Transgenic White Clover Plants With a Molecular Stack of Traits for Delayed Leaf Senescence, Aluminium Tolerance and *Alfalfa mosaic virus* Resistance. Generation and Molecular and Functional Characterisation

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List of abbreviations

p	*Promoter*
t	*Terminator*
ACT11	*Actin 11*
Al	aluminium
Al^{3+}	Trivalent cation of aluminium
AMV	alfalfa mosaic virus
ANOVA	analysis of the variance
At*myb32*	*Arabidopsis thaliana MYB 32*
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BLASTN	Nucleotide BLAST
bp	base pairs
Bt	*Bacillus thuringiensis*
°C	degree Celsius
C-terminal	carboxy-terminal
Cas9	CRISPR-associated protein-9 nuclease
CS	Citrate synthase,
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CaMV35S	Cauliflower mosaic virus 35S
cDNA	complementary DNA
CK	Cytokinin
CP	coat protein
Cq	Quantification cycle
CYP	*Cyclophilin*
CYVV	clover yellow vein virus
cZ	cis-zeatin
ddH2O	sterile double-distilled water
ddPCR	droplet digital PCR
RT-ddPCR	reverse transcriptase droplet digital PCR
EF1β	*Eukaryotic elongation factor 1-beta gene*
EF1α	*Eukaryotic elongation factor 1-alpha gene*
DZ	dihidrozeatin
DMSO	dimetilsulfoxide
DNA	deoxyribonucleic acid deoxyribonucleotide
dNTP	di-nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
EST	expressed sequence tags
gus	*B-glucuronidase gene*
FRG	Final root growth

FAM	fluorescein amidite
G6PD	*Glucose-6-phosphate dehydrogenase*
GFP	green fluorescent protein
GM	Genetically modified
GOI	Gene of interest
HEX	hexachloro-fluorescein
ha	hectares
hr	hours
hph	*hygromycin phosphotransferase*
IPT	isopentenyl transferase
iP	N 6-(2-isopentenyl)-adenine
IRG	Initial root growth
kg	kilogram
LB	Luria-Bertani medium
µg	microgram
µL	microlitre
µM	micromolar
M	molar
Mn	Manganese
mg	milligrams
Mha	million hectares
min	minutes
mM	millimolar
MP	movement protein
mRNA	messenger RNA
N	Nitrogen
NSRG	Net stress root growth
OA	organic acids
OAA	oxaloacetic acid
OD	optical density
PEPC	Phosphoenolpyruvate carboxylase
P	Phosphate
PCR	polymerase chain reaction
PDH	*Pyruvate dehydrogenase*
qPCR	quantitative PCR
ROS	reactive oxygen species
rpm	revolutions per minute
RIM	root-inducing medium
RNA	Ribonucleic acid
RSRG	relative stress root growth
RWC	relative water content
SAG	*Senescence associated gene*
SARK	*Senescence-associated receptor-like kinase*
PSARK	*SARK promoter*
s	seconds
SDS	sodium dodecyl sulphate
SWC	soil water content

SMG	selectable marker gene
SSR	simple sequence repeats
TALEN	transcription activator-like effector nucleases
T-DNA	transfer DNA
TCA	tricarboxylic acid
Tr*neMDH*	*Trifolium repens nodule-enhanced Malate dehydrogenase* gene
TrPT1	*Trifolium repens phosphate transporter gene*
TUA	*Tubulin alpha-5 gene*
tZ	trans-zeatin
WClMV	white clover mosaic virus
UBQ	*Polyubiquitin gene*
UBQ10	*Ubiquitin 10 gene*
ZFN	zinc finger nuclease

Statement of authorship

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Summary

White clover (*Trifolium repens* L.) is a perennial forage legume widely used in temperate areas of the world. In Australia, the use of the species is an important component in farming systems of the south-east region. However, stresses such as aluminium toxicity, drought stress and virus infection reduce white clover productivity.

In order to circumvent these limitations, genetically modified white clover plants for delayed leaf senescence, Aluminium tolerance and Alfalfa mosaic virus (AMV) resistance were independently generated in previous studies. Transgenic events carrying At*myb32*:*IPT* cassette exhibited delayed leaf senescence. Transgenic white clover events carrying the Ca*MV35S:*CP-*AMV* cassette exhibited durable resistance to AMV under field conditions. Double stacked transgenic events with Tr*Pt1*:Tr*neMDH::*Ca*MV35S:*CP-*AMV* construct exhibited superior root growth to wild type plants under aluminium stress, and resistance to AMV.

In the present work, a construct with the three aforementioned cassettes, At*myb32:IPT,* Tr*Pt1*:Tr*neMDH,* and Ca*MV35S:*CP-*AMV* in a single T-DNA was engineered, and transgenic white clover plants were generated by *Agrobacterium tumefaciens*-mediated transformation. A molecular characterization was performed, comprising screening for T-DNA insertion, evaluation of gene copy number and transcription levels of the three genes of interest. The transgenic events generated were phenotyped for delayed leaf senescence, drought stress and Aluminium tolerance.

Thirty triple stacked events were confirmed and approximately half of the events were estimated to have single T-DNA insertions. Functionality of the transferred transgene was confirmed by transcript abundance analysis. Thirteen events exhibited delayed leaf senescence and two showed increased yields under drought stress. Although plants were not phenotyped for AMV resistance in this work, CP-*AMV* transcription levels were used as indicative of potential for resistance to AMV. Five events with low copy number, functional T-DNA inserts and the desired phenotypes were identified as promising for the next steps of the breeding program.

1 Introduction

1.1 White clover

1.1.1 Origins and taxonomy

White clover (*Trifolium repens* L.) is a perennial legume widely used in grazing animal production systems in temperate areas. It belongs to the Fabaceae family and the genus *Trifolium*, which is composed of 255 species (Ellison et al., 2006). Centres of diversity of the *Trifolium* genus are described in the eastern Mediterranean, East Africa and South America (Ellison et al., 2006). The centre of origin of white clover is likely to be the Mediterranean region of Europe, with further spread to Asia and other parts of Europe by migrating animals (Lane et al., 1997; Ellison et al., 2006).

White clover was domesticated 400 years ago in the low countries of Europe. Dutch landraces were used in other parts of Europe, and the adapted races generated were later used in these areas (Zeven, 1991). The species extended to North America, New Zealand and Australia in the 18th century through European settlement, and further dispersion took place by grazing animals (Zeven, 1991; Lane et al., 1997). In the 19th century naturalization of white clover occurred, together with the expansion of pastures. Later in the 20th century imported cultivars were widely planted, especially during the "pasture revolution" of the 1950s (Lane et al., 1997).

The species is tetraploid, with a chromosome number of 2n=4x=32; it was described as an allotetraploid, which means that genetic background was generated from hybridisation between related diploid taxa (Leitch and Bennett, 1997). It was recently reported that the most possible diploid progenitors are *Trifolium occidentalis* D.E. Coombe (Western clover) and *Trifolium pallescens* Schreber (Ellison et al., 2006).

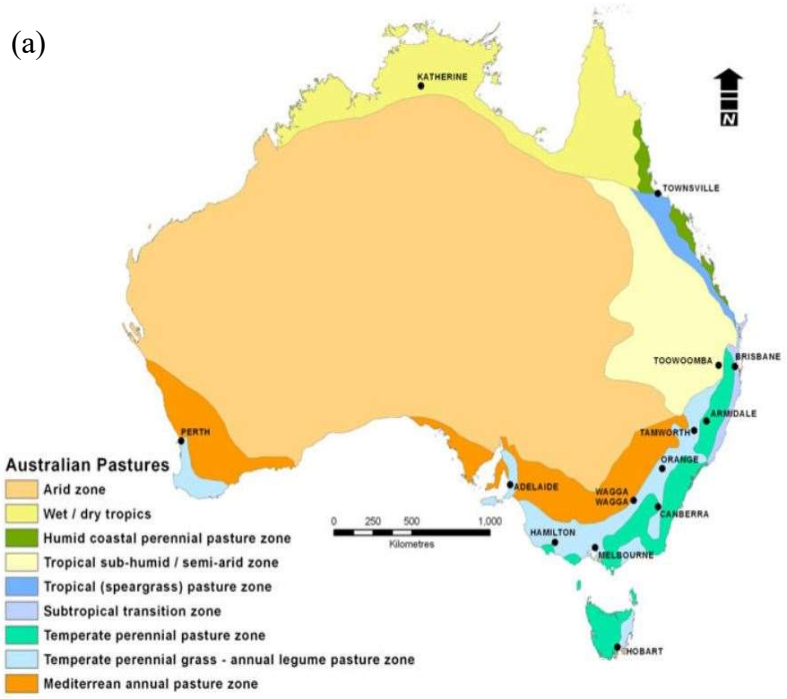
As an allopolyploid, white clover has a disomic inheritance, and therefore it behaves as a diploid as a consequence of an exclusive pairing between homeologous chromosomes (Sears, 1976). White clover is an outbreeding species and highly self-incompatible, with a very low number of self-compatible plants within a population (Forster et al., 2014). Therefore, a high genetic variability between and within populations, and a high level of heterozygosity is observed in the species (Gustine and Huff, 1999; Van Treuren et al., 2005; George et al., 2006). This high level of variability contributes to the adaptability of the species to different geographical regions and environments (Gustine and Huff, 1999).

1.1.2 White clover use in Australia

The white clover zone in Australia is predominant in south-eastern Australia, covering different agro-geographical regions. By 1998 it was reported that white clover use reached approximately 7.8 Mha (Hill and Donald 1998).

Although in relative terms of area white clover is considered of secondary importance as a pasture in Australia, the species is relevant in temperate areas comprising Victoria, New South Wales, Tasmania and southern Queensland (Wolfe, 2009). According to Wolfe (2009), most grazing systems in Victoria can be classified as 'Temperate perennial pasture zones' and 'Temperate perennial grass annual legume pasture zone' (Figure 1.1). The 'Temperate perennial pasture zones', with an annual median rainfall of 700-1000 mm, is mainly composed of mixed swards of perennial ryegrass (*Lollium perenne*) and white clover (Wolfe, 2009). This area contains a high proportion of Victoria's dairy industry, which is one of the major contributors of dairy production in Australia (Dharma and Martin, 2010). Recent estimations on white clover pastures use in Victoria report 8.7% of white clover-ryegrass mixed swards from the total grazing area (Figure 1.1) (Donald et al., 2011). Besides the high impact on dairy production, white clover use is also important in Victoria for its contribution to lamb and beef production (Lane et al., 1997).

(a)



(b)

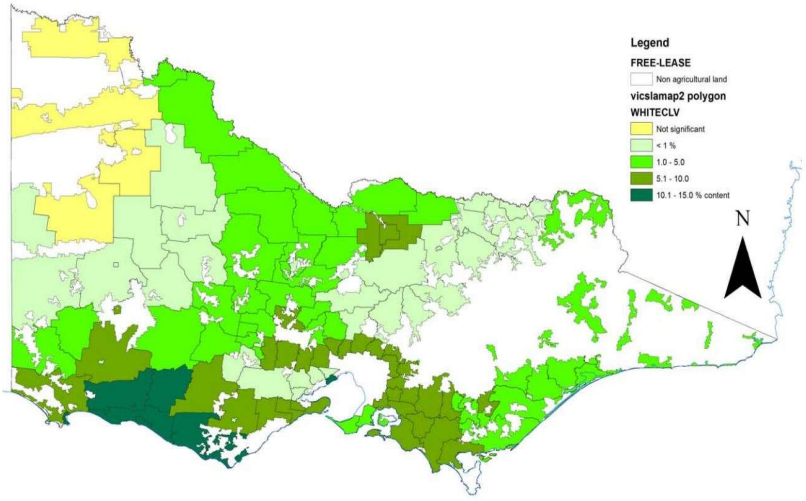


Figure 1.1. (a) Pastures of Australia based on the limits to the adaptation of tropical and temperate pasture (Wolfe 2009). (b) Distribution of white clover use in Victoria (Donald, 2012).

1.1.3 Benefits of white clover use

There are a number of beneficial features of white clover that explain its adoption by farmers. White clover exhibits a higher feeding value when compared to other forage crops. Dairy cow production increases significantly when cows are fed white clover and forage grass mixtures containing 500 or 800 g clover/kg of total dry matter compared to 200 g clover/kg of total dry matter (Harris et al., 1998). In addition, incorporation of a forage legume component in the diet can enhance meat and milk quality (Forster et al., 2014).

Also, significant increases in wool production, and beef and lamb weight gain have been observed when white clover was compared to exclusively forage grass systems (Ayres and Turner, 1998). This has been explained by a higher efficiency (gain per unit of intake) and higher intake rates associated with a preference of cows for white clover over other forage crops (Caradus et al., 1996; Rutter et al., 2004).

White clover, like many leguminous crops, has the ability to convert atmospheric N_2 into plant accessible nitrogen (N), in symbiosis with soil bacteria of the genus *Rhizobium*. Nitrogen fixation can range between 45 and 315 kg N ha^{-1} in intensively managed white clover pastures (Ledgard, 2001). As a consequence, pastures based on leguminous species require less N fertilization. Furthermore, nitrogen fertilization requirements for grain crop cultivation can be significantly reduced when grown after a leguminous crop or by inter-cropping grain and leguminous crops (Chalk, 1998; Kumar and Goh, 2000). With a proper management, perennial ryegrass/white clover pasture with no nitrogen fertilization and a perennial ryegrass pasture receiving 200 kg N ha^{-1} annum $^{-1}$ can produce similar plant dry matter and milk (Williams et al., 2003).

Applying inorganic N fertilizers can add a considerable direct cost to producers which can be ameliorated by use of leguminous pastures. According to Angus and Peoples (2012), if we consider a contribution of 87 kg N ha^{-1} of N_2 fixation for a New South Wales mixed lucerne-grass pasture and a retail price of A$1.4 for fertilizer N, an estimation of savings are A$122 ha^{-1}. With these numbers, the authors estimated an additional 29% to the gross margins of A$425 ha^{-1} for a pasture destined for wool production in New South Wales (Angus and Peoples, 2012). However, given price fluctuations over time, these estimations can vary. According to recent prices in Australia of A$225 per tonne of urea (Dairy Australia, May 2017), savings can be estimated at A$78 ha^{-1} if we consider the example mentioned above of 87 kg N ha^{-1} of N_2 fixation.

Extensive use of N fertilizers is a major pollutant in farming. Thus, the substitution of N fertilized pastures for N fixing pastures could contribute to more ecological and sustainable production systems (Bohlool et al., 1992). Global greenhouse emissions caused by N fertilizer production, transport and application is estimated at around 1%. Additionally, it was reported that legumes reduce N losses by nitrous oxide emissions compared to N fertilized crops (Reckling et al., 2016).

Legumes are more efficient in N use compared to N fertilized pastures due to biological N fixation (Reckling et al., 2016). The use of legumes produce N that is less susceptible to be lost through volatilization and leaching compared to N fertilizer, as it can be conserved as part of organic matter for later mineralization (Peoples et al., 1995; Peoples and Baldock, 2001). There are, however, reports of comparable leaching levels in legume-grass mixed pastures and grass pastures (Ridley et al., 2004). This issue can be addressed by using perennial legumes, such as white clover, as they exhibit a slower release of mineralized N over annual pastures (Ridley et al., 2004). Overall, an analysis of the whole system suggests a smaller environmental impact compared to N fertilised pastures (Unkovich, 2012).

Nitrogen fixed by legume-based pastures can contribute to recovery of nitrogen caused by leaching, grazing or cropping. White clover fixation of 20 kg N ha^{-1} in farms in south-western Victoria can return 69% of the N outputs in milk (Riffkin et al., 1999). Furthermore, Pakrou and Dillon (2000) reported that pastures containing 25% to 57% clover fixed sufficient N to balance the N lost through volatilization and leaching, and removed in milk. This supports the observation of a similar gross-margin between crops systems and mixed farming systems using legume pastures in southern Australia (Angus and Peoples, 2012).

Rotations using legumes can help to control weeds, improve soil structure and contribute to disruption of pest life cycles in cropping systems (Nichols et al., 2012). Legumes can have a positive impact on soil microbial diversity, and could favour the rhizosphere communities that compete and negatively affect the presence of pathogens (Peoples et al., 2009). Also, the use of legumes can favour mycorrhizal associations, which may contribute to an increase in the activity of a number of soil organisms such as earthworms, while they can reduce the survival of some nematodes (Lupwayi and Kennedy, 2007).

1.1.4 Productivity limitations and opportunities

Nichols et al. (2012) identify as a primary objective for white clover breeding in Australia to improve the reliability of the species where there is currently predominant (annual rainfall of 850-1000 mm), and enhance its adaptability to pasture areas where annual rainfall is around 650-850 mm, considered to be marginal for the species.

In order to reach these goals, long-term persistence and reliable forage yields throughout the year are the central aims for improving white clover pastures (Smith and Fennessy, 2011; Jahufer et al., 2012). In line with this, Smith and Fennessy (2011) identified the most important specific traits as selection criteria for white clover in Australia; these are drought tolerance, persistence, resistance to invertebrate pests, and tolerance of challenging soil conditions.

White clover has a dual behaviour as a perennial and annual species; while it can persist vegetatively through stolons, yearly seed production also contributes to maintenance of populations (Archer and Robinson, 1989; Hutchinson et al., 1995). Stolon survival has been identified as the main cause of increased persistence. However, in some circumstances such as post-drought in subtropical regions, seed production also makes an important contribution to white clover persistence (Archer and Robinson, 1989; Hutchinson et al., 1995).

A number of factors can hinder white clover persistence. The main component contributing to a low persistence is water limitation during summer (Archer and Robinson, 1989; Barbour et al., 1996). Drought stress can cause negative effects on herbage production and stolon survival, which leads to a decrease over time in forage production and quality, with a subsequent drop in animal productivity (Jahufer et al., 2012). Therefore, tolerance to drought stress, and specifically stolon survival during low soil moisture periods is one of the main factors to focus in white clover breeding in order to increase persistence (Lane et al., 2000).

Hostile soil conditions were also identified as a major contributor to a limited forage productivity and persistence of white clover in Australia (Hochman and Helyar, 1989; Smith and Fennessy, 2011). Among those limiting soil conditions, one of the most important are soil acidity and associated aluminium toxicity (Smith and Fennessy, 2011). In Australia, approximately 12-24 Mha of soils, which constitutes 13-24% of agricultural soils are acidic (with pH equal or below 4.8). In addition, within this area, 5Mha exhibit subsoil acidity (30-40 cm deep, pH equal or below 4.8) (Dolling et al. 2001).

Plant-parasitic nematodes and viruses, can also contribute to a decrease in white clover persistence (Barnett and Gibson, 1975; Lane et al., 2000). Among the viruses affecting white clover in Australia, Alfalfa mosaic virus (AMV) was reported as the most widespread in the years 1991-1992 (Norton and Johnstone, 1998). Also high levels of incidence of white clover mosaic virus (WClMV) and Alfalfa mosaic virus (AMV) were observed in southern Australia (Norton and Johnstone, 1998). AMV can cause a significant decrease in forage yields by reducing the numbers of rooting nodes, stolons, and leaves (Gibson et al., 1981). It also can cause a reduction in nitrogen fixation by hindering nodulation (Gibson et al., 1981).

Although all these factors contributed to a decrease in white clover use in Australia in the early 1980s (Lane et al., 1997), the species remains the most important perennial pasture legume in farming systems in temperate areas of Australia (Nichols et al., 2012).

In this project, transgenic white clover plants transformed with three genes for delayed leaf senescence, aluminium tolerance, and AMV resistance were generated. In the next sections, an exhaustive review on the state of art for research on the fundamental aspects on these traits, and their use in agriculture is developed.

1.2 Delayed leaf senescence

1.2.1 Leaf senescence

Leaf senescence is the final stage of leaf development. It is described as a type of programmed cell death which involves the degeneration of cellular and tissue structures (Guo and Gan, 2005; Schippers et al., 2015). At this stage leaves stop growing and change their function from photosynthesis to catabolism of macromolecules (Lim et al., 2007). One of the first and most characteristic signs of leaf senescence is the disassembly of chloroplasts which contain the majority of protein in leaves (Krupinska, 2007). Also, chlorophyll is degraded, which leads to the characteristic loss of green colour in the leaves (chlorosis) (Krupinska, 2007; Lim et al., 2007). Degradation of chloroplasts is followed by destruction of other organelles and degradation of macromolecules for redistribution of nutrients such as nitrogen, phosphorous and metals to new young leaves, growing seeds and fruits (Guo and Gan, 2005; Lim et al., 2007). The final steps of senescence, include chromatin condensation, internucleosomal fragmentation of nuclear DNA, and controlled vacuolar collapse (Simeonova et al., 2000; Lim et al., 2007). Finally, a

disruption of plasma membranes takes place, which leads to cell death. This orderly sequence of events supports the idea that organelle disintegration is an organized process, and that senescence is a type of a programmed cell death (Quirino et al., 2000; Lim et al., 2007).

The organized recycling of nutrients in the plant, as part of the leaf senescence process, is essential for an efficient use of resources. This makes leaf senescence essential for the normal generation of reproductive organs and seeds, which makes it a key process for survival and production of new offspring (Munné-Bosch and Alegre, 2004; Thomas, 2013; Schippers et al., 2015).

Several internal and external factors interact and influence the onset of senescence (Lim et al., 2007; Yoshida, 2003). There are many external factors that cause leaf senescence, such as shading, low and high temperatures, low nutrient availability, dehydration and pathogen attack (Schippers et al., 2015). Also, developmental age is one of the main factors determining the onset of leaf senescence. In addition, a number of internal factors influence the process. Crosstalk between phytohormones and molecular pathways play a crucial role at this level (Buchanan-wollaston et al., 2003; El-showk et al., 2013).

Although the apparent symptoms of leaf senescence appear similar regardless of senescence-inducing factors, there are differences at the molecular level depending on the element that induces senescence (Park et al., 1998). Generally, leaf senescence is observed starting from leaf margins towards the interior of the leaf blade. In other cases, for example in some pathogen infections, senescence can be localised (Lim et al., 2007).

What induces the start of senescence is not completely clear. However, the initiation of leaf senescence has been observed to be linked to high levels of sugar content in leaves. In line with this, a reduction in photosynthetic activity is observed as sugar content rises (Quirino et al., 2000; Guo and Gan, 2005). As young leaves develop, they generate photosynthetic machinery and thus act as sinks for assimilated carbon during this period. However, as leaves age, their demand for sugars is reduced. As a consequence sugar accumulates, which leads to the induction of senescence once the sugar levels reach a certain threshold (Yoshida, 2003).

Senescence is regulated at the molecular level. Some genes are induced in senescing leaves (named senescence associated genes SAGs) and are thus a target of study. These genes have been classified into six categories according to studies in *Arabidopsis thaliana* (Arabidopsis): those involved in developmental aging processes (class I), genes that control processes other than leaf senescence (class

II), genes that respond to environmental factors and affect senescence (III), genes that up-regulate senescence-associated activities or down-regulate cellular-maintenance activities (IV), genes involved in degradation of senescence regulatory factors (V), and genes involved in executing the senescence process (VI) (Lim et al., 2003). The study of the expression of these genes has made possible the identification of differences between age dependant-senescence and senescence induced by other factors and also helped to identify overlap between senescence types (Lim et al., 2003).

1.2.2 Cytokinins

Plant hormones play an important role in the regulation of leaf senescence. It has been observed that auxins, cytokinins, and giberellic acid inhibit senescence, while ethylene and abscisic acid promote it (Schippers et al., 2015). At the molecular and biochemical level, cytokinins play a critical role in the process. It's been reported that ectopic application of the hormone delays leaf senescence (Gan and Amasino, 1996). Furthermore, different transgenic crop species with increased cytokinin production show delayed leaf senescence. These were generated by overexpressing a gene encoding the enzyme isopentenyl transferase (IPT), from the cytokinin production pathway (Gregersen et al., 2013).

Cytokinins can affect plant development at many levels, which includes seed germination, vascular development, cell proliferation, bud and root differentiation, shoot meristem growth and leaf senescence (Ferreira and Kieber, 2005). A rise in cytokinin production by ectopic expression of the *IPT* gene alters leaf shape and generates a reduction in the apical dominance and root growth (Li et al. 1992). On the other hand, when cytokinin production is decreased, root development is boosted. Also, delayed leaf initiation, delayed onset of flowering and increased sterility are observed when cytokinin levels are reduced (Ferreira and Kieber 2005). Furthermore, it has been observed that cytokinins have an antagonistic function in regulation of growth, while cellular proliferation is repressed in roots, cytokinins induce cell proliferation in shoots (Werner et al 2003).

Cytokinins are known to be synthesized primarily at the root tip, but also in the cambium, the shoot apex, and immature seed (highest expression in seeds for the genes *AtIPT4* and *AtIPT8,* involved in cytokinin synthesis was reported in the chalazal endosperm) (Sakakibara, 2006). Changes in cytokinin levels in association with plant development and specifically with cell cycle have been reported (Li et al., 2006). Furthermore, environmental factors affect cytokinin levels. The presence of mineral nutrients, are generally positively correlated with levels of cytokinins (Takei et al., 2001). This correlation was

observed in tobacco (*Nicotiana tabacum*), nettle (*Urtica dioica*), barley (*Hordeum vulgare* L.) and maize (*Zea mays*). In addition, it was observed that ectopic application of cytokinin partially restored morphological adaptation in plantain (*Plantago major*) to low mineral nutrition (Sakakibara et al., 2006). On the other hand, cytokinin levels were observed to decrease under water stress (Yang et al., 2001). The levels of active cytokinins in plants are expected to be regulated by their rates of biosynthesis, interconversion, transport, and degradation (Kakimoto, 2003).

1.2.3 Cytokinin metabolism

Natural cytokinins are adenine derivatives with an N^6-side chain, and can be classified as isoprenoid or aromatic, depending on the structure of this chain (Mok and Mok, 2001; Kakimoto, 2003). The most abundant isoprenoid cytokinins in nature have an unsaturated isoprenoid side chain. Among these, a trans hydroxylated N^6-side chain, such as trans-zeatin (tZ) and its derivatives are very common in Arabidopsis, while N 6-(2-isopentenyl)- adenine (iP) and cis-zeatin (cZ) are the major forms encountered in maize and rice (*Oryza sativa*) (Sakakibara, 2006). On the other hand, some aromatic cytokinins were identified in a small number of plant species. These comprise Benzyladenine type cytokinins (BA) and BA-type cytokinins with minor modifications of the adenine ring, such as ortho-topolin, meta-topolin, their methoxy-derivatives (Figure 1.2) (Strnad, 1997; Mok and Mok, 2001).

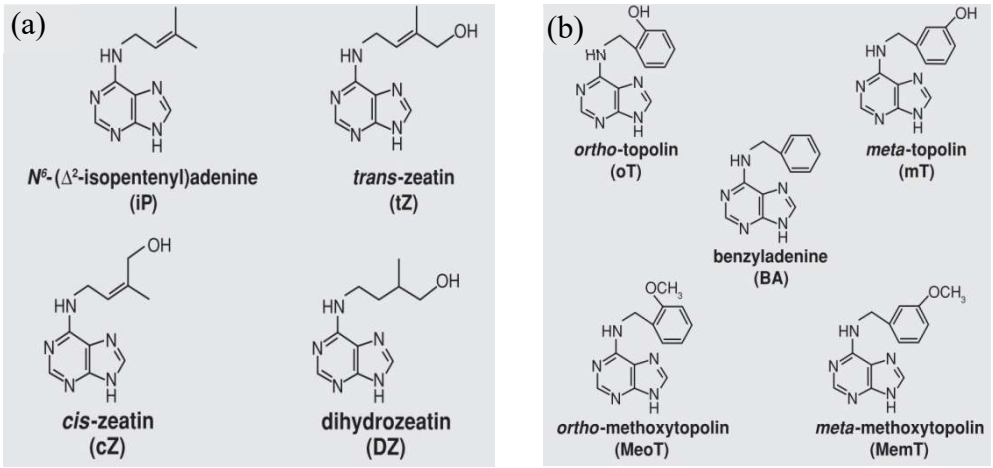


Figure 1.2. (a) Isoprenoid cytokinins; (b) Aromatic cytokinins (Sakakibara, 2006).

In Arabidopsis, cytokinins are most synthesized in the isoprenoid cytokinin pathway by the N-prenylation at the N^6-terminus of adenosine 5'-phosphates (AMP, ADP, or ATP) with dimethylallyl diphosphate (DMAPP), or hydroxymethylbutenyl diphosphate (HMBDP). This reaction is catalyzed by adenosine phosphate-isopentenyltransferase (IPT) (Figure 1.3) (Sakakibara, 2006). Seven *IPT* genes involved in prenylation of the adenine moiety were identified in Arabidopsis (*AtIPT1*, *AtIPT3-AtIPT8*), and eight in rice (*OsIPT1–OsIPT8*) (Sakakibara, 2006). However, *IPT* was first identified in the plant pathogen *Agrobacterium tumefaciens* (Agrobacterium). Agrobacterium has two *IPT* genes, *Tmr* integrated in the T-DNA region of the Ti-plasmid, and *Tmz*, present on the virulence region of nopaline-type Ti-plasmid. Once infection occurs, *Tmr* is inserted in the host genome and the enzyme competes with the plant IPTs for the substrate HMBDP in plastids. Interestingly, plants overexpressing *Tmr* produce almost only tZ cytokinins (Sakakibara, 2006; Kamada-nobusada and Sakakibara, 2009).

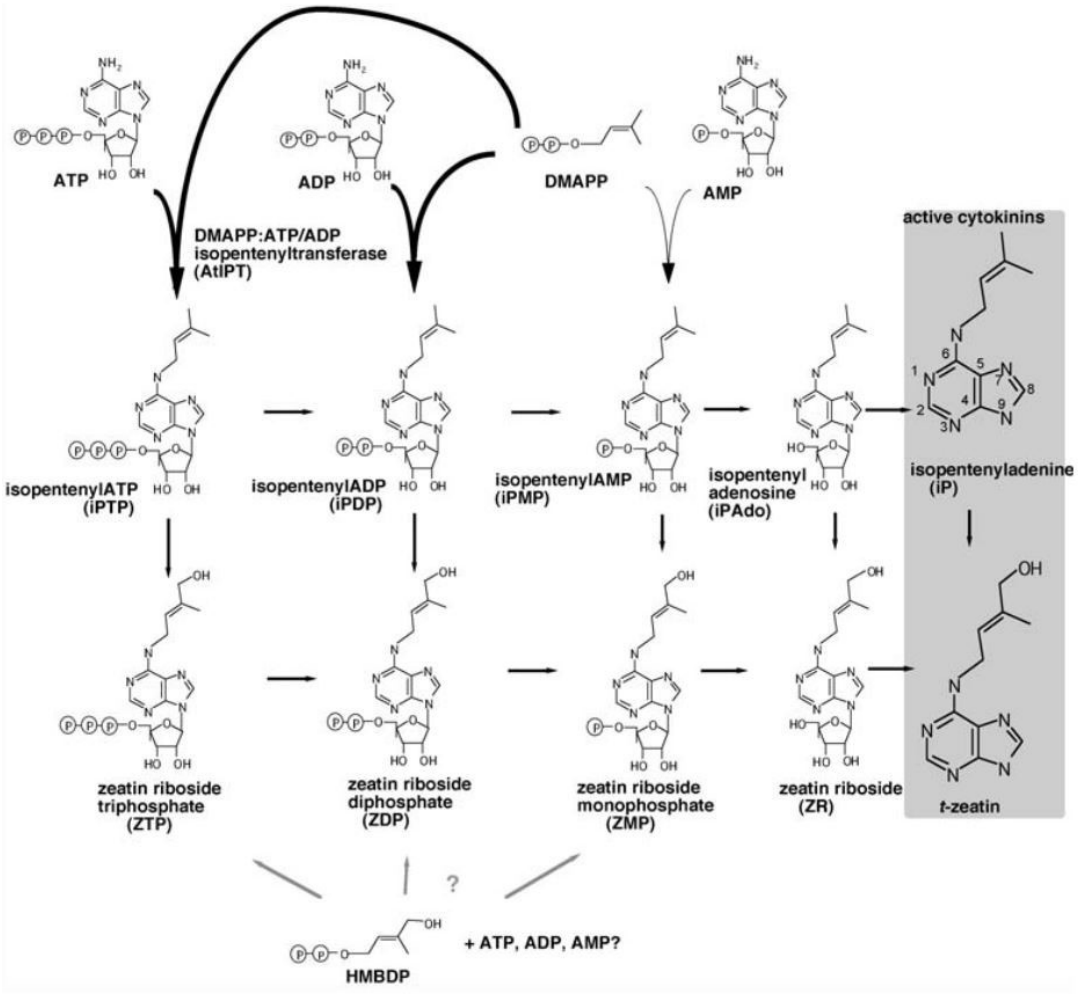


Figure 1.3. Model of isoprenoid cytokinin synthesis in plants (Kakimoto, 2003).

The naturally occurring isoprenoid cytokinins are tZ, iP, cZ, and dihidrozeatin (DZ). tZ and iP were reported as the most active cytokinin forms in Arabidopsis, while cZ presents low activity. In maize, cZ showed similar activity to tZ and iP. This suggests that cytokinin activities vary depending on the species (Kamada-nobusada and Sakakibara, 2009).

The aromatic cytokinins BA and topolins exhibit strong activity, but knowledge on the processes for biosynthesis and degradation pathways of aromatic cytokinins is very limited. A possible role of cytochrome P450 was reported for topolin biosynthesis (Sakakibara, 2006). Another possible source of aromatic cytokinins is tRNA, obtained by hydrolysis of some tRNA species containing an N^6-prenylated adenine (Kasahara et al., 2004). Therefore, an important step in this pathway is the prenylation of some tRNA species, catalyzed by tRNA-isopentenyltransferase (Sakakibara, 2006). However, it's been reported that the tRNA pathway is not the major contributor to cytokinin production (Kakimoto, 2003).

1.2.4 Delayed leaf senescence in agriculture

Delaying leaf senescence can be attractive in various crops for agricultural production purposes. An obvious advantage is its application in forage species that are used in hay production to feed animals. Delayed leaf senescence could be an advantage as hay would stay greener and retain nutrients longer (Calderini et al., 2007). Linked to this, production of leafy vegetables for human consumption with delayed leaf senescence may also be of interest for farmers, as the products would stay consumable for longer times after their harvest (Nam, 1997; Chen et al., 2001; McCabe et al., 2001). In this regard, lettuce and broccoli expressing *IPT* for delayed leaf senescence were generated by Chen et al. (2001) and McCabe et al. (2001).

The strategy of overexpressing the *IPT* gene encoding isopentenyl transferase for increasing cytokinin production has been developed in several species (Table 1.1). However, there are some limitations to this approach. The use of a constitutive promoter, such as the Ca*MV35S* promoter, generates systemic overproduction of the hormone, which causes low root growth, and loss of apical dominance (Smigocki and Owens, 1989). A fine balance of cytokinins is a determinant of normal plant growth. In order to limit overproduction of the hormone, Gan and Amasino (1995) transformed tobacco with an *IPT* controlled by the senescence inducible promoter *SAG12* from Arabidopsis. Using this approach, the inserted gene would be expressed only when the plant is starting to senesce, which leads to autoregulation of the

system, maintaining cytokinin overproduction to a minimum capable of inhibiting senescence. This strategy was applied in different species and delayed leaf senescence was reported (Table 1.1). Also a higher nitrate reductase activity and increased nitrate influx was observed in transgenic wheat, although no rise in grain yields was observed (Sýkorová et al., 2008). Some undesirable features were reported, such as delayed flowering (McCabe et al., 2001), and altered source sink relations explained by the lack of chlorophyll and protein degradation in source leaves (Jordi et al., 2000).

To overcome this issue, a slightly different strategy was conducted by Rivero et al. (2007) in tobacco, who used a promoter from a senescence-associated receptor-like kinase gene (SARK) from pea (Hajouj et al., 2000). This promoter is maturation-induced and stress-induced, and showed expression in all drought stressed tissues. Strikingly, besides delayed leaf senescence, plants also showed a delay in drought induced senescence, leading to a considerable drought stress tolerance (Rivero et al., 2007). Furthermore, there was minimal yield loss when tobacco plants were watered with 30% of the amount of water used under control conditions. Similar results were observed in rice (Peleg et al., 2011) and peanut (Qin et al. 2011) using the same promoter; here seed yields were almost stable after water stress. This may be explained by a change in the source/sink relationships induced by overproduction of cytokinins. It is hypothesized that the source strength may be maintained at high levels during drought stress. Higher cytokinins promote the storage of sucrose and starch in flag leaves under drought in pre-anthesis, which results in higher starch and sucrose content in grains (Peleg et al., 2011).

A different approach has been developed for canola and white clover. These species were transformed with the *IPT* gene from Agrobacterium controlled by the developmentally regulated promoter At*myb32* from Arabidopsis (Lin et al., 2003; Kant et al., 2015). It was reported that this promoter is induced in root and leaf vascular tissues, which facilitate the translocation of cytokinins to these tissues (Lin et al., 2003). Furthermore, the promoter used contains a modification in a motif which prevents the expression in the roots, thus avoiding the negative effect of high cytokinins in root growth. These transgenic canola events showed a delayed leaf senescence and increased seed yields compared to their controls in field trials, under rainfed and irrigated conditions (Kant et al., 2015).

Table 1.1. Examples of *IPT* overexpression for delayed leaf senescence in plants.

Recipient species	Promoter	Donor species	Reference
Tobacco	*SAG12*	Arabidopsis	(Gan and Amasino, 1995)
Lettuce	*SAG12*	Arabidopsis	(Mccabe et al., 2001)
Maize	*SAG12*	Arabidopsis	(Young et al., 2004)
Wheat	*SAG12*	Arabidopsis	(Sýkorová et al., 2008)
Bentgrass	*SAG12*	Arabidopsis	(Merewitz et al., 2011)
Tobacco	*PSARK*	Bean	(Rivero et al., 2007)
Rice	*PSARK*	Bean	(Reguera et al., 2013)
Cotton	*PSARK*	Bean	(Kuppu et al., 2013)
Peanut	*PSARK*	Bean	(Qin et al., 2011)
Maize	*PSARK*	Bean	(Oneto et al., 2016)
Ryegrass	*PSEE1*	Maize	(Li et al., 2004)
Chrysanthemum	*LEACO1*	Tomato	(Khodakovskaya et al., 2009)
Rice	*SAG39*	Rice	(Liu et al., 2010)
Tomato	HSP70	*Drosophila melanogaster*	(Ghanem et al., 2011)
Canola	*AT2S1*	Arabidopsis	(Roeckel et al., 1997)
Canola	At*myb32*	Arabidopsis	(Kant et al., 2015)
White clover	At*myb32*	Arabidopsis	(Lin et al 2010)

1.2.5 Delayed leaf senescence in white clover

White clover plants expressing *IPT* from Agrobacterium controlled by the promoter At*myb32* were developed with the primary objective of delaying leaf senescence (Ludlow 2000, Lin et al 2005). The events generated exhibited delayed leaf senescence compared to non-transgenic plants (Ludlow 2000,

Lin et al 2005). White clover plants expressing the *IPT* transgene evaluated under field conditions for four years revealed delayed leaf senescence, increased leaf number, higher stolon length and higher leaf area when compared to their non-transgenic controls. Furthermore, these transgenic plants exhibited 2-fold increases in seed production, and better summer survival rate compared to their non-transgenic controls (Lin et al 2007, 2010).

As mentioned above, drought stress tolerance and a better survival during summer are linked to an increase in white clover persistence and productivity. It is estimated that this trait could provide a 10% increase of a total ryegrass-white clover sward dry matter in the first year, in a marginal zone of south west Victoria with an average annual rainfall of approximately 650 - 800 mm (Lewis 2016).

In this project, together with the *IPT* gene for delayed leaf senescence, and the CP-*AMV* gene for AMV resistance, the Tr*neMDH* gene for aluminium tolerance was inserted. Aluminium stress in soils is directly associated to soil acidity. The impact in crop production that these stresses have in Australia, and particularly in Victoria are discussed in the next sections. Furthermore, the negative effects that aluminium stress have in plant growth and crop production are also described.

1.3 Soil acidity

Acidic soils (pH < 5.5) comprise approximately 30% (3950 million ha) of the Earth's ice-free land (Uexküll and Mutert, 1995). Furthermore, around 50% of the global area of arable soils is acidic. These soils extend for approximately 60% of the world's tropics and subtropics, and affect production of relevant grain crops such as rice and maize significantly (Uexküll and Mutert, 1995). In Australia, soil acidity is one of the main problems impacting productivity in agriculture (Ryan, 2018). Approximately 33 Mha have a pH lower than 4.8, and approximately half of these soils are in New South Wales and Victoria (Scott et al., 2000). An estimated 62% of Victoria's soils exhibit surface acidity (Figure 1.4) (Environment and Natural Resources Committee, 2004). Additionally, a reduction in productivity caused by soils acidity is observed in approximately 23% of agriculturally productive soils (Environment and Natural Resources Committee, 2004).

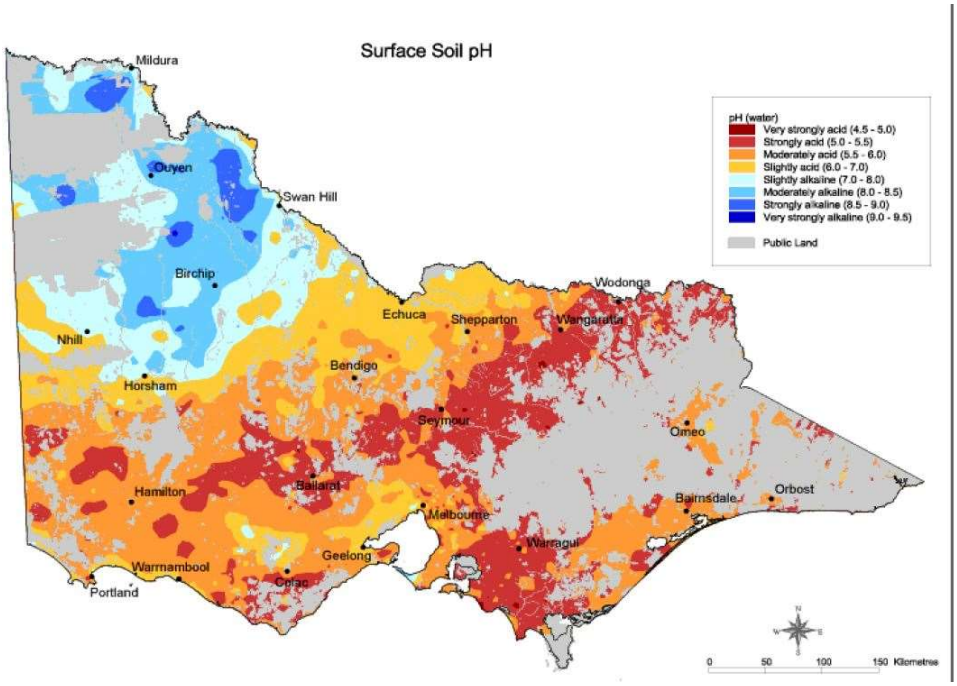


Figure 1.4. Distribution of soils acidity in Victoria (Environment and Natural Resources Committee, 2004)

Some practices in land management, such as the use of high doses of nitrogen fertilizer and intensive cropping lead to the acidification of soils (Guo et al., 2010). However, acidification can be reduced by the proper management of nitrogen use (Ju et al., 2009). Europe, and North America have circumvented this problem by lime application in soils to increase pH, but use of lime is expensive and thus generally not economically viable for the typical extensive grazing systems carried out in Australia (Scott et al., 2000).

There is limited knowledge regarding the direct effects of H^+ in plants (Samac and Tesfaye, 2003). However, soil acidity is not generally the direct cause of plant growth impairment. Soil acidity can generate a reduction in phosphate availability due to its fixation with aluminium (Al) and Fe oxides (Shen et al., 2011). This makes phosphate (P) availability a major factor limiting crop production in acidic soils (Curtin and Syers, 2001). Also aluminium and manganese (Mn) can hinder plant growth under acidic soil conditions (Sims, 1986; Kochian et al., 2004). Overall, aluminium toxicity and P availability are the two most important factors limiting crop production in acidic soils (Zheng, 2010).

1.4 Aluminium toxicity

Aluminium is the third most abundant element on earth and is present in soil in different forms (Foy, 1988; Kochian, 1995). Soils with neutral pH generally contain insoluble forms of Al such as Al oxides which are usually not toxic to plants. Soil acidity can lead to production of soluble Al forms that are toxic for plants. The predominant form of Al in acidic solutions is the trivalent cation $Al(H_2O)_6^{3+}$ (known as Al^{3+}) (Martin, 1986). As pH further increases, deprotonation leads to Al forms $Al(OH)^{2+}$ and $Al(OH)^{2+}$. The toxicity of the aforementioned cationic Al forms has been reported. However, there is not a complete consensus on the rank of toxicity of these species (Wright, 1989; Kinraide et al., 1990). The non-toxic form $Al(OH)_3$ forms at neutral pH, and as the pH of the solution gets closer to cytoplasmic pH (7.4), formation of the non-toxic form aluminate ion $Al(OH)^-_4$ takes place (Martin, 1986; Wright, 1989; Kochian, 1995). It is not clear whether Al^{3+} toxicity is governed by an apoplastic or a symplastic interaction, and there is limited knowledge of the possible Al forms that may traverse the root cell membrane (Kochian, 1995).

The most immediate observed effect of both symplasmic and apoplasmic Al^{3+} is a reversible inhibition of root growth (Barcelo & Poschenrieder, 2002; Kochian et al., 2004). These effects are mostly reduced to the transition zone of the root apex (Sivaguru et al., 1999). Aluminium effects observed as root inhibition can be detected after a 30 minutes period of exposure, and can affect some plant species severely at micromolar concentrations (Barceló and Poschenrieder, 2002). Given this, root growth inhibition is one of the symptoms used for identification of Al^{3+} stress in plants (Silva, 2012). The reduction in root growth causes a decrease in water and nutrient uptake which reduce plant growth and productivity (Ryan, et al., 1995).

There is not a complete concurrence about which are the primary factors contributing to inhibition of root growth. Some researchers reported a reduction in mitotic activity (Silva, 2012). The observation that cell division is inhibited in the root apex after 10 to 30 minutes of Al^{3+} exposure supports this claim. However, others argue the main element that contributes to root growth inhibition is the reduction in cell elongation rates (Ciamporova, 2002; Horst et al., 2010).

Nevertheless, a number of Al^{3+} molecular targets were proposed. Al^{3+} can cause abnormal cell shapes, cell division and mitosis patterns, which suggest an interaction of Al^{3+} with the nuclei, the cytoskeleton and actin filaments (Blancaflor et al., 1998; Silva et al., 2000). Al^{3+} can bind reversibly to a number of macromolecule, including proteins, polynucleotides, and glycosides (Kochian, 1995). Martin (1986)

reported that Al^{3+} can also form complexes with DNA. However, interaction between Al^{3+} and phosphate residues in DNA is weak, and it's possible that the interaction takes place with phosphorylated proteins such as histones which are associated with DNA (Kochian, 1995). In addition, the cation Al^{3+} interacts strongly and forms complexes with organic acids, inorganic phosphate, polyphosphates, and sulphate (Kochian, 1995).

It was also observed that Al^{3+} can affect microtubules and actin filament conformation (Horst et al., 2010). Additionally, Al^{3+} presence causes a reduction in expression of the actin-binding protein profilin, which causes disruptions in cytoskeleton conformation (Zhang et al., 2007a). Al^{3+} interacts with the generally negatively charged cell wall component pectin, and cell wall ligands, such as carboxylates and phosphates (Horst et al., 2010; Silva, 2012; Kochian et al., 2015). The interaction of Al^{3+} with pectin in the cell walls can reduce the activity of enzymes that favour cell wall elasticity (Wehr et al., 2004). The decrease in cell wall elasticity is associated with a decline in the cell elongation rates (Ma et al., 2004). Also linkage of Al^{3+} to hemicelluloses has been reported in Arabidopsis, which suggests an important role for these molecules in Al^{3+} toxicity and resistance (Yang et al., 2011; Kochian et al., 2015).

Al^{3+} also causes oxidative stress in plants. The oxidative stress or production of reactive oxygen species (ROS) causes the oxidation of macromolecules and consequential damage of cell structures such as cell membranes (Yamamoto et al., 2003). Al^{3+} can bind to cell membranes and cause peroxidation of lipids and oxidation of transmembrane proteins (Boscolo et al., 2003; Yamamoto et al., 2003). The high affinity of Al^{3+} to cellular membranes also favours the displacement of cations like Ca^{2+}, leading to changes in the cellular membrane charge. This can cause disruption such as depolarization of membranes, and a consequential modification to ion exchange dynamics through cellular membranes (Kochian et al., 2005; Horst et al., 2010).

The presence of Al^{3+} can produce the release of Ca^{2+} in the apoplast and activate Ca^{2+} channels, which leads to an immediate increase of cytosolic Ca^{2+} concentrations (Rengel and Zhang, 2003). Additionally, increase in Ca^{2+} concentration favours the synthesis of callose in the root tips, which is directly linked to inhibition of root growth (Rengel and Zhang, 2003). The induction of changes by Al^{3+} in cellular pH, K^{+} and Ca^{2+} concentrations, can trigger signalling cascades that leads to a plant response of resistance to Al^{3+} (Kochian et al., 2015). Furthermore, a similar timing in ROS production and expression of the gene *SbMATE* encoding for a transporter associated to Al^{3+} resistance, suggests a role of ROS in the initiation of signalling cascades that trigger the resistance to Al^{3+} in sorghum (Sivaguru et al., 2013).

1.5 Aluminium tolerance in plants

Some plant species have mechanisms for tolerance to Al^{3+}-induced stress. Aluminium tolerant cultivars have been developed using classical breeding, and a number of Quantitative Trait Loci (QTL) for Al^{3+} tolerance have been mapped in different species. Monogenic inheritance was reported in barley, sorghum (*Sorghum bicolor*), and wheat, where major dominant QTLs have been mapped (Delhaize et al., 1993; Minella and Sorrels, 1997; Magalhaes et al., 2004)**.** Three other QTL were mapped in wheat and a multigenic inheritance was proposed for this species (Kochian et al., 2015). Multigenic inheritance in rice and soybean (*Glycine max*) was reported based on the identification of nine and five QTL respectively (Nguyen et al., 2001).

