Transformation of *Medicago truncatula* with the Arginine Decarboxylase Gene to Modify Polyamine Metabolism toward Water Deficit Resistance

Ana Sofia Roldão Lopes Amaral Duque

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"E eu digo que sem movimento a vida é, com efeito, trevas
e que todo o movimento é cego sem o saber,
e que sem trabalho todo o saber é vão,
e que sem amor todo o trabalho é vazio..."

"And I say that life is indeed darkness save when there is urge,
and all urge is blind save when there is knowledge,
and all knowledge is vain save when there is work,
and all work is empty save when there is love…"

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Resumo:

O principal objectivo desta tese consistiu na transformação da leguminosa modelo *Medicago truncatula* e expressão de uma sequência codificadora do gene da arginina decarboxilase (*Adc*) de aveia (*Avena sativa*), que codifica uma enzima chave da via das poliaminas. Com esta manipulação genética pretendia-se alterar os níveis de poliaminas com intuito de produzir linhagens que apresentassem respostas melhoradas na resistência ao deficit hídrico.

Dentro dos stresses abióticos, o deficit hídrico conduz a uma limitação da produtividade vegetal e condiciona a área de distribuição das formações vegetais na superfície do globo terrestre. As leguminosas têm um papel importante nos ecossistemas, devido a sua capacidade de fixarem o azoto atmosférico em simbiose com bactérias do género rhizobium. O cultivo de Leguminosas pode contribuir para o melhoramento do conteúdo em azoto do solo, melhorando a sua fertilidade e favorecendo a introdução de novas culturas em áreas previamente desertificadas.

O facto dos mecanismos fisiológicos envolvidos na resistência ao deficit hídrico serem complexos e ainda pouco compreendidos bem como a dificuldade em obter marcadores moleculares associados a esta característica tem limitado as abordagens de melhoramento convencional nesta família de plantas.

O melhoramento através da obtenção de cultivares geneticamente modificados requer uma optimização prévia de sistemas de transformação e selecção de forma a assegurar a introdução e expressão de genes relacionadas com a resistência ao stresse nas variedades de leguminosas que se pretende melhorar.

A compreensão dos efeitos fisiológicos subjacentes aos genes introduzidos, bem como a avaliação da performance das plantas transgénicas quando sujeitas a condições de stresse são essenciais para o implemento com sucesso desta tecnologia. Nos últimos anos *Medicago truncatula* surge como sistema modelo para o estudo dos processos biológicos em leguminosas e progressos significativos têm sido conseguidos nos conhecimentos de fisiologia, genética e genómica desta espécie.

Neste trabalho desenvolveu-se um sistema simples e eficiente de transformação/regeneração na leguminosa modelo *M. truncatula* cv. Jemalong. Este método teve como base a grande capacidade embriogénica da linha M9-10a obtida no nosso laboratório e permitiu a recuperação de várias linhagens transgénicas de *M. truncatula* num período de tempo de aproximadamente 3-4 meses. Procedeu-se à co-cultura de folíolos de plantas M9-10a, mantidas *in vitro* por micropropagação, com a estirpe de *Agrobacterium tumefaciens* EHA105 contendo o vector de transformação p35S*Adc-Gus*. Os vectores de transformação construídos contêm uma sequência codificadora do gene da arginina decarboxilase (*Adc*) de aveia sobre controlo do promotor 2XCaMV 35S e os genes *Gus* e *nptII* como marcadores de transformação.

Inicialmente procedeu-se à optimização da concentração de canamicina a utilizar nos processos de selecção dos tecidos vegetais transformados. A utilização de 171.6 μM de canamicina permitiu a redução do número de embriões falsos-positivos, tornando o processo de selecção mais eficiente e menos laborioso. O estado de desenvolvimento a que se procedeu ao isolamento dos embriões (torpedo/cotilédonar) bem como a utilização de um meio com agar aliado a uma baixa concentração de carbenicilina, permitiu a redução do fenómeno de embriogénese secundária, evitou o aspecto hiper-hídrico e desta forma melhorou a taxa de conversão dos embriões somáticos.

A análise por *PCR* e *Southern blot* permitiu confirmar a integração do transgene *Adc* no genoma dos transformantes primários (transformantes T_0). Confirmou-se a expressão do transgene por RT-PCR e procedeu-se à segregação do transgene com base no fenótipo de resistência à canamicina por germinação das sementes (T_1) em meio contendo 686.6 μM de canamicina.

Uma metodologia alternativa para a selecção *in vitro* de plantas transformadas de *M. truncatula* cv. Jemalong foi também desenvolvida. Utilizou-se um vector de transformação contendo sequências codificantes fundidas do gene para a proteína fluorescente verde (GFP) e do gene para a proteína β-glucuronidase (GUS). Utilizando o processo de transformação mediado pelo *Agrobacterium* seguido de regeneração por embriogénese somática em condições de escuridão (para evitar a síntese e consequente auto-fluorescência da clorofila). Nestas condições foi possível tirar partido da GFP para selecção precoce dos embriões transformados. Os embriões seleccionados desta forma desenvolvem-se em plantas e

confirmou-se o seu estado transgénico por detecção de GUS nas folhas, caule e raiz das plantas transformadas. Esta selecção baseada no GFP-GUS constitui um processo alternativo para obter plantas transformadas de *M. truncatula* evitando a utilização de marcadores de selecção que conferem resistência a antibióticos ou herbicidas.

Estabeleceram-se também as condições de regeneração de plantas *via* embriogénese somática a partir de culturas de células em suspensão de *M. truncatula* cv. Jemalong. Utilizou-se uma linha de células em suspensão de M9-10a mantida por subcultura no nosso laboratório durante cerca de 3 anos. Verificou-se que durante o processo de manutenção, em meio contendo 2,4-D e Cinetina, as células adquirem competência embriogénica e são capazes de desenvolver embriões somáticos em massa quando transferidas para meio sem fitorreguladores. Este é um processo alternativo de regeneração de *M. truncatula* cv. Jemalong *via* embriogénese somática que permite solucionar problemas relacionados com a insuficiente quantidade de folíolos de M9-10a disponíveis para transformação/regeneração devido a limitações de espaço de cultura e condições fisiológicas alteradas decorrentes do ritmo biológico anual destas plantas.

Para a detecção e quantificação de aminas biogénicas nas plantas transformadas com o gene *Adc* e nas plantas controlo utilizou-se uma metodologia de RP-HPLC (*Reverse-Phase High Performance Liquid Chromatography*). Neste processo procedeu-se a uma derivatização pós-coluna com o reagente OPA/MCE (*o-phtaldialdehyde/2-mercaptoethanol)* o que permitiu a detecção e quantificação não só das aminas livres, bem como das conjugadas solúveis e conjugadas insolúveis.

Observou-se uma grande acumulação de Agmatina livre (o produto da enzima ADC e intermediário na biossíntese de Putrescina) na linhagem transgénica L108. Verificou-se o subsequente aumento dos níveis de Putrescina e Espermidina. O conteúdo total em Histamina na linhagem transgénica L108 comparado com o controlo foi também significativamente mais elevado. A possibilidade de detecção concomitante de Poliaminas, Agmatina e Histamina é uma vantagem da metodologia aplicada.

As linhas ADC transgénicas obtidas neste trabalho, em conjunto com o procedimento descrito para quantificação de aminas biogénicas, permitem estudos posteriores relacionados com a alteração do metabolismo das poliaminas bem como possibilitam o estudo do efeito dessas alterações na resistência ao stresse abiótico da leguminosa modelo *M. truncatula.*

Summary:

The main purpose of this thesis was to introduce and express in the model legume *Medicago truncatula* the arginine decarboxylase encoding gene (*Adc*) from *Avena sativa*, that codes for a key enzyme of the polyamine biosynthetic pathway, to modify polyamine metabolism aiming to alter the plant responses to water deficit.

Water deficit is the major abiotic stress that severely limits crop yields and controls the distribution of vegetation over the earth's surface. Due to their capacity to grow on nitrogen-poor soils, legumes are important candidates for improving saline soil fertility and helping to reintroduce agriculture to dehydrated lands. Efforts in developing legume crops with better drought/water deficit resistance through conventional breeding have been restricted, mainly because of underprovided understanding of the physiological mechanisms underlying stress resistance and to the lack of sufficient polymorphism for drought resistance-related traits. The alternative process of generating transgenic cultivars requires success in the transformation method and proper incorporation of stress resistance into plants. Further evaluation of the transgenic plants under stress conditions and understanding the physiological effect of the inserted genes at the whole plant level are also essential for the success of this technology. Towards the usefulness of developing a model system, *Medicago truncatula* has become increasingly relevant in latest years due to significant progress at the genetic and genomic levels in this specie.

A simple and efficient regeneration-transformation method was established to obtain transgenic plants of the model legume *Medicago truncatula* cv. Jemalong. This method takes advantage of a highly embryogenic line (M9-10a) isolated in our laboratory and allowed the recovery of several transgenic *M. truncatula* plants within 3-4 months. Leaflets of *in vitro* grown M9-10a plants were co-cultured with *Agrobacterium tumefaciens* EHA105 bearing the p35S*Adc-Gus* constructs. The plasmid constructs used for plant transformation contained: the oat arginine decarboxylase encoding gene (*Adc*) under control of a CaMV 35S promoter with duplicated enhancer sequences; the *Gus* reporter gene; and the *nptII* gene as selection marker.

Using 171.6 μM of kanamycin we were able to reduce the number of emerging false kanamycin-resistant embryos, which is an important improvement to the method, making it less laborious and very efficient. Isolation of late torpedo/cotyledonary-stage embryos to lower carbenicillin/agar media reduced secondary embryogenesis and prevented hyperhydricity, improving embryo conversion.

PCR and Southern blot hybridization analysis confirmed the integration of the *Adc* transgenes in the genome of the T_0 transformants. The oat *Adc* transgenic expression was analysed by RT-PCR and transgene inheritance based on the kanamycin resistance phenotype was confirmed by germinating seeds in 686.6 μM kanamycin-containing medium.

We developed an alternative methodology for *in vitro* selection of transgenic *Medicago truncatula* cv. Jemalong plants using a bifunctional construct in which the coding sequences for the green fluorescent protein (GFP) and the β-glucuronidase protein (GUS) are fused. An *Agrobacterium*-mediated transformation protocol was used followed by regeneration via somatic embryogenesis in the dark, to avoid the synthesis and the consequent autofluorescence of chlorophyll. GFP was used as an *in vivo* detectable marker for early embryo selection. All the somatic embryos selected as GFP positive were transformed, as subsequently verified by the detection of GUS activity in leaves, stems and roots of the regenerated plants. The GFP-GUS selection consists in an alternative methodology to obtain transgenic *M. truncatula* plants without marker genes conferring antibiotic or herbicide resistance.

In this thesis, we also established the conditions for plant regeneration *via* somatic embryogenesis from 3-year-old cell suspension cultures of *M. truncatula* cv. Jemalong M9-10a line. Although plant regeneration *via* somatic embryogenesis from *in vitro* cultured leaf-explants was extremely efficient, we sometimes experienced a constraint on availability of M9-10a leaflets for plant transformation experiments because of *in vitro* culture space limitations and physiological adequacy due to annual rhythms. We found that, when growing in medium supplemented with 2,4-D and Kin, cells acquire embryogenic competence and are able to generate a large number of somatic embryos upon transfer to growth regulator-free media. We consider this a useful alternative procedure for regeneration of *M. truncatula* cv. Jemalong *via* somatic embryogenesis.

An optimized ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) methodology was used to detect and quantify biogenic amines in leaves of both wild-type untransformed (M9-10a) and in the transgenic L108 line. This procedure uses a post-column derivatization with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) reagent and was shown to be suitable for detection and quantification of free, soluble bound and insoluble bond amines using.

A large accumulation of free Agmatine (the direct product of the ADC enzyme and intermediate in the Putrescine biosynthesis) in the transformed plants and a related increase in the levels of Putrescine and Spermidine was observed. The total Histamine content in leaves of transgenic L108 plants compared to wild-type was also significantly higher. The concomitant detection of Polyamines, Agmatine and Histamine, is an advantage of this technique. This ADC transgenic line coupled with the described procedure for quantifying biogenic amines will provide a powerful tool to perform further studies related to altered polyamine metabolism and to fulfill our broad purpose of studying abiotic stress resistance in the *M. truncatula* model legume.

List of most used abbreviations:

ADC- Arginine decarboxylase enzyme

Adc- Arginine decarboxylase encoding gene

ABA- Abscisic acid

ABRE- ABA responsive element

ACC- Aminocyclopropane

ACCS- ACC synthase

ACCO- ACC oxidase

Agm- Agmatine

AHI- Agmatine iminohydrolase

Amp- Ampicillin

ANOVA- One-way Analysis of Variance

AO- Amino oxidase

Arg- Arginine

Bar- Bialaphos resistance gene

Cad- Cadaverine

cDNA- Complementary DNA

CaMV- Cauliflower mosaic virus

CaMV 35S- Cauliflower mosaic virus 35S promoter

2X35S- 35S Promoter with duplicated enhancer sequences

Carb- Carbenicillin

CE- Capillary electrophoresis

Chl- Chlorophyll

CIAP- Calf intestinal alkaline phosphatase

CPA- *N*-carbamoylputrescine amidohydrolase

C_2- Stress inducible promoter from *C. plantagineum*

2,4-D- 2,4-Dichlorophenoxyacetic acid

DAH- 1,6-Diaminohexane

DAO- Diamine oxidase

dcSAM- Decarboxylated SAM

DH5α- *E. coli* strain used

DNA- Deoxyribonucleic acid

DNase I- Desoxyribonuclease I

dNTPs- Deoxyribonucleoside triphosphates

Dsp22- Dessication stress protein 22 encoding gene

DW- Dry weight

ECM- Embryo conversion medium

EDTA- Ethylenediaminetetraacetic acid

EIM- Embryo induction medium

EHA105- *Agrobacterium tumefaciens* strain used

ELIP- Early light induced protein

EPM- Embryo proliferation medium

EST- Expressed Sequence Tag

EtBr- Ethidium bromide

GABA- γ-Aminobutiric acid

GFP- Green fluorescent protein

Gfp- Green fluorescent protein encoding gene

GUS- β-glucuronidase protein

Gus- ß-Glucuronidase gene

FW- Fresh weight

His- Histamine

Hpt- Hygromycin phosphotransferase gene

HSP- Heat shock proteins

IP_3- Inositol 1,4,5-triphosphate

IPTG- Isopropyl ß-D-thiogalactopyranoside

IS- Internal standard

Kan- Kanamycin

Kan^R- Kanamycin resistant

Kan^S- Kanamycin sensitive

LA- LB solidified with microagar

Lac Z- ß–Galactosidase gene

LB- T-DNA left border

LB- Luria Broth rich medium

LEA- Late-embryogenesis-abundant proteins

LDC- Lysine decarboxylase

LHCII- Light-harvesting complex II

L108- *M. truncatula* transgenic line expressing heterologous oat *Adc* gene

MCS- Multiple cloning site

MS- Murashige and Skoog culture medium

M9-10a- *Medicago truncatula* cv Jemalong embryogenic line

NO- Nitric Oxide

*Npt*II- Neomycin phosphotransferase II gene

Nos- Nopaline synthase gene

ODC- Ornithine decarboxylase

OPA- *o*-phthalaldehyde

OPA/MCE- *o*-Phtaldialdehyde/2-mercaptoethanol

Orn- Ornithine

PAs- Polyamines

PAO- Polyamine oxidases

PEG- Polyethylene glycol

PCR- Polymerase chain reaction

pNos- Nopaline synthase promoter

Put- Putrescine

PYRR- Δ^1-pyrroline

qPCR- Quantitative real time PCR

*rab*17- Responsive to absicic acid 17 promoter

RB- T-DNA right border

RF- Response factors

Rif-Rifampicin

RNA- Ribonucleic acid

RNase A- Ribonuclease A

ROS- Reactive oxygen species

RP-HPLC- Reverse-Phase High Performance Liquid Chromatography

RT-PCR- Reverse transcription-PCR

RWC- Relative Water Content

R_{SWC}- Relative soil water content

SAM- S-Adenosylmethionine

SAMDC- SAM decarboxylase

SAMS- SAM synthetase

s-GFP-TYG- synthetic GFP red-shifted variant

Spd- Spermidine

SPDS- Spd synthase

Spm- Spermine

SPMS- Spm synthase

STD- Standard deviations

TAE- Tris acetate EDTA buffer

Taq- *Thermus aquaticus* DNA polymerase

Tet- Tetracycline

TBE- Tris borate EDTA buffer

TCA- Trichloroacetic acid

TCA Cycle- Tricarboxylic acid cycle or Krebs cycle

T-DNA- Transferred DNA region of Ti plasmid

TE- 10 mM Tris; 1 mM EDTA buffer

TGases- Transglutaminases

Ti- Tumour-inducing plasmid

TLC- Thin layer chromatography

tNos- Nopaline synthase terminator

Tyr- Tyramine

TYDC- Tyrosine decarboxylase

T_0- Primary transformants

T_1- First generation of transgenic plants (cross T_0 X T_0)

Ubi-1- Maize ubiquitin promoter

WD- Water deficit

X-Gal- 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

X-GlcA- 5-Bromo-4-chloro-3-indolyl-β-D-Glucuronide acid

Zea- Zeatin

Ψ_w- Water potential

Table of contents

I – General Introduction

1. General considerations

1.1. The legume family

Legumes (Leguminosae or Fabaceae) are the third largest family of flowering plants with an estimated 20,000 species (Cronk et al., 2006). The Leguminosae are second to cereal crops in agricultural importance based on area harvested and total production. In 2007 about 3.300 million tonnes of grain legumes have been produced on 3.900 million hectares around the world (According to FAO; UNdata accessed 25/10/2010). Grain legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance, 2003). In many places of the world, legumes complement cereals or root crops, the primary source of carbohydrates, in terms of amino acid composition (Wang et al., 2003). Legumes are also important forages in temperate (e.g. alfalfa, clovers (Trifolium spp.)) and tropical (Stylosanthes sp., Desmodium sp.) regions (Gepts et al., 2005).

The monophyletic subfamily Papilionoideae (pea-flowered legumes) is the largest and most widespread of the three legume subfamilies with an estimated 476 genera and 13,860 species (Wojciechowski et al., 2004) and includes the hologalegina (galegoid) legumes, which comprise the robinioids (including Lotus sp) and the inverted repeat loss (IRL) clade (including Medicago sp and Pisum) (Cronk et al., 2006).

One of the important features of legumes is the association between plants and rhizobial and mycorrhizal symbionts. The formation and development of nitrogen-fixing root nodules is the result of a symbiotic relationship between leguminous plants and soil bacteria collectively called rhizobia or *Rhizobium*, but including more specifically the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (reviewed in Kinkema et al., 2006). As for the formation of arbuscular endomycorrhiza (AM), most of the higher plants have the ability of the symbiotic association with fungi belonging to the order of *Glomales* (Albrecht et al., 1999). In AM symbiosis, the fungus also forms hyphae outside the plant and these provide a connection

between the soil and the inner part of the plant and they facilitate the uptake of nutrients such as phosphate (reviewed in Gianinazzi-Pearson, 1996; Harrison, 1997).

The development of nitrogen-fixing nodules results in the production substantial amounts of organic nitrogen fertilizer (e.g. Jensen and Hauggaard-Nielsen, 2003; Hirsch, 2004) that provides a major means of available nitrogen into the biosphere. In these nodules the bacteria are hosted intracellularly and there they find the ideal environment to reduce atmospheric nitrogen into ammonia, a source of nitrogen which can be used by the plant (for reviews see: Mylona et al., 1995; Long, 1996; Albrecht et al., 1999). Studies of the rhizosphere in legumes are among the most developed of all botanical families and can lead to significant advances in plant health and growth.

The large genome size and the polyploidy of some legumes have hampered the successful application of biotechnology to face biotic and abiotic constrains in legume crops. To solve some of these problems two species, *Medicago truncatula* Gaertner and *Lotus japonicus* (Regel) K. Larsen (subfamily Papilionoideae), have emerged as model plants to investigate the genetics of nodulation and other important processes such as resistance or tolerance to stresses (Young et al., 2005; Zhu et al., 2005, Dita et al., 2006). The application of genomics has led to substantial and rapid advances in our understanding of the molecular basis of the two types of symbioses in *M. truncatula* and *L. japonicus* (Oldroyd et al., 2005).

Presently these two models are used in a variety of biological fields from plant physiology and development to population genetics and structural genomics (reviewed in Ané et al., 2008).

1.2. *Medicago truncatula:* a model legume

Medicago truncatula Gaertn. (Barrel medic) is an annual legume with agronomic interest originated from Mediterranean regions (Ané et al., 2008). As a legume, it represents a source of protein-rich food and can be grown independently of exogenous nitrogen supply, due to its ability to form nitrogen fixing roots nodules in symbiosis with *Sinorhizobium meliloit* (previously named *Rhizobium meliloti*) (Albrecht et al., 1999), which is one of the best-characterized *Rhizobium* species at the genetic level (Galibert et

al., 2001). This plant is used for pasture, forage and crop rotation to improve soil fertility of cultivated lands, however, it is susceptive to pronounce drought periods that are usual in southern areas of Portugal.

Important characteristics that make *M. truncatula* a desirable experimental system for legume biology studies (Barker et al., 1990; Cook, 1999, Ané et al., 2008) includes diploidy (2n=16) and autogamous fertilization, small genome (500-600 Mdp/1C; Blondon et al., 1994), short life cycle and a number of available cultivars; being the Jemalong cultivar one of the most used in agricultural practices and pastures for animals.

At the beginning of this work, a whole genome sequencing project for *M. truncatula* was being carried out combined with a large-scale EST (Expressed Sequence Tag) sequencing program with over 170.000 entries in public databases (Lamblin et al., 2003) and the establishment of genetic and physical maps (Thoquet et al., 2002); with the global purpose of studying the genetic and biochemical events associated with the growth, development and environmental interactions of legumes (Bell et al., 2001, Lamblin et al., 2003). The *M. truncatula* genome sequencing by international consortiums is actually approaching completion (Young and Udvardi, 2009). The third assembly version was recently released (Medicago Genome Sequence Consortium, October 2009, http://www.medicago.org/genome/downloads/Mt3/). The current availability of a mutant *M. truncatula* population resource for reverse genetics, the *Tnt*1-tagged population, is also an important tool for further studies concerning the legume biology (Tadege et al., 2009).

Additionally, high degree of synteny at the genome level has been found between *Medicago* and others cool-season legumes genomes like pea (Kalo at al. 2004), lentil, chickpea, peanut and soybean (Gepts et al., 2005); contrary to *L. japonicus* that belongs to the Loteae and is more distant from cultivated cool season legumes (Ané et al., 2008).

As conclusion, *M. truncatula* emerged as an attractive model for studying the molecular genetics of the *Rhizobium*-legume symbiotic relation as well as gene expression regulation and physiological and developmental processes in plants (Barker et al., 1990; Covitz et al., 1998; Ané et al., 2008).

1.3. Transformation/regeneration in *Medicago truncatula*

The features referred above also enable *M. truncatula* to be used in transgenic approaches for fundamental studies or for expression of genes for crop improvement. These studies require efficient transformation/regeneration systems to generate a high number of transgenic plants with a low occurrence of phenotypic abnormalities. However, somatic embryogenesis in legumes is known to be genotype dependent (Nolan et al., 1989; Somers et al., 2003) and only some selected lines/genotypes of *M. truncatula* cv. Jemalong are found to have embryogenic potential, e. g. the reported J5, A17 and 2HA (Thomas et al., 1992; Chabaud et al., 1996; Rose et al., 1999; Kamaté et al., 2000; Zhou et al., 2004).

In our laboratory we have *M. truncatula* lines with different embryogenic capacity. A highly embryogenic line (M9-10a) was obtained from a line with very low embryogenic potential, named M9, after regeneration *via* somatic embryogenesis with an intermediate period of *callus* growth (Neves et al., 1999; Neves, 2000; Santos and Fevereiro, 2002). Briefly, folioles from the M9 line were placed on an embryo induction medium (EIM) containing 2,4-dichlorophenoxyacetic acid (2,4-D) and zeatin (Zea); after 21 days abundant friable *calli* growing along the incisions were transferred to the same medium without growth regulators, were few somatic embryos appeared. One of this embryos converted to a plantlet that was designated M9-10a (Santos and Fevereiro, 2002). This M9-10a line has been propagated by cuttings as described in Neves et al (2001) and maintains the embryogenic capacity until nowadays.

Furthermore, a system of repetitive somatic embryogenesis from leaf-explants was established for *M. truncatula*, which represents a permanent source of embryogenic material that could be used for the genetic modification (Neves et al., 1999; Neves, 2000). Additionally, *M. truncatula* plants have been transformed using particle bombardment with reporter and selectable marker genes (*Gus*, *Hpt* and *Bar* genes) (Neves et al., 1998).

We can conclude that in *M. truncatula* an important and necessary condition for both genetic transformation and other tissue-cultures derived techniques is, as recognized by Dita et al. (2006), the implementation of robust protocols for plant regeneration.

1.4. Importance of improving drought stress resistance

Plant productivity is strongly affected by abiotic stresses. This broad term includes multiple stresses such as heat, chilling, excessive light, drought, waterlogging, wounding, ozone exposure, UV-B irradiation, osmotic shock and salinity (Dita et al., 2006). Drought and salinity are two major environmental factors determining plant productivity and plant distribution and reduced soil water potential is a common consequence of both of them (Groppa and Benavides, 2008). Drought and salinity affect more than 10 percent of arable land, and desertification and salinization are rapidly increasing on a global scale declining average yields for most major crop plants by more than 50 percent (Bray et al., 2000; Bartels and Sunkar, 2005). There is the prediction that 30% of land will became unusable for agriculture in 25 years because of soil salinization (Kolodyazhnaya et al., 2009).

Plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth and development. The regulatory circuits include stress sensors, signaling pathways comprising a network of protein-protein reactions, transcription factors and promoters, and finally the output proteins or metabolites (reviewed in Bartels and Sunkar, 2005). As water and salt stresses occur frequently and can affect most habitats, plants have developed several strategies to cope with these challenges: either adaptation mechanisms, which allow them to survive the adverse conditions, or specific growth habits to avoid stress conditions (Bartels and Sunkar, 2005).

Drought stress can induce situations of photo oxidative stress due to the inhibition of the photosynthetic activity or confers an osmotic stress upon plants that will interfere with nutrient availability (reviewed in Chinnusamy et al., 2004, Kolodyazhnaya et al., 2009).

Several abiotic stresses have in common the fact that at least part of their detrimental effect on plant performance is caused by disruption of plant water status and this is of most obvious importance in drought (Verslues et al., 2006). The decreased water availability can be quantified as a decrease in plant water potential (Ψ_w) (Kramer and Boyer, 1995). According to Kramer and Boyer (1995), Ψ_w is the chemical potential of water divided by the partial molar volume that can be express in units of pressure and allows the assessment of the direction of water movement in the plant system. A diagram

based on Verslues et al. (2006) adaptation of stress avoidance/stress tolerance terminology proposed by Levitt (1972) is shown in figure 1.

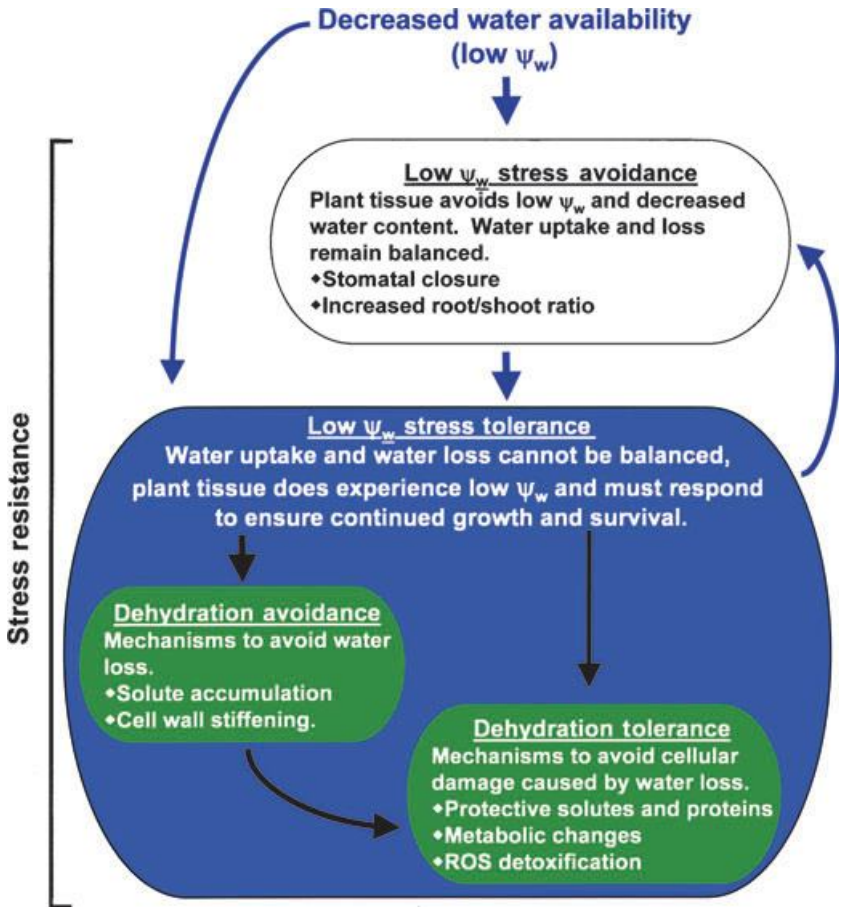

Figure 1. Conceptual diagram of the low water potential (Ψ_w) responses based on the stress avoidance/stress tolerance terminology of Levitt (1972) and integrally adapted from Verslues et al. (2006).

To avoid low-Ψ_w the first plant response is to balance water uptake and water loss to be close to an unstressed level (essentially, the stress is kept outside the plant tissue). Such phenomenon is achieved in the short term mainly by stomatal closure and in the longer term by changes in root and shoot growth (Verslues et al., 2006). It is known that the major effect of decreased water availability is the diminished leaf carbon fixation due

to stomatal closure (Chaves and Oliveira 2004). Plants redirect assimilates and energy, used for shoot growth, into protective molecules and/or to maintained root growth improving water acquisition (Chaves et al, 2003; Chaves and Oliveira 2004). Under such conditions, modifications like decreased stomatal conductance and increased root growth are observed. Growth arrest can be considered as a way to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief (Bartels and Sunkar, 2005).

If stress becomes more severe and low Ψ_w cannot be avoid the plant does experience low Ψ_w (the stress becomes internalized to the plant tissues) and stress responses occur that maintain a high water content despite a decreased Ψ_w (**dehydration avoidance**) or tolerate a reduced water content (**dehydration tolerance**) (Fig. 1). The term "**stress resistance**" is used in cases where it is not possible or not desirable to refer to a more specific mechanism (Verslues et al., 2006).

The main mechanisms of **dehydration avoidance** are accumulation of solutes and cell wall hardening (see fig.1). Many plants accumulate compatible solutes, amino acids, glycine betaine, sugars, or sugar alcohols in response to low Ψ_w. Compatible solutes are nontoxic molecules that do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress (reviewed in Chen and Murata, 2002). The properties of cell walls also play important role in dehydration avoidance since cell wall deformability request cells to maintain turgor even with large water loss (Verslues et al., 2006).

As low-Ψ_w stress become more severe, there is an increased difficulty for the plant to avoid dehydration and mechanisms to tolerate reduced water content become important (see Fig.1).

Dehydration tolerance mechanisms function to protect cellular structure from the effects of dehydration. This includes several types of protective proteins, like late-embryogenesis-abundant (LEA) proteins and heat shock proteins (HSP) (Verslues et al., 2006; Bartels and Sunkar, 2005). The accumulation of compatible solutes that can also protect proteins and membrane structures and, another aspect of dehydration tolerance, is the control of reactive oxygen species (ROS) or limitation of the damaged caused by ROS (Verslues et al., 2006).

Adaptation to salinity and drought is undoubtedly one of the complex processes, involving numerous changes including attenuated growth, the activation/increased expression or induction of genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. Several stress-associated genes have been evaluated and studies are in progress for their contribution to drought or salt tolerance in laboratory studies (reviewed in Bartels and Sunkar, 2005).

In the case of the Mediterranean climate zones it is important to study water deficit (WD) resistance in legumes because they are susceptible during the reproductive period (flowering and pod filling). Furthermore, the establishment and functionality of legume-*Rhizobium* symbiosis are compromised by WD (reviewed in Serraj et al., 1999). Additionally, in stress situations legumes become more vulnerable to pests and diseases (Sharma and Lavanya, 2002, Dita et al., 2006). Overall, the drought stress by water deficit results in yield losses, lower productivity and reduction of nitrogen fixation capacity (Serraj et al., 1999).

Two basic genetic approaches are currently being used to improve stress resistance: (1) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of QTLs (Quantitative Trail Loci) and subsequent marker-assisted selection and (2) generation of transgenic plants to introduce novel genes or alter expression levels of the existing genes to affect the degree stress resistance (Blumwald et al., 2004). Dita et al. (2006) provide an exhaustive review on the biotechnological approaches available to overcome abiotic and biotic stress constrains in legumes and among those genetic transformation is referred as an attractive possibility.

1.5. Transgenic technology to improve drought stress resistance

The advance in genetic engineering offers new ways to understand the genetic mechanisms of stress-related genes and their contribution to the plant performance under stress (Bajaj et al., 1999). However, while a great degree of success has been obtained in the production of herbicide-, virus- and fungal-resistant plants and plants with fortified

nutritional values using transgenic tools, the same has not been the case in production of abiotic stress-tolerant crops (Grover et al., 2003). This is largely because of the complex genetic mechanisms that govern abiotic stress tolerance. The genes that have proven somewhat effective in providing stress tolerance using a transgenic approach belong to both structural and regulatory gene categories (Grover et al., 2003).

The development of cultivars with improved phenotypes using genetic engineering has been shown to be one of the possible ways to achieve water deficit tolerance. However, there are several *lacunae* in the production of abiotic stress-tolerant transgenics and important considerations have to be taken for the success of the use of this technique (adapted from Grover et al., 2003):

1- It is likely that multiple gene introductions could be necessary to achieve abiotic stress tolerance, since stress response is a complex genetic mechanism regulated by several genes. This may be achieved by cloning vectors with different promoters (to avoid homology-based gene silencing) and selection marker genes (to individually select different genes) (Grover et al., 2003). Therefore one of the major challenges will be the introduction of multiples genes by pyramiding strategies or co-transformation (Cushman and Bohnert, 2000; Chaves and Oliveira, 2004).

2- The promoters that have been most commonly employed in the production of abiotic stress-tolerant plants include the CaMV35S (mostly used for dicot crops) and actin1 promoter (used for expression of transgenes in monocot crops) (Grover et al., 2003). As these promoters are constitutive, the downstream transgenes are by and large expressed in all organs and at all stages which is unnecessary as well as demanding on the energy reserves of the cell (Kasuga et al., 1999). The use of stress-inducible promoters was reported to minimize the negative effects that the constitutive expression usually induces in transgenic plants under un-stressed conditions (Su et al., 1998; Su and Wu, 2004). There is a strong need to obtain increased array of inducible promoters, which are expressed only when exposed to stresses, and to pair such promoters with the stress tolerance-related genes in the requisite cloning vectors (Zhu et al., 2010). Additional tests need to be performed to guarantee that obtained stress-inducible promoters work in heterologous plant systems.

3- Following the initial results with primary transformants which showed that a given protein appears important in conferring stress resistance, there is a need for extensive experimentation (taking in view issues such as segregation, production of homozygosity, analysis of expression levels, etc.) in stabilizing the transgene in the progeny of primary transformants (Grover et al., 2003).

4- The introduction of the transgene has to be examined in the context of the overall yield of the plant at the field-level as it is possible that a given transgene leads to stress tolerance but brings in certain traits that are not acceptable in cropping systems. For instance, there may be a penalty on biomass and yield or a change in plant phenotypic characteristics associated with increased stress-tolerance (Grover et al., 2003). In the case of crop plants, it is ultimately the yield of genetically altered plants under specific field conditions that will determine whether or not a specific gene, or metabolic or signaling pathway, is of technologic importance (Verslues et al., 2006).

1.6. Polyamines in plants

Polyamines (PAs) are small (low-molecular-weight), positively charged, aliphatic amines that are found widespread in living organisms. In plants, they have been implicated in a broad range of biological processes, including cell division, cellular growth, plant differentiation, senescence, somatic embryogenesis and response to environmental stresses (Kumar et al., 1997; Groppa and Benavides, 2008). These essential components of the plant cell are capable, like phytohormones, of fine regulation of diverse vital processes under both normal and stress conditions (Kuznetsov et al., 2006).

