GENETIC MANIPULATION OF AGRONOMICALLY IMPORTANT TRAITS IN *LILIUM*

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ABSTRACT

The ornamental industry has become an important economic force in recent years, in the UK alone this industry is estimated to be around £2.1 billion, while the international trade is around £60-75 billion (Chandler and Tanaka, 2007). The continued success of the floriculture industry depends on the introduction of new species/cultivars with major alterations in key agronomic characteristics, such as resistance to pathogens, novel flower colour and patterns or control of male fertility. *Lilium*, one of the most important bulbous ornamental crops, is an attractive and popular cut flower. However, the production of vast quantities of pollen that stains easily and is toxic to animals is not always desirable. The control of pollen release without affecting the appearance of the flower is therefore an important breeding goal. *Lilium* is also susceptible to several fungal pathogens, including *Botrytis cinerea*, which infects leaves, stem and flowers leading to a reduction of yield. New cultivars have tended to rely upon selective breeding as a mechanism for trait development. However approaches that utilise transgenes to manipulate traits of interest provide alternative opportunities for the ornamental industry provided that transformation and regeneration can be achieved efficiently.

A rapid, highly efficient and reproducible *Agrobacterium*-mediated transformation for *Lilium* has been developed. Successful transient GUS expression in callus, shoots and basal plate discs was achieved using *A. tumefaciens* strain AGL1 containing plasmid pBI121 harbouring intron-containing *GUS* and *NPTII* genes in cultivars "Beverly's Dream", "Star Gazer", "Night Flyer", "Acapulco", "Sweet Surrender" and Lilium leichtlinii.

Based on the same transformation protocol, transgenic plants of cv. "Star Gazer" overexpressing the *RCH10* chitinase gene from rice were generated. *In vitro* sporulation assays of these plants showed different levels of resistance to *Botrytis cinerea* correlated to the level of relative expression of the transgene. This is the first report of induced pathogen resistance in any *Lilium* cultivar by transgenic approach.

Experiments were also conducted to modify fertility and pollen release in *Lilium* by translating regulatory gene information from *Arabidopsis* to *Lilium*. Transgenic plants of cv. "Star Gazer" either overexpressing or silencing the *AtMYB26* gene, were generated. RNAi lines showed a delay in anther dehiscence suggesting that pollen development pathways could be conserved between *Arabidopsis* and *Lilium*. In addition, partial sequences of the putative orthologues of *AtMS1* and *AtMYB26* in *Lilium* were identified and cloned for future research.

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
µl	Microlitre
µm	Micrometre
µM	Micromolar
µg/µl	Micrograms per microlitre
AS	Acetosyringone
AT	Asiatic/Trumpet
BAP	6-Benzylaminopurine
bp	Base pairs
CC	Co-culture
CDS	Coding Sequence
CI	Callus induction
cm	Centimetre
cv	Cultivar
g	Gravity
GA	Gibberellic Acid
g/L	Grams per litre
GM	Genetically modified
GMO	Genetically modified organism
ITS	Internal transcribed spacer region
LO	*Longiflorum*/Oriental
LA	*Longiflorum*/Asiatic
M	Molar
mg	Milligrams
mg/L	Milligrams per litre

ml	Millilitre
mM	Millimolar
mm	Millimetre
MS	Murashige and Skoog
MSZ	Murashige and Skoog medium plus Zeatin
NAA	1-Naphtalene acetic acid
ng	Nanograms
ng/µl	Nanograms per microlitre
OA	Oriental/Asiatic
OD	Optical density
OT	Oriental/Trumpet
pmol	Picomolar
pmol/µl	Picomol per microlitre
psi	Pounds per square inch
rpm	Revolutions per minute
SC	Subculture
SL	Selection
UV	Ultraviolet
v/v	Volume to volume
w/v	Weight to volume

CHAPTER 1: GENERAL INTRODUCTION

1.1 Ornamental Industry Market.

The ornamental industry has been gaining in strength and importance over recent years. In the UK this industry is estimated to be around £2.1 billion, while the international trade is around £60-75 billion (Tanaka *et al.*, 2005; Chandler and Tanaka, 2007). The volume of ornamentals demanded worldwide has increased steadily for the past few decades. This has been strongly influenced by the rising disposable income of persons in the developed countries. Western Europe produces almost half of the world's supply of cut flowers (Pizano, 2005). The five nations with major demand for ornamentals are United States, France, United Kingdom, Germany and Netherlands, importing nearly 70% of the total worldwide.

