistance is the result of a mis-sense mutation in the endogenous corn EPSPS produced by site-directed mutagenesis (Dill 2005).

Naturally occurring glyphosate-tolerant enzymes have been isolated from bacteria that maintain a low K_m for PEP (Franz et al. 1997). These have been classified as Class II EPSPS enzymes. One of these, from *Agrobacterium tumefaciens* strain CP4, has been successfully utilised in the transformation of important crop species such as soybean, corn, cotton and canola (Dill 2005). The substrate and glyphosate binding region of CP4 EPSPS is identical to that found in the sensitive EPSPS enzymes found in most plant species (Dill 2005). Since the amino acid sequence homology of these enzymes is 49-59% similar and 23–41% identical (Bradshaw et al. 1997), it has been proposed that glyphosate may be kept from binding by an altered protein conformation due to amino acid sequence changes outside the active site of the CP4 EPSPS enzyme (Dill 2005).

Glyphosate-tolerant corn was produced by Heck et al. (2005) using dual *CP4 EPSPS* transgene cassettes, one under the control of the enhanced cauliflower mosaic virus 35S (P-e35S) promoter, and the other under the control of the rice actin 1 (P-Ract1) promoter. Expression of the *CP4 EPSPS* gene under the P-e35S promoter alone had not been fully constitutive and critical cell types in the sporophytic tapetum and early stages of gemetogenesis were damaged following commercial glyphosate applications rates, leading to male sterility. The P-e35S driven *CP4 EPSPS* expression was thus supplemented by P-Ract1 as it was shown to direct *CP4 EPSPS* expression in all cell types within the corn anther (Heck et al. 2005).

In 1996, soybean became the first commercially available glyphosate-resistant crop in the USA. In 2004, 85% of all soybeans grown in the USA were herbicide-resistant, as well as 60% of all cotton and 18% of all maize, with the vast majority being glyphosate-resistant (Dill 2005). Nearly all glyphosate-resistant crops currently available contain the CP4 EPSPS enzyme (Dill 2005).

Detoxification of the glyphosate molecule is another strategy that has been employed to confer glyphosate resistance via the transformation of plants with glyphosate metabolism genes (Pline-Srnic 2006). This method of glyphosate resistance is considered desirable as it reduces the likelihood of damaging levels of glyphosate accumulating in sensitive plant tissues such as reproductive sinks (Pline-Srnic 2006).

The *gat* gene codes for the enzyme glyphosate N-acetyl transferase which acetylates and deactivates glyphosate (Castle et al. 2004). The *gox* gene encodes the enzyme glyphosate oxidoreductase. This cleaves the nitrogen-carbon bond in glyphosate yielding aminomethylphosphonic acid (AMPA) and glyoxylate. However, AMPA is a phytotoxin that has been reported to cause injury to both glyphosate-tolerant and non-transgenic soybeans (Reddy et al. 2004).

Although detoxification by these methods has been shown to confer glyphosate resistance, detoxification alone fails to confer resistance to commercial rates of glyphosate applications (Tan et al. 2006). Currently all commercially available crops containing the *gox* or *gat* genes also contain a glyphosate-resistant form of EPSPS (Pline-Srnic 2006).

1.7. Issues with glyphosate tolerance

1.7.1. The use of glyphosate tolerant technology

Commercial production of glyphosate-tolerant crops, such as soybean, corn and cotton is already widespread in a number of countries, including the USA and Argentina. Utilisation of glyphosate-tolerant soybean crops in the USA has resulted in simplified spraying regimes with fewer herbicide applications (Carpenter and Gianessi 2000).

Glyphosate-tolerant soybean has been grown in vast quantities in Argentina with 800,000 hectares grown in 1996 increasing to 6.6 million hectares grown in the 1999/2000 season (http://www.mindfully.org/Farm/Argentina-GE-Ag.htm). However, as little or no crop rotation has been employed in the growth of these crops, farmers in Argentina are now

experiencing problems with resistant weed species and changes in soil microbe composition (Branford 2004). This demonstrates the necessity for integration of this technology into a suitable crop management system in order to achieve an improved weed control system. Onion is a rotational crop and, as such, should not impose constant glyphosate selection pressures onto the environment.

1.7.2. Spontaneous glyphosate resistance

Spontaneous glyphosate resistance is very rare in plants. As glyphosate inhibition of EPSPS is competitive with respect to PEP, glyphosate resistance due to an altered plant EPSPS enzyme would be accompanied by a decreased affinity for PEP. It follows that under conditions of limited PEP availability, aromatic amino acid biosynthesis would be severely reduced, impacting negatively on the competitive ability of the plant to survive (Bradshaw et al. 1997). Also, it is highly unlikely that a gene for a plant EPSPS will mutate into the CP4 EPSPS, which has a high glyphosate resistance while retaining a high affinity for PEP. This would require a large number of specific amino acid substitutions, as well as a change in the total number of amino acid residues of the enzyme (Bradshaw et al. 1997).

Hence, after over 20 years of widespread glyphosate use there have been reports of only 12 resistant plant species to date (http://www.weedscience.org/Summary/UspeciesMOA.asp?lstMOAID=12). The first published recording of glyphosate resistance arising due to the evolution of an altered EPSPS protein was observed in 2002 in goosegrass (*Eleusine indica*) (Baerson et al. 2002). The exact mechanisms of resistance are yet to be defined in the other plant species but are thought to involve systems aside from direct target alteration such as the translocation of glyphosate or interference with the transport of glyphosate to the target site (Dill 2005). Not much information is available detailing the fitness of these resistant plants. However, researchers have indicated that glyphosate-resistant rigid rye grass (*Lolium rigidum*) and glyphosate-resistant hairy fleabane (*Conyza bonariensis*) are both

ecologically less fit than the wild type plants of those species (http://www.weedscience.org/Summary/UspeciesMOA.asp?lstMOAID=12).

As glyphosate is soil-bound and readily degraded by soil microbes (Franz et al. 1997), there is no intensive selection pressure for resistance to develop. However, with the increased use of glyphosate-tolerant crops in farming regimes, the risk of poor management may increase the probability of glyphosate resistance appearing in weed species.

1.8. Biotechnology in New Zealand

1.8.1. Public concerns over genetic modification

The issue of genetic modification has raised much public concern. In New Zealand, a two year moratorium on the commercial release of genetically modified organisms was put in place in 2001 while an investigation was carried out by the New Zealand Royal Commission on Genetic Modification. After consultation with the public, the Royal Commission found that most people were comfortable with genetic modification for medical purposes, but many were strongly against the genetic modification of food and crops (Report of the Royal Commission on Genetic Modification 2002 http://www.mfe.govt.nz/publications/organisms/royal-commission-gm/). However, others considered conventional plant breeding techniques presented similar risks to those identified in transgenic crops (Conner and Jacobs 2000). The Royal Commission report concluded that New Zealand should proceed with caution as it would be unwise to ignore the potential benefits of this technology (Report of the Royal Commission on Genetic Modification 2002 http://www.mfe.govt.nz/publications/organisms/royal-commission-gm/).

1.9. Transformation of *Alliums*

The most commonly used methods for plant transformation are biolistics and *Agrobacterium tumefaciens*-mediated transformation.

Biolistics involves the use of DNA-coated microprojectiles, usually tungsten or gold, delivered into target cells by acceleration (Hansen and Wright 1999). The advantage of this method is that DNA can be introduced into almost any tissue of any cultivar (Hansen and Wright 1999). However, biolistics generally results in complex patterns of integration, and large segments of DNA are difficult to transfer as breakages can occur during DNA delivery (Hansen and Wright 1999).

Agrobacterium tumefaciens-mediated transformation of plants occurs in nature resulting in the formation of a crown gall. Initially, plant wounding provides an entry site for the bacteria. Phenolic and sugar compounds are produced by plant cells in response to the wounding which induces the transfer of DNA from the bacterium into the plant genome (Gelvin 2000). Enzymes encoded in the transferred DNA ensure the production of auxin and cytokinin, promoting plant cell division and expansion, and generating a crown gall (Hiei et al.1997). The advantages of *Agrobacterium*-mediated transformation over biolistics include the transfer of DNA with defined ends and with minimal rearrangement, the transfer of relatively large segments of DNA, and the integration of low numbers of gene copies into plant genomes (Dai et al. 2001).

Strains of *Agrobacterium* have been modified into a binary vector system for genetic engineering purposes. This system consists of two plasmid components: a disarmed tumour-inducing plasmid from *Agrobacterium* in which the genes responsible for tumour formation have been removed whilst retaining those necessary for T-DNA transfer; and a binary vector which contains the T-DNA region to be transferred into the plant (Ko et al. 2004, Coutu et al. 2007).

Agrobacterium-mediated transformation has been routinely used with dicotyledonous plants. However, monocotyledonous plants were initially thought to be recalcitrant to gene transfer using *Agrobacterium*-mediated transformation, as they are not the natural hosts of *A. tumefaciens*. In many monocots, the cells at sites of wounds do not divide but tend to become lignified, producing hardened cells around the wound site (Schlappl and Hohn, 1992). Also, the phenolic compounds required for the induction of DNA transfer

by the bacterium are either not released from the wound sites, or are released at insufficient levels (Roy et al. 2000). To overcome the lack of a wound response, tissue with actively dividing cells such as callus or immature embryos is used, and a phenolic compound such as acetosyringone is added to the bacterial cultures or co-cultures to induce DNA transfer in the bacterium (Hiei et al. 1997). Optimisation of these techniques has led to the transformation of a number of monocotyledonous species such as rice (Hiei et al. 1994), maize (Ishida et al. 1996), and wheat (Cheng et al. 1997) using *A. tumefaciens*.

Only three protocols have been published reporting successful onion transformation. Zheng et al. (2001) used *Agrobacterium*-mediated transformation of young callus induced from mature embryos of onions and shallots. Aswath et al. (2006) used *Agrobacterium*-mediated transformation of onion callus proliferated from the seedling radicle. Eady et al. (2000) used *Agrobacterium*-mediated transformation of immature embryos. Although all three systems produced transgenic plants, albeit at low frequencies, only that of Eady et al. (2000) has been shown to be effective with different cultivars, constructs and selective agents (Eady et al. 2003a, b). This technique, with slight adaptations, has also been applied to other *Allium* species leading to the first reported transformation protocols for leek (*A. porrum*) and 'true seed' garlic (*A. sativum*) (Eady et al. 2005).

1.9.1. *Agrobacterium*-mediated transformation of onion with the *CP4 EPSPS* gene construct

Glyphosate-tolerant lines of onion have been produced using *Agrobacterium*-mediated transformation of immature embryos with the *CP4 EPSPS* gene (Eady et al. 2003a). Three constructs were used, all containing different promoter regions. These plants were grown to maturity in a containment glasshouse and seed was produced. Analysis of subsequent generations has shown that inheritance of this gene is stable, and exhibits Mendelian properties within the first selfed cycle.

1.9.2. The expression of transgenes in onion

Transgene expression has been studied in onion plants transformed with the *gfp* reporter gene under the control of the cauliflower mosaic virus 35S (*CaMV35S*) constitutive promoter (Eady et al. 2003b). Inheritance was shown to follow a Mendelian pattern. Western blot analysis of GFP protein content in the roots of transgenic seedlings from selfed plants revealed zero levels in non-transgenic offspring and two different levels in the transgenic offspring consistent with the gene being present in the hemizygous and homozygous condition. Furthermore, when images of seedling roots were analysed for the intensity of green fluorescence, distinct groups of fluorescence were revealed that corresponded to expected Mendelian ratios for hemizygous and homozygous individuals. This suggests a probable link between copy number and gene expression level although research to date is lacking in this area, particularly with respect to field based evaluation.

1.9.3. *CP4 EPSPS* gene expression in glyphosate resistant plants

Characterisation and analysis of plants transformed with the *CP4 EPSPS* gene has been undertaken in glyphosate-resistant plant species such as soybean (Padgette et al. 1995), corn (Heck et al. 2005) and poplar (Meilan et al. 2002). These transgenic plants were also subjected to field trials mainly to assess the efficacy of glyphosate resistance in a glyphosate-based weed control regime, with plants scored visually for injury. However, it appears that little molecular analysis has been done on field-grown plant material.

The glyphosate-resistant soybean event 40-3-2 contained a single copy of the *CP4 EPSPS* gene under the control of a 35S constitutive promoter. Values of 415 to 443 µg of CP4 EPSPS protein g^{-1} FW was reported in field-grown leaf (Padgette et al. 1995).

In the glyphosate-resistant corn event NK603, the mean CP4 EPSPS protein level was 20.8 µg g^{-1} FW in the leaves of glasshouse-grown plants. This event contained a single copy of a dual cassette with one *CP4 EPSPS* gene under the control of an enhanced 35S promoter and another under the control of a rice actin 1 promoter to provide fully constitutive expression (Heck et al. 2005).

The mean CP4 EPSPS protein level in glyphosate-resistant poplar was 0-26 µg g^{-1} FM in the leaves of glasshouse-grown plants containing at least one intact copy of the *CP4 EPSPS* gene under the control of the caulimovirus figwort mosaic virus (FMV) promoter. Levels of CP4 EPSPS protein were negatively correlated with herbicide damage and positively correlated with growth in herbicide-treated plants. Further, growth of the transgenic plants was not significantly different from that of the non-transgenic plants in the unsprayed control rows. A slight increase in damage to the plants was observed following spray applications in the second year of trials, and this was attributed to possible gene silencing (Meilan et al. 2002).

1.9.4. Transgene silencing

In some instances transgenes are not expressed as would be expected in plant systems. Two general classes of silencing mechanisms have been identified.

The first is known as the 'position effect' in which the extent of expression of a single-copy transgene is influenced by the position in the plant genome where the T-DNA has inserted. Hence, if the T-DNA integrates into an area of the genome that is highly methylated and so only transcribed at low levels, then it is expected that the T-DNA will also be expressed at low levels (Kooter et al. 1999).

The second silencing mechanism is known as homology-dependent gene silencing (HDGS) and arises when there are multiple copies of the same sequence in the genome. These sequences can act *in trans* as silencers for homologous copies located elsewhere in the genome, often resulting in promoter methylation or post-transcriptional silencing by transcript degradation (Meza et al. 2002). This silencing complicates the idea that increasing gene copy number directly corresponds to an increase in expression (Kooter et al. 1999).

The integration of vector backbone sequences into the plant genome alongside T-DNA sequences has also been implicated in transgene silencing (Kooter et al. 1999). However,

this idea has been challenged by Meza et al. (2002) who suggest that vector sequences are not sufficient to induce transgene silencing.

1.10. The purpose of this investigation

The main purpose of this investigation was to assess expression levels of the *CP4 EPSPS* gene in field-grown herbicide-resistant onions. Initially, molecular characterisation was applied to verify their transgenic status, confirm copy numbers of the transgene, and also to ascertain if there had been any superfluous DNA integration. Molecular "fingerprints" were developed for individual lines. Following this, an attempt was made to identify homozygous and hemizygous individuals using molecular techniques. Inheritance ratios of the transgene were determined in the lines selected for field-testing in order to ascertain the agronomic usefulness of each line for breeding purposes. Finally, *CP4 EPSPS* transcript and CP4 EPSPS protein levels were examined in four of the field-grown onion lines. This assessment was carried out using the approaches outlined below.