Although a number of Al^{3+} tolerance mechanisms have been proposed, only two are understood in detail. One mechanism is based on the tolerance of Al^{3+} present in the root and shoot symplasm, possibly through detoxification of Al^{3+} via Al^{3+} complexing with organic acids (Ma, et al., 2001; Ma et al., 1997). This strategy was observed in hydrangea (*Hydrangea macrophylla*) and buckwheat (*Fagopyrum esculentum*)*,* where high concentrations of accumulated Al-oxalate and Al-malate respectively were found in shoots (Ma et al., 1997). The second mechanism is based on the exclusion of Al^{3+} from the apoplasm and the rhizosphere. This system relies on the exudation of organic acids from the root apex and formation of strong non-toxic complexes between organic acids and cationic Al (Figure 1.5) (Delhaize et al., 1993; Ma et al., 2001; Kochian et al., 2004). There is compelling evidence that root exudation of organic acids and exclusion of toxic Al^{3+} from the root-soil interface plays a key role in preventing accumulation of Al^{3+} not only in the aposplasm but also in the symplasm (Barceló and Poschenrieder, 2002).

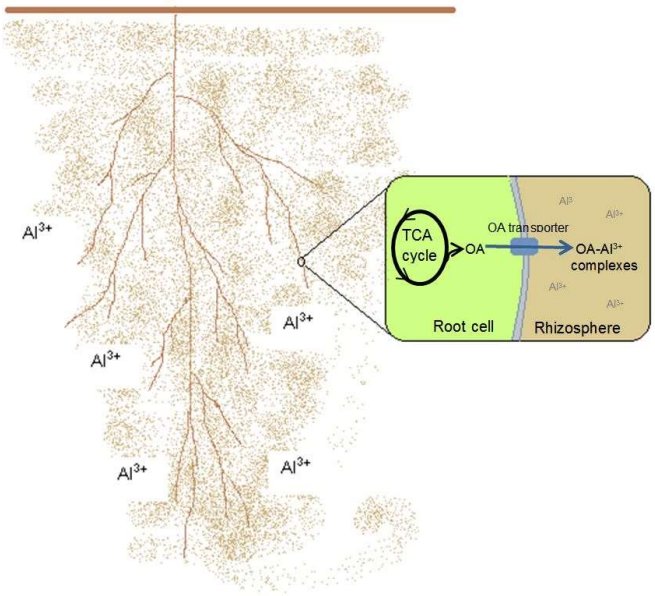


Figure 1.5. Al^{3+} exclusion mechanism model. Organic acids (OA) are transported by organic acid transporters from the root apex and strong non-toxic complexes between organic acids and cationic Al are formed in the rhizosphere. TCA cycle: Tricarboxylic acid cycle. Illustration adapted from Ma et al. (2001) and Kochian et al. (2015).

Several variants of the Al^{3+} exclusion mechanism have been reported. For instance, the organic acid excreted can be different depending on the species. It is known that malate is excreted in wheat and Arabidopsis, and citrate is excreted in maize, sorghum, oat (*Avena sativa*), radish (*Raphanus raphanistrum*), soybean, and tobacco (Ma et al., 2001; Kochian et al., 2004). Some species such as oilseed rape, rice and rye (*Secale cereale*) excrete malate and citrate combined. Also exudation of oxalate was reported in buckwheat (*Fagopyrum esculentum*) and taro (*Colocasia esculenta*) (Kochian et al., 2004).

Some transporters of organic acids have been characterized. The wheat aluminium-activated malate transporter gene from wheat (*Triticum aestivum*) (*TaALMT1*) encodes an anion channel on the plasma membrane that transports malate outside the root apices (Sasaki et al., 2004). This transporter is expressed constitutively and is activated in the presence of aluminium (Sasaki et al., 2004). Similar genes have been characterized in other species, but only *ALMT* genes from wheat, barley, rye, and *Brassica napus* have been shown to confer resistance to aluminium (Delhaize et al., 2012). Another group of genes from the MATE family, encode a transporter that facilitates the efflux of citrate from the roots and is

activated by Al^{3+}. These genes were characterized in sorghum (*sbMATE*) and barley (*HvAACT1*) and confer tolerance to Al^{3+} in these two species. In addition, *MATE* genes were reported as linked to Al^{3+} tolerance in wheat, maize, rye, Arabidopsis, rice, rice bean (*Vigna umbellata*) and bean (*Phaseolus vulgaris*) (Delhaize et al., 2012).

Al^{3+} can enter the cell via some Al^{3+} transporters, such as OsNrat1 in rice or aquaporin HmPALT1 in hydrangea, and interact with transcription factors and so regulate gene expression (Kochian et al., 2015). The transcription factor STOP1 was identified as a regulator of the genes that encode for AtALMT1 and AtMATE transporters in Arabidopsis (Hoekenga et al., 2006; Kobayashi et al., 2007). A similar gene called *ART1*, which regulates the expression of Al^{3+} tolerance genes, was identified in rice (Delhaize et al., 2012). Some examples of organic acid transporters that are regulated at the gene level are *OsSTAR1/2*, Os*Nrat1*, Os*FRDL4*, and Os*ALS1* in rice, and the *MATE* genes Sb*MATE*, Zm*MATE*, Vu*MATE*, and At*MATE*; *ALMT* and At*ALMT1* (Kochian et al., 2015).

Broadly, organic acid transporters are classified in two types. Some transporters are expressed constitutively and their activity is induced by direct interaction with Al^{3+}. Here, given that the response to Al^{3+} presence is rapid, the mechanism may not involve induction of gene expression. In these cases, no time lapse between Al^{3+} exposure and organic acid extrusion is observed. On the other hand, some transporters are only expressed in the presence of Al^{3+}. In this type of response, Al^{3+} may interact with a protein receptor in the membrane, or internally with a transcription factor, which in turn activates expression of genes expressing the related transporters. This type of interaction is evidenced by a delay between the presence of Al^{3+} and the release of organic acids (Ma, 2000; Kochian et al., 2015).

Given that organic acids are relevant intermediates in the key tricarboxylic acid cycle, the Al^{3+} tolerance mechanism based on organic acids release needs to be finely regulated in order to produce minor carbon costs to the plant (Kochian et al., 2015). This is evident with the observation in sorghum that the Al-inducible Sb*MATE* transporter expresses exclusively in the outer layers of root distal transition zone, where the highest Al^{3+} induced damage is observed (Sivaguru et al., 2013). Furthermore, it was reported that the transporters ALMT and MATE are induced only when certain Al^{3+} levels are reached. This enables a differential organic acid exudation in the rhizosphere dependent on Al^{3+} concentrations and the pH of the rhizosphere (Kochian et al., 2015).

A role for hormones in the response against Al^{3+} toxicity has been proposed. A rise in ethylene production was observed in stressed bean roots after 5 minutes of Al^{3+} exposure, followed by an increase in cytokinin production after 20 minutes (Massot et al., 2002). Furthermore, it has been proposed that Al^{3+} inhibits auxin transport from the meristem to root tips, reducing root elongation (Kollmeier et al., 2000). However, not enough published information is currently available to fully elucidate the role of hormones in response to Al^{3+} presence.

1.5.1 Generation of aluminium-tolerant plants by genetic engineering

A number of species have been genetically modified with the aim of generating Al^{3+} tolerant plants. A widely used strategy has been to overexpress genes that boost organic acid production using constitutive promoters or organ specific promoters (Samac and Tesfaye, 2003). A number of research studies were directed at overexpression of genes encoding enzymes that are part of the biosynthetic or regulatory pathways of organic acids, such as citrate synthase (CS), phosphoenolpyruvate carboxylase (PEPC), and malate dehydrogenase (MDH). PEPC catalyses the conversion of phosphoenolpyruvate and CO_2 to oxaloacetic acid and inorganic phosphate; CS converts oxaloacetate to citrate and modulates synthesis of other organic acids, and MDH catalyses the reversible conversion of oxaloacetate to malate (Figure 1.6).

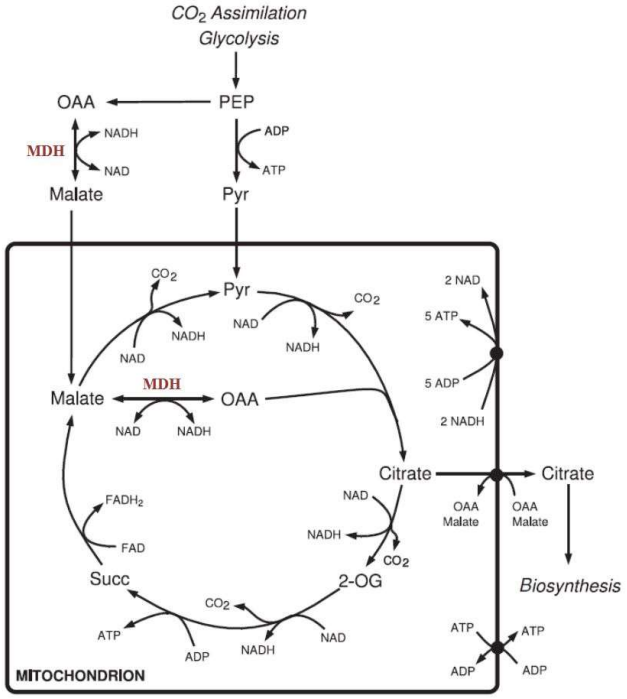


Figure 1.6. Tricarboxylic acid cycle scheme. In dark red are the steps where the enzyme malate dehydrogenase (MDH) catalyses the reversible reduction of oxaloacetate to malate (Steuer et al., 2007).

Al^{3+} tolerance was reported in papaya (*Carica papaya*) and tobacco by overexpression of *Pseudomonas aeruginosa CS* using the constitute promoter Ca*MV35S*. Here, root growth was evaluated in seedlings grown in Al-containing media with Al^{3+} concentrations ranging from 75 to 1000 µM (De la Fuente et al., 1997). However, these results couldn't be confirmed in subsequent experiments, and the strategy of generating Al-tolerant plants by overexpressing CS was questioned (Delhaize et al., 2001).

Other research projects support the use of this strategy for production of Al^{3+}-tolerant plants. Alfalfa (*Medicago sativa*) lines transformed with the *P. aeruginosa CS* gene controlled by the constitutive promoter *act2* or a root specific promoter *RB7* from tobacco exhibited increased tolerance to Al^{3+} when evaluated in soil. There were, however, inconsistent results when plants were evaluated in hydroponic conditions (Barone et al., 2008). Similarly, Al^{3+} tolerance was reported in alfalfa overexpressing *P. aeruginosa CS* controlled by the constitutive promoter Mt*HP* from *Medicago truncatula* in soil experiments (Reyno et al., 2013). Contrary to the results observed by Delhaize et al. (2001) and Barone et al. (2008), here an increase in citrate concentration in tissues was observed when transgenic plants were compared to their non-transgenic controls (Reyno et al., 2013).

Other research overexpressing *CS* from different sources supported the use of this strategy. Improved Al^{3+} tolerance has been achieved in carrot (*Daucus carota*) cells (Koyama et al., 1999) and Arabidopsis overexpressing a mitochondrial *CS* from carrot controlled by the constitutive promoter Ca*MV35S* (Koyama et al 2000). Canola with increased expression of a mitochondrial Arabidopsis *CS* gene (Anoop et al., 2003), and *Nicotiana benthamiana* overexpressing *CS* from Yuzu (*Citrus Junos* Sieb. ex Tanaka) (Deng et al., 2009), both genes controlled by promoter Ca*MV35S,* also exhibited increased Al^{3+} tolerance.

Plants transformed with a *malate dehydrogenase* (*MDH*) gene for Al^{3+} tolerance have been generated in a number of species. Tesfaye et al. (2001) engineered overexpression of the nodule specific genes isolated from alfalfa *MDH* (*neMDH*) and phosphoenolpyruvate carboxylase (*PEPC*) controlled by the constitutive Ca*MV35S* promoter in alfalfa. Compared to other MDH isoforms, the nodule enhanced isoform MDH has a higher turnover towards production of malate (Miller et al., 1998; Tesfaye et al., 2001). Transgenic alfalfa events carrying the *neMDH* transgene showed a considerable increase in citrate, oxalate, malate, succinate and acetate concentration in roots, and root exudates. In addition, transformed plants showed increased root and shoot growth compared to controls in Al^{3+} toxic soil. Oat plants transformed with *neMDH* from alfalfa were also developed. In this case, no significant change in malate exudation was observed, and events whose root growth increased under Al^{3+} stressed conditions in hydroponics exhibited impaired root growth in control conditions (no Al^{3+} solution) (Radmer et al., 2011).

Transgenic white clover was also generated using an *neMDH* isolated from white clover (Tr*neMDH*) controlled by a white clover root-tip-specific promoter from a gene encoding a phosphate transporter (*TrPT1*). The use of this promoter favours the expression of the transgene only in root tissue where toxic Al^{3+} effects are known to occur. The system has the advantage of working efficiently with a low fitness penalty (Labandera 2007, Rosello 2011). In this work, three events out of 15 evaluated showed an improved Al^{3+} tolerance compared to their non-transgenic isolines when tested in 10 µM Al^{3+} solution culture (Rosello 2011).

Wang et al. (2010) transformed tobacco plants with *MDH* genes from Arabidopsis and *E. coli* under the control of a leaf-specific light inducible promoter (Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit promoter, *PrbcS*) and observed a 120-130% increase in MDH enzyme activity by indirect measurements in leaves. A higher concentration of malate both in leaves and roots was verified and higher external concentrations of malate were detected in root exudates in transgenic to wild type plant comparisons (Wang et al., 2010). These results suggest that induction of *MDH* via a light-induced promoter may generate Al^{3+} tolerant plants (Wang et al., 2010).

Transgenic alfalfa plants overexpressing nodule enhanced *PEPC* controlled by Ca*MV35S* promoter were generated. No rise in organic acid in roots and root exudates was detected in *PEPC* transformed alfalfa plants, and no evident tolerance to Al^{3+} was reported (Tesfaye et al., 2001; Samac and Tesfaye, 2003). On the contrary, Begum et al. (2009) reported that the transfer of the intact maize *PEPC* gene and promoter to rice resulted in an increase in organic acid concentration in leaves and roots and a rise in exudation of oxalate. These rice plants exhibited increased tolerance to Al^{3+} in comparison to their non-transgenic controls (Ku et al., 1999; Begum et al., 2005).

Al-induced plant genes were identified for use in transformation and generation of Al^{3+} tolerant plants (Samac and Tesfaye, 2003). Ezaki et al. (2000) studied the performance for Al^{3+} tolerance of blue-copper-binding protein gene (*AtBCB*) from Arabidopsis, and the tobacco genes glutathione S-transferase gene (*NtparB*), peroxidase gene (*NtPox*), and GDP-dissociation inhibitor gene (*NtGDI1*). Transgenic Arabidopsis plants expressing these genes were produced and different levels of Al^{3+} tolerance were observed. Further research work revealed the possible function of these genes. *AtBCB* gene may suppress Al^{3+} absorption, whereas expression of the *NtGDI1* gene promotes a release of Al^{3+} in the root tip region of Arabidopsis. Meanwhile, lipid peroxidation caused by Al^{3+} stress was repressed in the lines transformed with *NtparB* or *NtPox*, suggesting that overexpression of these two genes diminishes oxidative damage caused by Al^{3+} stress (Ezaki et al., 2001).

Also, the overexpression of genes encoding Al-inducible organic acid transporters has proven successful for different crops. Transgenic Al-tolerant barley was generated using a gene encoding the malate transporter *ALMT1* from wheat (Delhaize et al. 2004). As mentioned above, this transporter is present on the plasma membrane of apical root cells of wheat, and is induced by Al^{3+} (Ryan et al. 1997, Zhang

et al. 2001, Delhaize et al. 2004). Similarly, Al^{3+} tolerant wheat plants were developed by overexpression of *TaALMT1* using a constitutive maize ubiquitin promoter (Pereira et al., 2010).

1.5.2 Plant phenotyping for Al^{3+} tolerance

Root growth inhibition is one of the symptoms used for identification of Al^{3+} stress in plants (Silva, 2012). As this symptom can be visible within a few days, a straightforward strategy for phenotyping for Al^{3+} tolerance usually involves root length measurements. Root growth inhibition under Al^{3+} stress can correlate with shoot growth in the long term (Foy et al., 1993; Narasimhamoorthy et al., 2007). Therefore, an alternative for Al^{3+} stress phenotyping can be shoot weight measurements.

Diverse systems have been used for evaluation of aluminium tolerance in different plant species. For example, hydroponics (nutrient solutions with Al^{3+}) was used in wheat (Zhang and Taylor 1989; Riede and Anderson 1995) maize (Cançado et al., 1999), rice (Wu et al., 2000), alfalfa (Zhang et al., 2007) and white clover (Rossello, 2011). Soil-based assays were performed in alfalfa (Reyno et al., 2013; Dall'agnol et al., 1996) wheat (Foy et al 1996), and tall fescue (Foy and Murray 1998), among others.

Compared to other assays, soil-based experiments have the main advantage of representing more accurately what happens in the field (Narasimhamoorthy et al., 2007). Soil experiments can be more time consuming than other methods. Generally, these can extend from approximately between one month, when plants are evaluated in pots in the glasshouse, to a whole growth season in field experiments (Samac and Tesfaye, 2003). Long-term soil experiments have the advantage of enabling the evaluation of plant growth at both early and late stages (Samac and Tesfaye, 2003). Tang et al. (2003) reported an exception to the high time demand of the technique, as soil-based experiments in wheat were performed in 6 days.

Some constraints to soil-based trials are observed. For instance, obtaining intact roots for length measurement can be difficult when the numbers of plants evaluated are large (Narasimhamoorthy et al., 2007). Also, in situations where the species studied is highly sensitive, the effects of Al^{3+} toxic acidic soils can be too severe to make root growth differences observable between genotypes (Foy et al., 1993; Samac and Tesfaye, 2003).

Field screening is the most representative approach for Al^{3+} tolerance phenotyping, as plants are evaluated in real soil and climatic conditions where plants are cultivated (Howeler, 1991). In this case, plants performance is assessed though their whole growth cycle (Howeler, 1991). Therefore this method is time consuming, and given that the use of large land areas are required, it can also be costly (Howeler, 1991). Other issues are the noise caused by nutrients effects, and the variation in soil composition. The effects of Al^{3+} and other nutrients present in the soil can be difficult to differentiate both in field experiments and soil assays in glasshouse conditions (Samac and Tesfaye, 2003). Also, a high variability in soil composition in the field is common, and genotype by soil interactions may take place (Narasimhamoorthy et al., 2007).

An alternative to soil assays are soil-on agar assays. This method has the advantage of being performed on seedlings within a short timeframe. While the time required for in soil assays extends to weeks, here it can be performed in days (Voigt et al., 1997). Fundamental to this experiment is that seedlings initially source nutrients from reserves rather than soil. Therefore, given that nutrients are obtained from reserves, external nutrients are not used by the seedling, and only the effects of Al^{3+} on the plants would be directly evaluated (Voigt et al., 1997). One constraint of this method is the possibility of excessive water exposure or flooding (Pan et al., 2008).

A widely used method for Al^{3+} tolerance screening is solution culture assay. Compared to soil assays, solution culture does not accurately reflect field conditions. However, the method has advantages that make it attractive. Solution culture assays require less time than soil experiments. Tesfaye et al. (2001) and Pan et al. (2008) reported the Al^{3+} tolerance screening of alfalfa cuttings in solution culture within 3 and one day after Al^{3+} application, respectively. Therefore, the method allows for screening of high number of plants in a short period of time. Another important advantage of solution culture methods is that they are non-destructive (Howeler, 1991). This makes it possible to perform multiple root measurements through the duration of an experiment. Furthermore, variables such as pH or Al^{3+} concentration are relatively easy to control in solution culture assays. It also avoids effects caused by other nutrients that may confound the results and ensure that only Al^{3+} tolerance is being evaluated (Pan et al., 2008).

Nevertheless, Narasimhamoorthy et al. (2007) mention that results may be affected by pH and interactions of Al^{3+} with other nutrients in the nutrient solution such as phosphate. In order to avoid nutrients effects, Al^{3+} solutions containing only $CaCl_2$ are used (Tesfaye et al., 2001; Pan et al., 2008). In these cases is necessary to evaluate 7-day old seedlings, given that young seedlings may not need nutrients from the extracellular medium (Pan et al., 2008). Pros and cons between soil-based experiments and solution culture experiments are summarized in Table 1.2.

Theoretically, compared to nutrient solution assays, soil-based experiments can be more effective for plants whose Al^{3+} resistance is based on the Al^{3+} exclusion mechanism. Soil-based assays could have the advantage of retaining particles in the soil located near the root at or adjacent to the site of organic acid excretion (Dong-Man Khu et al., 2012). An alternative to soil assays for evaluating plants whose Al^{3+} tolerance mechanism is based on Al^{3+} exclusion is the use of sand assays. By this method, organic acid exudates would thus be retained by sand particles. Nevertheless, many research studies report the successful use of solution culture for selection of Al^{3+} tolerant plants whose tolerance is based on Al^{3+} exclusion mechanism.

Other methods have been reported, such as staining with chemicals having affinity for Al^{3+}. This makes it possible to visualize and locate Al^{3+} in roots cells. Hematoxylin root staining patterns have been shown to correlate with Al^{3+} stress (Polle et al. 1978). Hematoxylin staining was used for screening of Al^{3+} tolerance in transgenic white clover (Labandera, 2007), and study of mechanisms involved in Al^{3+} tolerance in wheat (Delhaize et al., 1993). Lumogallion staining was used in soybean (Kataoka and Nakanishi, 2001) and pine (Moyer-Henry et al., 2005) to study the effects of Al^{3+} and Al^{3+} tolerance (Dong-Man Khu et al., 2012).

A callus biomass method has also been used. In this technique, callus biomass is measured after being grown in media with and without Al^{3+}. This was used for screening of Al^{3+} tolerance in alfalfa (Parrot and Bouton, 1990; Dall'Agnol et al., 1996; Dong-Man Khu et al., 2012). The principal constraint of this method is that it is not possible to study the effect of Al^{3+} on roots, which is the organ most affected by Al^{3+} toxicity.

Dall'Agnol et al. (1996), Dong-Man Khu et al. (2012) and Pan et al. (2008) compared methods in alfalfa. Pan et al. (2008) concluded that there are advantages and disadvantages to each screening procedure. As there are different Al^{3+} tolerance mechanisms and various genes may be involved, there is not a single

preferred method to study Al^{3+} tolerance, and a combination of methods is thus recommended (Narasimhamoorthy et al., 2007).

Table 1.2. Comparison of the two most used methods for Al^{3+} tolerance studies, soil assays vs solution culture assays.

Soil experiments	Solution culture
"Realistic" to what happens in agricultural systems	Less "realistic" to what happens in agricultural systems
Time consuming. Experiments can extend from weeks to an entire growth season in field conditions	Fast. Experiments can extend from 1 day to 1 or 2 weeks
Short and long-term effects can be studied	Short-term effects are studied
High labour demand	Low to medium labour demand
Medium to high cost (high extension of land is required for field experiments)	Low cost
Root measurements difficult, generally destructive sampling	Root measurements easy, non-destructive sampling
High variability of environmental conditions (for example pH)	Reduced variability of environmental conditions (for example, pH variations can be controlled)
Nutrients in soil can interfere with Al^{3+} tolerance evaluations	It's possible to evaluate exclusively the effects of Al^{3+}, without the interference of other nutrients
Need to find the appropriate Al^{3+} toxic soil according to the species and variety requirements	Possible to adjust Al^{3+} concentration according to the species and variety requirements
Theoretically more effective for evaluation of plants whose Al^{3+} resistance is based on "Al^{3+} exclusion" mechanisms	Theoretically not very effective for evaluation of plants whose Al^{3+} resistance is based on "Al^{3+} exclusion" mechanisms.

1.6 Alfalfa mosaic virus (AMV)

Alfalfa mosaic virus (AMV) was first identified in alfalfa in the year 1931. The virus belongs to alfamovirus genera from the bromoviridae family (Bol, 2005). Like the majority of plant viruses, AMV

has a genomic single RNA strand with plus-sense polarity, encapsidated by a single category of coat protein (CP) (Tenllado and Bol, 2000).

Four different virus particles were identified by fractionation of AMV by gradient centrifugation. Contained in each of these particles an RNA molecule was isolated and named 1, 2, 3, and 4. RNAs 1 and 2 encode the replicase proteins necessary for viral replication, whereas RNA 3 encodes the coat protein (CP) and a movement protein (MP). CP is encoded in RNA 3 but it is translated from the subgenomic RNA 4 (Figure 1.7) (Bol, 2005).

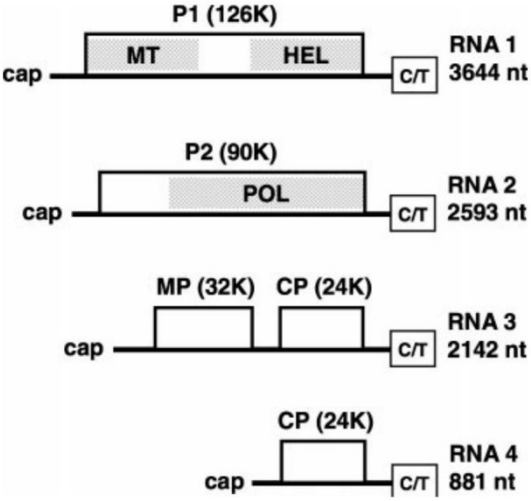


Figure 1.7. Genome structure of AMV. P1 and P2 encode replicase proteins. Shaded are Methyltransferase-like (MT), helicase-like (HEL) and polymerase-like (POL) domains. MP: Movement protein; CP: Coat protein (Bol, 2003).

Both MP and CP play important roles in cell-to-cell movement (Bol, 2005). It was also reported that CP helps in the leaf to leaf spread of the virus through vascular systems (Tenllado and Bol, 2000). Overall, CP has the main function of protecting the viral RNA inside the plant cell, and in favouring the spread of the virus between plants (Bol, 2005).

It was observed that infectivity happened when the three particles carrying the RNAs 1, 2 and 3 were put together. However, when the three RNA alone were mixture together infectivity didn't occur. Only infectivity was re-established when molecules of RNA 4 or CP proteins were added to the mixture, which supports that CP or its messenger RNA 4 is necessary for the infection start (Bol, 2003).

1.7 AMV in white clover

Alfalfa mosaic virus (AMV) was reported as the most widely occurring virus in Australia by 1998 (Norton and Johnstone, 1998). It infects white clover in the states of Tasmania, Victoria, Australian Capital Territory (ACT), New South Wales (NSW), West Australia (WA), and Queensland (Jones, 2013). Australian dairy industry losses caused by AMV incidence were estimated at A$30 million by year 1991 (Garrett 1991). Furthermore, the combined effects of the three main viruses affecting white clover in Australia, alfalfa mosaic alfamovirus (AMV), white clover mosaic potex virus (WCMV) and clover yellow vein potyvirus (CYVV), could cause a production reduction of 30% per year (Kalla et al., 2001).

It has been reported that AMV can reduce leaf dry weight yields to 60%, and that at some sites in Australia infected plants can reach up to 90% of total (Gibson et al., 1981; Norton and Johnstone, 1998). Ten white clover pastures from 19 surveyed between years 1994-96 exhibited an AMV infection incidence over 69% (Mckirdy and Jones 1997), while most of the 26 white clover pastures examined in south-west Australia exhibited an AMV infection of 100% (Jones, 2013). AMV infection can cause yield reduction by a decrease in stolon branching, and the number of leaves per plant. Also there is a considerable impact on root nodulation, which leads to a decrease in nitrogen fixation (Gibson et al., 1981).

AMV can cause interveinal light green or yellow mottle on leaves, and plant stunting (
Figure 1.8). The symptoms can change from light to severe depending on environmental conditions, cultivar and AMV strain (Jones, 2013).

Figure 1.8. Symptoms of white clover infected by AMV (Jones, 2013).

AMV is transmitted by different species of aphids in a non-persistent manner (Hull 1969; Garran and Gibbs, 1982). As the virus is seed-borne in lucerne and can remain latent, it can be spread to white clover from lucerne swards, or through volunteer lucerne plants from previous cultivation (Mckirdy and Jones, 1995). Additionally, AMV can remain latent through annual medic wild species, such as the annual weeds flatweed (*Hypochaeris glabra* L.), and rufous stonecrop (*Crassula decumbens* Thunb.) (Jones, 2013). The incidence of the virus was observed to increase in irrigated systems (Mckirdy and Jones, 1995). AMV spread is favoured in south-east zones in Australia by summer and winter rains, as it contributes to an increase in aphid numbers (Jones, 2013).

1.7.1 Genetic resistance to AMV

AMV control through dispersion avoidance is difficult, and there is no effective chemical or biological control for the virus. Given this, the alternative of generating genetic resistant plants is attractive. There are some reports of the existence of germplasm with genetic resistance to AMV in white clover (Barnett and Gibson, 1975; Gibson et al., 1989). Some works mention the availability of genetic resistance in the white clover relative *Trifolium ambiguum* (Barnett and Gibson, 1975; Pederson and Mclaughlin, 1989), but Wijkstra and Guy (1996) reported this species as susceptible. Also Martin et al., (1997) reported the presence of resistant plants in red clover *Trifolium pratense.* However, wide range resistance to AMV has not been identified in white clover (Panter et al., 2012). In addition, development of *Trifolium* resistant hybrids has some constraints and no commercial varieties have been produced (Abberton, 2007).

Plant transformation for generation of resistant plants against AMV has been applied as an alternative to classical breeding in some species. The development of resistant plants against viruses was sometimes based on the concept of pathogen derived resistance and the observation that plants expressing one or more *CP* genes from a given virus can become resistant to infection by that virus (Sandford and Johnston, 1985; Tepfer, 2002). The possible mechanism for the resistance may be explained as a block in the disassembly of the infecting virus mediated by the transgene-derived coat protein (Baulcombe, 1996; Goldbach et al., 2003). In other cases, the mechanism for plant virus resistance of transgenic plants expressing a virus RNA is explained by RNA silencing (Baulcombe, 1996; Vaucheret et al., 1998).

Virus resistance has been obtained in different plant species against various virus groups using this system. Some examples are ToMV and TMV in tomato, PVX, PVY, CMV and TSV in tobacco, PVX and PVY in potato, and WMV2 and ZYMV in squash (Beachy et al., 1990; Tepfer, 2002). In addition, transformed *Nicotiana tabacum* (Loesch-Fries et al., 1987), alfalfa (Hill et al., 1991) and pea (Timmerman-Vaughan et al., 2001) expressing the virus coat protein gene showed resistance to AMV. Also resistance to AMV has been observed in greenhouse and field conditions in transgenic *Medicago truncatula* (Jayasena et al., 2001) and white clover, described in next section (Panter et al., 2012). Some examples of plant virus resistance obtained by gene silencing are PSbMV in *Pisum sativum,* TYDV in *Petunia hybrid,* and PVX in *Nicotinia benthamiana* (Voinnet, 2001). Gene silencing was also applied in ryegrass for resistance to RgMV (Xu et al., 2001) and in white clover for resistance to WCMV (Ludlow et al., 2009).

1.7.2 Transgenic white clover resistant to AMV

Transgenic white clover was developed in order to produce plants with tolerance to AMV by expressing a coat protein from AMV (Kalla et al., 2001; Panter et al., 2012). White clover plants cv. Irrigation were transformed with the gene that codifies for a coat protein from AMV (CP-*AMV*) controlled by the constitutive Ca*MV35S* promoter. A molecular characterisation was carried out; copy number was determined by Southern blots, and gene expression of four events was evaluated by northern blots (Panter et al., 2012).

An exhaustive phenotypic evaluation of transgenic plants for AMV resistance was performed in glasshouse and under field conditions over 8 years in two geographically different sites at Hamilton and Howlong in Australia. Primary transformants (T_0) exhibited immunity to AMV under both glasshouse and field conditions (Figure 1.9). The following generations, T_1, and T_2 (homozygous for CP-*AMV* transgene), also showed immunity to AMV, while T_4 plants exhibited high levels of resistance, with viral infection under 5% (Table 1.3) (Panter et al., 2012).

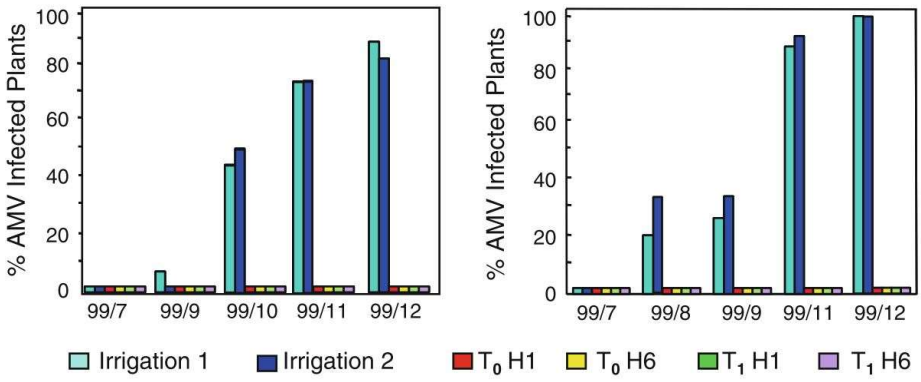


Figure 1.9. Field evaluations for AMV tolerance of T_0 and T_1 events of white clover carrying CP-*AMV* transgene (Panter et al., 2012). Irrigation 1 and 2 are non-transgenic control lines. In *x* axis are represented the different dates of evaluation during year 1999 season.

Table 1.3. Phenotyping results of AMV infection in the different generations of transgenic evaluated white clover (Panter et al., 2012).

Germplasm type	Transgene	No. tested genotypes	AMV Glasshouse	AMV Howlong	AMV Hamilton
cv. Irrigation, non-transgenic control	None	2		100%	75%
Event 1 T_0 (cv. Irrigation)	AMV CP	1	0	0	0
Event 6 T_0 (cv. Irrigation)	AMV CP	1	0	0	0
Event 1 T_1	AMV CP	6	0	0	0
Event 6 T_6	AMV CP	6	0	0	0
Event 1 and Event 6 T_2	AMV CP	1,300	0	NA	0
cv. Sustain, non-transgenic control	None	11	NA	NA	59%
Event 6 T_4 (cv. Sustain)	AMV CP	600	NA	NA	≤5%

AMV glasshouse: Percentage of infected plants evaluated in the glasshouse;
AMV Hamilton: Percentage of infected plants evaluated at Hamilton in field conditions;
AMV Howlong: Percentage of infected plants evaluated at Howlong in field conditions.

Furthermore, mitotic and meiotic stability of the inserted gene through the subsequent generations was confirmed, and biochemical analyses supported the "substantial equivalence" of events compared to their non-transgenic controls (Panter et al., 2012).

In further experiments, clover plants previously carrying the CP-*AMV* transgene were re-transformed with an Tr*neMDH* gene for Al^{3+} tolerance, and evaluated and confirmed for AMV resistance (Rossello, 2011). This work is described in section 1.10.1.

1.8 Gene Stacking

1.8.1 Transgenic crops expressing multiple genes for herbicide or insect tolerance

Globally, the most widely grown GM crops are soybean, maize, cotton and canola carrying genes for pest- or herbicide-resistance (James, 2010). The appearance of resistant weeds to herbicides has been reported over recent years, and development of new strategies for weed control are required (Owen and Zelaya, 2005; Powles and Yu, 2010). Among other strategies, researchers have focused on development of new transgenic plants with multiple genes for tolerance to different herbicides (Dill et al., 2008; Green et al., 2008; Que et al., 2010).

The use of different *Bacillus thuringiensis* (Bt) toxin-producing transgenic crops to control insects could face a similar problem with the appearance of resistant insects (Christou et al., 2006; Storer et al., 2010; Gassmann et al., 2011). To address this problem, a considerable proportion of the cultivated area is assigned for non-transgenic crops as refuges, which dilute resistant alleles with susceptible insects. Some limitations may appear using this strategy, such as Bt and non-Bt seed mixing in seed production, and economic losses associated with the use of large refuge areas (Bates et al., 2005).

Alternatively, research efforts to control insects are now focused on generation of transgenic crops with multiple genes (Christou et al., 2006; Gatehouse, 2008; Que et al., 2010). The potential emergence of resistant insects can be tackled by the insertion of multiple genes directed to different insect modes of action (Christou et al., 2006; Que et al., 2010). Additionally, gene stacking of pest resistance genes can contribute to the control of secondary pest species that affects crops carrying single Bt genes (Gatehouse, 2008).

1.8.2 Next generation of multigene GM crops

Genetic engineering in crops has had a positive global economic impact in crop production (Qaim, 2009; James, 2016). However, this effect is not as profound as expected after more than 10 years of commercialization. Public opposition and highly restrictive regulatory systems have resulted in a limited number of transgenic crops and traits used and commercialized globally (Qaim, 2009). The first generation of GM crops are beneficial for farmers but failed to provide direct benefits to the consumer. This could be one of the factors in public opposition to GM crops (Qaim, 2009). On the other hand, a second generation of GMO's could be more attractive to consumers, as it includes crops with increased nutritional quality and benefits for the environment, such as traits for phytoremediation or production of molecules by "green factories" (Halpin, 2005; Gonzalez et al., 2009; Onyango et al., 2016).

Nutritional quality traits are generally complex, governed by more than one gene, and may require the development of metabolic engineered crops with stacked genes (Halpin, 2005; Hirschi, 2009; Naqvi et al., 2009). An example of this is golden rice, in which a combination of transgenes that reconstructs the β-carotene biosynthetic pathway enabled biosynthesis of provitamin A in the endosperm (Ye et al., 2000).

Climate change is generating pressure for development of crops with tolerance to extreme environments (Varshney et al., 2011). Furthermore, world population growth justifies the need for increase in crop yields to produce more food (Godfray et al., 2010; Foley et al., 2011). Development of the next generation of GM crops for tolerance to challenging environments could require manipulation of multiple genes. For instance, agronomic traits like drought tolerance generally depend on many genes (Fukao and Xiong, 2013; Ricroch and Hénard-Damave, 2017). Gene stacking may also be needed when environmental stresses such as drought stress and heat stress, act in parallel (Mickelbart et al., 2015).

Furthermore, limitations in crop productivity are generally hindered by a number of factors. For example, white clover persistence depends on many elements, such as drought tolerance, pests resistance and tolerance to challenging soil conditions. Therefore, in order to address the numerous factors affecting persistence, the development of a more persistent white clover may require the insertion of multiple genes (Rossello et al 2010).

Additionally, the pressure for increasing yields could push future development of GM crops towards other highly complex agronomical gene traits that depend on multiple genes. Some examples are

nitrogen fixation in cereal crops (Oldroyd and Dixon, 2013), generation of apomictic plants (Spillane et al., 2004), or plants with increased photosynthetic activity (Zhu et al., 2010).

1.9 Gene stacking strategies

1.9.1 Sexual crossing

Pyramiding of transgenes is performed using various strategies that comprise both classical and molecular techniques. A frequently used method is crossing two parent plants carrying different transgenes to obtain progeny carrying the parent's genes. Among others, Amian et al. (2011) produced a double stacked transgenic pea with enhanced resistance to fungal diseases by crossing one transgenic line carrying a chitinase gene with another line expressing glucanase gene. Datta et al. (2002) generated a triple stacked rice for resistance to insects, and tolerance to sheath blight, by crossing two transgenic parental lines. Similarly, Wei et al. (2008) generated a triple stacked transgenic rice line by crossing one line expressing the *cry1Ab* gene (for insect resistance) and the *bar* gene (for tolerance of herbicide), with the another line expressing the *Xa21* gene (resistance to bacterial blight).

However, this method has limitations. As insertion of each transgene will take place at a random site in the genome, segregation in future crosses can cause the loss of one or more of the inserted genes. This leads to the need for maintenance of large populations and screening of progeny for transgenes in each generation, which makes the breeding process time consuming and expensive (Halpin et al., 2001; Halpin, 2005). Furthermore, crossing transgenic plants for gene stacking may require the initial generation of plants homozygous for the transgenes, which takes between two and three generations. If we consider the production of a transgenic plant with 4 transgenes, pairwise crosses between transgenic homozygous and a second self-pollination step are necessary. Homozygous plants become more difficult to achieve when several genes are inserted at various sites within the genome. Overall, it can take 4 to 6 generations to generate a transgenic plant containing 3 or 4 transgenes in one plant by sexual crossings (Halpin et al., 2001).

1.9.2 Re-transformation

Alternatively, gene stacking can be achieved by re-transforming a transgenic plant with new genes of interest. Re-transformation or sequential transformation, can be distinctly effective for some woody plants and trees, as they can be difficult to propagate by sexual crossing (Douglas and Halpin, 2009).

Some examples of use of this strategy are transgenic potato producing freeze-thaw-stable starch carrying three inhibited starch synthase genes (Jobling et al., 2002), Arabidopsis producing long chain polyunsaturated omega-3 and omega-6 fatty acids by expressing three genes encoding for the enzymes Δ^9-specific elongating activity, Δ^8-desaturase and Δ5-desaturase (Qi et al., 2004), or poplar containing antisense transgenes for two lignin biosynthesis genes in an attempt to modify lignin structure to improve wood quality for the pulp industry (Lapierre et al., 1999).

However, re-transformation is labour intensive, as it requires transformation and screening for insert presence at each generation, as well as characterization of the transformed events. Moreover, in order to select transformed plants in each transformation round, each integration will typically require a different selectable marker gene. The use of a wide range of selectable marker genes, make regulatory approval difficult (François et al., 2002; Halpin, 2005). Therefore, the application of systems that don't require the use of selectable marker genes or remove them (discussed in section 1.9) present an interesting alternative (Darbani et al., 2007).

1.9.3 Co-transformation

The simultaneous transfer of multiple genes, known as co-transformation, appears as a more favoured strategy than sexual crossings or re-transformation when integration of multiple genes is desired. Here, the genes of interest would be inserted in the first generation (T_0) at a single site in the genome, thus making gene stacking faster and less laborious than sexual crossing or re-transformation (Farre et al., 2014). Co-transformation can be either by insertion of constructs carrying two or more transgenes linked in *cis* (linked co-transformation) or by inserting unlinked genes placed in different plasmids (unlinked co-transformation) (Naqvi et al., 2009).

Some examples of linked co-transformation are the Agrobacterium mediated production of transgenic Arabidopsis, canola, and soybean expressing up to four genes for increased tocochromanol levels (Karunanandaa et al., 2005) or potato with increased carotenoid contents expressing the genes *phytoene*

synthase (*CrtB*), *phytoene desaturase* (*CrtI*) and *lycopene beta-cyclase* (*CrtY*) from *Erwinia herbicola* (Diretto et al., 2007). Double stacked transgenic white clover events expressing the CP-AMV and Tr*neMDH* genes were generated by linked co-transformation (Vala, 2012). A more detailed description of this work is presented in section 1.10.2. Variants of linked co-transformation include the use of split reading frames (Farre et al., 2014) and operons, feasible for genes inserted in plastids (Figure 1.10) (Lu et al., 2013).

Linked co-transformation becomes less efficient when more genes are incorporated, given that high molecular weight DNA transgenes are more prone to fragmentation and can make vectors unstable (Hamilton et al., 1996; Naqvi et al., 2009). Unlinked co-transformation by direct DNA transfer can be a solution to this constraint. A very successful case was reported by Chen et al. (1998), who inserted eleven genes by particle bombardment in rice using eleven different vectors simultaneously. On the other hand, the use of unlinked Agrobacterium mediated co-transformation of a high number of genes can be difficult. T-DNA integration efficiency decreases when the number of transferred inserts increases (Naqvi et al., 2009). The co-transformation strategies are not mutually exclusive, and a combination of linked and un-linked co-transformation systems can be used (Ye et al., 2000).

The estimated DNA size limit for Agrobacterium mediated linked co-transformation approximates to 50 kb, while for direct DNA transfer the approximate size limit can reach 80 kb (Naqvi et al., 2009). In order to overcome this constraint, Agrobacterium high capacity binary vectors bacterial artificial chromosomes (BIBACs) (Hamilton et al., 1996) and bacteriophage P1-derived transformation competent artificial chromosomes (TACs) have been developed (Liu et al., 1999). These vectors have an estimated maximum transfer limit of 150 kb (Hamilton et al., 1996) and 80 kb respectively (Liu et al., 1999).

This DNA size barrier for transformation was overcome by direct transfer of DNA fragments by minichromosomes (Liu et al., 1999; Carlson et al., 2007). Although this strategy is still experimental, it has promising prospects for future development of transgenic multigene crops. This method would solve issues such as the carryover of undesired linked sequences in further breeding processes, gene silencing or undesired levels of expression of the inserted transgenes (Birchler et al., 2010; Gaeta et al., 2012).

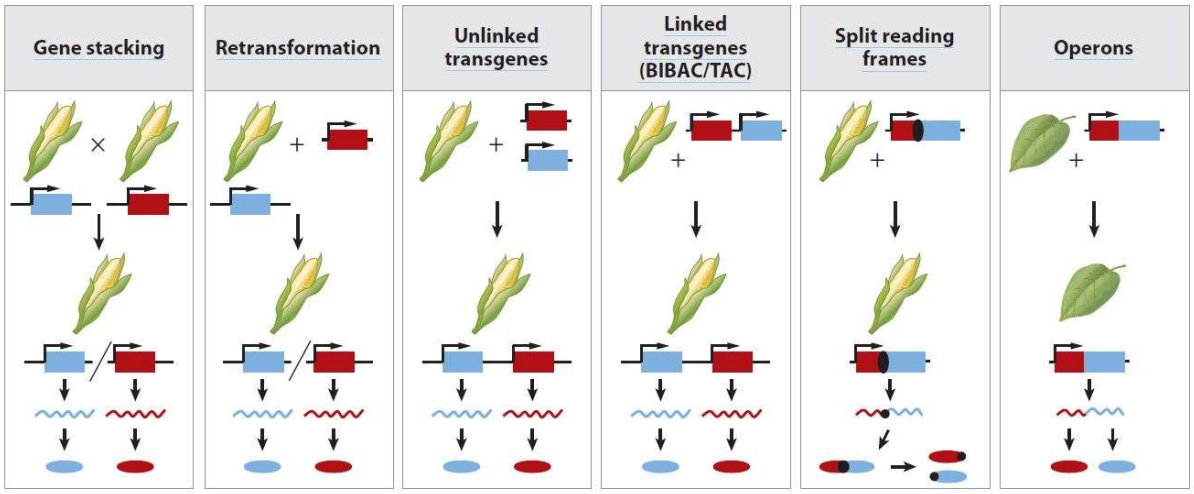


Figure 1.10. Illustration of different strategies used for co-transformation. Gene stacking: two transgenic lines carrying gene 1 and gene 2 respectively are crossed in order to generate a transgenic line carrying both genes 1 and 2. The genes are inserted in different locations in the genome, and they express independently (represented by the diagonal bar). Retransfromation: A transgenic line carrying gene 1 is transformed with gene 2. Similarly to the gene stacking method, the genes are inserted in different locations in the genome, and they express independently (represented by the diagonal bar). Unlinked transgenes: A non-transgenic line is transformed with genes 1 and 2 from two different vectors. the genes may be inserted in different locations in the genome, or linked in tandem (represented by a connector line). Linked transgenes: A non-transgenic line is transformed with genes 1 and 2 linked in tandem in a single vector. Therefore, the two genes are inserted linked in the genome. Split reading frames: A non-transgenic line is transformed with two genes linked by the 2A peptide from food-and-mouth-disease virus (black disc). This generates a polycistronic mRNA, which is translated in a polyprotein that is cleaved into proteins 1 and 2. Operons: Plant is transformed with a polycistron carrying genes 1 and 2. The two genes are thus in a single transcript, and separated proteins 1 and 2 are produced via internal ribosome entry sites (Farre et al., 2014).

1.9.4 Site specific gene stacking

One of the main limitations of gene stacking is the need for insertion of many genes in a single locus to ensure the co-segregation of the inserted genes throughout the breeding process. As aforementioned, although this problem could be solved by linked co-transformation, large insert sizes can make the process difficult. Additionally, the insertion site cannot be directed by Agrobacterium-mediated transformation or particle bombardment.

A number of site-specific DNA integration technologies that can address these issues have been developed. The insertion of multiple genes in a single site has been done in plants by using site-specific

recombination systems (Srivastava and Thomson, 2016). These include the bacteriophage Cre-lox (Gilbertson, 2003), the yeast FLP-FRT (Li et al., 2009b; Li et al., 2010), and R-RS systems (Ow, 2002).

'Gene editing' technologies allow a precise DNA modification in a defined genome site by a nuclease-mediated double strand break, and a subsequent insertion of the desired modification by a DNA repair mechanism (Urnov et al., 2010; Carroll, 2014). These methods comprise the Zinc finger nucleases (ZFNs), meganucleases, transcription activator-like effector nucleases (TALENs), and (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR- associated) systems.

The ZFN system depends on the combination of Zinc finger nuclease (ZFN) domains. When a number of ZFN domains are combined, they can recognize any specific DNA sequence site. This structure can be linked with the nuclease domain of the FokI restriction enzyme (Urnov et al., 2010; Carroll, 2014). Thus, a modular assemble of 4 to 6 ZFN nucleases that specifically recognizes a defined DNA site, directs the dimerization of C-terminal bounded restriction enzyme FokI for a specific DNA cleavage (Urnov et al., 2010; Carroll, 2014).

An alternative to the use of ZFNs are TAL effector nucleases (TALENs). Similarly to ZFNs, TALENs are composed of one domain that recognizes a specific DNA sequence fused to a nonspecific FokI nuclease domain. The DNA recognizing domain is formed from highly conserved repeats derived from transcription activator-like effectors (TALEs) (Joung and Sander, 2012).

Designer meganucleases are another interesting option for site directed genome editing. However, these are more difficult to design than ZFNs and TALENs, as their DNA recognition domain are not separated from their catalytic domain (Epinat et al., 2003; D'Halluin et al., 2013).

More recently, the discovery of CRISPR/Cas9 system has revolutionized genomics, and particularly, genome editing. Unlike meganucleases, ZFNs, and TALENs that recognize DNA based on protein domains, CRISPR/Cas9 system is very easy to design as DNA recognition depends on a RNA guide (gRNA) that base pairs with the target site (Zhang and Zhou, 2014; Bortesi and Fischer, 2015; Raitskin and Patron, 2016).

The use of 'gene editing' techniques is making possible the modification of crops by targeted mutagenesis, targeted transgene integration, gene replacement and genome rearrangements (Petolino et al., 2016; Weeks et al., 2016). Gene stacking for crop improvement is also in deployment. Targeted gene

stacking has been accomplished in maize using ZFNs combined with an adjacent recognition site or "trait landing pads" (TLPs) (Figure 1.11) (Ainley et al., 2013). Similarly, site specific gene stacking was developed in cotton by combining targeted meganuclease cleavage and homologous recombination repair (D'Halluin et al., 2013). Gene editing techniques are also of special interest for removal of selectable marker genes in generation of transgenic plants. This is discussed in the next section.