The major forms of polyamines (PAs) are putrescine (Put), spermidine (Spd) and spermine (Spm), although plants also synthesise a variety of other related compounds. Arginine (Arg) and ornithine (Orn) are the precursors of plant PAs. Ornithine decarboxylase (ODC; EC 4.1.1.17) converts Orn directly into Put. The other biosynthetic route to Put, via arginine decarboxylase (ADC; EC 4.1.1.19), involves the production of the intermediate agmatine (Agm) followed by two successive steps catalysed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA, EC 3.5.1.53) (Fig. 2). In animals and fungi Put is synthesised primarily through the activity of

ODC while in plants and bacteria the main pathway involves ADC. Recently, it has been suggested that ADC/ODC alternative pathways reflect their different evolutionary origins (reviewed in Alcázar et al., 2010a). Cadaverine (Cad), a diamine less known as compared to major PAs, is commonly found in legumes and is formed directly from lysine decarboxylase (LDC), an enzyme mainly localized in the chloroplasts (Kakkar and Sawhney, 2002). Aminopropyl groups, donated by decarboxylated S-adenosylmethionine (dcSAM), must be added to convert Put into Spd and Spm in a reaction catalysed by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), respectively (Fig. 2) (recently reviewed in Alcázar et al., 2010a).

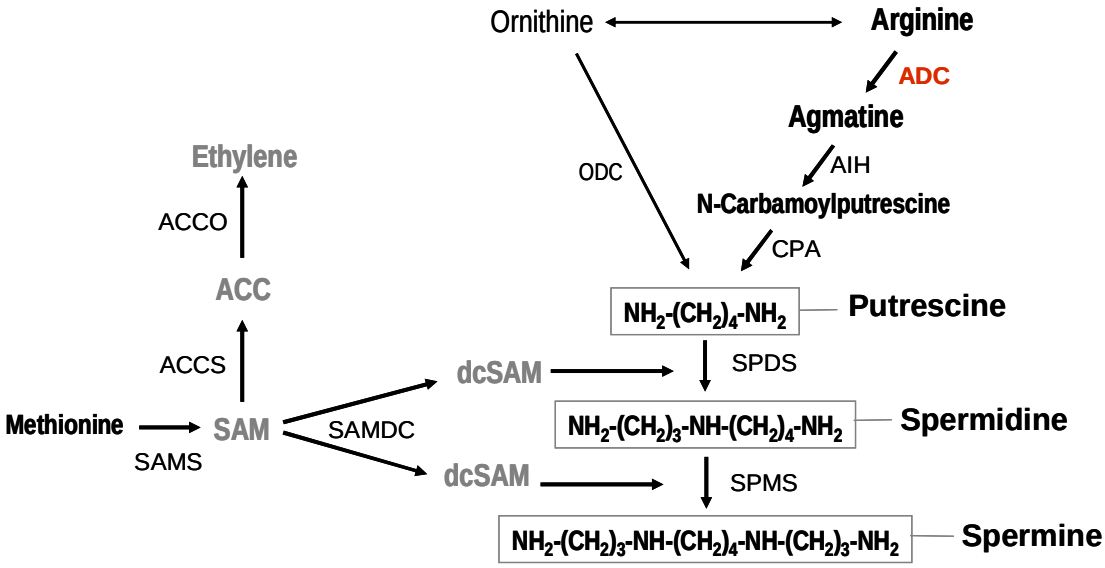

Figure 2. Diagram showing the PAs biosynthetic pathway in plants and its relation with Ethylene biosynthesis (adapted from Kuznetsov et al., 2006). Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; SPDS, spermidine synthase; SPMS, spermine synthase; SAM, S-adenosylmethionine; dcSAM, decarboxylated S-adenosylmethionine; SAMDC, SAM decarboxylase; SAMS, SAM synthetase; ACC, aminocyclopropane carboxylic acid; ACCS, ACC synthase and ACCO, ACC oxidase.

Metabolic studies indicate that the intracellular levels of PAs in plants are mostly regulated by anabolic and catabolic processes, as well as by their conjugation to hydroxycinnamic acids, fatty acids and macromolecules (Moschou et al., 2008; Alcázar et al., 2010a).

The catabolism of polyamines flows either through direct terminal oxidation or indirectly through back-conversion and subsequent oxidation (Fig. 3). Diamine oxidases are copper-containing enzymes (DAOs/CuAOs; EC 1.4.3.6) that catalyze the oxidation of the diamines Put and Cad at the primary amino groups. The reaction produces 4-aminobutanal (that spontaneously cyclizes to Δ^1-pyrroline -PYRR), hydrogen peroxide (H_2O_2) and ammonia (NH_3) (Tiburcio et al., 1997). Following the oxidation of Put, PYRR is catabolyzed into (GABA), which is ultimately converted into succinate, a component of the Krebs cycle (Gill and Tuteja, 2010).

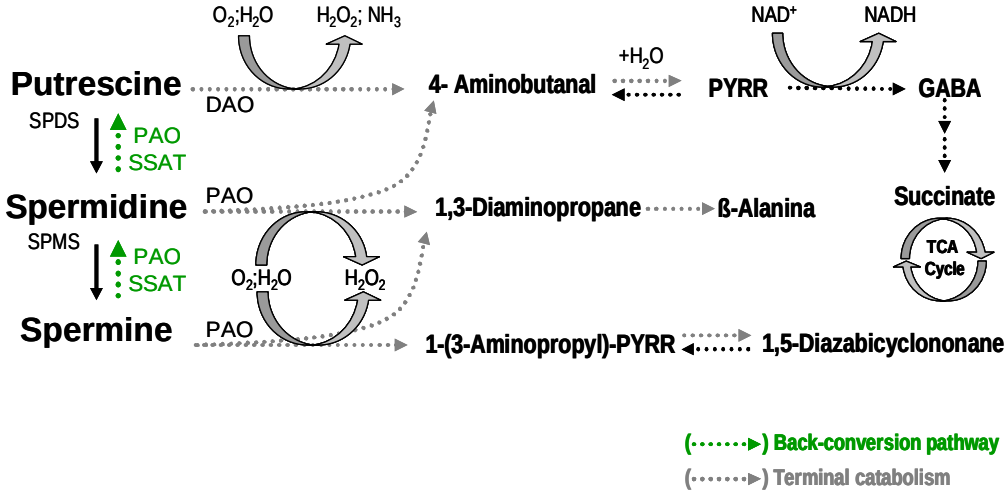

Figure 3. Schematic representation of polyamine catabolic pathways either by back-conversion (----->) or terminal oxidation (----->), respectively (adapted from Cona et al., 2006 and Angelini et al., 2010). Abbreviations: DAO/CuAO, diamine oxidase/copper-containing amine oxidases (CuAOs; EC 1.4.3.6); PAO, flavin-containing polyamine oxidases (PAOs; EC1.5.3.11); GABA, γ-aminobutyric acid; PYRR, pyrroline; SSAT, spermidine/spermine N^1-acetyltransferases; SPDS, spermidine synthase; SPMS, spermine synthase.

Flavin-containing polyamine oxidases (PAOs; EE 1.5.3.11) are responsible for the oxidation of Spm and Spd and/or their acetylated derivatives at their secondary amino groups (reviewed in Cona et al., 2006, Angeline et al., 2010). Additional PAOs, with novel subcellular localization in peroxisomes, are able to back-convert PAs instead of just being involved in their terminal catabolism (see Fig. 3). In this way, PAOs also act in back-convertion pathway, converting Spm to Spd and Spd to Put, in a two step reaction with an acetylation (SSATs) followed by an oxidation (PAOs) with the production of 3-acetamidopropanal and H_2O_2 (Mochou et al., 2008).

Recently, multiple functions of amino oxidases (AOs) have been proposed including, among others, the regulation of cell cycle through the modulation of PA cellular content and the production of (H_2O_2) during plant developmental and stress interactions (Angeline et al., 2010). Moschou et al. (2008) suggested that a crucial point in plant stress tolerance is the ratio of PA catabolism to PA anabolism.

Interactions of these processes with other metabolic pathways in particular with ethylene biosynthesis, which shares SAM as common precursor, are also determinant aspects to be considered in PAs regulation (see Fig. 2 and 4). Interestingly, the competition for the SAM pool between PAs and ethylene biosynthesis might explain its antagonic effects (Tiburcio et al., 1997) (Fig. 4).

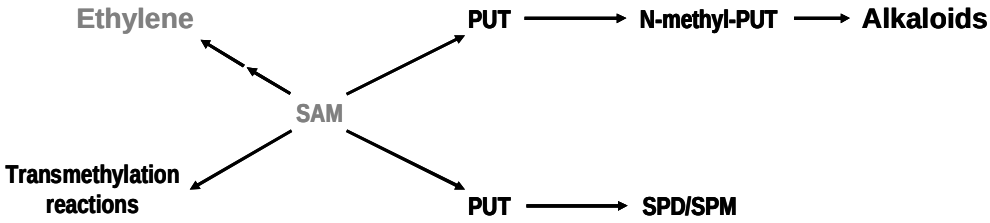

Figure 4. Key role of S-adenosylmethionine (SAM) in the metabolic fluxes leading to transmethylation reactions and to the biosynthesis of ethylene, polyamines and putrescine-derived alkaloids (adapted integrally from Tiburcio et al., 1997).

Additionally, PA biosynthetic pathway also interact with other pathways, such as those of alkaloids, amino acid metabolism including proline (in which glutamate is a central molecule), urea, γ-aminobutiric acid (GABA) and TCA cycle (Kakkar and Sawhney, 2002; Kuznetsov et al., 2006; Page et al., 2007; Forde and Lea, 2007; Alcázar et al., 2010a) (Fig. 4 and 5).

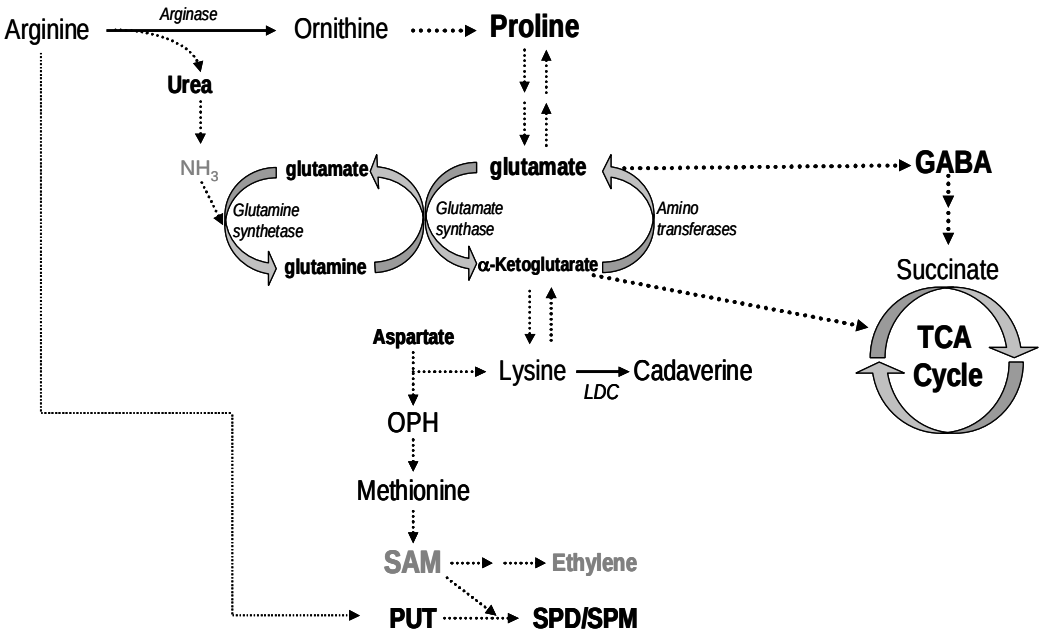

Figure 5. Interaction of PA biosynthetic pathway with other pathways, such as glutamate cycle, proline, urea, γ-aminobutiric acid (GABA) and TCA cycle. Methionine serves as a precursor for protein, S-methylmethionine (SMM) and S-adenosylmethionine (SAM) biosynthesis. LDC, lysine decardoxilase; OPH, *O*-phosphohomoserine. Dashed lines represent parts of the pathway in which detailed descriptions of the enzymatic steps have been omitted. (Based on several publications, including; Kavi Kishor et al., 2005; Forde and Lea, 2007; Alcázar et al., 2010a).

1.7. Involvement of polyamines in stress responses

For a long time, the only way to manipulate endogenous polyamine concentration was by using polyamine biosynthesis inhibitors. Some effects of polyamines on cell division, plant regeneration and somatic embryogenesis during tissue culture have been demonstrated in that way (Altamura et al., 1993, Bajaj and Rajam, 1995 and Minocha and Minocha, 1995). However, results of experiments using inhibitors are sometimes difficult to interpret because the commonly used inhibitors are not strictly specific to the target enzymes and their permeability and stability in cells are unclear.

A possible role for polyamines in the responses of plants to stress was proposed by Flores (1991). Cultivars demonstrating a higher degree of salt tolerance contained higher levels of polyamines. Salt-tolerant rice plants accumulate higher levels of polyamines compared to the salt-sensitive rice plants in response to salinity stress (Chattopadhyay et al., 1997, 2002). Exogenously supplied putrescine prevented stress damage and increased stress tolerance in *Conyza bonariensis*, wheat and rice (Ye et al., 1998; Ndayiragije and Lutts, 2006).

Polyamines levels in plants increase under a number of environmental stress conditions, including drought and high salinity (Kasinathan and Wingler, 2002). Plants subjected to osmotic stress show a rapid increase in putrescine levels due to transcription and activation of arginine decarboxylase (Borrell et al., 1996; Flores and Galston, 1982). Furthermore, in *Arabidopsis thaliana*, *AtADC2* expression was correlated with free putrescine accumulation under salinity and dehydration (Urano et al., 2003; 2004).

Genes from several enzymes of the polyamine biosynthetic pathway have been cloned and it has become possible to manipulate polyamine biosynthesis using transgenic approaches (for cloned genes available see Kumar et al., 1997 and more recently Liu et al., 2007). These strategies have been used to study the regulatory mechanisms controlling cellular polyamine levels in plants.

A resume of several works intending to change the PA metabolism by genetic engineering and their effects is shown in table 1.

Table 1. Changes in the PA metabolism by genetic engineering and their associated effects (adapted from Kakkar and Sawhney, 2002; Alcázar et al., 2010a).

Plant	Vector/approach	Phenotype/ biochemical alteration	Reference
tobacco	*S. Cerevisae* ODC, 2xCaMV 35S constitutive promoter	↑ ODC activity, Put and Nicotine	Hamill et al. 1990
tobacco	Human SAMDC cDNA, CaMV35S promoter	↑ SAMDC activity, Spd and Spm levels ↓ Put levels; thick leaves, stems and stunting	Noh and Minocha 1994
tobacco	Oat ADC cDNA, *Tet*-inducible promoter	↑ ADC activity; phenotype changes pp to Put levels: e.g. thin stems and leaves, leaf necrosis, chlorosis, short internodes and growth inhibition	Masgrau et al. 1997
tobacco	Oat ADC cDNA, 2xCaMV 35S	↑ ADC activity, ODC and SAMDC normal; ↑ Agm; Put, Spd and Spm normal	Burtin and Michael 1997
rice	Oat ADC cDNA, CaMV 35S	↑ Put levels in regenerated plants but not in seeds	Capell et al. 1998
rice	Oat ADC cDNA in antisense	↓ Put, Spd but not Spm in *callus* lines	Capell et al. 2000
rice	Oat ADC cDNA, ABA inducible promoter	↑ Put, ADC and biomass under salt-stress	Roy and Wu 2001
rice	*D. stramonium* ADC cDNA, inducible promoter	↑ Put, Spd and Spm levels drought tolerance	Capell et al. 2004
eggplant	Oat ADC cDNA, CaMV 35S	↑ Put, Spd and Spm multiple abiotic stresses; fungal resistance	Prabhavathi and Rajam 2007
arabidopsis	homologous ADC1 and ADC2	↑ Put, freezing and drought tolerance	Altabella et al. 2009; Alcazar et al. 2010b

In early experiments, putrescine levels were increased in tobacco roots as a result of expressing yeast *Odc* (Hamill et al., 1990). The expression of a mammalian *Odc* in transgenic carrot and tobacco resulted in a significant increase in cellular putrescine levels (DeScenso and Minocha, 1993; Bastola and Minocha, 1995). Ectopic expression of the oat *Adc* in tobacco and rice caused, respectively, an increase in agmatine and putrescine (Burtin and Michael, 1997; Capell et al., 1998). Roy and Wu (2001) presented evidences that transgenic rice plants with an enhanced expression of *Adc* increased their biomass under saline conditions, when compared to control plants. Capell and collaborators (2000) reported simultaneously the reduction of the activity of the two enzymes involved in early

steps of the polyamine biosynthetic pathway, by a single antisense cDNA in rice transformed *callus* lines. The heterologous expression of *Odc* in tobacco (Kumria and Rajam, 2002) has resulted in a significant increase in Put and Spd and conferred salt tolerance. The transgenic rice (Roy and Wu, 2002) and tobacco (Waie and Rajam, 2003) expressing heterologous *Samdc* have showed increased PAs levels and tolerance to salinity and drought and also conferred resistance against *Fusarium* and *Verticillium* wilts (Waie and Rajam 2003). Recently, the introduction of spermidine synthase (SPDS) encoding gene into tobacco (Franceschetti et al. 2004), arabidopsis (Kasukabe et al. 2004) and sweet potato (Kasukabe et al. 2006) has led to the increased tolerance against multiple abiotic stresses.

Lately, in Arabidopsis, the constitutive expression of homologous *Adc1* and *Adc2* genes resulted in freezing and drought tolerance, respectively (Altabella et al., 2009; Alcazar et al., 2010b). In arabidopsis the gene coding for ODC and the corresponding enzyme activity cannot be detected (Hanfrey et al., 2001), thus, *A. thaliana* is dependent totally on the ADC pathway for putrescine biosynthesis. Based on the relative frequency of *Adc* and *Odc* sequences in large EST collections from many different plant species, including soybean, *Medicago truncatula*, and *L. japonicus*, it appears that the ADC pathway may be the primary source of putrescine in plants (Flematakis et al., 2004).

In the beginning of this thesis, several biological roles were proposed for polyamines action in stress situations. In drought conditions, PAs could act as osmoprotectants, as scavengers of reactive oxygen species or by stabilizing cellular structures, such as thylakoid membranes (Tiburcio et al, 1994; Bouchereau et al., 1999; Martin-Tanguy, 2001). In fact, Capell and co-workers (1998) found that the constitutive overexpression of heterologous *Adc* in rice minimised chlorophyll loss during drought stress but affected *in vitro* development patterns. Recently, new insights into the role and regulatory function of polyamines in plant abiotic stress tolerance have been achieved (recently reviewed in Kuznetsov et al., 2006; Groppa and Benavides, 2008; Gill and Tuteja, 2010; Alcázar et al., 2010a).

Plants respond to changes in water status by accumulating low molecular-weight osmolytes such as proline and PAs (reviewed in Groppa and Benavides, 2008). These

molecules may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures. The polycationic nature of PAs at physiological pH is believed to mediate their biological activity, since they are able to bind to several negatively charged molecules, such as DNA, membrane phospholipids, pectic polysaccharides and proteins (Martin-Tanguy, 2001).

In respect to the antioxidant activity of PAs, the research data is contradictory; on one hand PAs have been suggested to protect cells against reactive oxygen species (ROS), on the other hand, their catabolism generates ROS (Groppa and Benavides, 2008). PA catabolism produces H_2O_2 (see Fig. 3), a signaling molecule that can act promoting activation of antioxidative defense response upon stress, but can also act as a peroxidation agent (Groppa and Benavides, 2008). In a recent study, the effect of increased putrescine accumulation was found to negatively impact the oxidative state of poplar cells in culture due to the enhanced turnover of Put (Mohapatra et al., 2009). Gill and Tuteja (2010) stated that, while increase Put accumulation may have a protective role against ROS in plants, enhanced Put turnover can actually make them more vulnerable to increased oxidative damage. The higher polyamines, Spd and Spm are believed to be most efficient antioxidants and are considered scavangers of oxyradicals (He et al., 2008).

As plants with elevated putrescine are able to tolerate drought stress because Put has a direct protective role in preventing the symptoms of dehydration, the higher PAs (Spd and Spm) appear to play an important in role in stress recovery (Peremarti et al. 2009). Recently, transgenic rice plants expressing SAMDC, with increased Spd and Spm levels, were found not drought tolerant, however showed a more robust recovery from drought compared to wild type (Peremarti et al., 2009). The *de novo* synthesis of Spd and Spm in transgenic plants under drought stress, at the expenses of putrescine, was responsible for the stress tolerance observed in these plants.

The covalent linkage of PAs to proteins appeared to be of extreme importance in plant light-induced stabilization of the photosynthetic complexes and Rubisco therefore exerting a positive effect on photosynthesis and photo-protection. Also in the cytosol, they are involved, mediated by TGase activity, in the modification of cytoskeletal proteins and in the cell wall construction/organization (reviewed in Serafini-Fracassini and Del Duca, 2008).

A role for PAs, especially putrescine (Put), in the protection of the photosynthetic apparatus have been proposed and, in a recent study, the characterization at the proteomic level of the chlTGase interaction with thylakoid proteins, demonstrated its association with photosystem II (PSII) protein complexes using maize thylakoid protein extracts (Campos et al., 2010). Binding of Put to thylakoid membranes has been proposed to be a photo-adaptation response under controlled stress conditions and Campos and collaborators (2010) results reinforce the importance of the chlTGase in photo-protection by polyamine conjugation to light-harvesting complex II (LHCII) proteins.

Recently, PAs were proposed to be components of signaling pathways and fulfill the role of second messengers (reviewed in Kuznetsov et al., 2006; Alcázar et al., 2010a). Studies with ABA-deficient and ABA-insensitive Arabidopsis mutants subjected to water stress (Alcázar et al., 2006) support the conclusion that the up-regulation of PA-biosynthetic genes and Put accumulation under water stress are mainly ABA-dependent responses.

A novel mode of action of PAs as regulators of ABA biosynthesis have been proposed since ABA and Put reciprocally promote each other's biosynthesis in response to abiotic stress (Alcázar et al., 2010a) (see Fig. 6). Moreover, to reinforce the fact that PAs biosynthesis maybe regulated by ABA, several stress-responsive elements, like drought responsive (DRE), low temperature-responsive (LTR) and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes (Alcázar et al., 2006).

Plants react to external stimuli by initiating a cascade which activates the expression of appropriate responses. These signalling pathways comprise a network of protein-protein reactions and signalling molecules [for example, Reactive Oxygen Species (ROS), Ca^{2+}, Nitric Oxide (NO), inositol 1,4,5-triphosphate (IP_3), etc.] (Bartels and Sunkar, 2005). Inositol 1,4,5-triphosphate (IP_3) is the major phospholipid-derived signalling molecules in the contest of osmotic stress; nitric oxide (NO) is an important signalling molecule involved in plant defence responses and in mammalian cells has been shown to protect against oxidative damage; and calcium signaling is well documented to be involves in osmotic and ionic stresses (Bartels and Sunkar, 2005). NaCl causes a rapid

and transient increased in cytosolic calcium ($[Ca^{2+}]_{cyt}$), that triggers many signal transduction pathways, including ion channel activity, and mediates salt adaptation (Bartels and Sunkar, 2005).

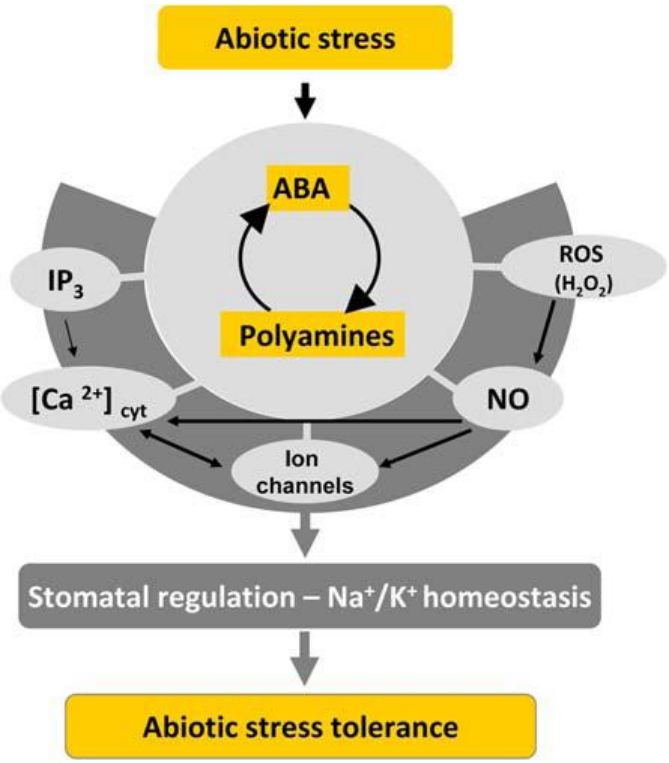

Figure 6. Simplified model for the integration of polyamines with ABA, ROS (H_2O_2), NO, IP_3, Ca^{2+} homeostasis and ion channel signaling in the abiotic stress response (adapted integrally from Alcázar et al., 2010a).

According to the scheme on Figure 6, it is likely that PAs participate in ABA-mediated stress responses involved in stomatal closure (Alcázar et al., 2010a). This is supported by the study of Liu et al. (2000) which found that inward potassium channels were targets for PA regulation of stomatal movements. Since ABA signalling pathway in stomata regulation involves many different components such signalling molecules, including the previously referred ROS, Ca^{2+} and NO (Kuppusamy et al, 2009 in Alcázar et

al., 2010a), evidences point to an interplay between ABA, polyamines, ROS (H_2O_2) and NO in stomata regulation.

Apparently, accumulation in excess of polyamines seems to be toxic to the plants under normal conditions, probably because polyamines are involved in such a broad range of processes in plant development (Tiburcio et al., 1997; Martin-Tanguy, 2001; Bartels and Sunkar, 2005). Additional concerns about the development of food plants with altered PA levels and altered nutritional and health values were also reported (Bardocz, 1993; Tiburcio et al., 1997). Several phenotypic alterations have been described in plants with altered PA levels like thin stems and leaves, wrinkled leaves, leaf necrosis, chlorosis, short internodes and growth inhibition (e.g. De Senzo and Minocha; 1993; Kumar et al., 1996; Masgrau et al., 1997; Panicot et al., 2002; Alcázar et al., 2005). However, polyamine over-accumulation, in a moderate level, could contribute to increased stress tolerance. Moreover, plant cells, contrarily to animal cells, can be less affected by excess PAs, as they can buffer this excess by binding PAs to TCA-soluble conjugates, such as cinnamic acids, or by storing them in the vacuole (Serafini-Fracassini and Del Duca, 2008). A recent work by Prabhavathi and Rajam (2007) showed that PA-accumulating in transgenic eggplants resulted in increased tolerance to multiple abiotic stresses (salinity, drought, low and high temperature and heavy-metal) and also fungal resistance. Prabhavathi and Rajam (2007) found increase in Put, but also in higher PAs, Spd and Spm. These authors concluded on the achievability of PA biosynthesis engineered for the production of stress-tolerant plants

2. Aims of the Thesis

The main goal of this thesis is to introduce and express in the model legume *M. truncatula* the arginine decarboxylase encoding gene (*Adc*) from *Avena sativa* that codes for a key enzyme of the polyamine biosynthetic pathway.

To achieve this goal, the embryogenic line M9-10a of *Medicago truncatula* cv Jemalong will be used which implies that appropriate regeneration/transformation systems have to be optimised for stable transformation of this legume specie and plant vectors bearing the oat *Adc* cDNA must be constructed for plant transformation purposes.

The heterologous expression of the oat *Adc* should introduce changes in polyamines levels in transgenic plants that ought to be evaluated using Chromatographic techniques. An ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) methodology will be optimised.

The transgenic lines of *Medicago truncatula* expressing the heterologous *Adc* coupled with a procedure for quantifying biogenic amines will provide a powerful tool to study the effects of altered polyamine metabolism on other metabolic processes including additional investigation on the role of polyamines in abiotic stress responses in plants.

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II – Construction of vectors p35S*Adc* and p35S*Adc-Gus* for plant transformation purposes

The description of the construction of plasmid p35S*Adc-Gus* was partially published in: Santos D.M, Araújo S.S., Duque A.S., Fevereiro P. (2003) Reverse transcription-PCR assay to verify gene integrity within plasmid constructs for plant transformation. Plant Cell, Tissue Organ Cult. 74:293-296.

1. Abstract

In this chapter we describe the procedures for the construction of two plasmid vectors, p35S*Adc* and p35S*Adc-Gus*. The vector p35S*Adc*, was constructed by cloning the arginine decarboxylase cDNA (*Adc*) from *Avena sativa* L., under the control of the cauliflower mosaic virus (CaMV) constitutive 35S promoter with duplicated enhancer sequences and the CaMV transcriptional termination region, into the binary plant transformation vector pGreen0000. Because pGreen0000 does not contain a selectable marker gene it was first necessary to introduce an antibiotic resistance gene. We cloned the neomycin phosphotransferase II gene (*Npt*II), under the control of the nopaline synthase promoter (pNos) and terminator (tNos). The presence of the *Npt*II gene confers resistance to aminoglycosides antibiotics like kanamycin and serves as a selection marker for recovering transformed plants. The vector p35S*Adc-Gus* was obtained from p35S*Adc* by introducing the reporter ß-glucuronidase gene (*Gus*A) under the control of a CaMV 35S promoter. The presence of the *Gus*A gene allows the fast histochemical analysis of putative transgenic plants for a first confirmation of transgenesis.

Key words: Plasmid constructs, plant transformation vectors, oat *Adc* gene, CaMV35S promoter

2. Introduction

The most used method to transfer foreign genes into plant cells and the subsequent regeneration of transgenic plants is based on the natural system of *Agrobacterium*-mediated plant transformation (de la Riva et al., 1998). *Agrobacterium tumefaciens* is a remarkable Gram-negative soil bacterium that has the ability to naturally infect dicotyledonous plants at wounding sites causing the formation of crown gall tumours. During the infection process, *A. tumefaciens* transfers a particular DNA segment (T-DNA) of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where is then stable integrated into the host genome and transcribed, causing the crown gall disease (Nester et al., 1984; Binns and Thomashaw, 1988). The T-DNA region is defined by the T-DNA

border sequences that are 25bp in length, highly homologous in sequence and are present in a directly repeated orientation (reviewed in Gelvin, 2003). These borders, referred as right border (RB) and left border (LB), are the only *cis* elements necessary to direct T-DNA processing. Any DNA between these borders will be transferred to a plant cell (Zupan and Zambryski, 1995).

Agrobacterium-mediated transformation has advantages over direct transformation methods, including a reduction in transgene copy number and the stable integration with fewer rearrangements of long molecules of DNA with defined ends; potentially leading to fewer problems with transgene co-suppression and instability (Koncz et al., 1994, Hansen et al., 1997).

Several plant transformation vectors have been constructed based on this natural occurring T-DNA plasmid by the removal of the genes responsible for the formation of crown gall tumours and maintenance of virulence (*vir*) genes, creating disarmed Ti plasmids. However, the fact that, Ti plasmids are very large (≥200 Kbp) and the T-DNA regions do not contain interesting restriction endonuclease sites, dictated the development of a number of cloning strategies to introduce foreign genes into the T-DNA (reviewed in Gelvin 1998, 2003). Two different approaches were developed: cloning the gene of interest into the Ti plasmid such that the new gene is on the same plasmid (in *cis*) with the *vir* genes; or cloning the gene in a T-DNA region that is on a separate replicon (in *trans*) from the *vir* genes, generating T-DNA binary vectors.

T-DNA regions were also engineered by the introduction of multiple cloning sites (MCS) to facilitate the insertion of transgenes but maintaining the sequence borders (RB and LB) involved in the transference of foreign DNA to the plant.

The basis of modern Ti plasmid vectors, termed binary vectors, resides on the possibility that the T-DNA and the *vir* region could exist in separate plasmids (Hellens et al., 2000a). The *vir* gene functions are provided by the disarmed Ti plasmids resident in the *Agrobacterium* strain and the T-DNA, within which are the gene(s) to be transferred is provided on a different vector. The basic *in vitro* manipulation techniques use *Escherichia coli*, consequently, binary Ti vectors replicate both in *E. coli* and *Agrobacterium* (Hellens et al., 2000a). This implies that antibiotic resistance genes for plasmid selection must be included to allow selection in both bacterial species. The most commonly used plasmids

contain marker genes encoding resistance to kanamycin (e.g. pBIN19 and pGreen). However, *Agrobacterium* strains are also marked with antibiotic resistance that are either chromosomal or Ti-plasmid localized (e.g. rifampicin resistance for *Agrobacterium* strain EHA105; Hood et al., 1993), resulting that the binary Ti vector and the bacterial antibiotic resistance markers are not duplicated (Hellens et al., 2000a).

Once a gene of interest has been isolated and cloned, it must undergo several modifications before it can be effectively inserted into the T-DNA for transfer to the plant genome:

1. A **promoter sequence** must be added for the gene to be correctly expressed, the promoter is the on/off switch that controls when and where in the plant the gene will be expressed. To date, most promoters in transgenic crop varieties are constitutive. The most commonly used constitutive promoter is CaMV35S, from the cauliflower mosaic virus, which generally results in a high degree of expression in plants (Benfey and Chua, 1990).
2. The **termination sequence** signals to the cellular machinery that the end of the gene sequence has been reached.
3. A **selectable marker gene** must be added to the construct in order to identify plant cells or tissues that have successfully integrated the transgene. Selectable marker genes encode proteins that provide resistance to agents that are normally toxic to plants, such as antibiotics or herbicides. Only plant cells that have integrated the selectable marker gene will survive when grown on a medium containing the appropriate antibiotic or herbicide. One of the most used is the *Npt*II gene encoding for neomycin phosphotransferase II (Beck et al., 1982) which confers resistance to aminoglycosides antibiotics like kanamycin, gentamicin or geneticin (G418). As for other inserted genes, marker genes also require promoter and termination sequences for proper function.
4. Additional **reporter genes** can be added to the construct; the use of a reporter gene system simplifies the expression analysis of the gene in transgenic plants. *E. coli*-originated ß-glucuronidase gene (designated *Gus*A or *Uid*A) (GUS, Jefferson et

al., 1987) is a widely used reporter gene and has the advantage that GUS expression can be easily assayed histochemically with almost no background activity in most plant species.

In this work we made plasmid constructs, transferable via *A. tumefaciens*, to express the arginine decarboxilase cDNA (*Adc*) from *Avena sativa* in the model legume *Medicago truncatula*. This *Adc* cDNA was kindly provided by Dr. Teresa Capell (John Innes Centre, Norwich) in a bacterial plasmid ($pAMC_2$) with a cauliflower mosaic virus (CaMV) constitutive 35S promoter with duplicated enhancer sequences. Before it can be used for plant transformation purposes it has to be transferred to a plant transformation vector. We used the pGreen binary system for insertion of the genes of interest into the plant genome. This binary system has the advantages of being reduced in size (pGreen: 3.3 Kbp) and of having the *Rep*A gene (pSa replicase gene) resident on a compatible plasmid (pSoup) in *Agrobacterium*, therefore providing pGreen replication functions *in trans* (Hellens et al., 2000 b). The strategies for the construction of this plant transformation vectors are described in detail in the Materials and Methods section.

3. Materials and Methods

3.1. Strategies for the construction of p35S*Adc* and p35S*Adc-Gus* vectors

To construct the p35S*Adc* and p35S*Adc-Gus* vectors we used the following strategies:

1. Construction of **p35S*Adc***: This construct was engineered in the pGreen0000 (Hellens et al., 2000b) (Figure 1), a binary plant transformation vector. Two-steps were involved in the cloning process (Figure 2):

A - To allow selection for plant transgenic tissues it was necessary to introduce a marker gene. We chose the *Nos-Kan* cassette (John Innes Centre, Norwich) containing the neomycin phosphotransferase II gene (*Npt*II), under the control of the nopaline synthase promoter (pNos) and terminator (tNos). The 1406-bp *Nos-*

Kan cassette was excised as an *Eco*RV fragment from plasmid p*Nos-Kan* and subcloned into the *Eco*RV site of pGreen0000.

B - The arginine decarboxilase cDNA (*Adc*) from *Avena sativa*, under the control of the cauliflower mosaic virus (CaMV) constitutive 35S promoter with duplicated enhancer sequences (2X35S) and a CaMV transcriptional termination region, were excised as a 3.6Kbp *Kpn*I/*Xho*I fragment from $pAMC_2$ (Capell et al., 1998) and inserted in the *Kpn*I/*Xho*I site of the previous construct.

2. Construction of **p35S*Adc-Gus***: The basis of this vector was the previously constructed p35S*Adc*, in which the GUS cassette [containing the *E. coli* β-glucuronidase reporter gene (*Gus*A) under the control of the CaMV35S promoter and with a CaMV transcriptional termination region] was introduced to allow the histochemical detection of the transgenic plants. This cassette was excised as a 2.5Kbp *Eco*RV fragment from the binary vector pBI121 (Jefferson et al., 1987) obtained from Clontech (USA) and subcloned into the *Hpa*I site of pGreen (Figure 3).