1.10.1. Molecular characterisation of glyphosate-resistant onion lines

Molecular characterisation of the transgenic onion lines was necessary in order to satisfy ERMA requirements prior to inclusion in the field trial. These requirements state that the onion line to be tested must contain only one or two insertion events of the *CP4 EPSPS* gene, and only lines with one insertion event could be used for large scale trial plots. Also, that molecular analysis of the transgenic parental lines used in the large scale trial plots must demonstrate the absence of vector-derived genes outside the left and right T-DNA borders. These stipulations were satisfied by carrying out PCR for identification of the *CP4 EPSPS* gene followed by Southern analysis probing for the *CP4 EPSPS* gene, and also PCR testing for the presence of the spectinomycin resistance selectable marker (*aadA* gene) as this is the only gene present on the backbone of the constructs used in the transformation process.

1.10.2. Molecular "fingerprinting" of glyphosate resistant onion lines

Further molecular analysis was carried out in order to create a DNA "fingerprint" for individual glyphosate-resistant onion lines. During the onion transformation process, it is often difficult to distinguish regenerating plants that may be clonal from those that originate from independent insertion events. The left border T-DNA/genomic DNA junction sequence will be unique for each transgenic event providing the means for specific detection of independent T-DNA insertion sites. Although Southern blotting can be used to distinguish independent events, a PCR approach would be rapid and inexpensive, enabling easy identification of large numbers of plants. Thermal Asymmetric Interlaced (TAIL)-PCR (Liu & Whittier 1995) was used to identify such unique junction sites. TAIL-PCR consists of three sequential reactions, utilising three specific nested primers within the T-DNA, and a short, arbitrary degenerate primer which binds randomly in the unknown onion genomic sequence, to amplify a region spanning the T-DNA/genomic DNA junction. The first two specific primers are designed quite close together, but the third primer is designed around 100 bp upstream from the second. This primer placement is shown in Figure 1.6. When visualised on a gel, a successful TAIL-PCR reaction is indicated by a step-down of the correct size between the TAIL 2 and TAIL 3 PCR products.

Sequencing of TAIL-PCR products enabled the design of primers for use in conventional PCR to detect specific T-DNA junctions. A T-DNA border primer was used as the forward primer and a genomic return primer was designed from onion genomic DNA in the TAIL-PCR product (Figure 1.6).

1.10.3. Determination of zygosity

In order to simplify the plant breeding of these transgenic onion lines, it would be useful to be able to identify which individual plants are hemizygous or homozygous for the *CP4 EPSPS* gene. If genomic sequence was obtained for the onion lines on one side of the inserted T-DNA, then employing TAIL-PCR from this sequence in the reverse direction in non-transgenic individuals would allow sequence to be obtained that should be on the

other side of the T-DNA in transgenic siblings. Primer placement for this TAIL reaction is shown in Figure 1.7.

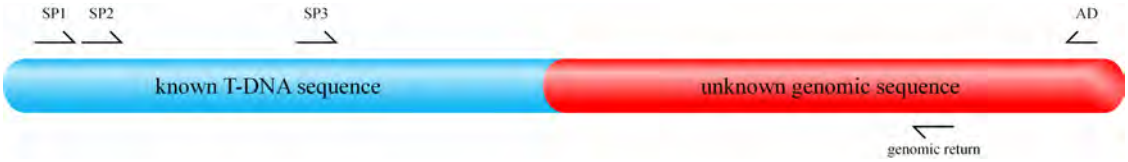

Figure 1.6. Schematic diagram of primer placement in Thermal Asymmetric Interlaced (TAIL)-PCR. TAIL-PCR consists of three sequential reactions, utilising three specific nested primers (SP1, SP2 and SP3) designed within the known T-DNA sequence of the transgenic plant, paired with an arbitrary degenerate primer (AD) that binds randomly within the unknown onion genomic sequence in order to amplify the T-DNA/genomic DNA junction site. Sequencing of this junction allows a genomic return primer to be designed within the now-known onion genomic sequence. Specific T-DNA junctions can be detected via a conventional PCR reaction using this genomic return primer paired with one of the specific nested primers designed within the T-DNA.

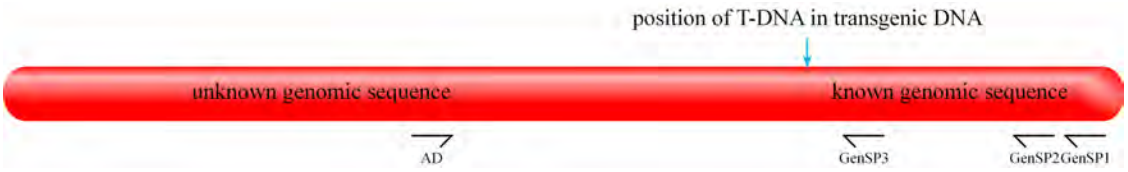

Figure 1.7. Schematic diagram showing the primer placement for the TAIL-PCR reaction on non-transgenic siblings of plants with identified T-DNA/genomic DNA junctions. Three specific nested primers (GenSP1, GenSP2 and GenSP3) were designed within the known onion genomic sequence and paired with an arbitrary degenerate primer (AD) which binds randomly in the unknown onion genomic sequence that would be on the other side of the T-DNA in transgenic plants of this line. The position of the T-DNA in transgenic siblings is indicated by a blue arrow

A conventional PCR pairing a primer designed within this newly identified sequence (a hemizygosity-determining primer) with any of the genomic return primers for this line should amplify the sequence in the non-transgenic siblings that spans the location of the T-DNA insert in the transgenic plants. As the T-DNA insert size is around 3.5 kb in size, a good test for homo/hemi-zygosity would be to do a multiplex PCR on DNA from a transgenic plant using a genomic return primer paired with both a hemizygosity-determining primer and a specific primer located within the T-DNA. Figure 1.8 shows the primer placement for this PCR reaction. Two PCR products of different sizes would be obtained in the hemizygous individuals, whilst only one PCR product should be observed in those that are homozygous.

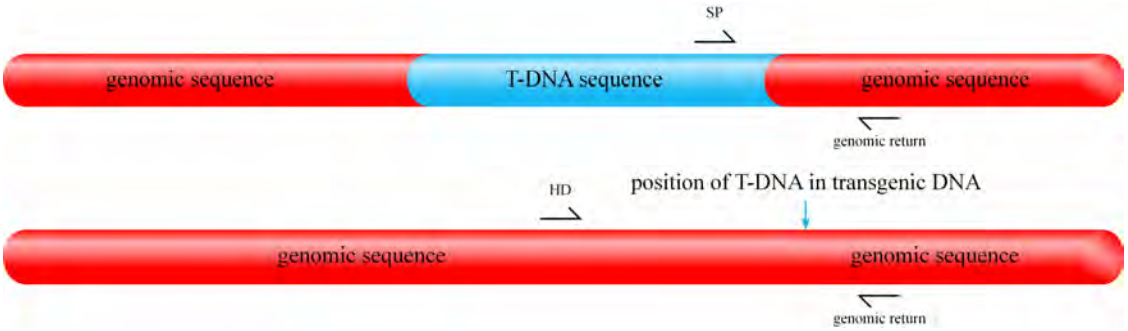

Figure 1.8. Schematic diagram showing the primer placement in a multiplex PCR for determination of homozygosity or hemizygosity in transgenic onion lines. A genomic return primer designed within the known onion genomic DNA sequence is paired with both a specific primer (SP) located within the T-DNA, and a hemizygosity-determining primer (HD) which is also designed within onion genomic sequence. Two PCR products of different sizes would be obtained in the hemizygous individuals, whilst only one PCR product should be observed in those that are homozygous.

1.10.4. Inheritance of the *CP4 EPSPS* gene

Inheritance of the transgene was assessed by glyphosate spray tests and scoring the phenotype. It is hypothesised that the insertion of a single copy of the *CP4 EPSPS* transgene will not be detrimental to the health of the plant. For lines with single copy insertions, the *CP4 EPSPS* gene will be inherited in the seedlings from selfed parent plants in a Mendelian fashion with a ratio of 3:1.

1.10.5. Expression of the *CP4 EPSPS* gene

In the glyphosate-resistant onions it is necessary for the *CP4 EPSPS* gene to be transcribed at all stages of onion development in order to allow glyphosate to be applied whenever weed control is required. Investigation into the expression levels of the introduced *CP4 EPSPS* gene within and between lines at different stages of plant development may provide information about the potential agronomic usefulness of the glyphosate-resistant onion lines.

Expression of the *CP4 EPSPS* gene was examined. It was hypothesised that the levels of *CP4 EPSPS* transcript and expressed CP4 EPSPS protein will vary within different tissues and between transgenic onion lines due to differing T-DNA integration sites in the onion genome. Also, within a line, it is thought that expression levels will be affected by copy number (homozygous vs hemizygous) and genomic background. It was expected that the individuals homozygous for the *CP4 EPSPS* gene will show higher levels of transcript and will also show higher levels of CP4 EPSPS enzyme than the hemizygous individuals. A 1:2 ratio of homozygous:hemizygous expression levels will emerge within lines. It was also expected that *CP4 EPSPS* transcript levels will correspond in a directly proportional manner to CP4 EPSPS protein levels within the same tissue samples. The levels of *CP4 EPSPS* transcript and protein observed in the glyphosate-resistant onions will be similar to that seen in other glyphosate-resistant plant species.

Chapter Two

Materials and Methods

2.1. Plant material

Glyphosate-resistant lines of onion were produced in a range of germplasm backgrounds, between the years 2000-2002 via *Agrobacterium*-mediated transformation of immature embryos (Eady et al. 2003a). The plants were transformed with one of three constructs, all containing different promoter regions. This plant material was regenerated in tissue culture and grown to maturity in the GMO containment glasshouse.

For Southern analysis and PCR reactions, genomic DNA was isolated from 2 g young leaf tissue from glasshouse-grown onion plants using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham) following the manufacturer's instructions. DNA was resuspended in 1 ml sterile water and quantified with λHindIII DNA ladder (Invitrogen) on a 1% (w/v) agarose gel.

For TAIL-PCR reactions, genomic DNA was isolated from 100 mg young leaf tissue from glasshouse-grown onion plants using a urea extraction method (Shure et al. 1983) and treated with RNase A (Qiagen) following the manufacturer's instructions.

Ten different glyphosate-resistant onion lines were selected for field trial evaluation in Canterbury (ERMA permit no. GMF03001). Lines B, C, D, E, F are transgenic events in short day hybrid germplasm, line G is a transgenic event in a parental breeding line, and lines H, I, J, K are transgenic events in open-pollinated PLK-type germplasm. F_1 or F_2 seed from these lines was germinated in the containment glasshouse. When seedlings reached 10 cm in height, they were subjected to an application of glyphosate at the commercial application rate for broad spectrum weed control following the manufacturer's instructions. To determine inheritance patterns for each line, the total

number of seeds sown was recorded, as was the number of germinated seedlings present before, and three weeks after, the herbicide treatment.

For the 2004/2005 field trial, resistant onion seedlings were transplanted into the field in plots of 40 seedlings randomised within a block. There were three blocks of 12 plots in the trial. The field trial plan is shown in Figure 2.1. Seedlings were sprayed with a glyphosate solution at twice the commercial application rate for broad spectrum weed control on 19/11/2004 and 5/1/2005. Non-transgenic control onions were sprayed with Twin-Star (Elliott Chemicals), a systemic broadleaf weed killer for post-emergent use in onions at the commercial application rate following the manufacturer's instructions, on 19/11/2004, 29/11/2004 and 7/1/2005.

Block 1

1 **I**	2 **F**	3 **E**	4 **G**
5 **K**	6 **A**	7 **H**	8 **J**
9 **D**	10 **B**	11 **G**	12 **C**

Block 2

13 **D**	14 **B**	15 **A**	16 **H**
17 **F**	18 **G**	19 **C**	20 **I**
21 **J**	22 **K**	23 **E**	24 **G**

Block 3

25 **K**	26 **J**	27 **G**	28 **C**
29 **E**	30 **B**	31 **G**	32 **D**
33 **F**	34 **I**	35 **A**	36 **H**

Figure 2.1. 2004/2005 Field trial plan consisting of three blocks of 12 plots with 40 seedlings in each. Small numbers denote plot numbers. There were three replicates of 11 onion lines (10 transgenic + one control line) with one line double-replicated. Each block contained a complete replicate, and there was also a complete replicate in each row of plots across the three blocks.

Four lines, which were a representative sample of the range of germplasm in the field trial, were chosen for sampling. Lines C and D are plants from selfed seed from transgenic events in short day hybrid germplasm and lines H and J are plants from selfed seed from transgenic events in open-pollinated PLK-type germplasm. Nine plants were randomly selected for analysis from each of these four lines.

Leaf material was sampled in the field during the early afternoon. Leaf samples were cut and immediately placed on ice in the field prior to immersion in liquid nitrogen and storage at -80°C. Young leaf (less than 10 cm emerged from sheaf) and old leaf (second to fourth outer-most leaf) samples were taken at 10-14 weeks post-transplanting, and 14-18 weeks post-transplanting, respectively. Samples were frozen in liquid nitrogen, ground into a fine powder and stored at -80°C prior to analysis.

Bulb samples were taken after harvest and following six weeks of curing. Bulbs were decapitated and the top diced before immersion into liquid nitrogen and storage at -80°C. This bulb material was then freeze-dried for three days before grinding into a fine powder, and stored at -20°C prior to analysis.

A summary of which glyphosate-resistant onion lines were used in each stage of analysis in this project is given in Figure 2.2.

2.2. Polymerase Chain Reaction (PCR)

All PCR reactions for gene detection were in 25 µl volume, and were carried out in a Gene Amp® PCR System 9700 thermocycler (Applied Biosystems). PCR products were run on a 1% (w/v) agarose gel containing 0.01 mg ml^{-1} ethidium bromide (Invitrogen). Gels were run in ½ × Tris-borate-EDTA (TBE) buffer and visualised under UV light.

For detection of the *CP4 EPSPS* gene, PCR reactions consisted of 1 × ReddyLoad™ PCR Buffer (ABgene), 2.0 mM $MgCl_2$ (ABgene), 0.2 mM dNTPs (Roche), 20 µM each of Gly1 (5'-TCTCGCTAGCGGTGAAACT-3'), and Gly2 (5'-TTGAGCGGAAGCCATAGGT-3') primers (Monsanto, St Louis, Missouri, USA) and 1 U Taq polymerase (ABgene). Denaturing was done at 95°C for 1 min followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C with a final extension cycle of 7 min at 72°C. Expected product size was 400 bp.