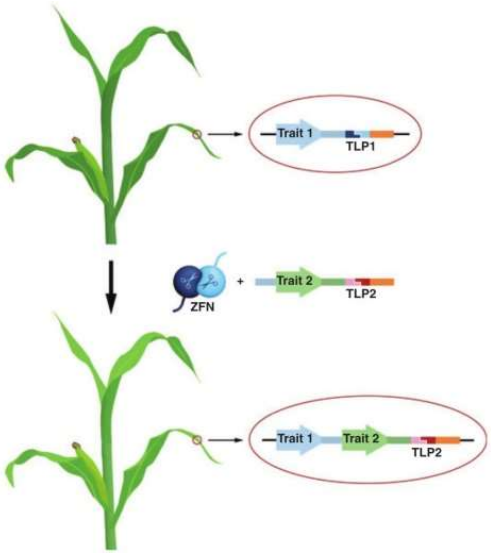


Figure 1.11. Gene stacking by ZFN nucleases. Sequential transgene stacking using trait landing pads (TLPs). TLP1 is composed of a target sequence for a ZFN flanked by sequences homologous to the next transferred construct containing trait 2. The transgenic plant is re-transformed with a ZFN-1 and a cassette containing trait 2 and TLP2. ZFN-1 cuts into TLP-1 and insertion of trait 2 and TLP-2 occurs (Ainley et al., 2013).

1.10 Gene stacking in white clover for delayed leaf senescence, aluminium tolerance, and AMV resistance

Comprehensive work has been carried out by Agriculture Victoria Research into gene stacking of genes for delayed leaf senescence, aluminium tolerance, and AMV resistance in white clover. A brief description of this work is provided in the next two sections.

1.10.1 White clover expressing aluminium tolerance and AMV resistance

Transgenic white clover plants double stacked for the genes Tr*neMDH* for aluminium tolerance and CP-*AMV* for AMV resistance were previously generated. The two transgenes were stacked by Agrobacterium-mediated re-transformation of a transgenic event expressing CP-*AMV* with the gene Tr*neMDH* (Rossello, 2011).

Molecular characterization was carried out, which comprised confirmation of presence of the transgenes, copy number determination, and transcript abundance studies. Transcript abundance of the inserted Tr*neMDH* gene was confirmed for a number of transgenic plants, and determined to be 100 times higher in roots than in leaves. This supported the specific expression in roots of the promoter *TrPt1*.

The T_o plants generated were assessed for aluminium tolerance and re-evaluated for AMV resistance. Fifteen transgenic events were tested for aluminium tolerance in solution culture containing 10 µM aluminium. Three events with potential increased tolerance to aluminium were identified, evidenced by root growth measurements under Al^{3+} stressed conditions (Figure 1.12) (Rossello, 2011).

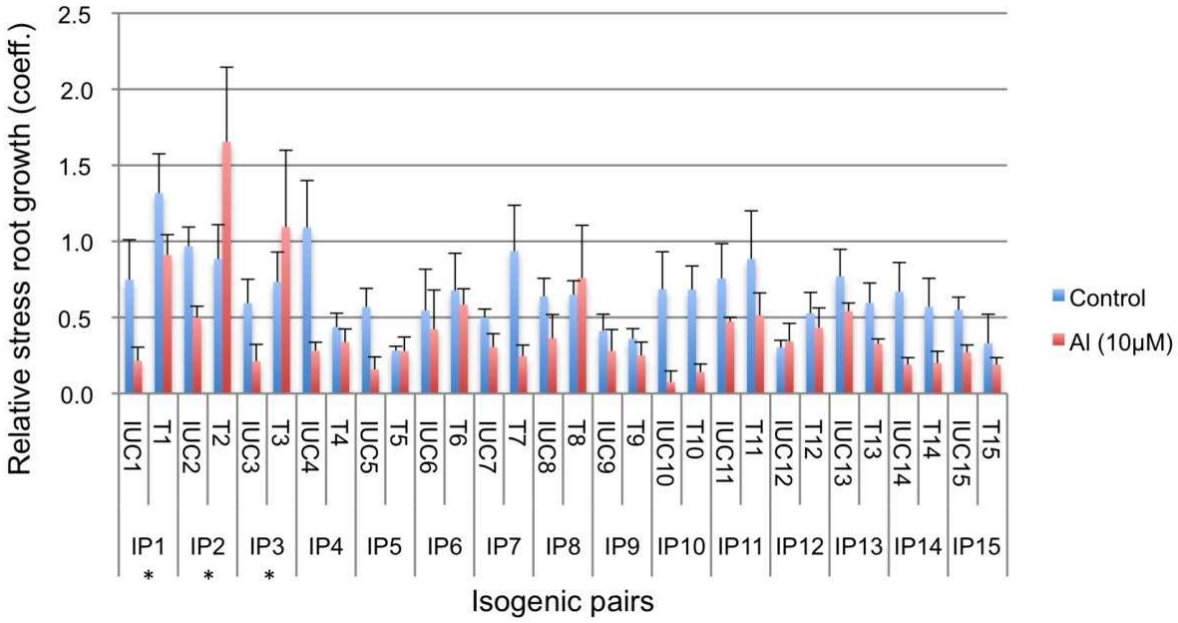


Figure 1.12. Relative stress root growth (coeff.) (RSRG) analysis of double stacked transgenic Trne*MDH::*CP-*AMV* white clover to control and Al^{3+} stress conditions under solution culture. error bars denote ± SEM (n = 8). Asterisks denote statistically significant differences ($P < 0.05$) within the isogenic pairs assessed. IP: isogenic pair; IUC: isogenic untransformed control line; T: transgenic line (Rossello, 2011).

Additionally, re-evaluation of AMV resistance of the transgenic double stacked events was carried out by infectivity bioassays using *C. amaranticolor* and cowpea as indicator plants, and untransformed 'Mink' and 'sustain' cultivar plants as susceptible controls. Maintenance of the resistance to AMV after re-transformation was confirmed (Rossello, 2011).

1.10.2 White clover expressing aluminium tolerance and delayed leaf senescence

Double stacked transgenic white clover plants expressing *IPT* for delayed leaf senescence and Tr*neMDH* for aluminium tolerance were produced by linked co-transformation. In this case, Tr*neMDH* and *IPT,* controlled by Tr*Pt1* and At*myb32* promoters respectively, were placed in a single T-DNA. Molecular characterization was performed, which comprised evaluation of insertion of the genes of interest, and copy number estimations (Vala, 2012).

Phenotyping of the transgenic events was carried out for the two traits of interest. Delayed leaf senescence was observed by detachment of leaves and visual inspections of colour change as a sign of

senescence in Petri dishes (Figure 1.13). Two events from eight evaluated exhibited a significant increase in shoot dry weights, and one of these showed increased root dry weights compared to their isogenic controls in solution culture containing aluminium (Figure 1.14) (Vala, 2012). One of these events exhibited also delayed leaf senescence.

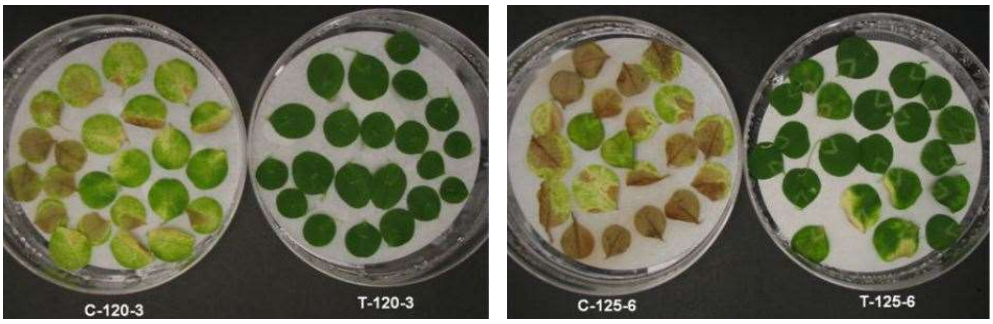


Figure 1.13. Detached leaf experiments of transgenic white clover. Delayed leaf senescence was evaluated by leaf detachment and colour change assessment in Petri dishes in transgenic lines carrying Tr*PT1:*Tr*neMDH* + At*myb32:IPT* and their respective non-transgenic isogenic lines (Vala, 2012). T: transgenic, C: non-transgenic isogenic control.

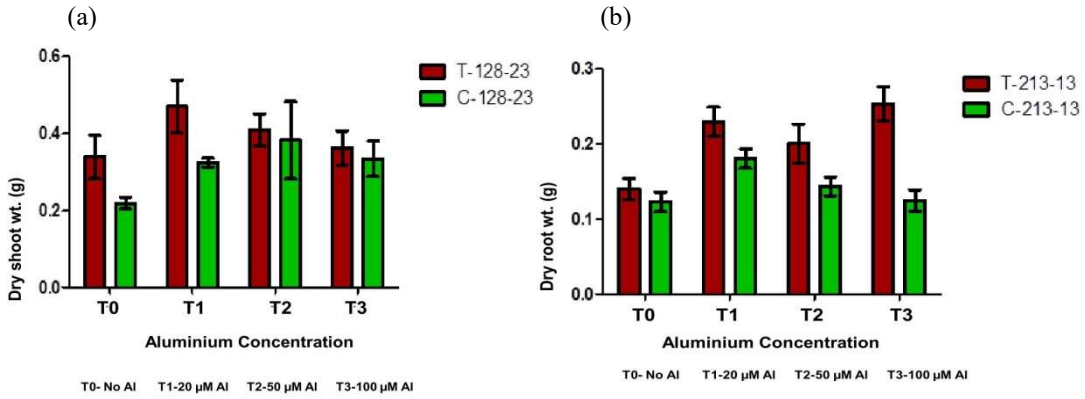


Figure 1.14. Results of hydroponics evaluation under different aluminium concentrations of white clover plants. (a) Dry shoot weights of transgenic line carrying Tr*PT1:*Tr*neMDH* + At*myb32:IPT* and its non-transgenic isogenic line (T-128-23 and C-128-23 respectively) and (b) transgenic line carrying Tr*PT1*:Tr*neMDH*:Tr*neMDH* and its non-transgenic isogenic line (T-213-13 and C-213-13 respectively) (Vala 2012).

1.11 Strategies for generation of selectable marker genes free transgenic plants

The generation of transgenic plants usually requires the use of a selectable marker gene, which generally consists of an antibiotic or herbicide resistance gene. Their use has raised concerns from regulatory

institutions and public opinion about possible human or environmental hazards. Potentially, antibiotic resistance genes could be transferred horizontally in the field from a GM plant to soil bacteria, or from a GM plant to clinical bacteria in the animal or human intestinal tract (Demaneche et al., 2008). However, there are many barriers that can impede such events, and no scientific evidence that supports a theoretical transfer from GM plants to bacteria have been reported (Bradford et al., 2005; Ramessar et al., 2007; Capita and Alonso-Calleja, 2013). Additionally, according to the European Food Safety Authority (EFSA), given that the antibiotic resistance genes currently used as selectable marker genes in transgenic plants are widespread in natural bacterial populations, their use does not pose a human or animal health risk (European Food Safety Authority, 2009). Nevertheless, public concerns persist and regulatory commissions apply pressure for removal of antibiotic resistance selectable marker genes (Breyer et al., 2014).

A more likely event is the introgression of transgenes from GM crops to related wild species by sexual hybridization. Depending on the trait, this phenomenon could increase fitness of the wild relatives and contribute to their weediness (Stewart et al., 2003; Warwick et al., 2009; Kwit et al., 2011). Of particular concern is the transference of herbicide resistance from GM crops to wild relatives, particularly when the related species are agricultural weeds. This could a have negative impact on weed management practices, and a consequent need for development of new control measures (Stewart et al., 2003; Baucom, 2004). Some cases support this concern. For example, hybridization between the glyphosate tolerant transgenic canola (*Brassica napus*) and the wild relative *Brassica rapa* was observed in Canada. In this case, the presence of *B. rapa* plants expressing glyphosate resistance, and the persistence of herbicide resistance for 6 years were reported (Warwick et al., 2008). Also, introgression of a transgene expressing herbicide resistance from creeping bentgrass (*Agrostis stolonifera*) in wild *Agrostis* species was reported in United States (Reichman et al., 2006).

Nevertheless, the occurrence of such events depend on the existence of wild relatives in the region of cultivation, and the sexual compatibility between the crop and the related species (Stewart et al., 2003; Warwick et al., 2009).

1.11.1 Removal of selectable marker genes by gene editing

A number of techniques have been used in order to remove selectable marker genes from transgenic plants. Among these, gene editing techniques provide the prospect of precise gene removal. Petolino et

al. (2010) used ZFNs for deleting a transgene encoding for the *gus* marker gene in tobacco. These authors crossed a transgenic plant transformed with a ZFN construct and the plant carrying the target gene *gus*, and elimination of the *gus* marker gene in the T_1 generation was confirmed (Petolino et al., 2010).

One of the most extended techniques used for selectable marker genes removal is the Cre-lox system. Here, the excision of the selectable marker gene is produced by a recombinase (cre) that produces a recombination-mediated event on two sites (lox sites) that flank the marker gene (Gidoni et al., 2008). Transfer of the *cre* gene to the target transformant can be achieved by hybridization or re-transformation. Similar to Cre/lox, the recombinase systems FLP/FLT and R/RS were also widely used for selectable marker gene elimination (Gidoni et al., 2008). Meganucleases were also successfully used for removal of selectable marker genes. Siebert and Puchta (2002) removed a selectable marker gene by inducing double strand breaks mediated by the meganuclease I-SceI. The construct consisted of two I-SceI cutting sites flanking the target marker gene (Siebert and Puchta, 2002). Furthermore, the recently developed gene editing technique CRISPR/Cas9 appears to be a promising technique for removal of selectable marker genes (Breyer et al., 2014).

1.11.2 Transposon-based SMG removal

Selectable marker genes may also be removed using transposon systems. Here, the gene of interest (GOI) and the selectable marker gene are flanked by transposase-recognizing sites, and next to the gene of interest a gene encoding the enzyme transposase is placed. The transposase cuts in the recognition sites, and the gene of interest or the selectable marker gene are repositioned in a different locus in the genome. This allows the definitive removal of the selectable marker genes by segregation in further generations (Ebinuma et al., 1997; Cotsaftis et al., 2002; Yau and Stewart, 2013).

1.11.3 Transformation without selection

A realistic option to obtain marker free transgenic plants is to perform transformation without selection. Given that selectable marker genes are not used, screening of all the plants for presence or absence of the inserted transgene needs to be carried out by PCR (Yau and Stewart, 2013). Since a high proportion of the plants will be negative for the transgene presence by this technique, the process could be very laborious and time consuming (Yau and Stewart, 2013). Additionally, generation of chimeric shoots make the PCR screening more difficult, and false positive PCRs are possible given that Agrobacterium

can persist inside the transformed plant (Li et al., 2009a). Considerably lower transformation frequencies were reported for this technique, compared to transformation with selection in barley (0.8 and 3.1% respectively) (Holme et al., 2012), and tobacco (2.2 and 90% respectively) (Li et al., 2009a).

1.11.4 Selectable marker genes removal by co-transformation

Co-transformation by Agrobacterium or by particle bombardment is another effective alternative for generation of marker free transgenic plants. This technique involves the separated transfer of the gene of interest and the selectable marker genes using two different transformation vectors (Yau and Stewart, 2013; Breyer et al., 2014). It is expected that when the selectable marker gene is transferred to the plant, the gene of interest will also be transferred (Yau and Stewart, 2013; Breyer et al., 2014). Furthermore, un-linked insertion in the genome of the two T-DNAs need to occur with high frequency (Komari et al., 1996). If the gene of interest and the selectable marker gene are inserted in different sites of the genome, the selectable marker gene can be removed by hybridization and segregation in the subsequent generations (Komari et al., 1996).

There are three variants of Agrobacterium mediated co-transformation: (1) the vectors carrying the selectable marker gene and the GOI can be in two different Agrobacterium strains, (2) the two vectors can be in one Agrobacterium strain, or (3) more than one T-DNA can be placed in a single vector in a single Agrobacterium strain (Yau and Stewart, 2013; Breyer et al., 2014). These methods were extensively used in both monocotyledonous and dicotyledonous plants (Manimaran et al., 2011). Some examples with relevance to the present work are selectable marker gene elimination by co-transformation in the forage species white clover (Rossello, 2011) and alfalfa (Ferradini et al., 2011).

In contrast to methods such as Cre/lox, R/Rs, or FLP/FRT, this strategy has the main advantage of not leaving behind undesired residue sequences (Breyer et al., 2014). However, the technique has some limitations. For this strategy to succeed, the two T-DNAs need to be inserted at separate loci, and a simultaneous insertion of the two T-DNAs in a single transformation event should occur. These issues lead to a decrease in the transformation frequency when compared to single T-DNA transformations in some species (Ferradini et al., 2011; Breyer et al., 2014).

Given that a high number of plants are generally generated by this method, the screening by PCR to confirm the gene of interest insertion can be work and time demanding (Yau and Stewart, 2013; Breyer

et al., 2014). Furthermore, as the removal of selectable marker genes depends on sexual crossings, the use of this system can't be applied to plants with asexual propagation or species whose sexual cycles are extremely long such as trees (Darbani et al., 2007; Manimaran et al., 2011).

1.12 Phenotyping by digital imaging

This project has an important component in plant phenotyping. White clover was phenotyped using digital images for delayed leaf senescence, drought stress, and root growth under aluminium stressing conditions. In this section, we introduce phenotyping by digital imaging and its uses.

The 'genomic revolution', produced as a consequence of the development of next generation sequencing techniques, has enabled generation of a vast amount of genomic information. This lead to the deployment of new tools, such as marker assisted selection (MAS) and genomic selection, which enable to overcome classical plant breeding limitations (Varshney et al. 2009). Marker assisted selection (MAS) and genomic selection are based on combining genetic and molecular information with phenotypic data. However, the quantity and rate of production of phenotypic data has not kept the pace of the generation of genomic and transcriptomic data (Furbank and Tester 2011; Araus and Cairns 2014).

Phenotyping usually requires the screening of a high number of plants, grown in different environments, which can be costly and time consuming (Furbank and Tester 2011; White et al. 2012). Furthermore, typically, generation of phenotypic information is based on human observation, and involves high labour demand. Also, the accuracy of quantification of some phenotypic traits is low by conventional methods, as it often relies on subjective observation by humans. Also, usually, conventional phenotyping involves destructive yield measurements at fixed times during plant development (Furbank and Tester 2011).

Therefore, the rate of generation of phenotypic data is considered a limitation for plant breeding (Furbank and Tester 2011; Fiorani and Schurr 2013; Araus and Cairns 2014). This constraint has resulted in increased inputs into research directed at improving phenotyping techniques (referred to as 'phenomics') (White et al. 2012). The integration of modern technologies such as varied sensors, high resolution digital imaging, software for digital image analysis, and automated systems, used both in the glasshouse and the field, has allowed for the rapid development of this new area (Fiorani and Schurr 2013). Modern phenotyping technics involve imaging of plants under controlled conditions in the glasshouse or in the field. This can be performed manually, by delivering the plants to the camera station, or automatically

or semi-automatically by using conveyor systems in the glasshouse, or vehicles carrying sensors or cameras in the field (White et al. 2012).

Different components of plant structure can absorb, reflect or transmit light, and therefore images obtained at different wavelengths can provide a range of information (Li et al. 2014). Within the visible range (400-700 nm), parameters such as plant architecture, plant size, leaf area, canopy size, leaves number, leaf colour, and root architecture can be estimated (Fiorani and Schurr 2013; Li et al. 2014). Briefly, the plant outline is identified, in order to separate the plant from the background, and pixels enclosed within the plant outline are computed from the acquired image by using a software for image analysis. Pixels can also be classified based on colour, in order to quantify colour changes in the plant, caused by various stresses (Hairmansis et al. 2014).

Usually, fresh and dry weights are estimated by projection of plants shoot area from the acquired images (Tackenberg 2007; Golzarian et al. 2011). However, these estimations are largely dependent on the species analysed, as their plant structure variates. For rosette plants, such as Arabidopsis, images are usually acquired from the top, while for main cereal crops, images are usually acquired from different directions, by rotating the plants relative to the camera. Therefore, imaging protocols need to be designed for specific species or groups of species, depending on their morphology and growth habits (Fiorani and Schurr 2013).

The optimization of plant digital image-based phenotyping protocols requires considerable amount of time, and is identified as an important limitation of the technique (Walter et al. 2012). Overlap of leaves and/or canopies occurs, which can have a negative impact on plant size estimations. Additionally, plant colour in images can vary based on lighting, and standardization of lighting to allow colour classification should be performed. Background colours should also be clearly different from the evaluated plant in order to separate the subject from the background (Walter et al. 2012).

Non-invasive methods for root growth and root architecture evaluation can be performed by digital imaging. In order to perform non-destructive evaluations, plants need to be grown on agar or paper surfaces, or in clear gel media (Zhu et al. 2011). Alternatively, plant can be grown in liquid media using rhizotrons. However, these methods may not represent the root behaviour in soil. Furthermore, the described methods are generally performed in young plants, which may not correlate with root growth in later developmental stages (Zhu et al. 2011). In recent years, non-invasive techniques such as magnetic

resonance imaging (MRI) and X-ray computed tomography (CT) were developed, and may represent an important step forward in root phenotyping. X-ray CT can provide volumetric data on soil moisture and root biomass. MRI has been used, among other applications, for visualization of the symptoms caused by cyst nematodes of sugar beet, observation of root nodulation in bean, and to study the shoot-to-root carbon fluxes (Fiorani and Schurr 2013). However, these techniques are still restricted to use in containers (Araus and Cairns 2014).

Thermal imaging use has extended in controlled conditions and in the field. Transpiration rate and stomatal conductance can be measured by this method. Leaf temperature can provide data on leaf water status, and canopy temperature can be associated to drought stress tolerance, and grain yields in some cereals (Fiorani and Schurr 2013). Changes in plant temperature caused by heat flux, influenced mostly by wind, can limit the accuracy of the results generated in the field (Fiorani et al. 2012).

Light can be absorbed by plant compounds, and can provide informative parameters on the plant composition and physiological state (Fiorani et al. 2012). Spectral imaging is usually performed with multispectral and hyperspectral cameras at different wavelengths, such as 550 nm, where green reflectance occurs (Fiorani et al. 2012). Photosynthetic efficiency can be estimated by photochemical reflectance, and the normalized difference vegetation index (NDVI), which compares the red and the near-infrared reflectance, and can provide an approximation to the chlorophyll content (Humplík et al. 2015). Furthermore, various visible-near infrared indices can also provide an estimation on water content in tissues (Humplík et al. 2015). Imaging spectroscopy opens new possibilities for extracting spectral features related to plant health and disease status (Fiorani et al. 2012).

Fluorescence imaging can be a useful method for plant phenotyping. Chlorophyll fluorescence, detected in the red to far-red region, can be an indirect strategy for measuring excited electrons in the photosynthetic light reactions. Therefore, it can be used for measuring quantum efficiency of the photosystem II and electron transport rate, which are useful parameters for early estimation biotic and abiotic stress (Fiorani and Schurr 2013). Separation of chlorophyll emission from reflected light is a challenge of this technique. Furthermore, given that an application of homogeneous light to the evaluated plants is required for obtaining robust data, chlorophyll fluorescence measurements are usually restricted to single leaves (Fiorani et al. 2012; Fiorani and Schurr 2013).

1.13 Aim

The aim of this work is to generate novel triple stacked white clover events with delayed leaf senescence, aluminium tolerance and AMV resistance (Figure 1.15).

Objectives

- Generate transgenic white clover events carrying genes for delayed leaf senescence, aluminium tolerance and AMV resistance by Agrobacterium-mediated transformation
- Carry out a molecular characterisation of the transgenic events
- Optimise molecular techniques for copy number determination and transcript abundance analysis using droplet digital PCR (ddPCR) in white clover
- Phenotype and identify events that exhibit delayed leaf senescence and drought stress tolerance
- Phenotype and identify events that exhibit aluminium tolerance
- Develop image analysis based phenotyping of white clover for delayed leaf senescence and consequent drought stress tolerance
- Confirm the insertion and transcriptional activity of CP-*AMV* as a preliminary approach to AMV resistance assessment
- Integrate molecular and phenotyping results to select between 5 and 10 events with potential for increased agronomic performance

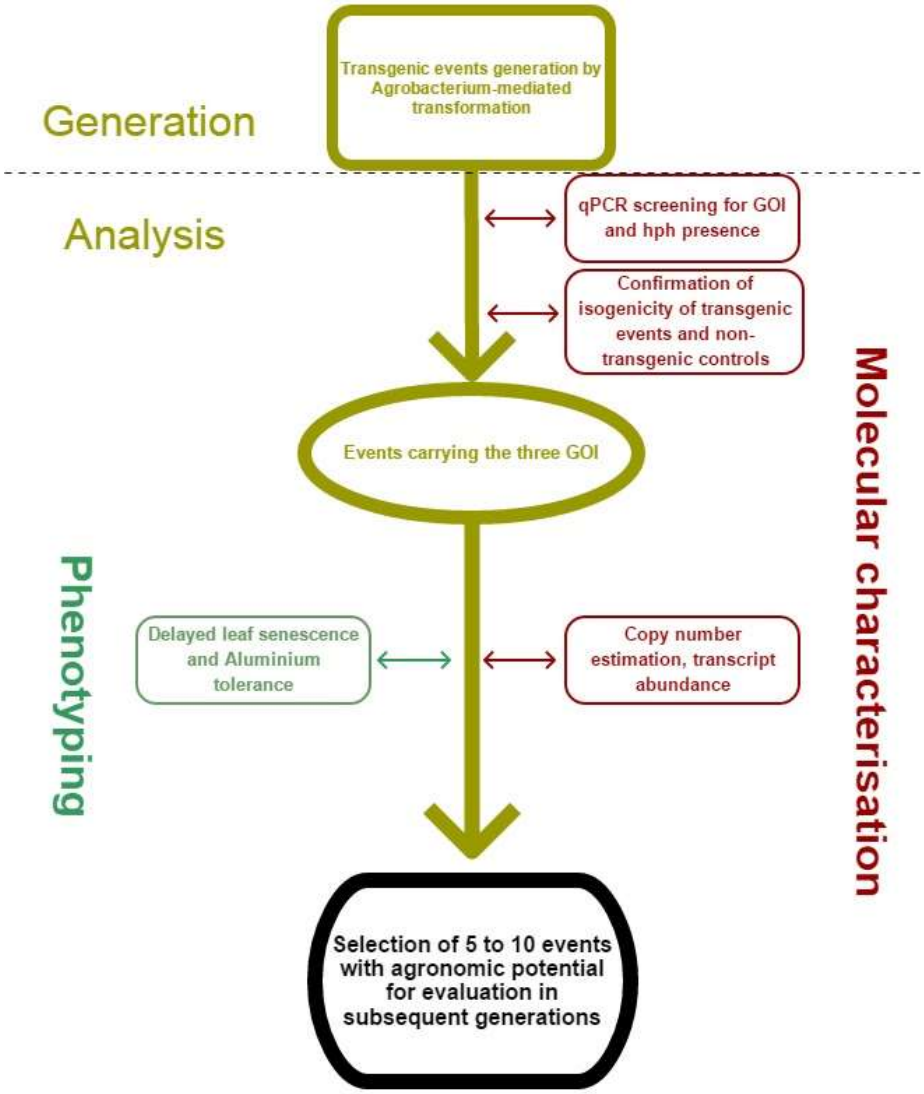


Figure 1.15. Workflow plan. GOI: Genes of interest.

2 Generation of transgenic white clover plants

2.1 Introduction

Transformation of white clover can be a useful tool for transferring agronomically important traits not available in the white clover germoplasm, or not possible to be transferred from related species by conventional methods. As previously mentioned, no sources of AMV resistance and tolerance to aluminium has been reported in white clover or the *Trifolium* genus. Therefore, plant transformation was used to overcome this limitation, and white clover plants with increased tolerance to aluminium (Labandera, 2007), delayed leaf senescence (Ludlow 2000; Lin et al 2005) and resistance to AMV were generated independently (Panter et al., 2012). In addition, double stacked transgenic plants combining AMV resistance with aluminium tolerance (Rossello, 2011), and delayed leaf senescence with aluminium tolerance were generated (Vala, 2012).

In this project the construction of a single T-DNA insert containing the three genes of interest in tandem (defined in Chapter 1 as linked co-transformation), each controlled by different promoters, was proposed. Thus, we expect to generate transgenic events containing the three genes integrated in a single site in the genome. This enables stacking of genes in a single transformation step, without the need for re-transformation strategies or subsequent crossing for gene introgression.

In addition to linked co-transformation, we proposed the use of unlinked co-transformation in order to produce selectable marker gene free events. Here, two independent vectors, one carrying the genes of interest and a second vector carrying the selectable marker gene were used. This strategy allows for the possible generation of marker free events both at T_0 and by recombination in future generations.

In this chapter, we describe the transformation of white clover by Agrobacterium combining co-transformation, and the insertion of multiple genes in a single T-DNA.

2.2 Materials and methods

2.2.1 Vector construction

The RNA4 coding region from AMV (*AMV-CP* transgene) was cloned according to Panter et al. (2012) and the cassette Ca*MV35S*-p*_AMV-CP_nos-t* was created. The *TrPt1* promoter sequence and the nodule-enhanced *Malate dehydrogenase* coding sequence (Tr*neMDH* transgene) from white clover were isolated and cloned according to Labandera (2007) and the *TrPT1-p_TrMDHb_TrMDH-t* cassette was generated. The cassette At*MYB32-p_ipt_ocs-t* was generated by cloning *Isopentenyl Transferase* (*IPT transgene*) from Agrobacterium *tmr*-gene encoded by the octopine Ti plasmid (Li, Hagen, & Guilfoyle, 1992), under the control of the At*MYB32* promoter from Arabidopsis (Preston, Wheeler, Heazlewood, Li, & Parish, 2004). The pPZP200 binary vector (Hajdukiewicz, Svab, & Maliga, 1994) was digested with PmeI and GatewayTM-enabled by adding the GatewayTM RfA cassette (Invitrogen) to this site, generating pDPI000001. The At*MYB32-p_ipt_ocs-t* cassette was cloned into the multiple cloning site of the pDPI000001 binary vector backbone, creating pCLV000031. The Ca*MV35S-p_AMV-CP_nos-t* cassette was transferred to pDONR221TM P5-P2, and the *TrPT1-p_TrMDHb_TrMDH-t* cassette was transferred to pDONR221TM P1-P5 by a GatewayTM BP ClonaseTM reaction, generating pCLV000030 and pCLV000029 respectively. A multisite GatewayTM LR ClonaseTM reaction was then performed to combine pCLV000031, pCLV000030 and pCLV000029 into the final transformation vector, pCLV000032 containing the At*MYB32-p_ipt_ocs-t*, *TrPT1-p_TrMDHb_TrMDH-t*, and Ca*MV35S-p_AMV-CP_nos-t* cassettes (Figure 2.1). The pDPI000080 vector contains a *CaMV 19S-p_hph*(D)*_AtORF1*-t selection cassette in a pBIN backbone (Bevan, 1984). The pDPI000081 vector contains a *CaMV 19S-p_hph*(D)*_AtORF1*-t selectable marker cassette and a Ca*MV35s_turboGFP*(D)*_nos-t* reporter gene cassette in a pBIN backbone (Figure 2.1). The construction of these vectors was performed by Dr. Heather Anderson. Vector sequences were verified by restriction endonuclease analysis and independent Sanger sequencing by the Agriculture Victoria Science Support QA team and sequences were compared to those available in public databases using BLAST searches. Diagnostic restriction endonuclease digestions were performed with enzymes *Apa*LI, *Eco*RI, *Eco*RV and *Xmn*I for the pCLV000032 vector, *Ava*I, *Eco*RI and *Pvu*II for pCLV000080 vector, and *Eco*RV, *Xmn*I and *Pvu*II for pCLV000081 vector.

Agrobacterium strain EHA105 was co-transformed according to Ding et al. (2003) with pCLV000032 and either pDPI000080 or pDPI000081. Bacterial cultures were then grown in selection in 50 mg L^{-1} kanamycin, and 20 mg L^{-1} spectinomycin.

2.2.2 Agrobacterium-mediated transformation

The elite cultivar Storm (Heritage seeds, Dandenong South, Australia) was co-transformed with an Agrobacterium isolate of strain EHA105 containing vectors pCLV000032 and either pDPI000080 or pDPI000081 (Figure 2.1). A -70°C stock of transformed Agrobacterium was transferred to LB medium containing 20 mg L^{-1} rifampicin, 50 mg L^{-1} spectinomycin and kanamycin and incubated at 29°C for 48 h. In the morning of the day of plant transformation, 25 mL MGL medium containing 20 mg L^{-1} Rifampicin, 50 mg L^{-1} kanamycin and 50 mg L^{-1} spectinomycin was inoculated with one loop of bacteria from the 48 h preculture. This culture was incubated at 29°C and the estimated concentration of bacteria adjusted to OD 0.2-0.4 using MGL medium and 200 µm l^{-1} acetosyringone added.

For sterilization, 3 g of seeds were washed under running tap water for 5 minutes, and rinsed in 70% ethanol for 5 minutes. Seeds were then rinsed with 1.5% sodium hypochlorite for 16 minutes, followed by one step of 5 minutes wash in 70% ethanol, and 8 steps of distilled water wash. Sterilized seeds were kept in distilled water overnight at 4°C. The seedcoat and endosperm of cotyledonary explants were removed and the hypocotyl was cut leaving 1-2 mm of petiole segment intact. Cotyledons and 1-2 mm of hypocotyls were bisected to obtain two isogenic plants from each explant. Cotyledonary explants were incubated for 40 min at room temperature in an Agrobacterium culture (OD_{600} = 0.2 - 0.4) on a rotary shaker. After incubation explants were transferred to culture medium RM73 without antibiotics. Explants were co-cultivated for 3 days and transferred to fresh selective media RM73 + 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime at 25°C under a 16/8 h photoperiod at a photon flux density of 80 µM m^{-2} s^{-1}. Explants were transferred to fresh selection media every 2 weeks for subculture. Four rounds of selection were performed (8 weeks in total). Explants were examined for GFP signal during selection stages and when grown in soil using a MZ-FLIII fluorescence stereomicroscope (Leica Microsystems, Heidelberg, Germany). After selection of explants on media containing antibiotic, two rounds of regeneration on non-selective RM73 media containing 250 mg L^{-1} cefotaxime were performed. Regenerated shoots were transferred into containers with root-inducing medium RIM73 containing 250 mg L^{-1} cefotaxime and grown at 25°C under a 16/8 h photoperiod at a photon flux density of 80 µM m^{-}

^{2} s^{-1}. Transformations were performed by Yihan Lin, Yonglin Ding and the author. The author contributed on one third in the transformation process.

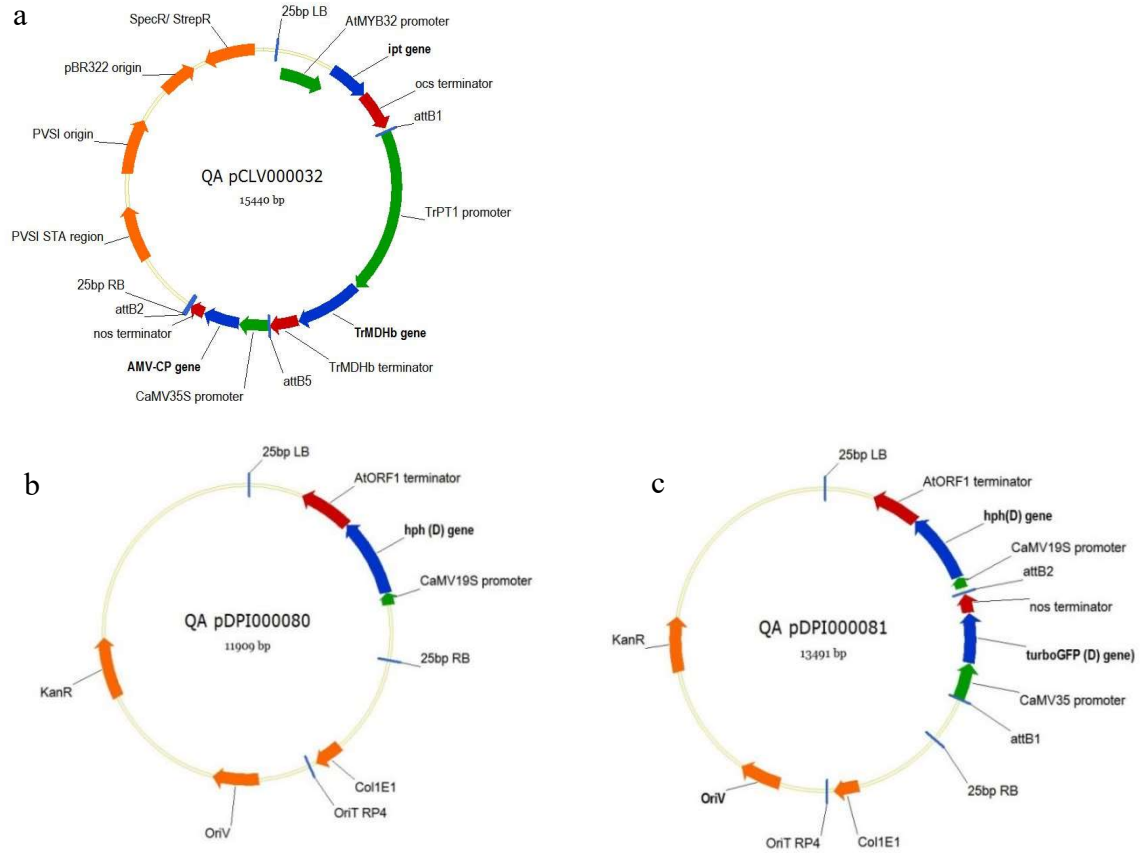


Figure 2.1. Vectors used for white clover transformation. (a) vector pCLV000032 containing the three genes of interest (b) vector pCLV000080 containing the selectable marker gene *hph* for resistance to hygromycin (c) vector pCLV000081 containing *hph* and the reporter gene *green fluorescent protein* (*GFP*). attB1 and attB2: Gateway att sites; RB: right border; LB: left border; *KanR*: *kanamycin resistance gene*; SpecR/StrepR: *spectinomycin/streptomycin* resistance gene; OriV: origin of vegetative replication. OriT: Origin of transfer; *IPT*: *Isopentenyl transferase*; *TrMDH: T. repens Malate dehydrogenase; AMV-CP: Alfalfa mosaic virus coat protein gene. ocs terminator: Octopine synthase terminator. nos terminator: Nopaline synthase terminator. AtORF1 terminator: Agrobacterium tumefaciens* open reading frame 1 3′ untranslated region.

2.2.3 Screening for presence or absence of genes of interest

High throughput DNA extraction was performed using a DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Presence of insert, and selectable marker sequences

was confirmed in putative events by Quantitative PCR (qPCR) using both SYBR Green and Taqman probes. Quantitative SYBR green PCR reactions were performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) in 20 µl of final volume, 600 nM of forward and reverse primers, and 1x of master mix. Taqman qPCR reactions were performed using Qiagen QuantiTect Probe PCR kit (Qiagen, Valencia, CA, USA) in 20 µl of final volume, 600 nM of forward and reverse primers, 200 nM of probes and 1x of master mix. The reaction conditions were the same for both SYBR and Taqman reactions: 10 min at 95°C, and 40 cycles of 10 s at 95°C, 30 s at 60°C, and 10 s at 72°C. A melting step was added for SYBR Green reactions in order to detect nonspecific PCR products. Primer sequences used for *IPT,* CP-*AMV,* Tr*neMDH* and *hph* detection, and the internal reference gene (*Translation initiation factor-related,* Id: GU443963.1, BAC 88b23) are presented in Table 2.1.

Table 2.1. List of primers and carboxyfluorescein (FAM)-labelled probes (Taqman probes), used for qPCR screening of insertion of the three genes of interest and the selectable marker (*hph*).

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe (5'-3')	Product size (bp)
IPT	TGGAGTGCAGATTTTCGTTG	CTGCTTAACTCTGGCCTTGG	TCGCCACAAGTTACCCGACCAA	90
Tr*neMDH*	TGCAGGAAACTAGGTAGCAGA	TGAGGAAGTGACCTCCCTGT	CCATGGCAGCATCAGCAGCA	95
CP-*AMV*	TCGGGTTTGAGTTGGTCTTC	ACCCAAACTTCGTTGAATCG	CTTCGATGCGCAGCCTGAGG	151
Hph	CTTACATGCTTAGGATCGGACTTG	AGTTCCAGCACCAGATCTAACG	CCCTGAGCCCAAGCAGCATCATCG	119
Translation initiation factor (internal reference gene)	GGTTCGTCTACTGGTGGTGG	GTTCCGACTCAACAGCACCT	TGTGGTCGATGAGGAAGGAGGTGGC	76

2.3 Results

2.3.1 Plant transformations and tissue culture

The vectors used to transform strain EHA105 of Agrobacterium were pCLV000032 containing the three genes of interest, and either vectors pCLV000080 or pCLV000081 carrying the selectable marker genes

(Figure 2.1). Vector pCLV000080 carried the selectable marker gene *hph*, and pCLV000081 carried *hph* and the reporter gene *green fluorescent protein (GFP)*. Transformation with *GFP* allowed preliminary screening of transformed plants in the early days of explant selection. Additionally, different T-DNA sizes used in vectors pCLV000080 and pCLV000081, 3344 bp and 4926 bp of length respectively, allowed for the evaluation of co-transformation frequencies for different cassette sizes.

White clover cotyledonary explants were transformed with Agrobacterium strain *EHA105* carrying either pCLV0080/pCLV000032 or pCLV000081/pCLV000032 vectors combinations. Cotyledonary explants were grown in selection media for 8 weeks, and putative transgenic explants (explants that survived selection) were transferred to recovery media (Figure 2.2). Putative transgenic explants were grown for 4 weeks in recovery media, and transferred to rooting media. Plants were grown in rooting media for approximately 4 weeks, until roots were formed. Only plantlets whose roots were developed were transferred to soil in the glasshouse. Two hundred and thirty-one putative transgenic events were transferred to soil.

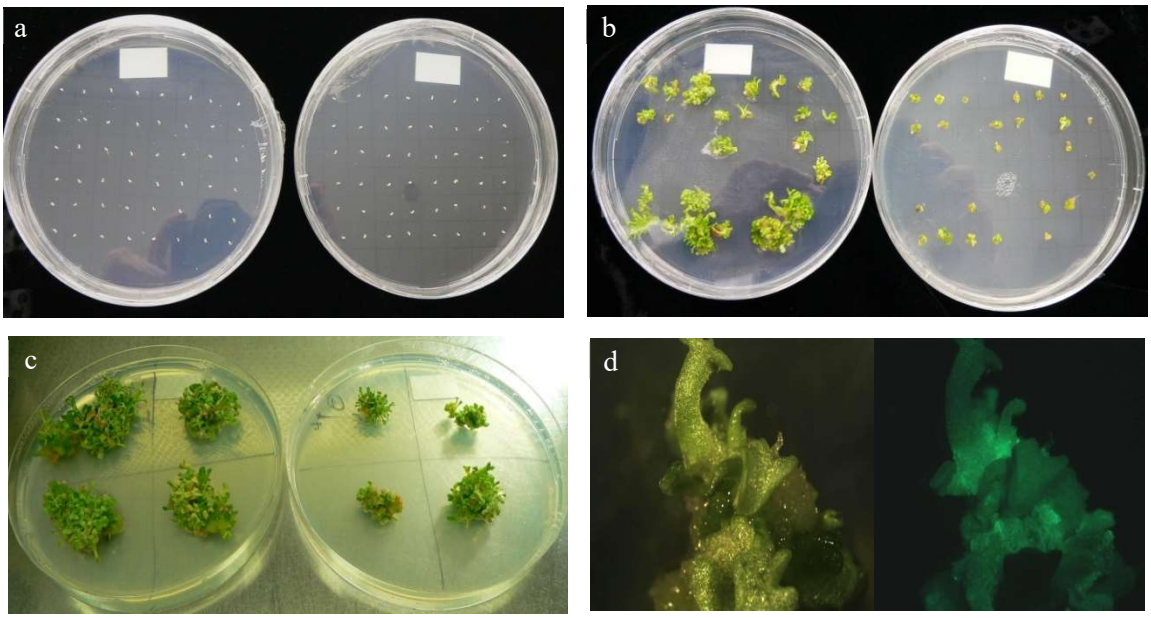


Figure 2.2. White clover tissue culture (a) Cotyledonary explants on the first day post-inoculation with Agrobacterium. (b) Explants growing in selection media (c) Explants growing in recovery media after selection (d) right, GFP signal in explants after growth in selection media; left, explant under visible light.

Screening for genome integration of the selectable marker gene *hph* and the three genes of interest was performed by qPCR in putative transgenic plants growing in soil in the glasshouse (section 2.3.2). Plants whose qPCR amplification was positive for sequences from any of the genes of interest or the selectable marker gene were considered transgenic. It is important to note that a single qPCR per gene of interest was performed, and that each qPCR does not span the entire cassette (Figure 2.5) (for more details on the molecular aspects of this work see section 2.3.2). For the purposes of this work, the integration of an intact cassette was assumed when qPCR for the three genes of interest was confirmed positive. However, further assays such as Southern blots or sequencing should be performed in order to confirm without unambiguously the integration of the entire intact cassette. Detection of mRNA transcribed from the genes of interest in transgenic events is further evidence that intact cassettes were transferred to the genome.

Twenty-six events were confirmed transgenic for the three genes of interest when co-transformed with vectors pCLV000032 and pCLV000081, and 14 events were validated transgenic for the three genes of interest when transformed with vectors pCLV000032 and pCLV000080. Plant transformation frequencies, calculated as number of independent transgenic plants produced per one hundred inoculated cotyledons are presented in Table 2.2. Only transgenic plants positive for the three genes of interest were

included in further molecular and phenotype characterization. Of a total of 40 transgenic events generated, 30 had their respective non-transgenic isogenic controls, and were used for further evaluations.

Table 2.2. Summary of results for plant transformations and qPCR screening for integration of the genes of interest and the selectable marker gene (*hph*). Transformation frequencies were calculated as number of independent transgenic plants produced per one hundred inoculated cotyledons. For co-transformation frequency calculation, only plants positive for the three genes of interest and *hph* were considered transgenic. GOI: Gene of interest.

Vectors used	No. of explants inoculated	Number of transgenic events (for the three GOI)	*hph* transformation frequency (%)	Co-transformation frequency (%)
pCLV000032 /pCLV000080	6,288	14	1.76	0.22
pCLV000032 /pCLV000081	11,067	26	1.29	0.23

Previous work in white clover showed that selection in 75 mg L^{-1} hygromycin produced 85% lethality of untransformed white clover cotyledonary explants within two weeks (Rossello, 2011). Similarly to Rossello (2011), a lower hygromycin concentration of 50 mg L^{-1} was used for selection in order to allow survival of plants lacking the selectable marker. A high proportion of plants which did not carry the selectable marker gene *hph* survived antibiotic selection. From 231 putative events transplanted to soil, 143 carried the selectable marker, while 88 did not. Two events transformed with vectors pCLV000032 and pCLV000081 were confirmed positive for the three genes of interest and lacked the selectable marker gene. The transformation frequency of selectable marker gene free events was estimated at 0.018% for the pCLV000032/pCLV000081 vector combination, with no marker gene free events identified for the pCLV000032/pCLV000080 vector combination.

Transformation frequencies for the selectable marker gene *hph*, estimated as the number of positive transformants for gene of interest divided by the total number of explants inoculated, were determined at 1.76% and 1.29% for pCLV000081 and pCLV000080 vectors respectively. Co-transformation frequencies, estimated as the number of positive transformants for the three genes of interest and *hph* divided by the total number of inoculated explants, were determined at 0.22% and 0.23% for pCLV0032/pCLV000081 and pCLV0032/pCLV000080 combinations, respectively (Table 2.2).

Although vectors pCLV000080 and pCLV000081 have different T-DNA sizes, transformation frequencies were similar for the transformation processes using the two vectors combinations (Table 2.2).

Some putative events were qPCR positive for one or two of the genes of interest (Figure 2.3). The co-transformation frequency calculation is estimated at 0.41 if these events were included in the estimation. No information on the integrity and functionality of the inserted genes was generated, and these events were not included in further molecular and phenotyping experiments.

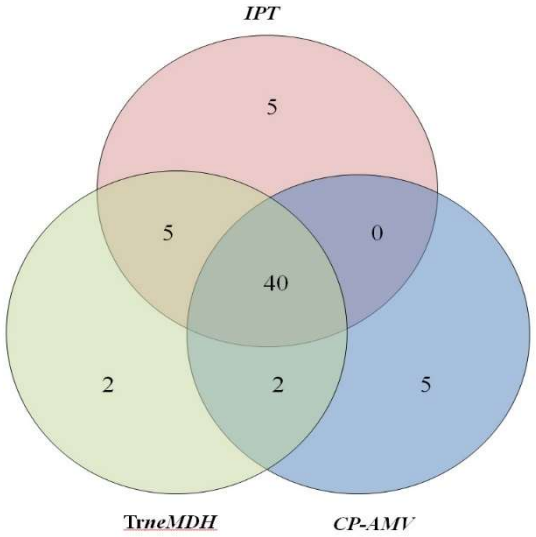


Figure 2.3. Venn diagram summarizing numbers of events with one, two or three genes of interest.

Ten explants exhibited GFP signal from 24 explants analyzed following the last round of selection. From 10 exhibiting a GFP signal, only four were transferred to soil and confirmed as transgenic (identified as PCR-positive for *hph* sequence). Leaves from transgenic events qPCR positive for the three genes of interest and *hph* exhibited GFP signal; no signal was observed for a selectable marker gene free transgenic event (Figure 2.4).

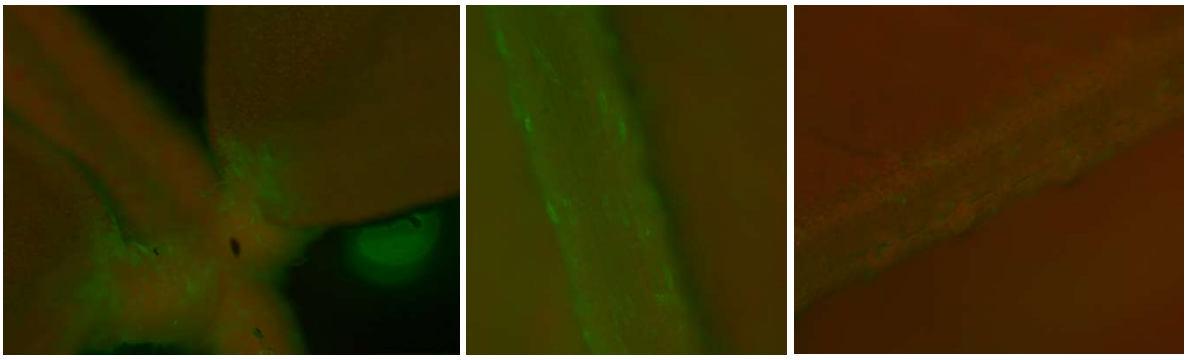


Figure 2.4. GFP signal of leaves from transgenic events. (a) and (b) two different transgenic events positive for the three genes of interest and selectable marker gene *hph*; (c) transgenic event carrying the three genes of interest and selectable marker gene free without GFP signal.