In the production of ornamental plants, countries like Netherlands, Colombia, Israel and Kenya are the global leaders, with Ecuador, India and Malaysia rapidly growing in importance. The annual sales from potted plants worldwide are approximately £13 billion and are increasing each year. In 2002 the wholesale flowers, nursery stock and florist supplies contributed $ 2879 Million in the United States (Tanaka *et al.*, 2005). Quality and diversity of ornamental plants and flowers has also increased recently due to an increased knowledge and demand from the final consumer. This diversity is not always easy to achieve. The main innovations that the market seeks are the development of new varieties and an attractive price to encourage consumers. Their choice is influenced mainly by flower colour and pattern, plant architecture and by resistance to pests and disease.

The Netherlands controls 93% of the world trade in the ornamental bulb industry, including bulbs, corms and tubers and by 2005 was exporting

$92 million in bulbs (Grassoti and Gimelli 2011). The genera that are most widely produced, utilising more than 20,921 hectares of land are: *Gladiolus*, *Hyacinthus*, *Iris*, *Lilium*, *Narcissus* and *Tulipa*. These six genera effectively represent 90% of the world's flower production from bulbs (Chandler and Tanaka, 2007).

The tulip is the major ornamental flowering bulb with more than 10,800 hectares globally under production with the Netherlands dominating the tulip market with about 88% of global production. There are more than 800 cultivars of tulip grown in The Netherlands (Le Nard and De Hertogh, 1993). Dutch tulip production in 2005 was 4.3 billion bulbs [2.3 billion bulbs (57%) used as starting material for cut flowers] that represented $1.32 billion turnover (Grassoti and Gimelli, 2011).

Lilium presents attractive, large flowers and for some cultivars these are highly scented. These attributes make the genus a perfect target for exploitation by the ornamental industry. *Lilium* is one of the six major bulb genera crops in the world (Le Nard and De Hertogh, 1993). *Lilium* has three important markets: fresh-cut flowers, potted flowering plants and bulbs for domestic gardens and formal landscapes.

In The Netherlands in 2005, 4,280 hectares were dedicated to *Lilium* bulb production giving a yield of 2.2 billion bulbs for that year; an additional 200 hectares were dedicated to the cultivation of *Lilium* for cut flower production, giving more than 410 million stems. Increases in production are principally due to the availability of novel varieties produced by breeders primarily in upright Asiatic and Oriental lilies (van Tuyl and van Holsteijn, 1996). Japan is the second most important *Lilium* bulb producer after The Netherlands, dedicating 430 hectares for production of about 67 million bulbs, and 550 hectares to produce 157 million cut stems (Grassoti and Gimelli 2011). Japan has played an important role in breeding and developing Easter lily, Asiatic and Oriental hybrids. In Italy, all the *Lilium*

bulbs are imported from The Netherlands, but it is estimated that about 280-300 hectares of land are dedicated for *Lilium* cut flower production, producing a turnover of $71 million (Okazaki, 1996).

1.2 Description of the genus *Lilium.*

The interest in *Lilium* as an ornamental started in the 18th Century. Authors such as Parkinson in 1689 and Kaempfer in 1712 were among the first to describe several species like *Lilium martagon, L. bulbiferum, L. speciosum* and *L. trigrinum*. Fifty years later Linneus, in 1762 described nine species, *L. candidum, L. bulbiferum, L. pomponium, L. chalcedonicum, L. superbum, L. martagon, L. canadense, L. philadelphicum,* and *L kamtchatkense.* In 1784, Thunberg published "Flora japonica" describing 14 species of lilies.

The first monograph of the genus *Lilium* was published by Henry Elwes in 1880; this was based upon on publications by Duchartre (1870) and Baker (1871) and his personal experience on the subject. Although he was not a botanist, this monograph was so extensive and detailed that it has formed the basis of the classification of this genus.