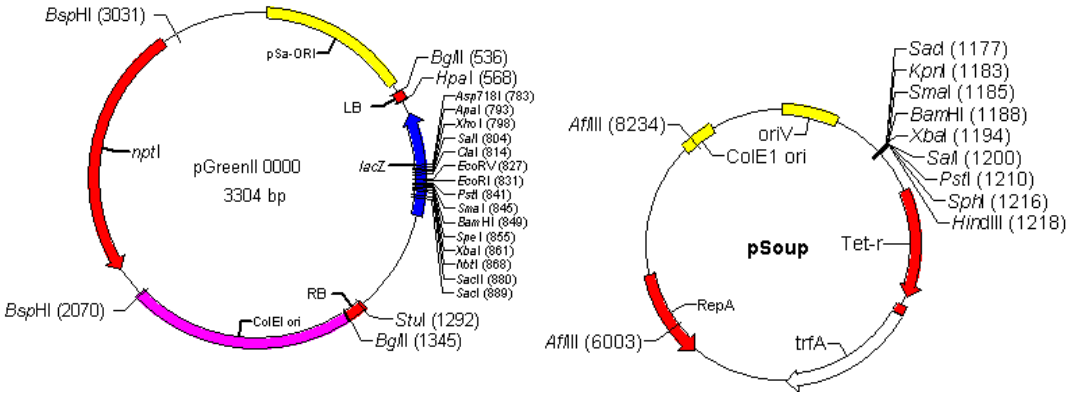

Figure 1. Binary plant transformation vectors (pGreen system): **A** - pGreen0000; **B** - helper plasmid pSoup (provides replication functions in *trans* for plasmid pGreen). (http://www.pgreen.ac.uk).

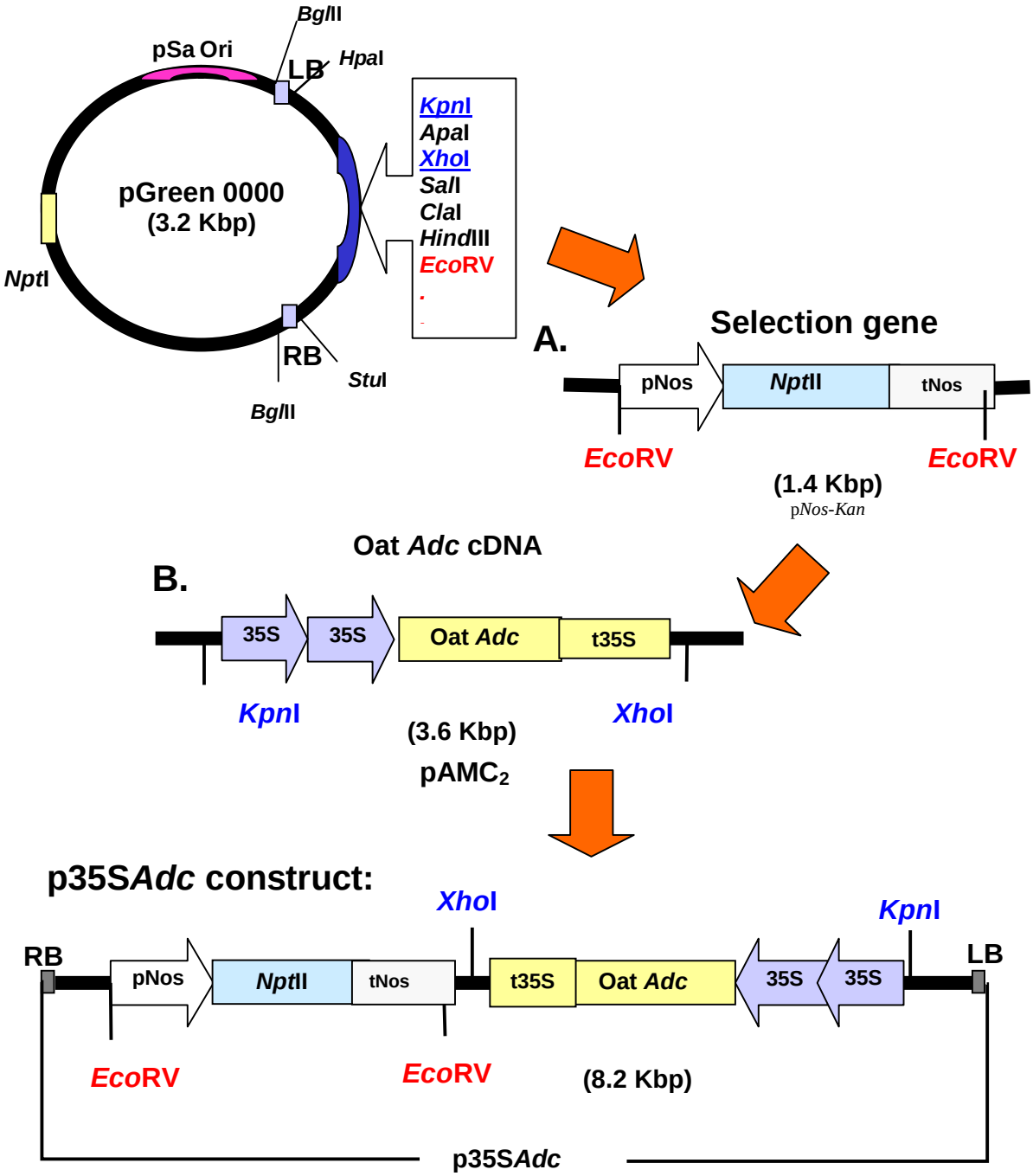

Figure 2. Diagram showing the two steps for the construction of vector p35S*Adc*: **A** - The 1406-bp *Nos-Kan* cassette was excised as an *Eco*RV fragment and subcloned into the *Eco*RV site of pGreen0000. **B** - A fragment that contains the 2124-bp oat *Adc* cDNA, a CaMV 35 S promoter with duplicated enhancer sequences and a CaMV transcriptional termination region, was excised from pAMC2 as a *Kpn*I/*Xho*I 3.6Kbp fragment and cloned into the *Kpn*I/*Xho*I sites of pGreen.

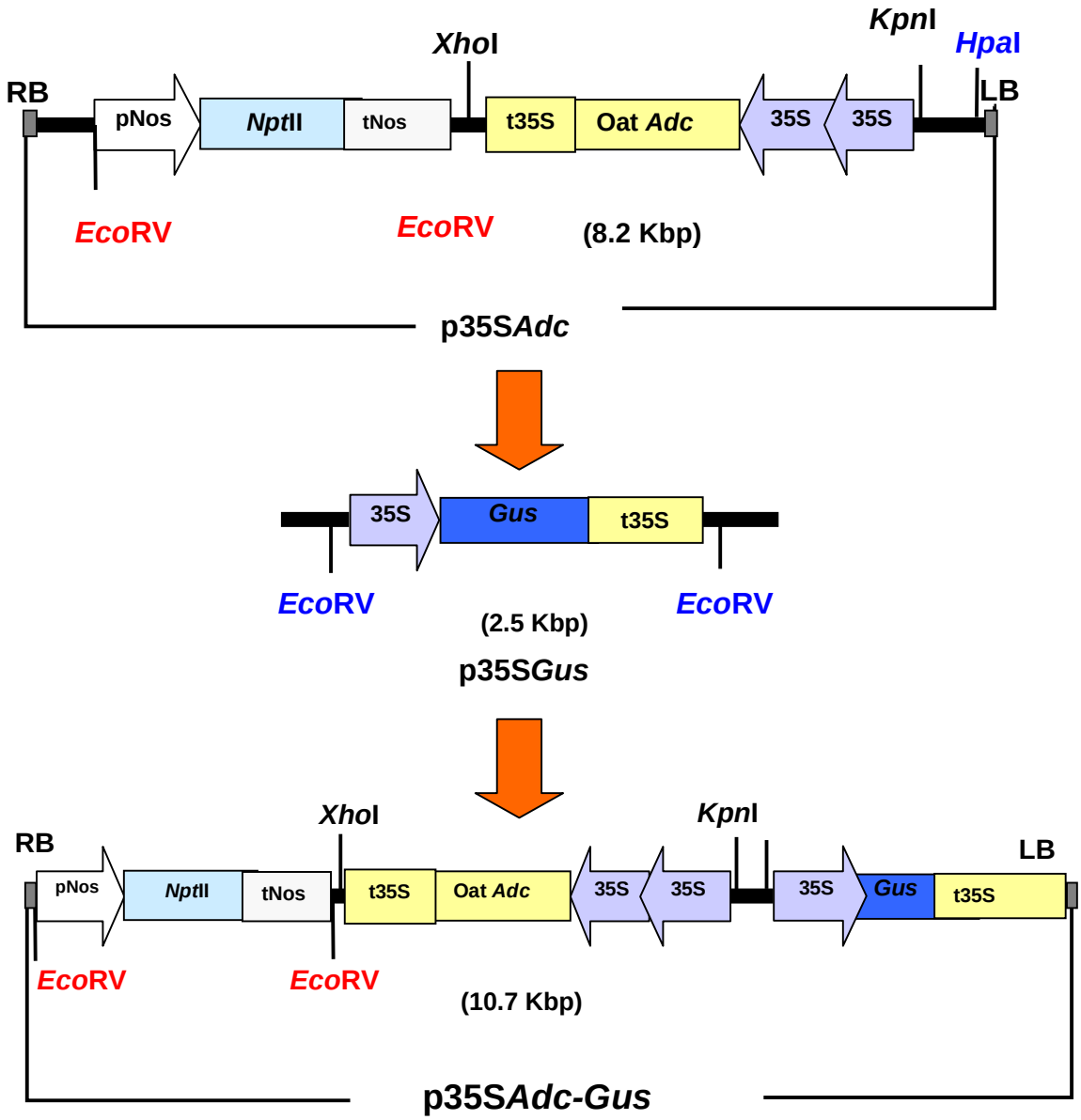

Figure 3. Diagram showing the steps for the construction of the plant transformation vector p35S*Adc*-*Gus*: The 2.5 Kbp 35S*Gus* cassette was excised as an *Eco*RV 2.5Kbp fragment from pBI121 and subcloned into the *Hpa*I site, near the left-border (LB) of pGreen T-DNA.

The following table are describes the most important characteristics of the plasmids used in this work.

Table 1. Characteristics of all the plasmids used throughout this work:

Plasmid	Size (Kbp)	Selection marker	Description	Reference
pAMC$_2$	5.8	Amp ^{R}	Contains the *Adc* encoding gene with p2x35S and a t35S	Capell et al., 1998
p*NosKan*	-	Amp ^{R}	Cassette pNos-*NptII*-tNos	JIC, Norwich, England
pBI121	13.0	Kan^R	Cassete p35S-*Gus*-t35S	Jefferson et al., 1987
pGreen0000	3.2	Kan^R	Plant transformation vector	Hellens et al., 2000b
pGreen*NosKan*	4.6	Kan^R	Plant transformation vector contains the pNos-*NptII*-tNos cassette in the T-DNA region	-
p35S*Adc*	8.2	Kan^R	Plant transformation vector contains the pNos-*NptII*-tNos cassette and the p2x35S-*Adc*-t35S in the T-DNA region	-
p35S*Adc-Gus*	10.7	Kan^R	Plant transformation vector contains the pNos-*NptII*-tNos, the p35S-*Gus*-t35S cassettes and the p2x35S-*Adc*-t35S in the T-DNA region	Santos et al., 2003 ; Araújo et al., 2004
pSoup	9.2	Tet^R	Contains the trans-acting genes of the pGreen vector	Hellens et al., 2000b

Amp^R – Ampicillin resistant; Kan^R – Kanamycin resistant; Tet^R – Tetracycline resistant.

3.2. Bacterial strains and culture media

Escherichia coli strain DH5α was the host for all the plasmids used in the cloning steps of the p35S*Adc* and p35S*Adc-Gus* plant transformation vectors.

E. coli DH5∝ were grown in *Luria Broth* rich medium (*LB*: 10 $g.l^{-1}$ tryptone, $5g.l^{-1}$ NaCl and 5 $g.l^{-1}$ yeast extract) or *LB* solidified with 15 $g.l^{-1}$ microagar (hereafter designated as *LA*) (Duchefa, The Netherlands). Different antibiotics were added to the media for bacteria selection according to the selection markers of the different plasmids used in this work (see Table 1).

3.3. Extraction of plasmid DNA

Bacteria harbouring the desired plasmids were inoculated in *LA* (with appropriate antibiotic supplementation) and grown over-night at 37ºC in order to obtain isolated colonies. Single colonies were isolated and inoculated in 3 ml of *LB*, with the same antibiotic supplementation, in 15ml tubes. Cultures were grown over-night at 37ºC under shaking (220rpm). The bacterial suspension was centrifuged at 3500 rpm for 5 min at 4ºC and supernatant discarded.

For small-scale extraction of plasmid DNA (*miniprep*) we used the alkaline lysis method (Sambrook et al., 1989). Following centrifugation cells were resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH=8 and 10mM EDTA). After, 200 µl of Solution II (0.2 M NaOH and 1% SDS) was added and the tubes were mixed by inversion. Tubes were then incubated for 5 minutes on ice. After, 150 µl of solution III (5M Potassium Acetate pH=4.8) was added and tubes were mixed by vortexing, followed by an additional centrifugation (5 minutes, 13 000 rpm). The supernatants were transferred to new Eppendorf tubes. Two additional extractions with Phenol:Chloroform:Isoamylic Alcohol (25:24:1) and one with Chloroform:Isoamylic Alcohol (24:1) were performed. Plasmid DNA was precipitated with two volumes of chilled absolute ethanol and recovered by centrifugation (5 minutes, 13 000 rpm). Precipitated DNA was resuspended in 50 µl of 1/10 TE (1XTE: 10mM Tris, 1mM EDTA; pH 8.0). RNA was digested by incubation with 20µg/ml of RNase A, for 30 min at 37ºC.

With this extraction protocol it is usually possible to obtain 1000 to 2500ng of plasmid DNA per 3 ml of bacterial culture. When a higher plasmid concentration and purity was needed plasmids were extracted with *MiniPrep Extraction Kit* (Qiagen, Germany) according to manufacturer's instructions

3.4. Preparation of DNA fragments for cloning

For the construction of p35S*Adc* and p35S*Adc-Gus* the DNA of both vector and insert were digested with appropriate restriction enzymes to generate compatible ends for cloning. The insert DNA was isolated by electrophoresis on agarose gels and purified from the agarose using the *QIAquick Gel Extraction Kit* (Qiagen, Germany) according to manufacturer's instructions.

The vector also had to be prepared for cloning. If the ends of the digested vector are identical (i.e., following a single digestion) it was advantageous to remove the phosphate groups from the 5'-ends to prevent self-ligation.

Strategies for preparation of vector and insert for p35S*Adc* and p35S*Adc-Gus* construction are summarised in Tables 2, 3 and 4.

Table 2. Strategies for preparation of DNA fragments for cloning of the *Nos-Kan* cassette in pGreen0000.

DNA fragment	Restriction enzyme	Generated ends	Treatment prior ligation
Vector (pGreen0000)	*Eco*RV	Blunt ends	Dephosphorylation of 5'-ends
Insert (*Nos-Kan* cassette)	*Eco*RV	Blunt ends	Purification by electrophoresis

Table 3. Strategies for preparation of DNA fragments for cloning of 2X35S*Adc* in pGreen0000, already bearing *Nos-Kan* cassette (pGreen*Nos-Kan*)

DNA fragment	Restriction enzyme (double digestion)	Generated ends	Treatment prior ligation
Vector (pGreen*Nos-Kan*)	*Kpn* I *Xho* I	Different cohesive ends	Purification by electrophoresis Dephosphorilation of 5'-ends
Insert (2X35S*Adc*)	*Kpn* I *Xho* I	Different cohesive ends	Purification by electrophoresis

Table 4. Strategies for preparation of DNA fragments for cloning of p35S*Adc-Gus*.

DNA fragment	Restriction enzyme	Generated ends	Treatment prior ligation
Vector (p35S*Adc*)	*Hpa* I	Blunt ends	None
Insert (35S*Gus*)	*Eco*RV	Blunt ends	Purification by electrophoresis

3.4.1. Digestion with restriction enzymes

About 800 to 1000ng of insert and vector DNA were digested with 1 to 4 U of appropriate restriction enzyme at 37ºC over-night.

To test for complete digestion one aliquot (1-2 µl) of the digested plasmid DNA was subjected to electrophoresis on 0.8-1% agarose (Merck, USA) gels of TAE 1X or TBE 1X (TAE: 0.4M Tris, 10mM EDTA, pH adjusted to 8.0 with Acetic Acid; TBE: 0.45M Tris, 10mM EDTA, 0.45M Boric Acid) with 0.5µg.ml^{-1} Ethidium bromide. Images of the gels were acquired using the system/software Gel Doc (BioRad).

3.4.2. Purification of DNA

Insert DNA and vector (when necessary) were purified by electrophoresis in agarose gels of 0.8-1% in TAE 1X or TBE 1X. Fragments of the expected size were isolated and extracted using the *QIAquick Gel Extraction Kit* from Qiagen (Germany), according to manufacturer's instructions.

3.4.3. Dephosphorylation of 5'-ends

Digested DNA vector (300-700ng), directly or after purification, was used for dephosphorylation of 5'-ends with *Calf Intestinal Alkaline Phosphatase* (CIAP). The following components were added to the DNA: 10µl of CIAP 10X reaction buffer, 1U of CIAP and nuclease-free water to a final volume of 100µl. Incubation conditions depended on the type of ends present:

1. For 5'-protruding ends: Incubation for 30 min at 37ºC, followed by addition of another 1U of CIAP and re-incubation for 30 min at 37ºC.

2. For 5'-blunt ends: Incubation for 15 min at 37ºC followed by 15 min incubation at 56ºC. Addition of another 1U of CIAP was followed by 15 min incubation at both temperatures.

Reactions were stopped by addition of 2μl of EDTA and tubes were heated at 65ºC for 20 min. After dephosphorylation, DNA was purified by phenol extraction and ethanol precipitation or alternatively using the *PCR Purification Kit* (Qiagen, USA), according to manufacturer's instructions.

3.4.4. Quantification of DNA concentration

When necessary (e.g., after extraction, after dephosphorylation, after purification) quantification of DNA concentration was estimated by electrophoresis on agarose gels (0.8-1%) in TAE 1X or TBE 1X. One aliquot (1-2 μl) of DNA solution was used for quantification, along with molecular weight standards of phage lambda (λ) of known concentrations.

3.5. Ligation of plasmid vector and insert DNA

Different vector:insert molar ratios were tested (1:1, 1:2, 1:3 and 1:4) in order to find the optimum ratio. The following formula allows the conversion of molar ratios to mass ratios depending on the size of the vector and the fragment to be inserted:

$$\text{ng of Insert} = \frac{\text{ng of Vector X Kbp size of Insert}}{\text{Kbp size of Vector}} \text{ X Molar Ratio (I/V)}$$

In order to monitor the efficiency of ligation and transformation steps and the yield of plasmid re-circularization, DH5α cells were transformed respectively, with supercoiled plasmid and with linear vector DNA.

3.5.1. Blunt ends ligation

For the subcloning of *Nos-Kan* cassette in pGreen0000, it was necessary to attach vector and insert blunt ends generated by digestion with *Eco*RV (see Table 2).

In the final cloning steps, when subcloning the 35S*Gus* fragment in p35S*Adc*, it was also necessary to connect vector and insert blunt ends (see Table 4). In this case, for the vector, an enzyme (*Hpa*I) that generates blunt ends near the RB of the plasmid was used. As for the insert, we chose the enzyme *Eco*RV that excise the 2.5 Kb fragment that contains the 35S*Gus* cassette. This enzyme also generates blunt ends, which facilitates the ligation. The vector was not subjected to dephosphorylation of its 5´ends.

Vector and insert were prepared as previously described. Calculation of insert mass, according to vector concentration, was made using the formula described in 3.5.

Table 5 and 6 summarise the components of ligation reaction mixture. These tubes were incubated at 22ºC for 4h and then stored at 4ºC.

Table 5. Components of the reaction mixture for subcloning of *Nos-Kan* cassette in pGreen0000

Tube	Ligation reaction	Vector (ng)	Insert (ng)	Molar ratio	Buffer 2X (μl)	T4 DNA Ligase (U)
1	pGreen + *Nos-Kan*	20	17.5	1:2	7	0.5
2	pGreen + *Nos-Kan*	20	26.3	1:3	7	0.5
3	pGreen x *Eco*RV (dephosp.)	10	0	_	7	0.5
4	pGreen (supercoiled)	10	0	_	7	0.5

Note: Reaction mixture final volume was 14 μl; sterile distilled water was added when necessary to adjust the final volume.

Table 6. Components of the reaction mixture for subcloning of 35S*Gus* in p35S*Adc*.

Tube	Ligation reaction	Vector (ng)	Insert (ng)	Molar ratio	Buffer 10X (μl)	T4 DNA Ligase (U)
J1	p35S*Adc* + 35S*Gus*	100	30	1:1	1	0.5
J2	p35S*Adc* + 35S*Gus*	100	60	1:2	1	0.5
J3	p35S*Adc* + 35S*Gus*	100	90	1:3	1	0.5

Note: Reaction mixture final volume was 10 μl; sterile distilled water was added to adjust the final volume.

3.5.1. Cohesive ends ligation

For the cloning of 2X35S*Adc* in pGreen*Nos-Kan*, to generate the vector p35S*Adc* (see Table 3), it was necessary to perform the ligation of vector and insert cohesive ends generated by double digestion with *Kpn*I and *Xho*I. Preparation of vector and insert for cloning was done as previously described. Calculation of insert mass, according to vector concentration, was done using the formula described in 3.5. Table 7 summarises the components of the reaction mixture for ligation. These tubes were incubated at 4ºC overnight.

Table 7. Components of the reaction mixture for cloning of 2X35S*Adc* in pGreen *Nos-Kan* (to generate p35S*Adc*)

Tube	Ligation reaction	Vector (ng)	Insert (ng)	Molar ratio	Buffer 5X (μl)	T4 DNA Ligase (U)
1	pGreen*Nos-Kan* + 2X35S*Adc*	40	80	1:3	4	0.5
2	pGreen*Nos-Kan* + 2X35S*Adc*	40	107	1:4	4	0.5
3	pGreen*Nos-Kan* X *Kpn*I/*Xho* I (dephosp.)	40	0	_	4	0.5
4	pGreen*Nos-Kan* (supercoiled)	40	0	_	4	0.5

Note: Reaction mixture final volume was 20 μl; sterile distilled water was added when necessary to adjust the final volume.

3.6. Transformation of *E. coli* DH5α with plasmid DNA

3.6.1. Preparation of DH5α competent cells

Competent cells were prepared using the $CaCl_2$ method described in Sambrook et al. (1989). Briefly, a single colony of DH5α from a fresh *LA* plate was inoculated in 5 ml of *LB* medium and grown overnight at 37ºC under vigorous shaking (200 rpm). One ml of this culture was inoculated in 100 ml of *LB* medium and incubated at 37ºC under vigorous shaking until reaching an OD 600nm=0.6 (usually for 3-4 hours). After, tubes were centrifuged for 10 minutes at 5ºC. Supernatant was discarded and the pellet was resuspended on 20 ml of ice-cold 100mM $CaCl_2$. Cell suspensions were maintained on ice for 1 hour and then, cells were again centrifuged. Pellet was resuspended on 2.5 ml of ice-cool 100mM $CaCl_2$ and 0.5 ml of sterile 80% Glycerol was added to each tube. Glycerol stocks of competent cells were flash-frozen in liquid nitrogen and stored at –80ºC, in 400µl aliquots.

3.6.2. Transformation of DH5α competent cells

Aliquots of 1-2 µl of supercoiled DNA or 2-10 µl of ligation reaction mixture were added to 200 µl of competent cells. These bacterial suspensions were incubated on ice for 1h, minimum, to allow the adsorption of plasmids to bacterial cell wall. After, cells were subjected to 42ºC for 90 sec (heat shock), followed by addition of 800 µl of *LB* and incubation for 1h at 37ºC, with gentle shaking (150rpm). Cells were pelleted by centrifugation (8000 rpm, 1min) and 800 µl of supernatant was removed. Cells were resuspended in the remaining medium (200µl) and spread onto plates containing *LA* with the appropriate selection antibiotic. Plates were incubated over-night at 37ºC.

3.7. Selection for recombinant plasmids

In order to select for recombinant plasmids the following strategies were used:

1. α-Complementation: Many of the commonly used plasmids have a structure that allows direct visual selection of colonies containing vectors with donor DNA inserts (the pGreen plasmid has this feature). They contain a small portion of the *E. coli* ß–galactosidase gene (*Lac Z*). Into this region has been inserted a piece of DNA called multiple cloning site (MCS), which contains many convenient restriction sites useful for inserting DNA fragments. The host cells (in our case DH5α) have a ß–galactosidase gene lacking the fragment present on the plasmid. The partial proteins coded by the two fragments unite to form a functional ß–galactosidase (α-complementation). If a colourless substrate for ß–galactosidase, called X-Gal, is added to the medium the functional enzyme converts it to a blue dye, which colours the colony blue (bacteria with phenotype Lac^+). If donor DNA is inserted into the multiple cloning site, then the ß–galactosidase activity is lost and colony is white (bacteria with phenotype Lac^-). Hence, selection for white kan^r colonies selects directly for vectors containing inserts, and such colonies are isolated for further study.

When using this selection feature it is necessary to previously spread in LA plates, already containing culture medium, 20 µl of 50 mg/mL X-Gal (Duchefa, The Netherlands) and 100 µl of 100mM IPTG. The IPTG (Isopropyl ß-D-thiogalactopyranoside) is one analogue of galactose that induces expression of *lac* function in *E. coli*.

2. DNA Extraction and Electrophoresis on Agarose Gels: After transformation and selection of possible transformed colonies plasmid DNA was extracted by *miniprep* and directly subjected to electroforesis on 0.8-1% agarose gels (with $0.5 \mu g.ml^{-1}$ Ethidium bromide) in TAE 1X or TBE 1X buffer. To determine the size of the DNA plasmid, a ladder of supercoiled DNA was used (*Supercoiled DNA ladder*).

The presence of restriction sites in the plasmids used for cloning allows searching for recombinants by single digestion of plasmid with an appropriate restriction enzyme

that linearises DNA. Plasmid DNA could also be digested to excise a fragment or fragments which size(s) can be predicted by consulting the plasmids restriction maps.

3. PCR Amplification: The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. The following components are necessary: a segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking it, a DNA polimerase (we used *Thermus aquaticus* - Taq - DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTPs), buffer and salts ($MgCl_2$). Once assembled, the mixture is cycled many times (usually around 30) through temperatures that permit denaturation, annealing, and synthesis to exponential amplify a DNA product of specific size and sequence (Ausubel et al., 1987) The PCR products can be displayed on an appropriate gel and examined for yield and specificity.

For the oat *Adc* gene amplification the following primers were used:
Oligo 1 – $^{5'}$CGGCGATGTGTACCATGTCGAGGG$^{3'}$
Oligo 5 – $^{5'}$GCGGGTGCAGCGGCATCGTCTCGG$^{3'}$

These primers amplify a fragment of 1.5Kb corresponding to an internal portion of oat *Adc* gene. PCR conditions were 96ºC/ 2 min for denaturation, followed by 34 cycles of 96ºC/ 40 sec, 60ºC/ 30 sec for annealing, 72ºC/ 2 min and 30 sec for polymerisation and 72ºC/ 15 min for final extension. Composition for PCR mixture is presented in Table 8.

The PCR technique can be used directly in bacterial cultures to verify the presence of a specific plasmid avoiding the need for plasmid extraction. For confirmation of the presence of the *Gus* gene, bacterial suspensions (DH5α) were used.

For the *Gus* gene amplification the following primers were used:
Forward (Gus 1) – $^{5'}$ ACGTCCTGTAGAAACCCCAA$^{3'}$
Reverse (Wo1) – $^{5'}$CATTACGCTGCGATGGATTCC$^{3'}$

These primers amplify a fragment of 517bp corresponding to an internal portion of the *Gus* gene. PCR conditions for the *Gus* gene amplification were 98ºC/ 1 min for denaturation, followed by 38 cycles of 94ºC/ 50 sec, 63ºC/ 1 min for annealing, 72ºC/ 50 sec for polymerisation and 72ºC/ 3 min for final extension. The composition for the PCR is presented in Table 8.

Table 8. Composition of PCR mixture for *Adc* and *Gus* amplification.

PCR mix.	**Final Volume=50µl**
Buffer	1X
[DNA] or Bact. Susp.	100ng 10µl
dNTPs	0.1mM
Primers	30 pmol/ each
[$MgCl_2$]	2mM
Taq Polymerase	1 U

Note: sterile distilled water was added to adjust the final volume.

4. Results and Discussion

4.1. Subcloning of *Nos-Kan* cassette in pGreen0000

As previously described, the binary plant transformation vector pGreen0000 was chosen for the cloning of the oat arginine decarboxylase encoding gene (*Adc*). Because the earlier versions of the pGreen vectors (like pGreen0000) did not include a selectable marker gene it was necessary to introduce one in the construct in order to allow selection for plant transgenic tissue. Being the antibiotic kanamycin the selective agent most widely used in *Agrobacterium*-mediated transformation of *M. truncatula*, we selected the *Nos-Kan* cassette, containing the *Npt*II gene encoding for neomycin phosphotransferase II, to be introduced in the construct.

The *Nos-Kan* cassette was excised as a 1.4Kb fragment from plasmid p*Nos-Kan*, by digestion with *Eco*RV, and electrophoresis allowed the recovery of this fragment (Fig.4.A).

The fragment was purified as described in section 3.4.2. The vector pGreen0000 was also digested with *Eco*RV and complete reaction monitorised by agarose gel electrophoresis (Fig.4B). Dephosphorylation of 5'-ends of pGreen vector resulted in major DNA lost (75-80%) if the process for purification after CIAP treatment was done by phenol extraction followed by ethanol precipitation, but if we used the *PCR Purification Kit* (Qiagen) DNA could be recovered with an efficiency of almost 95%. The results from quantification of DNA fragments (before ligation) are shown in Figure 4.B.

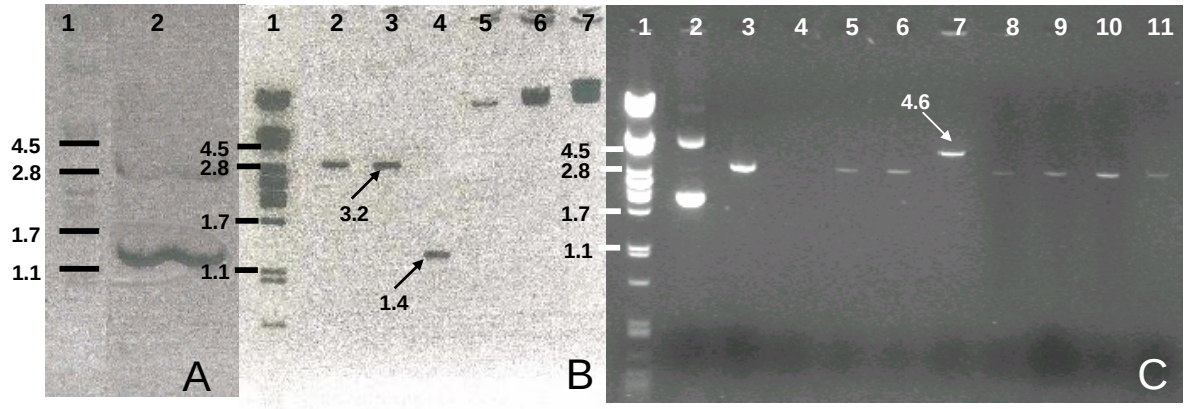

Figure 4. Agarose gel electrophoresis to monitor the subcloning of the *Nos-Kan* cassette into vector pGreen0000.
A – Recovery of fragment *Nos-Kan* 1-λ*Pst*I; 2- p*Nos-Kan* digested with *Eco*RV to excise a 1.4Kb fragment (*Nos-Kan* cassette). **B** - DNA quantification; 1- λ*Pst*I; 2 and 3 - pGreen digested with *Eco*RV and dephosphorylated; 4 - *Nos-Kan* cassette after gel extraction and purification; 5, 6 and 7 - respectively, 10ng, 25ng and 50 ng of molecular weight standards of phage λ. **C** - Selection for recombinants by digestion with *Kpn*I; 1- λ*Pst*I; 2 - pGreen supercoiled; 3 - pGreen x *Eco*RV; 4, 5, 6, 8, 9, 10 and 11 - non recombinants; 7 - recombinant with expected size (4.6 Kb).

Ligation of blunt ends was performed as described in section 3.5.1 and the resulting colonies were counted (Table 9).

Table 9. Results from ligation reactions for subcloning of *Nos-Kan* cassette in pGreen0000

Tube	Ligation reaction	Molar ratio	Nº Colonies (Total)	Nº white colonies	Nº growing colonies	Nº recomb. recovered
1	pGreen +*Nos-Kan*	1:2	75	5	5	0
2	pGreen +*Nos-Kan*	1:3	78	8	4	1
3	pGreen x *Eco*RV (dephosp.)	-	12	4	-	-
4	pGreen (supercoiled)	-	*	*	-	-

* Uncountable blue colonies; Nº growing colonies: Nº of colonies that were able to grow in antibiotic supplemented liquid medium; Nº recomb recovered: Nº of recombinant colonies recovered.

Because the fragment *Nos-Kan* was inserted in the Lac operon, direct visual selection of colonies containing vectors with DNA inserts is possible (bacteria with phenotype Lac^-). Hence, all the white colonies resulting from tubes 1 and 2 (see Tables 5 and 9) were amplified and plasmid DNA was extracted. DNA was linearised with restriction enzyme *Kpn*I and results from electrophoresis are shown in Figure 4.C. Only one recombinant with the expected size (recombinat 7) was selected. To confirm successful cloning, recombinant 7 (rec. 7) was further digested with *Eco*RV, which allows recovering the 1.4Kb fragment corresponding to *Nos-Kan* cassette (Figure 5.A).

All the white colonies formed from original tube 1 grown on *LB* medium supplemented with kanamycin, but no recombinant was recovered (they contained only pGreen0000) (Fig 4C). Half of the white colonies obtained from ligation mixture 2 were false positives, because were not able to grown in liquid antibiotic-supplemented medium. One recombinant was recovered from the 4 colonies that grew in liquid medium (rec. 7) (Fig 4C). It seems that 1:3 vector:insert molar ratio is the best for blunt end ligation of this specific plasmid and insert.

Self-ligation of dephosphorylated vector occurred as demonstrated by the high number of blue colonies that appeared in ligations 1 and 2, and also by the presence of 12 colonies resulting from tube 3. Nevertheless, the percentage of pGreen self-ligation when vector is not subjected to dephosphorylation is much higher than when treated with CIAP.

4.2. Cloning of 2X35S*Adc* in pGreen*Nos-Kan*

DNA from recombinant 7 (pGreen*Nos-Kan*) was amplified and extracted by *miniprep* (see section 3.3). For confirmation of the construct, recombinant 7 (rec. 7) was digested with *Eco*RV (Fig. 5A). After, this plasmid was double digested with *Kpn*I and *Xho*I to create compatible cohesive ends to the insert, also digested with the same enzymes. As previously explained the insert (2X35S*Adc*) contains the 2124-bp oat *Adc* cDNA, a CaMV 35S promoter with duplicated enhancer sequences and a CaMV transcriptional termination region, with a final size of 3.6Kbp. The presence of these two consensus sequences (enhancer sequences) preceding the initiator codon on β-glucuronidase gene resulted in three times more GUS activity in tobacco mesophyll protoplasts (Guerineaux et al., 1992).

Both vector and insert DNA were excised from the gel (Fig. 5.B and C) and purified with *Gel Extraction Kit* (Qiagen, USA). Quantification of DNA fragments, before ligation, was performed as described and results are shown in Figure 5.D.