2.3.2 qPCR screening for genes of interest presence/ absence

All plants transferred to the glasshouse were tested for presence of the three genes of interest and the selectable marker gene by SYBR green-based assays. Primer pairs were designed to amplify a section of the coding sequences of the two genes of interest *IPT* and CP-*AMV*. Selectable marker gene integration was tested by using primers targeted to the *hph* coding sequence. In the case of Tr*neMDH*, primers flanked the promoter-gene junction in order to avoid amplification of the Tr*neMDH* gene present in the white clover genome (Figure 2.5). The gene *Translation initiation factor-related* (Id: GU443963.1, BAC 88b23) (Hand et al., 2010) was used as an endogenous reference serving as a positive control for the reactions.

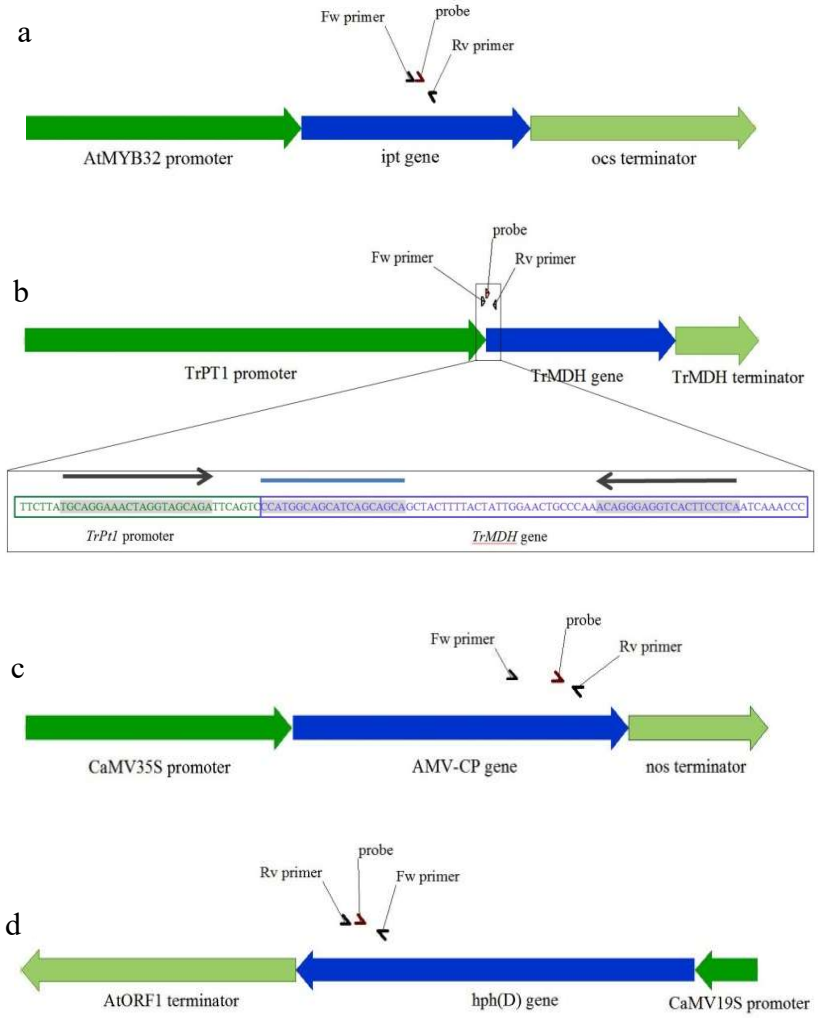


Figure 2.5. Representation of qPCR designed for detection of insertion of genes of interest and the selectable marker gene. (a) *ipt* gene (b) Tr*neMDH* gene (Tr*neMDH* primers were targeted to the promoter-gene junction) (c) CP-*AMV* (d) selectable marker gene (*hph*). *ocs terminator: Octopine synthase terminator. nos terminator: Nopaline synthase terminator. AtORF1 terminator: Agrobacterium tumefaciens* open reading frame 1 3′ untranslated region.

Relative Fluorescent Units (RFU) after each amplification cycle, from either SYBR green dye or carboxyfluorescein (FAM)-labelled probes were measured in the qPCR assay. Samples with quantification cycle (Cq) < 30 were assigned as transgene positive, and with Cq ≥ 30 as transgene negative (Figure 2.6). The Cq values are determined as the cycle number where RFU signal crosses an arbitrary threshold (Schmittgen and Livak, 2008). Plants whose qPCR amplification was positive for at

least one of the genes of interest were re-sampled and re-assessed by qPCR using Taqman probes in order to confirm presence of integrated genes.

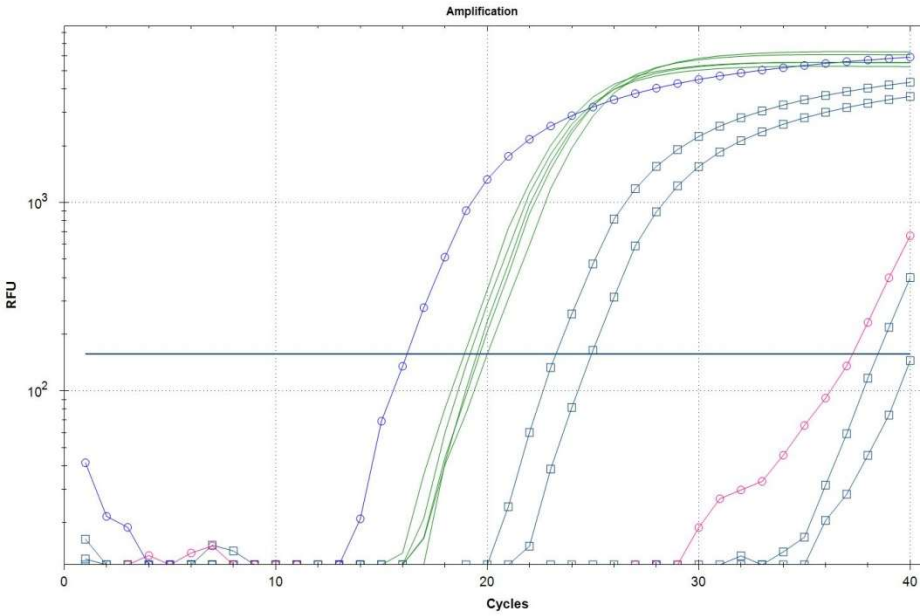


Figure 2.6. Example of outcome of qPCR screening for insertion of genes of interest. Tr*neMDH* screening is presented in this figure. qPCR amplification curves of positive samples and negative samples (blue curves with squares); non-transgenic negative control (pink curve with circles); positive control, vector pCLV000032 (blue curve with circles); internal reference gene control (green curves). Samples whose Cq were under 30 were considered positive or transgenic, and samples whose Cq ≥ 30 were determined as negative or non-transgenic.

2.4 Discussion

Three genes linked in a single T-DNA were successfully transferred by Agrobacterium-mediated transformation. This result supports the feasibility of using linked co-transformation to transfer multiple genes to the white clover genome. As discussed in Chapter 1, the use of linked co-transformation allows the circumvention of some of the constraints associated with methods such as re-transformation or unlinked co-transformation (Farre et al., 2014). Linked co-transformation also enables the insertion of genes of interest at the same locus. Thus, these genes will segregate together in subsequent generations. On the other hand, un-linked co-transformation and re-transformation methods typically generate insertion of genes of interest at different loci. As a consequence, segregation of inserted genes in the

following generations can occur. Additionally, linked co-transformation allows transfer of the genes of interest in one generation, which saves time and resources.

The absence of the selectable marker gene (*hph*) was confirmed in two events. Thus, the use of multiple plasmid co-transformation or unlinked co-transformation was shown to be an effective strategy for production of selectable marker gene free events in this project. Although the number of selectable marker free events was low, crossings would also enable the removal of the selectable marker gene in events with selectable marker by recombination.

The production of selectable marker gene free events is a result of the high percentage of escapes (38% of the putative transgenic events transferred to soil) as a consequence of selection in relatively low hygromycin concentrations (50 mg L^{-1}). A balance between escapes and a high selectable marker gene transformation frequency is necessary in order to obtain enough transgenic events for later phenotypic evaluations, and allow for the generation of marker free events in T_0 generation. Transformation frequencies for *hph* were 1.76% and 1.29% for pCLV000032/pCLV000080 and pCLV000032/pCLV000081 vector combinations respectively. These values were in the 1.30-2.49 range reported by Vala (2012) and Rossello (2011). The results support the effectiveness of *hph* as a selectable marker in the experimental conditions of this work.

This work is the first where the elite cultivar *Storm* was used for white clover transformations by the Agriculture Victoria Research group. The *hph* transformation frequency values fell inside the rank observed in previous work with cultivars *Grassland sustain* (1.30-2.49) (Rossello, 2011; Vala, 2012), and *Haifa*, *Huia*, *Irrigation* and *Mink* (0.3-6%) (Ding et al., 2003; Ashtiani, 2015). All these transformations were performed using the transformation protocol described by Ding et al. (2003). The results support the suitability of using cultivar Storm for Agrobacterium-mediated transformation.

Some limitations of transferring multiple genes by linked co-transformation were observed in this work. As previously reported by Hamilton et al. (1996) the transfer of large T-DNAs occurs at low frequency. In this work, co-transformation frequencies were lower than in previous reports by Vala (2012) and Rossello (2011). Rossello co-transformed the cultivar *Grassland Sustain* with genes Tr*neMDH* and selectable marker gene *hph* using two different vectors and reported a co-transformation frequency of 1.1. Vala co-transformed cultivar *Grassland Sustain* with either *TrPt1*:Tr*neMDH*::At*myb32*:*ipt* or At*myb32*:*ipt* and the selectable marker gene *hph*, and reported a co-transformation frequency of 0.54%

and 1.59% for the two different vector combinations (Figure 2.7). In both cases Agrobacterium strain AGL and the cultivar Sustain were used. Although elements such as Agrobacterium strain, plant cultivar and operator variation are potentially confounding, comparison between these studies can bring an approximation to the effects of T-DNA size on transformation frequencies in white clover. A negative correlation between the total length of transferred T-DNAs and co-transformation frequency is observed when these works are compared (Figure 2.7). Transformation frequencies and length of the total T-DNA integrated approximates to an exponential relationship, described by the equation $y=ab^x$ ($r^2=0.97$).

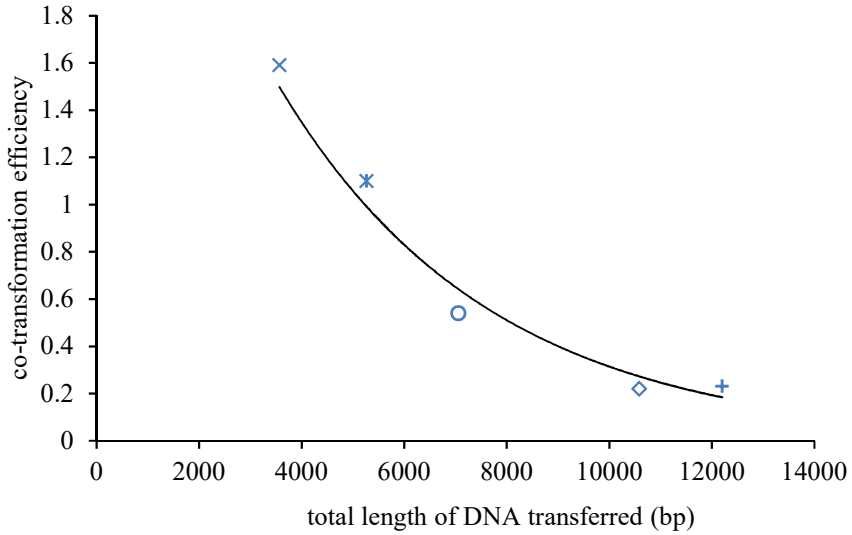


Figure 2.7. Co-transformation efficiencies presented as a function of total T-DNA transferred, calculated as the sum of sizes of the two T-DNAs transferred. (x) At*myb32:ipt* + *35S:hph* (*cv*. Sustain) (Vala, 2012); Each symbol represent a different experiment: (*) Tr*PT1*:Tr*neMDH* + *35S:hph* (*cv*. Sustain) (Rossello, 2011); (o) Tr*Pt1:*Tr*neMDH::*At*myb32:ipt* + *35S:hph* (*cv*. Sustain) (Vala, 2012); (◊) At*myb32:ipt::*Tr*Pt1:*Tr*neMDH::35S:*CP-*AMV* + *19S:hph::35S:GFP* (*cv*. Storm) (Current project); (+) At*myb32:ipt::*Tr*Pt1:*Tr*neMDH::35S:*CP-*AMV* + *19S:hph* (*cv*. Storm) (Current project).

T-DNA size needs to be considered at the time of selecting the most adequate transformation strategy. The results presented here can provide a guide to a T-DNA size limit where alternatives to linked co-transformation need to be considered in white clover. If total T-DNA transferred was 16,000 bp, transformation frequencies are estimated at 0.07 according to the function $y=ab^x$. If T-DNA size over 16000 kb are to be transferred to white clover, the use of strategies such as combining linked co-transformation and re-transformation, or linked co-transformation and sexual crossings may need to be deployed.

A total of 19 transgenic events positive for one or two of the genes of interest were identified by qPCR. In these cases, only a section of the whole T-DNA was integrated. This can be explained by an extensively documented phenomenon of T-DNA breakages or deletions following delivery (Tzfira et al. 2004; Hamilton et al. 1996). The absence of positive events for only the Tr*neMDH* gene, located between *IPT* and CP-*AMV* genes on the construct T-DNA, supports this hypothesis. Generation of events carrying only the Tr*neMDH* gene would only happen if two breaks in the T-DNA flanking the gene take place.

A *GFP* marker was useful for preliminary screening of transformed explants. However, not all of the explants examined after selection exhibited a GFP signal. This can be explained as escapes or low expression of the reporter gene at early stages of tissue culture. Nevertheless, GFP signal was detected in events growing in soil. Given that GFP expression is not always detectable, and that screening of a high number of plants for GFP can be laborious, the use of this reporter gene is not the best strategy for selection. However, it can be a useful tool for an early tracking of the transformation process.

3 Molecular characterisation of transgenic events

3.1 Introduction

After generation of putative transgenic white clover events, molecular characterisation was carried out. Screening for presence of the three genes of interest, and the selectable marker *hph*, was carried out as described in the previous chapter. Further molecular characterization, described in this chapter, involved confirmation of isogenicity of transgenic events and the non-transgenic controls, copy number estimation, and determination of transcript abundance of the three genes of interest, each of which is described in this chapter.

3.1.1 Confirmation of isogenicity of transgenic events and respective non-transgenic controls

The production of non-transgenic isogenic controls is crucial for use as references in molecular or phenotyping characterisation of the transgenic events. Given that white clover is an outbreeding highly self-incompatible species, non-transgenic isogenic controls identical to the transgenic plant were generated by 'isogenic transformation' (Ding et al., 2003). Once transgenic plants were identified by qPCR, confirmation of isogenicity of these transgenic events and their respective non-transgenic isogenic controls was required. This task was performed by using Simple Sequence Repeat (SSR) markers.

3.1.2 Copy number estimation of the genes of interest

Copy number estimations for the genes of interest of the generated events was also performed and is described in this section. A high number of T-DNA insertions can bring difficulties in further generations, as segregation of the inserted genes occurs, and the required transcription levels and desired phenotypes could be lost (Meyer 1998). Therefore, events with low transgene copy number are typically a priority for selection of transgenic plants for the following steps of breeding. Furthermore, information on the number of transgene insertions is a requirement of regulatory authorities for any commercial release of genetically modified crops (EFSA, 2011).

Transgene copy number of genetically modified crops has been traditionally determined using a Southern blot (Southern, 1975). However, the technique can be laborious and time consuming (Wang et al., 2014; Głowacka et al., 2016). More recently, the use of qPCR has been proposed as an alternative

(Ingham and Money, 2001; Wang et al., 2014). When compared to qPCR, Southern blots are considered more reliable as they generate more unequivocal results (Głowacka et al., 2016). On the other hand, qPCR enables high throughput copy number determination in a shorter timeframe, and much lower DNA quantities are required in comparison with Southern blots (Ingham and Money, 2001; Bubner and Baldwin, 2004; Wang et al., 2014).

In addition to copy number, determination of zygosity of the transgene is also important. Identification of homozygous plants in white clover is required in the T_2 generation if introgression of the transgene in a different elite cultivar is required (Spangenberg et al., 2001). The zygosity of the transgene can be determined by a test cross. This involves crossing the transgenic plants produced in T_2 with non-transgenic plants and analysing the segregation ratio of the transgene by Southern blot or qPCR (Spangenberg et al., 2001). This method can be laborious and time consuming, as an extra generation is needed to determine zygosity (Bubner and Baldwin, 2004). More recently, the use of qPCR to determine zygosity in transgenic plants has been implemented (Ingham and Money, 2001; Bubner and Baldwin, 2004; Wang et al., 2014). This method is faster and less labour demanding than a test cross. However, given that this technique depends on a logarithmic template quantification, the accuracy for identifying small copy number differences, such as differentiating a homozygous from a hemizygous transgenic plant, can be limited (Bubner and Baldwin, 2004).

3.1.3 Use of droplet digital PCR (ddPCR) for copy number estimation

Droplet digital PCR (ddPCR) is a new technology (Hindson et al., 2011) that allows highly accurate absolute DNA quantification. Similarly to qPCR, the method relies on a fluorescent dye or probe. However, ddPCR is based on partition of template DNA at a given dilution in multiple small reactions contained in oil droplets. At the appropriate dilution, there will be reactions containing one or more template DNA molecules of the target of interest and others without template of the target of interest (Figure 3.1). Thus, end-point reactions containing one or more template will amplify (positive) and reactions without template will not amplify (negative). Using this information the number of template DNA molecules of the target of interest can be estimated using a Poisson probability distribution (Hindson et al., 2011).

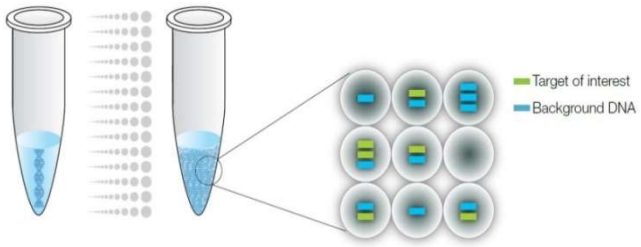


Figure 3.1. Illustration of the fundamentals of droplet digital PCR (ddPCR) technique. ddPCR is based on partition of template DNA at a given dilution in multiple small reactions contained in oil droplets.

Droplet digital PCR was reported to be a more accurate technique than qPCR for transgene copy number estimation in transgenic tobacco (Głowacka et al., 2016). Also, it was reported to be much faster and to have similar levels of reliability to a Southern blot (Głowacka et al., 2016). In this work we propose to use qPCR and ddPCR as an alternative to Southern blots for copy number estimation in white clover.

Given that the copy number estimation using these techniques are performed by relative quantification, the use of a gene whose copy number remains constant among samples to use as a reference is required. Ideally, this reference gene should be single copy and the number of copies should remain constant among genotypes of the studied species (Wang et al., 2014). Although there are a number of single copy genes identified for use as a reference in some species (Ingham and Money, 2001; Wang et al., 2014; Głowacka et al., 2016), there are no detailed reports on searches for reference genes for copy number estimations in white clover. Work on identification of a single copy gene to use as a reference in white clover for copy number estimation is described in this chapter.

3.1.4 Transcript abundance by using RT-ddPCR

Reverse transcription droplet digital PCR (RT-ddPCR) can be a powerful technique for transcript abundance measurements. This technique is considered to have a higher sensitivity and precision for transcript abundance determination than RT-qPCR (Baker, 2012). Here, RT-ddPCR was implemented for transcript abundance estimation in white clover as an alternative to the widely used RT-qPCR. Transcript abundance was evaluated for the three genes of interest using this technique.

The presence of transcripts from the genes of interest are proof of the intactness of the inserted cassettes. Additionally, transcription levels of the genes can be a relevant factor for selection of the events with

the desired phenotype. For example, only events whose Tr*neMDH* transcript abundance is higher than their respective non-transgenic isogenic controls are planned to be selected for Al^{3+} phenotyping, as they are considered more likely to produce more malate, and thus they have more chance of exhibiting increased tolerance to Al^{3+}.

Although phenotyping for AMV resistance was outside the scope of this work, a basic molecular characterisation was performed. This comprised gene presence at the genome level (described in Chapter 2), and copy number and transcript abundance estimations. Given that white clover plants carrying the CP-*AMV* gene exhibited resistance to AMV (Panter et al., 2012), we proposed that confirmation of transcript presence and transcript abundance estimation would represent a first step to further confirmation of the desired phenotype.

3.2 Materials and methods

3.2.1 Plant material, sampling and DNA extractions

Plant tissue was sampled from newly developed leaves from plants growing in the glasshouse under 22°C day/16°C night, 16 h light/8 h dark. High throughput DNA extraction was performed using a DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

3.2.2 Confirmation of isogenicity of transgenic events and respective non-transgenic isogenic controls

PCR was performed using GoTaq Green Master Mix (Promega, USA). Amplification reactions were carried out in a 25 µL reaction volume containing GoTaq Green Master Mix (2) 12.5 µL, 380 nM of each primer, and 10-50 ng template DNA. Reaction conditions were as follows according to George et al. (2006): one step of denaturation at 94°C for 10 min followed by 30 cycles at 94°C for one minute, 55°C for 30 s and 72°C for one minute, with 0.3°C decrements per cycle until 45°C, and a final extension at 72°C for 10 min. Primer sequences were obtained from Jones et al. (2003) and George et al. (2006).

SSR products were precipitated with 0.1 volume of 7.5 M ammonium acetate and 2.75 volumes of 100% ethanol followed by centrifugation at 3000g for 30 min. The supernatant was removed and the pellet was washed with 70% (v/v) ethanol before air drying for 2–3 min and resuspension in sterile distilled water, followed by storage at –20°C (George et al., 2006). PCR products were combined with the ABI GeneScan LIZ500 size standard and analysed using a capillary electrophoresis platform ABI3730xl

(LifeTechnologies Australia Pty Ltd, Victoria, Australia) according to the manufacturer's instructions. Allele sizes were scored using GeneMapper®3.7 software package (LifeTechnologies Australia Pty Ltd, Victoria, Australia).

Table 3.1. SSR markers used for isogenicity confirmation of transgenic and control plants.

Name	Forward	Reverse	Size (bp)		Modification (5' Forward primer)
TRSSRB02D12	CTGTAATTCAGGAAGAAAGCAAGG	ATAAGCATTTGTAGTAATCCACT	325	301	FAM
TRSSRA04B06	ACCTTTCTTCTCATTGCGTTTC	TCTAGAATTTCTCGTTTTCATC	152	149	FAM
TRSSRB01A07	CCTACGATTCGTCTATAATTTGTT	ACTCAAGTTATGCTCATTTGGTTC	254	236	FAM
TRSSRA02G08	AGAAAAGAAGAACACCCAGA	ACTTTAAGGACATGTTTGGC	177	175	FAM
TRSSRA02H09	CCACAACTACAAGTAGGTTT	CGTGAATGGTGTTCTATTCT	150	238	HEX
TRSSRA01C10	GTACCTGGAAATGTTGATT	GAGCAGCCATGACCTCTG	207	311	HEX
TRSSRA04F01	TCCTTCGCCAGTCGTTTCAA	CGATCGCTATCCTATGCTGTT	158	272	HEX
TRSSRA01H11	AGAAAGGTGAATGATGAAA	TCTAATTCTTCCAATAGGG	146	258	HEX

3.2.3 Copy number determination of genes of interest

Copy number of the insert was estimated by ddPCR and qPCR. Duplex PCR were performed including an internal reference gene in both qPCR and ddPCR. Single copy gene *Pyruvate dehydrogenase* (*PDH*) and *ATP-dependent protease La (LON) domain protein* (*ATP-dependent protease*) were used as the internal references. Primers and probes sequences used are presented in Table 3.3 and Table 3.4. For qPCR, reactions were performed using Qiagen QuantiTect Probe PCR kit (Qiagen, Valencia, CA, USA) in 20 µL of final volume, 600 nM of forward and reverse primers, 200 nM of probes and 1x of master mix. Reaction conditions were the following: 10 min at 95°C, and 40 cycles of 10 s at 95°C, 30 s at 60°C, and 10 s at 72°C.

Reaction mixtures were prepared for ddPCR in 24 µL final volume, 600 nM of forward and reverse primers, 200 nM of probes, 12 µL of ddPCR 2x Master Mix (Bio-Rad, Hercules, CA, USA) and 20-50 ng of DNA per reaction mixture. Emulsified 1 nL reaction droplets were generated using a droplet generator AutoDG™ (Bio-Rad, Hercules, CA, USA) and a DG8 cartridge (Bio-Rad, Hercules, CA, USA) containing 20 µL of reaction mixture and 70 µL of ddPCR droplet generation oil (Bio-Rad, Hercules, CA, USA) per well. The droplet emulsions generated were transferred to 96-well PCR plates and PCR performed for 10 min at 95°C, and 40 cycles of 30 s at 95°C, 1 min at 60°C with 0.2°C

increments per cycle until 60°C, and 30 s at 72°C in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The fluorescence of each thermal cycled droplet was measured using a QX100 droplet reader (Bio-Rad, Hercules, CA, USA).

3.2.4 Transcript abundance analysis

Plant tissue was sampled from newly developed leaves from plants growing in the glasshouse under 22°C day/16°C night,16 h light/8 h dark. Samples were flash frozen and stored at -80°C until RNA extraction. RNA extraction was performed using RNeasy kit (Qiagen, Valencia, CA, USA). RNA quantity was determined by NanoDrop N-D 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity estimated by 2% agarose gel electrophoresis by monitoring 25S/18S ribosomal RNA integrity. RNA was treated with DNase I (Sigma) to remove traces of DNA before cDNA synthesis. cDNA was synthesized with iScript cDNA synthesis kit for RT-ddPCR (Bio-rad, Hercules, CA, USA). Dnase I treated No RT controls were included in the assays in order to check for absence of amplification of genomic DNA.

For each gene of interest, a duplex PCR was performed using a pair of primers and a FAM-labelled probe directed to the target gene and another pair of primers and a HEX-labelled probe directed to the endogenous reference gene *Elongation Factor 1-alpha* (*EF1α*) (Table 3.6). Reaction mixtures were prepared for ddPCR for each gene of interest, in 24 µL final volume, 600 nM of forward and reverse primers, 200 nM of probes, 12 µL of ddPCR 2x Master Mix (Bio-Rad, Hercules, CA, USA) and 3 µL of cDNA per reaction mixture. cDNA was previously diluted 1:20 for CP-*AMV* and Tr*neMDH* assays, and undiluted cDNA was used for *IPT* assay. Emulsified reaction droplets were generated, PCR performed and thermal cycled droplets measured using the same conditions as used for copy number assays.

Three to six biological replicates were used for this assay, and samples were obtained from complete randomized block designs for each gene of interest assay. Statistical significance for Tr*neMDH* transcript abundance when comparing transgenics to non-transgenic lines was evaluated for gene Tr*neMDH* by one-tailed and two-tailed *t* tests assuming equal variances by GenStat for Windows 14th Edition, VSN International, Hemel Hempstead, UK.

3.3 Results

3.3.1 Confirmation of isogenicity of transgenic pairs

Eight different molecular markers were used for confirmation of isogenicity of transgenic and their respective non-transgenic isogenic controls. Markers were selected based on their polymorphic nature, previously reported by Jones et al., 2003 and George et al., 2006. Identical genetic background of transformed and untransformed cotyledons was confirmed for each transgenic event generated by Simple Sequence Repeat (SSR) molecular markers (Figure 3.2). Additionally, characteristics (leaves size, leaves and stolon colour) of isogenic pairs plants were inspected visually, and similar morphology was observed.

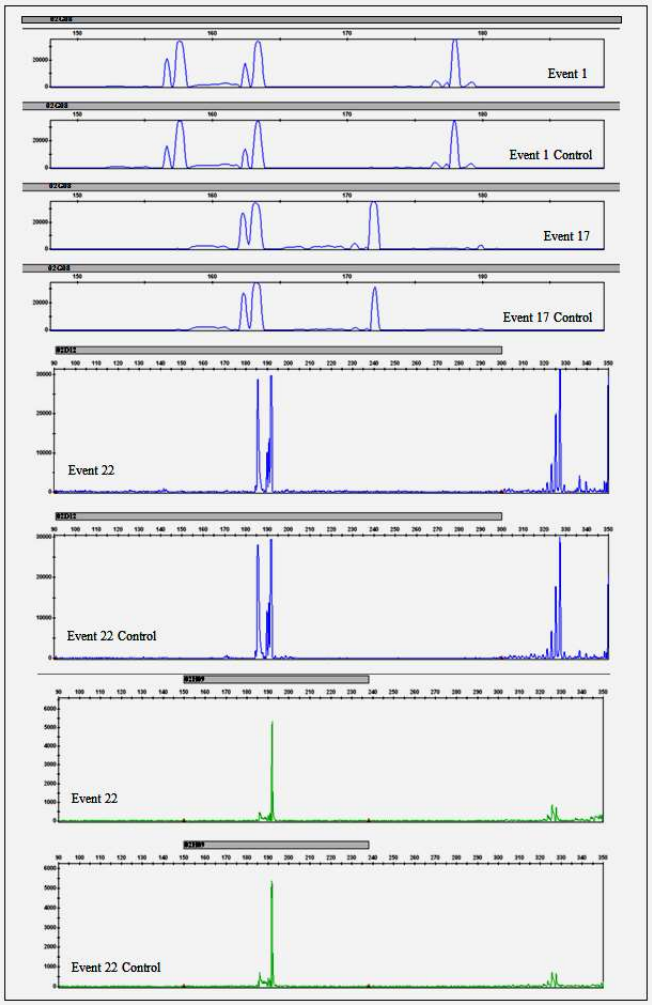


Figure 3.2. Confirmation of isogenicity of transgenic/non-transgenic pairs by SSR markers. Chromatograms of events 1, and 17 and their respective non-transgenic isogenic controls (marker TRSSRA02G08), and event 22 and its respective isogenic non-transgenic control (marker TRSSRB02D12 in blue, and marker TRSSRA02H09, in green). *x* axes represent product sizes (bp) and *y* axes represent fluorescence signal amplitude.

3.3.2 Copy number estimation

Copy number of the three genes of interest was estimated for the generated transgenic events by relative quantification using qPCR and ddPCR. The use of ddPCR for copy number assays was reported in tobacco (Głowacka et al., 2016), and developed in wheat and canola by our group (Kay et al. unpublished). However, no protocols were developed for other crops, such as relevant forage crops, including white clover. Given the convenience of using an accurate and high throughput method for copy number estimation, a ddPCR assay in white clover for the three genes of interest was implemented.

In order to perform this task, identification of an endogenous gene to use as a single copy reference was required. Reference genes for copy number estimations have to comply with some requirements. Ideally, a reference gene for copy number studies should be present in single or low copy number, and remain constant in the evaluated species (Baeumler et al., 2006; Wang et al., 2014). To our knowledge, no work has previously been reported on searches for reference genes for copy number estimation in white clover. Furthermore, given that available genomic data on white clover is limited (Hand et al 2010), little information on candidate genes for this purpose is available. In order to find a reference gene whose copy number remains constant among different white clover genotypes, a search for single copy genes was carried out. A BLASTN search on the NCBI white clover EST database was performed to find the putative orthologues of single copy genes reported in Arabidopsis (Table 3.2) (Smet et al., 2013).

Table 3.2. List of single copy candidate reference genes. BLASTN search on the NCBI white clover EST database was performed to find the putative orthologues of single copy genes reported in Arabidopsis.

White clover EST accession no.	Arabidopsis gene	Arabidopsis gene description	BLAST N score E value	Sequence identity (%)
FY466505.1	AT1G01090	*Pyruvate dehydrogenase E1 alpha subunit*	2e-81	78%
FY463177.1	AT1G03030.1	*P-loop containing nucleoside triphosphate hydrolases superfamily protein*	3e-59	76%
FY455018.1	AT1G20575.1	*Dolichol phosphate mannase synthase*	2e-88	78%
FY466817.1	AT1G30300.1	*Metallo-hydrolase/oxidoreductase superfamily protein*	4e-73	73%
FY462563.1	AT1G61520.3	*Photosystem I light harvesting complex gene 3*	2e-159	81%
FY464051.1	AT1G75460	*ATP-dependent protease La (LON) domain protein*	2e-77	78%
FY468581.1	AT3G11830	*Cpn60 chaperonin family protein*	2e-136	80%
FY465631.1	AT3G45300	*Isovaleryl-coa-dehydrogenase*	1e-168	82%
FY458968.1	AT5G06360	*Ribosomal protein S8e family protein*	7e-124	81%

Table 3.3. Primers and probes directed to candidate reference genes evaluated for copy number estimations. All probes were labelled with HEX.

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe (5'-3')	Product size (bp)
Chaperonin	AACTGTAGTTGCCGGTGGAG	TGTCACATAATTGGCGAGGA	CCTAAGGCAACATGCACGCACA	146
Pyruvate dehydrogenase subunit	CAATGTGCTTGGTGGTTTTG	CAAGTTCCGTCTCCGAAAAA	TCCAGTGGCAACCGGTGCAG	144
P-Loop containing nucleoside	AAAACTCGCTGCATTGCTTT	GTTGGGGATCCAGTTGAAGA	TCCAGACCCCATCTTCCAGGAGC	170
Nucleotide diphosfo-sugar transferase	GCGCATAAGATTCCATCCAT	TGATTTATCGCACCATCCAA	CACACCACCGCCTCTAACATAACG	127
LHCA3 photosystem I	GGCAACTGCTACTCCTCCTG	AGGCTGCCATCCAAGTAAGA	GGCCTTTGTGGTTTGCATCAAAGC	93
ATP-dependant protease	CGAACGACTCGTCGATGATA	GCCACACGAGCAACAAGATA	GGTCAAGAGCGTTTTCGTGTCACG	105
Isovaleryl Coa dehydrogenase	CAGGGGAAAGTTGCTGACAT	CACAATCCTTCGGGTCAACT	GCTCGGGACTGTGACAATGGGA	106
Ribosomal protein	ATGTCGGTATCCCAATCCAA	GCTGTTCGCATCATTCTTGA	TTCGCCATTTTTCCCAGACAAACA	82

Nine white clover ESTs with high similarity to single copy genes from Arabidopsis were identified (Figure 3.2). Primers pairs and HEX-labelled probes targeted to the identified sequences were designed (Table 3.3). A plot of Cq versus log cDNA dilution was generated and the efficiency was calculated using the formula E = 10(-1/slope) (Pfaffl, 2001). Genes whose PCR efficiencies were inside the range E = 0.8-1.2 (Schmittgen and Livak, 2008) and exhibited a consistent amplification among all the transgenic events were selected for further analysis. Only *Pyruvate dehydrogenase E1 alpha subunit* (*PDH*) and *ATP-dependent protease La (LON) domain protein* (*ATP-dependent protease*) genes met these requirements and were selected as candidate genes for use as references for copy number estimations.

Quantitative PCR copy number results were estimated by the ΔΔCt method (Schmittgen and Livak, 2008) and ddPCR results by calculating the ratios between positive droplets for the gene of interest vs positive droplets for the reference gene multiplied by four copies of the reference gene in tetraploid white clover (Hindson et al., 2011). Duplex reactions were adjusted for both qPCR and ddPCR assays. Probes targeted for the genes of interest were labelled with FAM, and probes targeted to the reference genes were labelled with HEX. Two different primer/probe combinations for each gene of interest were evaluated based on signal/noise ratios obtained in duplex ddPCR reactions (Figure 3.3 and Figure 3.5). Signal/noise ratios were calculated by dividing the mean amplitude of positive droplets over the mean amplitude of negative droplets. This parameter provides an estimation of the significance of positive reactions versus negative reactions. High signal/noise ratios are an indication of better separation between positive and negative droplets fluorescence signals, which allows a more reliable copy number estimation (Figure 3.4). Low signal/noise ratios, can be a sign of unspecific products generated in the PCR, such as primer dimers. For the purposes of our experiment, reactions whose signal/noise ratios were higher than 1.5 were considered optimal. All primer/probe combinations evaluated exhibited signal/noise ratios higher than 1.5. IPT combination 1, TrneMDH combination 1 and CP-AMV combination 1 exhibited the highest ratios and were selected for use in copy number analysis (Figure 3.5). Also, the primers and probes targeted to the two reference genes exhibited signal/ratios higher than two, which makes these reliable for use as references in copy number estimations (Figure 3.5).

Table 3.4. Primers/probes combinations assessed for copy number estimations. All probes were labelled with FAM.

Combination	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe (5'-3')	Product size (bp)
IPT combination 1	IPT1: TGGAGTGCAGATTTTCGTTG	IPT1: CTGCTTAACTCTGGCCTTGG	IPT: TCGCCACAAGTTACCCGACCAA	90
IPT combination 2	IPT2: TAGGCTGATCGAGGAGGTGT	IPT1: CTGCTTAACTCTGGCCTTGG	IPT: TCGCCACAAGTTACCCGACCAA	190
TrneMDH combination 1	TrMDH1: TGCAGGAAACTAGGTAGCAGA	TrMDH1: TGAGGAAGTGACCTCCCTGT	TrMDH: CCATGGCAGCATCAGCAGCA	95
TrneMDH combination 2	TrMDH3: GCGATCGCTACTATAACCACAA	TrMDH3: GAAGTGACCTCCCTGTTTGG	TrMDH: CCATGGCAGCATCAGCAGCA	151
CP-AMV combination 1	CP-AMV1: TCGGGTTTGAGTTGGTCTTC	CP-AMV1: ACCCAAACTTCGTTGAATCG	CP-AMV: CTTCGATGCGCAGCCTGAGG	151
CP-AMV combination 2	CP-AMV2: GATGACGTGACGACTGAGGA	CP-AMV1: ACCCAAACTTCGTTGAATCG	CP-AMV: CTTCGATGCGCAGCCTGAGG	242

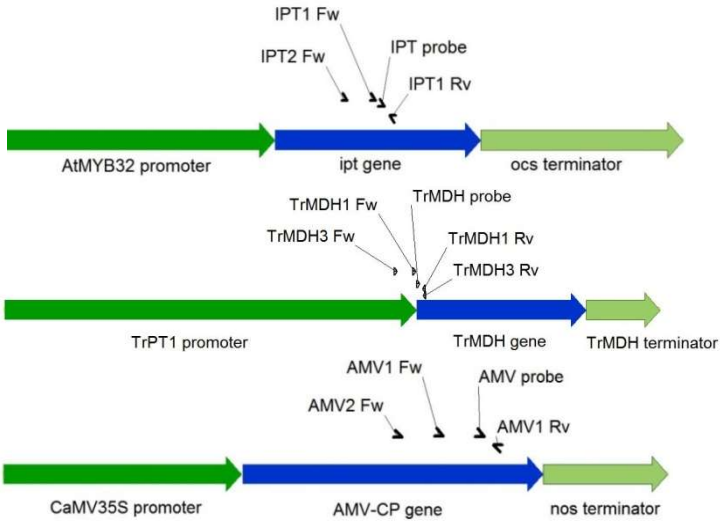


Figure 3.3. Location of primers and probes evaluated for copy number estimations.

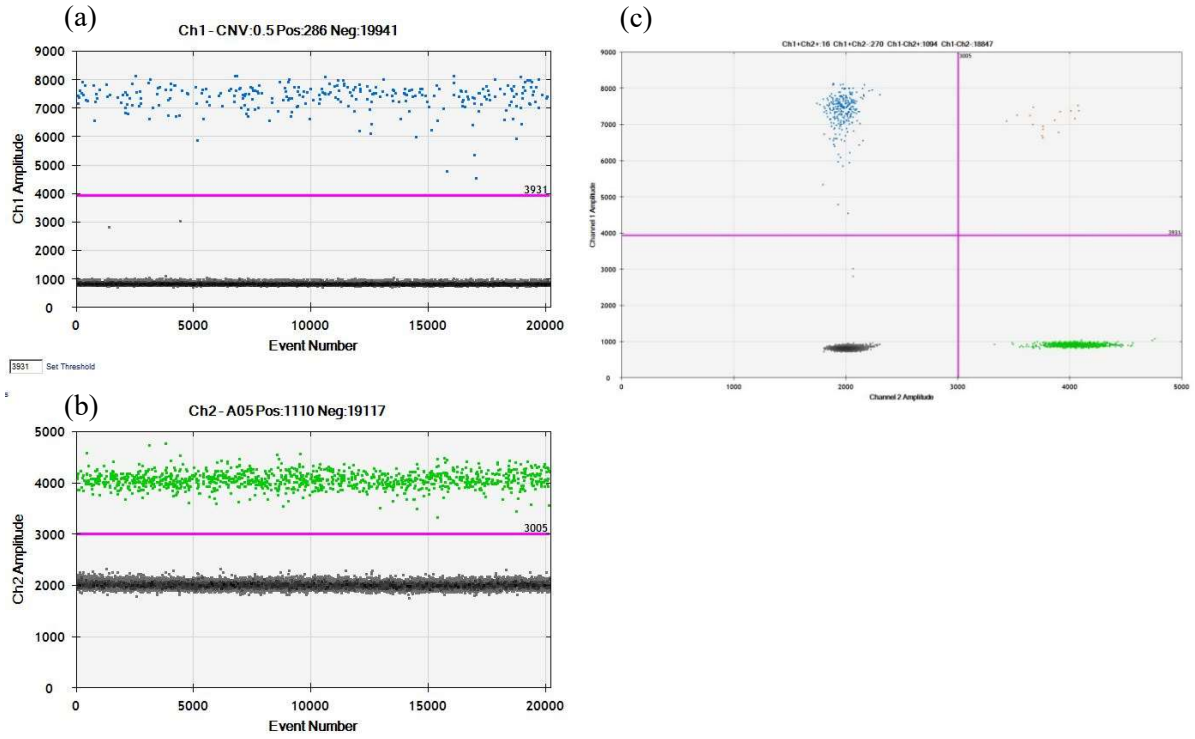


Figure 3.4. Example of an optimal duplex ddPCR. Here ddPCR results for event 1 directed to the target gene Tr*neMDH* and the reference gene *Pyruvate dehydrogenase* (*PDH*) are presented. (a) FAM positive reactions for Tr*neMDH* gene (blue dots) and negative reactions (black dots) are represented. (b) HEX positive reactions for the reference gene *Pyruvate dehydrogenase* (*PDH*) (green dots) and negative reactions (black dots) are represented. (c) Results for channels 1 and 2. Orange dots represent double-positive reactions (positive for both FAM and HEX). Pink lines represent the threshold separating positive from negative reactions.

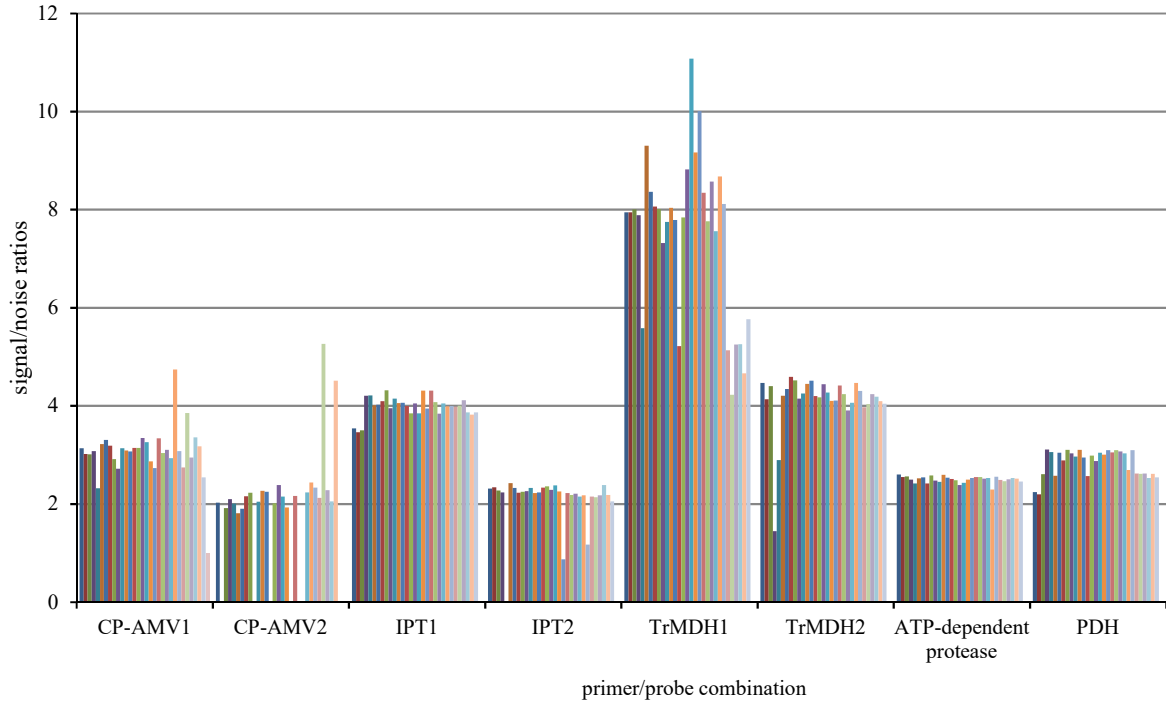


Figure 3.5. Signal/noise ratios of the different primers combinations tested for copy number studies. Signal/noise ratios were calculated by dividing the mean amplitude of positive droplets over the mean amplitude of negative droplets. Each bar represents the different transgenic events.

Copy number for the three genes of interest was estimated (Figure 3.6) and (Table 3.5). Single copy for the three genes of interest was estimated for 13 of the 30 events generated. A single copy number for the three genes of interest in these events suggests a single T-DNA insertion. Copy number estimations for the three genes of interest ranged between one and two for seven events, and the remaining ten events exhibited copies of 3 or higher, with copy numbers higher than 5 for events 8, 11, and 14.

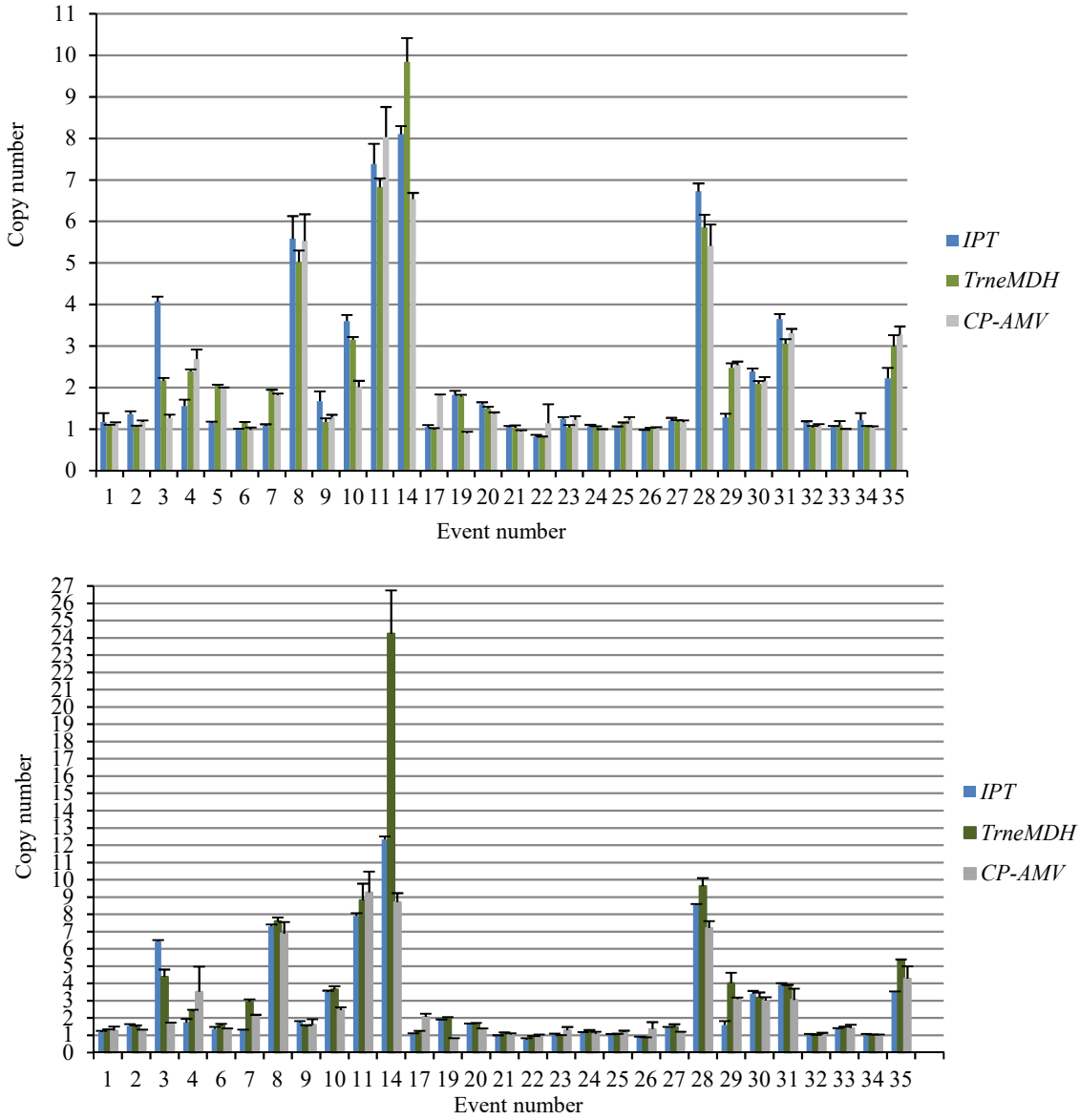


Figure 3.6. Copy number estimation. (a) ddPCR results. Copy number was estimated by calculating the ratios between positive droplets for the gene of interest vs positive droplets for the reference gene multiplied by four copies of the reference gene in tetraploid white clover (b) qPCR results. qPCR copy number results were estimated by the ΔΔCt method (Schmittgen and Livak, 2008). For both ddPCR and qPCR, probes targeted for the genes of interest were labelled with FAM, and probes targeted to the reference genes were labelled with HEX. Reference genes used were *Pyruvate dehydrogenase E1 alpha subunit* (*PDH*) and *ATP-dependent protease La (LON) domain protein* (*ATP-dependent protease*).

Table 3.5. Estimated copy number of the three genes of interest by ddPCR.