The Genus *Lilium* is composed of approximately 100 species distributed mainly throughout the cold and temperate regions of the Northern Hemisphere (Nishikawa *et al.,* 1999). The first *Lilium* classification was made by Endlicher in 1836 and he divided the genus into five sections: *Amblirion, Cardiocrinum, Eulirion, Martagon and Pseudolirium.* Baker in 1871 modified Endlicher's classification establishing *Archelirion* as a new section. In 1925, the classification was modified again, this time by Wilson who divided this genus into four subgenera, with all the true lilies belonging to *Eulirion,* which comprised four sections, *Leucolirion, Archelirion, Pseudolirium,* and *Martagon.*

In 1949, Harold Comber proposed a classification of this genus based upon 13 morphological characteristics and two germination types. He divided the genus into seven sections and nine subsections: section *Martagon,* typed by *L. martagon*; section *Pseudolirium,* by *L. philadelphicum*; section *Liriotypus,* by *L. candidum*; section *Archelirion,* by *L. auratum*; section *Sinomartagon,* by *L. davidii*; section *Leucolirion,* by *L. longiflorum*; and section *Daurolirion,* by *L. dauricum.* Sections *Pseudolirium, Sinomartagon,* and *Leucolirion* were divided into four, three, and two subsections, respectively. Recently more accurate classifications have been proposed for the genus *Lilium* (McRae, 1998), and with the aid of molecular analysis (Nishikawa *et al.,* 1999) sections of the genus *Lilium* are being revised periodically (Brodie 2009). Figure 1.1 shows a phylogenetic tree based on the Internal Transcribed Spacer region (ITS) of ribosomal DNA (Nishikawa *et al.,* 1999).

The Royal Horticultural Society is the international registration authority for cultivated lilies and it has established nine divisions, and subdivisions that are based on flower form (trumpet, bowl, flat or recurved) and floral habit (out-, up- or facing-down). Table 1.1 describes some of the main characteristics of these divisions.