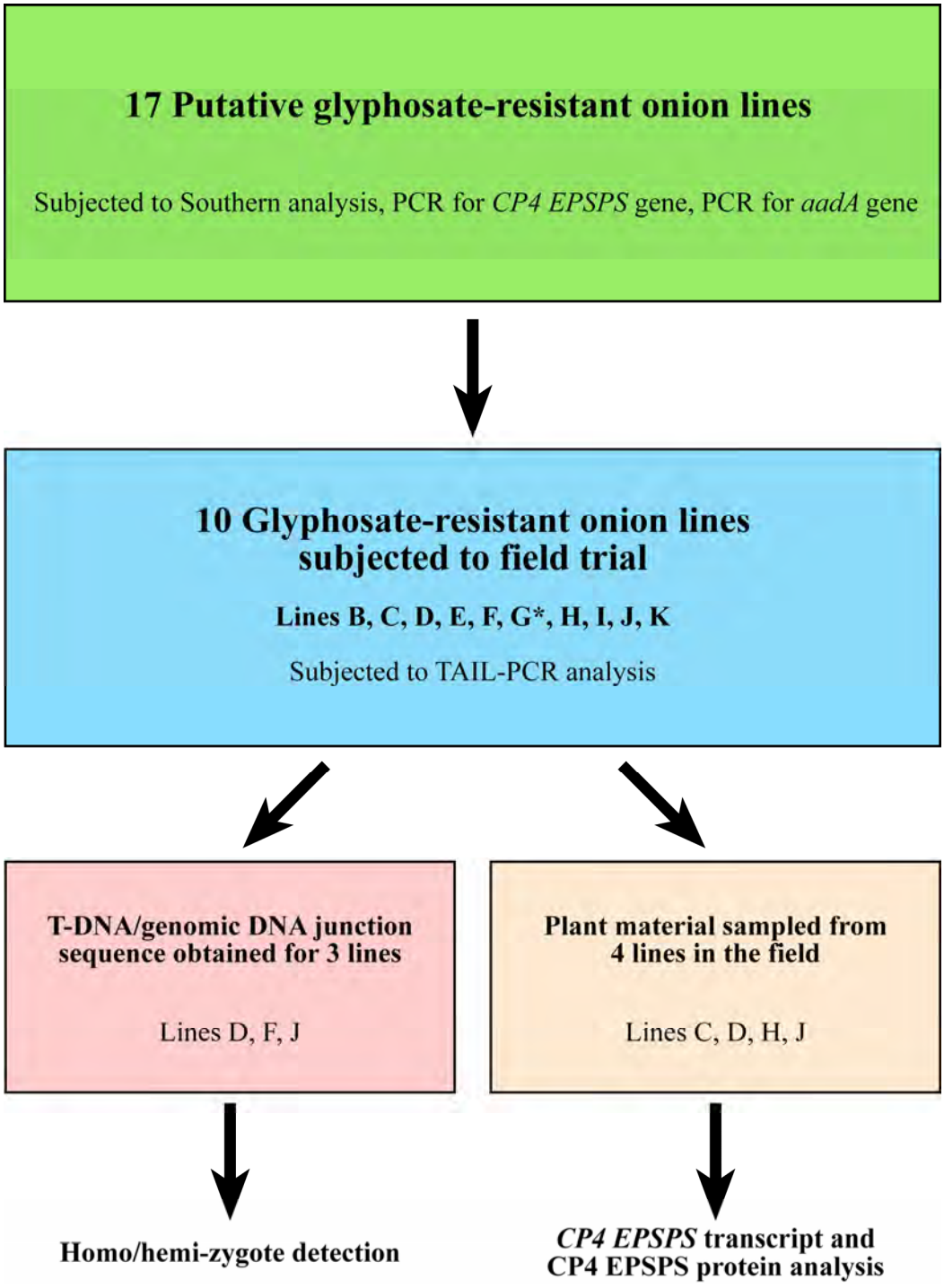

Figure 2.2. Summary of glyphosate-resistant onion lines used in each stage of analysis in this project.

* Data from line G were not included in this thesis

For detection of the spectinomycin resistance gene (*aadA* gene), PCR reactions consisted of 1 × ReddyLoad™ PCR Buffer (ABgene), 2.0 mM $MgCl_2$ (ABgene), 0.2 mM dNTPs (Roche), 5 µM each of aadA1 (5'-TGATTTGCTGGTTACGGTGAC-3') and aadA2 (5'-CGCTATGTTCTCTTGCTTTTG-3') primers (Clark et al. 1999), and 1 U Taq polymerase (ABgene). Denaturing was done at 95°C for 10 min followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C with a final extension cycle of 10 min at 72°C. Expected product size was 284 bp.

2.3. Thermal Asymmetric Interlaced (TAIL)-PCR

TAIL-PCR was used to identify T-DNA/genomic DNA junction sites in transgenic glyphosate-resistant onion lines. The nested sequence specific primers used for TAIL-PCR were TAILsp1 (5'-GACAACATGCATCAATCGACC-3'), TAILsp2 (5'-GACCTGCAGCCACTCGAAGC-3'), and TAILsp3 (5'-TTGGACGTGAATGTAGACACG-3') (all designed by Andrew Catanach of Crop & Food Research). Figure 2.3 shows the binding sites for these primers in relation to the left border in a Roundup Ready plasmid. A range of short arbitrary degenerate primers were used as return primers and are listed in Table 2.1. The TAIL-PCR protocol of Liu et al. (1995) was followed with some modifications.

The primary reaction was done in 25 µl volume and consisted of the following components: 1 × ReddyLoad™ PCR Buffer (ABgene); 1.5 mM $MgCl_2$ (ABgene); 0.2 mM dNTPs (Roche); 0.2 µM TAILsp1; 3 µM of an arbitrary degenerate primer; 1.25 units Taq polymerase (ABgene); and 2 µl transgenic genomic DNA. The primary PCR reaction was diluted 1:50 and 1 µl used as the template for the secondary PCR reaction.

The following components were used for the secondary reaction in a final volume of 20 µl: 1 × ReddyLoad™ PCR Buffer (ABgene); 1.5 mM $MgCl_2$ (ABgene); 0.2 mM dNTPs (Roche); 0.2 µM TAILsp2; 2 µM arbitrary degenerate primer; and 0.75 units Taq polymerase (ABgene). The secondary reaction was diluted 1:50 and 1 µl used as the template for the tertiary PCR reaction.

The following components were used for the tertiary reaction in a final volume of 20 µl: 1 × ReddyLoad™ PCR Buffer (ABgene); 1.5 mM $MgCl_2$ (ABgene); 0.2 mM dNTPs (Roche); 0.2 µM TAILsp3; 2 µM arbitrary degenerate primer; and 0.75 units Taq polymerase (ABgene).

The thermocycler programs for each of the TAIL-PCR reactions was as follows. Primary reaction: 2 min at 95°C; 5 cycles of 30 s at 94°C, 1 min at 62°C, and 2.5 min at 72°C; 30 s at 94°C; 3 min at 25°C, ramping to 72°C over 2.5 min; 2.5 min at 72°C; 15 cycles of 30 s at 94°C, 1 min at 62°C, 2.5 min at 72°C, 30 s at 94°C, 1 min at 62°C, 2.5 min at 72°C, 10 s at 94°C, 1 min at 44°C, 2.5 min at 72°C; 5 min at 72°C, then hold at 4°C. Secondary reaction: 15 cycles of 30 s at 94°C, 1 min at 62°C, 2.5 min at 72°C, 30 s at 94°C, 1 min at 62°C, 2.5 min at 72°C, 10 s at 94°C, 1 min at 44°C, 2.5 min at 72°C; 5 min at 72°C, then hold at 4°C. Tertiary reaction: 30 cycles of 10 s at 94°C, 1 min at 44°C, 2.5 min at 72°C; 5 min at 72°C, then hold at 4°C.

Each TAIL-PCR experiment included a water blank and a plasmid control. The secondary and tertiary PCR reactions were run on a 1% (w/v) agarose gel containing 0.01 mg ml^{-1} ethidium bromide (Invitrogen). Gels were run in ½ × TBE buffer and the products were visualised under UV light. Potential left border T-DNA/genomic DNA junctions were identified by a difference in the step-down between the secondary and tertiary PCR bands of 79 bp.

A gel fragment from either the secondary or tertiary PCR product was used as DNA template in a repeated tertiary TAIL-PCR reaction in a 50 µl volume. This was then run out on a gel to determine purity. Pure single bands were further purified using a High Pure PCR Purification kit (Roche) following the manufacturer's instructions. If a single band was not produced, the desired band was isolated and purified using a Qiaquick Gel Extraction kit (Qiagen) following the manufacturer's instructions. Cleaned up PCR product was run on a 1% agarose gel next to Low Mass Ladder (Invitrogen) to quantify and estimate product size. PCR products were sequenced by standard Big Dye cycle

sequencing with primer TAILsp3 at the University of Waikato DNA Sequencing Facility, Hamilton, New Zealand.

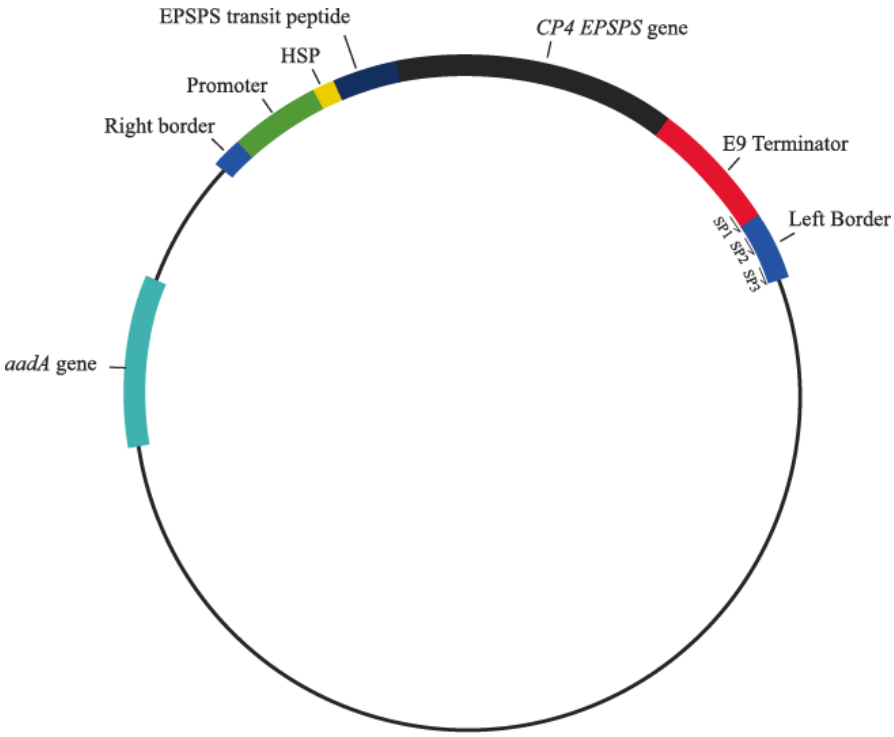

Figure 2.3. Schematic diagram indicating the positions of the specific nested primers (SP1, SP2 and SP3) for TAIL-PCR in the DNA sequence of the plasmids used in the production of the glyphosate-resistant onion plants.

Table 2.1. List of arbitrary degenerate primers used in TAIL-PCR reactions. N, S and W indicate degenerate bases. N = (A/C/G/T), S =(C/G), W = (A/T).

Primer name	Primer length	Sequence (5' - 3')	From:
AD1	15	NTC GAS TWT SGW GTT	Liu and Whittier 1995
AD2	16	NGT CGA SWG ANA WGA A	Liu and Whittier 1995
AD3	16	WGT GNA GWA NCA NAG A	Liu and Whittier 1995
AD4	16	NGT ASA SWG TNA WCA A	Liu and Whittier 1995
AD5	16	TCS TNA GWA CNT WGG A	**AD5** Qin et al. 2003
AD6	15	NAC GTS AWT SCN AGA	**AD1-1** Qin et al. 2003
AD7	15	NTC GAS TWT NGW GAA	**AD1-2** Qin et al. 2003
AD8	15	NTC GTS WGA NAW GTT	**AD2-1** Qin et al. 2003
AD9	16	NCA GCT SWC TNT WGA A	**AD2-2** Qin et al. 2003
AD10	16	NCT CGT SWG ANT WGA T	**AD2-3** Qin et al. 2003
AD11	16	NGT CGA SWC ANT WCT A	**AD2-4** Qin et al. 2003
AD12	16	NGT CGA SWC TNA WCA A	**AD2-5** Qin et al. 2003
AD13	16	AGW CAN GWT NCA WGA A	**AD4-1** Qin et al. 2003
AD14	16	AGW GNA GWA NCA WAG G	**AD4** Qin et al. 2003

2.3.1. Genomic return PCR using TAIL-PCR sequence

Genomic return primers were designed using DNAMAN software within the genomic section of the known T-DNA/genomic DNA junction sequence of onion lines. The genomic return primer GenRetJ (5'-GTTCTTTGAGCACAACATCCC-3') (Andrew Catanach, C&FR) was used for line J, the genomic return primer GenRetD (5'-GCTATTTAATTTAAAGCGGAAACC-3') was used for line D, and the genomic return primer GenRetF (5'- GGTGGCAAGAGGAAGTGAAG -3') was used for line F. PCR reactions were carried out in 25 µl volume and consisted of 1 × ReddyLoad™ PCR Buffer (ABgene), 1.5 mM $MgCl_2$ (ABgene), 0.2 mM dNTPs (Roche), 5 µM each of TAILsp1 primer and a line specific genomic return primer, 1 U Taq polymerase (ABgene), and 1µl of DNA template. Multiplexed PCR reactions were the same as above

but with 5 µM of each additional line specific genomic return primer. Denaturing was done at 95°C for 1 min followed by 40 cycles of 40 s at 94°C, 40 s at 62°C, and 40 s at 72°C, with a final extension cycle of 7 min at 72°C.

2.4. Hemi/homo-zygote detection using TAIL-PCR

Specific nested primers were designed using DNAMAN software within the genomic section of the known T-DNA/genomic DNA junction sequence of onion lines D, F and J. TAIL PCR was carried out and analysed following the same conditions outlined in Section 2.3.

Line D was analysed using GenSP1D (5'-CATGAAAACGTGCTACTGCATG-3') in the primary reaction, GenSP2D (5'-GCAAGCATACACATTGTTGTCC-3') in the secondary reaction, and GenSP3D (5'-GACACTAGAACTTCATGCACTCGG-3') in the tertiary reaction. The expected step-down between the secondary and tertiary PCR reactions was 112 bp.

Line F was analysed using GenSP1F (5'-CCTATAGATCGACCCGAAAAGCTAC-3') in the primary reaction, GenSP2F (5'-GTCCAAAGGTGGCAAGAGGAAG-3') in the secondary reaction, and GenSP3F (5'-CGCGTGTTCTTTATGTTCAGAAAC -3') in the tertiary reaction. The expected step-down between the secondary and tertiary PCR reactions was 92 bp.

Line J was analysed using GenSP1J (5'-CGTGCCTTTAAGACTGCCACAC-3') in the primary reaction, GenSP2J (5'-CTTTCGCTCCTAAGAGCCACTG-3') in the secondary reaction, and GenRetJ in the tertiary reaction. The expected step-down between the secondary and tertiary PCR reactions was 106 bp.

2.5. Southern analysis

Genomic DNA (20 µg) was digested overnight at 37ºC in a 900 µl reaction with 400 U HindIII restriction endonuclease (Roche) and 1 × Buffer B (Roche). Samples were then dried down to 150 µl in a AES2000 SpeedVac (Savant) at 42°C. The restriction digests were size-fractionated by electrophoresis in 1% (w/v) agarose gels in TBE buffer and transferred to zeta probe membrane (Biorad) by capillary action (Mann and Reed 1985). Membranes were left to air-dry, and then baked at 80°C for 1 h in a SGD210D SpeedGel System (Thermo Savant) before being stored at -20°C.

Membranes were washed in 2 × SSC at 65°C for 15 min. Herring sperm DNA (900 µl) was heated to 65°C for 5 min, added to 30 ml of pre-hybridisation buffer and placed in a hybridisation tube with the membrane. The tube was incubated for 2-4 h. The probe DNA was prepared by PCR amplification of the *CP4 EPSPS* gene from plasmid template DNA, then purified using a High Pure PCR Product Purification Kit (Roche) following the manufacturer's instructions, and quantified by running on a 1% (w/v) agarose gel against a λHindIII DNA ladder (Invitrogen). ^{32}P-labelling was performed in a Mega Prime DNA Labeling System (Amersham) according to the manufacturer's instructions but with half the reaction size specified, the same amount of enzyme and 50 ng of probe DNA. The labelling reaction was left for 2 h at 37°C. Unincorporated radio-nucleotides were removed using a Sephadex Quick Spin Column (Roche) according to the manufacturer's instructions. The probe was denatured by adding 15 µl 1M NaOH and incubated at room temperature for 10 min. The reaction was neutralised by adding 15 µl of 2 M Tris pH 7.5. The probe was added to the pre-hybridisation solution and incubated overnight at 65°C. The following day, membranes were washed with 2 × SSC + 0.1% SDS for 30 min at 65°C, followed by 1 × SSC + 0.1% SDS for 15 min at 65°C. The membrane was then exposed to BioMax MR film (Kodak) at -80°C for one week.