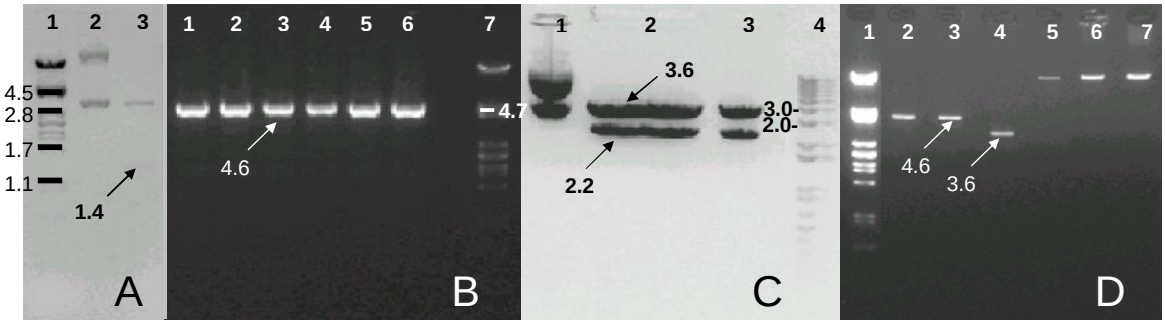

Figure 5. Agarose gel electrophoresis to monitor the subcloning of 2X35S*Adc* into vector pGreen*Nos-Kan*.
A - Confirmation of recombinant 7 (rec. 7) by restriction with *Eco*RV. **1** - λ*Pst*I; **2** - pGreen*Nos-Kan* supercoiled (rec. 7); **3** - pGreen*Nos-Kan* (rec. 7) digested with *Eco*RV that releases a 1.4Kb fragment corresponding to the *Nos-Kan* cassette; **B** - Purification of linearised vector pGreen*Nos-Kan* prior to ligation. **1 to 6** - pGreen*Nos-Kan* (rec. 7) double digested with *Kpn*I and *Xho*I to be excised from the gel; **7** - λ*Pst*I. **C** - Preparation of insert 2x35S*Adc*. **1** - $pAMC_2$ supercoiled; **2 and 3** - $pAMC_2$ double digested with *Kpn*I and *Xho*I to yield a 3.6Kb fragment (2x35S*Adc*); **4** - 1Kb plus DNA ladder. **D** - Quantification of vector and insert DNA before ligation. **1** - λ*Pst*I; **2 and 3** - pGreen*Nos-Kan* (rec.7) x *Kpn*I/*Xho*I, after gel extraction and purification; **4** - 3.6Kb fragment (2x35S*Adc*) after gel extraction and purification; **5, 6 and 7** - respectively, 10ng, 20ng and 30 ng of molecular weight standards of phage λ.

Ligation was performed as described in 3.5.2 and results are presented in Table 10.

Table 10. Results from ligation reactions for cloning of 2X35S*Adc* in pGreen*Nos-Kan*

Tube	Ligation reaction	Molar ratio	Nº Colonies (Total)	Nº growing colonies	Nº recomb. recovered
1	pGreen*Nos-Kan* + 2X35S*Adc*	1:3	10	2	2
2	pGreen*Nos-Kan* + 2X35S*Adc*	1:4	8	4	2
3	pGreen*Nos-Kan* X *Kpn*I/*Xho* I (dephosp.)	-	15	-	-
4	pGreen*Nos-Kan* (supercoiled)	-	*	-	-

* Uncountable colonies; Nº growing colonies: Nº of colonies that were able to grown in antibiotic supplemented liquid medium; Nº recomb recovered: Nº of recombinant colonies recovered.

All the colonies resulting from tubes 1 and 2 of ligation reactions (see Table 7 and 10) were amplified and plasmid DNA was extracted by *miniprep*. One aliquot of 2-4 µl of plasmid DNA was directly subjected to electrophoresis on agarose gel 0.8-1% in TAE 1X or TBE 1X buffer. To determine the size of non-digested DNA plasmid a ladder of supercoiled DNA was used (Fig.6. A). Four recombinants with the expected size were selected, E1 and H8 (lane 4 and 7, Fig. 6.A.) resulting from 1:3 molar ratio, and I3 and I4 (lane 8 and 9, Fig. 6.A) resulting from 1:4 vector insert ratio. Plasmids from lanes 5 and 6 were non-recombinants (Fig. 6.A).

Only one recombinant plasmid from each vector insert molar ratio was tested (H8 and I3). These putative recombinants were digested with *Xba*I for plasmid linearization (Fig. 6.B., lane 5 and 8). They were also both digested with *Eco*RI that should generate three fragments: 1st – a 3.9Kbp fragment corresponding to pGreen and the 35S promoter with duplicated enhancer sequences (2X35S); 2nd – a 2.2Kbp fragment generated by *Nos-Kan* cassette and the CAMV term; 3rd – the 2.1Kbp fragment of oat *Adc* gene. H8 and I3 contained both the expected construct: p35S*Adc* (Fig. 6.B., lane 6 and 9). Clone H8 was further tested by PCR with the primers described in section 3.7. (see Figure 9A; section 4.3.).

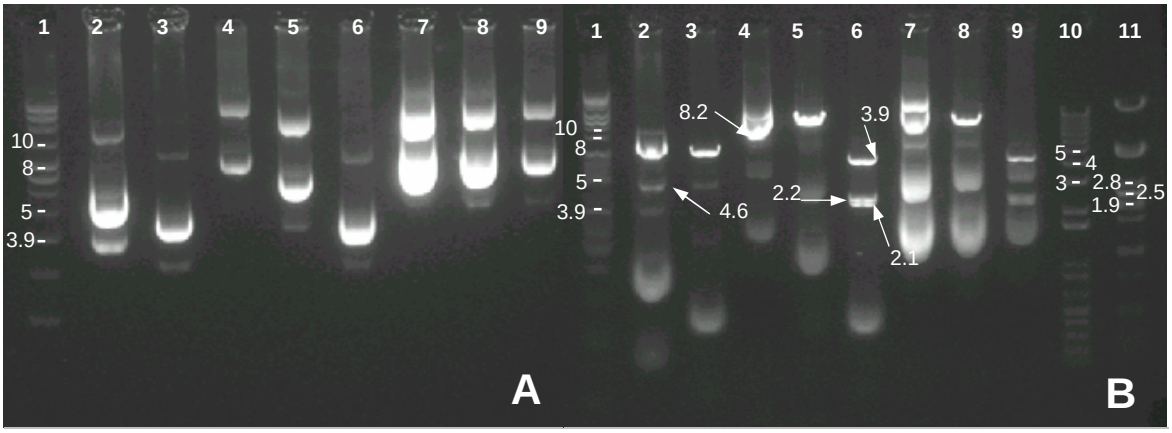

Figure 6. Agarose gel electrophoresis to screen for recombinant bacterial clones containing the vector p35S*Adc*.
A - Electrophoresis of non-digested putative recombinants. **1** - *Supercoiled DNA ladder*; **2** - supercoiled $pAMC_2$; **3** - pGreen*Nos-Kan* (rec. 7) supercoiled; **4, 7, 8 and 9** - supercoiled putative recombinants (p2x35S*Adc*); **5 and 6** - supercoiled non-recombinants.
B – Electrophoresis of *Xba*I or *Eco*RV-digested recombinants. **1**- *Supercoiled DNA ladder*; **2** - pGreen*Nos-Kan* (rec. 7) supercoiled ; **3** - pGreen*Nos-Kan* (rec. 7) digested with *Xba*I; **4 and 7** - supercoiled putative recombinants H8 and I3; **5 and 8** - putative recombinants H8 and I3 digested with *Xba*I; **6 and 9** - putative recombinant H8 and I3 digested with *Eco*RV; **10** - *1Kb plus DNA ladder*; **11** - λ*Pst*I.

Most of the colonies that grew in *LA* plates were wild type DH5α as they were not able to grow in liquid antibiotic-supplemented medium. The 1:3 vector:insert molar ratio seemed to be the most effective, as 100% of the growing colonies were recombinants. The increase of vector and insert concentrations, compared to those used for blunt-ends ligation, resulted in higher ligation efficiency (100% for reaction 1 and 50% for reaction 2) (see Table 10).

Dephosphorylation was not necessary in this case because two different enzymes generating different cohesive ends were used.

One of the problems resulting from this cloning strategy was the selection of those two enzymes that are too close in the plasmid MCS (only 11bp distant). In the case of adjacent restriction sites, the second digestion may be compromised. It was also difficult to monitor the efficiency of the second digestion and it was necessary to remove the small fragment generated by digestion with the second enzyme, to avoid re-ligation of the plasmid vector (see Figure 5B).

4.3. Insertion of *Gus* reporter gene in the construct p35S*Adc*

The 35S*Gus* cassette was excised as a 2507bp fragment, by digestion with *Eco*RV (Fig.7.A.). This fragment was purified as described in section 3.4.2. The vector p35S*Adc* was digested with *Hpa*I and complete reaction monitorised by agarose gel electrophoresis (Fig.7.B). The results from quantification of DNA fragments (before ligation) are shown in Figure 7 C.

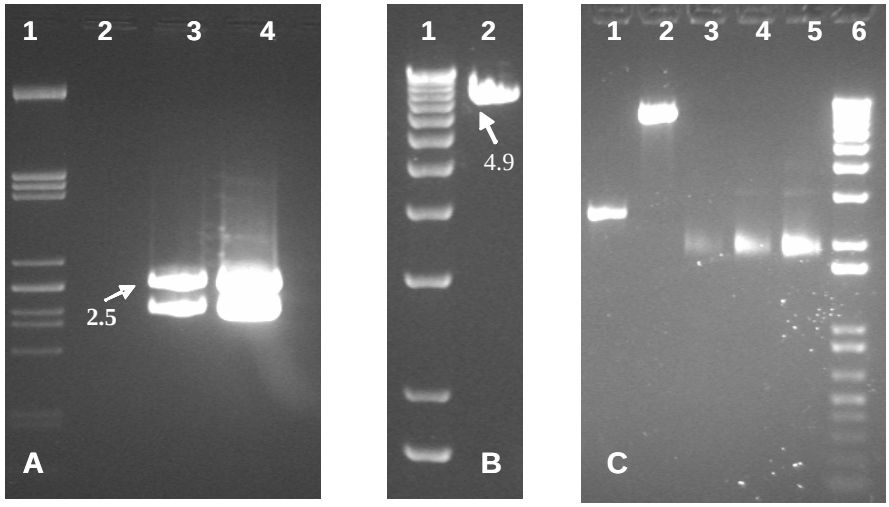

Figure 7. Agarose gel electrophoresis to monitor the subcloning of 35S*Gus* cassette into vector p35S*Adc*.
A - Plasmid p35S*Gus* digested with *Eco*RV that releases a 2.5Kbp fragment corresponding to the 35S*Gus* cassette. **1** - λ*Pst*I; **2** - empty lane; **3 and 4 –** p35S*Gus* digested with *Eco*RV to excise a 2.5Kb fragment. **B** - Confirmation of p35S*Adc* linearization. **1** - *1Kb plus DNA ladder*; **2** - p35S*Adc* linearised with *Hpa*I. **C** - Quantification of vector and insert DNA before ligation. **1** - 35S*Gus* cassette excised as a 2.5Kbp fragment; **2** - p35S*Adc* linearised with *Hpa*I; **3, 4 and 5** - 25ng, 50ng and 100ng, respectively, of molecular weight standards of phage λ DNA; **6** - *1kb plus DNA ladder*.

Ligation of blunt ends was performed as described in section 3.5.1 and the resulting colonies were counted (Table 11). All the colonies resulting from tubes J1 to J3 (see Tables 6 and 11) were inoculated in liquid *LB* medium supplemented with kanamycin to amplify the plasmid DNA. Not all the colonies were able to grow in liquid *LB* kanamycin-supplemented medium (see Table 11; Nº growing colonies).

Table 11 – Results from ligation reactions for subcloning of 35S*Gus* cassette in p35S*Adc*.

Tube	Ligation reaction	Molar ratio	Nº Colonies (Total)	Nº growing colonies	Nº recomb. recovered
J1	p2X35S*Adc* + 35S*Gus*	1:1	5	4	0
J2	p2X35S*Adc*+ 35S*Gus*	1:2	0	0	0
J3	p2X35S*Adc*+ 35S*Gus*	1:3	6	4	1

Nº growing colonies: Nº of colonies that were able to grown in antibiotic supplemented liquid medium; Nº recomb recovered: Nº of recombinant colonies recovered.

Plasmid DNA, from liquid growing colonies, were extracted by *miniprep* and digested with restriction enzyme *Bgl*II. Three fragments with different size were expected: 1st – a 5.9Kbp fragment corresponding to oat *Adc* gene, the double promoter (2X35S), ½ of the CAMV terminator and the 35S*Gus* cassette; 2nd – a 2.4Kbp fragment corresponding to the backbone of pGreen0000; 3rd – the 2.3Kbp fragment generated by *Nos-Kan* cassette and ½ of the CAMV terminator. Only one recombinant with the expected fragments (rec. J3.1) was selected corresponding to the expected construct: p35S*Adc-Gus*. Results from electrophoresis are shown in Figure 8.

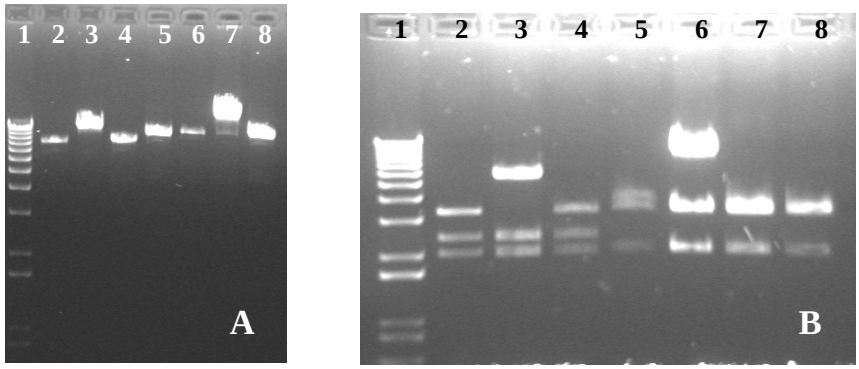

Figure 8. Agarose gel electrophoresis to screen for recombinant bacterial clones containing vector p35S*Adc-Gus*.
A – Electrophoresis of non-digested putative recombinants. **1 –** *Supercoiled DNA ladder*; **2 to 7 –** supercoiled putative recombinants; **3 –** recombinant J3.1 with the expected size. **B** – Electrophoresis of *Bgl*II-digested recombinants. **1 -** *1Kb plus DNA ladder*; **2 to 8 –** putative recombinants digested with *Bgl*II; **3 –** recombinant J3.1 revealing the bands with the expected size.

Only one recombinant was recovered from the colonies that grew in liquid medium (recombinant J3.1). It seems that 1:3 vector:insert molar ratio is the best for blunt end ligation of this specific plasmid and insert.

To confirm successful cloning, recombinant J3.1 was subjected to PCR amplification for the *Adc* and *Gus* genes, directly using bacterial cultures without plasmid extraction. The expected bands were obtained for both genes *Adc* (results not shown) and *Gus*. A fragment of 517bp corresponding to the amplification of an internal portion of the *Gus* gene can be observed in Figure 9 B.

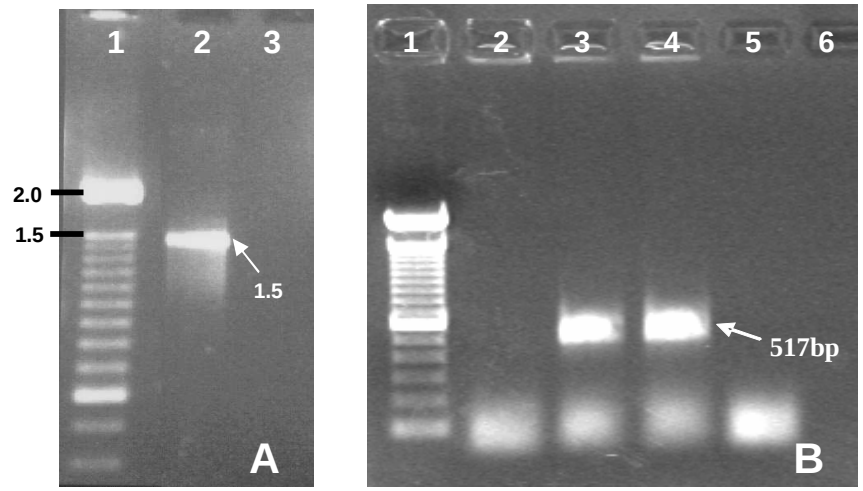

Figure 9. Agarose gel electrophoresis to confirm recombinant bacterial clones containing vector p35S*Adc* and p35S*Adc-Gus* by PCR amplification for the *Adc* and *Gus* genes, respectively.
A – Confirmation of successful cloning of p35S*Adc* (recombinant H8) by PCR amplification of plasmid *miniprep*. **1** - *100bp DNA ladder*; **2**- PCR amplification of 1.5 Kb corresponding to the oat ADC fragment in p35S*Adc*; **3** - master mix without DNA template. **B** - Amplification by PCR using bacterial suspensions without plasmid extraction **- 1 -** *100bp DNA ladder*; **2 -** bacterial suspension without the plasmid (negative control); **3 and 4 –** PCR amplification of 508 bp fragment corresponding to an internal portion of *Gus* gene in bacterial suspensions bearing the recombinant plasmid J3.1 (replicates); **5 -** master mix without DNA template.

From these results, we confirmed that recombinant J3.1 contains the required construct (p35S*Adc-Gus*) and can be used for future plant transformation experiments.

Vector p35S*Adc* was used in a first attempt to genetically transform *M. truncatula* line M9-10a but no transgenic lines were obtained (see Chapter III). Lately, vector p35S*Adc-Gus* obtained from the insertion of the *Gus* reporter gene into p35S*Adc* was used

for the subsequent M9-10a transformation experiments (Chapter IV). The insertion of the *Gus* gene in vector p35S*Adc* facilitates the confirmation of transgenesis by performing an easy histochemical detection of expression of the reporter gene for β-glucoronidase in transgenic plants.

Before being used for plant transformation the integrity of the chimeric genes *Adc*, *Gus* and *Npt*II in this construct was checked by Santos et al. (2003) by reverse transcription-PCR assay coupled to *A. tumefaciens*-mediated transient expression.

5. Conclusions

We have generated two plasmid constructs to be used for plant transformation: p35S*Adc* and p35S*Adc*-*Gus*. Cloning of p35S*Adc* involved two sub-cloning steps: insertion of *Nos-Kan* cassette in pGreen0000 followed by the insertion of 2x35S*Adc* cassette. Cloning of p35S*Adc*-*Gus* involved a third sub-cloning step, since p35S*Adc* was used as the basis to insert the 35S*Gus* cassette. All the sub-cloning steps were made relatively easy by the use of the versatile pGreen system.

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III – Optimisation of kanamycin concentration for selection of *M. truncatula* cv Jemalong lines transformed with the *Npt*II gene

This work was accomplished in collaboration with Susana Araújo from the plant Cell Biotechnology Laboratory (ITQB) and a version of this chapter was published as:

Duque A.S.*, Araújo S.S.*, Santos D.M., Fevereiro P. (2004). Optimisation of a selection scheme using kanamycin to improve transformation of *Medicago truncatula* cv. Jemalong. Plant Cell, Tissue Organ Cult. 78:227-280.

*Both authors contributed equally to this publication

1. Abstract

We developed an efficient method for *in vitro* selection of *Medicago truncatula* cv Jemalong lines transformed with the *Npt*II gene and for subsequent confirmation of phenotype inheritance in these lines. For *in vitro* selection, the concentration of kanamycin inhibitory to embryogenic *callus* development and somatic embryo differentiation was identified by placing wounded leaves of non-transformed *M. truncatula* cv Jemalong on Embryo Inducing Medium (EIM) supplemented with 0, 85.8, 128.7, 171.6, 214.6, 257.5 and 343.3 µM of kanamycin (corresponding to 0, 50, 75, 100, 125, 150 and 200 $mg.l^{-1}$, respectively). Differentiation of somatic embryos was inhibited with 171.6 µM of kanamycin but *callus* development was not altered. To confirm transgene inheritance, the kanamycin concentration to distinguish between resistant and non-resistant seedlings was found by germinating non-transformed seeds of *M. truncatula* cv Jemalong on MS medium containing 0, 171.6, 343.3, 514.9 and 686.6 µM of kanamycin (corresponding to 0, 100, 200, 300 and 400 $mg.l^{-1}$, respectively). These concentrations did not impair seed germination since all the seedlings exhibited green cotyledons. The effect of kanamycin was only observed at 514.9 and 686.6 µM and on the first pair of leaves, which became white. Due to the high level of kanamycin resistance presented by the wild type seedlings, we thought to use the highest concentration to assure the efficient selection of the transformed seedlings. This optimised antibiotic selection scheme eliminates the regeneration of non-transformed escapes and discriminates between resistant (transformed) and non-resistant (non-transformed) seedlings, confirming the inheritance of the phenotype in *M. truncatula* cv Jemalong transgenic lines.

Key words: Antibiotic selection, barrel medic transformation, seedlings, somatic embryos

2. Introduction

Medicago truncatula is accepted as a model plant for studying biological questions that are unique to legumes (Barker et al., 1990; Cook, 1999). Its autogamic and diploid character, small genome and short life cycle enable the use of this plant in functional

genomic studies that require efficient transformation systems (d'Erfurth et al., 2003). Kanamycin is the selective agent most widely used in *Agrobacterium*-mediated transformation of *M. truncatula*. In such cases, the *Npt*II gene encoding for neomycin phosphotransferase II is co-transferred with the gene of interest to the plant genome and the transformed plant cells become resistant to aminoglycosides antibiotics like kanamycin and gentamycin.

A highly embryogenic *M. truncatula* cv Jemalong line (M9-10a) was isolated in our laboratory (Neves, 2000). Preliminary experiments of *Agrobacterium*-mediated transformation of the Jemalong M9-10a genotype using the process described by Neves (2000) and construct p35S*Adc* (containing the *Npt*II gene as selection marker, see Chapter II) demonstrated to be inefficient. In fact, in our first transformation experiments, a high number of kanamycin-resistant non-transgenic embryos were recovered (1186 somatic embryos isolated from initial *callus* tissue) when *in vitro* selection was performed using 85.8 μM of kanamycin. However, no transgenic lines were obtained regardless of the reasonable number of plants (a total of 60) that were able to survive and root in kanamycin supplemented medium. Therefore, since all the embryos that converted to plantlets proved to be "escapes" (confirmed by Southern blot analysis), it was necessary to optimise the selection efficiency to reduce or avoid the emergence of non-transformed embryos without compromising the embryogenic capacity of M9-10a.

To our knowledge, there are no reports describing the use of kanamycin to study the transgene inheritance pattern of the phenotype in transformed *M. truncatula* progeny, probably because *M. truncatula* wild type seedlings display a high level of kanamycin resistance (Trieu et al., 2000). In this context, it was also necessary to determine the optimal kanamycin concentration to distinguish resistant (transformed) seedlings from non-resistant (non-transformed) ones.

The aim of this study was to optimise a couple selection/regeneration method to be used in the recovery of transgenic *Medicago truncatula* lines. For the M9-10a line we determine the optimal concentration of kanamycin to be used for *in vitro* selection of transformants and for the analysis of seed segregation of the transformed lines.

3. Materials and Methods

3.1. Effect of kanamycin concentration on the development of embryogenic *calli* and differentiation of somatic embryos.

Plants of M9-10a micropropagated *in vitro* on growth regulator-free medium (MS030A) as described in Neves et al. (2001) were used in this study. Somatic embryogenesis was induced from young well-expanded wounded leaves on Embryo Induction Medium [EIM- MS (Murashige and Skoog, 1962) basal salt and vitamins, 3% (w/v) sucrose, 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 9.1 μM zeatin (Zea), 0.2% (w/v) gelrite (Duchefa, Haarlem, The Netherlands), pH 5.8] supplemented with 85.8, 128.7, 171.6, 214.6, 257.5 and 343.3 μM of kanamycin A monosulphate (Duchefa, Haarlem, The Netherlands). Kanamycin and growth regulators were filter sterilised through 0.2μm filters (Whatman, Maidstone, England) and added to pre-sterilised media before platting. For each treatment seven leaf-explants were used per Petri dish. Two replicates were made. Cultures were maintained in a growth chamber (Phytotron EDPA 700, Aralab, Parede, Portugal) under 16-h photoperiod at 100 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent light and a day/night temperature of 24º/22ºC.

After 21 days on EIM, embryogenic *calli* were transferred to Embryo Proliferation Medium (EPM- identical to EIM but without growth regulators) (Neves et al., 1999) supplemented with the different concentrations of kanamycin. To maintain the selective pressure, the growing *calli* were transferred every week to fresh selective medium. The response of *M. truncatula* M9-10a leaf explants to kanamycin was observed after 40 days of treatment. We specifically evaluated the effect of kanamycin concentration on the development of embryogenic *calli* and differentiation of somatic embryos. Leaf-explants of M9-10a not subjected to kanamycin treatment were used as control.

3.2. Effect of kanamycin concentration on seed germination

Seeds of *M. truncatula* M9-10a genotype were germinated in four different kanamycin concentrations (171.6, 343.3, 514.9 and 686.6 μM). Scarification was

performed by immersion in concentrated anhydrous sulphuric acid (H_2SO_4) with intermittent agitation during 5-10 min. Sulphuric acid was removed and seeds were washed three times in a large volume of ice-cold sterile distilled water. Seeds were further surface sterilised by immersion in 50% (v/v) commercial bleach with detergent (Domestus 3) for 5 min under continuous stirring and thoroughly washed with sterile distilled water until all detergent was removed. Before plating, seeds were immersed in 70% (v/v) ethanol for 2min, rinsed with distilled water and sown on 0.8% (w/v) water-agar plates supplemented with the kanamycin concentrations previously referred.

As control, seeds were sown in water-agar plates without kanamycin. After four days at 4ºC under dark conditions, seeds were transferred to the growth chamber to germinate (16-h photoperiod of 100 $\mu molm^{-2}s^{-1}$; 24º/22ºC, day/night temperature). Seedlings were sub-cultured every week to fresh kanamycin-supplemented medium to maintain the selective pressure. The effect of kanamycin on M9-10a seed germination was observed a week after the beginning of the treatment.

4. Results and Discussion

4.1. Effect of kanamycin concentration on the development of embryogenic *calli* and differentiation of somatic embryos.

Percentages of explants that developed *callus* and percentages of explants that developed embryogenic *callus* (embryo-forming *callus*) in the different kanamycin concentrations tested are summarised in Table1.

Non-treated leaf explants presented a vast proliferation of somatic embryos (Figure 1A). The growth and proliferation of embryogenic *calli* were partially inhibited by kanamycin concentrations up to 171.6 µM (Table 1, Figure 1 B, C) and arrested by concentrations of 214.6 µM or higher (Table 1, Figure 1 D and E). Embryo differentiation was not completely inhibited with 171.6 µM of kanamycin but embryos (1-2 somatic embryos per embryo-forming *callus*) never developed over the globular stage and rapidly became bleached (Figure 1C). The callogenic capacity of the explants was not affected by this kanamycin concentration. The use of 214.6 and 257.5 µM of kanamycin reduced *calli*

development and no embryo formation was observed. Higher concentrations of kanamycin (343.3 µM) proved to be lethal to leaf explants. In this condition, *calli* formation was completely suppressed and necrotic areas in all the explants were observed (Figure 1E).

Table 1. Callogenic and embryogenic responses in leaf explants of *Medicago truncatula* cv Jemalong (M9-10a genotype) after 40 days in media with different kanamycin concentrations

Treatment	Results			
Kanamycin concentration ($mg.l^{-1}$)	Callogenic capacity* (%)		Embryogenic capacity** (%)	
	Assay 1	Assay 2	Assay 1	Assay 2
0	100	100	100	100
50	100	100	57	57
75	100	100	57	43
100	100	100	29	14
125	71	71	0	0
150	71	57	0	0
200	0	0	0	0

* Number *calli* formed/Total of explants

** Number embryo forming *calli* /Total of explants

(adapted integrally from Duque A. S., Araújo S. S., Santos D. M., Fevereiro P. (2004). Optimisation of a selection scheme using kanamycin to improve transformation of *Medicago truncatula* cv. Jemalong. Plant Cell, Tissue Organ Cult. 78: 227-280)

Protocols for the transformation of leaf-explants of *M. truncatula* 2HA (Jemalong) and R-108 genotypes (Chabaud et al., 1996; Trinh et al., 1998) use 68.7 to 85.8 µM of kanamycin to select for transformants. However these concentrations did not efficiently inhibit the development of non-transformed embryos in our embryogenic genotype. In agreement with our preliminary experiments, d´Erfurth et al. (2003) also reported a high number of regenerated escapes when selection was performed under such conditions. It is generally accepted legume plants exhibit a natural resistance to aminoglycosides antibiotics like kanamycin and this aspect may affect selection efficiency (Christou, 1994). Our data suggest that 171.6 µM of kanamycin does not affect M9-10a callogenic capacity but prevents the development of somatic embryos. Importantly, this concentration eliminates the regeneration of non-transformed escapes and only transgenic plants are recovered, as confirmed by Southern blot analyses (see Chapter IV of this thesis).

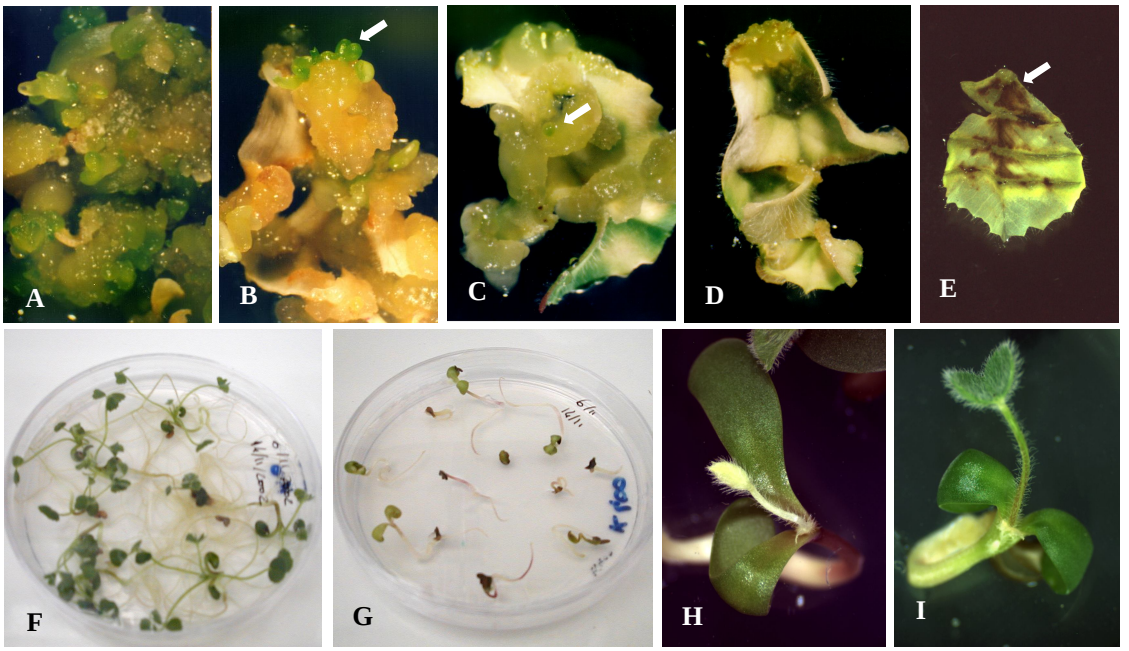

Figure 1. Effect of the kanamycin supplement on the embryogenic response of leaf explants (A - E) and seedling development (F – I) of *M. truncatula* M9-10a genotype.
(**A**) Proliferation of somatic embryos observed in medium without kanamycin (control); (**B**) Embryogenic *callus* in 85.8 µM kanamycin, embryo development is still observed (*arrow*); (**C**) Treatment with 171.6 µM of kanamycin still allows the formation of 1-2 somatic embryos per *callus* (arrow); (**D**) Treatment with 257.5 µM reduces callogenesis and prevents embryo differentiation; (**E**) Treatment with 343.3 µM of kanamycin inhibits regeneration (note the necrotic areas in the leaf explants); (**F**) Seed germination and seedling development in medium without kanamycin (control); (**G**) Seed germination and seedling development in 686.6 µM of kanamycin (germination percentage is not affected; (**H** and **I**) Seedling development with 171.6 µM of kanamycin. At this concentration, 89% of the seedlings presented the sensitive phenotype, with the first pair of leaves bleached (**H**), and 11% still display natural resistance to kanamycin, with the first emerging pairs of leaves green (**I**).

4.2. Effect of kanamycin concentration on seed germination

The percentage of seed germination (emergence of radicle and cotyledons) was not affected by the increasing kanamycin concentrations (Figure 1F and G). In all concentrations tested, germination was close to 80%. Kanamycin effect on seedling development was observed only after 3 weeks treatment. Usually, seedlings exhibited green cotyledons and the effect of kanamycin was only observed in the first emerging pair of leaves that became bleached (Figure 1 H and I). Kanamycin concentrations ranging between 171.6 and 343.3 µM still allowed the development of 11% of the seedlings with non-bleached leaves Figure 1I). At 514.9 µM of kanamycin, none of the seedling produced green leaves. The same effect was observed with 686.6 µM.

Due to the high level of kanamycin resistance displayed by the M9-10a seedlings, the highest concentration (686.6 µM of kanamycin) was chosen to be used in future transformation experiments, to assure the efficient selection of transgenic plants. In fact, our transformation experiments with line M9-10a confirms that the transgenic progeny is easily and rapidly confirmed by screening seedling resistance with 686.6 µM of kanamycin (see Chapter IV).

5. Conclusions

The optimisation of a selection strategy is crucial for improving transformation efficiency. Current *M. truncatula* transformation protocols use 50 $mg.l^{-1}$ of kanamycin but this concentration does not efficiently inhibits the development of non-transformed embryos in our embryogenic genotype. We also observed that the kanamycin concentration used to select embryogenic *calli* was not appropriate to carry out selection of the seedlings. These aspects led us to believe that kanamycin resistance is potentially genotype and tissue-dependent, and an optimisation for each situation is necessary. It is well reported that the use of an appropriate concentration of kanamycin makes the selection process more effective in terms of time, costs and labour. The study here presented provides the data necessary to establish the suitable antibiotic concentration to

be used in crucial steps of the transformation methodology and should be always performed when using a new type of explants or genotype.

6. Acknowledgements

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IV – *Agrobacterium*-mediated transformation of *Medicago truncatula* with arginine decarboxylase gene from *Avena sativa*

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Chabaud M., Ratet P., Araújo S.S., Duque A.S.R.L.A., Harrison M.J., Barker D. (2006) *Agrobacterium tumefaciens*-mediated transformation and *in vitro* plant regeneration. The *Medicago truncatula* Handbook. (ISBN 0-9754303-1-9) [http://www.noble.org/ MedicagoHandbook/]

*Both authors contributed equally to this publication

1. Abstract

A simple and efficient regeneration-transformation method was established to obtain transgenic plants of the model legume *Medicago truncatula* cv. Jemalong. This method takes advantage of a new highly embryogenic line (M9-10a) isolated in our laboratory. Leaflets of *in vitro* grown M9-10a plants were co-cultured with *Agrobacterium* t*umefaciens* EHA105. A plasmid construct containing the oat arginine decarboxilase gene *Adc* and the *Gus* reporter gene (p35S*Adc-Gus*) was used. This construct include the *NptII* gene as selection marker. Embryogenic *calli* (100-97%) were obtained on embryo induction medium containing 171.6 µM (100 $mg.l^{-1}$) kanamycin and 1.2 mM (500 $mg.l^{-1}$) carbenicillin. Using a two-fold increase in kanamycin concentration, instead of 85.8 µM (50 $mg.l^{-1}$) usually used, we reduced the number of emerging false kanamycin-resistant (Kan^R) embryos, which is an important improvement to the method, making it less laborious and very efficient. Isolation of late torpedo/cotyledonary-stage embryos to lower carbenicillin/agar media reduced secondary embryogenesis and prevents hyperhydricity, improving embryo conversion. Primary transformants (T_0) were regenerated within 3-4 months and those that were able to root in an 85.8 µM (50 $mg.l^{-1}$) kanamycin medium were transferred to the greenhouse to produce seeds. Southern blot hybridisation analysis confirmed the integration of the *Adc* transgenes in the genome of the T_0 transformants. Detection of ß-glucuronidase (GUS) activity and RT-PCR analysis in *Adc-Gus* T_0 plants demonstrated the expression of the inserted transgenes. Inheritance of the transgenes was shown to be stable in the T_1 generation.

Key Words: *Agrobacterium*-mediated transformation, Jemalong M9-10a, Somatic Embryogenesis

2. Introduction

Legumes are a large family of plants that, because of their capacity to fix atmospheric nitrogen, are essential components of different terrestrial ecosystems and a source for production of food, feed, forage and other compounds with industrial and

commercial uses (Somers et al., 2003). Understanding their biological questions and the molecular basis of nitrogen fixation is important and led to the development of legume experimental model systems like *Medicago truncatula* (Barker et al., 1990; Cook, 1999).