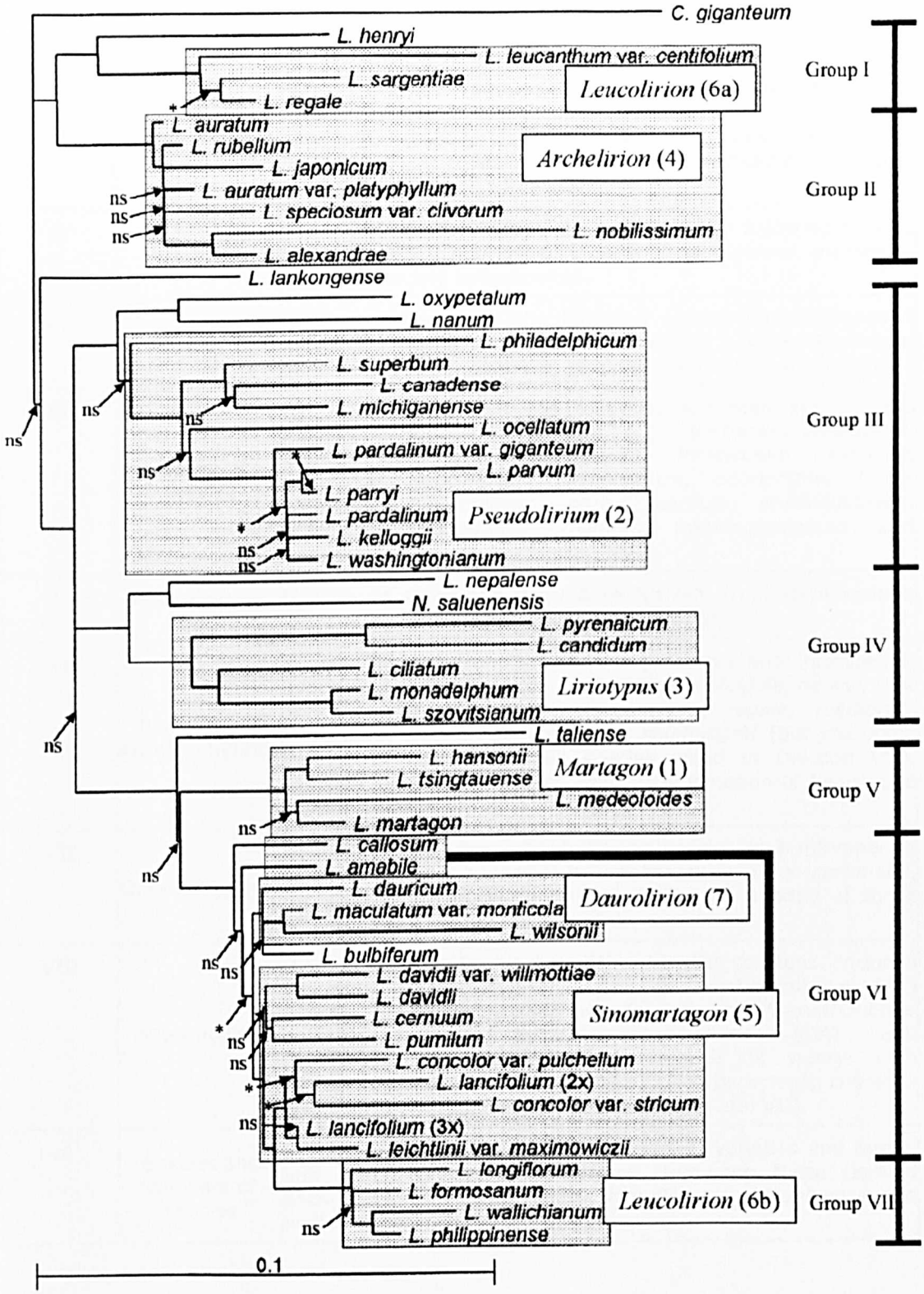

Figure 1.1 Phylogenetic tree constructed from ITS sequences of 55 *Lilium* species by the maximum-likelihood method. (Nishikawa *et al.*, 1999)

DIVISION	NAME	DESCRIPTION
I	Asiatic hybrids	Hybrids derived from the following species and interspecific hybrids: *amabile, bulbiferum, callosum, cernuum, concolor, dauricum, davidii, L.* × *hollandicum, lancifolium* (syn. *tigrinum*), *lankongense, leichtlinii, L.* × *maculatum, pumilum, L.* × *scottiae, wardii* and *wilsonii.*
II	Martagon hybrids	Hybrids of martagon type derived from the following species and interspecific hybrids: *L.* × *dalhansonii, hansonii, martagon, medeoloides* and *tsingtauense.*
III	Euro-Caucasian hybrids	Hybrids derived from the following species and interspecific hybrids: *candidum, chalcedonicum, kesselringianum, monadelphum, pomponium, pyrenaicum* and *L.* × *testaceum.*
IV	American hybrids	Hybrids derived from the following American species and interspecific hybrids: *bolanderi, L.* × *burbankii, canadense, columbianum, grayi, humboldtii, kelleyanum, kelloggii, maritimum, michauxii, michiganense, occidentale, L.* × *pardaboldtii, pardalinum, parryi, parvum, philadelphicum, pitkinense, superbum, vollmeri, washingtonianum* and *wigginsii.*
V	Longiflorum lilies	Hybrids or selections derived exclusively from *formosanum, longiflorum, philippinense* and *wallichianum.*
VI	Trumpet and Aurelian hybrids	Hybrids derived from the following species and interspecific hybrids: *L.* × *aurelianense, brownii, L.* × *centigale, henryi, L.* × *imperiale, L.* × *kewense, leucanthum, regale, rosthornii, sargentiae, sulphureum* and *L.* × *sulphurgale* (but excluding hybrids of *henryi* with all species listed in Division VII). Aurelian hybrids are derived from a combination of *henryi* and trumpet lilies.
VII	Oriental hybrids	Hybrids derived from the following species and interspecific hybrids: *auratum, japonicum, nobilissimum, L.* × *parkmanii, rubellum* and *speciosum* (but excluding all hybrids of these with *henryi*).
VIII	Other hybrids	Hybrids not covered by any of the previous divisions. Includes all interdivisional hybrids, such as Asiatic/Trumpet (AT) hybrids, *longiflorum*/Asiatic hybrids (LA), *longiflorum*/Oriental hybrids (LO), Oriental/Asiatic hybrids (OA) and Oriental/Trumpet hybrids (OT). Hybrids of *henryi* with *auratum, japonicum, nobilissimum, L.* × *parkmanii, rubellum* and *speciosum* (excluded from Divisions VI and VII).
IX	Species and cultivars of species	Includes all species and their subspecies, varieties and forms, and cultivars selected therefrom (excluding those derived exclusively from *formosanum, longiflorum, philippinense* and *wallichianum.*

Table 1.1 Horticultural classification of cultivated lilies by The Royal Horticultural Society. Modified from "The International Lily Register and Checklist", 2007.