2.6. *CP4 EPSPS* transcript analysis

RNA was extracted using an RNeasy Plant RNA Extraction Kit (Qiagen) following the manufacturer's instructions. For leaf samples, 50 mg of crushed frozen tissue was used.

For bulb samples, 15 mg of crushed freeze-dried material was used. RNA was eluted into 80 µl RNase-free water and treated with RNase-Free DNase I (Ambion) following the manufacturer's instructions.

RNA samples were initially quantified on a ND1000 spectrophotometer (Nanodrop). RNA (400 ng) was run on a 1% (w/v) agarose gel and further quantification was undertaken by summation of the four major peak areas depicted on the gel by use of Phoretix 1D gel analysis software. Samples were subsequently adjusted to 20 ng μl^{-1}.

CP4 EPSPS transcript was measured and normalised relative to 18S ribosomal subunit amplification via multiplex RT-PCR using Ready-To-Go™ RT-PCR Beads (Amersham). DEPC-treated water (36 µl) was added to each bead and these were incubated on ice for 15 min. 2 µl of RNA template (20 ng μl^{-1}) and 2 µl of the random primer Pd(N)6 (200 ng μl^{-1}), for first strand cDNA synthesis, were then added to each tube. The samples were aliquoted into PCR plates in duplicates of 20 µl per well. Samples were placed in a Gene Amp® PCR System 9700 thermocycler (Applied Biosystems) at 42°C for 30 min, then incubated at 95°C for 5 min to inactivate the reverse transcriptase and completely denature the template. Samples were then brought down to 4°C and 5 µl of specific primer mix (1 µl of each of 10 µM Gly 1 and 10 µM Gly 2 primers, 1 µl water and 2 µl of 5 µM Classic 18S PCR primer pair (QuantumRNA™ 18S Internal Standards, Ambion) was added to each PCR well. Samples then underwent PCR amplification of 94°C for 3 min, 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1min, then 72°C for 7 min. PCR product was run on a 1% (w/v) agarose gel and band volumes were determined using Phoretix 1D gel analysis software. Relative peak area ratio = *CP4 EPSPS* PCR product/18S PCR product.

2.7. CP4 EPSPS protein analysis

Total protein was isolated and CP4 EPSPS levels were quantified in leaf and bulb tissue using a GMOChek Soya Test Kit (Strategic Diagnostics) following the manufacturer's instructions with some modifications. Total protein was quantified using Coomassie Plus

– The Better Bradford Reagent (Pierce) following the standard microplate protocol but using a 1:5 dilution of leaf protein extract and a 1:2 dilution of bulb protein extract. Bovine serum albumin was used as the standard. For leaf samples, total protein was isolated from 50 mg of crushed frozen tissue in 450 µl of supplied extraction buffer, and all samples were adjusted to a value of 1000 µg ml^{-1}. For bulb samples, total protein was isolated from 30 mg of powdered freeze-dried bulb material in 450 µl of supplied extraction buffer. Samples from lines C and H were adjusted to 1000 µg ml^{-1}, samples from line J were adjusted to 100 µg ml^{-1}, and samples from line D were adjusted to 50 µg ml^{-1}. Soya protein isolate standards (Strategic Diagnostics) were used as references for CP4 EPSPS protein quantification. Samples were read on a Spectromax 190 Plate Spectrophotometer and data reported with Softmax Pro data management software.

2.8. Statistical analysis

All statistical analysis was carried out by Ruth Butler at Crop & Food Research.

Inheritance data was analysed using a Chi-square goodness of fit test with 1 d.f. testing the hypothesis that transmission of the *CP4 EPSPS* gene will follow a 3:1 Mendelian ratio in the glyphosate-resistant onion lines used in the field trial.

Relative peak area ratio data was analysed with analysis of variance, excluding lines for which there were no data. The ratios were log-transformed before analysis to stabilise the variance. The analysis took account of the nested structure of the data, i.e. blocks, plots within blocks, plants within plots and duplicates within plants.

Chapter Three

Results

3.1. Characterisation of transgene integration by PCR and Southern analysis

Genomic DNA was isolated from 17 putatively transgenic *Allium cepa* L. lines transformed with the *CP4 EPSPS* gene. The same DNA extract was used for both PCR and Southern analysis.

Gel analysis of PCR amplification products of the *CP4 EPSPS* gene showed that the *CP4 EPSPS* containing plasmid control and plant samples from all onion lines tested, except the untransformed control, produced bands of the expected size of 400 bp (Figure 3.1b).

Southern analysis probing for the *CP4 EPSPS* gene following HindIII digestion of genomic DNA produced a single band for all lines, except for line L, which produced two bands. No bands were produced in the non-transgenic control (Figure 3.1a).

PCR amplification of the spectinomycin resistance selectable marker (*aadA* gene) present on the backbone of the binary vector constructs showed that two lines contained imperfect integration events (Figure 3.1c). Line C and line I produced bands of the expected size of 284 bp at the same position as the binary vector control. There was no amplification of the non-transgenic control.

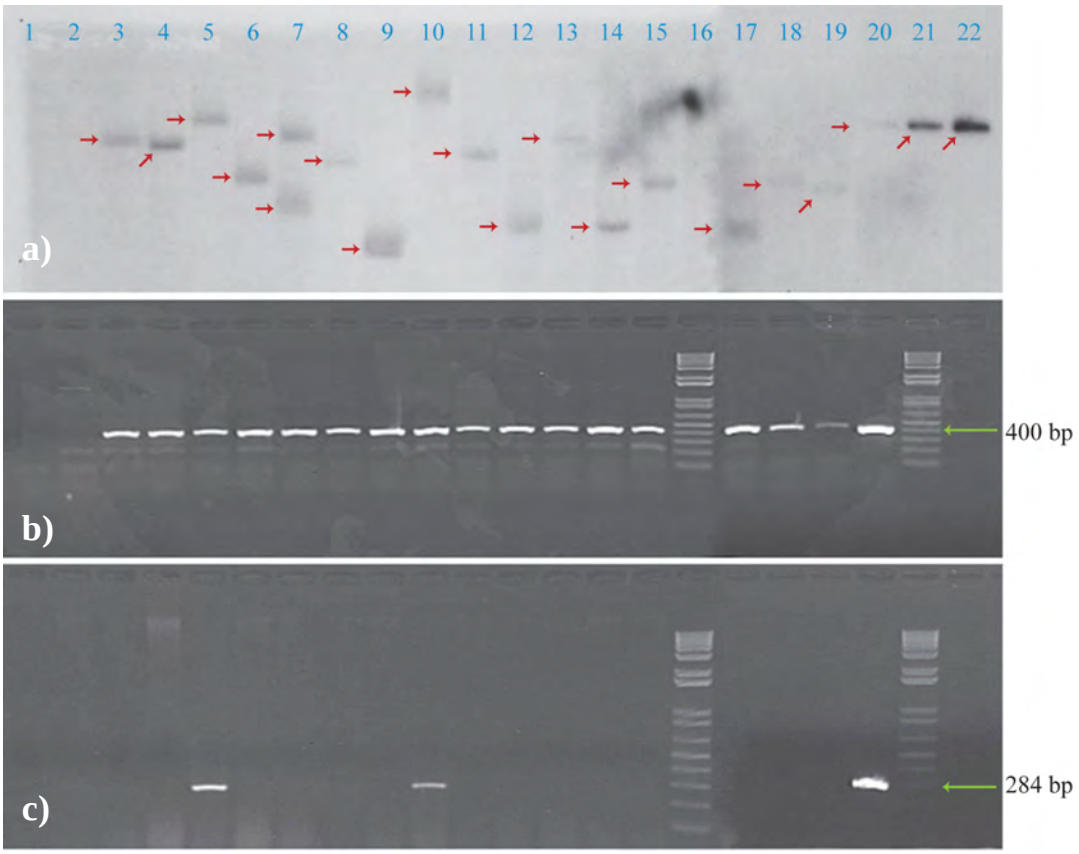

Figure 3.1. Results from **a)** Southern blot probing for *CP4 EPSPS* gene, **b)** PCR for *CP4 EPSPS* gene and **c)** PCR for *aadA* gene in putatively transgenic *Allium cepa* L. plants transformed with the *CP4 EPSPS* gene. Lane 1: water blank; lane 2: non-transgenic control; lanes 3–15: lines B; J; I; F; L; M; K; C; E; N; O; P; Q respectively; lane 16: a) blank, b) & c) 1kb^{+} DNA ladder; lane 17: line R; lane 18: line D; lane 19: line S; lane 20: a) 1 copy number plasmid control, b) & c) plasmid control; lane 21: a) 5 copy number control, b) & c) 1kb^{+} DNA ladder; lane 22: a) 10 copy number control. Positive bands in the Southern blot analysis are indicated by red arrows.

3.2. Identification of left border T-DNA/genomic DNA junctions and utilisation of this sequence for distinguishing independent insertion events

TAIL-PCR was performed on nine of the ten onion lines that had been selected for field trial evaluation in order to isolate T-DNA/genomic DNA junction sequence (the sequence of the T-DNA/genomic DNA junction for line J had been obtained prior to this body of work by Andrew Catanach of Crop & Food Research). TAIL-PCR reactions were screened for a step-down of 79 bp between the secondary and tertiary PCR products.

The T-DNA/genomic DNA junction sequences were identified for lines D and F. Figure 3.2 shows the successful TAIL-PCR reaction for line F using the arbitrary degenerate primer AD3. Many step-downs were present, however, they were in the same position across all samples, including plasmid, non-transgenic onion and water controls. Therefore these bands were dismissed as background and not isolated. A product of approximately 400 bp was isolated from the tertiary TAIL product and re-amplified for sequencing. Figure 3.3 shows the successful TAIL-PCR reaction for line D using the arbitrary degenerate primer AD4. A product of approximately 800 bp was isolated from the tertiary TAIL product and re-amplified for sequencing.

These isolated products showed partial sequence alignment to the left border sequence of the plasmid used for transformation.

To enable easy identification of these lines, genomic return primers were then designed within the onion genomic DNA sequence. Figures 3.4, 3.5, and 3.6 show the T-DNA/genomic DNA junction sequences obtained from lines F, D and J, respectively.

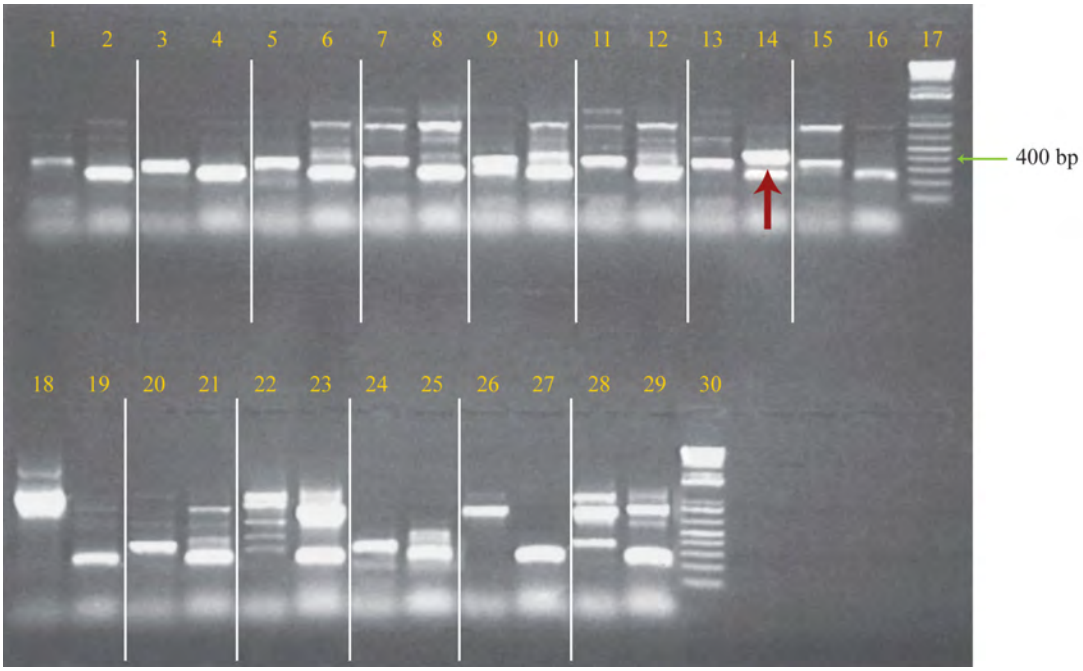

Figure 3.2. TAIL-PCR reaction using arbitrary degenerate primer AD3 showing positive result for line F. Each sample occupies two lanes as the TAIL 2 reaction is run immediately left of the TAIL 3 reaction. Lanes 1–12 & 15-21: all unsuccessful TAIL reactions using onion DNA samples from various lines; lanes 13 & 14: successful TAIL reaction for line F (A 79 bp step-down was observed in the PCR products between lanes 13 & 14, however the TAIL 2 PCR product was very faint); lanes 22 & 23: plasmid control; lanes 24 & 25: non-transgenic onion control; lanes 26 & 27: water control; lanes 28 & 29: no sample control; lanes 17 & 30: 1 kb^+ DNA ladder. The ~400 bp TAIL 3 product from lane 14, indicated by the red arrow, was used as template for a re-amplification reaction using TAIL 3 PCR reaction conditions.

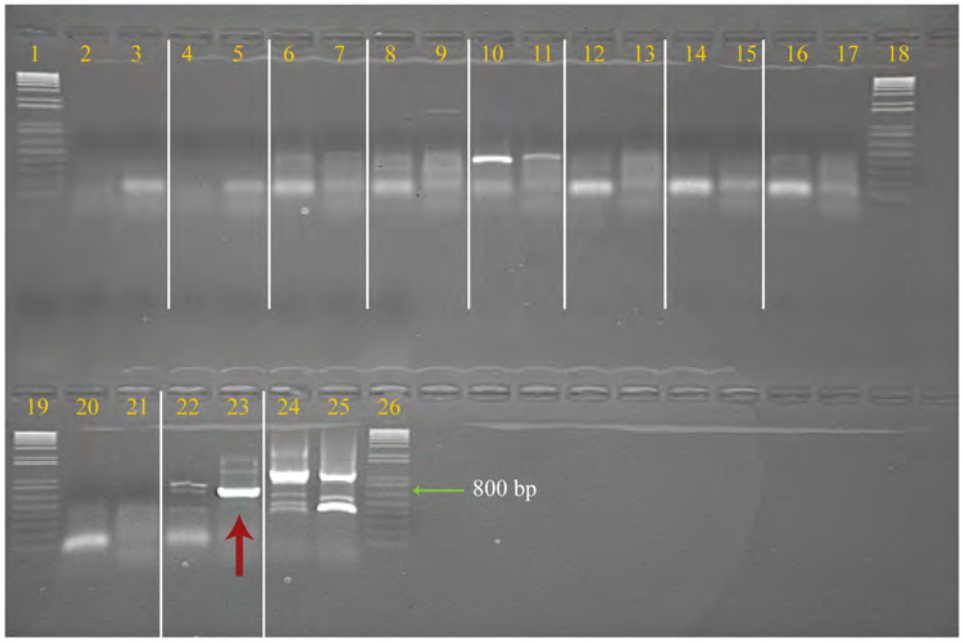

Figure 3.3. TAIL-PCR reaction using arbitrary degenerate primer AD4 showing positive result for line D. Each sample occupies two lanes as the TAIL 2 reaction is run immediately left of the TAIL 3 reaction. Lanes 1, 18, 19 & 26: 1 kb^{+} DNA ladder; lanes 2 & 3: no sample control; lanes 4 & 5: water blank control; lanes 6–17, 20 & 21: all unsuccessful TAIL reactions using onion DNA samples from various lines; lanes 22 & 23: successful TAIL reaction for line D (observe the 79 bp step-down in PCR product between lanes 22 & 23); lanes 24 & 25: plasmid control. The ~800 bp TAIL 3 product from lane 23, indicated by the red arrow, was used as template for a re-amplification reaction using TAIL 3 PCR reaction conditions.