Event	*AMV-CP*	*IPT*	Tr*neMDH*	*Estimated inserted T-DNA copies*
1	1.17	1.08	1.14	1
2	1.36	1.07	1.17	1
3	4.07	2.17	1.27	1-4
4	1.55	2.39	2.69	2-3
5	1.15	2.06	1.99	1-2
6	0.99	1.14	0.98	1
7	1.08	1.92	1.82	1-2
8	5.58	5.02	5.53	5-6
9	1.67	1.17	1.28	1-2
10	3.60	3.15	2.01	2-3
11	7.38	6.83	8.03	7-8
14	8.10	9.84	6.54	7-10
17	1.05	0.98	1.81	1-2
19	1.83	1.78	0.90	1-2
20	1.60	1.50	1.37	1-2
21	1.05	1.03	0.95	1
22	0.82	0.81	1.14	1
23	1.24	1.04	1.23	1
24	1.06	1.05	0.99	1
25	1.06	1.14	1.22	1
26	0.96	1.03	1.03	1
27	1.20	1.18	1.16	1
28	6.72	5.85	5.41	5-7
29	1.28	2.47	2.54	1-3
30	2.38	2.09	2.16	2
31	3.65	3.06	3.31	3-4
32	1.17	1.04	1.08	1
33	1.05	1.09	0.99	1
34	1.21	1.05	1.05	1
35	2.22	2.99	3.28	2-3

Similar copy number results were observed when qPCR and ddPCR were compared, and a good correlation was observed between the techniques (Table 3.7). A higher spread was observed for events with high copy number (specially in events showing more than 3 copies). For high copy events, estimated copy number by qPCR were in general higher than that estimated by ddPCR.

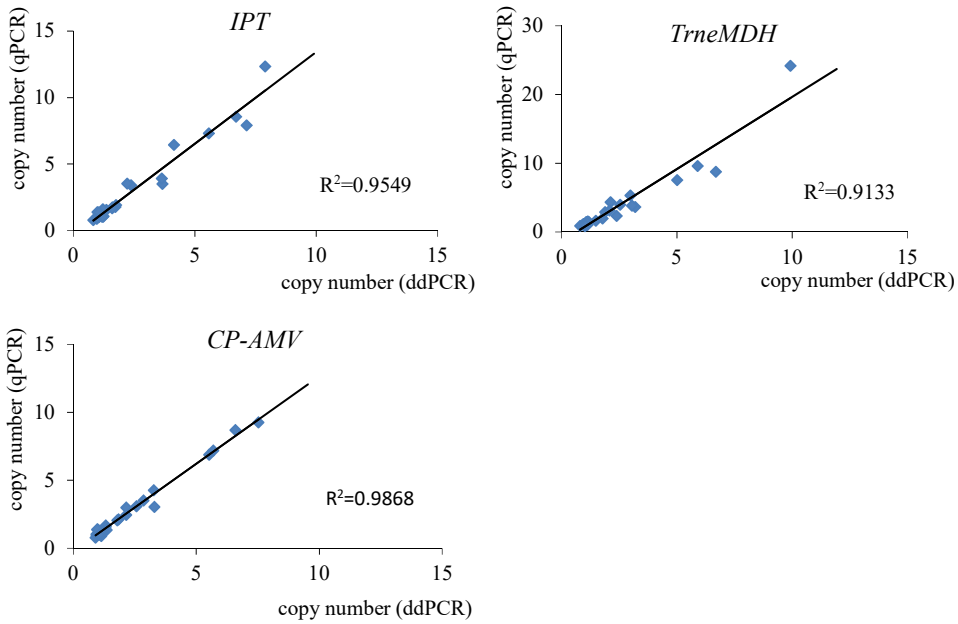


Figure 3.7. Scatter plots of copy number results generated for each transgene by quantitative PCR (qPCR) vs. droplet digital PCR (ddPCR).

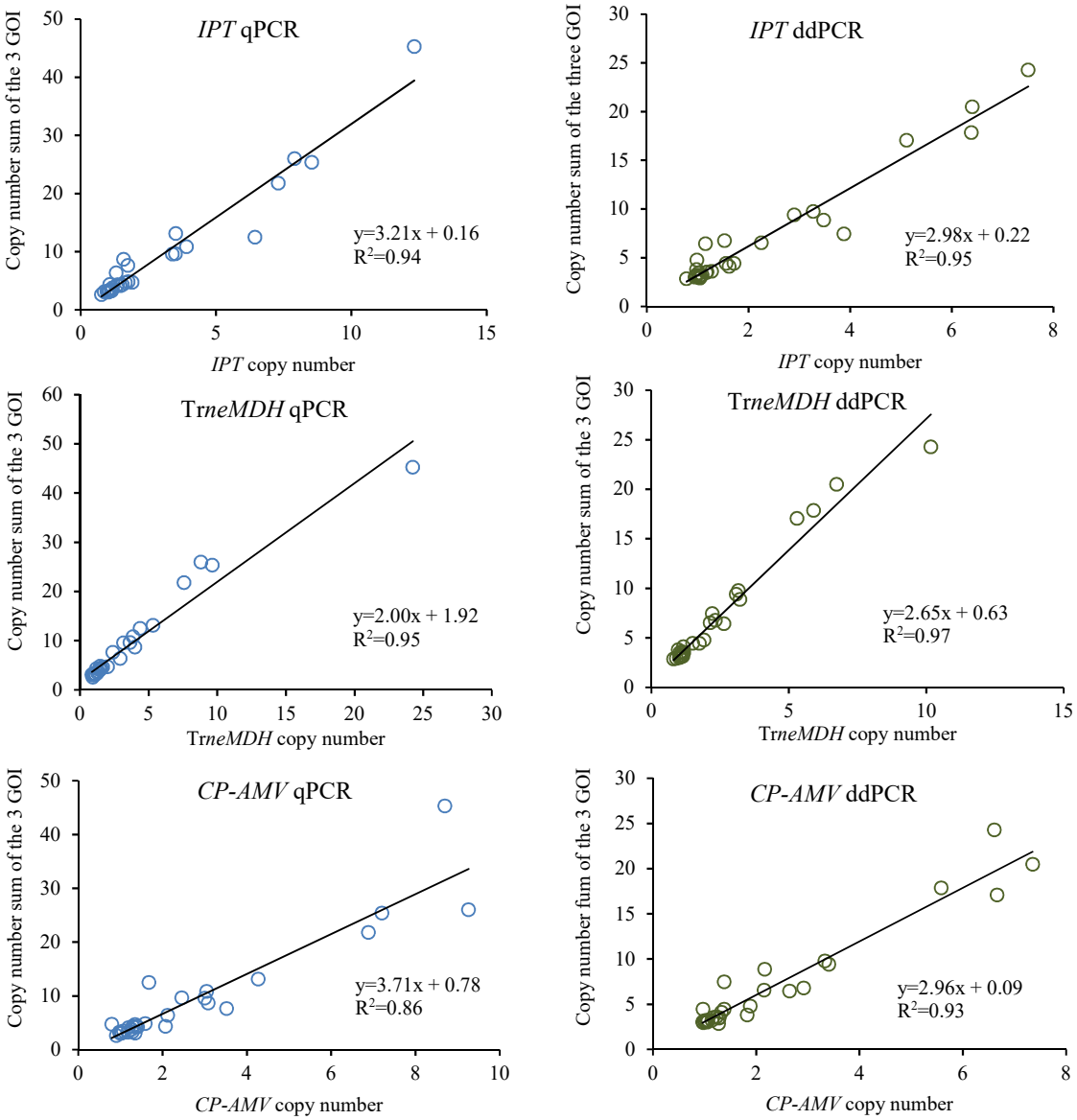


Figure 3.8. Scatter plots of copy numbers of each gene versus the copy number sum of the three genes for each event. Blue circles represent ddPCR results and green circles represent qPCR results.

Given that the three genes of interest are linked in a single T-DNA, we can assume that a perfect copy number measurement in a single event occurs when the number of *IPT*, Tr*neMDH* and CP-*AMV* genes are equal, and the sum of the three genes is a triple. Therefore, the correlation between copies of each gene versus the sum of the three genes for each event should follow a linear relationship, and would enable the evaluation of the accuracy of the techniques used. Furthermore, the slope of the linear equation generated should approximate to 3. When this relationship was evaluated, a stronger correlation was found for ddPCR assays when compared to qPCR assays (Figure 3.8). In addition, the slopes of the

generated equations approximated more accurately to 3 in ddPCR assays that in qPCR assays (Figure 3.8).

3.3.3 Transcript abundance assays

Transcript abundance was measured by RT-ddPCR for the three genes of interest. For genes *IPT* and CP-*AMV* genes, transcript abundance was evaluated in leaves from five events. These events were selected based on the observation of delayed leaf senescence and their potential for drought tolerance. This work is described in Chapter 4. Relative quantification was performed by calculating the ratio of concentration target (target copies/µL)/concentration reference (reference copies/µL) (Heredia et al., 2013). A duplex experiment was designed: a pair of primers and a FAM labelled probe were targeted to the gene of interest and another pair of primers and a HEX labelled probe were targeted to the reference gene (Figure 3.9 and Table 3.6). Previous work was carried out in order to identify a gene with stable transcript abundance among samples, and *Elongation factor 1-α* (*EF1α*) was selected as the reference gene for transcript abundance estimation (work described in Chapter 6).

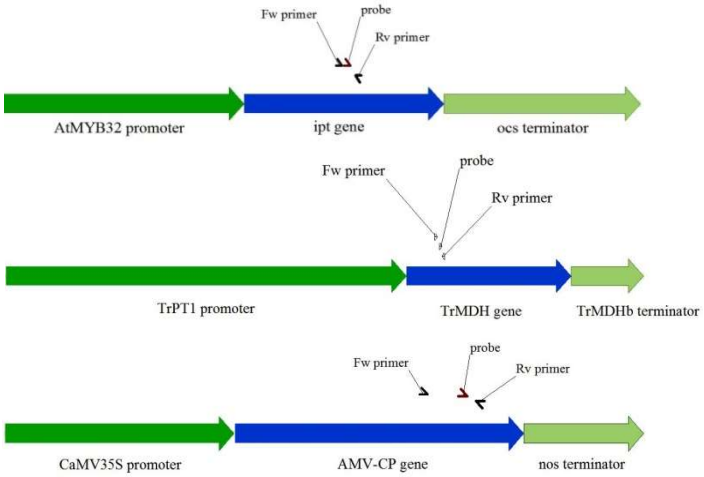


Figure 3.9. Location of primers and probes used for transcript abundance analysis.

Table 3.6. Primers and probes sequences used for transcript abundance analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe (5'-3')	Product size (bp)
IPT	TGGAGTGCAGATTTTCGTTG	CTGCTTAACTCTGGCCTTGG	TCGCCACAAGTTACCCGACCAA	90
Tr*neMDH*	CACCCAAAGCTCAAAAGGAA	AACCGCCACTTTGTAGGATG	CCGCAATTTGCAGCCTCAGG	74
CP-*AMV*	TCGGGTTTGAGTTGGTCTTC	ACCCAAACTTCGTTGAATCG	CTTCGATGCGCAGCCTGAGG	151
EF1α	AGGTCCACCAACCTTGACTG	TGGCTTGTCTGAGGGTCTCT	CAAGGGACCAACCCTCCTTGAAGC	90

IPT transcripts were detected in leaves of 3 of the 5 events evaluated. In those events where *IPT* transcripts was detected, the measured *IPT* transcript abundance was low. Transcript abundance was estimated between 380 and 1200-fold lower than that of the reference gene. No *IPT* transcripts were detected in events 21 and 25. No correlation was observed between copy number and transcript abundance. The highest levels were measured in event 34 (conc. target/ conc. reference = 0.002623) whose T-DNA copy number is one, and the lowest levels were observed for event 11 (conc. target/ conc. reference=0.000847), whose copy number is approximately 7 (Figure 3.10).

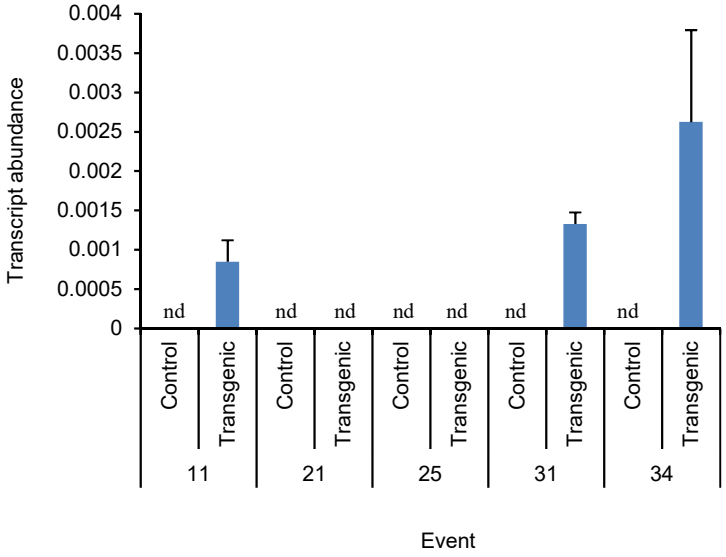


Figure 3.10. *IPT* transcript abundance. Transcript abundance was estimated by relative quantification using droplet digital (ddPCR), by calculating the ratio of concentration target (*IPT* transcript copies/µL)/concentration reference (*EF1α* transcript copies/µL) (n=3). nd: transcript not detected.

Transcript abundance was also evaluated for Tr*neMDH* in 28 of the 30 events generated. Since the promoter used is root specific, and the primary toxicity effect of Al^{+3} takes place in the root tips, transcript abundance was evaluated in root tips.

Given that Tr*neMDH* is an endogenous gene from white clover, two strategies for transcript abundance quantification are possible. Rossello (2011) designed a set of primers targeted to the promoter 5' untranslated region (UTR)-coding sequence junction, using a left primer directed to the Tr*PT1* promoter 5' untranslated region (UTR) in order to avoid the amplification of the Tr*neMDH* endogenous gene. This strategy allowed confirmation of the functionality of the inserted cassette, and a comparison of the performance of the Tr*neMDH* cassette in the different events and tissues.

However, the method is not suitable to evaluate whether the insertion of the Tr*neMDH* cassette had an effect on increasing Tr*neMDH* transcript abundance in the transgenic when compared to the endogenous gene of the non-transgenic isogenic control. In order to evaluate this, a comparison of Tr*neMDH* transcripts levels among the transgenic and the non-transgenic isogenic control is necessary. Therefore, an assay that target the Tr*neMDH* coding sequence was designed, where both the transgene and the endogenous Tr*neMDH* genes are amplified (Figure 3.9, Table 1.1). The main limitation of this method is that it's not possible to differentiate between transcripts from the endogenous gene and the inserted cassette. Therefore, it is assumed here that an increase in Tr*neMDH* transcripts would be a result of transcription from the inserted cassette.

Twelve events exhibited increased Tr*neMDH* transcript abundance when compared to their respective non-transgenic isogenic controls. Three events showed lower Tr*neMDH* transcript abundance when compared to the non-transgenic isogenic controls. Six events showed a statistically significant Tr*neMDH* transcript level increase when compared to their controls (p-value=0.05), and event 26 exhibited a statistically significant decrease (Figure 3.11).

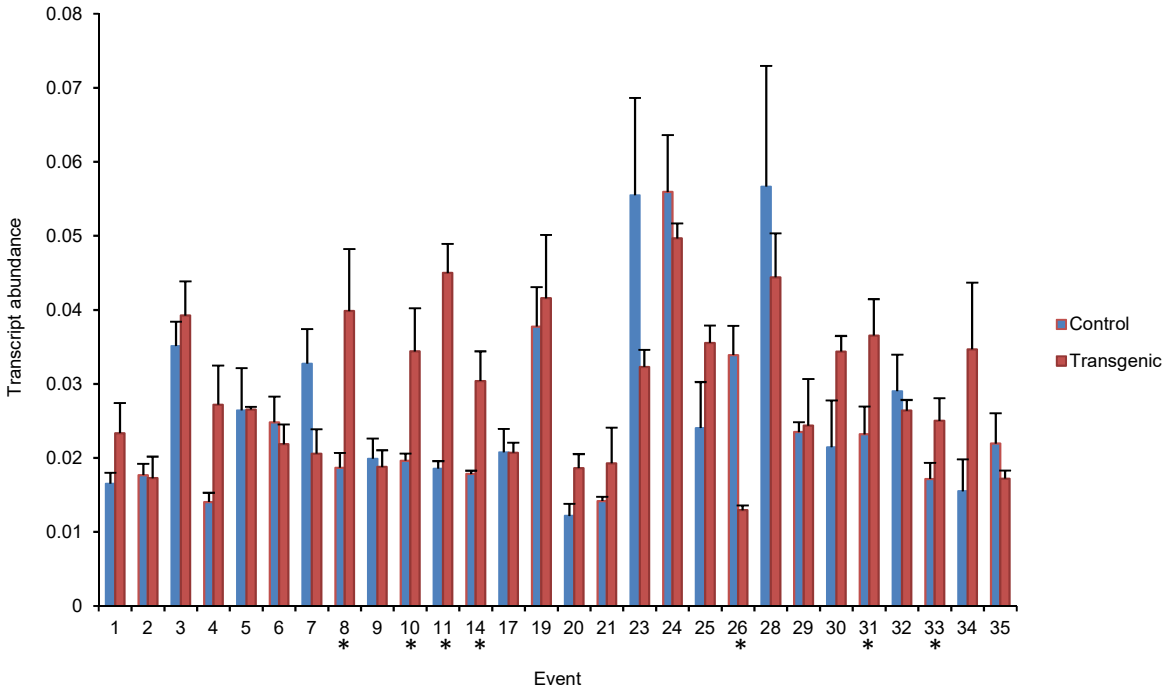


Figure 3.11. Tr*neMDH* transcript abundance. Transcript abundance was estimated by relative quantification using droplet digital PCR (ddPCR), by calculating the ratio of concentration target (Tr*neMDH* transcript copies/µL)/concentration reference (*EF1α* transcript copies/µL). (*) statistically significant at p values<0.05 (between 3 to 6 replicates were analysed for this experiment).

CP-*AMV* transcript abundance was also evaluated (Figure 3.12). A duplex experiment was designed for CP-*AMV* transcript abundance estimations: a pair of primers and a FAM labelled probe were targeted to the gene of interest, and another pair of primers and a HEX labelled probe were targeted to the reference gene (Figure 3.9 and Table 3.6).

Interestingly, CP-*AMV* transcript levels show a high variation among events. While events 31 and 34 show high transcript abundance, with levels almost as high as to the reference gene, event 11 exhibited a ratio of 0.00389, which is 250-fold lower than the reference gene. No transcripts were detected for event 21, and almost undetectable transcript levels were measured for event 25.

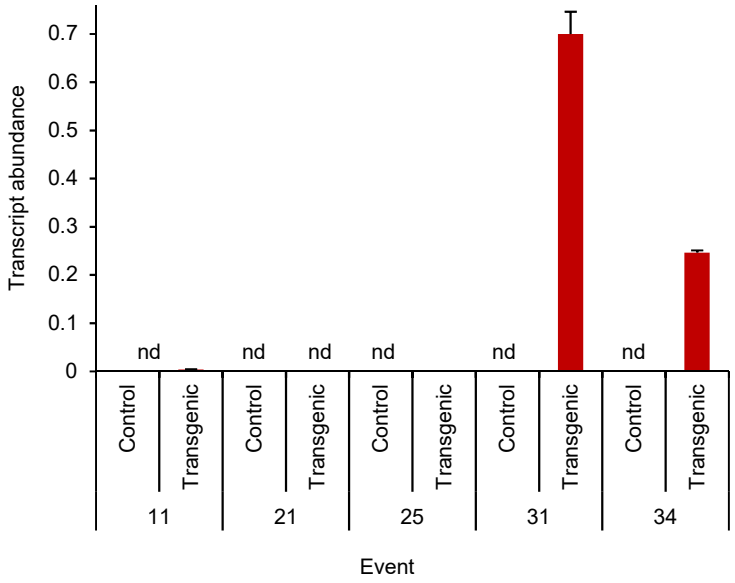


Figure 3.12. CP-*AMV* transcript abundance. Transcript abundance was estimated by relative quantification using droplet digital PCR (ddPCR), by calculating the ratio of concentration target (CP-*AMV* transcript copies/µL)/concentration reference (*EF1α* transcript copies/µL).

No evident correlation was observed between CP-*AMV* and *IPT* gene expression when events were compared. While CP-*AMV* transcript abundance was one order of magnitude higher than *IPT* in event 11, this relation was two orders higher in events 31 and 34 (Table 3.7). In order to compare the transcription levels between the three genes of interest, Tr*neMDH* transcript abundance caused by the inserted cassette was estimated by the difference in Tr*neMDH* transcript levels between transgenic and their respective non-transgenic controls. Some level of correlation in transcript abundance for the three cassettes was observed in events 31 and 34. While CP-*AMV* levels were close to the transcript levels of the reference (between 1.4 and 4-fold lower for events 31 and 34 respectively), Tr*neMDH* was approximately 2 orders lower (75 and 52-fold lower in events 31 and 34 respectively), and *IPT* was approximately 3 orders lower (753 and 381-fold lower in events 31 and 34 respectively).

Table 3.7. Transcript abundance of the three genes of interest. Values presented in this table are the ratios of concentration of target (target copies/µL)/concentration of reference (reference copies/µL). Tr*neMDH* transcript abundance presented here are the difference between Tr*neMDH* transcript levels of the transgenic events versus their respective non-transgenic isogenic controls. The gene used as reference was *EF1α*. (*) significant difference (p value < 0.05).

Event number	*IPT*	Tr*neMDH*	CP-*AMV*
11	0.000847	0.026450*	0.003897
21	n.d.	0.005100	n.d.
25	n.d.	0.011516	n.d.
31	0.001327	0.013333*	0.700000
34	0.002623	0.019167	0.246333

3.4 Discussion

Copy number of the three genes of interest was estimated for the thirty events in this study. Forty three percent of the events exhibited the putative insertion of a single copy T-DNA. Previous work reported a 50% frequency of single T-DNA insertions in white clover by Agrobacterium-mediated transformation (Rossello, 2011). A high frequency of single T-DNA insertions on Agrobacterium-mediated transformation in different species was reported. A frequency of 30-40% was observed for single T-DNA insertions in rice (Sallaud et al., 2003), approximately 40% was reported in maize (Ishida et al., 1996), and 31.5% was observed in soybean (Olhoft et al., 2004). Compared to these species, the frequency of single T-DNA insertions is slightly higher in white clover. However, in addition to the species transformed, many elements such as target tissue used, cultivar or genotype, Agrobacterium strain and transformation method can affect the frequency of single T-DNA insertion (Grevelding et al., 1993; Kohli et al., 2003).

Twenty events of the 30 generated exhibited one or two T-DNA copies. As mentioned previously, low copy number is a desired feature for selection of the most suitable events. Low copy number events minimize the difficulties that segregation of the inserted genes in the following generations after T_0 would bring. Therefore, the production of a high number of low copy number events in this work is highly relevant.

The current work doesn't provide a definitive confirmation of the single copy nature of the endogenous genes used as reference for copy number estimation. However, some elements support the possibility that these genes are single copy in white clover. If the endogenous gene used was present in more than one copy, all the events whose copy number was estimated at one would exhibit an unlikely result of copy numbers lower than one. Additionally, copy number estimated for the genes of interest using either *PDH* or *ATP-dependent protease* generated identical results. Given that it's highly unlikely that changes in copy number occurs for both reference genes used in the same genotypes/events, these results support the notion that copy number of the reference genes remains constant among the genotypes evaluated. Nevertheless, the performance of Southern blots on the generated events would be needed in order to confirm unambiguously the results generated.

Given that the three genes of interest are linked in a single T-DNA, the same copy number for the three genes in each event is expected. However, dissimilar copy number was estimated for the three genes of interest in some events. For example, event 3 exhibited 4 copies of *IPT*, 2 copies of Tr*neMDH*, and 1 copy of CP-*AMV*, and event 29 showed 2, 4 and 3 copies of *IPT*, Tr*neMDH* and CP-*AMV* respectively. These results can be explained by the occurrence of T-DNA breakages and insertion of sectioned T-DNA. Additionally, copy number dissimilarities between genes of interest in events can be explained by inherent variation.

A higher variation of qPCR results as copy number increases has previously been reported (Bharuthram et al., 2014). Additionally, higher accuracy of ddPCR results when compared to qPCR results was observed (Bharuthram et al., 2014). When analysing the correlation between each gene of interest copy numbers and the sum of the three genes copy numbers per event in this work, a similar tendency was observed. Also, the slopes of the linear equations generated approximated more accurately to 3 in ddPCR assays in relation to qPCR assays. These results support the implementation of ddPCR for high throughput copy number studies in white clover.

RT-ddPCR technique was implemented successfully for transcript abundance assays in white clover. RT-ddPCR was observed to be more sensitive than qPCR for transcript abundance measurements, in accordance to the higher sensitivity observed by this technique when compared to qPCR in previous studies (Hindson et al., 2011; Baker, 2012). In the case of the *IPT* gene, transcript levels that were undetectable or exhibited high Cq values (Cq~40) by qPCR were detected and measured by RT-ddPCR.

Therefore, the limitation of measuring transcripts in low concentration by qPCR can be overcome by using RT-ddPCR.

The detection of *IPT* transcripts in the transgenic plants generated in this work is in line with the earlier reports of Lin et al. (2003) of the active role of At*myb32* promoter in white clover leaves. However, no quantitative data on the promoter activity in white clover was previously reported. The quantification of *IPT* transcripts performed in this work supports the extensive phenotypic information generated on delayed leaf senescence of transgenic events carrying At*myb32:IPT* cassette in white clover in this project (Chapter 4), and previous studies (Lin et al 2007, 2010) and in canola (Kant et al., 2015).

Although *IPT* transcripts were detected, their levels were very low. Semi-quantitative RT-PCR performed in Arabidopsis for At*myb32* transcript quantification, revealed low transcript levels in leaves compared to flowers (Preston et al., 2004). These results suggest a low activity of the At*myb32* promoter in leaves in Arabidopsis. The low transcription abundance observed for this work in white clover events can be explained by a localized activity of the At*myb32* promoter, mostly confined to vascular tissues (Lin et al., 2003).

High cytokinin production could induce negative effects in plant development, such as root growth impairment and loss of apical dominance (Smigocki and Owens, 1989). Therefore, in order to produce transgenic events with the desired phenotype, cytokinin levels should not be too high, to avoid negative effects, but high enough to produce delayed leaf senescence. Arguably, the low *IPT* transcript levels observed here are in line with these requirements, and would provide a result with the desired phenotype. Thus, *IPT* transcript abundance assays can provide an approximation to the transcript levels required in order to have plants with delayed leaf senescence without the deleterious effects of high cytokinin levels.

As mentioned in section 1.2.4, different promoters whose activity is limited to certain conditions, such as senescence inducible *PSARK* and the maturation-induced and stress-induced *SAG12* were tested in order to avoid such negative effects (Gan and Amasino, 1995; Rivero et al., 2007). The use of the tissue-specific promoter At*myb32* for expression of *IPT* follows a different strategy, by limiting the transcription to certain tissues at low levels. There is further discussion on these aspects in the following chapter, where phenotyping for delayed leaf senescence and the relation to transcript abundance is assessed.

The significant increase in Tr*neMDH* transcript levels observed in six events, supports the use of the Tr*Pt1:*Tr*neMDH* cassette to increase malate production in roots. Furthermore, it would allow to select the events with more potential for expressing Al^{3+} tolerance. The information generated also provides further evidence on the functionality of the inserted T-DNA for the events whose transcription levels are increased.

Given that CP-*AMV* is controlled by the constitutive promoter Ca*MV35S*, high levels of CP-*AMV* transcript were expected in the generated transgenic events. However, although this was observed in events 31 and 34 (0.70 and 0.25 respectively), it did not occur in the other three events evaluated. Most notably, no transcripts were detected in event 21 and almost undetectable transcript levels were measured in event 25.

There was no clear correlation between the five events when transcript abundance were compared for the three genes of interest (Table 3.7). Nevertheless, although highly variable, CP-*AMV* show higher levels than the other two genes of interest. Tr*neMDH* is ranked second according to its transcript levels, with values of 37-fold (event 11), 52-fold (event 34) and 75-fold (event 31) less than the reference gene. The lowest transcript abundance was measured for *IPT*, with fold changes of 753, 381, and 1180 (events 31, 34 and 11 respectively). This trend occurs in events 31 and 34. However, this does not happen in event 11, where CP-*AMV* is very low.

Events 21 and 25 do not show detectable transcription for either CP-*AMV* or *IPT*. Additionally, the difference in Tr*neMDH* transcript levels between transgenic and control for these two events is the lowest of five events. This may indicate that the three genes of interest are silenced in these events. A lack of expression of integrated T-DNA has been previously reported (Gelvin, 2003). T-DNA can be inserted in different regions of the genome, which could be either transcriptionally active or silent. Transcription can also be positively or negatively affected depending on whether T-DNA is inserted in proximal or distal from transcriptionally activating elements (Birch 1997; Gelvin, 2003). Furthermore, transgenes silencing can be triggered by DNA methylation (Matzke and Matzke, 1998), or change of environmental conditions (Zhong, 2001).

Furthermore, it has been previously observed that when plants are transformed with a transgene sharing homology to an endogenous gene, silencing of some or all of the inserted copies can occur (Flavell, 1994; Zhong, 2001). Given that Tr*neMDH* was isolated from white clover, homology-dependent gene

silencing is a possibility in the generated events. This could explain the outcome observed for some events (events 23 and 26), where Tr*neMDH* transcription is reduced in transgenic plants when compared to their non-transgenic controls.

Lack of expression of the genes of interest can be a sign of insertion of a non-functional T-DNA. Given that intactness of the T-DNA for the events generated wasn´t confirmed in this project, transcription of the three genes of interest in an event provides evidence that a T-DNA is intact and functional.

Integration of the molecular characterisation results can provide a guidance to selection of the events with the desired characteristics. Presence of the three genes of interest, their low copy number, and the absence of the selectable marker gene are the primary elements to be considered. Only one selectable marker free event and its respective non-transgenic isogenic control was generated. Transcription of the genes of interest provides the ultimate proof of functionality of the inserted T-DNA. The information generated in this chapter provides relevant data for decision on the events with higher potential for the desired agronomic performance. However, beyond low copy number, and transcription for the three genes of interest, the ideal event should exhibit and express the desired phenotypic traits delayed leaf senescence and aluminium tolerance. The phenotyping for these traits is described in Chapters 4 and 5.

4 Phenotyping of triple stacked transgenic white clover plants for delayed leaf senescence and tolerance to water limitation

4.1 Introduction

4.1.1 Use of the IPT gene for conferring delayed leaf senescence and drought tolerance

Leaf senescence is the final stage of leaf development, characterized by organelle disassembly and subsequent cell death. Macromolecules such as proteins and DNA obtained from organelles are degraded, and nutrients produced as by-products of degradation are remobilised from old to young leaves and reproductive organs, which allows the survival of the subsequent progeny (Lim, Kim, & Nam, 2007; Yoshida, 2003). At the macroscopic level, colour change from green to yellow or brown, caused by chlorophyll degradation, is one of the most characteristic symptoms of leaf senescence. Both environmental and internal factors form a complex regulatory network that determine the timing and rate of senescence (Yoshida, 2003; Lim et al., 2007). Leaf age is one of the inducers of senescence onset, together with external factors such as light, temperature, nutrient availability and water stress (Gan and Amasino, 1997).

At the molecular and biochemical level, cytokinins play an important role in senescence. It has been reported that ectopic application of the phytohormone delays leaf senescence, while transgenic plants overproducing cytokinins show similar responses (Gan and Amasino, 1996). Different crop species transgenic for enhanced production of the enzyme Isopenthenyl transferase (IPT), involved in the cytokinin production pathway, exhibit delayed leaf senescence. However, the use of constitutive promoters such as the Ca*MV35S* promoter, generate systemic overproduction of the hormone, which causes a decrease in root growth, and loss of apical dominance (Smigocki and Owens, 1989). Concomitantly, given that loss of apical dominance induces branching (Müller and Leyser, 2011), this effect is not necessarily detrimental in a forage species such as white clover, as branching can favour plant survival under drought stress (Brock and Kim, 1994). Nevertheless, a fine balance of cytokinins levels in conjunction with auxins levels are necessary for normal plant growth (Smigocki and Owens, 1989). In order to limit overproduction of the hormone, Gan and Amasino (1995) transformed tobacco with the *IPT* gene controlled by a senescence inducible promoter (*SAG12*) from Arabidopsis. This approach was applied in different species, and delayed leaf senescence was reported (Calderini et al.,

2007; McCabe et al., 2001; Sýkorová et al., 2008). Also, a higher nitrate reductase activity and increased nitrate influx was observed in transgenic wheat overexpressing *IPT*, although no rise in grain yields was observed (Sýkorová et al., 2008). However, some negative effects, such as inhibition of flowering and reduced yield, were also noted in various species using the *SAG12* promoter (Gan and Amasino, 1996; Peleg and Blumwald, 2011). These negative effects were circumvented by using an alternative senescence inducible promoter from a *senescence-associated receptor-like kinase* gene (SARK) from pea (Hajouj et al., 2000) Using this strategy, tobacco and cotton plants exhibiting delayed leaf senescence and drought stress tolerance were generated (Rivero et al., 2007; Kuppu et al., 2013).

In the current project, the At*MYB32* promoter from *A. thaliana* was used. This promoter confers expression mainly in root and leaf vascular tissues, which may facilitate translocation of cytokinins to different tissues (Lin et al., 2003). A modified version of the At*MYB32* promoter was used in which a promoter motif that is specific for root expression was removed in order to reduce the negative effects of cytokinin production on root development and growth in tissue culture (Spangenberg et al., 2008; Kant et al., 2015). It was hypothesized that these features of the construct are crucial for an appropriate localized *IPT* expression that would help to circumvent undesired effects on plant growth (Spangenberg et al., 2008; Kant et al., 2015).

As mentioned in Chapter 1, previous work in generation of transgenic white clover expressing *IPT* under the control of At*myb32* promoter was carried out by our group. These events, evaluated under field conditions for four years, revealed delayed leaf senescence, increased leaf number, higher stolon length and higher leaf area when compared to their non-transgenic controls. Furthermore, white clover overexpressing *IPT* exhibited increased seed production, and better summer survival rate compared to their non-transgenic controls (Lin et al 2007, 2010).

As previously described, here white clover plants were co-transformed with three genes in a single T-DNA: an Agrobacterium *IPT* gene controlled by the promoter At*MYB32,* a nodule enhanced *Malate dehydrogenase (*Tr*neMDH)* gene from white clover controlled by the root specific white clover phosphate transporter 1 (Tr*Pt1*) promoter sequence, and *AMV* (Alfalfa mosaic virus) coat protein gene driven by the constitutive Ca*MV35S* gene promoter.

Phenotyping for events carrying one or two of the three genes of interest was performed in previous works. However, no work on evaluating the effects of the three genes linked in a single T-DNA has been

carried out until the current work. Although each gene is controlled by a different promoter, there is no certainty on whether the levels of expression of these would be affected by their neighbouring genes. Ideally, we expect to identify transgenic events concomitantly expressing the desired phenotypes conferred by each of the three genes of interest.

Although white clover plants expressing the *IPT* transgene were previously evaluated under field conditions, no conclusive reports on drought stress tolerance of these events were generated. However, all the information available from white clover (Lin et al 2007, 2010), canola (Kant et al., 2015), and other crops transformed with the *IPT* gene (Rivero et al., 2007; Kuppu et al., 2013), suggest the possibility of observing tolerance to water limitation in the events generated in this work. Also, as previously reported in canola, the use of a non-stress-inducible promoter could favour the generation of plants showing increased forage yields under irrigated conditions (Kant et al., 2015). Production of drought tolerant white clover plants holds particular importance, as it could add an agronomically important trait to the available white clover germplasm.

4.1.2 Drought stress tolerance under glasshouse conditions

Generally, in the T_0 generation, phenotyping experiments of transgenic plants are usually performed in conditions different to the ones observed in the field; frequently, small scale experiments, during short periods of time, in controlled environments are carried out (Saint Pierre et al., 2012). Consequently, the correlation between phenotyping results from glasshouse and field can be weak, and phenotyping experiments that relate to what happens in the field are required in order to select the best performing events (Bhatnagar-Mathur et al., 2008; Saint Pierre et al., 2012). Considering this, a drought stress trial was set up in the glasshouse under conditions that aim to mimic a controlled field trial. The development of a protocol for a scaled glasshouse trial performed in this work, with more similarities to what happens in the field, would allow to have a more accurate selection of events with potential for a better agronomical performance under drought stress in the field.

4.1.3 Phenotyping by digital imaging

The development of next generation sequencing techniques has enabled generation of a vast amount of genomic information. As a consequence, this 'genomic revolution' allowed the deployment of tools, such as marker assisted selection (MAS) and genomic selection, that help to overcome plant breeding

limitations (Varshney et al., 2009). These breeding strategies are based on the integration of genetic and molecular information with phenotypic data. However, although there is extensive genomic information generated, the amount and rate of production of phenotypic data is still limited (Furbank and Tester, 2011; Araus and Cairns, 2014). Thus, the slower rate of generation of phenotypic information relative to that of genomic and transcriptomic data generated is currently identified as a bottleneck for plant breeding (Furbank and Tester, 2011; Fiorani and Schurr, 2013; Araus and Cairns, 2014).

Phenotyping usually requires the screening of a high number of plants, grown in different environments, which can be costly and time consuming (Furbank and Tester, 2011; White et al., 2012). Also, the accuracy of quantification of some phenotypic traits is low by conventional methods. Usually, conventional phenotyping involves destructive yield measurements at fixed times during plant development (Furbank and Tester, 2011).

This constraint has resulted in increased inputs into research directed at improving phenotyping techniques (referred to as 'phenomics') (White et al., 2012). The integration of modern technologies such as varied sensors, high resolution digital imaging, software for digital image analysis, and automated systems used both in the glasshouse and the field, has allowed for the rapid development of this new area (Fiorani and Schurr, 2013). In a nutshell, modern phenotyping technics involve imaging of plants under controlled conditions in the glasshouse or in the field. This can be performed manually, by delivering the plants to the camera station, or automatically or semi-automatically by using conveyor systems in the glasshouse, or vehicles carrying sensors or cameras in the field (White et al., 2012).

Different components of plant structure can absorb, reflect or transmit light, and therefore images obtained at different wavelengths can provide a range of information (Li et al., 2014). For example, infrared wavelengths above 1000 nm can provide information about canopy conductance, whilst data on transpiration and canopy water use can be obtained by thermographic analysis of canopy temperature (Walter et al., 2012). Within the visible range (400-700 nm), parameters such as plant architecture, plant size, leaf area, canopy size, leaves number, leaf colour, and root architecture can be estimated (Fiorani and Schurr, 2013; Li et al., 2014). This requires identification, manually or automatically, of the structures of interest and the background, and deriving pixel counts (Walter et al., 2012).

4.1.4 Phenotyping by digital imaging of transgenic white clover

As mentioned in Chapter 1, white clover plants expressing the *IPT* gene were phenotyped for delayed leaf senescence in earlier studies. This was performed in detached leaf experiments, which consists of removing leaves from a similar location in the plant and evaluating their colour change over time in Petri dishes. This method relies on visual colour change estimations. Although in some cases the colour change difference between the transgenic and their respective non-transgenic controls is evident to the naked eye, the development of a quantitative method would be valuable. Digital image analysis provides a valuable tool for these purposes. Therefore, a quantification method of analysis for delayed leaf senescence was developed and is described in this chapter.

Given that plant structure and morphology changes between species, phenotyping by digital imaging methods need to be designed and optimised for particular groups of species with similar morphology and growth habits (Fiorani and Schurr, 2013). For example, plant size can be accurately estimated from side images in cereal crops (Golzarian et al. 2011). However, given that leguminous species have spread growth habits, this method can be inaccurate, and top view images might be a better strategy. The use of hyperspectral and aerial images for high throughput phenotyping in white clover in the field for cold tolerance was developed by Inostroza et al. (2016). To our knowledge, no protocols for phenotyping by digital imaging of white clover under water limitation in the glasshouse have been described until the current work.

4.2 Materials and methods

4.2.1 Detached leaf bioassay

Detached leaf bioassays were performed according to Vala (2012), with some modifications. Petri dishes with detached leaves were placed inside a tissue culture growth chamber at 21°C 14 h light and 8 h dark. In order to evaluate leaves with a similar age, second leaves from the tip of the stolons were detached and placed in Petri dishes on filter paper soaked in distilled water. Nine replicates were used for the analysis, and significance was evaluated by a two-tailed *t* test. Digital images were taken when a clear colour contrast was observed with the human eye between transgenic and isogenic controls. Colour analysis was performed using Lemnagrid software (Lemnatec GmbH, Würselen, Germany) in order to estimate the proportion of green and senescing tissue (Figure 4.2). Briefly, a first step of digital image analysis was carried out, where the object of analysis (foreground) and background pixels were identified, followed by a step for noise reduction. Another step that assigns the colours for senescing and non-senescing tissue area was included, and pixels corresponding to the two tissue groups were counted in order to estimate the leaf senescence. A more extended description of the digital image-based analysis is described in section 4.3.2.

4.2.2 Delayed leaf senescence and drought stress in soil

Twelve transgenic events were selected based on copy number and delayed leaf senescence results. Single stolons were grown for 15 d in 85 mm pots, similar sized plants were selected, and two plants were transplanted to large tubs (35 cm long, 26 cm wide, and 17 cm deep), and grown for a further 20 days. The trial was performed in the glasshouse at 18/26°C minimum/maximum temperatures, under 14 h of supplemental light during daytime (103 μmol m^{-2} s^{-1}). Two rounds of drought with one round of regrowth evaluation, in between, were performed (Figure 4.4). Plants were watered to saturation, and water was then withheld for 42 d until soil water content (SWC) was 25%. Plants were watered up to 90% SWC once they reached day 42 of water limitation, and shoots were harvested to measure fresh and dry mass 7 days later. After the first drought stage, tubs were rewatered and maintained at 70% SWC for 21 days to evaluate regrowth. Shoots were harvested to measure fresh and dry mass at this point. Following regrowth evaluation, plants were watered up to 90% SWC, and similar to the first drought stage, water was withheld for 35 days until water content (WC) reached 25%. Up to this time point plants were watered up to 90% SWC and harvested 7 days after (day 42). Soil water content (SWC) was

maintained at 30% between days 20 and 30, and at 25% from day 30 until the end of each water limitation stages. Every harvest was performed by cutting shoots one cm above the ground without removing stolons rooted to the soil. Harvested leaves and stolons were separated and only leaves were weighted. Tubs were weighed on the first day and then two times per week in order to measure water loss.

Plants were arranged in a complete block design, and four blocks per event were evaluated. Two plants were present in each tub and four tubs (one in each block) per event were evaluated. Transgenic plant performance was evaluated relative to non-transformed isogenic control plants. Significance was evaluated by one-factor ANOVA by GenStat for Windows 14th Edition, VSN International, Hemel Hempstead, UK.

Top view digital images were taken twice weekly using a Canon 500d camera mounted on a stand (Canon, Tokyo, Japan) to follow colour change in leaves and to estimate forage yields (Figure 4.5). Camera settings were F Stop=8.0, ISO=100, and lighting=tungsten. Digital images were taken 110.5 cm from the top of the pots. Images were analysed by Lemnagrid software (Lemnatec, Würselen, Germany).

4.3 Results

4.3.1 Detached leaf bioassay

Delayed leaf senescence in detached leaf bioassays was estimated using digital image analysis. Images were acquired at the moment a visual trend of colour contrast was observed between the transgenics and their respective non-transgenic controls. Pixel counts from non-senescing (colour green) and senescing (colour brown and yellow) leaf areas was acquired by using Lemnagrid software (for more details see next section). Statistically significant difference in delayed leaf senescence was estimated by comparing green pixel counts between transgenics and their respective non-transgenic controls by *t* test (p value<0.01). Delayed leaf senescence was observed in 13 of the 30 transgenic events evaluated relative to their respective non-transgenic isogenic controls (Figure 4.1 and Figure 4.3). Conversely, no statistically significant difference in green pixel counts between transgenics and their respective non-transgenic controls was observed in 15 events, and 2 events showed accelerated senescence. The contrast observed was consistent and event dependant. In some events delayed leaf senescence was maintained for periods of 10 to 20 days, but in others it was observed for shorter periods of 3 to 5 days. An exceptional case was observed in event 32, where a defined colour contrast was still observed after 30

days of detachment (Figure 4.1). However, this level of difference in delayed leaf senescence could not be repeated in subsequent assays. A number of events initially showed delayed senescence but could not be replicated in subsequent assays. These were considered as events that did not exhibit delayed leaf senescence.

Events to be included in drought stress tolerance trial were selected according to whether consistent delayed senescence was observed on detached leaf assays, and based upon molecular screening results. Regulatory authorities demand low copy number insertions in order to permit the commercial release of a transgenic event (EFSA, 2011). Also, single T-DNA copy events are required in order to avoid segregation in further generations when plants are crossed. Therefore, we considered low copy number a desirable feature for event selection in phenotyping evaluations.

Twelve events were selected for drought stress phenotyping based on copy number and delayed leaf senescence results. Of the 13 events showing delayed senescence, events 11, 14 and 28 were of high copy number (Table 3.5). Although this is an undesired feature, we included event 11 to evaluate correlation between copy number, gene expression and phenotype. An event showing accelerated senescence was also included (event 3).

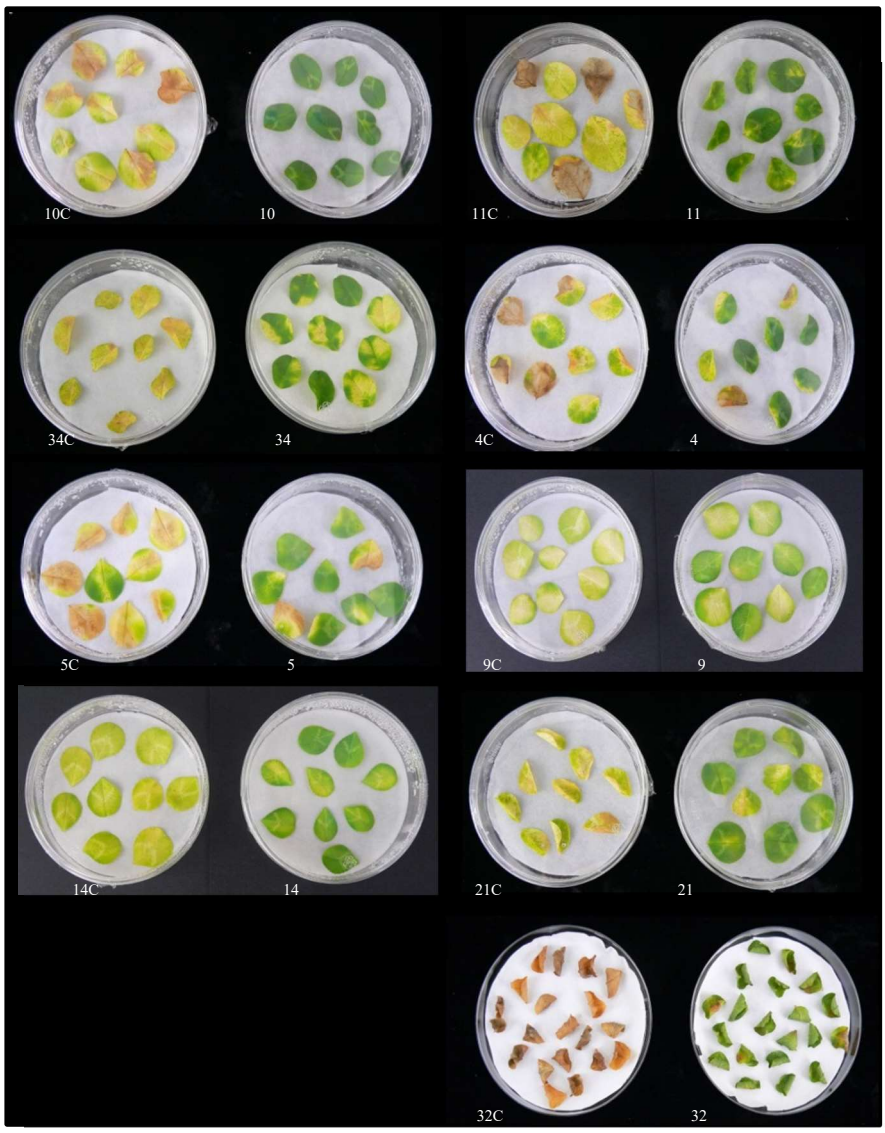


Figure 4.1. Detached leaves bioassays. Digital images of detached leaves of events (At*Myb32:IPT::* Tr*Pt1:*Tr*neMDH::*Ca*MV35S:*CP-*AMV*) and their respective non-transgenic isogenic controls. Image timepoint relative to removal varied for each event.

4.3.2 Phenotyping by digital imaging: delayed leaf senescence

Until the current work, delayed leaf senescence estimations have been performed by visual scoring of detached leaf experiments. However, no quantitative scores are generated by this method, and inaccuracies associated to subjective visual observations can occur. The development of high quality digital imaging and software packages for image analysis allowed us to implement a quantitative analysis method of delayed leaf senescence.

Digital images were analysed by Lemnagrid software (Lemnatec, Würselen, Germany). First, identification of the outline of the leaves was performed using the feature 'nearest neighbour foreground/background colour separation'. Here colours are classified as part of the region of interest (foreground), or as background. Only pixels contained within leaf margins were included as the region of interest (Figure 4.2). Subsequently, digital image modification was performed, where small background unwanted areas were excluded from the analysis by a modification called 'multi step morphological', and all the object or leaves are set as different objects by a step 'object decomposition'. Next, the 'object cutter' feature is used, which separates adjacent objects (in this experiment two adjacent leaves) primarily identified as single objects. Finally, a 'colour classification' step was included in order to classify image colours as associated with non-senescing and senescing tissue. This made it possible counting pixels classified as either non-senescing or senescing in the analysed image, and to derive an accurate estimation of the level of senescence in the leaves studied (Figure 4.3).