1.2.1 *Morphology.*

Lilium has white-yellow, non-tunicated bulbs composed of numerous modified leaves or scales and a compressed stem or basal plate. The bulb is globose and the scales contain stored reserves. The scape, or stalk, is erect and stout. The number of leaves depends on the cultivar (Miller, 1993). Leaves can be lanceolate, linear or elliptical. Flowers are white for *L. longiflorum* and fragrant, funnel-shaped and multi-coloured for Oriental hybrids. The perianth segments, or tepals, are composed of three sepals and three petals with an oblanceolate shape. The six anthers are yellow-orange; the stamens are shorter than the pistil (Fig. 1.2). The colour of flowers varies from deep purple to flaming red and white; there are no blue floral forms to date. Flowers can be heavily scented, with diverse shapes; flowering times have been considerably extended due to selection for diversity in modern hybrids (Beattie and White, 1993).

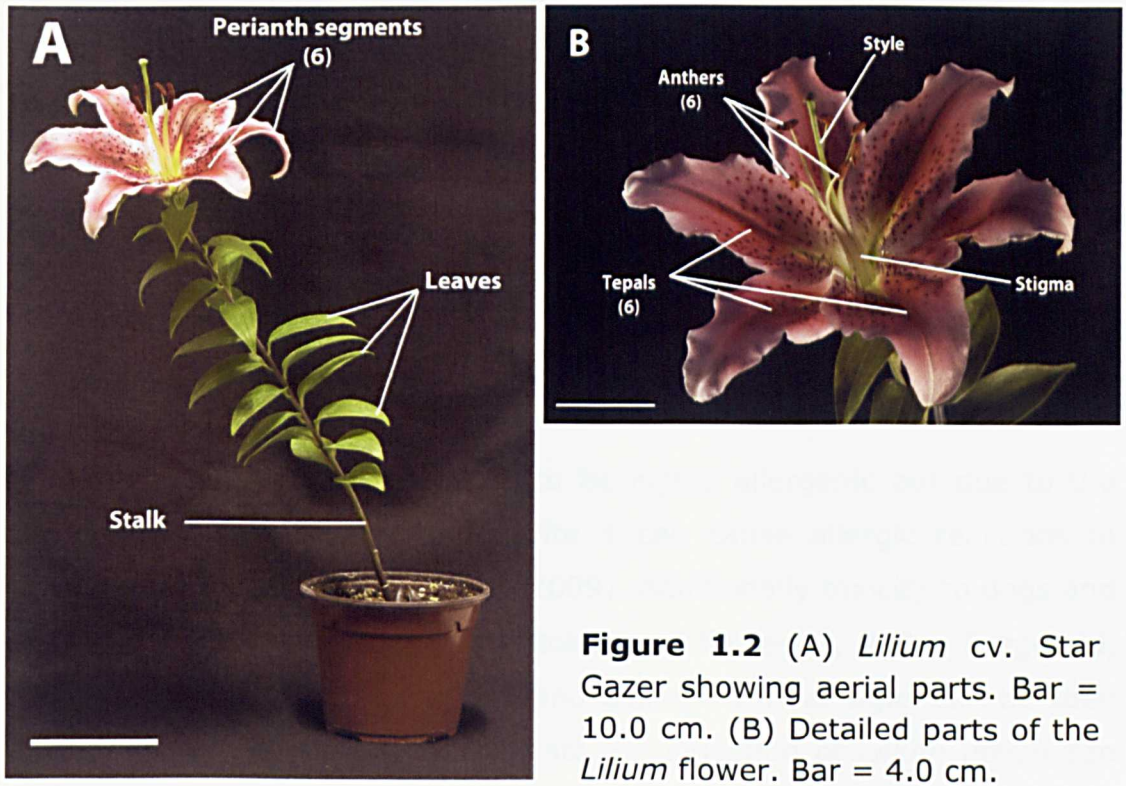

Figure 1.2 (A) *Lilium* cv. Star Gazer showing aerial parts. Bar = 10.0 cm. (B) Detailed parts of the *Lilium* flower. Bar = 4.0 cm.