Medicago truncatula is a diploid (2n=16), autogamous species with a relatively small genome ($1.8x10^9$ bp for the Jemalong cultivar) (Barker et al., 1990) and short life cycle of 3-5 months. These characteristics enable this species to be used in molecular genetic studies like analysis of gene expression, promoter functional analysis, T-DNA mutagenesis and expression of genes for crop improvement. However, these studies require an efficient transformation system to generate a high number of transgenic plants with a low occurrence of phenotypic abnormalities. Legume plants have been regarded as recalcitrant to transformation, and regeneration *in vitro* is highly genotype dependent and only rarely cultivated varieties are amenable to regeneration (for reviews see Somer et al., 2003). The Jemalong cultivar is one of the most used in agricultural practices and pastures for animals, therefore the need to develop an efficient method to transform and regenerate *M. truncatula* plants of this genotype. Protocols of coupled regeneration via somatic embryogenesis and *Agrobacterium*-mediated transformation have been published using Jemalong genotypes of *M. truncatula*: 2HA and J5 (Chabaud et al., 1996; Rose et al., 1999; Kamaté et al., 2000). However, for these Jemalong lines the transformation-regeneration process is time consuming and inefficient (Kamaté et al., 2000; d'Erfurth et al., 2003).

Here we describe a successful regeneration-transformation method with a new highly embryogenic genotype of *Medicago truncatula* cultivar Jemalong. Isolated in our laboratory and designated M9-10a, this genotype has an embryogenic capacity similar to the well reported R-108-1(c3) genotype (Trinh et al., 1998) and allowed us to obtain a high number of transgenic plants. Transformation was carried out with *Agrobacterium* t*umefaciens* harbouring plasmid p35S*Adc-Gus*. The results obtained for this experiment were comparable to those of the transformation with plasmid p35S-*Dsp22* (Araújo et al., 2004), indicating the reproducibility and reliability of this *Agrobacterium*–mediated transformation method.

3. Materials and Methods

3.1. Plant material and culture media

Plants of the M9-10a genotype of *M. truncatula* Gaertn cv Jemalong were used in this study. The M9-10a genotype was selected due to its high embryogenic potential (Neves, 2000; Santos and Fevereiro, 2002). M9-10a plants are maintained in *in vitro* culture conditions and micropropagated in growth-regulator-free medium: MS030A – MS (Murashige and Skoog, 1962) basal salts and vitamins, 3 % (w/v) sucrose, 0.7% (w/v) agar (Microagar, Duchefa, The Netherlands), as described in Neves et al. (2001). Indirect somatic embryogenesis was induced in an embryo induction medium: EIM - MS basal salts and vitamins, 3% (w/v) sucrose, 0.45 μM (0.1 $mg.l^{-1}$) of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.91 μM (0.2 $mg.l^{-1}$) of zeatin (Zea), 0.2% (w/v) gelrite (Duchefa, The Netherlands). After 21 days in EIM, embryogenic *calli* were transferred to an embryo proliferation medium: EPM – MS basal salts and vitamins, 3% (w/v) sucrose, 0.2% (w/v) gelrite. Embryos ready to be isolated were transferred to an embryo conversion medium (ECM = MS030A). The pH of all media was adjusted to 5.8 before autoclaving (121ºC, 20 min.). Growth regulators and selective agents were filter sterilised through 0.2μm filters (Whatman, England) and added before dispensing the media. Cultures were maintained in a growth chamber (Phytotron EDPA 700, Aralab, Portugal) with 16-h photoperiod of 100 μmol $m^{-2}s^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24º/22ºC.

3.2. Plasmid constructs

The construction of plasmid p35S*Adc-Gus* is described in detail in Chapter II. However, for information purposes a brief explanation and a schematic representation of the construct (Figure 1) is given. The base binary plant transformation vector for this construct is pGreen (Hellens et al., 2000; versions 0000) (www.pgreen.ac.uk). Plasmid p35S*Adc-Gus* contains in its T-DNA the p35S-*Gus* cassette and the oat arginine decarboxylase (ADC) cDNA (2.1 Kbp, GenBank Accession nº X56802) fused to the

CaMV 35S promoter with duplicated enhancer sequences and the CaMV transcriptional termination region. This plasmid also contain in its T-DNA the 1406 bp Nos-*Kan* cassette that encodes for the neomycin phosphotransferase II gene (*Npt*II) that confers kanamycin resistance. The integrity of the chimeric genes *Adc*, *Gus* and *Npt*II were checked by reverse transcription-PCR assay coupled to *A. tumefaciens*-mediated transient expression (Santos et al., 2003).

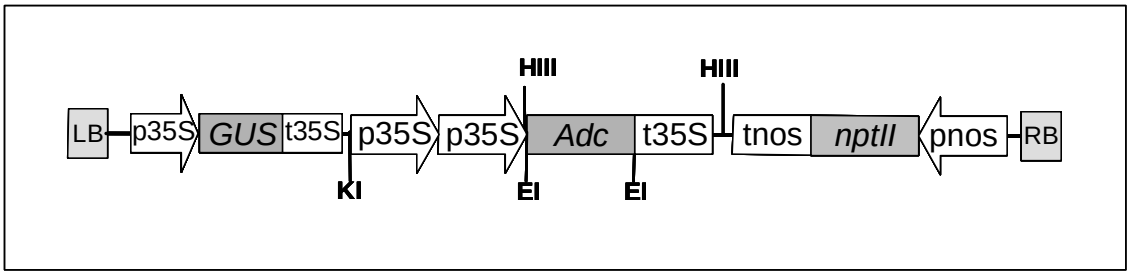

Figure 1. Schematic representation of T-DNA region of plasmid constructs p35S*Adc*-*Gus* used for *M. truncatula* transformation. Arrows indicate the direction of transcription. EI, *Eco*RI; HIII, *Hind*III; KI, *Kpn*I. For Southern blot hybridisation analysis, total DNA was digested with *Hind*III that cleaves inside the T-DNA region but outside of the oat *Adc* gene, generating a fragment of approximately 2.9 Kbp, that comprises the promoter 35S CaMV-*Adc* transgene.

3.3. Transformation of *A. tumefaciens* (strain EHA105)

3.3.1. Preparation of EHA105 competent cells

Agrobacterium tumefaciens disarmed succinamopine strain EHA105 (Hood et al., 1993) was used in all transformation experiments.

For preparation of EHA105 competent cells the $CaCl_2$ method (Sambrook et al., 1989) with some modifications was used. Briefly, an isolated colony from a plate containing solid *Luria Broth* (*LA*: 10g.l^{-1} triptone, 5 g.l^{-1} NaCl, 5g.l^{-1} yeast extract) medium was inoculated in 5 ml of *LB* supplemented with 61 µM (50 mg.l^{-1}) rifampicin and grown for 48h at 28ºC under vigorous shaking (200 rpm). Three ml of this culture was inoculated in 50 ml of fresh *LB* antibiotic supplemented medium and left under shaking until $O.D_{600nm}$ reached 0.8 to 1.0 (approximately 4h). This cell culture was centrifuge at 3000 rpm for 15

min at 4ºC. The pellet was resuspended in 1 ml of ice-cooled 10 mM $CaCl_2$ with addition of 250µl of sterile 80% glycerol. Aliquots of 100µl of this suspension were distributed in Eppendorf tubes (100µl per tube), freeze in liquid nitrogen and stored at –80ºC.

3.3.2. Transformation of EHA105 competent cells

Plasmid p35S*Adc-Gus* was mobilised to $CaCl_2$-competent cells of *Agrobacterium* by the freeze-thaw method (Walkerpeach and Velten, 1994) according to the protocol used for transformation of competent *E. coli* cells , previously described in Chapter II. *A. tumefaciens* competent cells were first transformed with helper plasmid pSoup (Hellens et al., 2000) and selection carried out on *LA* medium containing 50 $mg.l^{-1}$ rifampicin and 5 $mg.l^{-1}$ tetracycline.

After selection for transformed bacteria (by digestion of plasmid DNA with the appropriated restriction enzymes) competent *Agrobacterium* cells harbouring pSoup were prepared by the same freeze-thaw method. This bacterial suspension was again transformed with p35S*Adc-Gus* and selection for both co-resident plasmids was carried out in *LA* medium containing 50 $mg.l^{-1}$ rifampicin and 50 $mg.l^{-1}$ kanamycin.

3.3.3. Screening for transformed EHA105 colonies

For screening of transformed EHA105 colonies, aliquots (5-10µl) of 48h grown bacterial cultures (in 5 ml *LB* medium with the appropriate antibiotic supplementation) were directly used for polymerase chain reaction (PCR) amplification. Confirmation of the presence of plasmid p35S*Adc-Gus* in *A. tumefaciens* was performed by PCR amplification of a 1.5Kb fragment for the oat *Adc* gene and PCR amplification of a 517bp fragment corresponding to an internal portion of *Gus* reporter gene. PCR amplification conditions for the oat *Adc* and *Gus* transgenes are as described in Chapter II. The products of the *Adc* and *Gus* amplification reactions were visualised by 1% and 2%, respectively, agarose gel electrophoresis stained with 0.5 $\mu g.ml^{-1}$ ethidium bromide (EtBr).

3.3.4. Preparation of *A. tumefaciens* for plant transformation

An *Agrobacterium* colony harbouring both plasmid (pSoup and p235S*Adc-Gus*) was inoculated in 5 ml of *LB* medium supplemented with 61 μM (50 mg.l^{-1}) rifampicin and 85.8 μM (50 mg.l^{-1}) kanamycin. After overnight incubation at 28ºC, 200 rpm, the bacterial culture was diluted 1:100 in *LB* medium and incubated overnight at 28ºC, 200 rpm. This cell culture was centrifuged for 10 minutes at 3500 rpm and the pellet was resuspended at A_{600nm}= 1.5-1.6 in liquid EIM supplemented with 100 μM (196,2 mg.l^{-1}) acetoseryngone (Sigma-Aldrich Inc., USA) for an incubation period of 30 min to activate the *Agrobacterium* virulence mechanisms.

3.4. Coupled transformation/regeneration procedure

Neves et al. (1999) established a two step protocol for the regeneration of selected *M. truncatula* genotypes. This procedure was adapted for the transformation/regeneration of the embryogenic line M9-10a. Leaflets of *in vitro* grown M9-10a plants were used as explants for transformation. Leaflets were put onto a wet sterile filter paper in a Petri-dish to prevent excessive desiccation and wounded perpendicularly to the central vein using a scalpel blade previously dipped into the *Agrobacterium* suspension.

After a co-culture period of 5 days on solid EIM with 100 μM of acetoseryngone, in a growth chamber, at 23ºC in the dark (Heraeus, Germany), the infected explants were transferred to EIM containing 171.6 μM (100 mg.l^{-1}) of kanamycin as selective agent for transformed tissue and 1.2 mM (500 mg.l^{-1}) of carbenicillin to eliminate *Agrobacterium*. Embryogenic c*alli* cultures were maintained in a growth chamber (Phytotron EDPA 700, Aralab, Portugal) with 16-h photoperiod of 100 μmol.m^{-2}.s^{-1} applied as cool white fluorescent light and a day/night temperature of 24º/22ºC.

To maintain the selective pressure, explants were removed to fresh selective medium every week. As control, to monitor the effectiveness of the selection applied and regeneration process, non-infected leaflets were also subjected to the regeneration process in the presence of 171.6 μM (100 mg.l^{-1}) kanamycin and 1.2 mM (500 mg.l^{-1}) of carbenicillin and in the absence of these antibiotics.

Twenty-one days after infection, embryogenic *calli* were transferred to EPM supplemented with 171.6 µM (100 $mg.l^{-1}$) kanamycin and 1.2 mM (500 $mg.l^{-1}$) carbenicillin. *Calli* were maintained in this medium until somatic embryos could be isolated. Somatic embryos were isolated in a late torpedo/dicotyledonary stage onto ECM supplemented with 171.6 µM (100 $mg.l^{-1}$) kanamycin and 0.6 mM (250 $mg.l^{-1}$) carbenicillin. About 40-50 embryos were placed per Petri-dish, in a vertical position. Embryos were maintained in the light/temperature/humidity conditions described above.

Every 2 weeks, green somatic embryos resistant to kanamycin were transferred to fresh selective ECM until conversion to plantlets. When plantlets developed roots, carbenicillin was eliminated from the medium and kanamycin concentration reduced to 85.8 µM (50 $mg.l^{-1}$). Only well-rooted plantlets that demonstrated to be kanamycin-resistant (Kan^R) after 2 successive cycles of subculture in selective medium were selected as putative transgenic plants (T_0 generation) and transferred to soil in the greenhouse to produce seeds.

3.5. Histochemical GUS assay of *Adc-Gus* transgenic plants

The histochemical assay to detect β-glucuronidase (GUS) activity was performed essentially as described by Jefferson et al. (1987) using as substrate 5-Bromo-4-chloro-3-indolyl-β-D-Glucuronide acid (X-GlcA , Duchefa, The Netherlands). X-GlcA was dissolved with dimethylformamide (approximately 10µl per mg of X-GlcA) and directly prepared in 50 mM sodium phosphate buffer pH 7.0. The assay buffer was prepared with 1mM X-GlcA in 50 mM sodium phosphate buffer pH 7.0; 10 mM EDTA and 0.1% (v/v) Triton X-100. Leaflets, stems and roots were covered with the assay buffer and vacuum infiltrated at 0.8 bar for at least 1h. The reaction was incubated at 37°C in a wet chamber for 24h and excess chlorophyll was removed from stained explants with 70% (v/v) ethanol.

3.6. Molecular analysis

3.6.1. Plant genomic DNA extraction and PCR amplification

Total plant DNA was isolated from control non-transformed *M. truncatula* M9-10a and putative T_0 transgenic lines using a small scale DNA preparation protocol adapted for *Arabidopsis thaliana* (Edwards et al., 1991) with two additional phenol:chloroform:isoamyl alcohol (25:24:1) extractions steps, followed by one chloroform:isoamyl alcohol (24:1) extraction step. DNA was precipitated with two volumes of absolute ethanol and resuspended in TE (10mM Tris-HCl, pH 7.5, 1 mM EDTA). Treatment with 100 $\mu g.ml^{-1}$ of RNase A (Sigma-Aldrich Inc., USA) was optional. DNA concentration was estimated in 0.8% agarose gel stained with EtBr (0.5 $\mu g.ml^{-1}$) using phage lambda DNA as standards.

The integration of the *Adc* gene in the genome of the T_0 plants was analysed by polymerase chain reaction (PCR) amplification on total DNA samples. PCR amplification conditions for the oat *Adc* transgene are as described in Chapter II. Briefly, PCR was carried out on a thermocycler UNO II (Biometra, Germany) with 200ng of template DNA, 50pmol of transgene specific reverse (Oligo5-$^{5'}$GCGGGTGCAGCGGCATCGTCTCGG$^{3'}$) and forward primers (Oligo1-$^{5'}$CGGCGATGTGTACCATGTCGAGGG$^{3'}$); 1.5 mM of $MgCl_2$, 1X PCR buffer and 0.5 Units of *Taq* polymerase (Invitrogen, USA) in a final volume of 50 µl adjusted with sterile MiliQ water. Oat *Adc* transgene specific primers amplify a fragment of 1500 bp.

3.6.2. Southern blot hybridisation

For total DNA gel blot analysis, 10µg of total DNA from *Adc* transformants were digested with 8 Units of restriction enzyme per µg of total plant DNA. *Adc* transformants were analysed by restriction with *Hind*III that cleaves inside the T-DNA region but outside the oat *Adc* gene, generating a fragment of approximately 2.9 Kbp corresponding to the oat *Adc* gene and the CaMV transcriptional termination region (for details see Figure 1). After overnight restriction, the DNA was concentrated by precipitation with 0.5 volume of 7.5M

ammonium acetate and 2 volumes of 100% ethanol. DNA digests were placed for few hours at –80ºC, centrifuged, resuspended in 30-35µl TE, separated by electrophoresis in 1% agarose gels and blotted to Hybond-N+ membranes (Amersham, UK) according to manufacturer's instructions. Southern blot hybridisation conditions were essentially as described in Santos and Fevereiro (2002). *Adc* transformants were hybridised to a DNA probe corresponding to the oat *Adc* cDNA excised from plasmid p35S*Adc-Gus* with *Eco*RI and labelled with α-dCTP^{32}.

3.6.3. Total plant RNA extraction and RT-PCR amplification

Total RNA was extracted from *in vitro* grown leaflets of *M. truncatula* T_0 transgenic lines, previously cheeked by Southern blot, using the RNAeasy Plant Mini Kit from Qiagen® according to manufacturer's instructions. The RNA samples were treated with 20 Units of DNaseI RNase free (Boehringer, Germany) for 1h at 25ºC, followed by heat inactivation of the DNaseI at 65ºC for 5 min.

One µg of each RNA sample was subjected to reverse transcription–polymerase chain reaction (RT-PCR), using the Ready-To-Go RT-PCR Beads from Amersham®, according to manufacturer's instructions (RT-PCR converts RNA into first strand cDNA, which is then used as a template for PCR). First strand synthesis (RNA-cDNA hybrids) was carried out at 45ºC for 50 min on a thermocycler UNO II (Biometra, Germany), followed by 15 min at 70ºC to denature the reverse transcription (RT) enzyme. The PCR amplification with specific primers for the oat *Adc* gene was performed as previously described (Chapter II). Briefly, PCR was carried out with 50pmol of transgene specific reverse (Oligo5-$^{5'}$GCGGGTGCAGCGGCATCGTCTCGG$^{3'}$) and forward primers (Oligo1-$^{5'}$CGGCGATGTGTACCATGTCGAGGG$^{3'}$); 1.5 mM of $MgCl_2$, 1X PCR buffer and 0.5 Units of *Taq* polymerase (Invitrogen, USA) in a final volume of 50 µl adjusted with sterile MiliQ water. Oat *Adc* transgene specific primers amplify a fragment of 1.5 Kbp.

3.7. Progeny production and segregation analysis

The T_0 plants with a well-developed root system were potted in a mixture 2:1 soil-vermiculite, covered with a polyethylene film (Silvex® wrap, Portugal) and placed in the greenhouse for acclimatisation. After 1 week, the T_0 plants were transferred to pots containing soil (Montemor soil, A Estufa, Portugal) to grow to maturity and produce seeds (T_1 generation). T_1 seeds were obtained by self-pollination of primary transformed *M. truncatula* (T_0) plants.

For segregation analysis, the T_1 seeds were scarified by immersion in concentrated anhydrous sulphuric acid (H_2SO_4) following the protocol previously described in Chapter III. Before plating, seeds were immersed in 70% (v/v) ethanol for 2 min, rinsed with sterile distilled water and sown on 0.8% (w/v) water-agar plates supplemented with 686.6 µM (400 $mg.l^{-1}$) of kanamycin. After 4 days at 4ºC in dark conditions, seeds were transferred to the 23ºC dark growth chamber were they germinated within 2-3 days. Germinated seeds were transferred to light conditions in a growth chamber. After scoring Kan^R and Kanamycin sensitive (Kan^S) T_1 seedlings, emerging Kan^R T_1 seedlings were subcultured to glass vials containing MS030A medium and transferred to light conditions in a growth chamber to develop into plants (T_2 generation).

Observed segregation ratios for Kan^R:Kan^S were compared to Mendelian segregation models using chi-square (χ^2) analysis. At least 19 random T_1 seedlings were analysed from independent T_0 plant lines.

4. Results

4.1. Optimisation of plant transformation conditions

The data presented here concern one set of transformation experiments carried out with *A. tumefaciens* EHA105 carrying the plasmid construct p35S*Adc-Gus*. However, the results obtained for *A. tumefaciens* transformation with construct p35S*Dsp22* (Araújo et al., 2004) were comparable.

Prior to plant transformation, the presence of plasmid p35S*Adc-Gus* in *A. tumefaciens* EHA105 was confirmed by the amplification of a 1.5Kbp fragment for the oat *Adc* gene and by the amplification of a 517bp fragment corresponding to an internal portion of *Gus* reporter gene (Figure 2).

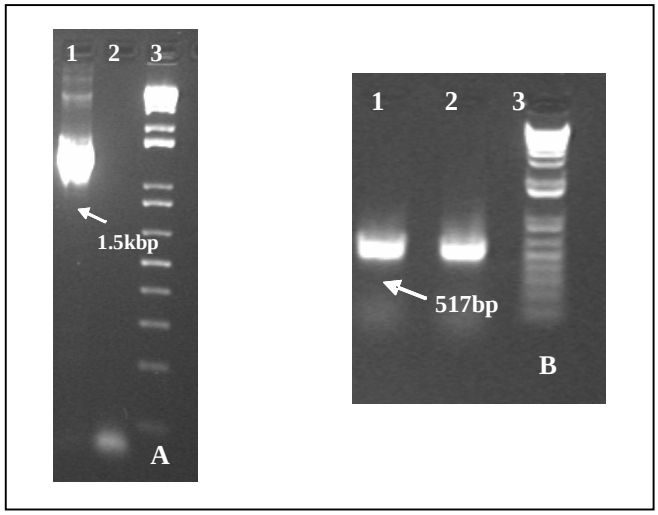

Figure 2. Confirmation of the presence of plasmid p35S*Adc-Gus* in *A. tumefaciens* (EHA105) by PCR amplification of the *Adc* and *Gus* transgenes.
(A) PCR amplification of the *Adc* transgenes. **Lane 1 -** PCR amplification of 1.5Kbp oat *Adc* fragment; **Lane 2 -** PCR amplification of an untransformed EHA105 colony; **Lane 3 -** *100bp DNA ladder*. **(B)** PCR amplification of the *Gus* transgenes. **Lanes 1 and 2 -** PCR amplification of 517 bp fragment corresponding to an internal portion of the *Gus* gene (2 different colonies); **Lane 3 -** *1Kb plus DNA ladder*.

Results from plant transformation experiments are presented on Figure 3. Young, well-developed leaflets from M9-10a *in vitro* cultured plants are used as explants for *Agrobacterium* transformation (Figure 3A). Approximately 97% of embryogenic *calli* are obtained on EIM containing 171.6 µM (100 $mg.l^{-1}$) kanamycin and 1.2 mM (500 $mg.l^{-1}$) carbenicillin, from which 25% produce (Kan^R) embryos. In our early transformation experiments (using plasmid construct p35S*Adc*), co-culture was performed in light-conditions as described in Neves (2000). In these conditions, bacterial overgrowth was often observed that led to leaf tissue death. When we shifted to co-culture in the dark, it was possible to overcome this bacterial overgrowth, without loss of infection efficiency.

The presence of 171.6 µM (100 $mg.l^{-1}$) kanamycin and 1.2 mM (500 $mg.l^{-1}$) of carbenicillin in control experiments did not affect the callogenic capacity of the explants

(Figure 3B), but no embryo formation was observed, as expected for non-transformed tissue. Without these antibiotics, M9-10a explants underwent the described process of regeneration and a vast proliferation of somatic embryos was observed (Figure 3C). The development of somatic embryos in these conditions was faster than in the *Agrobacterium* transformed explants. Several late-torpedo shaped embryos could be isolated after 3 weeks on EPM. In the *Agrobacterium* infected explants, Kan^R embryos start to appear one month after initiating the selective pressure. The development of somatic embryos was asynchronous and several stages of embryo development could be observed simultaneously (Figure 3D). In this way, and during 1 month, somatic embryos in a late-torpedo/dicotyledonary development stage were isolated every week. To maintain the selective pressure, Kan^R somatic embryos were subcultured every 2 weeks to fresh medium with antibiotics where they start to convert (Figure 3F). In some cases, it was observed that apparent Kan^R embryos bleached or became brown when they were put in direct contact with the selective medium (Figure 3E).

In previous transformation experiments, somatic embryos were transferred to a gelrite-containing medium. In this situation, a high percentage of hyperhydricity of the somatic embryos was observed. Twenty six to forty six percent of isolated embryos became hyperhydric and failed to convert. Hyperhydricity of converting embryos or emerging shoots (Figure 3G) could be reduced or even eliminated when these were isolated and transferred to an agar-solidified medium. Moreover, plantlets already hyperhydric in gelrite-containing medium could be rescued if transferred to an agar-solidified medium. Frequently, somatic embryos transferred to ECM undergo through several cycles of repetitive embryogenesis and usually fail to convert. This could be due to the carbenicillin concentration 1.2 mM (500 $mg.l^{-1}$) used in ECM reported to have a phytohormone-like behaviour that could affect somatic embryogenesis (Lin et al., 1995). A reduction to 0.6 mM (250 $mg.l^{-1}$) instead of the 1.2 mM (500 $mg.l^{-1}$) originally used, effectively minimised the occurrence of secondary somatic embryogenesis without compromising the effectiveness of *Agrobacterium* elimination. Together, the use of agar and reduction to half of the concentration of carbenicillin led to a successful increase in embryo conversion rates from 20% to 85% (data from comparison of previous transformation experiments with the present results).

The first plantlets (Figure 3H) appeared within 2-3 months after the co-cultivation step and 63% rooted well in a kanamycin selective medium (Figure 3I). Only these were selected as putative transgenic plants. These plants (T_0 generation) were successfully transferred to greenhouse where they flowered and produced seeds (Figure 3J). Transfer to greenhouse is not a limiting step of the process when we choose *in vitro* cultured plants with a well-developed radicular system.

In total, 22 transgenic lines carrying the p35S*Adc-Gus* construct were recovered. These T_0 plants show no altered external morphology.

4.2. Expression of the *Gus* gene in *Adc-Gus* transgenic plants

Leaf, stem and root tissues of 15 T_0 transgenic plants carrying the p35S*Adc-Gus* construct were tested histochemically for GUS activity. About 60% of the transgenic plants analysed showed blue staining, indicating the presence of GUS activity (Figure 3K). No GUS activity was detected in non-transformed control plants (Figure 3K, first well).

4.3. Molecular analyses of *Adc-Gus* transgenic plants

A total of 15 *Adc-Gus* putative T_0 transgenic lines were analysed by PCR and Southern blot analysis.

PCR analysis was performed in *Adc-Gus* transgenic lines both positive and negative for the *Gus* gene expression. In all cases, the specific 1.5 Kbp band corresponding to the oat *Adc* transgene was amplified (Figure 4A, lanes 2-10). The indication by PCR of the integration of the oat *Adc* transgene in all Adc-Gus transgenic plants tested was confirmed by Southern blot hybridisation analysis (Figure 4B). Neither PCR amplification nor hybridising signals were detected in non-transformed control M9-10a plants (Figure 4A, lane 11; Figure 4B, lane 13).

The observation that 40% of the *Adc-Gus* putative transgenic plants are negative for *Gus* gene expression but contain the *Adc* gene, suggest that some transgenic plants may contain a silenced *Gus* gene or contain truncated versions of the T-DNA lacking the *Gus* gene.

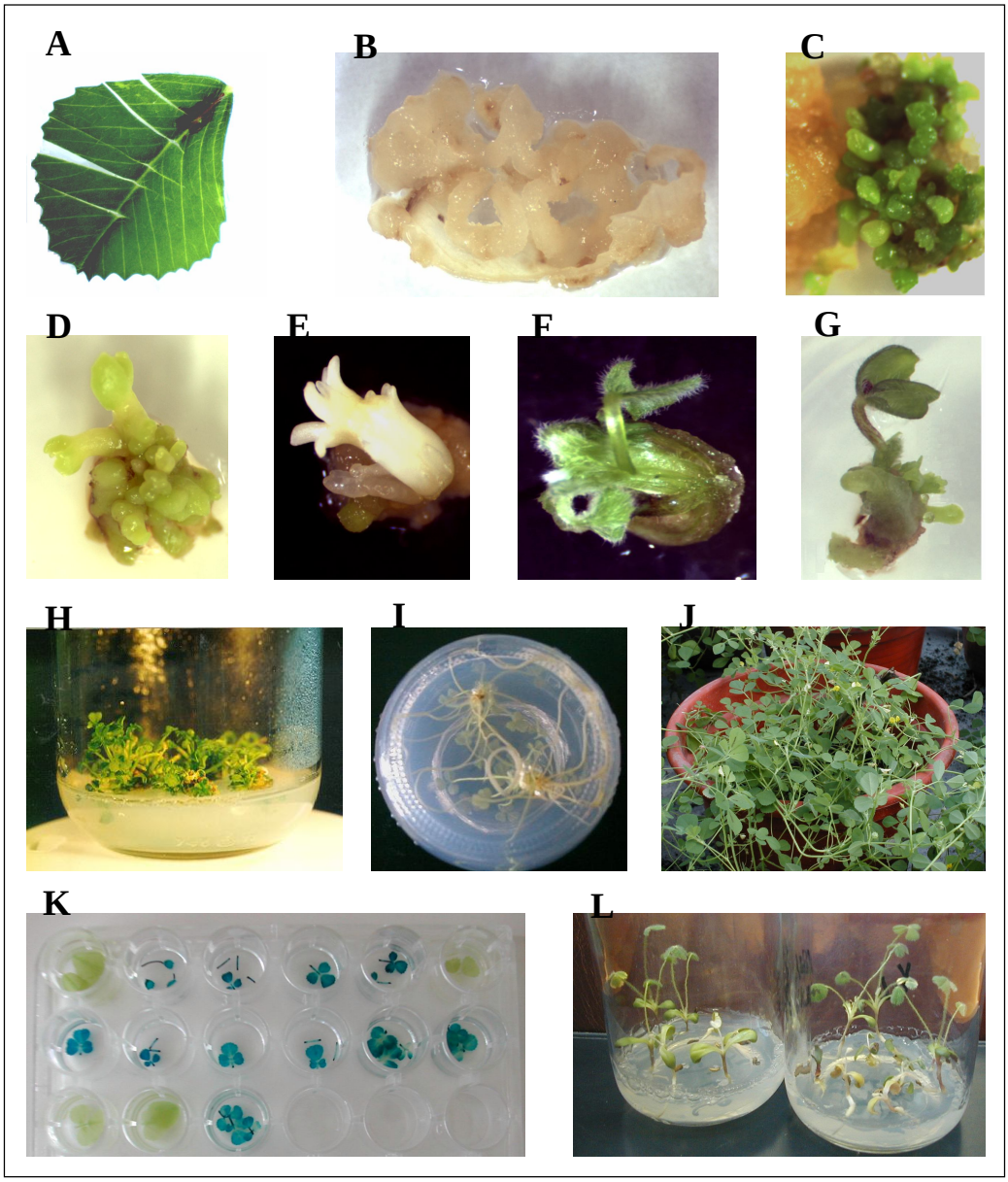

Figure 3. Transformation-regeneration in *M. truncatula* cv Jemalong M9-10a genotype.
(A) Wounded leaflet of the Jemalong M9-10a genotype; **(B)** Embryogenic *callus* originated from non-infected leaflet in selective conditions. No somatic embryos developed; **(C)** Embryogenic *callus* derived from a non-infected leaflet in EPM; **(D)** Kan^R clump of somatic embryos at different stages of development; **(E)** Kan^S embryo (bleached); **(F)** Kan^R embryo conversion; **(G)** Hyperhydric shoot in gelrite-containing medium. Note the occurrence of secondary embryogenesis; **(H)** Kan^R plantlets; **(I)** Transgenic T_0 line rooted in 85.8 µM (50 $mg.l^{-1}$)of kanamycin; **(J)** T_0 transgenic lines flowering in the greenhouse; **(K)** Histochemical GUS assay: positive (blue staining) and negative (bleached) results in leaves, roots and stems of putative *Adc-Gus* transgenic plants. Untransformed M9-10a used as negative control (bleached leaves, first well); **(L)** T_1 seedlings segregating in 686.6 µM (400 $mg.l^{-1}$) kanamycin-containing medium. Kan^R seedlings present green leaves; Kan^S seedlings present bleached leaves.

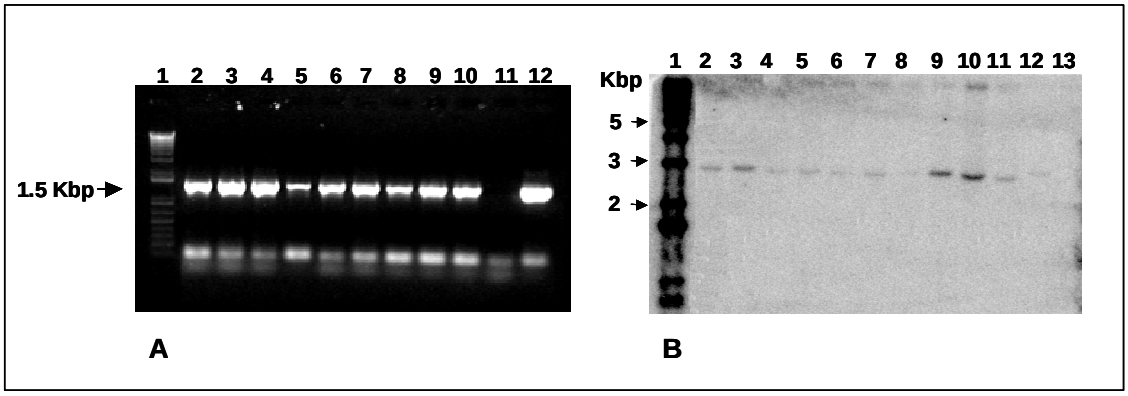

Figure 4. Molecular analyses of putative transgenic *M. truncatula* plants. **(A)** PCR with *Adc* gene-specific primers amplified a 1.5 kbp fragment; lane 1 - $1Kb^+$ DNA ladder; lanes 2 to 10 - putative *Adc-Gus* transgenic plants; lane 11 - untransformed M9-10a plant; lane 12 - positive control (plasmid containing oat *Adc* gene). **(B)** Southern blot hybridisation of *Adc-Gus* transgenic plants; lane 1 - $1Kb^+$ DNA ladder; lanes 2 to 12 - transgenic *Adc-Gus* plants; lane 13 - untransformed (M9-10a) control plant.

4.4. Expression of the *Adc* gene in oat *Adc-Gus* transgenic lines

The transgene expression was analysed by RT-PCR in several independent T_0 lines that were previously tested for the oat *Adc* gene integration (by Southern blot) and for the *Gus* gene expression (by histochemical GUS assay). Results from RT-PCR amplification for two independent T_0 lines are presented in Figure 5. No amplification was found in non-transformed control M9-10a plants (Figure 5, lane 2). Additional controls to detect the presence of plant DNA contamination in the RNA preparations revealed no amplification product (Figure 5, lane 4). All lines tested presented the 1.5Kbp oat *Adc* RT-PCR amplification fragment demonstrating the expression of the oat *Adc* transgene at the transcript level.

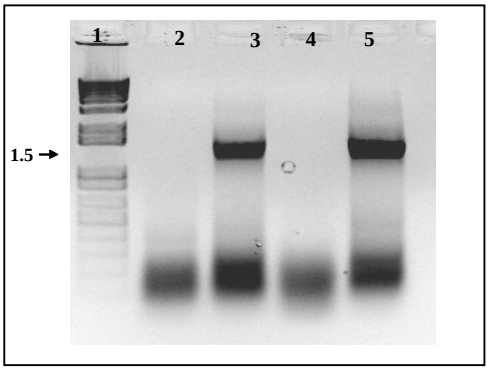

Figure 5. RT-PCR analysis for transgene expression at transcript level using specific primers for the *adc* gene in *M. truncatula* plants. Agarose gel (1%) stained with 0.5 $\mu g.ml^{-1}$ EtBr. **Lane 1 -** *1Kb plus DNA ladder*; **lane 2 -** untransformed (M9-10a) control plant; **lane 4 -** RT-PCR amplification of total mRNA after Reverse Transcriptase (RT) degradation for testing the presence of plant DNA contamination; **lanes 3 and 5 -** RT-PCR amplification of 1.5Kbp oat *adc* fragment (two independent T_0 lines; L104 and L108).

4.5. Transgene segregation analysis

After self-pollination of the transgenic *M. truncatula* T_0 lines, the stable inheritance of the transgenes was tested in six independent T_1 lines of *Adc-Gus* transgenic plants. These lines were earlier tested for oat *Adc* gene integration (by Southern blot) and 2 of them (L104 and L108) were additionally assessed for gene expression (by RT-PCR).

In previous experiments we established a suitable kanamycin concentration to visualise seed segregation (Duque et al., 2004; Chapter III). We found that 686.6 µM (400 $mg.l^{-1}$) allowed us to distinguish kanamycin resistant seedlings (first emerging leaves green) from kanamycin sensitive ones (first emerging leaves bleached) resulting from the progeny of self-fertilised transgenic plants. Based on these results, T_1 seeds were germinated in 686.6 µM (400 $mg.l^{-1}$) of kanamycin and seedlings were screened for their resistance to the antibiotic (*Npt*II gene expression). The results are presented in Figure 3L. These lines showed a Mendelian segregation pattern of 3:1, corresponding to a dominant gene at a single *locus* (Table 1).

Table 1. Chi-square (χ^2) analyses of segregation ratios for kanamycin resistanT/sensitive seedlings among six T_1 lines obtained from self-pollination of transgenic *M. truncatula* primary T_0 independent lines.