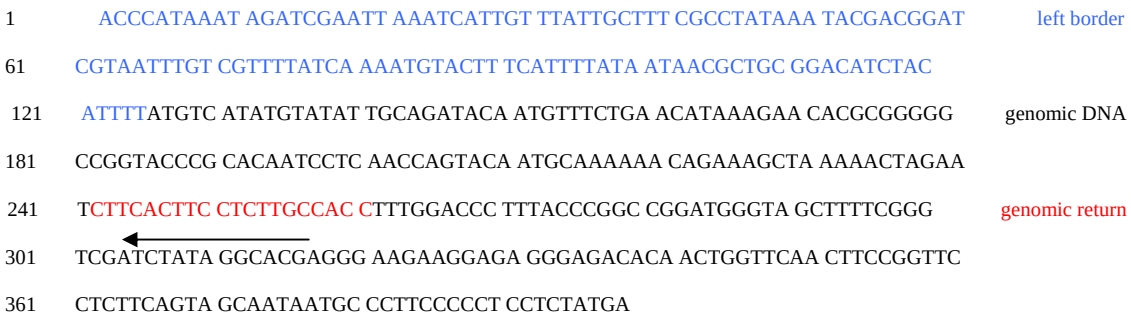

Figure 3.4. Left border sequence alignment and position of the genomic return primer in the 399 bp T-DNA/genomic DNA junction sequence for line F. Blue letters represent plasmid left border sequence, black letters represent onion genomic sequence and red letters represent the position of the genomic return primer. The orientation of the genomic return primer is indicated by the direction of the arrow.

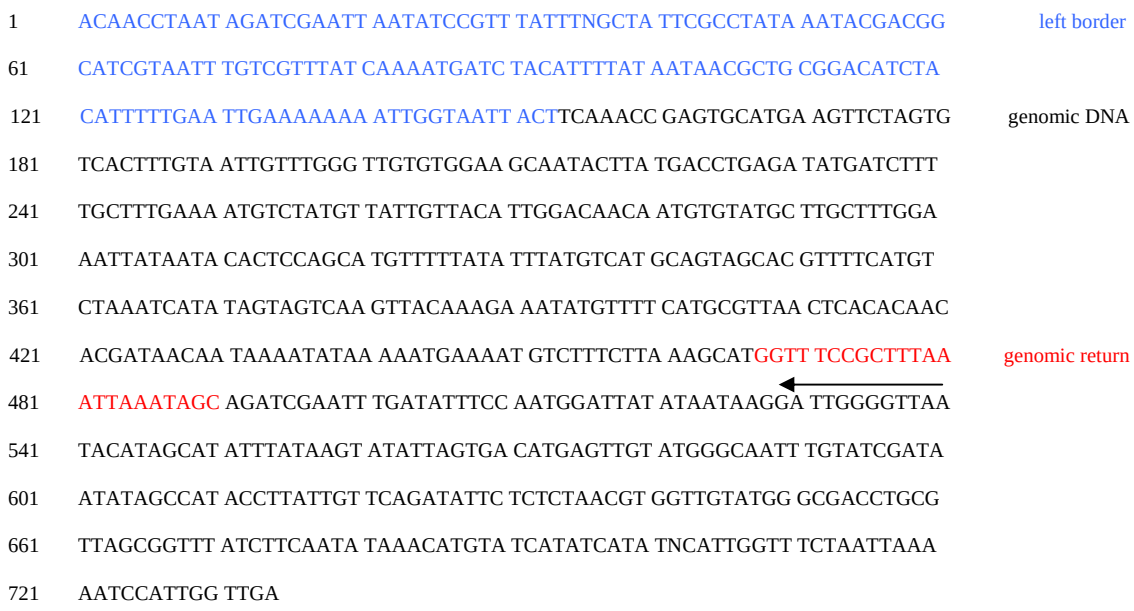

Figure 3.5. Left border sequence alignment and position of the genomic return primer in the 734 bp T-DNA/genomic DNA junction sequence for line D. Blue letters represent plasmid left border sequence, black letters represent onion genomic sequence and red letters represent the position of the genomic return primer. The orientation of the genomic return primer is indicated by the direction of the arrow.

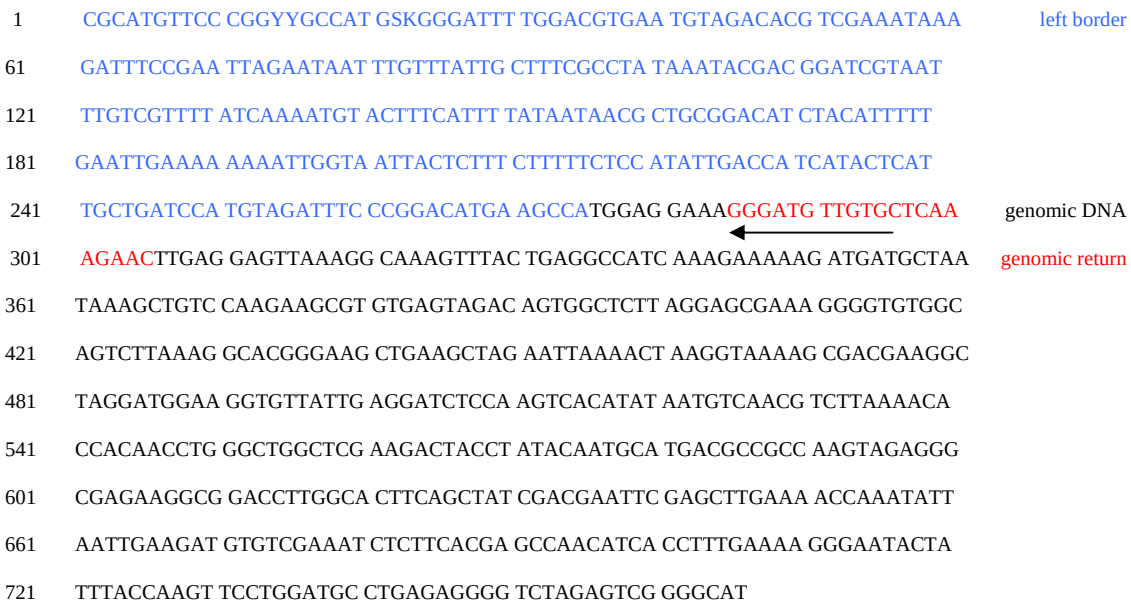

Figure 3.6. Left border sequence alignment and position of the genomic return primer in the 766 bp T-DNA/genomic DNA junction sequence for line J (sequence obtained and primer designed by Andrew Catanach at C&FR). Blue letters represent plasmid left border sequence, black letters represent onion genomic sequence and red letters represent the position of the genomic return primer. The orientation of the genomic return primer is indicated by the direction of the arrow.

Each of these genomic reverse primers was paired with TAILsp1, the specific primer placed within the left border sequence present in all of the transgenic onion lines, in a PCR reaction. Genomic DNA was isolated from all onion plants putatively from these three lines using the urea extraction method, and subjected to a PCR test using the appropriate genomic reverse primers. All transgenic plants tested were found to be positive for their respective line PCR indicating clones from a single insertion event. Figure 3.7 shows that genomic return primers were extremely specific for each line, producing amplified products of the expected sizes from the correct transgenic lines. There was no amplification of DNA from plasmid, non-transgenic onion or from other transgenic lines.

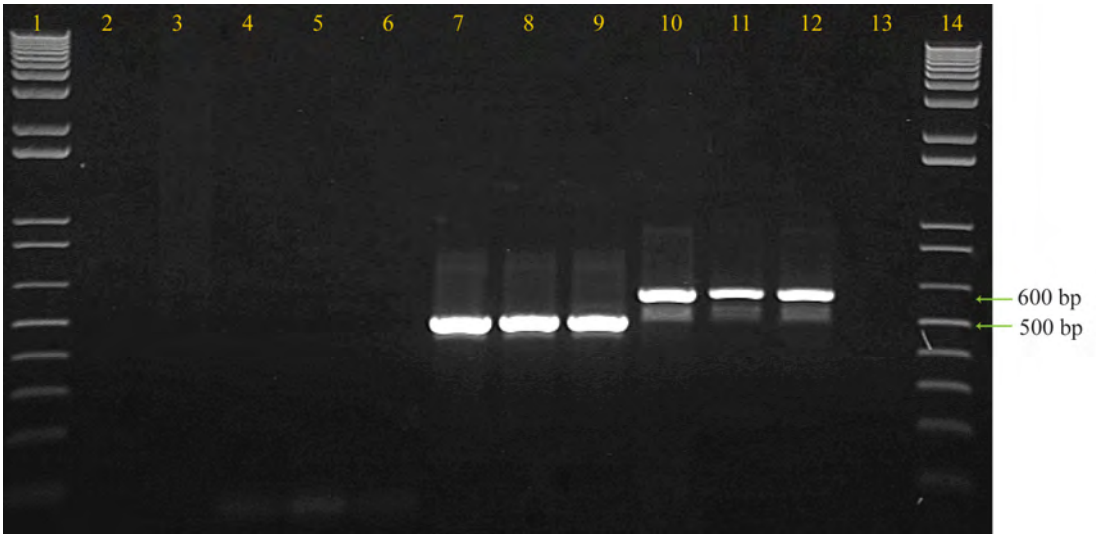

Figure 3.7. PCR reaction demonstrating specificity of genomic return primers using the T-DNA specific TAILsp1 primer and a mixture of genomic return primers from lines D & F. The expected size of the line D and line F PCR products were ~600bp and ~500 bp respectively. Lanes 1 & 14: 1kb^{+} DNA ladder; lane 2: no sample control; lane 3: non-transgenic onion DNA; lanes 4, 5 & 6: samples from line J; lanes 7, 8, & 9: samples from line F; lanes 10, 11 & 12: samples from line D; lane 13: plasmid control.

3.3. Hemi/homo-zygote detection using TAIL-PCR

Three nested primers were designed within the genomic sequences of lines F, D and J obtained in Section 3.2, heading towards the T-DNA/genomic DNA junction as shown in Figures 3.8, 3.9 and 3.10 respectively.

DNA was extracted from 20 F_1 seedlings of lines D and J, and 20 F_2 seedlings of line F using the urea extraction method. This DNA was subjected to a PCR testing for the absence of the *CP4 EPSPS* gene, to identify non-transgenic individuals. TAIL-PCR was then carried out on the DNA of the non-transgenic siblings of lines D, F, and J. TAIL-PCR products are shown in Figure 3.11.

Step-downs of the desired sizes were obtained in lines D and F using arbitrary degenerate primer AD1 and in line J using arbitrary degenerate primer AD2. Onion DNA samples were duplicated, so identical bands were obtained in TAIL reactions using the same AD primers and the most intense of these products was chosen for sequencing. A product of approximately 500 bp was isolated from the secondary TAIL product of line F but proved difficult to re-amplify, so sequencing was not done. A product of approximately 150 bp was isolated from the tertiary TAIL product of line J, re-amplified using TAIL 3 conditions, and sequenced using the primer GenSP3J. However, this product was very short and the sequence returned was not of good quality. A product of approximately 300 bp was isolated from the tertiary TAIL product of line D, re-amplified using TAIL 3 conditions, and sequenced using the primer GenSP3D. The PCR product from a non-transgenic sibling of line D returned a 281bp sequence. However, due to the placement of the GenSP3D primer within the known genomic sequence, this did not allow enough sequence to make an alignment, and hence the sequence obtained cannot be verified as correct. A hemizygous determining primer was designed within the 281bp potential genomic sequence of line D, as shown in Figure 3.12. This was paired with GenSP2D in a PCR using non-transgenic DNA from line D, but this failed to amplify any products.

Figure 3.8. The position of the specific nested primers for TAIL-PCR with respect to the left border in the 399 bp T-DNA/genomic DNA junction sequence for line F. Blue letters represent left border sequence, black letters represent onion genomic sequence. The orientation of primers GenSP1F (orange letters), GenSP2F (green letters), and GenSP3F (purple letters) are indicated by the direction of the arrows. The expected step-down size between the TAIL2 and TAIL3 products is 92 bp.

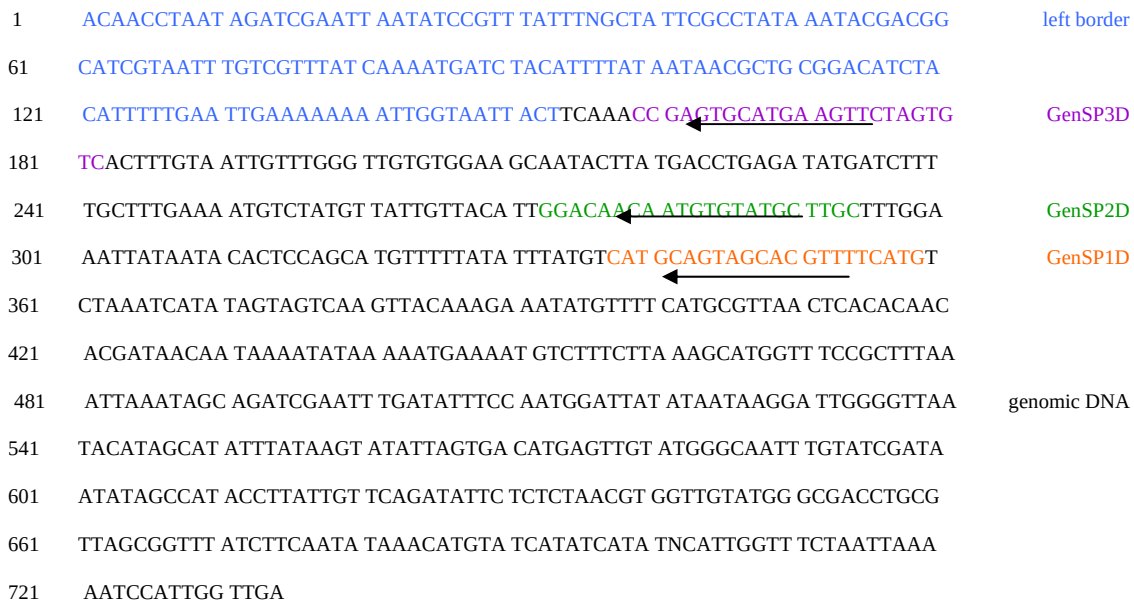

Figure 3.9. The position of the specific nested primers for TAIL-PCR with respect to the left border in the 734 bp T-DNA/genomic DNA junction sequence for line D. Blue letters represent left border sequence, black letters represent onion genomic sequence. The orientation of primers GenSP1D (orange letters), GenSP2D (green letters), and GenSP3D (purple letters) are indicated by the direction of the arrows. The expected step-down size between the TAIL2 and TAIL3 products is 112 bp.