1.3 Major breeding objectives and limitations of *Lilium.*

The main goals for lily breeding programmes are focused on forcing a shorter time for initiation of flowering, delayed senescence and an extended post-harvest life, low nutritional requirements, introduction of wider colours and forms in flowers, improved fragrance, elimination of staining pollen and pest, disease and virus resistance (Miller, 1993).

Dormancy of bulbs represents a delay in breeding programmes, so research has focused on breaking dormancy with cold treatments, or the use of ethylene, gibberellic acid or cytokinins (Roh *et al.,* 1996). The major problem for forced lilies is the abortion of floral buds, which is often related to low light intensity and prolonged cold storage of the bulbs.

Growth and flowering are polygenic traits making them difficult to breed for and manipulate under natural conditions. A different approach involving molecular breeding to modify plant architecture, by targeting a gene of interest in a cloned form, combined with an efficient plant regeneration and transformation protocol, may provide opportunities to overcome the difficulties found in traditional breeding techniques (Tanaka *et al.,* 2005). This can lead the way for the development of numerous attractive new varieties.

Pollen of *Lilium* is not considered to be highly allergenic but due to the production of nitric oxide and nitrite it can cause allergic reactions to sensitive individuals (Bright *et al.,* 2009). Additionally toxicity to dogs and cats has been well documented (Stokes and Forrester, 2004; Fitzgerald, 2010; Thompson 2012). Vomiting and gastrointestinal signs can be seen after pollen ingestion in dogs. In cats the ingestion of *Lilium* pollen can cause acute nephrotoxic syndrome that can lead to the dead of the animal, cats are extremely sensitive to the toxic effects of this pollen

although the precise toxic dosage and the exact mechanism of action of lily poisoning is unknown (Fitzgerald 2010). Thus manipulation of pollen production and release from *Lilium* flowers is of high commercial importance.

1.3.1 Pathogen Resistance.

One of the main objectives of breeding programs is the development of varieties that can resist, or be tolerant to pathogens. According to Lawson and Hsu (1996), the most important pathogens of lily are *Botrytis cinerea*, *Fusarium oxysporum f. sp. lilii, Botrytis elliptica* and *Phytophtora* spp.

F. oxysporum f. sp. lilii infects the bulb and roots. The infection starts in wounded parts of the bulb and the tissues of the bulb turns necrotic, if a section of the stem is under the soil, it turns an orange-brown colour and the infection spreads to all the aerial parts.

For *B. elliptica*, the symptoms start with grey or orange punctures in the damaged tissue and when the humidity increases, the spots grow to circular or elliptical zones (1-2 cm diameter); the perimeter of the infection zone is marked by an orange colouration. If the disease reaches the perianth it is marked with grey punctures. In extreme conditions, the plant becomes totally covered with a grey mould, ultimately killing the plant.

Phytophtora spp. affects the aerial parts of the plant, if the infection is established at an early stage of leaf development, this stops the growth of the stem and leaves keep emerging from the apex leading to a rosette phenotype; in later stages the main symptom is the formation of a violet-

brown band at the base of the stem causing breakage and loss of the floral parts.

1.3.2 Manipulation of Flowering.

The understanding and manipulation of flowering is another major research goal for *Lilium* and all plants. Flowers are the precursor of fruit, and if the flowering process can be regulated, plants can potentially be manipulated to remain in a vegetative or flowering state. Accelerated flowering could lead to a much shorter growing season, or to a specific harvest season, both of these are important targets for growers and plant breeders. The major developmental transition in flowering plants is the switch from vegetative to reproductive development (Fujioka *et al.*, 1983). The correct timing of this transition is essential to maximize reproductive success given the requirement for synchronous flowering in out-crossing species and the dependence on favourable conditions for optimal seed formation.

Several reproductive strategies have evolved in different plant species. Many plants respond to environmental cues to control flowering time, particularly those that indicate seasonal change. For example, flowering in *Arabidopsis* is accelerated by conditions that indicate the passage of winter and the onset of spring and summer, such as prolonged periods of cold temperature, elevated ambient growth temperatures, and day length increase. In contrast, flowering in rice is promoted by short days (Aksenova *et al.*, 2006). Flowering is also triggered by response to stress such as overcrowding, nutrient deficiency, heat, and drought; endogenous signals also regulate the floral transition.