Plant lines	Total seedlings	Observed Kan^R	Observed Kan^S	Expected Kan^R	Expected Kan^S	Test ratio (Kan^R: Kan^S)	χ2 (df=1)
L12	45	34	11	33.75	11.25	3:1	**0.007**
L55	32	27	5	24	8	3:1	**1.5**
L56	20	15	5	15	5	3:1	**0**
L64	24	16	8	18	6	3:1	**0.889**
L104	41	35	6	30.75	10.25	3:1	**2.350**
L108	19	15	4	14.25	4.75	3:1	**0.158**

Km^R = kanamycin-resistant seedlings; Km^S = kanamycin-sensitive seedlings; Test ratio = Mendelian expected ratio of hemi-to homozygous lines; χ^2 = Results from Yates chi-square (χ^2) test using the formula [χ^2= Σ (Observed value-Expected Value) ^{2}/Expected value]; with 1 degree of freedom (df=1); by statistical convention we use the 0.05 probability level as our critical value.
Note: If calculated χ^2 value is less than the value obtained from a chi-square (χ^2) table with 0.05 probability level and 1 df (in the present case 3.84), we accept the null hypothesis (the null hypothesis of the test is that the progeny segregates in the 3:1 ratio).

These data together with the results from the Southern blot analyses prove that the T-DNA region is stably inserted in the genome of the *M. truncatula* primary transformants and was transferred to the T1 progeny.

5. Discussion

We present here an efficient regeneration-transformation method using a highly embryogenic genotype of *M. truncatula* cv. Jemalong designated M9-10a. This new line of the cultivar Jemalong was obtained after regeneration via somatic embryogenesis from the very low embryogenic line M9 (Neves, 1999; Santos and Fevereiro, 2002). This line has been propagated by cuttings and seeds for the last 4 years keeping the same embryogenic capacity. The *Agrobacterium*-mediated transformation protocol previously developed for the M9-10a line (Neves, 2000) was modified in order to set-up a more efficient transformation scheme that generates a high number of transgenic plants. Aspects like the

effectiveness of bacteria infection, applied selection and recovery of transgenic plantlets were improved and are here discussed.

The effectiveness of bacteria infection ensures the success of plant transformation. We selected the commonly used hypervirulent *A. tumefaciens* strain EHA105 (Hood et al., 1993) that already proved to transform another genotype of *M. truncatula* (R-108-1(c3), Trinh et al., 1998). However, we observed that bacterial overgrowth during the co-culture in light conditions was particularly detrimental to *callus* formation and led to the loss of leaf explants. Chabaud et al. (1996) verified the same effect with strain LBA4404 in their transformation procedure and they included a washing step after co-cultivation to remove the excess of *Agrobacterium*. We showed that it was possible to overcome this bacterial overgrowth, without loss of infection efficiency by performing co-culture in the dark, thus avoiding excessive manipulation of the explants.

An effective selection system should establish a good compromise between the regeneration capacity of the explants and the effectiveness of the selection applied. With our M9-10a genotype, the use of 68.7-85.8 µM (40-50 $mg.l^{-1}$) kanamycin usually reported in the literature (Thomas et al., 1992; Chabaud et al., 1996; Wang et al., 1996; Trinh et al., 1998; Kamaté et al., 2000) leads to the isolation of a high number of false Kan^R embryos. Other authors working with *Medicago* also verified that a large percentage of regenerated plants escaped the selection with 68.7 µM (40 $mg.l^{-1}$) of kanamycin (d'Erfurth et al., 2003). These results indicate that these kanamycin concentrations are ineffective for selection of transgenic tissues containing the *Npt*II gene. This might be because legumes have a natural resistance to aminoglycoside antibiotics like kanamycin (Christou, 1994). In fact, a two-fold increase of the kanamycin concentration drastically reduced the number of false Kan^R embryos (Duque et al., 2004; Chapter III). With this tight selection we were able to reduce the number of emerging false Kan^R embryos, thus avoiding future exhaustive work analysing non-transgenic plants that escaped selection.

Recovery of transgenic plantlets is affected by the rate of embryo-to-plant conversion that is an inefficient step of the *M. truncatula* transformation-regeneration process (Chabaud et al., 1996; Wang et al., 1996). One of the problems is secondary formation of somatic embryos. These can be useful when used as a source of morphogenetic material, however in the transformation procedure and during plant

regeneration, somatic embryos that undergo repetitive embryogenesis usually fail to convert by arresting shoot and root development (Neves et al., 1999). In our hands, isolation of late torpedo/dicotyledonary stage embryos to a lower concentration of carbenicillin reduced repetitive embryogenesis without compromising their conversion to plants. In our previous transformation protocol, another limitation to the embryo-to-plant conversion was the hyperhydricity of somatic embryos. Although embryogenic *calli* must be subcultured to a gelrite-containing medium to develop somatic embryos (Neves et al., 1999), some become hyperhydric and fail to convert when transferred to gelrite. As suggested by others authors (Trinh et al., 1998) we transferred somatic embryos to an agar-containing medium and we decreased hyperhydricity and also promoted embryo conversion to plantlets.

With these improvements to the method, primary *Adc-Gus* transformants (T_0 generation) were regenerated within 3-4 months of *in vitro* culture. A total of 22 *Adc-Gus* T_0 transgenic lines rooted in 85.8 μM (50 mgl^{-1}) kanamycin containing-medium and were transferred to the greenhouse to produce seeds. The 15 *Adc-Gus* T_0 transgenic lines that were analysed by Southern blot hybridisation showed integration of the *Adc* transgene.

We also observed in this experiment that 40% of the *Adc-Gus* putative transgenic plants were negative for *Gus* gene expression but contained the *Adc* gene. This might suggest that some transgenic plants could contain a silenced *Gus* gene or contain truncated versions of the T-DNA lacking the *Gus* gene. Taking in consideration that three different genes were introduced on the T-DNA region of the plasmid vector (total T-DNA size of 7.5 Kbp) it is possible that truncated versions could appear. Moreover, since the DNA transfer initiates at the right border (RB) and proceeds in a polar direction to the left border (LB) (Zupan and Zambryski, 1995) it is possible that the genes nearer the LB (in our case the *Gus* gene) would be more likely not transferred to the plant genome. Therefore, the possible advantage of an easy histochemical detection of the transgenic plants by the introduction of the *Gus* gene in p35S*Adc-Gus* was prevented by the appearance of truncated versions. Taking these results in consideration, smaller T-DNA fragments should be assembled in future constructs and the selection genes (e.g. *NptII* gene) should be placed nearer the LB, to ensure that the selected plants contain the entire T-DNA (and consequently, all the genes present therein).

Two independent T_0 transgenic lines were further analysed by RT-PCR and demonstrated to express the oat *Adc* transgene. The progeny of 6 independent T_0 transgenic lines showed a Mendelian segregation pattern of 3:1, corresponding to a dominant gene at a single *locus*. An average of 1-2 T_0 transgenic plants could be obtained per Kan^R embryo producing *callus*, independently of the plasmid construct used for plant transformation (Araújo et al., 2004).

6. Conclusions

In about 4 months of *in vitro* culture, we could recover a high number of transgenic plants that are fertile and resulted from independent transformation events. The use of a highly embryogenic genotype (M9-10a) together with a strict selection of transgenic tissues with 171.6 μM (100 $mg.l^{-1}$) of kanamycin and the stage that somatic embryos are isolated to an agar-solidified medium with low concentration of carbenicillin are major improvements to the method, making it less laborious and very efficient.

7. Acknowledgments

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V – Biogenic amines determination by ion-pair RP-HPLC in *Medicago truncatula* plants overexpressing the oat Arginine decarboxylase

A version of this chapter was submitted to publication as:

Duque A.S., Leitão M.C., Araújo S.S., Santos D.M., Fevereiro P. (2010) One Step determination of Polyamines, Agmatine and Histamine by RP-HPLC in *Medicago truncatula* plants overexpressing the oat Arginine Decarboxylase.

1. Abstract

We characterized primary transformants (T_0) of the transgenic line (L108) of *M. truncatula* cv Jemalong, that expressed constitutively an oat arginine decarboxylase cDNA (*Adc*), using an optimized ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) methodology. This procedure was shown to be suitable for free, soluble bound and insoluble bound amines in leaves of both wild-type untransformed and transgenic (L108) lines. Polyamines (PAs) were analysed by RP-HPLC with post-column derivatization with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) reagent. The PAs putrescine (Put) and spermidine (Spd) were detected in both, wild-type and transgenic L108 lines. Importantly, other biogenic amines such as the monoamine histamine (His) and the agmatine (Agm), the direct product of the ADC enzyme and intermediate in the Put biosynthesis, were also detected which represents an advantage of this methodology. A large accumulation of free Agm in the transgenic plants and a related increase in the levels of Put and Spd was observed. The total His content in leaves of transgenic L108 plants compared to wild-type was also significantly higher. Significant increases in Put in the soluble bound fraction and the appearance of linked Agm in the insoluble bound fraction were also observed. Other amines, cadaverine (Cad) and tyramine (Tyr), were only detected in the insoluble bound fraction of both wild-type and transgenic L108 line. These ADC transgenic lines coupled with the described procedure for quantifying biogenic amines will provide a powerful tool to explore further the altered polyamine metabolism effects on plant legumes including abiotic stress tolerance phenotypes.

Key Words: biogenic amines; agmatine; polyamines; histamine; transgenic barrel medic; RP-HPLC; *Avena sativa* ADC

2. Introduction

Biogenic amines belong to a group of low-molecular-mass active organic bases that are usually synthesized by the decarboxylation of amino acid or by the amination and transamination of aldehyde and ketones. Biogenic amines are conveniently divided into aliphatic monoamines, aliphatic di- and polyamines and aromatic amines (Bouchereau et al., 2000).

The major forms of the commonly denominated Polyamines (PAs), the diamine putrescine (Put) and the polyamines spermidine (Spd) and spermine (Spm), are negatively charged aliphatic amines that are found widespread in living organisms. In plants, PAs have been implicated in a broad range of biological processes, including cell division, cellular growth, plant differentiation, senescence, somatic embryogenesis and response to environmental stresses (reviewed in Kumar et al., 2006). Arginine (Arg) and ornithine (Orn) are the precursors of plant PAs. Ornithine decarboxylase (ODC; EC 4.1.1.17) converts Orn directly into Put. The other biosynthetic route to Put, via arginine decarboxylase (ADC; EC 4.1.1.19), involves the production of the intermediate agmatine (Agm) followed by two successive steps catalysed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53) (Fig. 1). In animals and fungi Put is synthesized primarily through the activity of ODC while in plants and bacteria the main patway involves arginine decarboxylation. Recently, it has been suggested that ADC/ODC alternative pathways reflect their different evolutionary origins (reviewed in Alcázar et al., 2010a). Aminopropyl groups, donated by decarboxylated S-adenosylmethionine (dcSAM), must be added to convert Put into Spd and Spm in a reaction catalysed by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), respectively (Fig. 1) (reviewed in Alcázar et al., 2010a).

Metabolic studies indicate that the intracellular levels of PAs in plants are mostly regulated by anabolic and catabolic processes, as well as by their conjugation to hydroxycinnamic acids, fatty acids and macromolecules (Moschou et al., 2008; Alcázar et al., 2010a). Interactions of these processes with other metabolic pathways in particular with the ethylene biosynthesis, which shares SAM as common precursor, are also determinant aspects to be considered in PAs regulation (Fig. 1).

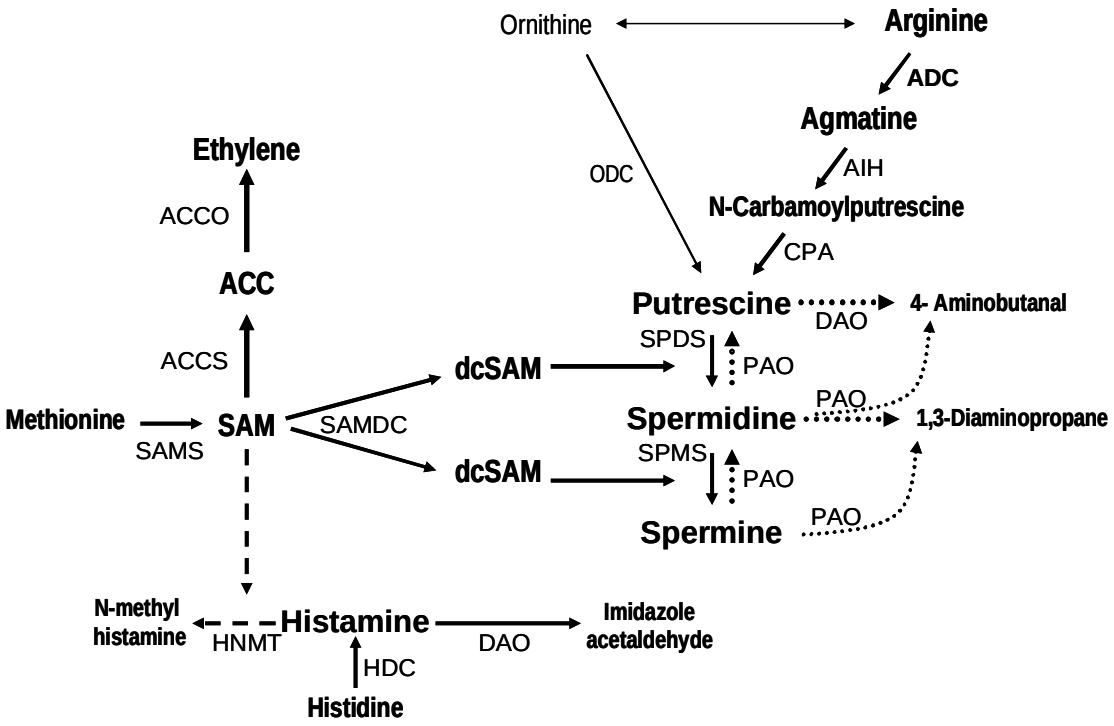

Figure 1. Resumed scheme of polyamine metabolic and catabolic pathways and interaction with ethylene and histamine (adapted from Abrighach et al., 2010 and Alcázar et al., 2010a). *Continuous lines*: Biosynthetic pathways for polyamines and related metabolites in plants. *Doted lines*: Polyamine catabolism in plants. *Dashed lines*: Interplay between polyamine and histamine metabolism in mammalian cells. Enzymes and other by-products involved: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; SPDS, spermidine synthase; SPMS, spermine synthase; SAM, *S*-adenosyl methionine; SAMS, *S*-adenosyl methionine synthase; SAMDC, *S*-adenosyl methionine decarboxylase; dcSAM, decarboxylated SAM; ACC, amino cyclopropane carboxylic acid; ACCS, amino cyclopropane carboxylic acid synthase; ACCO, amino cyclopropane carboxylic acid oxidase; DAO, diamine oxidase; PAO, polyamine oxidase; HDC, histidine decarboxylase; HNMT, histamine *N*-methyltransferase.

Earlier studies demonstrated that plants subjected to osmotic stress show a rapid increase in putrescine levels due to transcription and activation of arginine decarboxylase (Flores and Galston, 1982; Borrell et al., 1996). Additionally, exogenously supplied putrescine was shown to prevent stress damage and increase stress tolerance in *Conyza bonariensis* and wheat (Ye et al., 1998), and lately, soybean (Nayyar et al., 2005) and alfalfa (Zeid and Shedeed, 2006). It was also reported that stress-tolerant plants accumulate higher levels of polyamines in response to several stresses, comparatively to sensitive plants (Chattopadhyay et al., 1997; 2002). These results lead to the presumption that the manipulation of the PAs biosynthetic pathway could lead to an increase in the level of Put, Spd and Spm and that this increase could contribute to improve stress tolerance in transgenic plants (Flores, 1991; Chattopadhyay et al., 2002; Bhattacharya and Rajam, 2006; Prabhavathi and Rajam 2007; and references recently review in Alcázar et al., 2010a).

The adaptability and productivity of legumes are limited by major abiotic stresses including drought, heat, frost, chilling, water logging, salinity and mineral toxicities (Dita et al., 2006). Additionally, crops subjected to abiotic stress become more susceptible to weeds, insects and diseases, which decrease the productivity (Ready et al., 2004 in Dita et al., 2006). Furthermore, the establishment and functionality of legume-Rhizobium symbiosis are compromised by abiotic stresses.

The transformation and regeneration of *Medicago truncatula* Gaertn. (Barrel medic) cv. Jemalong line M9-10a (Araújo et al. 2004; Chapter IV and Duque et al. 2007; Chapter VI), was used for expression of genes related to drought tolerance (Araújo et al,. 2004; Chapter IV) and also proved to be a good system for metabolic engineering of the triterpene saponin pathway (Confalonieri et al, 2009). We produced transgenic lines of *M. truncatula* harbouring the oat ADC coding sequence (Chapter IV) aiming to alter polyamine levels and observe improved responses toward abiotic stresses.

Polyamine quantification in plants usually employs High Performance Liquid Chromatography (HPLC) of precolumn derivatives such as dansylchloride (5-dimethylaminonaphtalene-1-sulphonyl) or benzoylchloride. Also Thin-Layer Chromatography (TLC) of dansylderivatives is often the method of choice, although it does not provide the sensitivity or the reliability of HPLC separation (Smith, 1991).

Despite both methods drawbacks polyamine analysis in transgenic plants over-expressing enzymes of PAs biosynthetic pathway commonly employs TLC or HPLC separation of dansylchoride derivatized PAs.

Since agmatine (Agm) is the immediate product of ADC we were interested not only in the Put, Spd and Spm quantification but also in the changes in the Agm pool, not quantified in previous experiments using dansylchloride derivatives (Duque, unpublished data) as derivatization of Agm by dansylchloride results in the formation of three fragmentation products, including mono-dansyl agmatine, *bis*-dansyl agmatine and *bis*-dansyl putrescine (Smith, 1991).

Additional aromatic biogenic amines can be encountered in plant extracts and generally belong to classes of compounds derived from histamine and other imidazole-alkylamines, tryptamine and other indole-alklamines or phenetylamine and other phenylalkylamines. Histamine (His) is formed by decarboxylation of the amino acid histidine and is found both in plants and in animals, however few reports on determination of histamine in plant material are available and their actual function in plant tissues has yet to be elucidated (Bouchereau et al., 2000). Most of the reports concerns determination of histamine in food, especially on those processes obtained by fermentation, like wines and cheeses (e.g. Vidal-Carou et al. 2003) and also those related to food poisoning (such as fish, e. g. Frattini and Lionetti 1998) and meat product fermentation and storage (e.g. Smělá et al., 2003).

Research on plant biogenic amines has focused mostly on the pools of free compounds; however, it is becoming clear, that in many situations bound forms of aliphatic polyamines and aromatic amines can account for the major proportion of the metabolic pools (Bouchereau et al., 2000) and for that, the detection of conjugated polyamines is of extreme importance. **Free amines** are present either inside or outside the plant cells as free cations or involved in ionic interactions; **soluble bound or soluble conjugated** amines are presumed to be linked with low molecular mass compounds (e.g. hydroxycinnamic acids); and **insoluble bound or insoluble conjugated** amines are the ones linked to high molecular-mass compounds and cell walls (Yadav and Rajam, 1997; Bouchereau et al., 2000). In this late fraction we can find PAs covalentely bound to proteins glutamyl residues through transglutaminases (TGases), which appear to be of

extreme importance in plant light-induced stabilization of the photosynthetic complexes and Rubisco, therefore exerting a positive effect on photosynthesis and photo-protection (Serafini-Fracassini and Del Duca, 2008; Campos et al., 2009) . They are also implicated in cell wall construction and organization and involved in abiotic and biotic stresses, senescence and programmed cell death (review in Serafini-Fracassini and Del Duca, 2008).

In a recent study, an improved ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) method with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) post-column derivation was described for polyamines and other biogenic amines in wine and other alcoholic beverages, with the samples being only filtrated before the analytical determination (Vidal-Carou et al., 2003). We have adapted this method for the quantification of biogenic amines in *M. truncatula* aiming to understand the modifications in the metabolism induced by the expression of the heterologous oat *Adc*.

3. Materials and Methods

3.1. Plant material and culture conditions

Transgenic *Medicago truncatula* plants overexpressing the oat arginine decarboxylase (ADC) coding sequence were generated as described in Chapter IV. The plasmid construct used for plant transformation contained: the oat arginine decarboxylase coding region (*Adc*) (2,1Kbp, GeneBank Accession N^0 X56802) fused to the CaMV 35S promoter with duplicated enhancer sequences and the CaMV transcriptional termination region; the ß-glucuronidase (GUS) reporter gene; and the neomycin phosphotransferase II gene (*Npt*II), as selection marker. Integration of the construct in the *M. truncatula* genome was confirmed by Southern blot analysis (Chapter IV) and primary transformants of a transgenic line of *M. truncatula* overexpressing the oat *Adc* (L108) was used in this study. The non-transformed (wild-type) M9-10a line of *M. truncatula* cv Jemalong was used as control. Before moving to RP-HPLC analysis, the expression of the *Adc* gene in the L108 line was confirmed by RT-PCR (see Chapter IV). Plant lines M9-10a and L108 were micropropagated *in vitro* according to previously established protocols (Neves et al.,

2001). Three weeks before the beginning of the assays, each line was sub-cultured and plant material amplified by cutting stem segments with approximately 2 cm long (including 2 axillary buds) onto growth-regulator-free medium: MS030A – MS (Murashige and Skoog, 1962) basal salts and vitamins, 3 % (w/v) sucrose, 0.7% (w/v) agar (Microagar, Duchefa, The Netherlands). Plants were maintained in a growth chamber (Phytotron EDPA 700, Aralab, Portugal) with 16-h photoperiod of 100 μmol $m^{-2}s^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24º/22ºC.

Only the 2 youngest well-expanded 3-week-old leaves presenting dark green colour were used. Three biological replicates were taken for the amine extraction; each replica contained 4 to 6 leaves obtained from different *in vitro* plants. Leaves were randomly collected from several flasks always at the same daytime, corresponding to the end of the dark phase of the photoperiod, since light and daily rhythm are suggested to affect and change polyamine content (Bernet et al., 1999; Gemperlová et al., 2006). The fresh weight (FW) of each composite leaf sample was measured (FWs vary from 150 to 350 mg), then samples were immediately frozen in liquid nitrogen and dried under a lyophilizer MicroModulyo (Edwards, U.S.A.) for 168h according to Pedroso et al. (1997). Liophilized samples were weighted to obtain the dry-weight (DW) and stored at -80ºC for posterior analysis.

3.2. Biogenic amines analysis by ion-pair RP-HPLC

3.2.1. Extraction procedures

Procedures for the polyamine extraction were based in different published protocols (Minocha et al., 1990; Smith 1991; Burtin and Michael, 1997; Neves et al., 2002) and are further described in detail.

Dried leaf samples, were ground in liquid nitrogen in Eppendorf tubes with quartz sand and extracted with 500μl of 10% (W/V) cold trichloroacetic acid (TCA; Merck, U.S.A). Homogenized tissue was maintained on ice for 30 to 60 min. The homogenate was centrifuged at 4ºC, for 15 min at 20000G, and supernatant was collected to a new Eppendorf tube. For quantitative purposes an internal standard of 1,6-diaminohexane

(hexanediamine) (DAH; Sigma-Aldrich, U.S.A.) was added to the supernatant at the final concentration of 3.5 $mg.l^{-1}$. Pellets were washed 3 times with clean 10% TCA and resuspended in the original volume of 10% TCA (500µl containing DAH internal standard to 3.5 $mg.l^{-1}$ final concentration) by vortexing.

Aliquots of supernatants were directly analysed for **free amines** and portions of the supernatants were further treated to release **soluble bound amines**. Resuspended pellets were also treated to obtain **insoluble bound amines**. Treatments consisted in the hydrolysis of the suspensions in 6N HCl for 22h at 110°C in a Waters Pico-Tag Workstation (Waters, U.S.A.), with vacuum and nitrogen connections for drying and sealing the samples. For that purpose 100 µl of HCl (12N) was added to 100µl aliquots of supernatants and resuspended pellets, in glass capillary tubes, before hydrolysis. After heating and cooling samples were filtered through nylon membranes (0.2µm) (Millipore, U.S.A.), dried under a SpeedVac® (Savant SpeedVac® Plus SC110A, Savant, U.S.A.) at low/medium drying rate until complete evaporation (45 min to 1h). Samples were additionally ressupended in 100µl of 10% TCA and further analysed for amine content.

3.2.2. Separation and quantification

Biogenic amines were separated and quantified by ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using an adaptation of the method described for wines by Vidal-Carou et al. (2003). The RP-HPLC analysis was carried out in a Waters 717 Plus Autosampler with a fluorescence detector Waters 474 Scanning Fluorescence Detector (excitation wavelength of 340 nm and emission wavelength of 425 nm) and a Waters 510 HPLC Pump, all from Waters Chromatography, Milford, MA, U.S.A. The separations were performed on a Waters Nova-Pak C_{18} column with 3.9x150 mm and 4 µm particle size (Waters, U.S.A.). Data acquisition was accomplished with the Millenium^{32} version 3.05.01, 1998 system (Waters Chromatography, U.S.A.). The on-line post-column derivatization was performed with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) (Merck, U.S.A.) prepared according to Vidal-Carou et al. (2003). The column temperature was maintained at 40°C by a Waters Temperature Control Module (Waters, U.S.A.) and the post-column equipment was maintained at room temperature.

Aliquots of the polyamine fractions (10, 20 or 40 µl) were applied to the column and eluted with solvent A/solvent B (v/v) gradient adapted from (Vidal-Carou et al., 2003) over 43 min at a flow rate of 1 $ml.min^{-1}$. The gradient program was: time 0 min, A-B (78:22); time 5 min, A-B (74:26); time 22 min, A-B (46:54); time 26 min, A-B (20:80); time 31 min, A-B (20:80) ; time 33 min, A-B (78:22); time 43 min, A-B (78:22). Solvent A was a solution of 0.1 M sodium acetate (Calbiochem; Merck, U.S.A.) and 10mM sodium octanesulfonate (Fluka, Switzerland), pH was adjusted to 5.3 with acetic acid (Merck, U.S.A.); and solvent B was a mixture of a solution of 0.2M sodium acetate and 10 mM sodium octanesulfonate, (pH adjusted to 4.5 with acetic acid) with acetonitrile (6.6:3.4). The decrease/increase of eluents A/B was programmed according to a second-order exponential curve.

Standards stock solutions of each amine (Agm; Put; Spd; Spm and His) and the internal standard (DAH) were prepared in 0.1 M HCl in adequate concentrations and diluted in the extraction medium for injection to a final concentration of 0.125, 0.25, 1.25, 2.5, 5.0 and 10.0 $mg.l^{-1}$. Standards were purchased from Sigma (Sigma-Aldrich, U.S.A.) and concentrations were corrected on the basis of purity and free base content.

Concentration of each biogenic amine was obtained by direct interpolation of the peak area in the correspondent linear calibration curve (peak area versus amine concentration) and was further corrected using the internal standard (IS=DAH), according to the following formula (Smělá et al., 2003):

$C_x = RF_x \times (C_{IS} \times A_x)/A_{IS}$

Where: RF_x – response factor of the amine

($RF_x = A_x/A_{IS} \times C_x/C_{IS}$)

A_{IS} – peak area of the internal standard

A_x – peak area of biogenic amine

C_{IS} – concentration of the internal standard

C_x – concentration of the biogenic amine

3.3. Statistical analysis

One-way Analysis of Variance (ANOVA) was carried out to test the significance between the means of different polyamines quantification in lines M9-10a (control) and L108 at a significant level of $p < 0.05$. Results were expressed in $ng.g^{-1}$ dry weight (DW) as mean values ± standard deviation of three replicate samples.

4. Results and Discussion

4.1. Extraction and chromatographic procedures

Polyamines in wild-type (M9-10a) and transgenic plants (L108 and L104) expressing oat *Adc* were previously quantified by HPLC procedures (unpublished results), using precolumn dansylchloride derivatization with fluorescence detection according to Minocha et al. (1990). Preliminary results showed higher free Put levels in leaves of transgenic lines compared to M9-10a plants. Only line L108 was selected for further polyamines studies because it showed the highest increase in free Put and Spd and displayed a Mendelian segregation pattern of 3:1 corresponding to a dominant gene at a single locus [Yates (1934) chi-square (χ^2) test: $\chi^2= 0.158$; with 1 degree of freedom (df=1) and P-*value*= 0.05; see Chapter IV].

The *o*-phthalaldehyde (OPA) in the presence of reducing agents such as 2-mercaptoethanol (ME) reacts rapidly with primary amines to form strong fluorescent derivatives and is more selective than other fluorogenic agents (Smith 1991). Corbin et al. (1989) described a pre-chromatographic derivatization method, for crude plant extracts, with OPA/ME followed by RP-HPLC and fluorometric detection. However, there was a disadvantage of faster decay of the fluorescent product that required the immediate injection of the prepared samples and did not allowed automation of the process.

The post-column modification of polyamines is often preferred because OPA derivatives are more stable (Smith 1991). The automatic accomplishment of the derivation reduces time and effort of analysis (Vidal-Carou et al., 2003) and simplifies the sample preparation. In our samples it also permitted the separation of other important biogenic

amines besides PAs, like the monoamine histamine (His) and the significant precursor agmatine (Agm). Additional amines, cadaverine (Cad) and tyramine (Tyr) were also detected with this process.

In the special case of Agm this methodology allowed to overcome dansylderivatives detection/quantification limitations reported by others (Slocum et al., 1989; Burtin and Michael 1997; Bencsik et al., 1998).

Oguri (2000) provided an overview of the various electromigration methods for biogenic amines and aromatic amines determination, including histamine determination by capillary electrophoresis (CE) in rat peritoneal mast cells. More recently, Zhang et al. (2005) described concomitant determination of histamine and polyamines, using CE with 4-fluor-7-nitro-2,1,3-benzoxadiazole derivatization and fluorescence detection in the lysate of tobacco mesophyll protoplasts. An additional advantage of our methodology is the possibility of simultaneous determination of PAs and His by RP-HPLC with an OPA/ME post-column procedure for the first time adapted to plant extracts.

Representative chromatograms of biogenic amines, for the wild type (M9-10a) and the transgenic (L108) *M. truncatula* plants, obtained by RP-HPLC of different extraction fractions, are displayed in Figure 2.

The fluorescence calibration exhibited a linear correlation with the concentration within the range tested (0.125-10.0 $mg.l^{-1}$), with correlation coefficients (R^2) superior to 0.9998 for all the standards used.

The separation time with the adapted gradient elution program was relatively faster compared to the reported by Vidal-Carou et al. (2003), since within 43 min all biogenic amines were resolved.

Chromatograms were straightforward and the peaks of biogenic amines were satisfactorily resolved without interferences (Fig. 2). The only exception was for the insoluble bound fractions, in which a second peak appeared narrowly after the Spd elution peak, corresponding to a compound that we were not able to identify (Fig. 2, E and F).

The use of DAH as an internal standard was important for estimating the percentage of biogenic amine recovery (especially in the case of hydrolysed samples) and the obtained response factors (RF) found (RF range from 1.23±0.03 to 1.96±0.1, Table 1) are within the scope reported in the literature (Smělá et al., 2003).

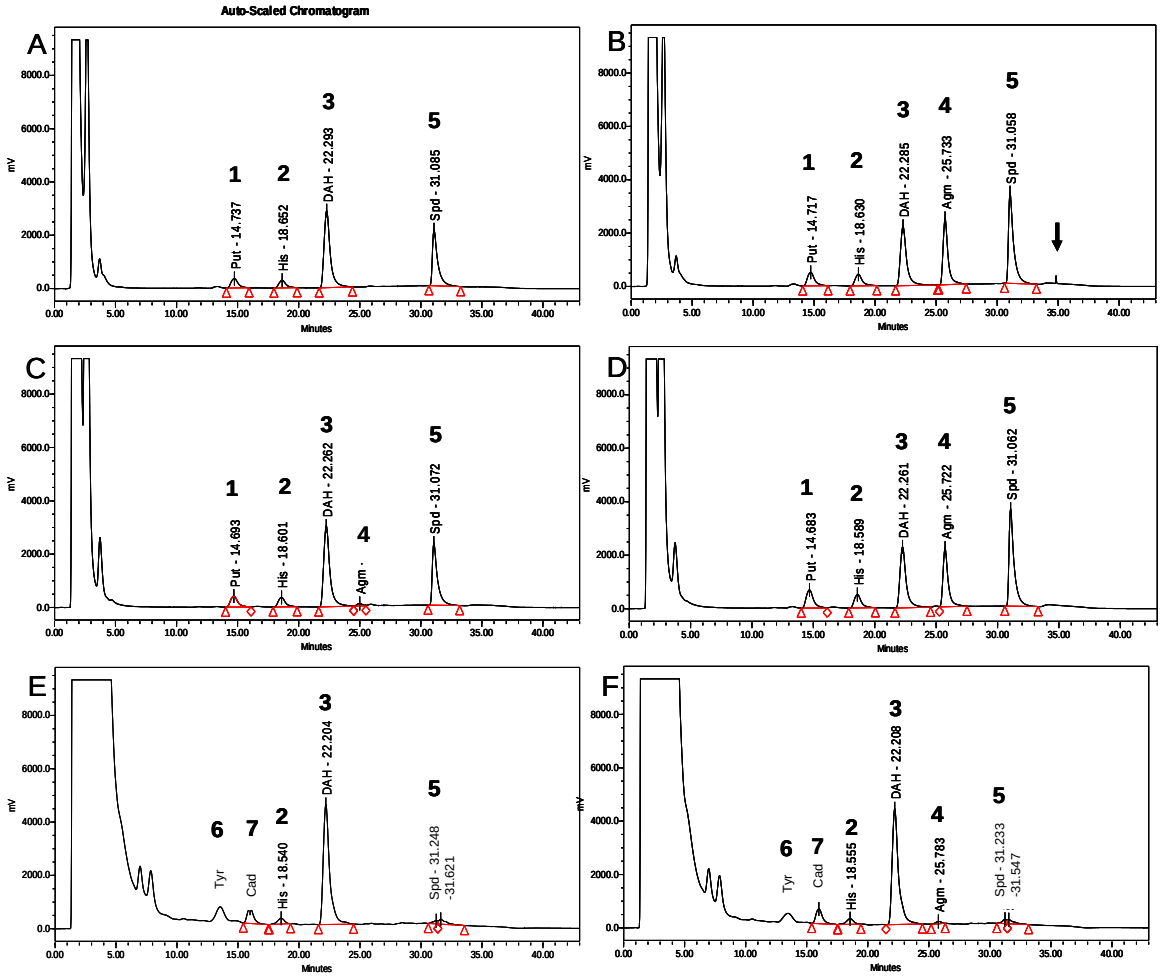

Figure 2. Representative chromatograms of biogenic amine separation by ion-pair RP-HPLC on a 4 µm C_{18} column (3.9x150 mm i.d) using a gradient of sodium acetate/sodium octanesulfonate at a flow rate of 1 ml.min^{-1} and post-column derivatisation with OPA/ME. Separation of the different fractions from control and transgenic *Adc M. truncatula* plants: Fraction resulting from the supernatant, **free biogenic amines** (10µl injection volume), in leaves of control (M9-10a) (**A**) and transgenic (L108) (**B**); fraction from supernatant hydrolysis, **free + soluble bound amines** (20µl injection volume), in leaves of control (M9-10a) (**C**) and transgenic (L108) (**D**), respectively; fraction resulting from pellet hydrolysis, **insoluble bound amines** (40µl injection volume), in leaves of control (M9-10a) (**E**) and transgenic (L108) (**F**). 1- (Put) Putrescine, 2- (His) Histamine, 3- (DHA) hexanediamine -internal standard, 4- (Agm) Agmatine, 5- (Spd) Spermidine, 6- (Tyr) Tyramine, 7- (Cad) Cadaverine; (arrow) putatively Spermine.

4.2. Biogenic amines quantification

The results of the quantification of free, soluble bound and insoluble bound amines, determined in leaves of transgenic *M. truncatula* L108 and wild-type, using ion-pair RP-HPLC with post-column derivatization with OPA/MCE reagent, are presented on Table 1**.** Concerned with the possibility that increased PAs concentration could function as osmolytes and change leaf water status, as suggested by Farooq et al. (2009) and contrasting with most previous reports, we presented our results in function of leaf dry weight (DW).

Table 1. Distribution of free, soluble bound and insoluble bound fractions of polyamines putrescine (Put) and spermidine (Spd); of the intermediate agmatine (Agm) and the monoamine histamine (His) (ng.g^{-1} DW); in leaves of control (M9-10a) and transgenic (L108) *M. truncatula in vitro* grown plants.