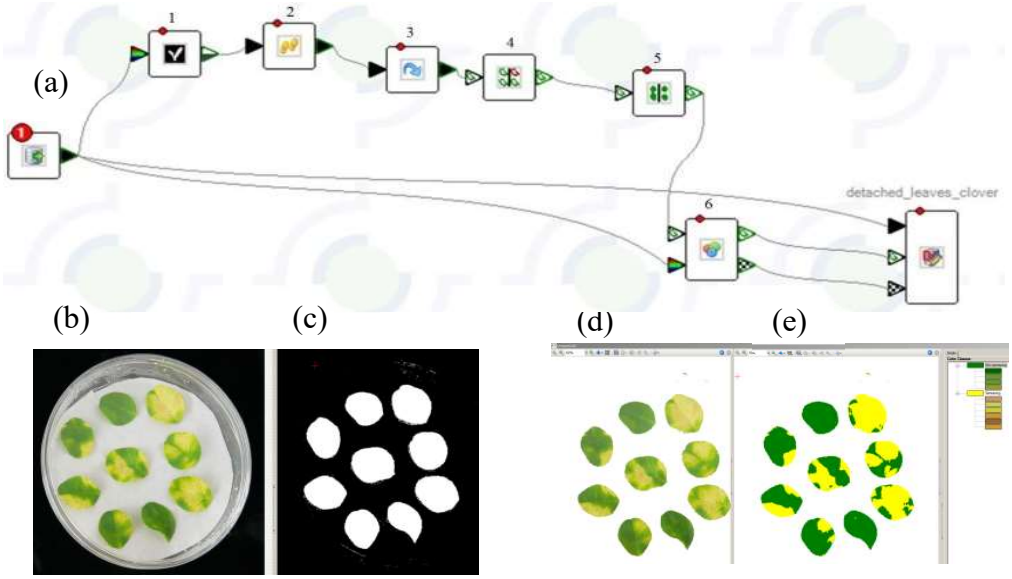


Figure 4.2. (a) Digital image analysis pipeline of detached leaf assays for evaluation of delayed leaf senescence from Lemnagrid software (Lemnatec, Würselen, Germany). Numbers identify the different steps of image analysis. (1) 'nearest neighbour foreground/background colour separation', colours are classified as part of the region of interest (foreground), or as background (panel c) from the original image (panel b). (2) 'multi step morphological', small unwanted areas from background are excluded from the analysis. (3) 'converter', allows to switch over different kinds of data formats. (4) 'object decomposition' object or leaves are separated as different objects. (5) 'object cutter' separates adjacent objects primarily identified as single objects (panel d) (6) 'colour classification', classify image colours as non-senescing and senescing tissue (panel e).

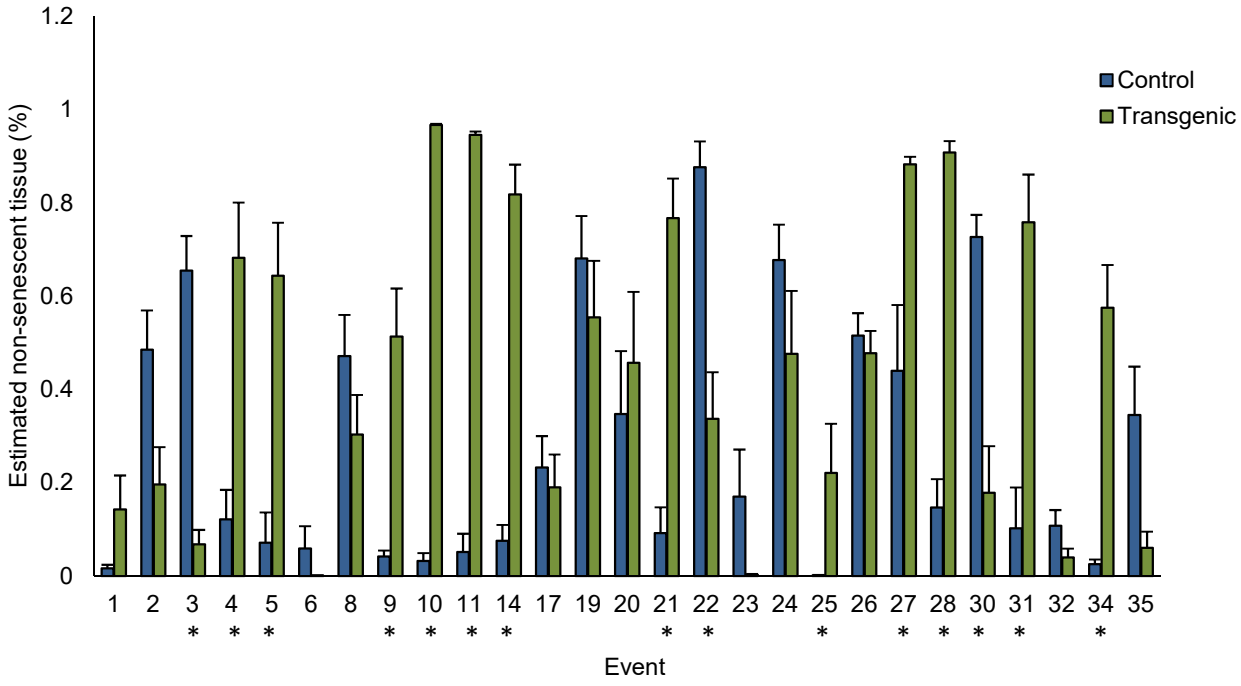


Figure 4.3. Detached leaf experiments results obtained by digital image analysis for delayed leaf senescence evaluation. Green tissue areas were classified as non-senescent and yellow and brown tissue areas were included as senescent. Percentage of green pixels count from total pixels count is represented in y axis. (*) significant at p value<0.01.

4.3.3 Drought stress trial

An experiment directed to identify events that exhibit tolerance to water limitation was performed. For the design of this trial, some elements in order to simulate field conditions were considered. In order to evaluate the performance of the events in their mature stages, during a period similar to the plant reproductive cycle, this experiment was executed in a long term. The experiments were performed over 112 days (almost four months), which consisted of: one stage of water limitation, a first harvest (harvest 1), one stage of regrowth, a second harvest (Harvest 2), a final stage of water limitation, and a third harvest (Harvest 3) (Figure 4.4).

Similarly to the work described by Bhatnagar-Mathur et al. (2008), a slow progression in water limitation applied to the plants was carried out during the trial, which attempts to simulate water availability in the field (Figure 4.5). Plants were grown in large tubs (35 cm long, 26 cm wide, and 17 cm deep) tubs where stolons could grow over the soil surface, similarly to what occurs in the field.

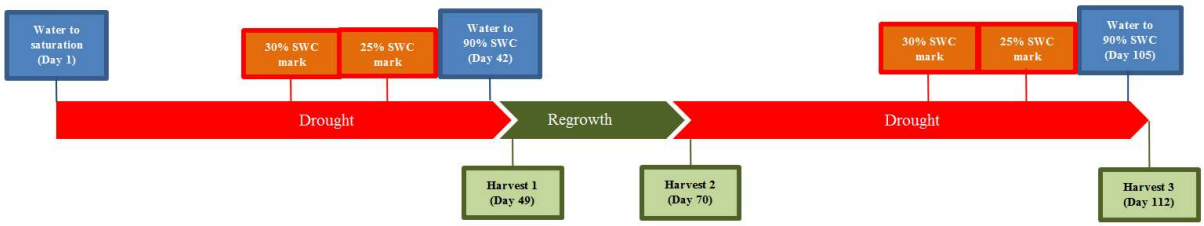


Figure 4.4. Drought stress trial timeline.

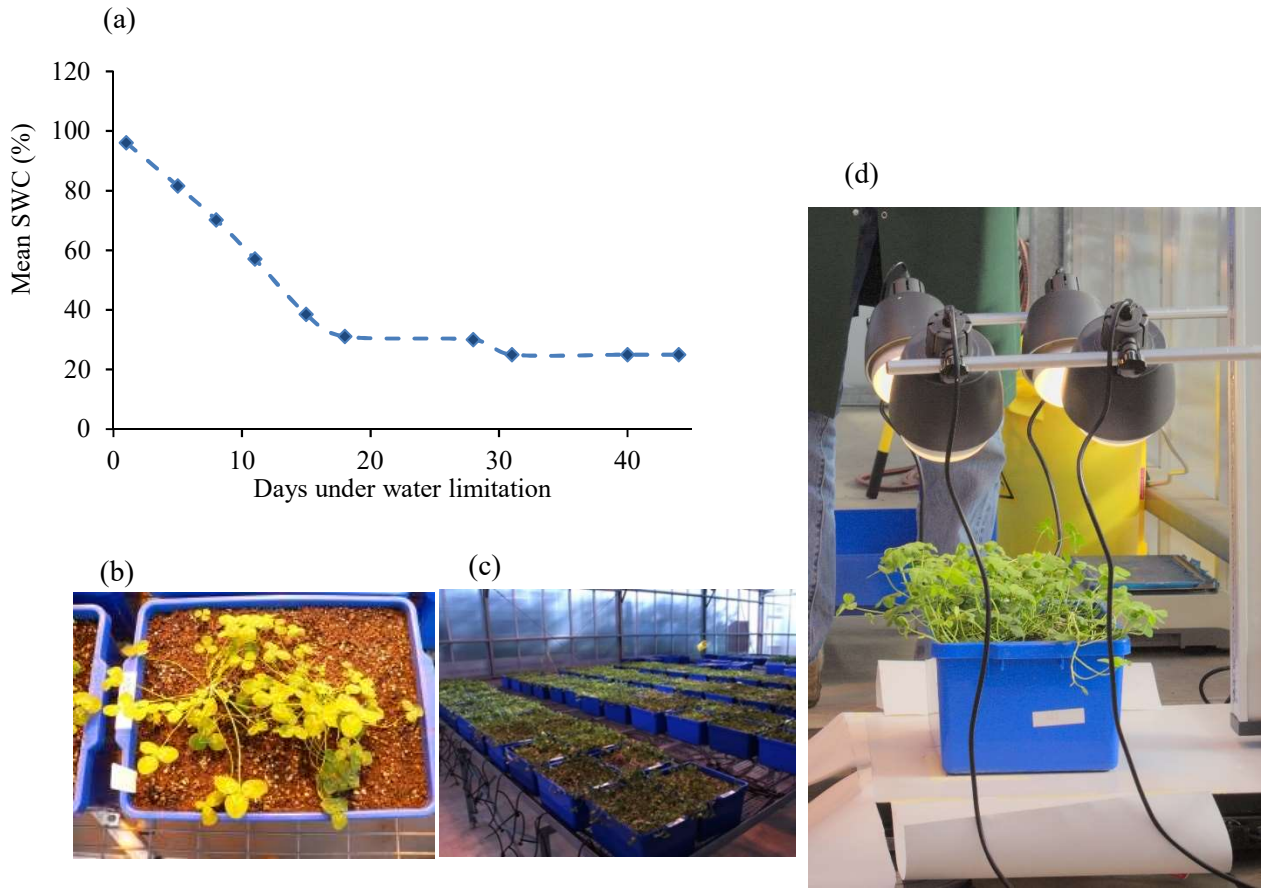


Figure 4.5. (a) Watering progress during the two drought stages. Soil water content (SWC) was maintained at 30% between days 20 and 30, and at 25% from day 30 until the end of each water limitation stages. (b) Image of one tub containing two plants used in the drought stress tolerance trial. (c) Image of drought stress tolerance trial. Plants were arranged in a complete block design, and four blocks were evaluated. Four pots (one in each block) per event were evaluated (n=4). (d) Stand used for imaging of plants for digital image-based phenotyping (Picture by Dr. Adam Dimech).

Twelve events were evaluated for survival under water limitation and regrowth upon rewatering. Higher forage yield was observed after 42 days of water limitation in the event 31 in the first drought stage, and in event 11 in the second drought stage. The observed difference for forage yield estimation of these events was confirmed for both fresh and dry weights (Figure 4.6 and Figure 4.7). Only event 31 exhibited a statistically significant difference in forage yield in the first drought stage for both fresh and dry weights (p value < 0.01), while event 11 exhibited significantly higher yields after the second drought stage (p value<0.05) for both fresh and dry weights (Figure 4.7 and Supplementary figure 4.1).

transgenic non-transgenic control

Figure 4.6. Effects of drought stress on transgenic plants. Images were taken after 40 days of water limited conditions, with a final soil water content of 25%. left: transgenic plants (two per tub), event 31 (At*Myb32:IPT::*Tr*Pt1:*Tr*neMDH::*Ca*MV35S:*CP-*AMV*). right: non-transgenic isogenic control plants.

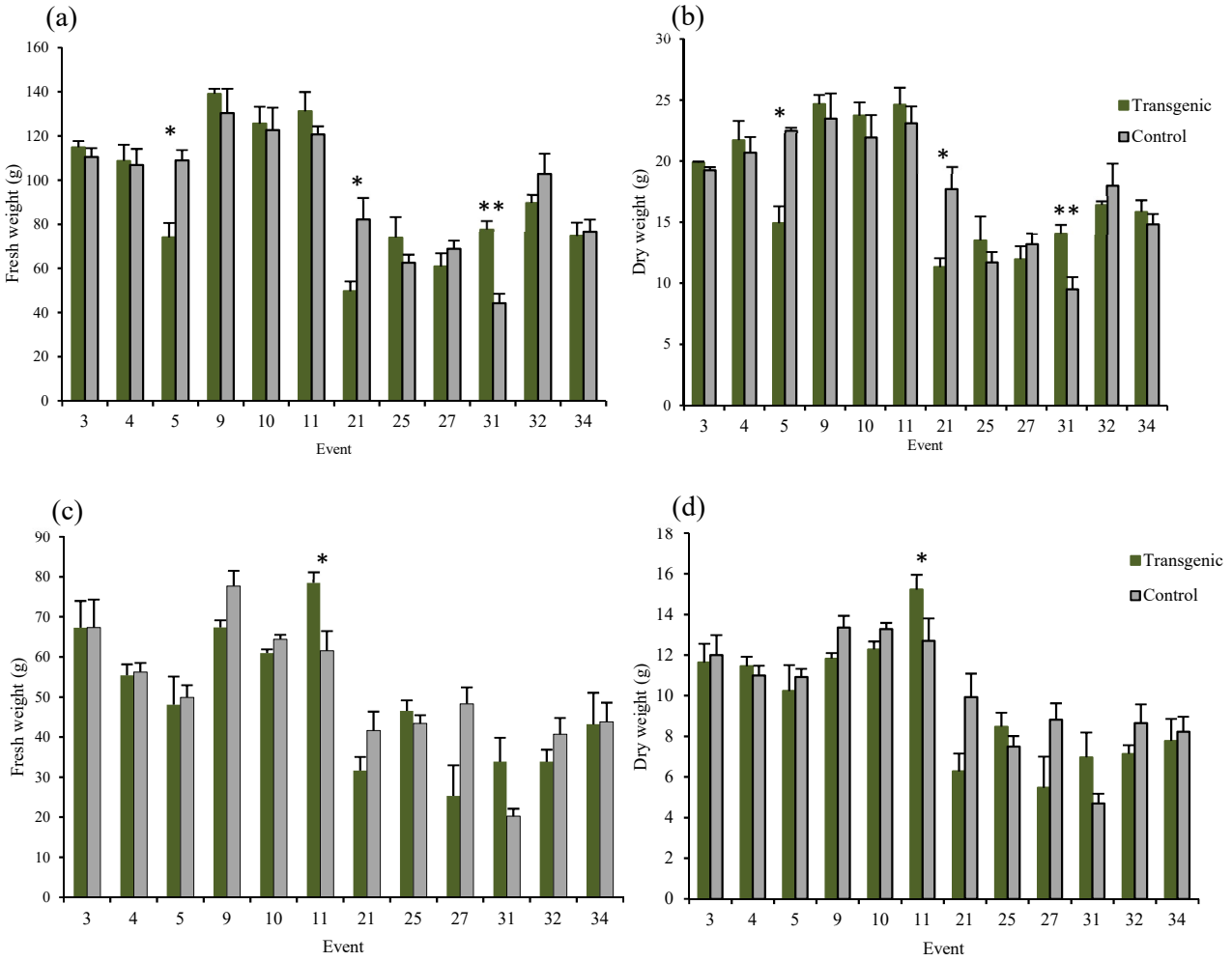


Figure 4.7. Forage yields of transgenic events under drought stress. Means of fresh and dry shoots weights, measured after a first round of drought stress (42 days of water limitation) (harvest 1), (a) and (b) respectively, and fresh (c) and dry weights (d) of second round of drought stress (harvest 3) (35 days of water limitation). In both drought rounds, water was withheld up to a final soil water content of 25%. (*) significant difference between transgenic plant and their respective non-transgenic control ($P<0.05$); (**) significant difference (p value <0.01).

Herbage recovery after defoliation caused by grazing or cuttings are of high relevance in white clover productivity (De la Hoz and Wilman, 1981). Therefore, harvests during the trial were performed in order to simulate intensive grazing or mechanical cutting in the field whilst under replete water. Only event 31 exhibited increased regrowth relative to its non-transgenic control (p value<0.01) (Figure 4.8).

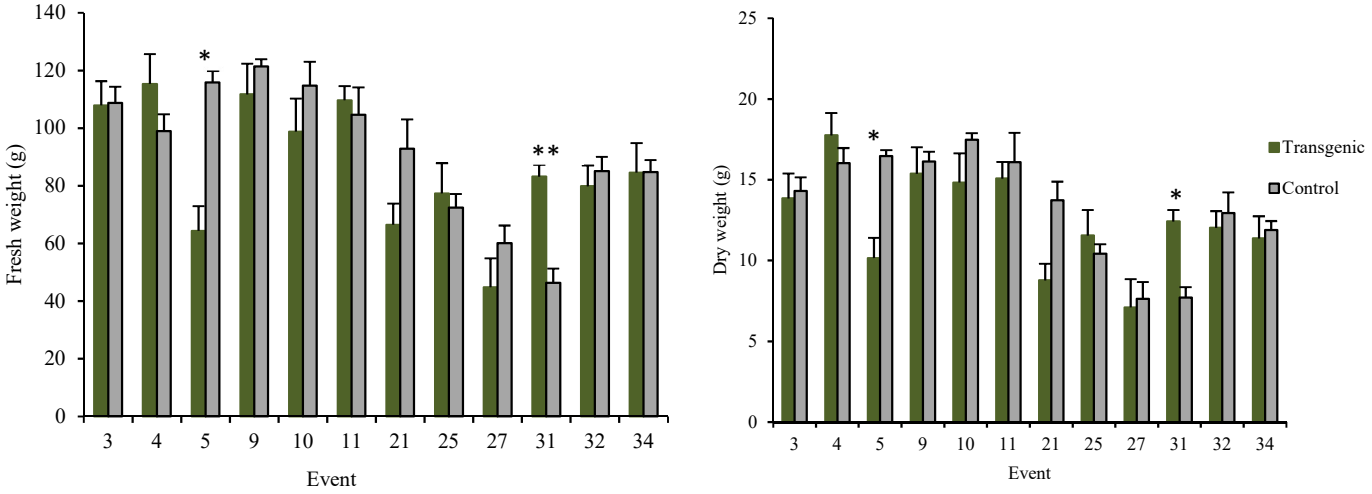


Figure 4.8. Forage yields of transgenic events in regrowth evaluation. Means of fresh and dry shoots weights, measured after a round of regrowth (harvest 2) under constant soil water content of 70%, (a) and (b) respectively. (**) significant difference between transgenic plant and their respective non-transgenic control($P<0.01$); (*) significant difference between transgenic plant and their respective non-transgenic control($P<0.05$).

Events 5, and 21 exhibited a decrease in forage yields when compared to their respective non-transgenic controls. This difference was significant for fresh and dry weights in both events in the first stage of the trial at p value<0.05 (Figure 4.7), and in the regrowth stage in event 5 (Figure 4.8). No significant difference was observed for events 3, 4, 9, 10, 25, 27, 32 and 34 in any of the three stages.

4.3.4 Phenotyping by digital imaging of drought stress trial

Phenotyping by digital imaging of the plants evaluated enabled the estimation of forage yields without the need of performing destructive harvests. Top view digital images were taken regularly (at 3 to 6 days intervals) during the trial and analysed by Lemnagrid software. First, a 'demosaicing' image processing was applied. In this step, a colour image is reconstructed from a bayer colour pattern, defined as the immediate output produced by a digital camera. A separation step between the analysed plant and the background followed, using the feature 'nearest neighbour foreground/background colour separation', where colours are classified as part of the region of interest (foreground), or as background. Only pixels contained within plant material (leaves and stolons) were included as the region of interest (Figure 4.9). A subsequent image modification was performed, where small unwanted areas from the background were excluded from the analysis by a modification called 'multi step morphological'. Similarly to what was done in detached leaf analysis, all the object or leaves were set as one object by a step 'object composition'. In parallel to these image analysis steps, a process called 'mean shift' was performed. This

feature clusters regions and applies the mean colour value to that specific area. Finally, and similarly to image analysis of detached leaf assays, a 'colour classification' step was included in order to classify image colours as non-senescing and senescing tissue. With this information, pixels were classified as senescing and non-senescing in the studied images, which would allow to estimate the level of senescence of the analysed plants. Furthermore, the broad horizontal extension of the plants in the tubs enabled a more accurate phenotyping by imaging from above.

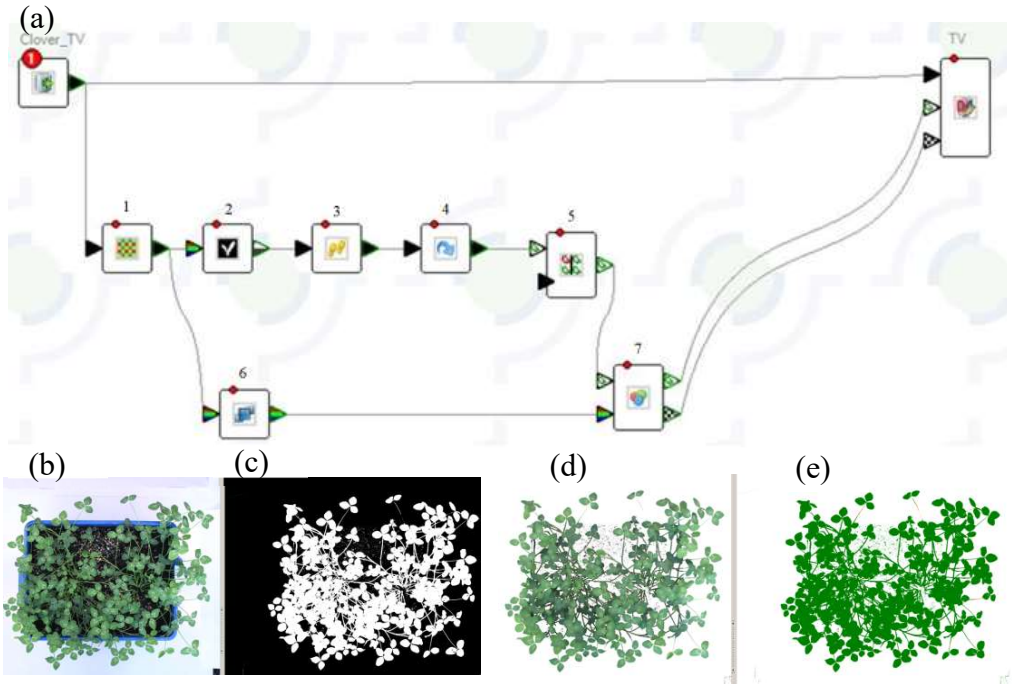


Figure 4.9. Digital image analysis of drought stress tolerance trials using Lemnagrid software (a) Image analysis pipeline of drought stress tolerance trials from Lemnagrid software (Lemnatec, Würselen, Germany). Numbers identify the different steps of image analysis. (1) 'demosaicing', reconstructs a colour image from a bayer colour pattern, defined as the immediate output produced by a digital camera (2) 'nearest neighbour foreground/background colour separation', colours are classified as part of the region of interest (foreground), or as background (panel c) from the original image (panel b). (3) 'multi step morphological', small unwanted areas from background are excluded from the analysis. (4) 'converter', allows to switch over different kinds of data formats. (5) 'object composition' merges a set of spatially independent objects within one region of interest to one bigger object (panel d). (6) 'mean shift', clusters regions and applies the mean colour value to that specific region. (7) 'colour classification', classify image colours as non-senescing and senescing tissue (panel e).

Limited leaf senescence (identified as colour change from green to yellow and brown) was observed in the plants grown under drought stress. Furthermore, although imaging was carried out on a stand using

artificial light in order to minimize light variations, changes in background lighting conditions in the glasshouse depending on time of the day, and sunlight fluctuations, restricted the detection of slight colour changes in the plants, such as green to light green. This limited the measurement of leaf senescence by digital imaging of plants during the trial.

Forage yields were estimated as counts of the pixels classified as green or non-senescing. In order to evaluate whether the yield estimations were accurate, correlations between green pixel count and fresh and dry weights was measured. A correlation of r^2=0.8122 (p value=1.7E^{-35}) for fresh weights and 0.7638 (p value=2.8E^{-30}) was estimated for dry weights (Figure 4.10). In fresh weights data, a higher dispersion of the data was observed in bigger plants, where superimposition of leaves is higher. Conversely, a higher accuracy in the pixel counts for smaller plants, where superposition of leaves is reduced. However, this tendency is not clearly observed in dry weights (Figure 4.10).

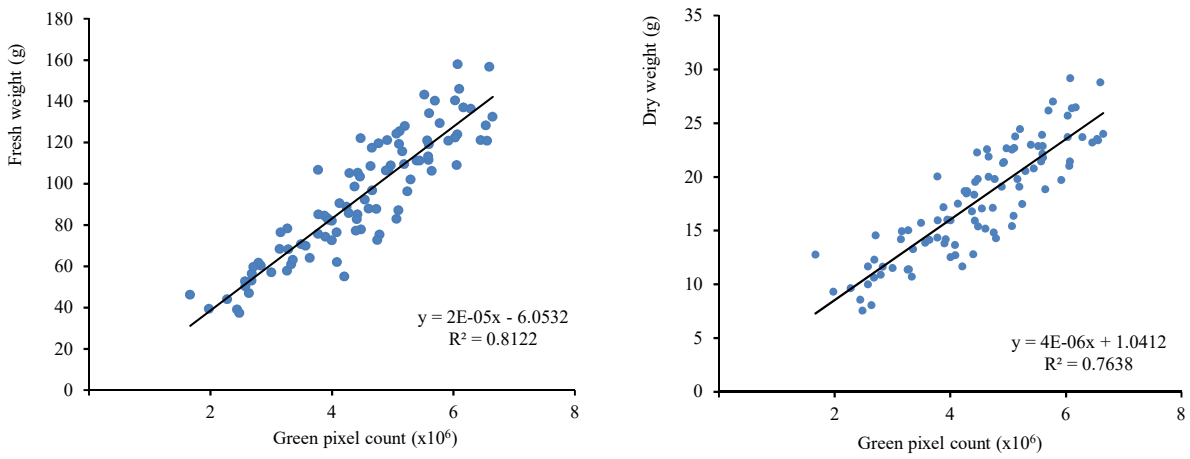


Figure 4.10. Relationship between weights and estimated yields by digital image analysis.

Associated to this, when estimated forage yields were followed during time, plants with lower sizes showed less fluctuations than plant of higher sizes. This is exemplified in Figure 4.11: for event 11, whose plants sizes are high in comparison with the average of plants analysed in the trial, fluctuations of estimated forage yields were observed during time. Conversely, events whose plant sizes were smaller, such as 21 and 31, these oscillations are minimized (Figure 4.11 and Figure 4.12).

Nevertheless, and most importantly, a higher forage production was estimated for event 11 against its non-transgenic control by digital imaging, which is correlated to the higher fresh weights measured in the second drought stage. Similarly, event 31 exhibited increased growth in all the three stages of evaluation when compared to its non-transgenic control. In the first stage of drought, the estimated forage yield of event 31 actually increases after day 31 of drought.

Plants were selected based on similar size and transplanted to large tubs (35 cm long, 26 cm wide, and 17 cm deep) 20 days before drought stress application, and maintained at water at 90% SWC during this period. However, event 31 exhibited higher yielding plants at the trial commencement at day 1 (Figure 4.11). Given that similar sized plants were selected for the trial, this difference can be explained by an increased growth of transgenic relative to the non-transgenic control growing under replete water in the 20 days prior to trial start. In addition, the estimated forage yield difference to non-transgenic controls rose as SWC decreased. These results are consistent with a higher forage production both under well-watered and drought stress conditions in event 31 (Figure 4.11). However, in event 11 the increase in yield was first observed when SWC was approximately 30%. As aforementioned, events 5 and 21 exhibited decreased forage yield compared to their non-transgenic controls. Digital image-based analysis enabled to observe that the forage yield was higher for the non-transgenic control at day one of the experiment commencement for events 5 and 21 (Figure 4.12). This suggests that the yield reduction for these two events was not caused by drought stress, and that vigour of these plants was already reduced under well-watered conditions on previous days of the trial start.

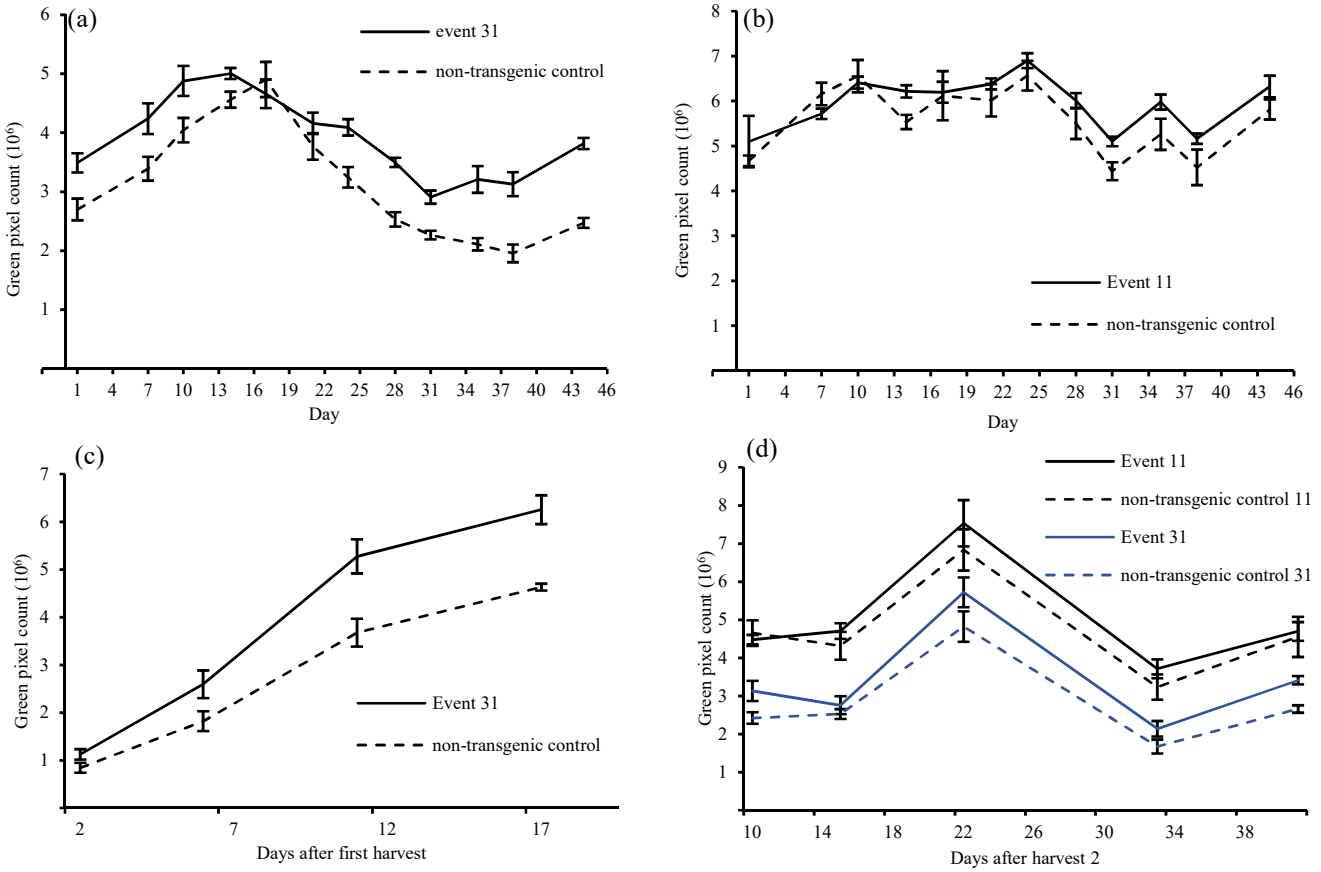


Figure 4.11. Forage yield estimations for transgenic events by digital image analysis. (a) Forage yield estimations in event 31 in the first round of drought. (b) Results for event 11 from the first round of drought. (c) Forage yield estimations during regrowth stage for event 31 (d) Forage yield estimations for events 11 and 31 at the second round of drought. Green pixel counts were obtained by using Lemnagrid software.

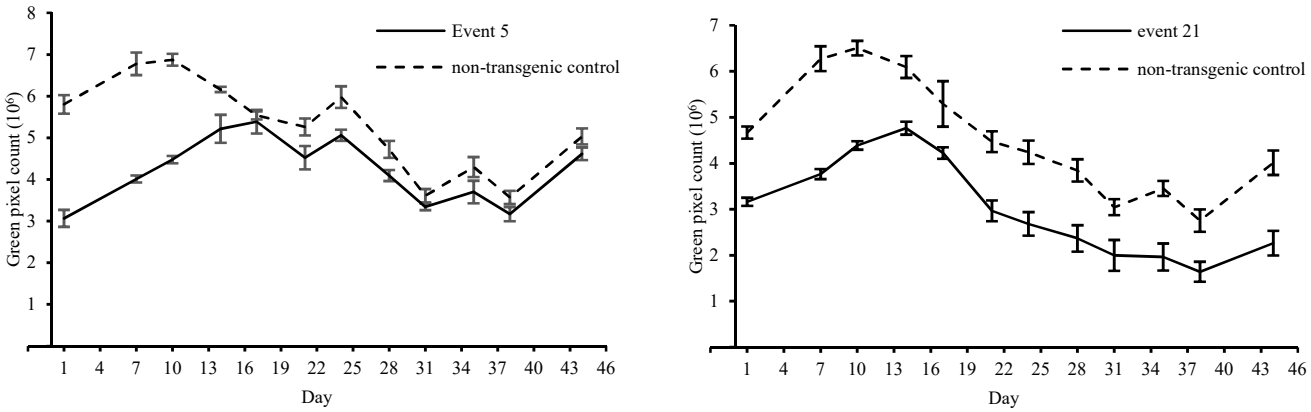


Figure 4.12. Forage yield estimations for events 5 and 21 by digital image analysis during the first round of drought. Green pixel counts were obtained by using Lemnagrid software.

No clear correlation between the response of the transgenic events and *IPT* transcript abundance was observed. However, similar transcription levels were measured for the two events that exhibited increased growth under water limitation (events 11 and 31) (Figure 3.10). Transcript abundance of event 21 was measured in order to elucidate if its decrease in forage yields was caused by a high cytokinin production. However, no *IPT* transcripts were detected for this event. Interestingly, highest *IPT* transcript levels were observed for event 34, which did not show increased growth under drought stress. Nevertheless, these differences are not statistically significant.

4.4 Discussion

The observed of delayed leaf senescence in 13 events of the 30 generated, and the apparent absence of negatively affected phenotypes when grown in the glasshouse under well-watered conditions, is evidence of the effectiveness of At*myb32:IPT* cassette with two additional linked expression cassettes in a single T-DNA.

Event 31 exhibited better regrowth and increased yields under fully watered conditions. Similar results were observed previously in *IPT* alfalfa controlled by the *SAG12* promoter, where a higher forage production after regrowth was observed (Calderini et al., 2007). Also higher seed yields under rainfed and irrigated conditions were previously observed in canola expressing *IPT* controlled by the same promoter that used in this work (Kant et al., 2015). In the present work, two independent events, 31 and 11, exhibited higher yields under water limiting conditions. Increased tolerance to drought stress was also previously observed in a number of studies where different promoters were used in a variety of species (Rivero et al., 2007; Kuppu et al., 2013). However, as far as we are aware, this is the first drought stress tolerance evaluation in a forage crop expressing an *IPT* transgene. These results provide support for the qualified application of expression of an *IPT* transgene controlled by the At*MYB32* promoter to generate white clover plants expressing higher forage yields under both well-watered and water limited conditions.

The absence of penalty yields in 10 of the 12 events evaluated under water limited conditions in the glasshouse, is further evidence of the utility of the At*myb32:IPT* cassette for delayed leaf senescence and drought stress tolerance. There were, however, two events from the 12 evaluated, 5 and 21, that exhibited decreased vigour from the start of the experiment. A number of hypotheses could explain this result. Cytokinin production in high concentrations could be an element contributing to a negative effect.

Although different studies show that expression of an *IPT* transgene could bring benefits to crop production, the selection of an effective promoter is crucial. High cytokinin production can generate undesired phenotypes, such as plants with reduced mature plant and leaf size and impaired root growth (Gan and Amasino, 1996). Additionally, many published reports describe a reduction in yields in transgenic events with high expression of transgenes controlled by constitutive promoters such as Ca*MV35S* (Cominelli et al., 2013). However, the non-detectable levels of *IPT* transcripts may rule out this explanation for event 21. Other possible negative effects include undesirable mutations which may arise during tissue culture, or insertional mutations caused by transgenesis (Xiao et al., 2007).

Although no visible symptoms were evident when plants were grown in small tubs under non-stress conditions, evaluating these plants over a long timeframe in large pots under stress, exposed differences in forage production. Therefore, the evaluation of the events in a large-scale experiment under drought stress contributed to a more stringent selection of events, and enabled discard of events with possible yield penalties.

Low *IPT* transgene transcript levels in relation to the reference gene were measured in the events whose transcripts were detected (0.0013, 0.0026, and 0.00085 ratios in events 11, 31 and 34 respectively). The observation of delayed leaf senescence, the potential observation of drought stress tolerance, and the absence of undesired effects in these events, suggest that low transcription levels for a limited increase in cytokinin production is necessary in order to produce the desired phenotype. Nevertheless, increases in *IPT* transgene transcription levels from the inserted transgene do not confirm increases in cytokinin production. Therefore, cytokinin production measurements for these events is necessary to confirm this hypothesis.

When transcript abundance was compared between events, intermediate values were observed in events 11 and 31. However, event 34 exhibited delayed leaf senescence and the highest transcript abundance of the *IPT* transgene in the events analysed, but no drought stress tolerance was detected. Interestingly, the two events with the most similar transcription levels are those that exhibited drought tolerance. This suggests that a fine tuning of the levels of *IPT* transcription are necessary to generate plants with potential for drought stress tolerance. However, as the observed differences between transcription levels for events 11, 31 and 34 were not statistically significant, this observation is not conclusive. In order to generate enough information that support this observation, a higher number of events should be evaluated.

Events 21 and 25 exhibited delayed leaf senescence in the detached leaf experiments. However, no *IPT* expression was detected for these events. The absence of, or very low levels of transcription measured in events 21 and 25 suggests the occurrence of transgene silencing, and it could be explained by a number of factors discussed in Chapter 3. Furthermore, although *IPT* transgene transcript levels were not measured for all of the events generated, the absence of delayed leaf senescence for a number of events may be due to a low or inexistent *IPT* transgene transcription, which may translate in no increase in cytokinin production. The erratic observation of delayed leaf senescence in some events, and the absence of transcription for events that previously exhibited delayed leaf senescence, lead us to speculate that transcription levels may vary depending on many factors, such as time of day or developmental stage of the plants. A wide variation in transcripts levels between events for the three transgenes was observed, and may have contributed to a reduction in the number of the events exhibiting the altered phenotype. However, the generation and evaluation of a large number of events made possible the identification of some that exhibited the desired phenotype.

The weak correlation between transcript abundance and phenotypes observed can be explained by differential *IPT* transgene transcription activity at different plant developmental stages, time of day, and environmental conditions. Samples for transcript abundance were obtained under well-watered conditions. However, ideally, in order to capture the potential transcription variation, transcript abundance measured at different time-points and in a range of tissues would be recommended. In addition, estimation of transcript abundance in a higher number of events would be a sensible strategy in order to shed light on the levels of *IPT* transcription required for display of the altered phenotype. Nevertheless, cytokinin level measurements would provide more definitive answers on correlations between transcript abundance, hormone levels and the phenotypes observed. Also, information on cytokinin production in different organs and at various environmental conditions, would shed light on the physiological dynamics of leaf senescence in white clover expressing *IPT*. The reduction on the number of events analysed, based on *IPT* transgene transcript abundance analysis and delayed leaf senescence results, would enable to perform a detailed cytokinin production analysis. However, such a study was beyond the scope of this project.

Many published reports show drought stress tolerance by expressing *IPT* in different species, while others report delayed leaf senescence in association with undesirable phenotypes (Smigocki and Owens, 1989). A variety of promoters were used, some inducible by senescence while others were constitutive.

Nevertheless, it seems to be a common factor that promoters are not associated with high *IPT* expression, and their expression is confined to particular organs or tissues, and/or specific environmental conditions.

The development of phenomics in recent years has allowed the development of protocols for phenotyping using digital image analysis. The high correlation observed between fresh weights and green pixel counts demonstrates the applicability of the protocol described here for white forage clover yield estimation. By this method, an estimation of shoot development and plant growth progress can be performed during the course of the trial.

A protocol for digital image analysis in the drought stress trial was developed in this work, and forage yields were estimated during the course of the trial with relative accuracy. This allowed the evaluation of growth changes in plants under stress without the need of harvest. However, optimization of some factors such as plant size, background colors, and consistent lighting are needed to increase accuracy of white clover yield estimations.

The digital image analysis approach was also applied for evaluating color change in detached leaf experiments. Previously, delayed leaf senescence has been evaluated by visual inspection of detached leaves, and manual scoring. The use of digital image analysis enabled us to quantify accurately color change of leaves and thus have a better estimation of delayed leaf senescence. This innovation has set the stage for future delayed leaf senescence and drought tolerance assays on transgenic white clover plants in glasshouse conditions. Moreover, similar experiment protocols can be transferred to other related species, such as alfalfa or other *Trifolium* species.

The previous reports of increased seed yields observed in white clover transgenic for *IPT* support the need for seed yield evaluations of the selected events during subsequent generations of elite events production. Additionally, evaluations of advanced elite events should be performed in field conditions over multiple years and ideally at multiple sites, in order to study the effects on phenotype of the inserted genes over the long term. Considering that leaf senescence can be triggered by many factors such as shading, heat, and frost, and given the positive results previously reported by Lin et al (2007, 2010) in a long-term field trial, we speculate that a greater impact on white clover persistence would be observed in long-term field trials.

5 Phenotyping of triple stacked transgenic white clover plants for tolerance to aluminium

5.1 Introduction

Aluminium toxicity in soils is a major factor affecting plant growth and crop production. This problem is directly associated to acidic soils, which comprises approximately 30% of the world's ice-free land (Uexküll and Mutert, 1995). These soils extend for approximately 60% of the world's tropics and subtropics, and affect production of relevant grain crops such as rice and maize significantly (Uexküll and Mutert, 1995). In Australia, soil acidity is one of the main problems impacting productivity in agriculture (Ryan, 2018). Approximately 33 Mha have a pH lower than 4.8, and approximately half of these soils are in New South Wales and Victoria (Scott et al., 2000). The acidity of soils can generate the solubilisation of Al forms that are toxic to plants, such as the trivalent cation $Al(H_2O)_6^{3+}$ (known as Al^{3+}) and $Al(OH)_2Al^{3+}$ (Kochian, 1995; Martin, 1986). The most observable negative effect of Al^{3+} is inhibition of root growth, which causes a decrease in water and nutrient uptake, and consequently, a reduction in crop production yields (Delhaize and Ryan, 1995).

Plants can tolerate Al^{3+} by different mechanisms. The best understood involves the exudation of organic acids into the rhizosphere. Organic acids can form stable complexes with Al^{3+}, thus reducing uptake of toxic forms of aluminium by the roots (Delhaize et al., 1993; Kochian et al., 2004). Also, some species such as hydrangea (*Hydrangea macrophylla*) and buckwheat (*Fagopyrum esculentum*) show tolerance through detoxification of Al^{3+} via Al^{3+} complexing with organic acids within cells (Ma, et al., 2001; Ma et al., 1997).

The combination of Al^{3+} toxicity, acidity of soils and low phosphate availability can hinder white clover production significantly (Lowther, 1980). Particularly in Australia, acidity of soils and its associated Al^{3+} toxicity is an major limitation in crop production (Scott et al., 2000). In Europe, and North America this problem has been ameliorated by application of lime to soils to increase pH. However, use of lime is expensive and thus generally not economically viable for extensive grazing systems (Scott et al., 2000). As an alternative, researchers have focused on generation of Al^{3+} tolerant crops. Although white clover lines with potential tolerance to aluminium have been identified, the results generated in the field are not conclusive (Caradus et al., 2001). Given these constraints, the development of Al^{3+} tolerant white clover plants through transgenesis appears an attractive option.

Most of the work to obtain Al^{3+} tolerance in other species through genetic engineering (or employing plant transformation-based strategies) has focused on overexpression of the genes coding for enzymes involved in the organic acid metabolism, principally for malate dehydrogenase and citrate synthase, in order to increase organic acid production. Potential Al^{3+} tolerant plants were obtained by expression of malate dehydrogenase (Tesfaye et al., 2001) in alfalfa, and citrate dehydrogenase in canola (*Brassica napus*) (Anoop et al., 2003), Arabidopsis (Koyama et al., 2000), tobacco (*Nicotiana tabacum*) (De la Fuente et al., 1997) and alfalfa (*Medicago sativum*) (Barone et al., 2008).

Tesfaye et al. (2001) overexpressed the nodule enhanced isoform of *MDH* from alfalfa controlled by the constitutive promoter Ca*MV35S* and observed increased production of organic acids and Al^{3+} tolerance in both soil and hydroponic culture conditions. As described in previous chapters in this work, transgenic white clover plants were generated by insertion of single T-DNA containing a previously isolated nodule enhanced isoform of *MDH* from white clover, controlled by the root specific promoter *TrPt1* from white clover (Labandera, 2007), together with two other genes, CP-*AMV*, for alfalfa mosaic virus (AMV) resistance (Panter et al., 2012), and *IPT* from Agrobacterium, for delayed leaf senescence. The molecular characterisation of the gene CP-*AMV* was described in Chapters 2 and 3, while the phenotyping and molecular characterisation of *IPT*, responsible for the trait delayed leaf senescence, was described in Chapters 2, 3, and 4. The current chapter focus on phenotyping for Al^{3+} tolerance, and comparing the molecular information generated for the Tr*neMDH* gene (described in Chapters 2 and 3) with the phenotyping results generated.

By overexpressing Tr*neMDH* in roots, we expect to increase malate production and exudation to the rhizosphere. Earlier observations of root-tip staining with hemotoxylin in transgenic white clover expressing the Tr*Pt1:*Tr*neMDH* cassette provides evidence that the Al^{3+} tolerance mechanism of these transgenic plants is based on Al^{3+} exclusion (Labandera, 2007). As previously discussed, this mechanism is based on quelation of Al^{3+} by the exuded organic acids and a consequential immobilisation of toxic Al^{3+} in the soil.

As discussed in Chapter 1, solution culture-based methods for Al^{3+} tolerance evaluation of events whose tolerance mechanism is based on Al^{3+} exclusion may have its limitations. Arguably, a rapid diffusion of excreted malate into the solution may occur (Dong-Man Khu et al., 2012). On the other hand, excreted organic acids would be retained in the rhizosphere matrix in soil-based experiments. Therefore, we proposed to perform soil-based evaluations of the transgenic events. We hypothesized that using this

method would enable the tolerance mechanism to work more efficiently and therefore would facilitate identification of Al^{3+} tolerant lines.

Usually, soil-based experiments for Al^{3+} tolerance evaluation are performed in the long term (Samac and Tesfaye, 2003). In these cases, instead of using indirect methods such as root tip growth measurements to estimate Al^{3+} toxicity, a direct estimation of shoot and root production yields can be performed. However, long-term soil-based experiments can be difficult to perform, as they require the availability of large cropping areas, and demand high labour inputs. In addition, root weights are only measured at the end of the trial, without considering initial root development, and initial root size variability between replicates.

A different approach was reported by Tang et al. (2003), who performed soil-based Al^{3+} tolerance assays in wheat over 6 days, in which root length was measured. Considering this precedent, we attempted to carry out a soil-based experiment in over a relatively short period (15 days), in order to measure net root growth during the trial. The performance of such experiment would allow the development of a simple and short-term soil-based trial for transgenic white clover evaluations.

A preliminary approach to a digital image-based evaluation of root growth in soil of white clover plants is described in this chapter. Usually, non-soil-based assays in liquid media, agar, or paper are performed in order to facilitate access to roots and minimize root destruction (Zhu et al., 2011). However, these approaches may not accurately represent root growth in field conditions as soil-based assays (Zhu et al., 2011). Setting up a short-term assay in soil, such as the one described here, would enable the performance of root imaging from an Al^{3+} tolerance soil-based assay, whilst minimizing the difficulties of root imaging in soil conditions.

Preliminary screening of transgenic and non-transgenic control lines for Al^{3+} tolerance in hydroponic conditions was performed. This was designed to complement the soil-based experiment and provides additional information toward the identification of events with increased tolerance to Al^{3+} toxic conditions. A similar approach to that used by Tesfaye et al. (2001), where root growth was evaluated in a short term, without the addition of nutrients was implemented, based on the assumption that the presence of nutrients could interfere with the observation of Al^{3+} toxic effects. Also, chemical interaction between Al^{3+}, and phosphate and sulphur could occur when using complete nutrient solutions (Samac and Tesfaye, 2003). Therefore, the experiment was performed using stolon cuttings in their early

developmental stages, where storage reserves are used for growth and therefore that addition of nutrients was unnecessary.

5.2 Materials and methods

5.2.1 Plant material

Transgenic events (T_0) were multiplied by stolon cuttings in order to obtain the necessary number of replicates for phenotyping experiments. Plants were maintained in the glasshouse under 22°C day/16°C night, 16 h light/ 8 h dark.

5.2.2 Al tolerance experiments

Soil-based assay

Eleven transgenic events showing increased Tr*neMDH* transcript levels respective to the non-transgenic lines, were evaluated in soil for Al^{3+} tolerance by measuring root growth. Stolons were grown in vermiculite for two weeks until primary roots were generated. Plants were grown in the glasshouse under 22°C day/16°C night, 16 h light/ 8 h dark in 85 mm pots in high aluminium soil for 15 days. The soil used was Dermosol; with soil pH, 4.02; $CaCl_2$-exchangeable Al (ICP), 54.3 mg kg^{-1}; Colwell-P, 3.2 mg kg^{-1}; PBI, 739; total C, 38.8 mg g^{-1}; total N, 1.74 mg g^{-1}, kindly provided by Professor Caixian Tang (Dept. of Animal, Plant and Soil Sciences, La Trobe University). Transgenic plant root growth in Al^{3+} toxic conditions was evaluated relative to that of non-transformed isogenic control plants. At the end of the experiment, plants were removed from soil and the roots washed. Shoot and root fresh and dry weights were measured. Plants were placed horizontally on a stand, and root digital images were taken at the start and at the end of the experiment using a Canon 500d camera mounted on a stand at a height of 107 cm (Canon, Tokyo, Japan). Camera settings were F Stop=8.0, ISO=100, and lighting=tungsten. Images were analysed by Lemnagrid software (Lemnatec, Würselen, Germany).