Figure 3.10. The position of the specific nested primers for TAIL-PCR with respect to the left border in the 766 bp T-DNA/genomic DNA junction sequence for line J. Blue letters represent left border sequence, black letters represent onion genomic sequence. The orientation of primers GenSP1J (orange letters), GenSP2J (green letters), and GenRetJ (purple letters) are indicated by the direction of the arrows. The expected step-down size between the TAIL2 and TAIL3 products is 106 bp.

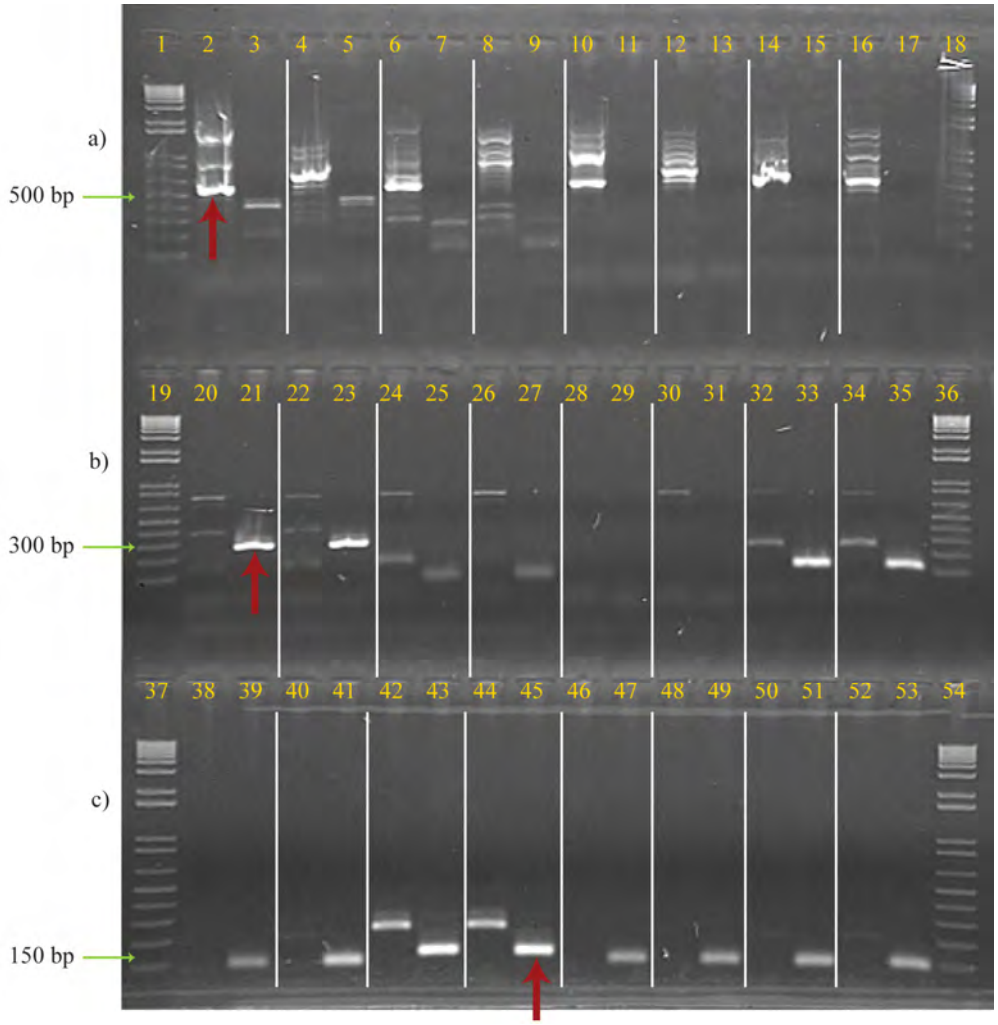

Figure 3.11. TAIL-PCR results of non-transgenic onion samples from **a)** line F, **b)** line D and **c)** line J. Each sample occupies two lanes as the TAIL 2 reaction is run immediately left of the TAIL 3 reaction. Lanes 2, 3, 4, 5, 20, 21, 22, 23, 38, 39, 40, 41: using arbitrary degenerate primer AD1; lanes 6, 7, 8, 9, 24, 25, 26, 27, 42, 43, 44, 45: using arbitrary degenerate primer AD2; lanes 10, 11, 12, 13, 28, 29, 30, 31, 46, 47, 48, 49: PCR using arbitrary degenerate primer AD3; lanes 14, 15, 16, 17, 32, 33, 34, 35, 50, 51, 52, 53: using arbitrary degenerate primer AD4; lanes 1, 18, 19, 36, 37, 54: 1kb+ DNA ladder. The 500 bp line F TAIL 2 product from lane 2, 300 bp line D TAIL 3 product from lane 21, and 150 bp line J product from lane 45, all indicated in the figure by red arrows, were used as template in re-amplification reactions using TAIL 3 PCR reaction conditions.

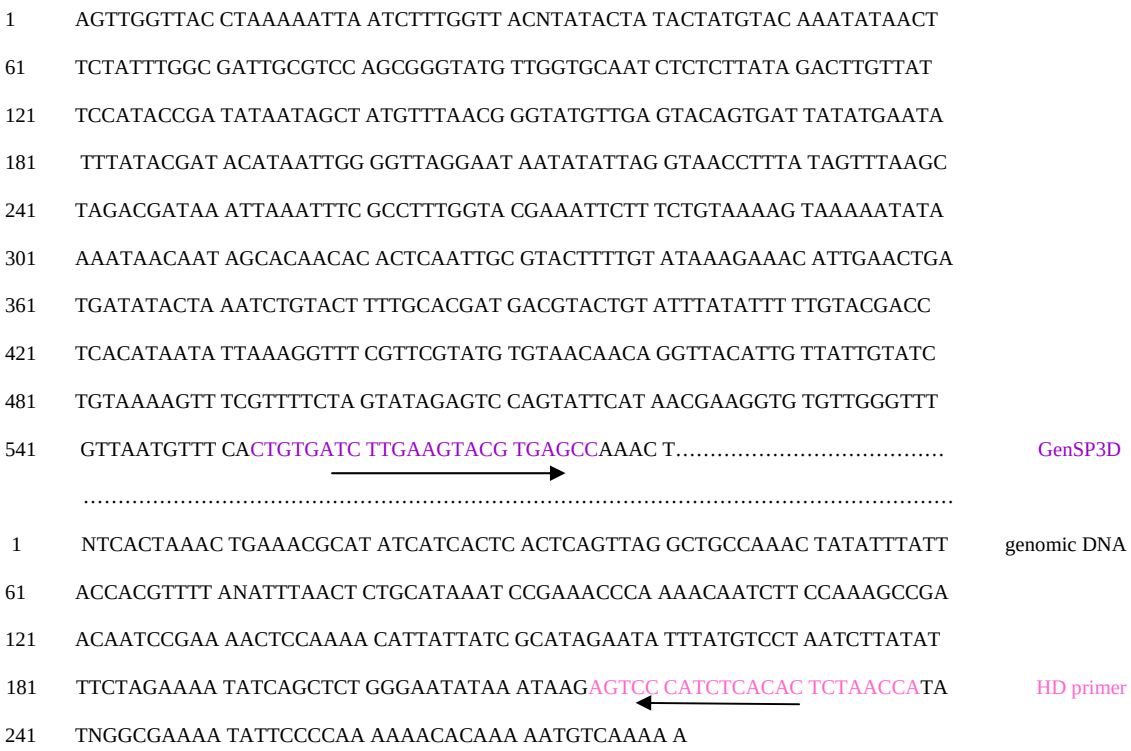

Figure 3.12. The 281 bp putative genomic sequence obtained from the non-transgenic sibling of line D in relation to the genomic sequence obtained from the T-DNA/genomic DNA junction sequence for line D (Figure 3.9). Black letters denote genomic sequence. Dots indicate missing sequence. The orientation of the line D genomic TAIL primer GenSP3D (purple letters) and the hemizygote determining (HD) primer (pink) are indicated by the direction of the arrows.

3.4. Inheritance of the *CP4 EPSPS* gene

Inheritance data for the *CP4 EPSPS* gene in the glyphosate-resistant onion lines is presented in Table 3.1. Only line D returned a result that was not significantly different from the expected ratio, indicating that transmission of the *CP4 EPSPS* gene followed a 3:1 Mendelian ratio. Transmission of the *CP4 EPSPS* gene in all other lines differed significantly from the expected 3:1 ratio.

Table 3.1. Chi-square goodness of fit test with 1 d.f. testing the hypothesis that transmission of the *CP4 EPSPS* gene will follow a 3:1 Mendelian ratio in the glyphosate-resistant onion lines used in the field trial. Transmission of the glyphosate-resistance trait to seedlings was assayed by glyphosate spray tests and scoring the phenotype.

		Observed		**Expected**			
Line	**% germinated**	CP4 positive	CP4 negative	CP4 positive	CP4 negative	**X^2**	**p-value**
B	73.5	133	125	193.5	64.5	75.664	p<0.001
C	49.44	118	60	133.5	44.5	7.199	p=0.007
D	96.3	253	85	253.5	84.5	0.004	p=0.950*
E	56.77	175	106	210.75	70.25	24.257	p<0.001
F	41.04	131	98	171.75	57.25	38.674	p<0.001
H	72.08	143	110	189.75	63.25	46.072	p<0.001
I	78.98	167	558	543.75	181.25	1044.160	p<0.001
J	60.07	193	153	259.5	86.5	68.166	p<0.001
K	75.35	222	212	325.5	108.5	131.641	p<0.001

3.5. Determination of *CP4 EPSPS* transcript levels

All ten field-grown onion lines displayed glyphosate tolerance at a level considered to be agronomically useful when subjected to a glyphosate-based weed control regime (Eady pers. comm.). A demonstration of this glyphosate tolerance in a single plot in the field trial is shown in Figure 3.13.

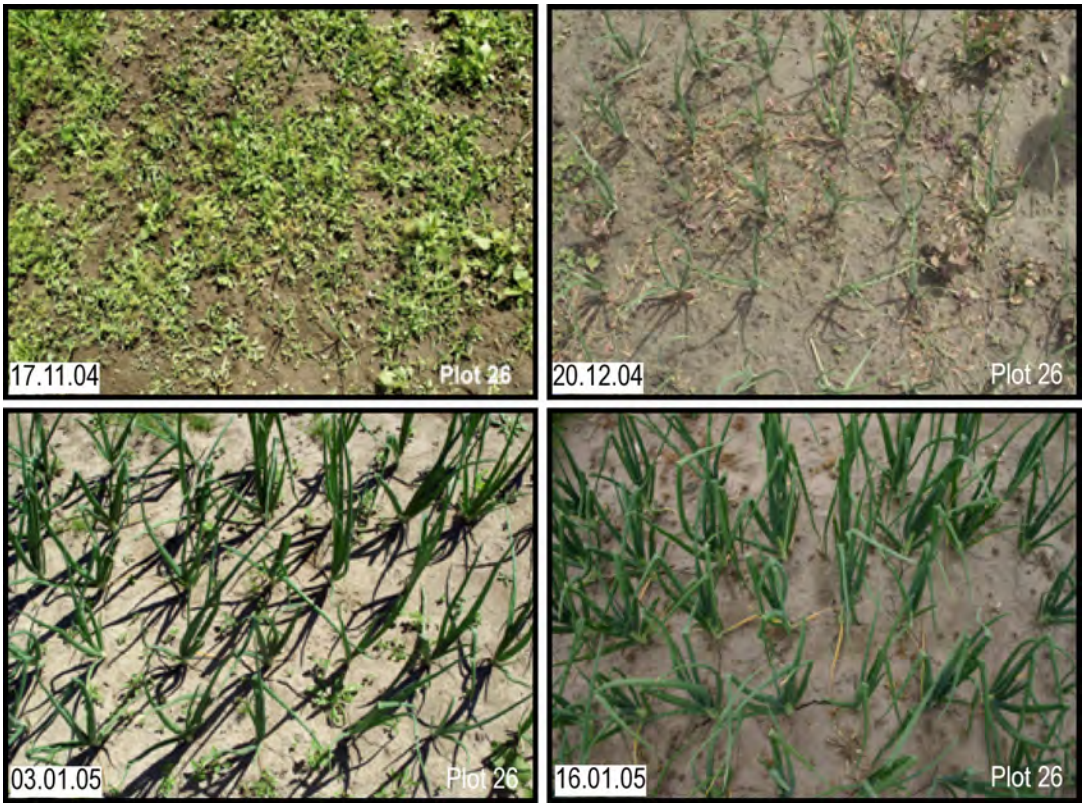

Figure 3.13. A demonstration of the weed control efficacy of a glyphosate-based spray regime on a single field-grown plot of glyphosate-resistant onions from line J. Glyphosate was applied to the plants in the field trial on 19/11/04 and 5/1/05.

Four lines were chosen for sampling that were a representative sample of the range of germplasm in the field trial. Lines H and J are plants from selfed seed from transgenic events in open-pollinated PLK-type germplasm, and lines C and D are plants from selfed seed from transgenic events in short day hybrid germplasm. The phenotypes of these lines are shown in Figure 3.14. The hybrid germplasm segregated into two different parental types. Hence lines C and D showed a greater overall variation in bulb size than the more uniformly sized open-pollinated-derived onions from lines H and J.

Figure 3.14. Phenotypes of field-grown glyphosate-resistant onion bulbs from segregating hybrid derived lines C and D, and open-pollinated lines H and J, after harvest and six weeks curing.

Overall, the *CP4 EPSPS* transcript level was relatively high in the glyphosate-resistant onion lines as no competimer was required for the highly expressed 18S internal control in the multiplexed RT-PCR reactions. Thus, expression of the *CP4 EPSPS* gene was in the same order of magnitude as the 18S. An example of a multiplex RT-PCR gel is shown in Figure 3.15.

Transcript levels were stated as a ratio of *CP4 EPSPS* transcript / *18S* transcript. The ratios were log-transformed before analysis to stabilise the variance. Statistical analysis was carried out by Ruth Butler (Crop & Food Research) using analysis of variance. Output from the analyses of variance for the three sets of log-transformed ratios is shown in Figure 3.16, with a summary in Table 3.2.