		Free fraction (ng.g^{-1} DW)		**Soluble bound fraction** (ng.g^{-1} DW)		**Insoluble bound fraction** (ng.g^{-1} DW)		**Total** (ng.g^{-1} DW)	
	RF_x	M9-10a	L108	M9-10a	L108	M9-10a	L108	M9-10a	L108
Put	1.23±0.03	45.9±1.5^{a}	82±7.7^{b}	6.5±0.3^{c}	11.3±0.9^{d}	0^{e}	0^{e}	56.4±1.8^{f}	93.3±8^{b}
Spd	1.85±0.03	297.8±2.3^{a}	537.2±11.9^{b}	24.1±21.6^{cd}	56.6±29.5^{c}	15.5±10^{d}	13.2±10.1^{d}	321.9±33.9^{a}	607±51.5^{e}
Agm	1.96±0.1	0^{a}	367.6±29.9^{b}	18±0.6^{c}	14.1±5.3^{cd}	0^{a}	10.9±0.8^{d}	18±0.6^{c}	392.6±31.3^{b}
His	1.83±0.23	57.4±3.1^{a}	90.7±4.9^{b}	11.2±3.8^{c}	16±5.9^{cd}	24.8±3.7^{de}	27.1±3.7^{e}	93.4±10.2^{b}	133.8±12.3^{f}

Values represent the mean (± standard deviation) of three replicate samples. Different superscripts within rows indicate statistical significance ($p<0.05$) (represented in blue colour). DW- dry weight; RF_x - response factor of the amine.

The first PA, the diamine **putrescine**, was detected in the free and soluble bound fraction of wild type and transgenic L108 line. Free Put levels were increased significantly in L108 (82±7.7 ng.g^{-1} DW) compared to the wild type (45.9±1.5 ng.g^{-1} DW), as a result of the expression of the oat *Adc* gene (Table 1, Fig. 2). In the soluble bound amine fraction, Put concentration was also significantly higher than in the control (11.3±0.9 ng.g^{-1} DW; Table 1). No covalently bound Put was detected in both wild type and transgenic line (Table 1).

Spermidine was detected in wild type and transgenic L108 line, with L108 showing a significant increase in the free fraction (537.2±11.9 $ng.g^{-1}$ DW; Table 1). In the soluble and insoluble bound fractions no significant differences were found for Spd concentration, but a great variability within the samples tested was obtained, with concomitant higher standard deviations (respectively STD=21.6-19.5 and STD=10-10.1; Table 1), which was not observed for the quantifications of the other biogenic amines. **Spermine** was not quantified in the free amine fraction of L108 extracts, in spite of the presence of a small peak corresponding to the correct spermine retention time (comparing with those of the standards), indicating that the concentration for this amine was below the detection limit of this method (<0.2 $mg.l^{-1}$) Vidal-Carou et al. (2003) (Fig. 2B, arrow).

The most striking difference detected between the wild type and the transgenic L108 line was the concentration of the biogenic amine **agmatine**, the direct product of the ADC enzyme (see Fig. 1). Agmatine was not present in the free amine fraction of wild type plants but a high concentration was found in L108 (367.6±29.9 $ng.g^{-1}$ DW; Table 1). This is not surprising, having in mind the accumulation of the oat *Adc* transcripts in L108 and being Agm the direct product of the ADC enzyme (see Chapter IV, Figure 5). There was no significant difference in the Agm soluble bound fraction between the wild type and L108 line. However, we could quantify 10.9±0.8 $ng.g^{-1}$ DW of Agm in L108 insoluble bound fraction, suggesting the sequestration of part of the Agm pool to form covalent bounds to high molecular mass compounds.

In barley plants, the conjugated forms of Agm have been shown to exert an anti-fungal effect and are also a likely constituent of cell walls (Stoessl et al., 2001; Kristensen et al., 2004). In recent research with animal cells, specifically in kidney mitochondrial preparations, Agm demonstrated attributes of a free radical scavenger by protecting against the oxidation of sulfhydryl groups and decreasing hydrogen peroxide content**.** Agmatine was implicated in depletion of intracellular polyamine content that in excess in animal cells may be toxic, and its antioxidant capacity afforded protection from mitochondrial insult and resistance to cellular apoptosis (Arndt et al., 2009)**.**

Burtin and Michael (1997) reported that, in tobacco plants, expressing the oat *Adc* cDNA, the elevated Agm accumulation (20-65-fold) (also correlated with oat *Adc* transcript accumulation) did not result in increased free Put, Spd and Spm, neither

conjugated and bound PAs, or increased nicotine levels. Polyamine biosynthesis and related pathways were not affected. Given that the enzymes AIH and CPA, which are necessary for the formation of Put from Agm via N-carbamoylputrescine, are not thought to be rate-limiting they suggest there was a metabolic block preventing the accumulation of Put in tobacco 35S*Adc* transgenic plants.

In our study, we found that a significant accumulation of free **agmatine** may be associated with the increased free Put and Spd levels. Moreover, additional determinations of PAs in soluble and insoluble bound fractions resulted in increased Put in the soluble bound fraction and the appearance of linked Agm in the insoluble bound fraction.

In transgenic rice plants constitutively expressing oat *Adc* cDNA driven by the CaMV 35S promoter, Put levels in vegetative tissue were twice that of controls, but no significant increase could be detected in seeds and the levels of higher PAs were also unchanged (Capell et al., 1998). In another study, rice transformation with oat *Adc* cDNA, under the control of the maize ubiquitin 1 (*Ubi-1*) promoter, one particular lineage showed very significant increases in Put levels in seeds, although Put levels in leaf tissue were not significantly different from controls. Additionally, Spd and Spm levels were also unchanged (Noury et al., 2000). Such findings suggest that the levels of Spd and Spm are under strict homeostatic regulation (Bhattacharya and Rajam, 2006).

Capell et al. (2004) developed a model which stipulates a minimum threshold in putrescine concentration prior to further conversion into the higher PAs, Spd and Spm. These authors suggested that the Put pool must exceed a certain threshold to induce the synthesis of Spd and Spm under stress. Such increases not only regulate Put levels, but also exert an anti-senescence effect at the whole plant level. These authors also discussed the influence of promoter strength in the polyamine metabolism. The CaMV 35S promoter being a moderately strong promoter for driving heterologous transgene expression in rice does not appear to be sufficient to reach the minimum threshold for Put accumulation, thus contrasting to the maize ubiquitin1 (*Ubi-1*) promoter, that enhanced the Put pool to levels that extend beyond the critical threshold required to initiate the conversion of excess Put to Spd and Spm (Bassie et al., 2000; Bassie et al., 2008). We can hypothesize that the Agm pool may be regulated in the same way as proposed by Capell et al. (2004) for Put. In this way, a minimum threshold in Agm concentration would be necessary for Put accumulation

prior to further conversion into the higher PAs, and we can presume that this concentration was not achieved in Burtin and Michael (1997) tobacco transformation experiments.

The aromatic biogenic amines, **cadaverine** (Cad) and **tyramine** (Tyr) were found in the insoluble bound fractions of both M9-10a and L108 (Fig. 2 E and F). Although not quantified, their OPA derivatives were identified by comparison of the absorption spectra of standards stored in the user's computer library. The diamine Cad, a penta homolog of Put, is mainly found in legumes and is produced by the decarboxylation of lysine catalyzed by lysine decarboxylase (LDC, EC 4.1.1.18) (Kuznetsov et al 2006). Tyrosine decarboxylase (TYDC, EC 4.1.1.25) is responsible for the biosynthesis of tyramine (Tyr) from tyrosine (Kang et al 2006). Tyramine is an important constituent of hydroxycinnamic acids that are believed to contribute to the active defense of plants against pathogen through their peroxidative incorporation into the cell wall to form covalently cross-linked polymers (Hagel and Facchini, 2005).

Significant **histamine** (His) increase in the free fraction was detected in L108 line (90.7±4.9 $ng.g^{-1}$ DW; Table 1) compared to wild type *M. truncatula* plants (57.4±3.1 $ng.g^{-1}$ DW; Table 1). Although histamine functions in plant tissues is still unknown (Bouchereau et al., 2000) in several mammalian cell types there are multiple lines of evidence suggesting an interplay between His and polyamines. Abrighach et al. (2010) results indicate a regulatory effect of His on the post-transcriptional expression of ornithine decarboxylase (ODC). Histamine can also interfere with polyamine metabolism since there are several metabolic connections between the synthesis and degradation pathways of both types of amines. Histamine degradation involves the action of histamine methyltransferase (a SAM dependent enzyme) and histaminase (EC 1.4.3.6), also known as diamine oxidase, that is also able to act on putrescine [(Tabor and Tabor, 1964; Abrighach et al., 2010); see Fig. 1]. Tissue transglutaminases (TGases) can also use Put and His as substrates (Abrighach et al., 2010) and this occurs also with plants TGases (Serafini-Fracassini and Del Duca, 2008). Our results suggest a relationship between augmented PAs in transgenic line L108 and a concomitant increase in His; but the interplay between these compounds still needs further investigation.

Recently, several studies have concluded on the feasibility of PA biosynthesis engineered for the production of stress-tolerant plants. The constitutive expression of

homologous ADC1 and ADC2 in Arabidopsis resulted in freezing and drought tolerance, respectively (Altabella et al., 2009, Alcázar et al 2010b). In transgenic eggplants with the oat *Adc* gene under the control of a CaMV35S promoter Prabhavathi and Rajam (2007) found an increase in Put, and also in higher PAs, Spd and Spm. Polyamine-accumulating transgenic eggplants exhibited increased tolerance to multiple abiotic stresses (salinity, drought, low and high temperature and heavy-metal) and also fungal resistance. Considering these results, further research concerning the PAs changes and the global response of our *M. truncatula* transgenic lines to multiple stresses should be developed in the near future.

In summary, and in respect to total amine content in *M. truncatula* cv Jemalong plants expressing heterologous oat *Adc*, we found that Put increased 1.7-fold, Spd was 1.9-fold higher, His augmented 1.4-fold and Agm had the higher increase (22-fold) when compared to control plants. Despite some constrains with the toxic effects of polyamine accumulation in plants (Tiburcio et al., 1994), plant cells, contrarily to animal cells, are less affected by excess PAs, as they can buffer this excess by binding PAs to TCA-soluble conjugates, such as cinnamic acids, or by storing them in the vacuole (Serafini-Fracassini and Del Duca, 2008). Our work shows that, even though there are several experiential changes in biogenic amines levels, the ADC transgenic *Medicago truncatula* plants have normal visual phenotypes, are fertile and progenies will serve as useful germplasm for further studies.

5. Conclusions

We characterized primary transformants (T_0) of the transgenic line (L108) of *M. truncatula* cv Jemalong, expressing the heterologous oat *Adc*, and found significant increases of free Agm, Put, Spd and His. Significant increases in Put in the soluble bound fraction and the detection of linked Agm in the insoluble bound fraction were also observed. Cadaverine and Tyr were only detected in the insoluble bound fraction of both wild-type and transgenic plants. Overall, we can hypothesize that the accumulation of free

Agm may be associated with the increased free Put and Spd levels and that a minimum threshold in Agm concentration would be required for Put accumulation and further conversion into the higher PAs, Spd and Spm.

Here we report an optimized ion-pair RP-HPLC methodology for detecting and quantifying biogenic amines in vegetative tissues of *M. truncatula* cv Jemalong useful for the analysis of the role of these biogenic amines in abiotic and biotic stress responses and also in elucidating the interaction of polyamine metabolism with other metabolic routes.

The changes in biogenic amines levels in transgenic *Medicago truncatula* plants resulted in phenotypically normal and fertile plants and offspring population will serve as useful germplasm for further studies.

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VI - Use of fused *Gfp* and *Gus* reporters for the recovery of transformed *M. truncatula* somatic embryos without selective pressure

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1. Abstract

We developed an alternative methodology for *in vitro* selection of transgenic *Medicago truncatula* cv. Jemalong plants using a bifunctional construct in which the coding sequences for the green fluorescent protein (GFP) and the β-glucuronidase protein (GUS) are fused. An *Agrobacterium*-mediated transformation protocol was used followed by regeneration *via* somatic embryogenesis in the dark, to avoid the synthesis and the consequent autofluorescence of chlorophyll. This method is a clear advantage over antibiotic and herbicide selection in which survival of non-transformed tissue is commonly reported, with the reassurance that all the somatic embryos selected as GFP positive are transformed. This was subsequently corroborated by the detection of GUS activity in leaves, stems and roots of the regenerated plants. Without antibiotic selection, and performing the embryo induction in the dark, it was possible to attest the advantage of using GFP as an *in vivo* detectable reporter for early embryo selection. The fusion with the GUS coding sequence provided additional evidence for the transformation of the previously selected embryos.

Key words: Agrobacterium-mediated transformation, Antibiotic-free, Dark embryo induction, GUS, GFP.

2. Introduction

An *Agrobacterium*-mediated transformation protocol was previously established to obtain stable transgenic *Medicago truncatula* plants from the cv. Jemalong line M9-10a (see Chapter IV). Although transformation of line M9-10a using *in vitro* leaf explants with an optimised kanamycin selection is very efficient, to fulfil our broad aim of studying the expression of drought-related genes using the model legume *M. truncatula*, alternative methodologies for the selection of transformed plants could be of great interest.

A major limiting factor in generating transgenic plants using a somatic embryogenesis regeneration system is the selection of transformed cells in *in vitro* culture. Selectable marker genes, namely the *Npt*II gene for antibiotic resistance, are widely used

but survival of non-transformed tissue is common in somatic embryo cultures under selective pressure and optimisation for each situation is required (Escobar et al., 2000; Duque et al., 2004).

The *Escherichia coli* gene *Gus*A encoding β-glucuronidase (GUS, Jefferson et al., 1987) is commonly used as a reporter in constructs for plant transformation because there is almost no endogenous GUS activity in most plant species, its activity can be analysed histochemically and easily quantified using fluorescent substrates (Quaedvlieg et al., 1998). Because GUS assay is destructive, only after the conversion of embryos to plantlets it is possible to remove plant portions to execute a test on organs (e.g. leaves or roots) and obtain a fast recognition of transformed plants, without their destruction.

For the early selection and isolation of the transgenic sectors (transformed cells and tissues/embryos) another process is needed. The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* has emerged as an *in vivo* detectable reporter for monitoring plant transformation (Chalfie et al., 1994; Sheen et al., 1995; Haseloff et al., 1996; Chiu et al., 1996) because the *Gfp* expression can be observed in real time in living cells, does not require the supply of a substrate and does not compromise cell viability. This opens the possibility to rescue the transgenic sectors and to regenerate transgenic plants without using selectable marker genes conferring antibiotic or herbicide resistance, which is currently a topic of great discussion for the commercialisation of transgenic plants (Ghorbel et al., 1999; Komemytsky et al., 2004). However, GFP has some limitations because its emission can be obscured by the chlorophyll autofluorescence present in light converted somatic embryos (due to the strong autofluorescence of the chlorophyll the expression of GFP is often detected as red-orange fluorescence). In fact, recent studies describe severe interference of chlorophyll with GFP fluorescence in *M. truncatula* as leaves age (Zhou et al., 2005).

In this study we describe an alternative methodology for *in vitro* selection of *M. truncatula* transformants using a bifunctional reporter construct (pMP2482; Quaedvlieg et al., 1998). In pMP2482 the coding sequences for GFP and GUS are fused and under the control of the same promoter, with the advantage that the detection of the resulting GUS/GFP protein product is strictly related with the gene expression (Quaedvlieg et al., 1998).

This construct, already tested for *Arabidopsis thaliana* and *Lotus japonicus* (Quaedvlieg et al., 1998), was transferred to *M. truncatula* M9-10a embryogenic line. Without the need for antibiotic or herbicide selective pressure, and performing the embryo induction in the dark, it was possible to combine the advantages of GFP as an *in vivo* detectable reporter with the high sensitivity of histochemical GUS staining to verify the efficiency of early selection of transformed somatic embryos.

3. Materials and Methods

The transformation of *M. truncatula* Gaertn. cv. Jemalong line M9-10a (Neves et al., 1999; Santos and Fevereiro, 2002) was carried out using the disarmed *A. tumefaciens* succinamopine strain EHA105 (Hood et al., 1993) harbouring the plasmid pMP2482 (Quaedvlieg et al., 1998), mobilised by the freeze-thaw method (Walkerpeach and Velten, 1994). This binary vector contains the *GusA::intr* and *Gfp* coding sequences fused, under the control of a CaMV35S promoter with duplicated enhancer initiation sequences. The presence of the potato *st-ls*I intron in the coding region of the *GusA* hinders its expression in *Agrobacterium*. The *Gfp* gene encodes a synthetic GFP red-shifted variant (s-GFP-TYG) with an optimised codon usage for humans shown to be highly expressed in plants (Chiu et al., 1996). In other region of the T-DNA, the presence of the *Npt*II coding sequence, under the control of the nopaline synthase promoter, confers resistance to aminoglycosides antibiotics like kanamycin, gentamicin or geneticin (G418) and could serve as a selection marker for transformed plants.

Plants of the M9-10a genotype were maintained *in vitro* and micropropagated in growth-regulator-free medium: Murashige and Skoog (1962) (MS) basal salts and vitamins, 3% (w/v) sucrose and 0.7% (w/v) agar (Microagar, Duchefa, The Netherlands) as described in Neves et al. (2001). Well-developed leaflets from 30 days-old *in vitro* grown M9-10a plants were used as explants for the transformation experiments. The preparation of *A. tumefaciens* EHA105 suspension for plant transformation was performed as described in Chapter IV. The *Agrobacterium* harbouriong pMP2482 were inoculated in 5 ml of *Luria Broth* (*LB*) with 61 μM of rifampicin and 147μM of kanamycin and incubated for 48h at 28ºC, 200 rpm. These cells were centrifuged (3500 rpm, 10 min), re-suspended

at $O.D._{600nm}$= 1.5 in embryo induction medium (EIM - MS basal salts and vitamins, 3% (w/v) sucrose, 0.45 μM of 2,4-D and 0.91 μM of zeatin) with 100 μM of acetoseryngone (Sigma-Aldrich Inc., USA) and incubated for 30 min to be used for transformation. The transformation procedure was done as described in Chapter IV with some modifications. Briefly, leaflets were placed onto a wet sterile filter paper in a Petri-dish to prevent desiccation and cut perpendicularly to the midrib using a sterile scalpel blade previously dipped into the *Agrobacterium* suspension.

A total of 120 leaflets were agro-infected and placed with the abaxial side-down on Petri-dishes containing EIM [MS (Murashige and Skoog, 1962) basal salts and vitamins, 3% (w/v) sucrose, 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.91μM zeatin (Zea), 0.2% (w/v) gelrite (Merck, USA)] with 100 μM of acetoseryngone, for a co-cultivation period of 5 days in the dark, in a growth chamber (Heraeus, Germany) at 23ºC.

After co-cultivation, the infected explants were transferred to EIM containing 1184 μM of carbenicillin to eliminate *Agrobacterium*. To maintain the antibiotic pressure for *A. tumefaciens* elimination, explants were removed every week to fresh medium containing carbenicillin. Fifteen Petri-dishes (90 leflets) were maintained in the dark during the embryo induction period and no kanamycin was added to the medium to select for transformed tissue. After 21 days in EIM, embryogenic *calli* were transferred to an embryo proliferation medium (EPM: EIM without growth-regulators) and again kept in dark conditions until embryo isolation. For comparison purposes, the remaining 30 agro-infected leaflets were subjected to the regeneration process in light conditions (as described in Chapter IV) also without kanamycin selective pressure.

The *Gfp* expression in the embryogenic cultures and during the development of somatic embryos was observed and followed over time under a dissecting stereomicroscope (Leica MZFLIII, Germany) equipped with GFP excitation and emission filters (excitation filter 480/40nm; barrier filter 510nm LP); which permits visualisation of GFP after blue light excitation.

Somatic embryos at the late torpedo/dicotyledonary stage were selected and recorded as gfp^+ (observed green fluorescence) and gfp^- (non-observed green fluorescence), placed on embryo conversion medium [ECM: EPM with 0.7% (w/v) agar (Microagar, Duchefa, The Netherlands) instead of gelrite, supplemented with 592 μM of

carbenicillin] and transferred to a 16-h photoperiod of 100 μmol.m^{-2}.s^{-1} applied by cool white fluorescent light and a day/night temperature of 24º/22ºC (Phytotron Edpa 700, Aralab, Portugal).

Every two weeks, somatic embryos were transferred to fresh ECM until conversion to plantlets. When plantlets developed roots, carbenicillin was eliminated and plants were transferred to 60 mm ∅ glass flasks containing MS growth-regulator-free medium covered with a plastic transparent sterilised film (SILVEX®, Portugal). The regenerated plants were either micropropagated (as described above for the M910a plant donor material) or transferred to the greenhouse.

The pH of all media was adjusted to 5.8 before autoclaving (121ºC, 20 min). Carbenicillin and growth regulators were filter sterilised through 0.2 μm filters (Whatman, England) and added to autoclaved (cooled to 40ºC) media.

Detection of β-glucuronidase (GUS) activity was performed essentially as described by Jefferson et al. (1987) using as substrate 5-Bromo-4-chloro-3-indolyl-*β*-D-Glucuronide acid (X-GlcA, Duchefa, The Netherlands). Leaflets, stems and roots were covered with the assay buffer and vacuum infiltrated at 0.8 bar for at least 1h. The reaction was incubated at 37^{o}C in a wet chamber for 24h and excess chlorophyll was removed from stained explants with 70% (v/v) ethanol.

4. Results and Discussion

After 5 days in EIM, either in light or dark conditions, it was possible to observe, using a stereomicroscope, the development of friable *calli* at the wounded edges of leaflets. After 15 days in EIM, the development of massive *callus* is observed in every responsive explant together with the development of green pro-embryogenic masses in the cultures growing in light conditions (Fig. 1 A). Some globular embryos differentiating at the top of the pro-embryogenic masses can be observed by the end of the induction period (21 days). In the explants growing under light conditions, it was difficult to visualise GFP expression, because the strong background of chlorophyll autofluorescence masks the green fluorescence of GFP (Fig. 1 B).

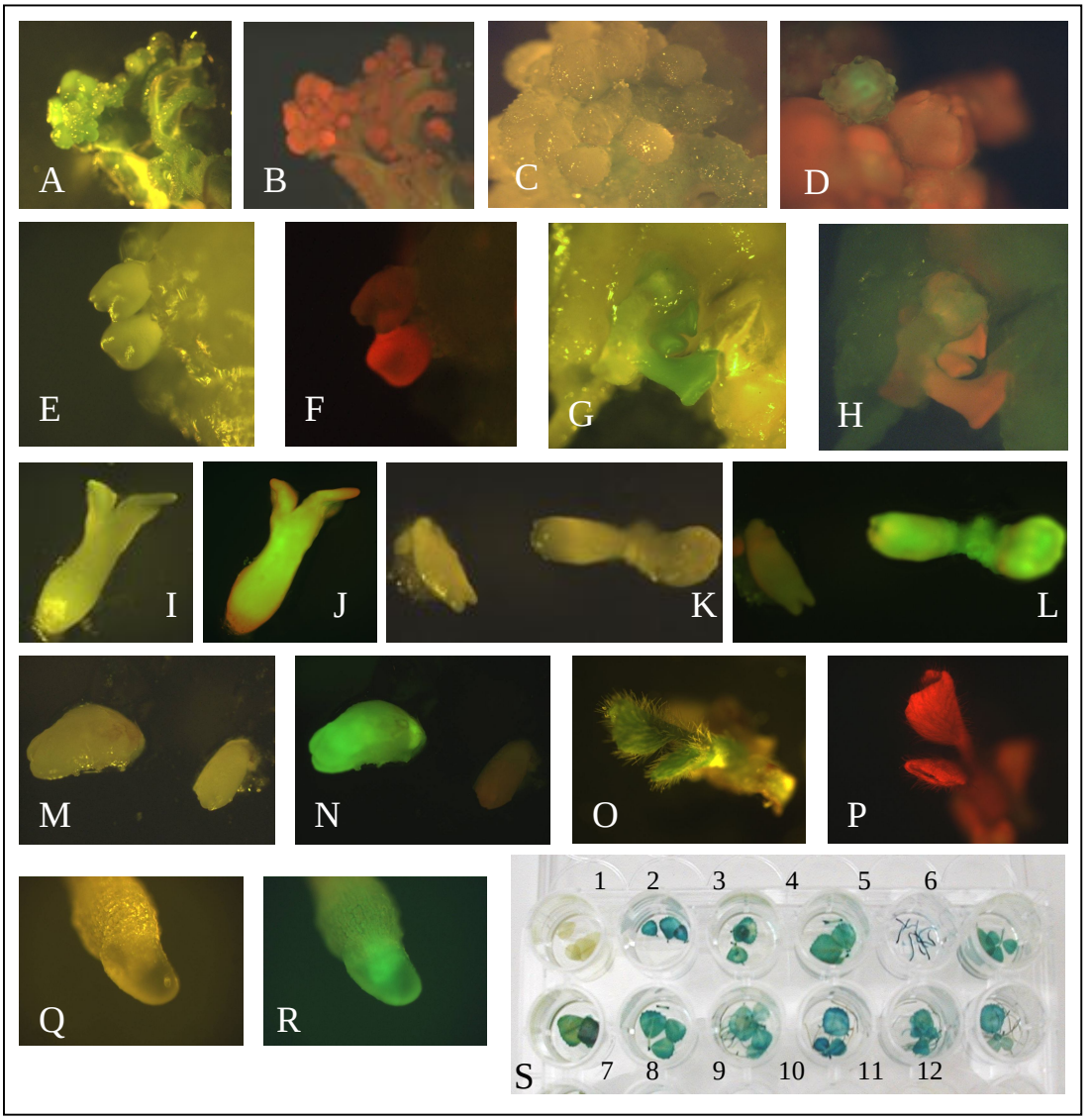

Figure 1. Transformation-regeneration of *M. truncatula* cv Jemalong M9-10a genotype using the bifunctional reporter gene construct pMP2482 containing fused *GusA::intr* and *Gfp* genes.
(A) Embryogenic *callus* originated from wounded edges of infected leaflets placed in light conditions, presenting green pro-embryogenic masses (photographed under white light); **(B)** The same embryogenic *callus* observed under blue light, showing high background of red chlorophyll autofluorescence; **(C)** Pale-yellow embryogenic *callus* derived from a infected leaflet placed in dark conditions; **(D)** Previous embryogenic *callus* observed under blue light, showing green fluorescent pro-embyogenic mass; **(E)** Pale-yellow embryogenic *callus* with heart-stage somatic embryos, derived from a infected leaflet placed in dark conditions; **(F)** Previous embryogenic *callus* grown in dark conditions observed under blue light, presenting gfp^- heart-stage somatic embryos; **(G)** Green somatic embryos at different stages of development, grown in light conditions and photographed under white light; **(H)** The same entity observed under blue light; note the chlorophyll autofluorescence that interferes with the GFP fluorescence; **(I)** Pale-yellow dicotyledonary stage

somatic embryos developed in dark conditions observed under white light; **(J)** The previous dicotyledonary stage somatic embryos photographed under blue light showing green fluorescence (GFP expression); **(K)** Pale-yellow somatic embryos developed in dark conditions observed under white light; **(L)** The previous somatic embryos photographed under blue light; note clear differences between gfp^+ (right side) and gfp^- (left side) somatic embryos; **(M)** Pale-yellow somatic embryos developed in dark conditions observed under white light; **(N)** The previous somatic embryos photographed under blue light; note clear differences between gfp^+ (left side) and gfp^- (right side) somatic embryos; **(O)** Plantlet developed from previously selected gfp^+ somatic embryo photographed under white light; **(P)** The previous plantlet photographed under blue light showing strong red chlorophyll autofluorescence; **(Q)** Developing root meristem of the same plant, photographed under white light; **(R)** Root meristem demonstrating to maintain the GFP expression, as shown by the fluorescence observed under the blue light excitation**, (S)** Histochemical Gus assay: positive (blue staining) results in leaves, roots and stems of *GusA::intr::Gfp* transformed plants. Untransformed M9-10a used as negative control (bleached leaves, well 1).

Pale-yellow *calli* developed in dark growing cultures with the same proliferation ability of those grown in light conditions (Fig. 1 C). Despite the emission of some weak autofluorescence it is easy to detect transformed, pro-embryogenic masses presenting green fluorescence (Fig. 1 D).

When embryogenic *calli* are transferred to the EPM, embryos differentiate. As previously described, embryo development is asynchronous and it is possible to observe embryo clumps with embryos at the globular, heart-shaped, torpedo and dicotyledonar stages. At these stages it is possible to detect green fluorescence (Fig. 1 E, F, G and H). However, in embryos developed under light conditions, as embryos convert, the chlorophyll autofluorescence interferes with the green fluorescence derived from GFP expression (Fig 1 G and H). Sixty somatic embryos in the late-torpedo/dicotyledonary stages were isolated from the *calli* growing under dark conditions, 30 recorded as gfp^+ and 30 as gfp^-, and transferred to fresh ECM until conversion to plantlets. Embryos that were maintained in the dark showed a light pale-yellow colour when observed under white light and those that were putatively transformed present strong green florescence under blue light excitation (Fig 1 I-N).

When previously selected gfp^+ somatic embryos convert to plantlets it was impossible to detect GFP and strong red autofluorescence was observed in the leaves and stem (Fig 1 O and P). However, when these same plantlets developed roots, these non-photosynthetic organs confirm the GFP expression as demonstrated by the fluorescence present under the blue light excitation (Fig 1 Q and R).

Embryo conversion rate was around 40% and a total of 14 gfp^+ plant lines were recovered from the 90 infected leaflets that were maintained in the dark condition. Similar conversion was observed for the embryos selected from the 30 leaflets used as control in which the complete regeneration process occurred in the light condition.

Assays for GUS activity were performed on leaves and roots of all converted plants. All plantlets previously recorded as gfp^+ showed blue GUS staining (Fig. 1 S), providing additional evidence for the transformation of the previously selected gfp^+ somatic embryos.

Binary vectors carrying either *Gus* or *Gfp* reporter genes were already used for *M. truncatula in vitro* transformation and regeneration *via* somatic embryogenesis (Kamaté et al., 2000; Chabaud et al., 1996). Kamaté et al. (2000) described difficulties in detecting GFP expression in *calli* at the embryo initiation phase. Recently, Zhou and co-workers (2004) transformed *M. truncatula* A17 with GUS and GFP constructs and regenerated transgenic plants by direct induction of shoots from cotyledonary nodes. They described severe interference of chlorophyll autofluorescence with GFP, suggesting that the application of GFP to plant research should deserve careful evaluation (Zhou et al., 2005). These authors commented on the difficulty of detecting GFP in the photosynthetic tissues of *M. truncatula*, *O. sativa* and *A. thaliana*. With their *M. truncatula* regeneration system, they observed strong GFP fluorescence in newly regenerating shoots from the cotyledonary node regions of the explants, but this was substantially masked shortly after leaf emergence. Similarly to our results they also observed no striking change in fluorescence for non-photosynthetic organs such as roots.

In our M9-10a transformation procedure using a plasmid containing the coding sequences for the GUS protein (p35S*Adc-Gus*) and a 147 µM kanamycin selective pressure, we were able to recover 22 transformed plant lines (see Chapter IV; Araújo et al., 2004) starting from 140 *in vitro* agro-infected leaflets. Despite the proved efficiency of this optimised kanamycin selection scheme, we found that several somatic embryos bleached or became brown when they were isolated and placed in direct contact with the selective medium. Selective pressure needs to be maintained and embryos ought to be subculture every 1-2 weeks to fresh antibiotic supplemented media to guarantee no escapes, which

imply a high-throughput of hand selection labour. Additionally, we found that the regeneration process in the presence of kanamycin was approximately 1 week slower comparatively to the control without selective pressure. Other authors also reporter the effect of some antibiotics in slower regeneration process (Komarnytsky et al., 2004). The effect of carbenicillin was only observed on the occurrence of secondary somatic embryogenesis and the positive outcome of lowering carbenicillin concentration was discussed (see Chapter IV).

In the present work, the 14 gfp^+ plant lines recovered from the 90 agro-infected leaflets represent similar regeneration competence to that of the selective pressure procedure (Chapter IV). However, the bifunctional reporter GUS-GFP method provides an advantage over the kanamycin selection given that it is faster, less time consuming (because untransformed tissues do not need to be maintained until the selective pressure is effected) despite the need for careful selection under the fluorescence stereomicroscope, and still allows the subsequent verification of the gfp^+ selection process by assaying for GUS activity. This process can benefit with the construction of a transformation vector based on pMP2482 carrying genes of interest within the T-DNA (replacing the *Npt*II gene for instance) together with these fused *Gus::Gfp* reporters.

The difficulty of detecting GFP in chlorophyll fluorescent tissue was overcome by obtaining somatic embryos in dark conditions, prior to selection and transfer to light for conversion to plantlets. For the M9-10a embryogenic line, the induction of somatic embryos in the dark does not compromise its embryogenic capacity or embryo conversion rate. The process of embryo selection can be accomplished at the time of subculture to fresh medium and maintaining the sterile conditions by placing the stereomicroscope (equipped with the appropriate filters) inside the flow cabinet.

5. Conclusions

In conclusion, the selection of transformed *M. truncatula* M9-10a lines using a bifunctional reporter construct is as an alternative scheme for *in vitro* recovery of transformed plants that avoids the use of selective pressure. Taking advantage of the embryogenic potential of the M9-10a line it is possible to perform the induction of somatic

embryogenesis in the dark, overcoming the interference of chlorophyll autofluorescence over GFP fluorescence.

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VII – Somatic embryogenesis and plant regeneration from long-term cell suspension cultures of *Medicago truncatula* cv. Jemalong

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Iantcheva A., Vlahova M., Atanassov A., Duque A.S., Araújo S.S., Santos D.F. and Fevereiro P. (2006). Cell suspension cultures. The *Medicago truncatula* Handbook (ISBN0-9754303-1-9) [http://www.noble.org/MedicagoHandbook/]

1. Abstract

Plants were successfully regenerated *via* somatic embryos from 3-year-old cell suspension cultures of *Medicago truncatula* Gaertn. cv. Jemalong line M9-10a. The cultures were originally initiated from *callus* induced in well-expanded leflets of 30 days *in vitro*-grown plants. Suspension cultures were established in stirred liquid Murashige and Skoog (MS) basal salts and vitamins supplemented with 2.3 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.3 µM kinetin (Kin) and subcultured weekly. Somatic embryogenesis induction step was conducted in liquid MS medium containing 0.45 µM 2,4-D and 0.91 µM zeatin (Zea), during 1, 2 and 3 weeks after subculture. Induced and non-induced cultures were transferred to solid Embryo Proliferation Medium [EPM- MS basal salts and vitamins solidified with 0.2% (w/v) gelrite]. Somatic embryos developed until the late torpedo/dicotyledonary stages. We found that the best condition for the development of somatic embryos was achieved when suspension cultures were not subjected to the induction step. Induction of 1 and 2 weeks led to a decrease in the recovery of somatic embryos and the 3-week treatment resulted in no differentiation of somatic embryos due to the oxidation of the cell suspension. Plant regeneration was obtained in all conditions (except for 3-week induction) when embryos were transferred to an Embryo Conversion Medium [ECM, similar to EPM but solidified with 0.7% (w/v) agar]. Embryo conversion rates were 54.5±1.6%, 52.5±18.5% and 41.6±8.4% for 0, 1 and 2 weeks induction treatments, respectively. These plants were successfully transferred to the greenhouse where they matured and produced seeds.

Key words: Embryogenic competence, cell suspension culture, *Medicago truncatula*, line M9-10a.

2. Introduction

Medicago truncatula Gaertn. become a model species for legume biology studies (Barker et al., 1990; Cook, 1999) due to its small genome [500-600 Mdp/1C (Blondon et al., 1994)], short life cycle, diploidy (2n=16), autogamous fertilization and the availability

of diverse germplasm. Jemalong is one of the most commonly used cultivar in agricultural practices.

Somatic embryogenesis in legumes is known to be strongly genotype-dependent (for review see Somers, 2003) in Jemalong only some selected lines/genotypes (e.g. A17, 2HA, J5, and M9-10a) have embryogenic capacity (Chabaud et al., 1996; Thomas et al., 1992; Rose et al., 1999; Kamaté et al., 2000; Araújo et al., 2004).

The line M9-10a was isolated in our laboratory and is currently used for genetic transformation (Araújo et al., 2004). Although transformation of M9-10a using *in vitro* leaf explants is very efficient, there is a requirement of large space in growth chambers to maintain an adequate stock. Moreover, *in vitro Medicago truncatula* plants follow the plant annual rhythms and their growth and physiological state decay during summer (unpublished results). We propose using embryogenic cell suspension as an alternative system for *in vitro* selection of mutants, mass propagation, and gene transfer.

The aim of this study was to establish the conditions for plant regeneration *via* somatic embryogenesis starting from a well-established cell suspension culture of the *M. truncatula* cv. Jemalong line M9-10a. We investigated the effect of different induction periods (1, 2 and 3 weeks) on the production of somatic embryos and on embryo conversion ability.

3. Materials and Methods

3.1. Plant material and culture media

Cell suspension cultures were established from *callus* derived from leaflet explants of *M. truncatula* Gaertn. cv. Jemalong line M9-10a (Neves et al., 1999; Santos and Fevereiro, 2002). Stock plants were maintained *in vitro* on a growth regulator-free medium, with a 16-h photoperiod of 100 $\mu mol.m^{-2}.s^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24º/22ºC (Neves et al., 2001). Murashige and Skoog (1962) (MS) basal salts and vitamins medium supplemented with 3% (w/v) sucrose was used in all experiments. The pH of the media was adjusted to 5.8 before autoclaving

(121ºC, 20 min.). Growth regulators were filter sterilised through 0.2 μm filters (Whatman, Maidstone, England) and added to autoclaved media.