Initial root growth (IRG), which is the length of the main longest root at the start of the experiment, and final root growth (FRG), which is the length of the longest main root at the end of the experiment, were measured. Net stress root growth (NSRG) was measured by subtracting IRG from FRG. Relative stress root growth (RSRG) was calculated by dividing the net stress root growth (NSRG) by the initial root growth (IRG) (Howeler, 1991; Rossello, 2011). Plants were arranged in a complete block design, and

five blocks per event were evaluated. Data was log transformed, and statistical significance evaluated by single-factor ANOVA with randomized blocks.

Solution culture assay

Nine events were evaluated in solution culture for Al^{3+} tolerance. Stolons were grown in vermiculite for two weeks until primary roots were generated. Five plants (replicates) per transgenic event, and 5 per each respective non-transgenic control were transferred to a container with 30 L of 0.5 mM $CaCl_2$ solution, pH 4.3, in a controlled environment room under 21°C and 14/10 h day/night. Plants were maintained for one day in these conditions for adaptation. Initial root growth was evaluated and plants were transferred to a container with 30 L 0.5 mM $CaCl_2$, 15 µM $AlCl_3$ solution, pH 4.3. Five plants per transgenic event, and 5 per each respective non-transgenic control were transferred to a control solution with no aluminium. pH was adjusted by HCl or NaOH addition. Final root growth was measured after 3 days of growth in aluminium containing solution. Percentage relative root growth (%RRG) was estimated as [(mean net root growth of transgenic)/ (mean net root growth of isogenic control)] x 100. Error propagation from RRG calculations was estimated using the equation $SE_{RRL} = RRG\ [(SE_x /x)^2 + (SE_y/y)^2]^{1/2}$. Plants were evaluated in a split plot design, and 3 to 5 replicates per event were evaluated. Statistical significance was evaluated by single factor ANOVA using GenStat for Windows 18th edition (VSN International, Hemel Hempstead, UK).

5.3 Results

As described in Chapter 3, Tr*neMDH* transcript abundance was evaluated in root tips of 28 transgenic events under non-challenging conditions. Six events showed a statistically significant Tr*neMDH* transcript level increase when compared to their non-transgenic controls (p-value=0.05), and event 26 exhibited a statistically significant decrease (Figure 3.11).

5.3.1 Soil-based experiment

Transgenic events that exhibited significant increase in Tr*neMDH* transcript levels, and some events showing non-statistically significant increases were selected for an aluminium tolerance trial in soil under glasshouse conditions. In this experiment, root growth of the transgenic plants and their respective

non-transgenic isogenic controls was evaluated after being growth for two weeks in acidic soil with elevated concentrations of Al^{3+}.

For some highly susceptible species, the use of Al^{3+} toxic soil can be too stringent for Al^{3+} tolerance evaluations (Foy et al., 1993; Samac and Tesfaye, 2003). Previously to this experiment, white clover plants were grown in Al^{3+} toxic soil in order to evaluate their survival in such challenging conditions (see Materials and Methods), and to estimate the levels of root growth impairment under these conditions. Root growth was interrupted for the first five days of the evaluation, but root growth was observed in the following 10 days. Furthermore, the plants evaluated exhibited affected shoot phenotypes in both transgenic and non-transgenic controls, including colour change such as leaf yellowing, and delayed growth.

Root growth of the transgenic plants and their respective non-transgenic isogenic controls was evaluated after being growth for 15 days in acidic, Al^{3+} toxic soil (Figure 5.1). Treated plants exhibited an estimated net root growth mean of 7.4 cm, with an initial 7.4 and a final root length of 14.8 cm in 15 days of the experiment duration. Although plants were obviously affected by the soil used, the observation of a measurable root growth validates the performance of Al^{3+} tolerance evaluations by root length measurements using the above described Al^{3+} toxic soil (see Materials and Methods).

Plantlets used in this experiment had similar sizes, and root length was similar at the start of the experiment. However, the variation in final fresh root weights between replicates was high. Furthermore, this variability extended to net stress root growth (NSRG), and relative stress root growth (RSRG). Nevertheless, some trends could be observed.

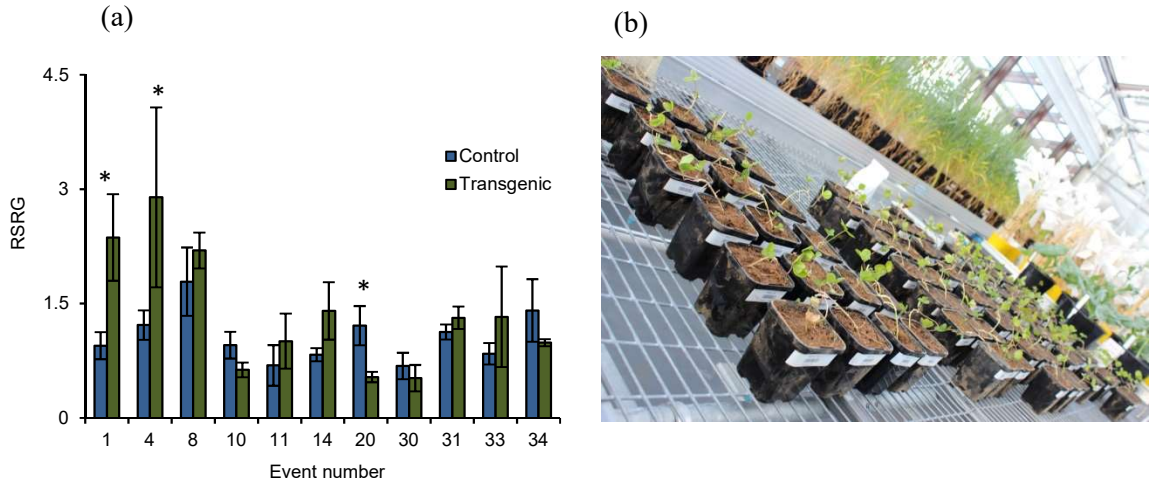


Figure 5.1. (a) Aluminium tolerance soil-based experiment results. Relative stress root growth (RSRG) was calculated by dividing the net stress root growth (NSRG) by the initial root growth (IRG). (b) Image of aluminium tolerance soil-based experiment. Plants were arranged in a complete block design, and five blocks per event were evaluated. Asterisks represent significant values (p value<0.05). Statistical significance was evaluated by single factor ANOVA.

Two transgenic events, 1 and 4, exhibited a statistically significant difference in RSRG (p value<0.05), when compared to their respective non-transgenic isogenic lines (Figure 5.1). On the contrary, a significant decrease of RSRG was observed for event 20 versus its non-transgenic control line (p value<0.05) (Figure 5.1). Although a trend toward a higher mean RSRG for transgenic versus non-transgenic controls was observed, this difference was not significant (p value>0.11).

5.3.2 Root imaging evaluation

Initial and final root length was determined in the above described soil-based assay as a way of studying the toxic effects of Al^{3+}. In addition, root weights were determined at the end of the assay. However, given that the initial root sizes between replicates can be high, determination of growth of the entire root by conventional methods such as root weight, may not be accurate. By using imaging, root growth can be estimated relative to the initial root size, which would enable standardization of the final values generated. Furthermore, although RSRG estimated by measuring the growth of the main root is a widely used method for Al^{3+} toxicity estimation, it may not reflect the effect of Al^{3+} on the entire root. Therefore, we employed a digital image-based method to estimate whole root growth.

A good correlation was observed between fresh root weights and estimated root size by digital image analysis, ranging from 0.80 to 0.98 for the different events evaluated (Supplementary figure 5.1). This result enabled a determination of root size by imaging. IRG, FRG and RSRG was determined using pixel counts from the roots images. The digital image-based root growth estimation enabled the identification of a significant image-based RSRG difference for event 31 when compared to the non-transgenic control (p value<0.05) (Figure 5.2).

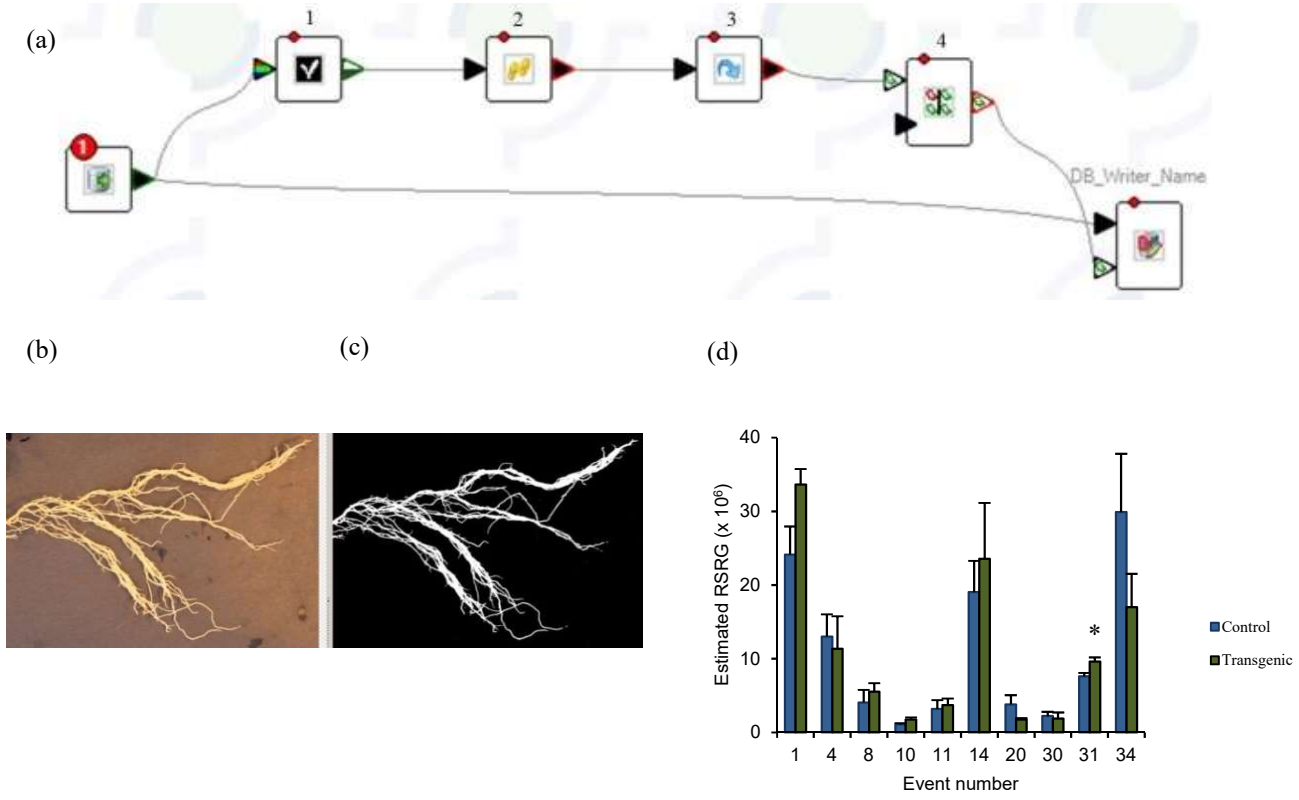


Figure 5.2. Digital imaging-based estimation of root growth. (a) (1) 'nearest neighbour foreground/background colour separation', colours are classified as part of the region of interest (foreground), or as background (panel c) from the original image (panel b). (2) 'multi step morphological', small unwanted areas are excluded from the analysis. (3) 'converter', allows to switch over different kinds of data formats. (4) 'object composition' merges a set of spatially independent objects within one region of interest to one bigger object. (panel d) Pixel counts were estimated for images taken at the start and the end of the trial and initial and final root growth (IRG) was estimated. Relative stress root growth (RSRG) was calculated by dividing the estimated net stress root growth (NSRG) by the estimated initial root growth (IRG). Asterisk denote significant difference at p value<0.05.

5.3.3 Solution culture experiment

Based on previous evaluations by Rossello (2011), where a higher contrast in root growth between the transgenic plants and their non-transgenic controls was observed using an Al^{3+} concentration of 10 µM, plants here were evaluated in a solution containing 15 µM of Al^{3+}. Percentage relative root growth (%RRG) was calculated. This parameter enables the estimation of root growth relative to the control without Al^{3+}. A split-plot design was used and 3 to 5 replicates per genotype were evaluated (Figure 5.3).

Higher %RRG values in events 10, 11, 20, and a lower value in event 31, were observed when compared to their respective non-transgenic controls (Figure 5.4). However, these differences were not significant. Contrary to what was observed in the soil-based experiment, where event 20 exhibited a significant RSRG decrease, an opposite trend was observed.

No significant differences were observed when grand means of RSRG of transgenic vs. non-transgenic plants grown in Al^{3+} treatment were compared. Furthermore, no significant difference was observed when comparing grand means of RSRG of transgenic vs. non-transgenic plants without Al^{3+}. Significant lower RSRG grand means were observed in transgenic plants when grown in Al^{3+} treatment versus no-Al^{3+} controls (p value<$1.74E^{-7}$). Similarly, significant lower RSRG grand means were observed in non-transgenic controls plants when grown in Al^{3+} treatment versus no-Al^{3+} controls (p value<$2.23E^{-8}$).

Figure 5.3. Al^{3+} tolerance assay of transgenic white clover in solution culture. Left: set up of solution culture assay. Right: Image of plants from a transgenic event (event 20) and its non-transgenic control at the end of the evaluation in solution culture. Al(+) treatment: 15 µM Al^{3+}; Al(-): No aluminium control.

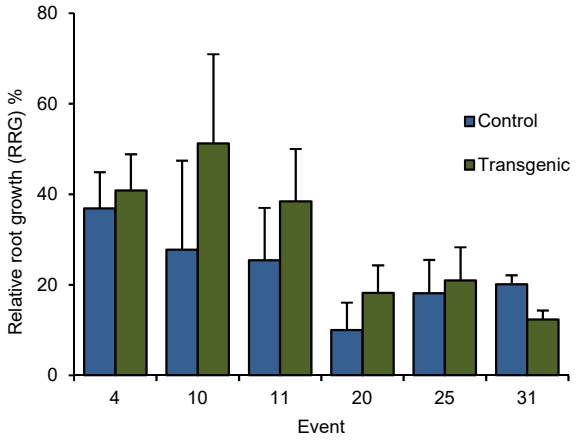


Figure 5.4. %RRG from events evaluated in Al tolerance assays in solution culture. Relative root growth (RRG) was calculated as (net root growth with Al^{3+}/net root growth without Al^{3+})*100. Plants were evaluated in a split plot design, and 3 to 5 replicates per event were evaluated.

5.4 Discussion

As previously discussed, the use of solution culture for evaluation of aluminium tolerance in transgenic plants, whose potential Al^{3+} tolerance is based on an Al^{3+} exclusion mechanism, can be difficult. Furthermore, and more importantly, the evaluation of plants in soil is more relevant to what happens in the field (Narasimhamoorthy et al., 2007). Therefore, soil-based trials undertaken in this work for aluminium tolerance evaluation were intended to overcome this limitation of solution culture trials. Aluminium tolerance of the transgenic events that exhibit increased Tr*neMDH* transcript levels was evaluated in soil experiments in the glasshouse. Overall, results from soil-based experiments and solution culture have a weak correlation. Consequently, Narasimhamoorthy et al. (2007) recommend the use of more than one method for Al^{3+} tolerance evaluation of transgenic events.

Root growth was measured as a metric for Al^{3+} toxicity stress in the soil-based trial. Some events exhibited an increase in root length, measured in RSRG. Significant RSRG differences were observed between transgenic and their respective non-transgenic controls for two events (1 and 4). This supports the use of Tr*Pt1*:Tr*neMDH* linked to the other two genes of interest in a single T-DNA to confer

aluminium tolerance. However, although these results may indicate an increased tolerance to Al^{3+} toxic conditions for events 1 and 4, the variability observed between replicates lead as to be cautious. The high variability observed could be explained by a high variation in soil composition from the different replicate pots. In this regard, previous studies report a high variation in Al^{3+} saturation in soils, even when they exhibit similar pH (Johnson et al., 1997; Samac and Tesfaye, 2003). Also, the toxic effects of Manganese (Mn) could mask the observation of Al^{3+} toxicity effects (Johnson et al., 1997; Samac and Tesfaye, 2003).

Excessive toxicity of soils containing Al^{3+} could also make difficult the identification of Al^{3+} tolerant plants. This limitation was observed by Foy et al. (1993), who studied Al^{3+} tolerance of sorghum in soil. However, in this case identification of tolerant sorghum plants was successful after liming Al^{3+} toxic soil in order to increase pH from 4.0 to 4.3. Similarly, liming the soil used in the current project in order to reduce Al^{3+} toxicity could be a sensible strategy toward a better separation of tolerant white clover plants.

A digital image-based system was developed for evaluation of roots in white clover. This enabled the estimation of the entire root growth, relative to initial root size. Although this information is valuable, as root growth is standardized in relation to initial growth, no conclusions should be reached in this study, due to errors associated with imaging, such as superposition of lateral roots, image quality, and error propagation associated to RSRG calculation.

In order to complement the results generated by soil-based assays, a solution culture assay was performed. The solution culture experiment was carried out in a solution without nutrients. This strategy was performed to avoid complexation of Al^{3+} with nutrients such as phosphate (Samac and Tesfaye, 2003). The assay was performed over a short timespan to counter negative effects of using nutrient-free media. The observation of root growth for the duration of the experiment indicates the feasibility of using such a system. The brief duration of the experiment allowed root growth based on storage nutrients from stolons.

Although no events with significantly increased Al^{3+} tolerance were identified by the solution culture assay, differences might be observed by evaluating a higher number of replicates. The production of a higher number of stolon cuttings, and selection of the most similar sized before the experiment start could increase the accuracy of this evaluation. Considering that this assay is less time consuming than

the performed soil-assay, using solution culture for the first screenings for selection the best performing events may be a sensible strategy.

A significant NSRG was estimated in event 20 against its respective non-transgenic counterpart in solution culture assays (Supplementary figure 5.2). Given variability between plants for both soil and solution culture experiments can be high, the effectiveness of using absolute root length and NSRG measurements for evaluation of Al^{3+} tolerance can be limited. However, from an agronomical point of view, this information can be valuable, and events that exhibited increased absolute root length or NSRG should be considered for evaluations in the following generations.

Different results were observed for some events when soil and solution culture experiments were compared. For example, a lower RSRG was observed for the non-transgenic line in event 20 in soil (Figure 5.1), and an opposite tendency was observed in solution culture assays (Figure 5.4). Similarly, a higher root growth was estimated by digital imaging for event 31 when compared to its non-transgenic control line in soil experiments (Figure 5.2), and lower %RRG (Figure 5.4) and NSRG (significant at p value<0.05) (Supplementary figure 5.2) was measured in solution culture in event 31 when compared to its control line. Although in some cases a lack of correlation between soil-based and solution culture methods for evaluating Al^{3+} tolerance occurs, these methods can be complementary, as root growth is evaluated at different stages of the plant (Narasimhamoorthy et al., 2007).

Additionally, confounding negative effects on root growth can be observed as a consequence of cytokinins overproduction and Al^{3+} toxicity when phenotyping the generated events for Al^{3+} tolerance. Nevertheless, except for event 20 when evaluated in soil and event 31 which showed a reduced NSRG when evaluated solution culture, no significant reduction in root growth was observed for the events generated. The absence of root growth reduction observed in the transgenic plants provides additional evidence that supports the transformation of white clover with the At*myb32:IPT::*Tr*Pt1:*Tr*neMDH::*CaMV*35S:*CP-*AMV* construct used in this work. These results also provide evidence on the effectiveness of using a At*myb32* promoter modified for reduced *IPT* expression in roots.

Reduced root growth between transgenic plants and their non-transgenic counterparts was observed for some events in alfalfa expressing *neMDH* controlled by the constitutive promoter Ca*MV35S* both in solution culture and field conditions (Tesfaye et al 2001). This outcome can be explained by the high

resource demand caused by the use of a constitutive promoter for expressing a highly relevant enzyme in the organic acid metabolism. In order to overcome this limitation, here the root specific promoter Tr*Pt1* was used. Previous work by Rossello (2011) using the same cassette as the current work in white clover reported the absence of negative effects in root growth. The absence of negative effects on root growth in the generated events provide additional information that supports the use of the Tr*Pt1:*T*rneMDH* cassette for Al^{3+} tolerance in white clover.

Some conclusions can be deployed for the next steps in the evaluation of transgenic events. The small-scale experiment of Al^{3+} tolerance in soil-based assays, performed here, enabled the identification of two events with significantly increased root growth in soil containing aluminium. Further to identifying events with the potential for aluminium tolerance, the results support the effectiveness of the soil-based approach used in this work. Nevertheless, as the variation between replicates is high, some adjustments of the trial for future evaluations may be necessary, such as increase of replicates, and the incorporation of limed soil controls.

In conclusion, a preliminary selection of events by solution culture assays, and subsequent confirmation by soil-based assays may be a sensible strategy for future evaluations. Then, in subsequent generations, evaluation of a low number of events using a high number of replicates in soil over a long period (at least one month) may allow a more accurate capture of shoot and root weights, and the identification of Al^{3+} tolerant events.

6 Selection of optimal reference genes for quantitative RT-PCR transcript abundance analysis in white clover (*Trifolium repens* L.)

6.1 Chapter preface

In this chapter, the search for reference genes in white clover for transcript abundance analysis is described. A number of 'housekeeping genes' whose transcript abundance stability was previously reported in various species were identified in white clover and evaluated. Different tests were performed, and genes with potential for use as reference genes in white clover were identified.

This chapter is presented in published format.

6.2 Publication details

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Selection of optimal reference genes for quantitative RT-PCR transcript abundance analysis in white clover (*Trifolium repens* L.)

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Abstract. Quantitative reverse transcription PCR (qRT-PCR) is a widely used method for transcript abundance analyses in plants. Relative quantification by qRT-PCR requires the use of a stably expressed reference gene. There are many 'housekeeping' genes reported in different plant species that are used as reference genes. However, it is important that the steady-state mRNA levels of these housekeeping genes are confirmed across different conditions and tissues in each species studied. Prior to this study, no comprehensive work had been performed in identifying optimal reference genes in white clover (*Trifolium repens* L.). To identify suitable reference genes in white clover, we analysed the transcript abundance stability of seven candidate genes in two organs (leaves and stolons) across two treatments (water-limited and well-watered). ΔCt, NormFinder and ANOVA tests were carried out to evaluate the mRNA level stability of candidate reference genes. According to the ΔCt results, the genes with the most stable mRNA levels were *EF1α* and *ACT11*. When stability among groups was evaluated by NormFinder, *UBQ* was the most stable across all organs and treatments. By multiple criteria, *EF1α*, followed by *ACT11* and *UBQ*, was the most stably-expressed gene across organs and treatments, and each of these are recommended as reference genes for transcript abundance studies in white clover.

Additional keywords: ΔCt, housekeeping genes, mRNA level stability, NormFinder.

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Introduction

Transcript abundance analysis is widely applied in biological studies and can be assessed using a variety of techniques. Although high-throughput techniques such as those based on microarrays and RNA sequencing allow for the simultaneous assessment of a large number of genes, other methods such as quantitative reverse transcription PCR (qRT-PCR) are more suited for transcript abundance studies of a small number of genes (Taniguchi *et al*. 2001; VanGuilder *et al*. 2008). Other quantitative techniques such as northern blotting, semiquantitative reverse transcription PCR and ribonuclease protection assays are also used to assess transcript abundance. However, they can be time-consuming and are not as sensitive as qRT-PCR. qRT-PCR is considered to be a reliable method for transcript abundance estimations and is one of the most extensively employed (VanGuilder *et al*. 2008).

However, qRT-PCR is not without its limitations. The most significant is the need for a reliable normalisation method in order to take variability in RNA concentration and quality into account. A straightforward strategy can be to normalise RNA concentrations in each reaction, but this can be inaccurate (Bustin 2002; Ginzinger 2002). A high proportion of the RNA obtained in extractions is rRNA, which makes the accurate estimation of total mRNA difficult (Vandesompele *et al*. 2002). Furthermore, RNA quality (purity and intactness), which affects reverse transcription and PCR efficiency, is not addressed by this method (Vandesompele *et al*. 2002). Absolute quantification using an external standard can also be used but the instability of such standards can limit the utility of this approach (Dhanasekaran *et al*. 2010). The best approach is to quantify transcripts relative to an internal reference gene. This requires the identification of suitable internal reference genes that are constitutively expressed.

Good internal references are typically genes that are necessary for maintaining basic cell function ('housekeeping' genes, HKG), as it is postulated that their transcript levels remain stable across tissues, development, and different environmental conditions. Some of the most frequently used are HKGs encoding actins, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin, rRNA, elongation factors and ubiquitins (Nicot *et al*. 2005; Jian *et al*. 2008). Nevertheless, variability can be found with these genes when comparing transcript abundance among tissues and experimental conditions, caused by differential rates of transcriptional activity and/or mRNA degradation. Genes that are considered stable in certain conditions or tissues may not be in others (Thellin *et al*. 1999). Furthermore, transcript abundance results can vary depending on the reference gene used. As a consequence, a systematic search for genes

whose transcription levels remain stable among tissues and conditions is crucial for reliable transcript quantification by qRT-PCR (Gutierrez *et al*. 2008).

Work directed at identifying optimal reference genes has been conducted in human cells (Silver *et al*. 2006), animals (Nygard *et al*. 2007; Robinson *et al*. 2007) and some plant species. The latter include *Arabidopsis thaliana* (L.) Heynh. (Czechowski *et al*. 2005), soybean (*Glycine max* (L.) Merr.) (Jian *et al*. 2008; Hu *et al*. 2009), and the forage crops, ryegrass (*Lolium perenne* L.) (Martin *et al*. 2008; Lee *et al*. 2010) and red clover (*Trifolium pratense* L.) (Mehdi Khanlou and Van Bockstaele 2012). Most of these species have considerable genomic and transcriptomic sequence resources that can facilitate the identification of reference genes. However, there are limited sequence data available for the forage legume white clover (*Trifolium repens* L.) (Hand *et al*. 2010). Although some work has been directed at finding a reliable reference gene in white clover (Abeynayake *et al*. 2012), more comprehensive work, taking spatial and environmental conditions into account, is needed in order to identify the optimal reference genes. The objective of this work was to identify candidate reference genes in white clover and compare their suitability across organs and conditions.

Materials and methods

Plant materials

Five different plants were used, named 5, 8, 9, 10 and 11, obtained from seeds of white clover (*Trifolium repens* L. cv. Storm). Given that white clover is self-incompatible, each plant is considered a distinct genotype. The plants were maintained in the glasshouse under 22°C day: 16°C night, 16 h light:8 h dark conditions, and each genotype vegetatively propagated via stolon cuttings. These plants were grown in 85-mm pots in standard potting mix soil. Samples for RNA extraction were collected from leaves and stolons of the five genotypes, which were grown in both water-limited and well-watered conditions, making a total of 20 samples. Thus, four groups were analysed: well-watered leaves (LWW), water-limited leaves (LWL), well-watered stolons (SWW) and water-limited stolons (SWL), with each group including the five aforementioned genotypes. Samples were taken from newly emerged leaves and from 1-cm stolon cuttings of the first internode. Drought stress was applied by withholding water for 3 days, after which visible wilting signs were observed as a symptom of drought. The well-watered treatment consisted of plants watered at field capacity.

RNA extraction

Samples were flash-frozen in liquid N and stored at 80 C until RNA extraction. RNA extraction was performed with an RNeasy Plant Mini Kit (Qiagen). RNA concentrations were determined by absorbance at 260 nm, and RNA quality was estimated by absorbance 260:280 ratios using a NanoDrop N-D 1000 spectrophotometer (Thermo Scientific). Only samples whose absorbance 260:280 ratios were between 1.8 to 2.0 were used. RNA integrity was estimated via 2% agarose gel electrophoresis by checking 25S/18S rRNA band intensity and the absence of smears.

Quantitative reverse transcription–PCR

RNA was treated with DNase I (Sigma Poole) to remove traces of DNA before cDNA synthesis. Complementary DNA was synthesised by using 500 ng RNA per reaction with the iScript cDNA Synthesis Kit for qRT-PCR (Biorad) following the manufacturer's instructions in a BioRad T100 Thermal Cycler (Biorad). Before qRT-PCR, cDNA samples were diluted 1:50 with nuclease-free water (Thermo Scientific).

qRT-PCR reactions were performed in a 20-mL final volume, with 2 mL of cDNA, 600 nM each of forward and reverse primers (Table 1), and 1 of QuantiTect SYBR Green PCR kit master mix (Qiagen). Cycling conditions were 10 min at 95°C and 40 cycles of 10 s at 95°C, 30 s at 60°C and 10 s at 72°C. qRT-PCR was performed in triplicate for all samples across the eight genes evaluated in a CFX 384 Real-Time PCR System (Bio-Rad).

In silico gene identification, gene sequence search and primer design

Candidate reference genes were selected on the basis of previous work by (Jian *et al*. 2008) and (Brunner *et al*. 2004). Sequences were downloaded from the *Arabidopsis thaliana* (L.) Heynh. database (https://www.arabidopsis.com) and used as queries in BLASTN searches of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) white clover expressed sequenced tag (EST) database to find putative orthologous genes. Primers were designed with primer3 (Untergasser *et al*. 2012) (Table 1) and tested for efficiency on five twofold serial dilutions of bulked cDNA from white clover leaves. A plot of Ct versus log cDNA dilution was generated and the efficiency (E) was calculated using the formula $E = 10^{-1/\text{slope}}$ (Pfaffl 2001).

Amplification of single products of the expected size was verified by 2% agarose gel electrophoresis and SYBR safe staining (Thermo Scientific), and by dissociation curve analysis.

Table 1. Primers used for quantitative reverse transcription–PCR of white clover genes

Name	Forward primer (5'–3')	Reverse primer (5'–3')	Predicted product size (bp)
ACT11	TATCCAGGCGGTTCTTTCAC	TCACGTCCAGCAAGATCAAG	147
G6PD	GAATCGCGACAACATTGCTA	GGCTTTTCCATAGCAACCAA	153
EF1β	ATGTGAAGCCATGGGATGAT	TGTTGTCAACGGAAACCAAA	174
EF1α	AGGTCCACCAACCTTGACTG	TGGCTTGTCTGAGGGTCTCT	90
TUA	GCGAATGCGTAGACAGAACA	GTTCAATGCTGTTGGTGGTG	170
CYP2	CGGTCCTGGAATCTTATCCA	TTCACGACCTCCAATCCTTC	141
UBQ10	GGCGAACTTTGGCTGATTAC	CATACCTCCCCTCAAACGAA	74
UBQ	ATTCGTTTCTTCGCGACCTA	TCCTGGATCTTGGCTTTGAC	128

Data analysis and statistics

Stability in transcript abundance was assessed by using the ΔCt method (Silver *et al*. 2006), NormFinder (Andersen *et al*. 2004) and single-factor ANOVA (Kortner *et al*. 2011). Ct values of the triplicate reactions performed for each sample and gene combination were averaged. For NormFinder and ANOVA analysis, Ct data were transformed to relative quantities (*RQ*) using the equation $RQ = 2^{Ct_{min} - Ct_{sample}}$, where Ct_{min} is the lowest Ct value in the sample pool. *F*-values were calculated for ANOVA analysis. These are defined as the ratio of the mean variance between groups to the mean variance within groups. ANOVA was performed using Microsoft Excel's Analysis Toolpak (Microsoft Corp.). The coefficient of variation (CV) was calculated as the ratio of the s.d. to the mean.

Results

The sequences of the genes encoding elongation factor 1-α (EF1α), elongation factor 1-β (EF1β), actin 11 (ACT11), glucose-6-phosphate dehydrogenase (G6PD), tubulin α-5 (TUA), cyclophilin (CYP), ubiquitin 10 (UBQ10) and polyubiquitin (UBQ) from *A. thaliana* were used to find candidate white clover reference genes. Genes were selected on the basis of prior reports of reference gene transcript abundance stability in the legumes soybean (*Glycine max* (L.) Merr.) (Jian *et al*. 2008) and red clover (*Trifolium pratense* L.) (Mehdi Khanlou and Van Bockstaele 2012), and in *A. thaliana* (Gutierrez *et al*. 2008). A BLASTN search on the National Center for Biotechnology Information white clover EST database was performed to find the putative orthologues of the *A. thaliana* genes (Table 2). Given that a secondary PCR product was observed for *UBQ10*, this gene was discarded from the analysis.

The ΔCt *test*

The ΔCt method was used to evaluate stability in transcript levels. Similarly to the geNorm method developed by Vandesompele *et al*. (2002), the ΔCt method is based on the assumption that the difference in the transcript abundance of two genes remains constant in different samples only if the transcription levels of these two genes is constant among samples or if co-regulation occurs (Silver *et al*. 2006). To calculate the Ct difference, each gene was compared with the remaining six genes across the 20 different samples. The s.d. of ΔCt for the 20 samples was then calculated and the mean of the s.d. was estimated in order to select the gene whose transcript abundance was more stable among samples. *EF1α* was observed to be the most stable gene when compared with the other six genes, with a mean s.d. of 1.398, followed by *ACT11*, with 1.423. The least stable genes were *CYP* and *G6PD* with 2.133 and 1.788 respectively (Fig. 1. Table 3). Transcript abundance variation was also assessed across both organs and treatments. In this study, there were two organs and two water availability treatments, which provides four groups for analysis. *EF1β* and *EF1α* showed the highest stability in LWW and LWL respectively, whereas *ACT11* was the most stable in both SWW and SWL (Table 3).

The NormFinder test

The stability of transcript levels was also studied by using the NormFinder method (Andersen *et al*. 2004), which allows for comparison between or among groups. When transcript abundance was compared among the four groups, the gene with the most stable transcript levels was *UBQ*. Most of the genes showed more intragroup variability than among-group variability (File S1, available as Supplementary Material to this paper; Fig. 2). The rank order of the genes from the most to the least stable transcript abundance based on the stability index is shown in Table 4.

Consideration of intergroup variation is important, as it can help to identify candidate genes whose variation in transcript abundance is more affected by organ or treatment. Genes whose transcript abundance varies greatly between organs or treatments would not be suitable as references to quantify transcript abundance (Kortner *et al*. 2011). Low intergroup variation was observed for *UBQ* among the four groups. *ACT11* in LWL and SWL, and *EF1α* in LWW and LWL groups also exhibited a low intergroup variance (Fig. 2*a*). However, ideally, low variation should be exhibited in both organ and treatment comparisons simultaneously. *UBQ* is therefore the only gene that meets these stringent criteria.

A useful feature of NormFinder is a provision for calculating intragroup variation. This allows the identification of the genes that perform best in each treatment or organ. *ACT11*, followed by *EF1β* and *EF1α*, showed the most consistent low intragroup variation in leaves for the two treatments, whereas a similar tendency was observed for *EF1α* followed by *UBQ* in stolons.

Table 2. Candidate white clover genes

EST, expressed sequenced tag; E-value, expect value, this parameter provides with the count of hits one can 'expect' to see by chance when searching a database of a particular size (www.ncbi.nlm.nih.gov/)

Name	White clover EST accession no.	Arabidopsis gene	Arabidopsis gene description	BLASTN score E value	Sequence identity (%)
ACT11	FY464614	AT3G12110	Actin 11	0.0	85
G6PD	FY456502	AT5G40760	Glucose-6-phosphate dehydrogenase	7×10^{-175}	79
EF1β	FY460489	AT5G19510	Eukaryotic elongation factor 1-beta	2×10^{-114}	74
EF1α	FY461009	AT5G60390	Eukaryotic elongation factor 1-alpha	0.0	88
TUA	FY468756	AT5G19780	Tubulin alpha-5	0.0	83
CYP2	FY468141	AT2G21130	Cyclophilin	1×10^{-109}	77
UBQ10	FY460776	AT4G05320	Ubiquitin 10	0.0	82
UBQ	FY469289	AT4G05050	Polyubiquitin	0.0	84

When the intragroup mRNA abundance stability of well-watered groups was analysed, *EF1β* exhibited the most consistent stability among the two organs analysed (LWW and SWW), whereas *ACT11* followed by *EF1α* and *UBQ* were the most stable in water-limited plants (LWL and SWL) (Fig. 2*b*). In order to consider a gene as stable, low intragroup and low inter-group variation should be observed together. NormFinder combines these two selection criteria and calculates a stability value. *UBQ* exhibited the best stability value for the four groups analysed (Table 4). NormFinder also provides the best combination of genes if expression is to be assessed using two references. The best combination when the four groups were compared was *ACT11* and *UBQ*.

ANOVA test

Similar to NormFinder, ANOVA enables the examination of the effects of both organ and treatment on estimated transcript abundance. However, as ANOVA only compares values among groups, we calculated the CV to obtain information on the variation within group samples. Genes with lower CV values were considered to have more stable transcript abundance. High *F*-values, defined as the ratios between the intergroup and intragroup variation, may indicate an effect of the organ or treatment. The highest F values were observed for *UBQ* in three comparisons (LWW *vs.* LWL, SWW *vs.* SWL and LWW *vs.* SWW). These were statistically significant at *P*-values of 0.05. *F*-values were also statistically significant for *EF1α* and *EF1β* when the comparisons LWL *vs.* SWW and LWW *vs.* SWW respectively were assessed (Table 5). *G6PD* had low *F*-values when groups were compared; however, this gene was not ranked highest according to its CV values (Tables 5, File S2). Conversely, *UBQ* and *EF1α* exhibited high *F*-values but were ranked first and second according to their CV values (Table 5, File S2).

In addition to considering the results obtained by the different methods used, the raw Ct values can give an additional criterion for selection. When looking at these values in a box plot, the smallest percentiles were observed for the genes *UBQ* and *EF1α*, followed by *EF1β* and *ACT11* (Fig. 3). Variation of over one Ct unit was observed in all genes, even for samples in the same groups (File S1). *EF1α*, *CYP* and *UBQ* genes exhibited mean Ct values under 30, and the remaining genes showed mean Ct values between 30 and 37 (Fig. 3).

Table 3. ΔCt **analysis across all samples of white clover and within groups**

Means of s.d. of calculated ΔCts for each gene are shown. Genes were ranked (in brackets) from the most to the least stable. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Gene name	All samples	LWW	LWL	SWW	SWL
EF1α	1.398 (1)	1.072 (5)	0.926 (1)	1.479 (3)	1.309 (2)
EF1β	1.652 (5)	0.994 (1)	1.050 (3)	1.544 (4)	1.807 (5)
ACT11	1.423 (2)	1.017 (4)	1.295 (6)	1.341 (1)	1.206 (1)
CYP	2.133 (7)	1.325 (5)	1.452 (7)	2.346 (6)	2.500 (6)
G6PD	1.788 (6)	0.998 (2)	1.261 (5)	2.899 (7)	1.930 (5)
TUA	1.580 (4)	1.016 (3)	1.174 (4)	1.634 (5)	1.739 (4)
UBQ	1.437 (3)	1.393 (6)	1.016 (2)	1.430 (2)	1.378 (3)

Discussion

Transcript abundance stability was evaluated for seven genes in two organs under two treatments using three analysis methods. Varying results were obtained using these methods. Therefore, an understanding of the fundamentals and limitations of each method is necessary in order to select the optimal reference genes. Although the effect of organ and treatment groups on transcript abundance plays a critical role in selecting a reference gene, total variation should also be considered. ANOVA addresses the variation between and within groups but does not consider the total variation in the sample pool. To circumvent this, the CV for the sample pool can be calculated.

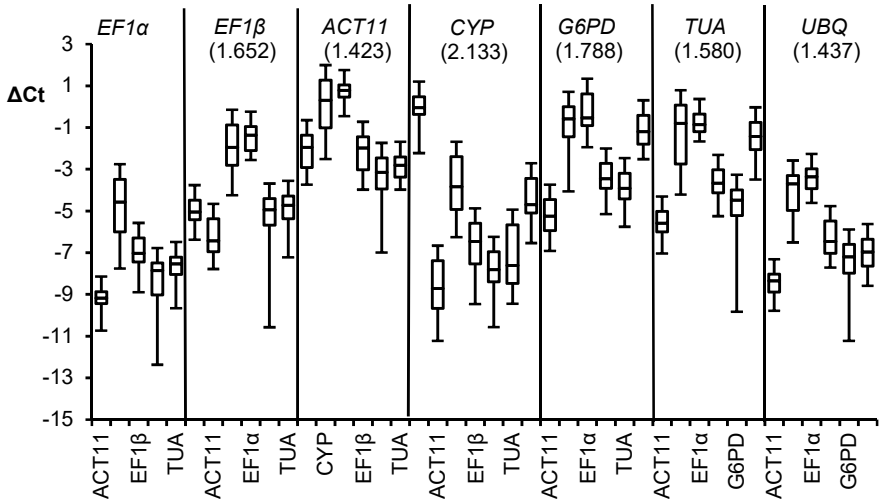

Fig. 1. White clover reference gene selection based on the ΔCt method. ΔCt values are shown as medians (horizontal lines), boxes represent the 25th percentile to 75th percentile and whiskers represent ranges. The values in brackets are the means of s.d. of calculated ΔCts for each gene.

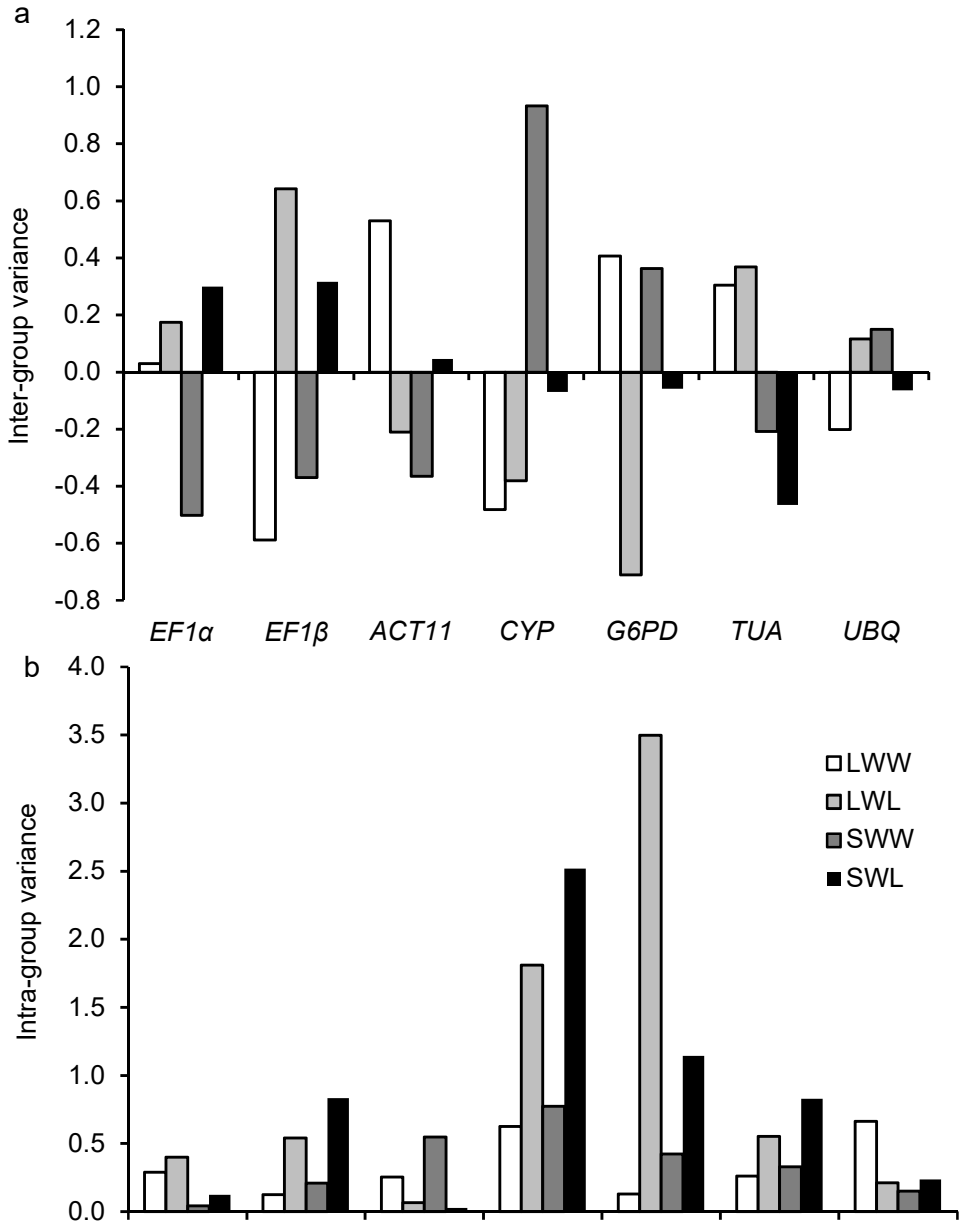

Fig. 2. NormFinder analysis results for potential white clover references genes. (*a*) Intergroup variances and (*b*) intragroup variances in four different conditions were plotted. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons.

Table 4. Ranking of white clover genes from least to most stably expressed, as determined by analysis with NormFinder

Gene name	Stability value
UBQ	0.395
ACT11	0.440
EF1α	0.463
TUA	0.566
EF1β	0.654
G6PD	0.678
CYP	0.778

In contrast, analysis of intergroup variation is not possible with the ΔCt method. However, intragroup variation and whole-sample group variation are evaluated in ΔCt. NormFinder, on the other hand, complements the results obtained by the other methods, as it provides a stability index that estimates both intragroup and intergroup variation.

As ΔCt is based on pairwise comparison between genes, transcript level stability is derived from the similarity of gene expression profiles. Hence, it is possible to inadvertently select stable genes that are coregulated. To guard against coregulation, this method is not recommended for genes belonging to the same family. The possibility of coregulation was taken into account at the time of selection of candidate genes for this analysis. Coregulation analysis with the database of coexpressed genes for *A. thaliana* ATTED-II (Obayashi *et al*. 2009) excluded the possibility of coregulation of the analysed genes. Furthermore, even for genes with different cellular functions, a similar expression trend caused by the treatment or environmental

Table 5. Coefficients of variation (CV) and ANOVA analysis of potential white clover reference genes
F-value, *F*-test of variance; LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons; *, significant at a *P*-value of 0.05

Gene	CV	*F*-values					
		LWW *vs.* LWL	LWL *vs.* SWW	SWW *vs.* SWL	LWW *vs.* SWL	LWW *vs.* SWW	LWL *vs.* SWL
EF1α	0.66	0.815	8.040*	1.848	1.662	5.278	4.761
EF1β	1.21	1.018	5.138	2.221	2.642	5.858*	1.728
ACT11	0.79	1.660	1.445	0.955	1.072	0.002	0.213
CYP	1.19	4.491	0.434	0.157	1.554	1.412	1.454
G6PD	0.99	0.417	0.140	0.385	0.011	0.529	0.176
TUA	1.15	0.361	3.059	3.004	0.333	2.205	0.012
UBQ	0.59	6.008*	1.378	5.905*	0.448	7.356*	4.138

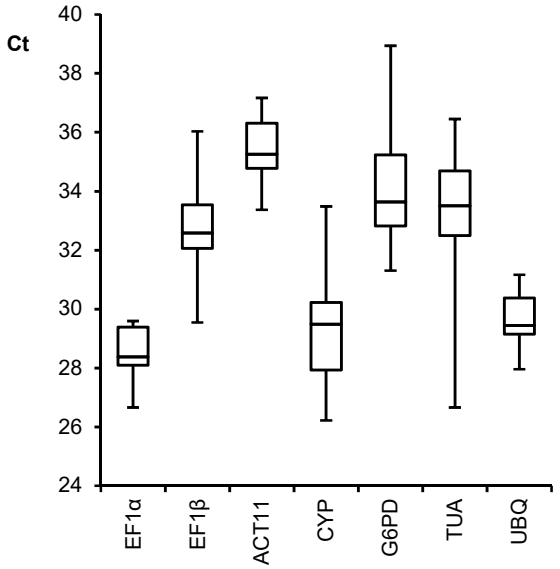

Fig. 3. Box and whisker plot of raw Ct values for potential white clover reference genes across all samples.

condition can be observed (Mehdi Khanlou and Van Bockstaele 2012). This problem is addressed by using NormFinder, which takes the possible systematic variation of the studied genes into account (Andersen *et al*. 2004).

Ideally, a reference gene for evaluating transcript abundance between organs or treatments should exhibit stable mRNA levels among samples and show minimal variation between groups. *UBQ* and *EF1α* were the most stable when overall variation was analysed by the ΔCt method (Fig. 1, Table 3). Additionally, CV values among all samples indicated low variation for these two genes (Table 5). Thus, their use as references is recommended if gene transcript abundance is to be studied in heterogenic samples, with some qualifications. Significant variation was observed in *EF1α* and *UBQ* when compared among groups by ANOVA (Table 5), making them undesirable for comparisons between treatments or organs. However, selecting a gene based on its *F*-values has its limitations, as addressed previously by Mehdi Khanlou and Van Bockstaele (2012) and Kortner *et al*. (2011). It was proposed that CV is a more important parameter than *F*-values for selecting a stable gene (Kortner *et al*. 2011). In line with this, here, we selected the genes with the lowest CV and intragroup variation, and we used F-values and intergroup variation as the final criteria (File S2). According to NormFinder analysis, *UBQ* was ranked first (Table 4). Although there may be an organ effect on *UBQ* expression, as shown by the *F*-values, the overall variation is low and is captured by the NormFinder method for the stability index calculation.

When genes were analysed within groups via the ΔCt method, different results were observed. Although *UBQ* was stable in LWL, SWL and SWW conditions, it did not perform well in LWW conditions (Table 3). Similarly, low transcript level variance was observed for *EF1α* within the LWL, SWL and SWW groups, being ranked first (LWL), second (SWL) and third (SWW), but low stability was observed in LWW (Table 3). This gene was previously used as a reference in white clover across the stages of flower development (Abeynayake *et al*. 2012). High mRNA level stability within both treatments in stolons (SWW and SWL) was observed for *ACT11*. However, transcription of this gene was ranked fourth and sixth when LWW and LWL were studied. If a gene expression study is to be performed in a single organ or tissue, consideration of the variation between groups is not highly relevant. In such cases, according to the ΔCt method results, the best genes for transcript abundance analysis are *EF1β* in leaves, and *ACT11*, *UBQ* and *EF1α* in stolons (Table 3, Fig. 2). However, according to the NormFinder results, *ACT11* in leaves and *EF1α* in stolons exhibit the most consistently low intragroup variation.

Given that each white clover plant represents a unique genotype, genotypic variability between plants could account, at least in part, for the observed variation in Ct values. Raw Ct values are other criteria that can be used in the selection of optimal reference genes. Higher Ct values, as observed for *ACT11*, *G6PD* and *TUA*, may indicate lower transcript abundance. However, the use of reference genes with low transcript abundance could be useful when genes with low levels of expression are studied (Czechowski *et al*. 2005).