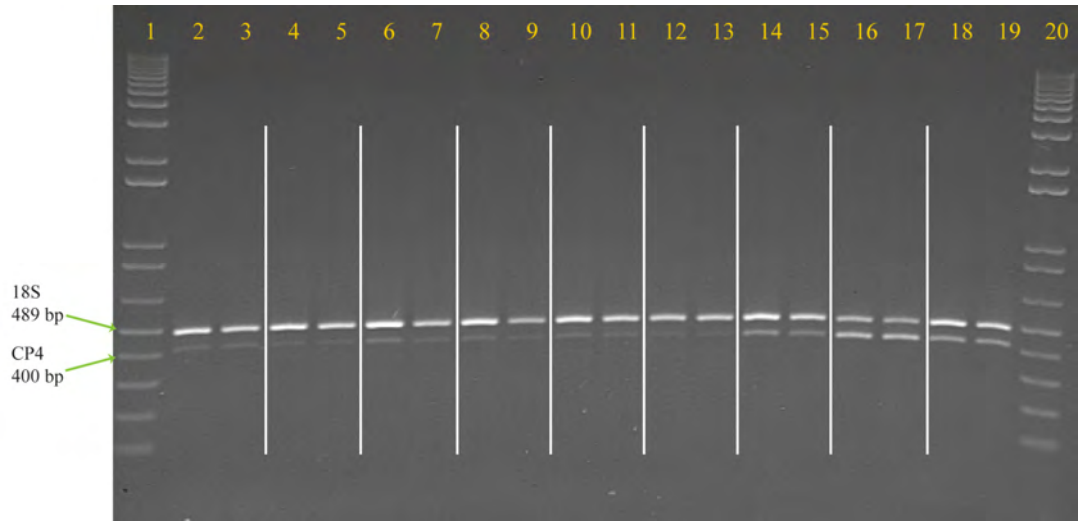

Figure 3.15. Gel showing multiplex RT-PCR of young leaf samples from field-grown glyphosate-resistant onion lines H and J. Each sample was loaded in duplicate so takes up two lanes. Lanes 1 & 20: 1kb^{+} DNA ladder; lanes 2–15: samples from line H; lanes 16–19: samples from line J. The top 489 bp band is the18S PCR product. The smaller 400 bp band is the *CP4 EPSPS* PCR product.

Significant differences in *CP4 EPSPS* transcript levels were observed between the lines for all three of the measurements ($p<0.05$ or smaller).

For young leaves, the mean log(ratios) were significantly smaller ($p<0.05$) for lines C and H than for lines D and J. The ratios for D and J were on average around three times as large as those for C and H.

For old leaves, the log(ratio) for line J was also significantly ($p<0.05$) larger than that for line H, again with the mean ratio for J being around three times that for line H.

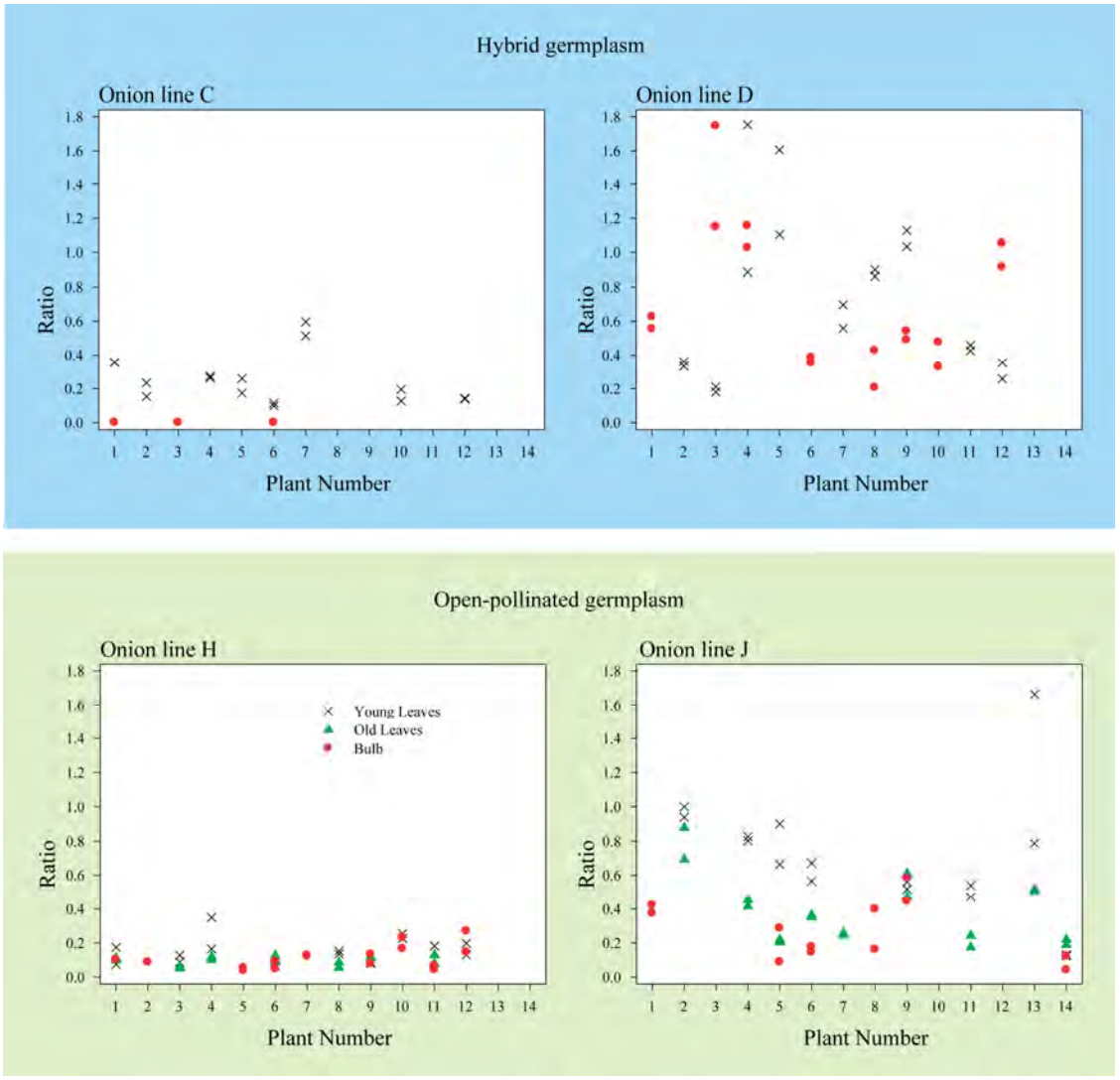

Figure 3.16. Scatterplots displaying *CP4 EPSPS* transcript levels stated as a ratio of *CP4 EPSPS* transcript/*18S* transcript for young leaves, old leaves and bulbs from each plant sampled in field-grown glyphosate-resistant onion lines C, D, H, and J. All measurements were done in duplicate for each sample.

Table 3.2. Mean *CP4 EPSPS* transcript levels stated as a ratio of *CP4 EPSPS* transcript /*18S* transcript for each line (natural log. transformed scale).

Line	Young leaf	Old leaf	Bulb
C	0.21 (-1.57)	-	0[†]
D	0.59 (-0.53)	-	0.57 (-0.56)
H	0.14 (-1.98)	0.10 (-2.30)	0.09 (-2.36)
J	0.59 (-0.53)	0.35 (-1.06)	0.19 (-1.65)
Lsd 5%[*]	(0.75)	(0.99)	(1.24)
d.f.[‡]	6	2	4

[†] All values zero, so these were excluded from the analysis

* Lsd 5%: least significant difference between two means (on the log. scale), at the 5% significance level

[‡] d.f: degrees of freedom associated with the LSD

For bulbs, a similar pattern was observed, with the ratio for J larger than that for H, but not significantly so. However, in this case the difference was not so great, with the ratio for J being around twice that for H. The log(ratio) for line D was greater than that for J and H (significantly for H only, $p<0.05$), being six times the ratio for H and nearly three times the ratio for J. The transcript from line C was not detected under the multiplex RT-PCR conditions, but was present when subjected to a non-competitive RT-PCR using only *CP4 EPSPS* primers.

There does not appear to be a direct correlation between transcript levels in young leaf, old leaf, and bulb measurements within individual plant samples.

3.6. Determination of CP4 EPSPS protein levels

Plant samples from lines C, D, H and J were subjected to an adapted ELISA assay to determine levels of the CP4 EPSPS protein. Where possible the same plants were sampled as those used in the transcript analysis. An ELISA assay is shown in Figure 3.17.

Figure 3.17. ELISA test plate used to quantify CP4 EPSPS protein in the glyphosate-resistant onions. Row 1: soya protein isolate control samples; row 2: young leaf samples of glyphosate-resistant onion samples, with a blank in the last two wells; rows 3 & 4: young leaf samples of glyphosate-resistant onions.

Statistical analysis was carried out by Ruth Butler (Crop & Food Research). The mean protein data for the measured plants are shown in Figure 3.18 with a summary given in Table 3.3.

The results here tend to mirror the patterns seen in the *CP4 EPSPS* transcript data, with CP4 EPSPS protein levels generally higher for lines D and J than for lines C and H.

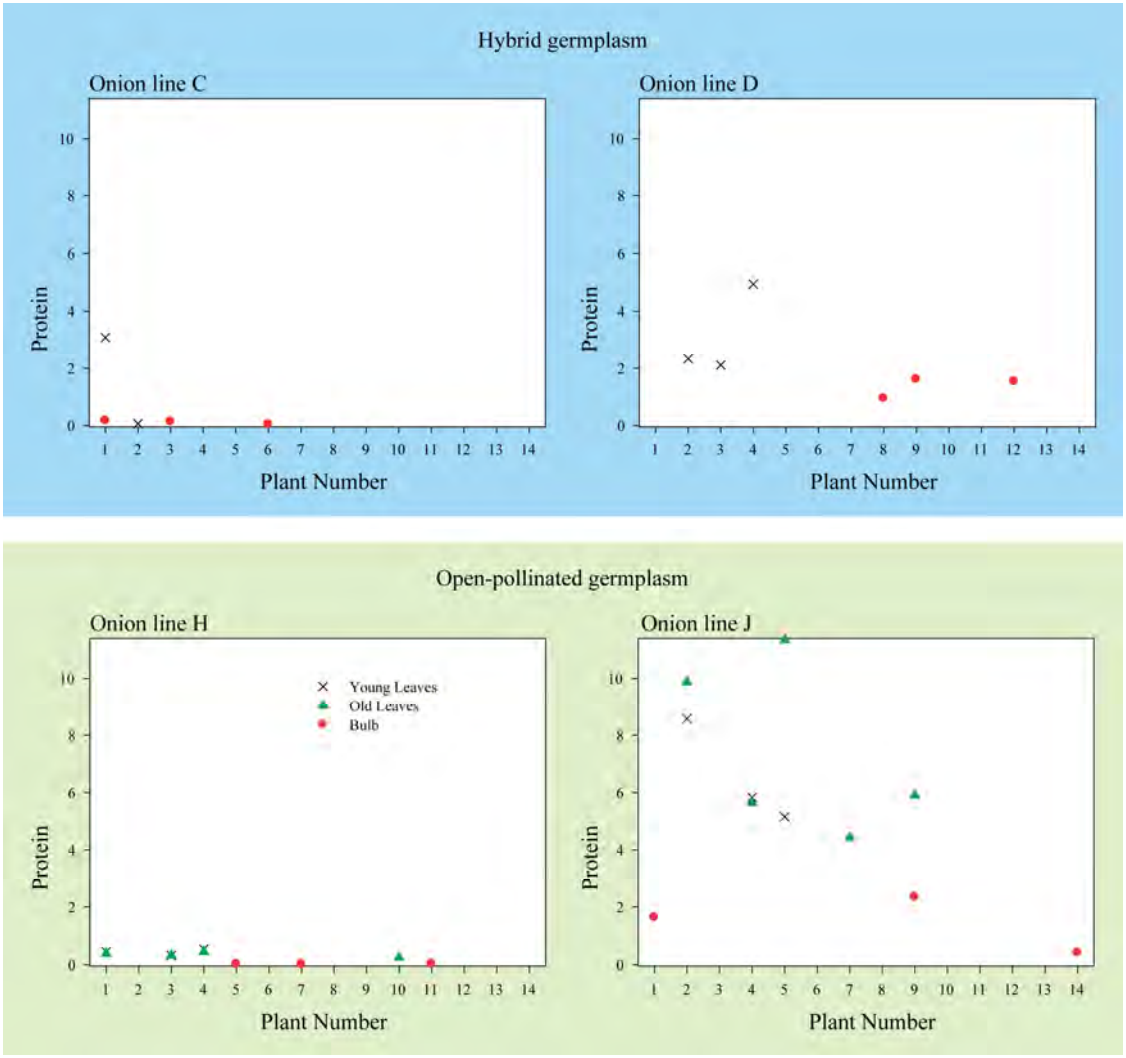

Figure 3.18. CP4 EPSPS protein (µg CP4 g^{-1} FW) for young leaves, old leaves and bulbs from each plant sampled in field-grown glyphosate-resistant onion lines C, D, H, and J.

Table 3.3. Mean CP4 EPSPS protein (µg CP4 g^{-1} FW) for each line.

Line	Young leaf	Old leaf	Bulb
C	1.58	-	0.12
D	3.13	-	1.36
H	0.44	0.36	0.02
J	6.52	7.44	1.46

Chapter Four

Discussion

This project presents the successful molecular characterisation of glyphosate-resistant onion lines transformed with the *CP4 EPSPS* gene. Ten of these onion lines were subjected to field trial analysis over the 2004/2005 growing season in Canterbury. The transcriptional and translational behaviour of the introduced *CP4 EPSPS* gene was investigated in four field-grown lines: two lines derived from selfed seed from open pollinated PLK-type germplasm; and two lines derived from selfed seed from short day hybrid germplasm. All field-grown onion lines displayed glyphosate tolerance at a level consistent with twice the agronomically useful application rates (Eady pers. comm.).

4.1. Molecular analysis of T-DNA integration events

The transgenic nature of all onion lines tested was confirmed by PCR amplification of the *CP4 EPSPS* gene, as shown in Figure 3.1b. Southern blot analysis showed that all of the glyphosate-resistant onion lines contained a single copy of the *CP4 EPSPS* gene integrated into their genomes, except for line L which had two copies of the gene (Figure 3.1a). This was to be expected as the transgenic onions were produced via the method of Eady et al. (2003a) using *Agrobacterium*-mediated transformation which usually facilitates the integration of low copy numbers of the transgene into the plant genome (Dai et al. 2001). These results are similar to Southern data reported for the *Agrobacterium*-mediated transformation methods of onion by Zheng et al. (2001) and Aswath et al. (2006), in which most transformants were found to have only a single copy of the transgene. For breeding and expression purposes it is considered desirable for transgenic plants to contain transgenes as a single copy. Homology-dependent gene silencing (HDGS) has been observed in plants containing multiple copies of transgenes, often resulting in unpredictable gene expression (Butaye et al. 2005).

Glyphosate-resistant onion lines C and I contained the spectinomycin resistance gene (*aadA* gene) that had been transferred to the plants from the backbone of the transformation vector (Figure 3.1c). However, as only a small-scale field-trial was being conducted, these lines were still able to be field-tested as ERMA requirements only preclude them from being involved in large scale trials.

This backbone integration was not unexpected as it is a relatively common event in genetically modified plant material produced via *Agrobacterium*-mediated transformation. It has been seen in many different species including *Arabidopsis*, tobacco, potato, and maize, with reported frequencies of between 30-75% (Kuraya et al. 2004, Lange et al. 2006). With *Agrobacterium*-mediated transformation, a vector is used that has two 25 bp imperfect repeats known as the left and right borders. DNA inserted between the two borders (T-DNA) is usually transferred into plant cells by illegitimate recombination with initiation at the right border and termination at the left border. Both repeats are recognised in the *Agrobacterium* by a virulence protein complex. Integration of vector-derived sequence found outside of the right and left borders (backbone sequence) is thought to occur by inefficient recognition of the left border resulting either in read-through or the initiation of DNA transfer from the left border (Kuraya et al. 2004). There are many factors that may influence the frequency of this backbone integration, such as plant species, co-cultivation method, *Agrobacterium* strain and binary vector used (Shou et al. 2004, Podevin et al. 2006). At 12% (2 out of 17), the glyphosate-resistant onions used in this study appear to have a low frequency of backbone integration compared with that published for other plant species.