The precise moment in a growing season when a plant initiates flowering is a critical developmental decision, if the plant is to ensure reproductive

success. In flowering plants, the timing of the transition from vegetative growth to flowering is controlled by the prevailing environmental conditions and/or intrinsic developmental signals. A brief summary of the genetic regulation of flowering in the model plant *Arabidopsis thaliana* is presented in Fig. 1.3.

1.3.2.1 Vernalization

Vernalization is the process of acceleration of floral initiation by a prolonged period of cold temperature; the exact conditions depend on the species or variant. A requirement for vernalization is a strategy adopted by many species to ensure they spend winter in a vegetative stage and flower under the favourable conditions of spring/summer. This is especially important to bulbous ornamental species like *Lilium* to trigger floral induction otherwise these plants remain in a constant vegetative stage. In *Arabidopsis*, this can be mapped as a monogenic trait with dominant alleles of *FRI* conferring a vernalization requirement, *FRI* encodes a protein that promotes the accumulation of *FLC* messenger RNA (mRNA) (Simpson and Dean, 2002).

FLC encodes a MADS box transcription factor that is a repressor of the floral transition. By promoting the accumulation of *FLC* mRNA, FRI represses the floral transition to such a degree that it overrides the influence of otherwise favourable conditions. Vernalization is permissive, not instructive, because it prepares the plant to flower rather than evoking the flowering itself. The mechanism of vernalization has been addressed through the identification of mutants defective in this process (*vrn* mutants) (Aksenova *et al.*, 2006).

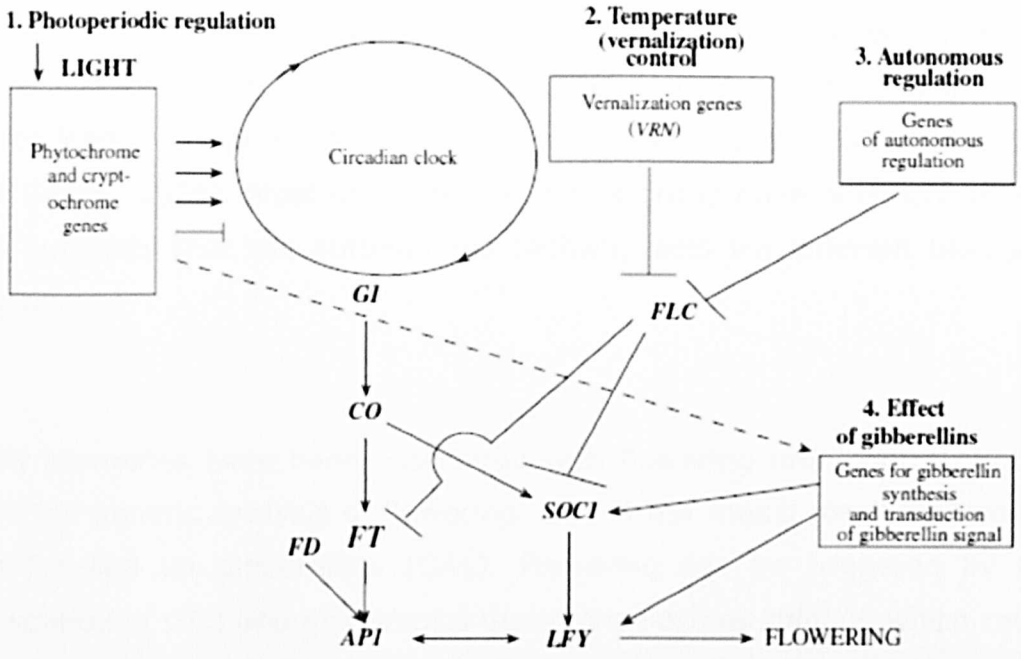

Figure 1.3 Genetic network controlling the transition to flowering in *Arabidopsis*. Different genes involved in flowering. AP1 (APETALA 1); CO (CONSTANS); FLC (FLOWERING LOCUS C); FT (FLOWERING LOCUS T): GI (GIGANTEA); LFY (LEAFY); SOC1 (SUPEREXPRESSOR OF OVEREXPRESSION OF CONSTANS 1). (Aksenova *et al.,* 2006)