There was no particularly striking correlation between the results generated here and those from studies on other species. Most notably, *EF1α* and *ACT11* were ranked sixth and seventh from 10 genes evaluated in different tissues from the legume soybean (Jian *et al*. 2008). However, similar to what was

observed here, more exhaustive research in soybean reported a low variation in *ACT11* transcript abundance (Hu *et al*. 2009). A high overall variation of *EF1α* in the related species red clover was reported when transcript abundance was compared with eight other genes. However, transcript abundance stability was observed for *EF1α* in red clover stolons (Mehdi Khanlou and Van Bockstaele 2012), which is recapitulated by the results observed in the current research. Consistent with our findings, *UBQ* was identified as the gene whose transcript levels were most stable in a study on trees from the *Poplar* genus (Brunner *et al*. 2004). To the best of our knowledge, there are not many reports evaluating this gene in other species. Given that its transcript levels are stable in the two distantly related species, white clover and *Poplar*, evaluating *UBQ* as a reference in other species could be a productive approach.

The use of transcript abundance data from heterologous species such as *A. thaliana* has the potential to identify reference genes that can be adopted across species. Such an approach was used by Tashiro *et al*. (2016) to identify reference genes in grapevine (*Vitis vinifera* L.), based upon evidence from *A. thaliana*, which might have broader application.

The genes *EF1α*, *UBQ* and *ACT11* stood out as the most stable when rankings from the ΔCt and NormFinder tests and CV values were consolidated. Of these three, *EF1α* was top ranked, followed by *ACT11* and *UBQ* (File S2). In order to define the most stable gene between *ACT11* and *UBQ*, *F*-values were compared and *ACT11* was ranked over *UBQ*. Furthermore, although *G6PD* exhibited low *F*-values, the CV and ΔCt values were high, which makes this gene unsuitable for use as a reference. In conclusion, although there was no complete consensus between the different methods used to assess gene transcript abundance stability in this study, *EF1α* emerged as the reference gene of choice for white clover, with *ACT11* and *UBQ* also being suitable. Given that variation in transcript levels can still be observed even in validated reference genes, the use of at least two genes as references is generally recommended (Gutierrez *et al*. 2008). Therefore, combining two of the three aforementioned most stable genes would be the ideal strategy.

Conflicts of interest

The authors declare no conflicts of interest.

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7 General discussion

White clover transformation

Triple stacked transgenic white clover events were generated by Agrobacterium-mediated transformation. The use of a single T-DNA containing three genes of interest facilitated the simultaneous insertion of these genes in a single transformation step, making the process faster than stacking genes by methods such as retransformation or crossing transgenic events. Furthermore, by linked co-transformation the genes of interest will segregate together when breeding subsequent generations, making processes such as introgression of the transgene in elite cultivars or production of homozygous plants less laborious.

To our knowledge, there are no reports of transfer of T-DNA of such a size (8697 bp) to white clover by Agrobacterium-mediated transformation. Overall, by combining linked and unlinked co-transformation, a total of five genes, three genes of interest, a selectable marker gene (*hph*), and a reporter gene (*GFP*) were delivered in a single transformation step. Although transformation frequencies were low (0.22-0.23), this work demonstrates the feasibility of Agrobacterium-mediated transfer of a large T-DNA with a number of genes to white clover in a single transformation step. In addition, the transfer of the selectable marker gene (*hph*) by unlinked co-transformation made it possible to produce selectable marker gene-free plants at the T_0 generation. Alternatively, it will enable the elimination of the selectable marker in subsequent crossings via segregation.

Almost 50% of the events generated were reported to have single T-DNA insertions. This was in line with previous work in white clover (Rossello, 2011) and other species (Ishida et al., 1996; Sallaud et al., 2003). The generation of single insert transgenic plants is of special interest. Regulatory authorities demand low copy number insertions in order to permit the commercial release of a transgenic event (EFSA, 2011). Also, single T-DNA copy events are desirable in order to avoid segregation in further generations when plants are crossed. This is particularly problematic in white clover, as it is a crossbreeding species.

Overall, combining linked co-transformation and unlinked co-transformation by Agrobacterium-mediated transformation was proven to be a successful method for obtaining

transgenic events with features that will facilitate later stages of transgenic plant evaluation, selection, and breeding before potential commercial release.

Selection of events with potential for agronomic use

The initial molecular characterization performed in this work, which included screening for insertion of the three genes of interest and copy number estimation, allowed us to narrow the number of events to be studied in subsequent phenotyping and molecular experiments. Additionally, detached leaf experiments in order to evaluate delayed leaf senescence provided a fast and efficient early approach for identifying events expressing one of the desired phenotypes. By combining these molecular and phenotyping results, we were able to focus on the events with greater potential for agronomic use. At this point, events carrying the three genes of interest, with low copy number, and expressing the phenotype of delayed leaf senescence were identified. Based on these results, twelve events were selected for drought tolerance experiments.

In parallel with evaluation of delayed leaf senescence and drought tolerance, determination of Tr*neMDH* transcript abundance of the 30 events provided information on the functionality of the transgene and an indication of which might display tolerance to Al^{3+}. The increased expression of Tr*neMDH* in 12 of the 30 generated events indicates the effectiveness of the Tr*Pt1:*Tr*neMDH* cassette for increasing Tr*neMDH* transcript levels in roots. Although this is not a confirmation of higher exudation of malate through the roots, and does not guarantee an increased tolerance to Al^{3+}, the results provide a tool for selecting the events with more potential for Al^{3+} tolerance. Therefore, events exhibiting increased Tr*neMDH* transcripts levels were selected for Al^{3+} tolerance trials.

A flowchart of the screening process and selection of events is presented in Figure 7.1.

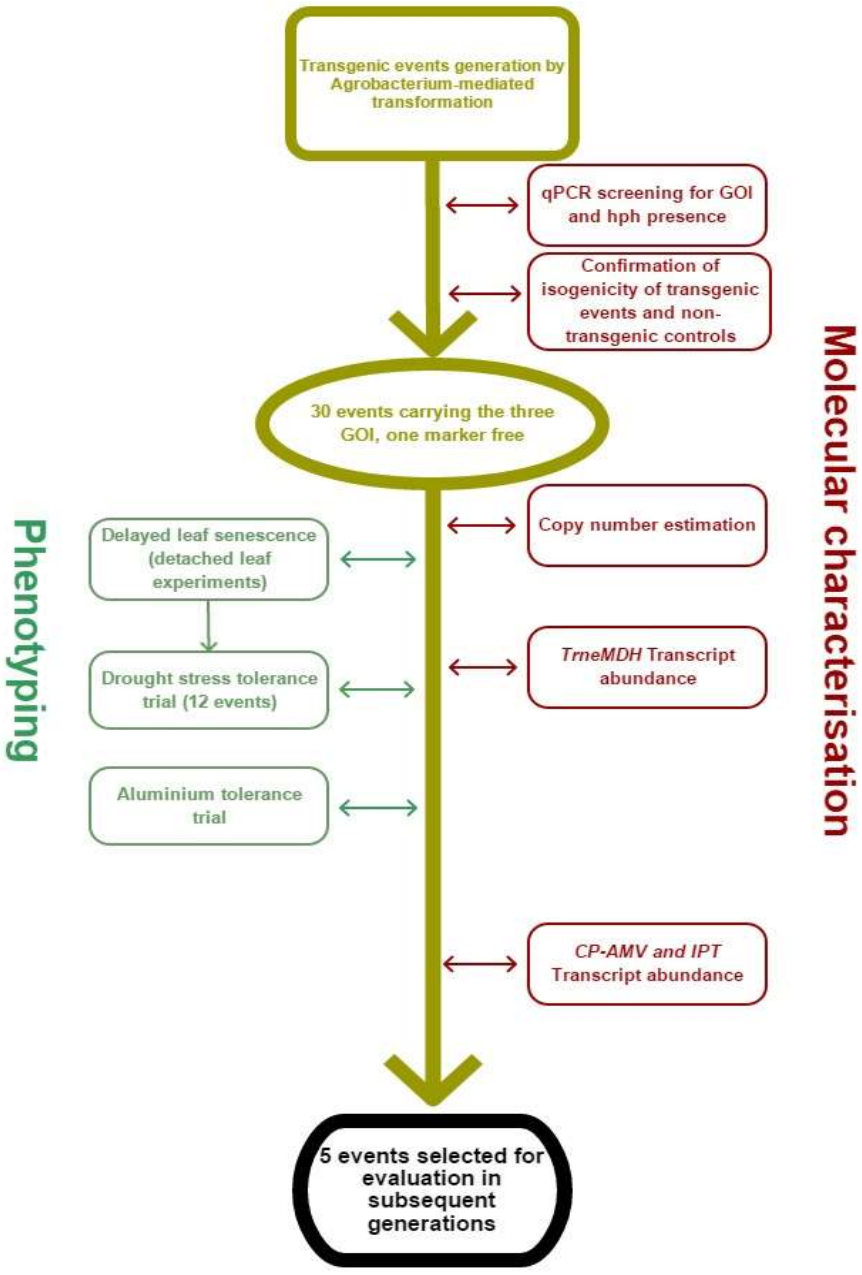


Figure 7.1. Flow chart of generation and selection of transgenic events with potential for agronomic use.

The delayed leaf senescence phenotype was observed in 13 events of the 30 generated. This supports the effectiveness of the At*myb32:IPT* cassette in a different context to that in which it was previously evaluated, including differences in species, various white clover cultivar and construct (Kant et al., 2015; Lin et al 2007, 2010). Theoretically, delayed leaf senescence could have a positive impact on hay production. Delayed leaf senescence would delay protein degradation and consequently forage with a higher nutritive value would result (Calderini et

al., 2007). More extensive research on this area is required, covering aspects such as evaluating delayed leaf senescence on a large scale, and measuring nutritive values of hay from white clover expressing the trait for delayed leaf senescence. This is particularly important in alfalfa, a species highly relevant for hay supplementation. Therefore, the transfer of this technology to this species may be of special interest (Calderini et al., 2007).

As previously described, transgenic plants expressing *IPT* exhibited drought stress tolerance in different species using various transgenes (Rivero et al., 2007; Kuppu et al., 2013; Kant et al., 2015). Additionally, previous results in white clover carrying the At*myb32:IPT* cassette showed better summer survival, consistent with an increased drought tolerance (Lin et al 2007, 2010). Based on these results, a drought trial was performed on 12 transgenic white clover events. As described in Chapter 4, two events, 11 and 31, exhibited potential for drought stress tolerance and one of these also showed better regrowth. These results further support previous observations of improved survival during summer in the field, and validate the use of this transgene to confer tolerance to water limitation in white clover. Considering that one of the main factors contributing to a decrease in white clover persistence is water limitation, these results suggest that overexpression of *IPT* may help to increase white clover persistence in the field.

IPT transcripts were detected in the two events that exhibited drought stress tolerance. Interestingly, the highest *IPT* transcript abundance was observed in event 34, which did not exhibit significant increases in drought tolerance. Given that transcript levels of the two events that showed drought tolerance are similar, we can speculate that the transcript abundance levels observed for events 11 and 31 are the levels required to observe the desired phenotypes. However, as *IPT* transcript levels can exhibit variations over time and development, and change depending on environmental conditions, using this parameter for selecting events can be uncertain. In order to generate more robust data, evaluation of a higher number of events would be necessary. Furthermore, cytokinin measurements would provide more definitive information on hormone levels required for producing events with the desired phenotypes.

No *IPT* transcripts were detected in events 21 and 25, which previously exhibited delayed leaf senescence. Different hypotheses could be attributed to this outcome. Arguably, the promoter used, At*myb32*, may exhibit temporal changes in activity, and these changes could be more or less pronounced depending on the genomic context of transgene integration. However, the lack of transcription of CP-*AMV* in these two events supports the hypothesis of transgene silencing,

described in a number of preceding studies (Chen et al., 2001; Sallaud et al., 2003). Furthermore, it has been observed that transgene silencing can occur when environmental conditions change (Zhong, 2001). Although a different strategy was followed here, assays for *IPT* and CP-*AMV* transcript presence as a first step for screening of the events generated could be a useful strategy to complement preliminary delayed leaf senescence data in order to select the most promising events for further evaluations.

The use of constitutive promoters driving transgenes in engineered plants can lead to a high energy resources demand, which can ultimately cause negative effects. This could ultimately lead to yield penalties (Cominelli et al., 2013). The constitutive expression of CP-*AMV*, together with increased cytokinins, which may cause undesired phenotypic effects, and the increased expression of a highly relevant enzyme in plant metabolism such as MDH, raised the concern of observing yield penalties in the transgenic events generated. The use of a tissue specific promoter for controlling Tr*neMDH*, which favors the limited use of plant reserves, and the use of an organ specific promoter to control *IPT* in order to produce a restricted increase in cytokinin concentrations was directed to address this issue. No evidence for negative effects in the events generated was observed when plants were visually inspected. However, as also described in Chapter 4, two events (5 and 21) exhibited reduced vigour when evaluated in the glasshouse trial, and a decrease in forage production under water limitation. Therefore, drought stress tolerance assays were also useful for identifying events that underperform in stress conditions. Furthermore, evaluation of forage production in large plants during the long-term, described in chapter 4, exhibited differences in vigour not observed when plants were grown at a smaller scale. Although the cause of yield reduction of these events is not clear, negative effects of high cytokinin production and any fitness penalty caused by overexpression of CP-*AMV* or Tr*neMDH*, or negative effects caused by the transgene genome context could explain this result. Potential transgene silencing in event 21, however, rules out the possible yield penalties being caused by overexpression of *IPT* or CP-*AMV* for this event.

As previously discussed, the potential Al^{3+} tolerance of the transgenic plants generated is based on an Al^{3+} exclusion mechanism, which is a product of quelation of Al^{3+} by excreted malate from the roots to the rhizosphere. Therefore, use of solution culture for aluminium tolerance evaluation of transgenic plants can be difficult, as excreted malate can diffuse rapidly when in the Al^{3+} solution where plants are evaluated. Soil-based trials for aluminium tolerance evaluation undertaken in this work were intended to overcome this limitation. The aluminium tolerance of the transgenic events that exhibit increased Tr*neMDH* transcript levels was

evaluated in soil experiments in the glasshouse. Root growth was measured as a symptom of Al^{3+} stress in these trials. Events 1, 4 and 14 exhibited higher root growth when compared to their respective non-transgenic controls. RSRG values were statistically significant for events 1 and 4. However, the variability in growth rate observed between replicates was high, meaning that caution should be exercised when considering these results.

The high variability observed between replicates, which may have been caused by changes in soil composition among pots and the relatively short evaluation period, likely contributed to the generation of inconclusive results. In order to complement results generated in this trial, an additional preliminary trial in solution culture assay was also performed. Although no significant differences were observed between transgenic plants and their respective controls, some conclusions were drawn from this analysis. Solution culture assay in a short term without nutrient addition was practical and easy to perform. Roots exhibited growth based on nutrients stored in stolons, which allowed the avoidance of interference from nutrients in the solution on Al^{3+} effects.

Therefore, for next steps in Al^{3+} tolerance evaluation, we propose integration of both solution culture and soil-based experiments. Ideally, screening of Al^{3+} tolerance in solution culture of a high number of events using a higher number of replicates, followed by evaluation of the best performing events in long-term soil-based trial, where shoot and root mass are measured, should be performed.

A reduction of the number of events evaluated in further steps of selection will allow to perform more exhaustive analyses that would shed light on the mechanisms of Al^{3+} tolerance involved. A more precise image-based analysis would enable to study architectural and morphological changes in roots under Al^{3+} stress. Also, hematoxylin staining in the generated events, previously used by Labandera (2007), and quantification of organic acids excreted from roots, previously measured by Rossello (2011), would provide answers on a potential involvement of an Al^{3+} exclusion mechanism in the tolerance of the events generated.

White clover events expressing CP-*AMV* were thoroughly evaluated in previous work, and resistance to AMV was confirmed in the field (Panter et al., 2012). Therefore, we hypothesize that confirming CP-*AMV* transcript abundance would provide a rapid method for preliminary identification of events with possible resistance to AMV. Hence, the most promising events were evaluated for CP-*AMV* transcript abundance, and CP-*AMV* transcript presence confirmed for events 11, 31 and 34. Although confirmation of transcript presence works as a proof of

concept for AMV resistance, evaluations of resistance to AMV will be necessary in subsequent generations.

The process of selection of events of potential commercial interest requires integration of the molecular and phenotypic information generated. The molecular and phenotypic information generated is summarized in Table 7.1. As discussed above, events with low transgene copy number, ideally single copy T-DNA are deemed necessary. A second level of priority when selecting events with agronomic potential was the occurrence of delayed leaf senescence and increased Tr*neMDH* transcription. As previously discussed, detached leaf experiments can be a fast and reliable method for evaluating the expression of delayed leaf senescence, while increased Tr*neMDH* transcription is the first step toward demonstrating increased localized MDH enzyme production and possible Al^{3+} tolerance.

Table 7.1 Summary of the molecular and phenotypic results for selection of the events of agronomic interest

Event	Estimated T-DNA copy number	Delayed senescence (detached leaf assay)	Possible drought tolerance	Increased Tr*neMDH* transcription	*IPT* transcription	CP-*AMV* transcription	Possible increased Al^{3+} tolerance	Events with agronomic interest
1*	1	0		+ (ns)			+	Y
2	1	0		0				
3	1-4	-	0	0				
4	2-3	+	0	+ (ns)			+	Y
5	1-2	+	-	0				
6	1	0		0				
7	1-2	0		-				
8	5	0		+			+(ns)	
9	1-2	+	0	0				Y
10	2-4	+	0	+			- (ns)	
11	7	+	+	+	+	+	0	
14	7-10	+		+			+ (ns)	
17	1-2	0		0				
19	1-2	0		0				
20	1-2	0		+			-	
21	1	+	-	+	nd	nd		
22	1	-						
23	1	+		-				
24	1	0		0				
25	1	+	0	+	nd	nd		
26	1	0		-			- (ns)	
27	1	+	0					
28	6-7	+		0				
29	1-3	0		0				
30	2	0		+			0	
31	3-4	+	+	+	+	+	0	Y
32	1	0	0	0				
33	1	0		+			0	
34	1	+	0	+ (ns)	+	+	0	Y
35	2-3	0		0				

**hph* marker gene free, (ns) not statistically significant; +, increased transcript levels (Tr*neMDH* transcript abundance, transcript detected (CP-*AMV* and *IPT*), or phenotype of interest not observed (detached leaf experiments and drought stress trial). 0, no transcription difference or phenotype of interest not observed; -, decrease in transcripts levels (Tr*neMDH* transcript abundance assays); nd, transcripts not detected; yield penalty observed (drought stress trials), accelerated senescence (detached leaf experiments);; Y, event selected with potential agronomical use.

Events 11 and 31 exhibited delayed leaf senescence, increased yields under water limitation, and transcription of the three genes of interest. However, their T-DNA copy numbers are high, especially in event 11. Event 34, on the other hand, appears to have a single T-DNA insertion, shows delayed leaf senescence, exhibited increased transcript levels for Tr*neMDH* (although not statistically significant), and transcripts were detected for the CP-*AMV* and *IPT* genes. Although event 31 has 3 or 4 inserted copies, its increased performance under water limiting conditions makes it an interesting event to evaluate in subsequent generations. Similarly, event 4, with possibly two T-DNA copies, may have potential for consideration as an elite event, as it exhibited delayed leaf senescence, increased Tr*neMDH* transcription, and potential for increased growth in soil with toxic levels of Al^{3+}.

The possible segregation of the inserted transgenes in the next generations could lead to changes in the transcription levels and/or loss of the observed phenotype. However, given their potential, screening for insert presence, detached leaf experiments, and transcript abundance of the genes of interest in progeny of events 4 and 31 may be worthwhile. Other events of interest include number 1, which shows attractive features, such as absence of the selectable marker gene, single T-DNA copy, increased Tr*neMDH* expression, and increased root growth in Al^{3+} toxic soil; events 9 and 32, which have a single T-DNA copy, and exhibited delayed leaf senescence. However, no difference in Tr*neMDH* transcript levels when compared to its non-transgenic control in these events.

Concomitant expression of the three traits of interest (delayed leaf senescence, aluminium tolerance, and AMV resistance) is required in order to generate an event with agronomical potential. Given the self-incompatibility of white clover, generation of transgenic plants in subsequent generations requires the performance of crossings with non-transgenic plants, and multiple pair crosses in order to maintain variability in the population (Woodfield and White, 1996; Spangenberg et al., 2000; Badenhorst et al., 2016). This implies the integration of the transgenes in different genomic backgrounds, which could lead to variations or loss of transgene expression. In addition, given that each gene is controlled by a different promoter, position effects could alter transcription of each gene differently. Transgene transcription could be altered or silenced by rearrangement or duplication of the transgene, recombination, epistatic interaction with other loci, or allelic interaction within a single locus (Zhong, 2001; Yin et al., 2004). Furthermore, a predictable inheritance of the transgene is required for

commercial use of transgenic events (Zhong, 2001). Therefore, selection of as many events as possible at the T_0 generation is a recommended strategy in order to offset these possible undesired outcomes, for further evaluation. In this regard, Badenhorst et al. (2016) recommend the selection of at least 5 events in the self-incompatible transgenic rye-grass. Five events were selected in this work in order to be evaluated in following generations for production of elite events (Table 7.1).

Plant phenotyping in the glasshouse

Usually, transgenic white clover plants are evaluated in highly controlled small-scale experiments in the T_0 generation. However, the results generated in such experiments can be misleading, and their associated results are not always transferable to performance of plants in the field (Bhatnagar-Mathur et al., 2008; Saint Pierre et al., 2012). Additionally, stringent government regulations on transgenic plants limit the establishment of field trials (Gómez-Galera et al., 2012). In response to these limitations, a drought stress trial for evaluating transgenic white clover plants in a system that simulates field conditions was carried out (Chapter 4). The development of a practical protocol for drought stress tolerance evaluations set the basis for future delayed leaf senescence and drought tolerance assays on transgenic white clover plants under glasshouse conditions. Moreover, similar experimental protocols can be transferred to other forage legumes, such as alfalfa or other *Trifolium* species.

Nevertheless, evaluation of the most promising events under field conditions should be carried out in the T_2 generation and beyond. A long-term evaluation would provide more complete answers on the impact of *IPT* expression on white clover persistence. Also, given leaf senescence can be triggered by various factors, such as shading or frost, we speculate that this trait could have a higher impact on the lifespan of the plant.

The establishment of phenomics technologies in the last decade has allowed the development of protocols for phenotyping using digital image analysis. A protocol for digital image analysis in a drought stress trial was developed in this work, and forage yields were estimated during the progression of the trial with relative accuracy. This allowed evaluation of any growth changes in plants under stress without the need for harvest. However, adjustment of parameters such as plant size, background color, and consistent lighting are needed to increase accuracy of white clover yield estimations.

The digital image analysis approach was also applied to evaluating color change in detached leaf experiments. In previous work, delayed leaf senescence has been evaluated by visual inspection of detached leaves, and manual scoring. The use of digital image analysis enabled us to accurately quantify color change in leaves and thus have a more precise estimation of the degree of delayed leaf senescence.

Adjustment of ddPCR and identification of reference genes for transcript abundance and copy number estimation

A number of molecular techniques were evaluated in this work, which pave the way for future research. The identification of single copy reference genes, enables the use of qPCR and ddPCR for copy number estimation in white clover. Furthermore, the identified reference genes in white clover could be evaluated for use in related *Trifolium* species.

As previously discussed, the development of protocols for copy number estimation by ddPCR makes it possible to perform more accurate quantifications than the typically used qPCR technique. Identification of homozygous events during the breeding process (for example in the T_2 generation) is necessary in the generation of elite transgenic events. This is usually performed by test crosses, which require the availability of high labour and time resources. The development of ddPCR use for copy number estimation, represents a more rapid and less labour intensive identification of homozygous plants than test crosses (Bubner and Baldwin, 2004).

Similarly, the work undertaken in identifying reference genes for transcript abundance in white clover will facilitate future transcript abundance analysis in the species, and could be transferred for transcript abundance analysis in white clover relatives. Furthermore, the use of ddPCR for transcript abundance analysis enabled the quantification of transcript at very low levels where qPCR is not sensitive enough.

7.1 Conclusion

The acidity of soils and associated aluminium toxicity are one of the main limitations for white clover use in Victoria (Environment and Natural Resources Committee, 2004). Moreover, water limitation has a negative impact on white clover survival during summer. This is particularly problematic in areas where annual rainfall is between 650-850 mm. Therefore, improving the adaptability of pastures in these areas, which are currently considered to be

marginal for white clover, and improving its reliability where it is currently predominant (annual rainfall of 850-1000 mm) are the primary objectives for white clover breeding in Australia (Nichols et al. 2012). Furthermore, AMV, along with other viruses, has a major impact on white clover productivity (Garrett 1991).

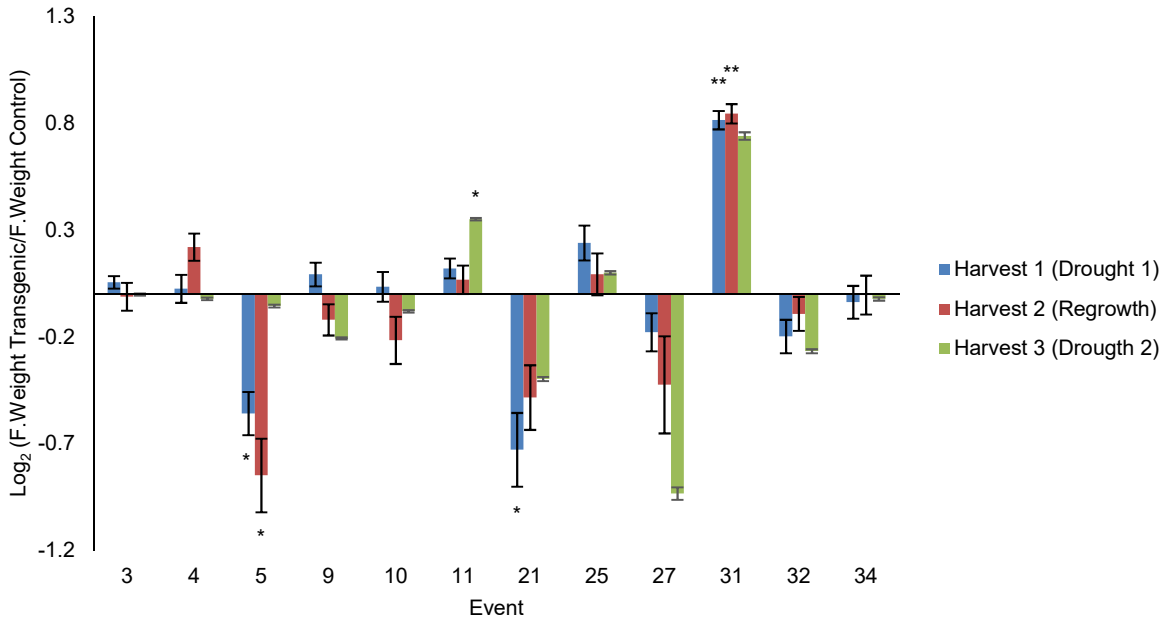
Improving white clover persistence was identified as one of the main objectives in white clover breeding in Australia (Smith and Fennessy, 2011). Persistence is mainly hindered, among other factors, by the three stresses mentioned above. The generation of transgenic events carrying the *IPT*, Tr*neMDH* and CP-*AMV* genes together, allowed the production of plants with characteristics that may contribute to overcoming these environmental limitations. This will lead to higher persistence of the species, and a more stable forage yield during the productive years under challenging conditions.

7.2 Future work

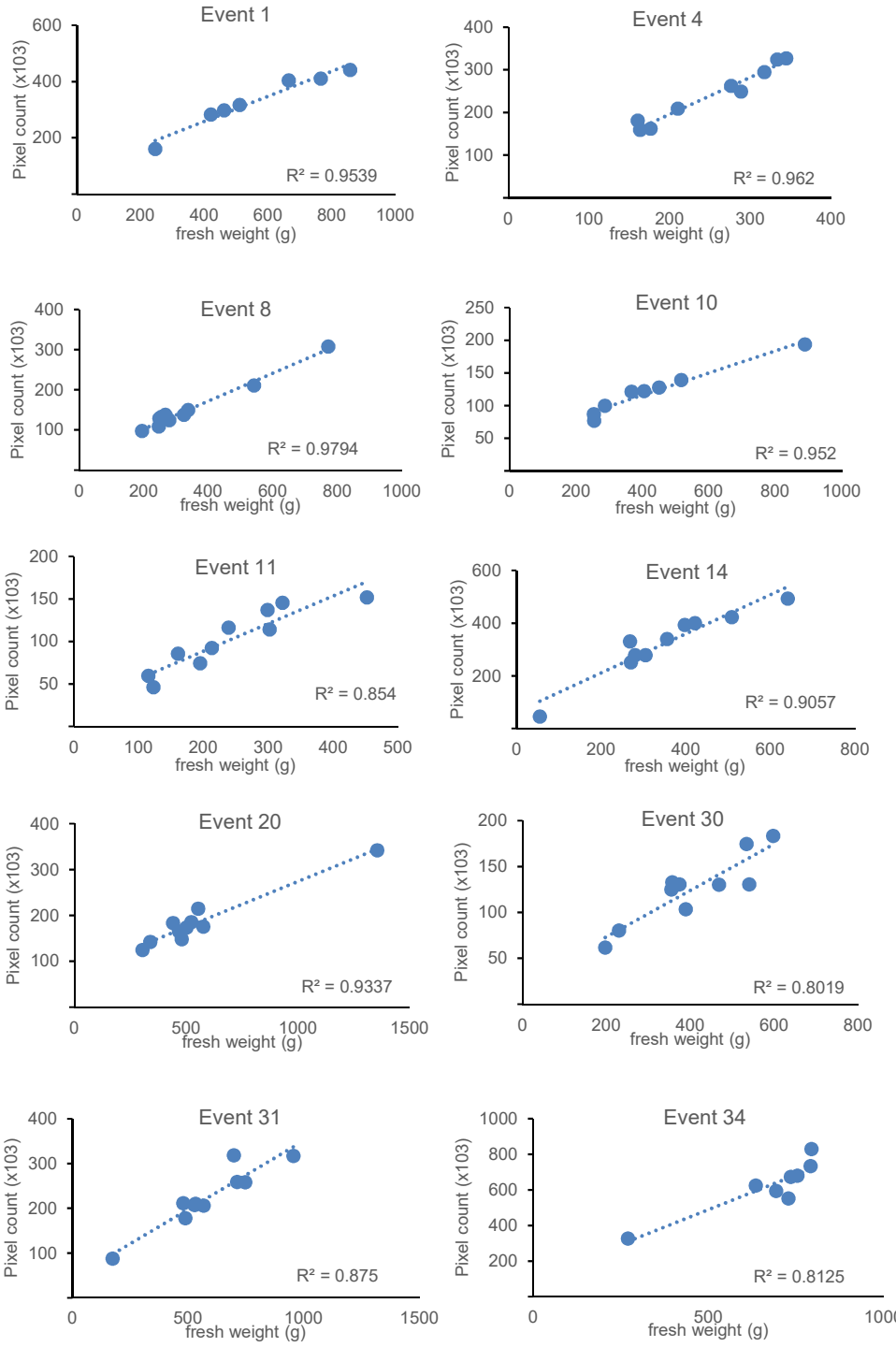
- Although CP-*AMV* transcript abundance analysis applied here as a proof of concept strategy for screening of AMV resistance, evaluating for resistance to AMV of the events selected in the subsequent generations is deemed necessary.
- The results generated from the soil-based assays in this work were not conclusive. Therefore, evaluation of transgenic plants in long-term soil-based assays, where root and shoot mass production are measured is proposed for generation of more definitive information about Al^{3+} tolerance of the produced events. Furthermore, field trials in advanced generations may be necessary in order to identify elite events for commercial use.
- The generation of events exhibiting delayed leaf senescence, drought stress tolerance and increased regrowth and vigour confirmed the effectiveness of the At*myb32:IPT* cassette for these purposes. However, the performance of field trials where forage production is measured over longer terms (multiple years) is required in order to estimate the impact of the inserted traits on white clover persistence may be required. Additionally, given that previous work reported that events expressing the *IPT* transgene exhibit an increase in seed production (Lin et al 2007, 2010), measurements of this parameter would be of special interest.
- The effects of each trait of interest can be confounded when evaluating the generated events under different stresses. Therefore, evaluation of events in controlled and field

conditions under combined stresses would be the ideal scenario in order to identify the contribution of each trait to the tolerance of different stresses.

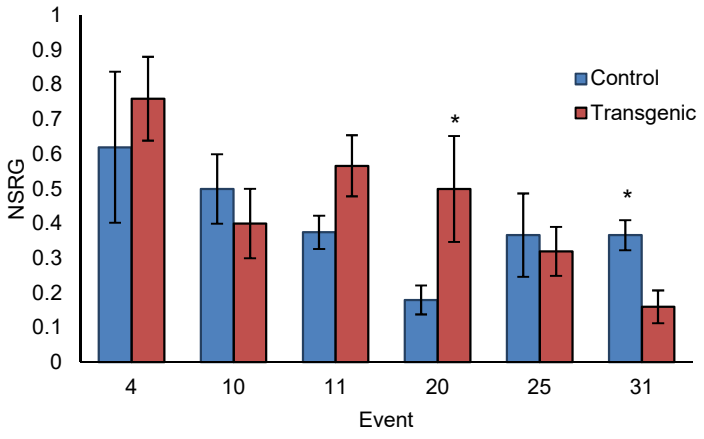
- Determination of stable inheritance of the transgene, transcription of the genes of interest, and expression of the desired traits in the subsequent generations are also needed in order to confirm agronomical value of the generated events. Techniques optimised in this project, such as transcript abundance analysis, and copy number estimations using ddPCR would be incorporated in these stages.
- The first steps toward digital image-based phenotyping of transgenic white clover were carried out in this work. Further development would involve the setup of fully automated phenotyping of transgenic white clover incorporating the information generated in the drought stress tolerance trial performed in this project.



Supplementary figure 4.1. Forage yields over drought trial of transgenic events versus non-transgenic control lines. Log_2(ratios) of fresh weights (g) of transgenic versus their respective non-transgenic lines are represented in y axis for the three harvests performed. Harvest 1 (Drought 1): first round of drought stress (42 days of water limitation), Harvest 2 (Regrowth) under constant soil water content of 70% for 21 days, (Drought 2) second round of drought stress (35 days of water limitation). Soil water content (SWC) was maintained at 30% between days 20 and 30, and at 25% from day 30 until the end of each water limitation stages. (*) significant difference between transgenic plant and their respective non-transgenic control ($P<0.05$); (**) significant difference (p value <0.01).



Supplementary figure 5.1. Correlation between fresh root weights and estimated root size by digital image analysis. Images were analysed by Lemnagrid software (Lemnatec, Würselen, Germany).



Supplementary figure 5.2. Net stress root growth (NSRG) from events evaluated in Al^{3+} tolerance assays in solution culture. NSRG was measured by subtracting initial root growth (IRG) from final root growth (FRG). Plants were evaluated in a split plot design, and 3 to 5 replicates per event were evaluated. (*) significant differences (p value<0.05).

Supplementary data 1: Analysis of housekeeping genes. Relative Ct values are presented in Table A. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Table A

Gene	Linearized data																			
	well-watered leaves (LWW)					water-limited leaves (LWL)					well-watered stolons (SWW)					water-limited stolons (SWL)				
	5 LWW	8 LWW	9 LWW	10 LWW	11 LWW	5 LWL	8 LWL	9 LWL	10 LWL	11 LWL	5 WW	8 SWW	9 SWW	10 SWW	11 SWW	5 SWL	8 SWL	9 SWL	10 SWL	11 SWL
EF1α	0.138	0.152	0.137	0.308	0.371	0.188	0.171	0.142	0.148	0.225	1.000	0.354	0.375	0.523	0.372	0.131	0.321	0.335	0.574	0.301
EF1β	0.011	0.054	0.052	0.153	0.062	0.063	0.119	0.139	0.085	0.066	1.000	0.165	0.380	0.688	0.124	0.153	0.487	0.209	0.114	0.030
ACT1 l	0.118	0.270	0.144	0.743	0.710	0.164	0.122	0.226	0.420	0.096	1.000	0.276	0.375	0.310	0.072	0.089	0.269	0.343	0.383	0.133
CYP	0.007	0.149	0.077	0.084	0.048	1.000	0.282	0.288	0.070	0.411	0.105	0.875	0.255	0.068	0.012	0.019	0.455	0.361	0.014	0.104
G6PD	0.030	0.339	0.117	0.234	0.226	0.536	0.176	0.326	0.154	0.069	1.000	0.383	0.227	0.005	0.020	0.015	0.442	0.056	0.411	0.076
TUA	0.050	0.086	0.086	0.246	0.336	0.226	0.086	0.050	0.118	0.135	1.000	0.291	0.164	0.661	0.051	0.016	0.057	0.209	0.277	0.023
UBQ	0.108	0.371	0.261	0.151	0.112	0.350	0.472	0.515	0.183	0.434	1.000	0.476	0.445	0.430	0.365	0.189	0.254	0.223	0.398	0.159

Supplementary data 1. NormFinder analysis results. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Best gene	*UBQ*
Stability value	0.395
Best combination of two genes	*ACT11* and *UBQ*
Stability value for best combination of two genes	0.270

Gene name	Stability value
UBQ	0.395
EF1α	0.463
ACT11	0.440
TUA	0.566
EF1ß	0.654
G6PD	0.678
CYP	0.778

Intragroup variation				
Group identifier	LWW	LWL	SWW	SWL
EF1α	0.290	0.401	0.042	0.122
EF1ß	0.124	0.541	0.210	0.833
ACT11	0.253	0.066	0.549	0.025
CYP	0.626	1.811	0.774	2.518
G6PD	0.129	3.500	0.424	1.142
TUA	0.260	0.551	0.329	0.827
UBQ	0.663	0.211	0.151	0.236

Intergroup variation				
Group identifier	LWW	LWL	SWW	SWL
EF1α	0.030	0.174	-0.503	0.299
EF1ß	-0.589	0.642	-0.369	0.316
ACT11	0.530	-0.210	-0.366	0.045
CYP	-0.482	-0.381	0.933	-0.070
G6PD	0.407	-0.711	0.363	-0.059
TUA	0.305	0.369	-0.208	-0.466
UBQ	-0.202	0.116	0.150	-0.065

Supplementary data 1. Delta Ct analysis results. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Gene	Sample																				StDev	StDev Mean
	5				8				9				10				11					
	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL		
TUA-																					--	
UBQ	3.686	3.197	2.566	6.089	4.681	5.020	3.278	4.713	4.163	5.944	4.005	2.664	1.859	3.192	1.944	3.088	0.977	4.249	5.408	5.362	1.411	
TUA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
G6PD	-1.540	0.457	-0.787	-0.915	1.198	0.243	-0.391	2.161	-0.348	1.931	-0.315	-2.681	-0.860	-0.408	-7.820	-0.219	-1.363	-1.753	-2.121	0.945	2.094	
EF1β	-1.185	-0.862	0.978	4.202	0.303	1.440	0.159	4.067	0.254	2.463	2.190	0.980	0.292	0.498	1.035	-0.308	-1.458	-0.046	2.265	1.354	1.533	
EF1α	5.330	3.592	3.860	6.854	4.689	4.845	4.145	6.346	4.522	5.375	5.053	4.544	4.185	4.184	3.523	4.910	4.001	4.593	6.727	7.580	1.132	
CYP	1.364	6.447	1.044	4.529	5.101	6.011	5.891	7.291	4.137	6.838	4.939	5.095	2.756	3.542	1.027	-0.025	1.481	5.906	2.202	6.481	2.268	
ACT11	-1.600	-3.307	-2.846	-0.415	-1.189	-2.342	-2.920	-0.617	-2.103	-0.660	-1.655	-2.127	-1.253	-1.020	-3.940	-2.379	-1.768	-3.348	-2.353	-0.311	1.043	1.354

Supplementary data 1. Delta Ct analysis results. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Gene	Sample																				StDev	StDev Mean
	5				8				9				10				11					
	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL		
UBQ-																						
UBQ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
TUA	-3.686	-3.197	-2.566	-6.089	-4.681	-5.020	-3.278	-4.713	-4.163	-5.944	-4.005	-2.664	-1.859	-3.192	-1.944	-3.088	-0.977	-4.249	-5.408	-5.362	1.411	
G6PD	-5.226	-2.740	-3.353	-7.004	-3.483	-4.777	-3.669	-2.553	-4.511	-4.013	-4.320	-5.345	-2.719	-3.600	-9.764	-3.307	-2.340	-6.003	-7.529	-4.417	1.878	
EF1β	-4.871	-4.059	-1.588	-1.887	-4.378	-3.581	-3.118	-0.647	-3.909	-3.481	-1.815	-1.685	-1.567	-2.694	-0.909	-3.396	-2.435	-4.296	-3.142	-4.008	1.240	
EF1α	1.644	0.395	1.294	0.765	0.008	-0.176	0.867	1.632	0.359	-0.568	1.049	1.880	2.325	0.991	1.579	1.822	3.024	0.344	1.319	2.218	0.913	
CYP	-2.322	3.250	-1.522	-1.559	0.420	0.990	2.613	2.578	-0.026	0.894	0.934	2.431	0.896	0.350	-0.917	-3.113	0.504	1.656	-3.206	1.118	1.879	
ACT11	-5.286	-6.505	-5.412	-6.504	-5.870	-7.362	-6.197	-5.331	-6.266	-6.603	-5.659	-4.791	-3.112	-4.212	-5.884	-5.467	-2.745	-7.597	-7.760	-5.673	1.303	1.437
G6PD-																						
UBQ	5.226	2.740	3.353	7.004	3.483	4.777	3.669	2.553	4.511	4.013	4.320	5.345	2.719	3.600	9.764	3.307	2.340	6.003	7.529	4.417	1.878	
TUA	1.540	-0.457	0.787	0.915	-1.198	-0.243	0.391	-2.161	0.348	-1.931	0.315	2.681	0.860	0.408	7.820	0.219	1.363	1.753	2.121	-0.945	2.094	
G6PD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
EF1β	0.355	-1.319	1.765	5.116	-0.895	1.196	0.550	1.906	0.602	0.532	2.505	3.660	1.152	0.906	8.855	-0.089	-0.094	1.707	4.387	0.409	2.355	
EF1α	6.870	3.135	4.648	7.769	3.491	4.601	4.536	4.185	4.870	3.444	5.369	7.225	5.045	4.592	11.343	5.129	5.364	6.346	8.848	6.635	2.005	
CYP	2.904	5.990	1.832	5.444	3.903	5.768	6.282	5.130	4.485	4.907	5.254	7.776	3.616	3.950	8.847	0.194	2.844	7.659	4.323	5.536	2.051	
ACT11	-0.060	-3.765	-2.059	0.500	-2.387	-2.585	-2.528	-2.778	-1.755	-2.591	-1.339	0.554	-0.393	-0.611	3.880	-2.160	-0.405	-1.594	-0.231	-1.256	1.684	2.011

Supplementary data 1. Delta Ct analysis results. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Gene	Sample																				StDev	StDev Mean
	5				8				9				10				11					
	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL		
EF1β-																					--	
UBQ	4.871	4.059	1.588	1.887	4.378	3.581	3.118	0.647	3.909	3.481	1.815	1.685	1.567	2.694	0.909	3.396	2.435	4.296	3.142	4.008	1.240	
TUA	1.185	0.862	-0.978	-4.202	-0.303	-1.440	-0.159	-4.067	-0.254	-2.463	-2.190	-0.980	-0.292	-0.498	-1.035	0.308	1.458	0.046	-2.265	-1.354	1.533	
G6PD	-0.355	1.319	-1.765	-5.116	0.895	-1.196	-0.550	-1.906	-0.602	-0.532	-2.505	-3.660	-1.152	-0.906	-8.855	0.089	0.094	-1.707	-4.387	-0.409	2.355	
EF1β	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
EF1α	6.514	4.454	2.883	2.653	4.386	3.405	3.986	2.279	4.268	2.912	2.864	3.564	3.893	3.686	2.488	5.218	5.459	4.639	4.461	6.226	1.200	
CYP	2.549	7.309	0.067	0.328	4.798	4.571	5.732	3.224	3.883	4.375	2.749	4.116	2.464	3.044	-0.008	0.283	2.939	5.952	-0.064	5.126	2.182	
ACT11	-0.416	-2.446	-3.824	-4.616	-1.492	-3.781	-3.079	-4.684	-2.357	-3.123	-3.844	-3.107	-1.545	-1.517	-4.975	-2.071	-0.310	-3.301	-4.618	-1.665	1.404	1.652
EF1α-																					--	
UBQ	-1.644	-0.395	-1.294	-0.765	-0.008	0.176	-0.867	-1.632	-0.359	0.568	-1.049	-1.880	-2.325	-0.991	-1.579	-1.822	-3.024	-0.344	-1.319	-2.218	0.913	
TUA	-5.330	-3.592	-3.860	-6.854	-4.689	-4.845	-4.145	-6.346	-4.522	-5.375	-5.053	-4.544	-4.185	-4.184	-3.523	-4.910	-4.001	-4.593	-6.727	-7.580	1.132	
G6PD	-6.870	-3.135	-4.648	-7.769	-3.491	-4.601	-4.536	-4.185	-4.870	-3.444	-5.369	-7.225	-5.045	-4.592	-11.343	-5.129	-5.364	-6.346	-8.848	-6.635	2.005	
EF1β	-6.514	-4.454	-2.883	-2.653	-4.386	-3.405	-3.986	-2.279	-4.268	-2.912	-2.864	-3.564	-3.893	-3.686	-2.488	-5.218	-5.459	-4.639	-4.461	-6.226	1.200	
EF1α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
CYP	-3.965	2.855	-2.816	-2.325	0.412	1.166	1.746	0.946	-0.385	1.463	-0.115	0.551	-1.429	-0.642	-2.496	-4.935	-2.520	1.313	-4.525	-1.099	2.224	
ACT11	-6.930	-6.900	-6.707	-7.269	-5.878	-7.186	-7.065	-6.963	-6.625	-6.035	-6.708	-6.671	-5.438	-5.203	-7.464	-7.289	-5.769	-7.941	-9.079	-7.891	0.911	1.398

Supplementary data 1. Delta Ct analysis results. LWW, well-watered leaves; LWL, water-limited leaves; SWW, well-watered stolons; SWL, water-limited stolons

Gene	Sample																				StDev	StDev Mean
	5				8				9				10				11					
	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL	LWW	LWL	SWW	SWL		
CYP-																					--	
UBQ	2.322	-3.250	1.522	1.559	-0.420	-0.990	-2.613	-2.578	0.026	-0.894	-0.934	-2.431	-0.896	-0.350	0.917	3.113	-0.504	-1.656	3.206	-1.118	1.879	
TUA	-1.364	-6.447	-1.044	-4.529	-5.101	-6.011	-5.891	-7.291	-4.137	-6.838	-4.939	-5.095	-2.756	-3.542	-1.027	0.025	-1.481	-5.906	-2.202	-6.481	2.268	
G6PD	-2.904	-5.990	-1.832	-5.444	-3.903	-5.768	-6.282	-5.130	-4.485	-4.907	-5.254	-7.776	-3.616	-3.950	-8.847	-0.194	-2.844	-7.659	-4.323	-5.536	2.051	
EF1β	-2.549	-7.309	-0.067	-0.328	-4.798	-4.571	-5.732	-3.224	-3.883	-4.375	-2.749	-4.116	-2.464	-3.044	0.008	-0.283	-2.939	-5.952	0.064	-5.126	2.182	
EF1α	3.965	-2.855	2.816	2.325	-0.412	-1.166	-1.746	-0.946	0.385	-1.463	0.115	-0.551	1.429	0.642	2.496	4.935	2.520	-1.313	4.525	1.099	2.224	
CYP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
ACT11	-2.965	-9.754	-3.891	-4.944	-6.290	-8.352	-8.811	-7.908	-6.240	-7.498	-6.594	-7.222	-4.009	-4.562	-4.968	-2.354	-3.249	-9.253	-4.554	-6.792	2.194	2.133
ACT11-																					--	
ACT11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	--	
UBQ	5.286	6.505	5.412	6.504	5.870	7.362	6.197	5.331	6.266	6.603	5.659	4.791	3.112	4.212	5.884	5.467	2.745	7.597	7.760	5.673	1.303	
TUA	1.600	3.307	2.846	0.415	1.189	2.342	2.920	0.617	2.103	0.660	1.655	2.127	1.253	1.020	3.940	2.379	1.768	3.348	2.353	0.311	1.043	
G6PD	0.060	3.765	2.059	-0.500	2.387	2.585	2.528	2.778	1.755	2.591	1.339	-0.554	0.393	0.611	-3.880	2.160	0.405	1.594	0.231	1.256	1.684	
EF1β	0.416	2.446	3.824	4.616	1.492	3.781	3.079	4.684	2.357	3.123	3.844	3.107	1.545	1.517	4.975	2.071	0.310	3.301	4.618	1.665	1.404	
EF1α	6.930	6.900	6.707	7.269	5.878	7.186	7.065	6.963	6.625	6.035	6.708	6.671	5.438	5.203	7.464	7.289	5.769	7.941	9.079	7.891	0.911	
CYP	2.965	9.754	3.891	4.944	6.290	8.352	8.811	7.908	6.240	7.498	6.594	7.222	4.009	4.562	4.968	2.354	3.249	9.253	4.554	6.792	2.194	1.423

Supplementary data 2. Rankings of genes including all tests.

Description: Genes were ranked based on their stability in the different organs and treatments (Table A), and when groups were compared (Table B). A ranking score was calculated for each gene by summing their ranking position in the different analyses.

Table A

Gene	Rankings including all samples		Rankings inside groups				NormFinder	Total score
	ΔCt	CV	ΔCt (LWW)	ΔCt (LWL)	ΔCt (SWW)	ΔCt (SWL)		
Ef1α	1	3	5	1	3	2	3	18
Act11	2	4	4	6	1	1	2	20
UBQ	3	2	6	2	2	5	1	21
TUA	4	6	3	4	5	6	5	33
Ef1β	5	8	1	3	4	5	7	33
G6PD	6	5	2	5	7	4	6	35
CYP	7	7	5	7	6	3	8	43

Table B

Gene	Rankings comparing groups						Total score
	ANOVA (F values)						
	LWW vs LWL	LWL vs SWW	SWW vs SWL	LWW vs SWL	LWW vs SWW	LWL vs SWL	
G6PD	2	1	2	1	3	2	11
ACT11	6	4	3	5	1	3	22
TUA	1	6	6	3	5	1	22
CYP	7	2	1	6	4	5	25
Ef1α	3	8	4	7	6	8	36
UBQ	8	3	7	4	8	7	37
Ef1β	4	7	5	8	7	6	37

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