The integration of vector backbone sequences is considered unfavourable for several reasons. Firstly, due to safety concerns regarding genetic modification, regulatory authorities and the public request that genetically modified plants are free of unnecessary DNA segments, especially vector backbone sequences containing bacterial selectable markers or bacterial origins of replication (Lange et al. 2006, Podevin et al. 2006). Secondly, the integration of vector backbone sequences into the plant genome alongside T-DNA sequences has been implicated in transgene silencing (Kooter et al. 1999).

Vector backbone sequences may be targeted for methylation within the plant genome due to an incompatibility of GC content with that of the integration site, consequently leading to reduced expression of the associated transgene (Kooter et al. 1999, Meza et al. 2002).

4.2. Molecular analysis of T-DNA/genomic DNA junction sites

TAIL-PCR was successfully used to obtain T-DNA/genomic DNA junctions from some glyphosate-resistant onion lines. However, the major limitation of TAIL-PCR is the small sizes of the products obtained. In this instance they ranged between 400 and 800 bp. These results are similar to those obtained by Liu et al. (1995) with DNA from transgenic *Arabidopsis*, and are comparable to those obtained by Dale (2004) using transgenic *Pinus radiata* DNA. Dale (2004) suggested that there appears to be a 1 kb limit on products recovered from TAIL-PCR, so this method will fail to amplify the T-DNA/genomic DNA junction in those plants where the degenerate primer binding sites are located outside this size limit. This is one possible explanation for why the junction sequence has been obtained in only some of the glyphosate-resistant onion lines. It is feasible that using a wider range of degenerate primers may lead to more TAIL-PCR products.

Alternatively, TAIL-PCR products may be obtained from lines that are yet to yield junction sequences by designing specific primers within the right border of the transformation vector sequence. The right border may be more faithfully transferred to the plant as this is the site of T-DNA transfer initiation. It is possible that, during integration of a functional *CP4 EPSPS* gene, that the left border has been truncated in some glyphosate-resistant onion lines. If this is the case then the specific primers will not be able to bind to an absent left border vector sequence and a TAIL-PCR product will not be obtained.

Another option for obtaining T-DNA/genomic DNA junction sequences from the glyphosate-resistant onion lines would be to use a method other than TAIL-PCR. White & Chen (2006) found inverse PCR to be a more efficient method than TAIL-PCR for

identifying T-DNA/genomic DNA junctions in transgenic *Ascochyta rabiei*. They attributed this to the use of only specific primers in inverse PCR as opposed to relying on random binding of the arbitrary degenerate primers in the TAIL-PCR procedure. SiteFinding-PCR described by Tan et al. (2005) appears to have several advantages over both TAIL-PCR and inverse PCR, such as only weak amplification of non-specific products, and the potential generation of longer amplification products. They obtained numerous products over 1 kb in size, with some even larger than 4.5 kb.

The genomic return PCR results, shown in Figure 3.7, illustrate successful detection of individual lines using a simple PCR method. So, for lines with a single copy T-DNA insertion and a known T-DNA/onion genomic DNA sequence, this method provides a more time-efficient and cheaper identifier than Southern analysis for screening large numbers of potentially clonal plants, and also provides traceability for these individual events.

While this genomic return PCR is very specific for each individual line, there appears to be some faint background banding visible on the gels. These superfluous faint PCR products could be produced by the genomic return primer binding less efficiently elsewhere in the genome. This may occur because, at 15,300 Mbp, the onion genome is extremely large, and the genomic return primers may potentially have been designed in one of many sequence repeats.

Adaptation of the TAIL-PCR method for the detection of hemi/homo-zygote individuals appears promising. Step-downs of the right size were identified for all lines tested, but the products were all very short. The TAIL-PCR should be repeated with a wider range of AD primers in order to generate a wider range of TAIL-PCR products for analysis.

The sequence obtained from the non-transgenic sibling of line D is shown in Figure 3.12. This DNA was sequenced with the primer GenSP3D, but as this primer is situated very close to the T-DNA junction it should have been sequenced with GenSP1D. This would have generated enough sequence overlap to align with the T-DNA junction sequence

from Figure 3.9 and hence determine the validity of the obtained sequence. This sequencing was not repeated due to time constraints.

Instead of using non-transgenic siblings of transgenic lines, the genomic sequence on the other side of the T-DNA could theoretically be obtained by carrying out TAIL-PCR from the right border of the T-DNA into the onion genome. Unfortunately, this approach would be difficult to apply to the glyphosate-resistant onions. Three constructs were used in the production of the glyphosate resistant onion lines. These constructs share terminator and left border sequence, but three different promoter regions were used, with one construct containing two copies of a particular promoter sequence. A large proportion of the glyphosate-resistant onions contain this double promoter in their T-DNA. The presence of this repeat sequence makes the design of primers for TAIL-PCR difficult. A better approach might be to use inverse-PCR which would simultaneously amplify both flanking genomic regions.

Alternatively, zygosity levels have been determined in transgenic plants such as maize (Ingham et al. 2001) and tomato (German et al. 2003) using a quantitative real-time PCR assay utilising TaqMan technology. Ji et al. (2005) has also used TaqMan real-time PCR to determine zygosity in transgenic zebrafish. This technique utilises the 5' - 3' exonuclease activity of the *Taq* DNA polymerase which causes the degradation of target-specific probe molecules during each PCR cycle, resulting in the accumulation of a fluorescence signal. The fluorescence levels directly correspond to the amount of PCR product and are detected during each cycle of the amplification (German et al. 2003). It was not possible to apply the real-time PCR approach to the glyphosate-resistant onions as the sequence of the *CP4 EPSPS* gene is commercially sensitive and so could not be obtained for this body of work. As a result, it was not possible to design the primers and probe required for TaqMan analysis.

4.3. Inheritance of the glyphosate-resistance trait

Transmission of the glyphosate-resistance trait to large numbers of seedlings was assayed by simple glyphosate spray tests and scoring the phenotype, as time and cost prevented large-scale molecular analysis. For breeding purposes, it is desirable to identify those glyphosate-resistant onion lines in which inheritance of the transgene follows a simple Mendelian pattern in their first selfed cycle. This would indicate that the transgene is transferred in a stable manner between generations and is unlikely to have integrated into a deleterious part of the genome.

Mendelian inheritance has been observed in CP4 EPSPS-based glyphosate-tolerant crops such as soybean (Padgette et al. 1995) and corn (Heck et al. 2005). Also 3:1 inheritance ratios for the *CP4 EPSPS* gene were obtained in the first selfing cycle of the glyphosate-resistant onion lines in previous years (C. Eady, unpublished). However, in this experiment, only line D demonstrated a 3:1 Mendelian inheritance ratio for the *CP4 EPSPS* gene. Plants were produced via selfing, but for most of the lines tested this was a second round of selfing. Onions are notorious for suffering inbreeding depression and so the observed reduced ratios from the 3:1 expected is possibly due to lethality linked to increased homozygosity.

4.4. Sampling limitations

Plant material was sampled from selected plants for lines C, D, H and J from young leaf, old leaf, and bulb material. The lines selected were a representative sample of the range of onion germplasm containing the *CP4 EPSPS* gene planted in the field trial. Lines H and J were plants from selfed seed from transgenic events in open-pollinated PLK-type germplasm. Lines C and D were plants from selfed seed from transformation events in short day hybrid germplasm, and they reached maturity much faster in the field than the other lines. As a result, old leaf samples could not be obtained for lines C and D as there was no fresh leaf material to take when the other lines were being sampled. Likewise, bulb samples from all four lines were taken after six weeks of curing, and samples could

not be taken from those bulbs that failed to store over that period. Where practical, the same plants were sampled at each stage, but this was not always possible.

Obtaining an accurate quantification of RNA was challenging. An OD reading using a GeneQuant was tried first, but this gave extremely unreliable results and was time-consuming. RNA was subsequently quantified by OD reading on an ND1000 spectrophotometer (Nanodrop). There are several advantages of using a Nanodrop over a GeneQuant. First, the RNA sample is added directly to the Nanodrop system, reducing both the time for analysis and the risk of contamination from the GeneQuant method in which the sample must be diluted and added to a cuvette. Second, the Nanodrop provides a spectra of the tested sample over 400–750 nm, presenting information about RNA integrity and possible contamination (Fleige and Pfaffl 2006). An OD 260/280 ratio greater than 1.8 is generally considered an acceptable indicator of good RNA quality, and most RNA samples tested had ratios around 2.0.

However, when visualised on an agarose gel, adjusted RNA samples did not appear uniform. Therefore, these samples were subjected to further quantification via gel peak analysis using Phoretix 1D gel analysis software. An extra benefit of this gel analysis was that it provided a method for ensuring that there was no DNA contamination and also that any degraded RNA samples could be identified.

New methods of RNA quantification are being developed that are high-throughput and more informative regarding RNA quality. Systems such as the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the Experion (Bio-Rad laboratories, USA) use on-chip technology where the RNA sample is loaded on to it, separated by capillary electrophoresis, and then detected using fluorescence (Fleige and Pfaffl 2006).

The use of the Ready-To-Go RT-PCR beads (Amersham) for determining transcript levels was also very problematic. Beads from opened kits, stored following the manufacturer's instructions, and well within the best-by date of the kits, failed to reproduce results obtained from a new kit. The sensitivity and reliability of the kits

appeared to decrease over time after opening, making it difficult to obtain consistent results. Including a standard sample in each analysis enabled relative results to be obtained. A more reliable alternative for quantitative analysis of transcript levels would be to use real-time PCR. But as mentioned before, the *CP4 EPSPS* gene sequence was not available, so it was not possible to design the primers and probe required for this method.

4.5. *CP4 EPSPS* transcript analysis

Lines C and H had significantly lower *CP4 EPSPS* transcript levels than those of lines D and J. This pattern was reflected throughout all tissue types tested. The low levels of *CP4 EPSPS* gene expression in line C may be due to the vector backbone sequence that has integrated into the genome along with the T-DNA cassette. Both lines C and D are from the same hybrid derived genomic background, and they both contain a single copy of the *CP4 EPSPS* gene under the control of the double promoter sequence. The presence of vector backbone may have induced methylation of the area of integration, resulting in the extremely low expression of the transgene that has been observed in line C (Kooter et al. 1999). However, there have been conflicting reports as to whether the presence of backbone does in fact induce silencing (Meza et al. 2002). It is more likely that this difference in expression is due to the individual chromosomal positions into which the transgene has integrated. This "position effect" cause of transcript expression variation is commonly observed (Zhong 2001).

Lines H and J are both from the open-pollinated derived background, but each contains the *CP4 EPSPS* gene under the control of a different promoter region. It is possible that one of the promoter regions is more effective in the glyphosate-resistant onions than the other, resulting in a higher level of gene expression. However, the sample size was too small to draw any firm conclusions from this, as the differences in expression may simply be due to position effects. It is possible that the T-DNA within line H has integrated into an area of the genome that is not highly transcribed, resulting in low levels of the *CP4*

EPSPS transcript, whilst that of line J has integrated into a more highly transcribed region of the onion genome.

Within-line variation demonstrates possible genotype specificity, with the hybrid derived lines showing more variation than the open-pollinated derived lines with respect to transcript expression levels. Lines C and H both express the *CP4 EPSPS* transcript at low levels. However, line C appears to demonstrate greater within-line variation than line H, with values ranging from 0-0.60 and 0.04-0.35 respectively. Lines D and J are both high expressing lines, but line D appears to demonstrate more within-line variation than line J, with values ranging from 0.18-1.75 and 0.04-1.0 respectively. This is to be expected as the hybrid germplasm segregated into two different parental types. The differing phenotypes observed between the more uniformly sized open pollinated-derived onions and the more variable hybrid-derived genomic backgrounds are shown in Figure 3.14.

There is a lot of variation within some of the sample duplicates in the variance tests shown in Figure 3.16. This variation is most apparent in samples at the higher end of detection. This may be due to experimental error with the samples perhaps not being mixed or aliquoted properly. A larger sample size is needed to obtain more statistically robust data.

If segregation of the *CP4 EPSPS* gene was occurring in an F_1 population exhibiting Mendelian inheritance, it would be expected that from the nine samples analysed in each line, there would be three corresponding to homozygous individuals which may show higher levels of expression than the other six samples corresponding to hemizygous individuals. Figure 3.16 shows that in each set of measurements (young leaf, old leaf, and bulb), there are groups of individuals in each line with elevated transcript levels around the expected proportions that may indicate homozygosity. However, these transcript levels do not appear to be correlated between the measurement sets, i.e. the individual plants that have the higher transcript in the young leaf measurements are not necessarily the same as those individuals that show higher values in the old leaf and bulb

measurements. "Position effect" could account for this difference, with the surrounding genomic region, and hence the inserted transgene, responding differently in different tissues types.

4.6. CP4 EPSPS protein analysis

While it is necessary for the *CP4 EPSPS* gene to be transcribed at all stages of onion development in the field, it is just as essential that this transcript is correspondingly translated into protein. The identification of lines in which the amount of CP4 EPSPS protein produced is relative to the amount of *CP4 EPSPS* transcript detected is desirable, as this would indicate the absence of any complicated controls at the level of translation that may reduce the efficacy of this transgene.

The patterns here tend to mirror the patterns seen in the *CP4 EPSPS* transcript analysis, with CP4 EPSPS protein levels generally higher for lines D and J than for lines C and H. This implies the absence of complicated translational controls in these transgenic onion lines.

However, because there were only a few measurements where both protein and transcript were measured on the same plant, it is not possible to get a meaningful summary of the relationship between protein and transcript on a plant by plant basis. Fewer plant samples were analysed for protein levels than were analysed for transcript levels due to the expense of the protein assay.

The CP4 EPSPS protein levels of the glyphosate resistant onion ranged between 0.36–7.44 µg CP4 g^{-1}FW in the leaf samples. This is a little lower than, but still similar to, values reported for glyphosate-resistant corn, which had a mean of 20.8 µg CP4 g^{-1}FW (Heck et al. 2005) and poplar, for which values ranged between 0 and 26 µg CP4 g^{-1}FM (Meilan et al. 2002). However, the four glyphosate-resistant onion lines analysed in this study are not the highest CP4 EPSPS expressing lines. CP4 EPSPS protein levels have been detected in glyphosate-resistant onion samples

(unpublished data) that are more comparable with those seen in the glyphosate-resistant corn and poplar, indicating that CP4 EPSPS expression is not species specific.

4.7. Conclusions

In conclusion, molecular characterisation of field-grown onion lines transformed with the *CP4 EPSPS* gene has shown that the gene is present as a single copy in most lines and that vector backbone integration occurred only at very low frequency. Molecular "fingerprints" were ascertained for some lines, allowing the creation of a simple PCR test for rapid identification of plants from these lines. Adaptation of the TAIL-PCR technique used in this "fingerprinting" appears promising for determination of homozygous and hemizygous individuals, however further work is required.

With the exception of one line, inheritance of the *CP4 EPSPS* gene did not occur at the expected 3:1 Mendelian ratio, which is in contrast to results obtained from previous growing seasons. This could be explained by possible lethality linked to increased homozygosity as a result of repeated selfing.

The *CP4 EPSPS* gene in the field-grown glyphosate-resistant onion plants was expressed at agronomically useful levels, with high amounts of transcript detected, and protein levels similar to those observed in other glyphosate-resistant plant species. However, a more detailed analysis of the onion plants could be undertaken if the gene sequence was available. This would allow the use of real-time PCR to gain a better idea of transcript levels and determine the zygosity of the plants.

This project reports the first in-depth analysis into the expression of the *CP4 EPSPS* gene in field-grown glyphosate-resistant onion lines.

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