Suspension cultures were obtained from *callus* induced in well-expanded leaflets from 30 days *in vitro*-grown M9-10a plants. For *callus* induction, leaflets were wounded perpendicularly to the midrib with a sterile scalpel blade and placed on solid MS medium supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.1 μM zeatin (Zea). *Callus* cultures were maintained in a growth chamber at 23^{o}C in the dark (Heraeus, Hanau, Germany). To initiate cell suspension cultures, 2 g of friable *callus* were separated with a sterile-scalpel blade and transferred to Erlenmeyer flasks containing 100 ml of liquid MS medium supplemented with 4.5 μM 2,4-D and 4,6 μM kinetin (Kin). Initially, and until an adequate cell density was obtained, the cells were pelleted and the medium was replaced by an equal volume of fresh medium every week. Throughout this process, *callus* began to dissociate into small cell clumps and single cells. Cell suspension cultures were then subcultured every 7-day in liquid MS medium supplemented with 2.3 μM 2,4-D and 2.3 μM Kin. These cultures were maintained in 500 ml Erlenmeyer flasks in 100 ml of medium per flask, agitated (124 rpm) in an orbital shaker (Innova 4900, New Brunswick Scientific, Nürtinger, Germany), in the dark, at 24^{o}C, for the last 3 years. Subcultures were carried out by transferring 10 ml of the cell suspension culture at exponential growth phase to 100 ml of fresh medium.

3.2. Somatic embryogenesis induction and plant regeneration

For somatic embryogenesis induction experiments, 1-week-old cell suspension cultures were filtered through a 1050 μm nylon mesh, rinsed twice with fresh medium and transferred to growth regulator-free liquid MS medium for one additional week. Previously, we were able to induce somatic embryogenesis from leaf-explants of line M9-10a by including a 3 week induction step on solid media supplemented with 0.45 μM 2,4-D and 0.91 μM Zea (see Chapter IV). In this study, induction of somatic embryos was tested in liquid MS medium (EIM - Embryo Induction Medium) supplemented with 0.45 μM 2,4-D and 0.91 μM Zea, during a period of 1, 2 and 3 weeks. Three samples of 1 ml from treated and non-treated embryogenic cultures were transferred to solid Embryo

Proliferation Medium [EPM – MS with 0.2% (w/v) gelrite (Duchefa, Haarlem, The Netherlands)] where somatic embryos developed until late torpedo/dicotyledonary stages. For dry weight (DW) assessment, three samples of 5 ml of each suspension culture were also transferred to paper filters (Whatman, Maidstone, England), dried at 70°C for 4 d and then weighed.

Embryos ready to be isolated were transferred to Embryo Conversion Medium [ECM, similar to EPM but solidified with 0.7% (w/v) microagar (Duchefa, Haarlem, The Netherlands) instead of gelrite]. Every 2 week, green somatic embryos were transferred to fresh ECM until conversion to plantlets. After 2-3 months, regenerated plants with a well-developed root system were potted in vermiculite, covered with a polyethylene film (Silvex® wrap, Benavente, Portugal) for acclimatisation and placed in a growth chamber. After 1 week, the plants were transferred to 2.5 L pots containing a commercial organic soil (Montemor soil, A Estufa, Lisbon, Portugal) and grown to maturity in the greenhouse.

During the process of somatic embryogenesis induction and embryo conversion, the growth behaviour of the cell suspension cultures was followed over the time under a stereomicroscope (Leica Wild MZ8, Heerbrugg, Germany).

For induction treatments, embryo development, *in vitro* plant growth and plant acclimatisation, cultures were maintained in light conditions with 16-h photoperiod of 100 $\mu mol.m^{-2}.s^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24º/22ºC (Phytotron EDPA 700, Aralab, Parede, Portugal).

3.3. Data analysis

For each treatment three replicates were performed. Single Factor Analysis of Variance (ANOVA) was carried out to test the significance between the means of different induction treatments and the control (cultures not subjected to induction treatment) at a significant level of $p < 0.05$.

4. Results and Discussion

Dark-yellow *callus* developed on the edges of inflicted wounds in leaflet explants after 30 days of culture on solid MS medium supplemented with 4.5 μM 2,4-D and 9.1 μM Zea. Cell suspension cultures (Fig. 1A, B) consisted of single cells and cell aggregates up to 2.5 mm of small spherical cells, containing dense cytoplasm (this type of cell is usually described as an embryogenic competent cell) and a few elongated cells. The same characteristics and growth patterns were observed in our laboratory over the past 3 years.

After 1 week of induction in all the tested conditions, embryogenic cell suspension cultures developed green proembryogenic masses (Fig. 1C, D). After transfer to gelrite-solidified medium without growth regulators, embryogenic cell clusters started to develop somatic embryos (Fig. 1E-G). Cultures not subjected to induction also presented green proembryogenic masses after transfer to solid medium (Fig. 1F). No somatic embryos could be obtained with the 3-week induction treatment due to the oxidation of the culture after transfer to solid medium, most likely because the cell suspension culture was already at the stationary growth phase and the amount of viable cells could be lower (see Fig. 2E).

In the other treatments, a vast proliferation of somatic embryos was observed on solid medium (Fig. 1G-I). The development of somatic embryos was asynchronous, and several stages of embryo development could be observed simultaneously (Fig. 1G, I). After 4 weeks on EPM, somatic embryos could be isolated and transferred onto ECM. In these conditions and for the subsequent 2 weeks, all somatic embryos developing to a late torpedo/dicotyledonary stage were transferred to ECM and counted. The total number of somatic embryos recovered, somatic embryos that developed secondary embryos and somatic embryos that were able to convert into plants were considered (Table 1). Somatic embryos converting to plants are presented in Figure 1 (J - L). The regenerated plants (Fig. 1M) were successfully acclimatised in vermiculite (Fig. 1N).

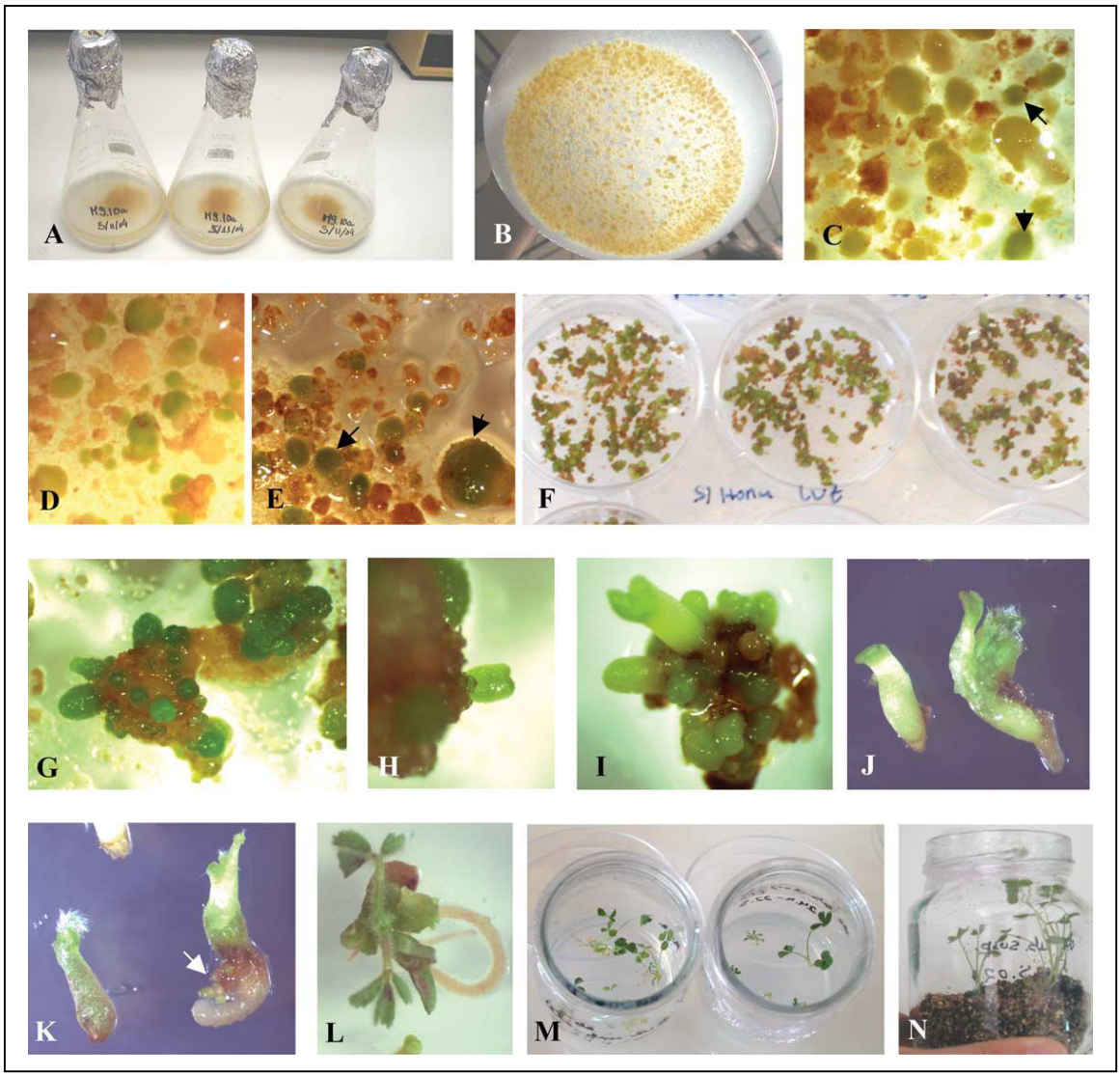

Figure 1. Somatic embryogenesis and plant regeneration from cell suspension cultures of *Medicago truncatula* cv. Jemalong line M9-10a.
(A) One-week-old cell suspension cultures; **(B)** Cell suspension culture after subculture; **(C)** Embryogenic cell suspension culture after 1 week induction treatment, presenting green proembryogenic masses (*arrows*); **(D)** Embryogenic cell suspension culture after 2-week induction treatment; **(E)** Transfer of 1 ml cell suspension to solid MS medium (without growth regulators) after 2-week induction treatment (*arrows:* several green proembryogenic masses); **(F)** Cultures not subjected to induction treatment, 2 weeks after transfer to solid medium; **(G)** Embryogenic clusters developing somatic embryos in gelrite-solidified medium without growth regulators; **(H)** Heart-stage somatic embryo; **(I)** Cluster of somatic embryos at different stages of development; **(J)** and **(K)** Somatic embryos on MS agar-solidified medium (*arrow* indicates secondary embryogenesis); **(L)** Embryo conversion to plant on agar medium; **(M)** Plantlets (3-week-old) on agar medium; **(N)** Rooted plants (2-month-old) in vermiculite before transference to the greenhouse.

We were able to obtain 84.5±9.1 (mean±SD) somatic embryos.ml^{-1} of suspension culture without the embryo induction step and 46.7±8.7 and 26.0±6.1, respectively, after 1 and 2 weeks of the induction treatment. Corresponding, respectively, to 5.8±1.0, 2.1±0.2 and 1.0±0.2 somatic embryos.mg^{-1} DW of suspension culture (Table 1).

The dry weight (DW) of the cells suspension cultures are graphically represented in Figure 2.

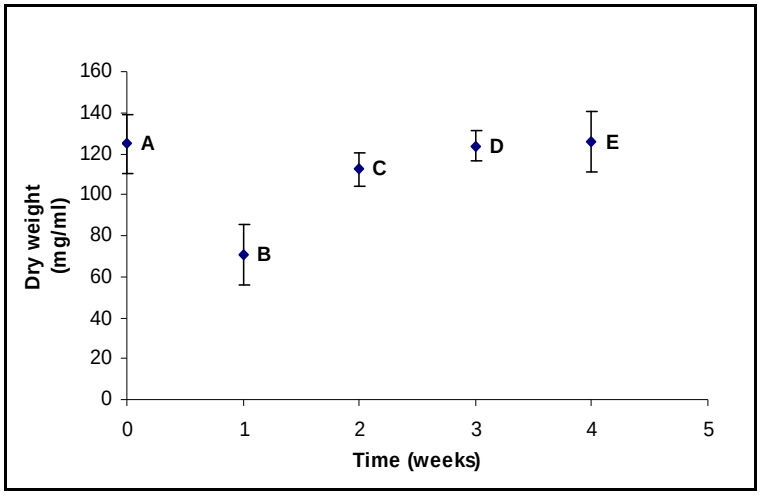

Figure 2. Dry weight (DW) of the cell suspension cultures (CSC). **(A)** One-week-old cell suspension cultures (CSC) before weekly subculture to fresh medium; **(B)** Non-induced CSC after 1 week in growth regulators-free medium, before transfer to solid medium; **(C)** CSC after 1 week induction treatment, before transfer to solid medium; **(D)** CSC after 2-week induction treatment, before transfer to solid medium; **(E)** CSC after 3-week induction treatment, before transfer to solid medium. Data represent the means of three replicated samples. Standard deviations were included.

Frequency of embryo-to-plant conversion from cell suspension cultures was not significantly different at $p<0.05$ for all situations (Table 1). Plant regeneration was achieved in all conditions except for the 3-week induction treatment. These plants showed no phenotypic abnormalities when transferred to the greenhouse where they were grown to maturity and produced seeds.

Table 1. Somatic embryogenesis from cell suspension cultures of *Medicago truncatula* cv Jemalong (line M9-10a) exposed to different induction conditions.

Weeks in EIM	Number of SE/ ml suspension culture	Number of SE/mg DW suspension culture	Secondary SE (%)	SE conversion (%)
0	84.5±9.1[a]	5.8±1.0[a]	19.6±1.2[a]	54.5±1.6[a]
1	46.7±8.7[b]	2.1±0.2[b]	23.4±8.0[a]	52.5±18.5[a]
2	26.0±6.1[c]	1.0±0.2[c]	29.3±6.9[a]	41.6±8.4[a]

Values represent the mean (± standard deviation) of three replicate samples. Different superscripts within columns indicate statistical significance ($p<0.05$). EIM-embryo induction medium; DW-dry weight; SE-somatic embryos.

In this work we established the conditions for plant regeneration *via* somatic embryogenesis from 3-year-old cell suspension cultures of *M. truncatula* cv. Jemalong line M9-10a.

Iantcheva et al. (2001, 2005) described the establishment of fine cell suspension cultures using leaf and root explants of *Medicago truncatula* cvs. R108-1 and Jemalong (obtained from seed collection). Somatic embryos of the R108-1 cv. were regenerated from cell suspension cultures initiated from root explants placed in EIM (Gamborg B5 liquid medium supplemented with 2,4-D and kin). These cultures maintained their embryogenic potential for only a few subcultures (4-5 passages) in the same medium (Ianthcheva et al., 2005). For the Jemalong cv. cell suspension cultures were also obtained with the same process but no plant regeneration was reported. In our study, 3-year-old well-established Jemalong cell suspension cultures maintain their embryogenic potential until now, suggesting that this ability is persistent in this line.

We found that the best conditions for somatic embryogenesis were achieved when cell suspension cultures were not subjected to induction. Apparently, when growing in the maintenance medium, which is supplemented with 2,4-D and Kin, cells acquire embryogenic competence and are able to develop a large number of somatic embryos upon transfer to growth regulator-free media. Exogenously applied auxins (preferably 2,4-D) for the induction of somatic embryos is well documented (for review, see Fehér et al., 2003).

The timing of subculture is especially critical in cell suspension cultures. Frequent subculturing can effectively minimize the extent of chromosomal changes in cell cultures and maintain embryogenic potentiality (Ammirato, 1984). Therefore, the transfer of the cells to fresh medium at an exponential growth phase could be responsible for the lasting embryogenic capacity in our cell line.

We could observe the development of secondary somatic embryos after subculture of embryos at the late torpedo/dicotyledonary stage, but no statistical significant difference was found among treatments. Various authors reported this phenomenon (e.g., Durham and Parrott, 1992; Neves et al., 1999; Little et al., 2000), and in our line, the development of secondary somatic embryos also affects the rate of embryo-to-plant conversion (Araújo et al., 2004). We could minimize this disadvantage by a strict 2-week subculture routine and by placing the somatic embryos in the upright position on the ECM.

5. Conclusions

Our group and other research groups are currently using line M9-10a for transformation purposes. Although plant regeneration via somatic embryogenesis from *in vitro* cultured leaf-explants is very efficient, there is a constraint on availability of leaflets of M9-10a, because of *in vitro* culture space limitations and physiological adequacy due to annual rhythms (observed in the *M. truncatula* plantlets maintained *in vitro*). Clear advantages of the present protocol are the constant availability of embryogenic-competent cells and the relative ease of scaling up this protocol. Moreover, an approximately 3 week reduction in the time to obtain plantlets is achieved compared to published protocols of somatic embryogenesis from leaves (Nolan et al., 1989; Chabaud et al., 1996; Araújo et al., 2004), due to the elimination of the induction step. For these reasons, we consider this an efficient alternative procedure for regeneration of *Medicago truncatula* cv. Jemalong *via* somatic embryogenesis.

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VIII – General Discussion

We used the *Adc* gene from *Avena sativa* to overexpress an heterologous ADC enzyme in *Medicago truncatula* cv. Jemalong aiming to increase the levels of polyamines in transgenic plants in order to assess its effect on plant stress resistance.

Here is provided a general discussion that integrates all the results of the research work presented in this thesis, connecting it to the information available in the literature. It is also drawn a summary of the main conclusions and the perspectives that are laid open for the future.

1. Construction of plant transformation vectors and optimisation of transformation/regeneration protocols

Several protocols of plant regeneration through somatic embryogenesis have been published for *M. truncatula* (Thomas et al., 1992; Chabaud et al., 1996; Rose et al., 1999; Kamaté et al., 2000), but regeneration is highly genotype dependent. In our laboratory we obtained a highly embryogenic line of the agronomically important cultivar Jemalong, named M9-10a (Neves et al., 1999; Santos and Fevereiro, 2002), which could be successfully used for plant transformation and regeneration *via* somatic embryogenesis. However, an optimised transformation/regeneration protocol was needed to fulfil our broad aim of studying the expression of drought-related genes in this cultivar.

The first vector to be constructed for *M. truncatula* transformation was plasmid p35SAdc (Chapter II). In the first transformation experiments, using the process described by Neves (2000), despite of the large number of somatic embryos isolated from initial *callus* tissue (1186) and of the reasonable number of plants (a total of 60) that were able to root in kanamycin supplemented medium, no transgenic lines could be obtained, as attested by Southern blot hybridization results (not presented in this thesis). The 60 putative transformed lines were escapes from the selection procedure due to the *M. truncatula* natural resistance to kanamycin (Christou, 1994; Trieu et al., 2000). These

results indicated that the kanamycin concentration originally used by Neves (2000) was not sufficient for a stringent selection of *M. truncatula* lines transformed with the *npt*II gene and prompted us to revise the original selection procedure (Chapter III).

The optimal concentration of kanamycin to be used for *in vitro* transformation of M9-10a was found to be the double of that previously tested (171.6 μM instead of 85.8 μM). For the analysis of seed segregation, we chose to use the highest concentration tested (686.6 μM) to assure the selection efficiency (Duque et al., 2004).

In parallel to the optimisation of the kanamycin concentration, we decided to introduce the β-glucoronidase reporter gene (GUS; Jefferson et al., 1987) in the original plasmid construct p35SAdc to assist in detection of the transgenic plants. The resulting vector was designated p35SAdc-Gus and was used for the subsequent transformation experiments of *M. truncatula* cv Jemalong line M9-10a, as described in Chapter IV.

Together with the kanamycin concentration, several other aspects were found to be critical to the successful transformation of *M. truncatula* cv Jemalong line M9-10a and were introduced in the original transformation/regeneration protocol described by Neves (2000). The use of the hypervirulent *A. tumefaciens* strain EHA105 and a co-culture period with acetoseryngone in the dark, together with a strict selection of transgenic tissues with 171.6 μM kanamycin and the stage that somatic embryos were isolated to an agar-solidified medium with low concentration of carbenicillin were major improvements to the original method, making it less laborious and more efficient. In about 4 months of *in vitro* culture, we could recover a total of 22 Adc-Gus T_0 transgenic lines that were fertile and resulted from independent transformation events. Seeds of six T_0 lines were germinated in 686.6μM kanamycin-containing medium and showed a Mendelian segregation pattern of 3:1, corresponding to a dominant gene at a single *locus*.

These data together with the results from the Southern blot analyses demonstrated that the T-DNA region was stably inserted in the genome of the *M. truncatula* primary transformants and was transferred to the T_1 progeny. Independent T_0 transgenic lines were further analysed by RT-PCR and demonstrated to express the oat *Adc* transgene. Using this improved method, an average of 1-2 T_0 transgenic plants could be obtained per Kan^R embryo producing *callus*, independently of the plasmid construct used for plant

transformation, as reported in a combined work using p35SAdc-Gus and p35SDsp22 (Araújo et al., 2004).

We also developed an alternative scheme for *in vitro* selection of transgenic *M. truncatula* M9-10a lines, that avoids the use of a negative selection, normally performed with antibiotics or herbicide, by using a bifunctional reporter gene construct in which the genes encoding the green fluorescent protein (GFP) and the beta-glucuronidase (GUS) were fused and placed under the control of a unique CaMV 35S promoter (Quaedvlieg et al., 1998). This alternative method exploits the advantage of GFP as a vital marker for early embryo selection coupled with the advantage of GUS for subsequent confirmation of the transgenic state of the selected plant lines (Chapter VI; Duque et al., 2007).

The same *Agrobacterium*-mediated transformation and regeneration protocol described in Chapter IV was used with some modifications. The modifications consisted on performing the induction of somatic embryogenesis in dark conditions until selection of the somatic embryos for conversion and also the exclusion of antibiotic for plant selection. The embryogenic potential of our line M9-10a was not affected by the induction in the dark and it was possible to overcome the interference of chlorophyll autofluorescence over GFP fluorescence. In conclusion, selection of transformed *M. truncatula* lines based on a bifunctional reporter construct could serve as an alternative scheme for *in vitro* selection of transgenic plants that avoids the usage of an antibiotic or herbicide for transgenic tissue selection. This process is called positive selection of transgenic plants (Joersbo and Okkels, 1996) in which non transformed cells are not killed, but transformed cells experience metabolic or development advantages (Hohn et al., 2001).

In all the experiments so far discussed, the M9-10a plant donor material was maintained by micropropagation as described in Neves et al. (2001). Advantage of the use of leaf-explants obtained from micropropagated material was the permanent availability of sterile explants for regeneration/transformation experiments without the need to carry out decontamination washing steps always detrimental to the good condition of the explants.

However, there is a requirement of large space in growth chambers to maintain an adequate stock of plants. Moreover, we found that *in vitro* grown *M. truncatula* plants

follow the annual rhythm of the plants in nature and their growth and physiological state diminish during summer, compromising any attempt to transform plants during this time of the year.

To overcome these constraints, we decided to find the conditions for plant regeneration *via* somatic embryogenesis starting from a well-established cell suspension culture of line M9-10a (Chapter VII) that have been maintained in our laboratory for over 3 years and were routinely used for protein production. We found that the best conditions for the development of somatic embryos were achieved when cell suspension cultures were not subjected to the growth-regulators-treatment to induce somatic embryogenesis. Apparently, when growing in the maintenance medium, which is supplemented with 2,4-D and Kin, cells acquire embryogenic competence and are able to develop a large number of somatic embryos upon transfer to growth regulator-free media. Advantages of this protocol are the constant availability of embryogenic-competent cells and the relative ease of scaling up. Moreover, an approximately 3 weeks reduction in the time to obtain plantlets is achieved due to the elimination of the induction step. For these reasons, somatic embryogenesis and plant regeneration from cell suspension cultures was considered an efficient alternative regeneration system that can be used for *in vitro* selection of mutants, mass propagation and gene transfer experiments (Duque et al., 2006).

The optimisation of an *Agrobacterium*-mediated transformation protocol starting from M9-10a cell suspension cultures was carried-out at the Plant Cell biotechnology Laboratory and, although not yet published, results show the possibility of mass production of transgenic *Medicago truncatula* somatic embryos using cell suspension cultures (Zhang and Fevereiro, in preparation) and a selection scheme based on fused GFP and GUS reporters (Duque et al., 2007; Chapter VI) for confirmation of transformed somatic embryos.

2. Analysis of the biogenic amines alteration in *Adc* transgenic *M. truncatula* plants

As far as we know this is the first time that the model legume *M. truncatula* is genetically engineered with genes that code for enzymes involved in the polyamine biosynthetic pathway.

A significant accumulation of free Agmatine is relatable to the increase of free Put and Spd levels in plants expressing the heterologous *Adc* transgene. Moreover, additional determinations of PAs in soluble bound (synonymous of conjugated) and insoluble bound fractions revealed the increase of Put in the soluble bound fraction and the detection of linked Agm in the insoluble bound fraction. Overall, and in respect to total amine content, we found that Put was increased 1.7-fold, Spd was 1.9-fold higher and Agm had the higher increase (22-fold) in *M. truncatula* cv Jemalong plants over expressing oat ADC compared to control plants. We were also able to separate other important biogenic amines, besides PAs, like the monoamine histamine (His) and additional amines, cadaverine (Cad) and tyramine (Tyr). Our results suggest a relationship between augmented PAs, in L108 transgenic line, and a concomitant increase in His (see Chapter V; Fig. 1); but the interplay between these compounds still needs further investigation.

The oat *Adc* cDNA under the control of a CaMV 35S constitutive promoter was previously transferred into rice plants (Capell et al., 1998), and these authors found increased putrescine levels in regenerated plants, however, Put accumulation affected *in vitro* development patterns of their transgenic rice plants. Apparently, no altered external morphology was observed in our *M. truncatula Adc* transgenic T_0 plants, that were successfully transferred to greenhouse were they developed without phenotypic visible alterations and produced seeds.

More recently, the same group, used a strong monocot maize *Ubi*-1 promoter to overexpress the *Datura Adc* gene and found that transgenic plants, with increased Put levels, were tolerant to drought stress (Capell et al., 2004). The *Ubi*-1 promoter is known to contain a number of stress-responsive elements that enhance transgene expression under drought stress (Christensen and Quail 1996 in Capell et al., 2004) and hence function as a stress-inducible promoter.

In a different system, Prabhavathi and Rajam (2007) found that transgenic eggplants (*Solanum melongena*) accumulating PAs exhibit increased tolerance levels to multiple abiotic stresses (salinity, drought, low and hight temperature, heavy-metal) and also biotic resistance against fungal disease caused by *Fusarium oxysporium*. They concluded on the feasibility of PA biosynthesis engineered for the production of stress-tolerant plants. These authors used a construct similar to ours, with the *Adc* gene from oat

under the control of the constitutive CaMV35S promoter and found that some transgenic eggplants lines showed an enhanced level of Put, Spd and in some cases also Spd. These lines also showed increase in ADC and also on the activity of the PA catabolic enzyme, DAO (Prabhavathi and Rajam 2007). There are several reports in which the plant response to abiotic stress (such as drought, salinity, heat and osmotic stress) is associated with stimulation of polyamine oxidation (review in Cona et al. 2006). However, the precise role of polyamine catabolism in the plant response to environmental stress remains elusive (Cona et al. 2006; Angelini et al., 2010).

Prabhavathi and Rajam (2007) analysed PAs levels in seedlings using TLC separation of dansylchoride derivatives and proceeded to the quantification of free, bound and conjugated PAs, although further discussion on the significance of those differences is lacking on their study. Unfortunately, in most of the studies no information is given about conjugated and bound PAs (Martin-Tanguy et al., 2001). This overall picture is of great importance, since an increase in bound polyamines, either cell wall bound or conjugated to phenolics, could account for a reduction in the levels of free PAs (Groppa and Benavides, 2008).

The ion-pair Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) method with *o*-phtaldialdehyde/2-mercaptoethanol (OPA/MCE) post-column derivation here reported allows detection and quantification of biogenic amines in plant vegetative tissues and the automatic accomplishment of the derivatiszation, which reduces time and effort of analysis and simplifies the sample preparation (see Chapter V). In our opinion, this procedure will be useful for further analysis of the role of biogenic amines in plants subjected to both abiotic and biotic stress responses and also in elucidating the interaction of polyamine metabolism with other metabolic routes.

3. Possible methodologies to evaluate water deficit resistance in *Adc* transgenic *M. truncatula* plants

Preliminary results of water deficit resistance in transgenic plants not present in this thesis, with ten replicates of control (M9-10a) and transgenic (L108) line, demonstrated that growth performance in well water conditions and after 10 days of water withdraw was not significantly different for both lines. Criteria like shoot and root length, number of internodes, and secondary brunches and also Relative Water Content (RWC) were evaluated. Preliminary assays also demonstrated that 10 days was the time needed for Relative Soil Water Content (Rswc) (calculated according to Pinheiro et al., 2005) lowered to 11.13±1.3 (mean values ± standard deviation of seven replicate samples) and for plants to present dehydration signals (wilted leaves and prostrated steams). The *M. truncatula* plants were grown in soil-containing pots and were subjected to slow and progressive water deficit, imposed by ceasing watering as described in Araújo (2007) and Nunes et al. (2008; 2009). Pot experiments are thought to produce results of water-deficit evaluation strongly correlated with those obtained in field trials (Khan et al., 2007).

Possible methodologies to evaluate water deficit resistance in transgenic plants include: measurement of chlorophyll *a* fluorescence parameters, leaf gas exchange analysis and quantification of photosynthetic pigments; these methodologies are widely used in the evaluation of plants and cultivars subjected to drought and could be applied to our *Adc* transgenic lines.

These methodologies were recently used with success for addressing abiotic stress tolerance in several lines of tobacco transformed with the trehalose-6-phosphate synthase gene from *Arabidopsis thaliana* (Almeida, 2005; Almeida et al., 2005; Almeida et al., 2007); and also to assess the physiological performance under water stress of transgenic *M. truncatula* expressing the ELIP-like Dsp22 from *Craterostigma plantagineum* (Araújo, 2007). In non-transgenic *M. truncatula* cv. Jemalong mature plants the mechanism of drought avoidance (that includes an efficient stomata regulation) and those of drought tolerance (involving processes at the cellular level, particularly osmotic adjustment) were evaluated through gas exchange and chlorophyll *a* fluorescence measurements as well as through water relations and membrane integrity determinations by Nunes et al. (2008).

This study contributed to identify useful physiological traits to be used as control for the behavior of plants transformed for increased drought resistance.

Photosynthesis light curves were also found to be an expeditious and non-destructive approach to discriminate plant photosynthesis performances and to screen and select water stress resistant or non-resistant *M. truncatula* lines (Nunes et al., 2009). Preliminary studies with two *M. truncatula* transgenic lines (Dsp22 and Adc) showed no improvement in photosynthetic performance under water stress induced by rapid dehydration (Nunes et al., 2009). However, it is possible that the transgenic lines may show a better performance under more prolonged periods of WD than those employed (in this experiment only 4–5 days of withholding water (WD) were tested) (Nunes et al., 2009). In fact, Araújo (2007) found both in M9-10a and Dsp22 transgenic line (A.27), that a severe level of water stress was only obtained after 10-12 days (in the described conditions of water deprivation and according to a relation established between Rswc and leaf RWC) when Rswc reach values below 15%.

It was found that in plants under a long period of water deficit re-watering could become more deleterious than the stress imposed by drought (Speer et al., 1988), in part due to the damage caused on membranes structure. The ability of a plant to recover from water deficit should be considered an important aspect when addressing plant improvement toward drought stress (Araújo, 2007) and should be taken in account when designing experimental procedures. Recently, transgenic rice plants expressing SAMDC, with increased Spd and Spm levels, were found not drought tolerant, however showed a more robust recovery from drought compared to wild type (Peremarti et al., 2009). As plants with elevated putrescine are able to tolerate water stress because Put has a direct protective role in preventing the symptoms of dehydration the higher PAs (Spd and Spm) appear to play an important role in drought recovery.

Several commonly used techniques for quantifying resistance to drought and salt, like: a) short-term avoidance of water using detached leaves; b) soil drying of pot-grown plants; c) low-Ψ_w treatment using PEG-infused agar plates; d) seed germination in salt-containing agar plates; e) salt-induced leaf damage using leaf disks; f) quantification of ions in plant tissue; g) root and shoot growth (root fresh and dry weight, leaf area, leaf

expansion, time of flowering, seed yield; among others); were recently reviewed in Verslues et al. (2006) and could be further applied to our transformed *Adc* lines.

4. Future perspectives and final conclusions

The use of tissue-specific or organelle-specific promoters for transgene expression is also an important consideration for future works. For example, osmolytes accumulated in leaf tissue would not be beneficial to root under osmotic stress (Bajaj et al., 1999). There is a possibility that the product of the transgene is needed in a specific cellular compartment or there may be a change in the compartmentalization of the concerned protein following stress (Grover et al., 2003). There is the future need to change PAs levels in various tissues by expression of genetic constructs with copies of genes encoding enzymes of PA syntheses and/or degradation under the control of tissue-specific promoters (Kuznetsov et al., 2006).

Another interesting approach could be the use of stress inducible promoters that might be a manner to overcome eventual problems related to the accumulation of polyamines. As defended by some authors, the constitutive overexpression may not be the appropriate way to obtain stress tolerance since in some situations polyamines appear to be toxic to the plants under normal conditions (Bartels and Sunkar, 2005). A stress inducible promoter, the promoter C_2 from the resurrection plant *Craterostigma plantagineum* (kindly provided by Dr. Dorothea Bartels, Institute of Molecular Physiology and Biotechnology of Plants, Bonn), is currently being tested in our *Medicago truncatula* system by transient expression to evaluate the possibility of using this promoter in future constructs for plant transformation. Additionally, the plant transformation vector pGreen0229-p*rab*17-t35S, that contains the stress inducible *rab*17 (responsive to absicic acid 17) promoter, was constructed in our laboratory by Araújo (2007) and is available for further genes constructs aiming the plant transformation using stress regulated genes. This promoter was previously tested in heterologous tobacco, rice and Arabidopsis systems (Vilardell et al 1991 and Vilardell et al., 1994 in Araújo 2007)

Studies like the described in Page et al. (2007) for poplar cell lines, in which the effect of genetic manipulation of Put was analysed in terms of transcription (by quantitative analyses of gene expression; qPCR) and activity of key enzymes involved in PA biosynthesis (ODC, ADC, SAMDC, SPDS and SPMS), will be important to elucidate mechanisms of PA metabolic regulation. Examining the effects of modulating a single step in PA metabolism on other branches of the pathway will provide powerful information for modelling PA metabolism regulation and the metabolism of related compounds; e.g. Proline, Arginine, γ-aminobutiric acid, ethylene (Page et al., 2007) and possibly, histamine.

The role of PAs metabolism for the abiotic stress tolerance is just beginning to be understood. High throughput analysis including microarray, transcriptomics, metabolomics, reverse genetics approaches will be essential to understand the involvement of PAs biosynthetic pathways in abiotic stress tolerance. Also, analysis of interacting partners of the PAs biosynthetic pathways will be fundamental to better understanding the mechanism of stress tolerance.

As a conclusion, the obtained Adc transgenic lines coupled with the optimized ion-pair RP-HPLC described procedure, for detecting and quantifying biogenic amines in vegetative tissues of *M. truncatula* cv Jemalong, will be useful for the analysis of the role of these biogenic amines in abiotic stress responses and also in elucidating interaction of polyamine metabolism with other metabolic routes. These transgenic lines were shown not to have any phenotypic alteration and were fertile, being available for further investigation on the role of polyamines in abiotic stress responses in plants.

The obtaining of these transgenic lines was possible because we developed an improved protocol for *Agrobacterium*-mediated transformation of *M. truncatula* cv. Jemalong that includes several aspects and considerations that are critical to the successful of transformation. Alternative systems for M9-10a plant selection (without antibiotics) and regeneration (from cell suspension cultures) were also developed.

Our group and others research groups are currently using with success the line M9-10a and the optimised protocol presented in this thesis for transformation purposes: with genes related to nodulation (Liang, 2006); for production of recombinant proteins (Sylvain, 2005; Abranches et al., 2005; Pires et al., 2008); for metabolic engineering studies related to the triterpene saponin pathway (Confalonieri et al, 2009); for the development of a *Medicago* P-DNA-based (plant-derived transfer DNA-based) vector system for production of backbone-free transgenic lines (Confalonieri et al., 2010); for expression of genes related to drought tolerance (Araújo et al., 2004; Araújo, 2007, Nunes et al., 2009) and also for modulation of stress responses by transformation with newly discovered *M. truncatula* miRNAs, differentialy expressed in plants subjected to water deficit (Trindade et al., 2009).

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