In some plants, for example *Arabidopsis,* acceleration of the floral transition is triggered by long days. The capability to sense the lengthening photoperiod requires an endogenous timer and the detection and transduction of the light signal. In *Arabidopsis*, light is perceived by phytochromes A through E and cryptochromes (CRY) 1 and 2 (Simpson and Dean, 2002); the timer that measures the duration of the day and night is the circadian clock. This clock controls many aspects of plant biology in addition to flowering time, as it has been proven in some *Arabidopsis* mutants (*lhy*, *cca1*, *gi*, *elf3*, *toc1*, *ztl*, *fkf1*) that disrupted day length dependent flowering also affects other circadian processes (Aksenova *et al.,* 2006). Photoreceptors contribute light input signals to the circadian system. Far-red (735 nm) and blue (440 nm) light promote flowering through PHYA and CRY1 and 2. Red light (660 nm) inhibits flowering through PHYB, D, and E function in a wide range of species independently of the circadian clock and do not involve transcriptional regulation of *CO*.

The mutations that delay flowering under any photoperiod and confer a response to vernalization were catalogued into the Autonomous pathway, these include *LD*, *FCA*, *FY*, *FPA*, *FVE*, *FLD* and *FLK*. Mutations in these genes lead to an increase in the levels of *FLC* mRNA and FLC protein (Tan and Swain, 2006). Most of the genes in this group have been cloned and this suggests that the autonomous pathway acts via different biological processes.

Plant hormones have been associated with flowering time control, but to date the genetic analysis of flowering time in the model plant *Arabidopsis* has focused on gibberellins (GAs). Flowering can be triggered by the application of GA3 and *Arabidopsis* mutations such as *spindly*, which cause constitutively active GA signalling. In contrast, mutants that blocks GA signalling (*gai*) or GA biosynthesis (*ga1-3*) delay flowering, especially in short days. The GA pathway is genetically distinct from the PHYB repression, vernalization, autonomous, and photoperiod pathways (Tan and Swain, 2006).

1.3.2.2 Flower development

Flower development in *Lilium* has been researched since the 1990's when 18 genes were characterized from microsporocytes of *L. longiflorum* cv. Hinomoto (Kobayashi *et al.*, 1994). To date a total of 8 MADS box genes have been found in lily; *LMADS1* has high sequence homology to *AP3* of *A. thaliana* (Tzeng and Yang, 2001), *LMADS2* has extensive similarity to *AGL11* in *A. thaliana* and *FBP7/11* from Petunia (Tzeng *et al.*, 2002). *LMADS3/4*; *LMADS3* is closely related to *SEP3* and its orthologs within the E function genes, *LMADS4* was found to be closely related to *SEP* in the AGL2 subclade of MADS box genes based on its protein sequence (Tzeng *et al.*, 2003).

More recently the *LMADS5*, *LMADS6* and *LMADS7* genes were characterized. *Lily MADS Box Gene 5* showed high sequence identity to *Elaeis guineensis SQUAMOSA3* gene (*EgSQUA3*). *LMADS6* was shown to be closely related to *LMADS5* whereas *LMADS7* is highly related to *DOMADS2*, a *Dendrobium* gene in the SQUA subfamily (Chen *et al.*, 2008). *LLAG1*, an *AG* homologue from Lily was isolated and characterized by Benedito *et al.* (2008); the deduced amino acid sequence revealed the MIKC structure and a high homology in the MADS-box among *AG* and other orthologues. A further phylogenetic analysis indicated a close relationship between *LLAG1* and *AG* orthologues from other monocot species (Benedito *et al.*, 2008).

Fukai and Goi (2001) analysed the process of flower initiation in *Lilium*, which takes around 4 weeks (Fig. 1.4). The first sign of this process is the swelling of one of the three axillary buds, followed by the swelling of the other two at the same time. Shortly after this the shoot apex can no longer be distinguished and each axillary bud develops into a floret primordium. *Lilium* florets originate from the axillary buds under natural conditions, differentiating acropetally. Each floret develops from a swelling apex of the axillary bud and produces a bract (Fig. 1.5). Later, three outer perianths appear on the top of the primordium and develop into broad leaf-like structures. Three inner perianths then appear in different directions. At this point the six stamens are differentiated and the apex becomes triangular in shape and forms the pistil primordium; the inner perianths have a large mid rib on the abaxial side. The top of the triangular structure is concaved at three independent points and grows upward and develops into the pistil.

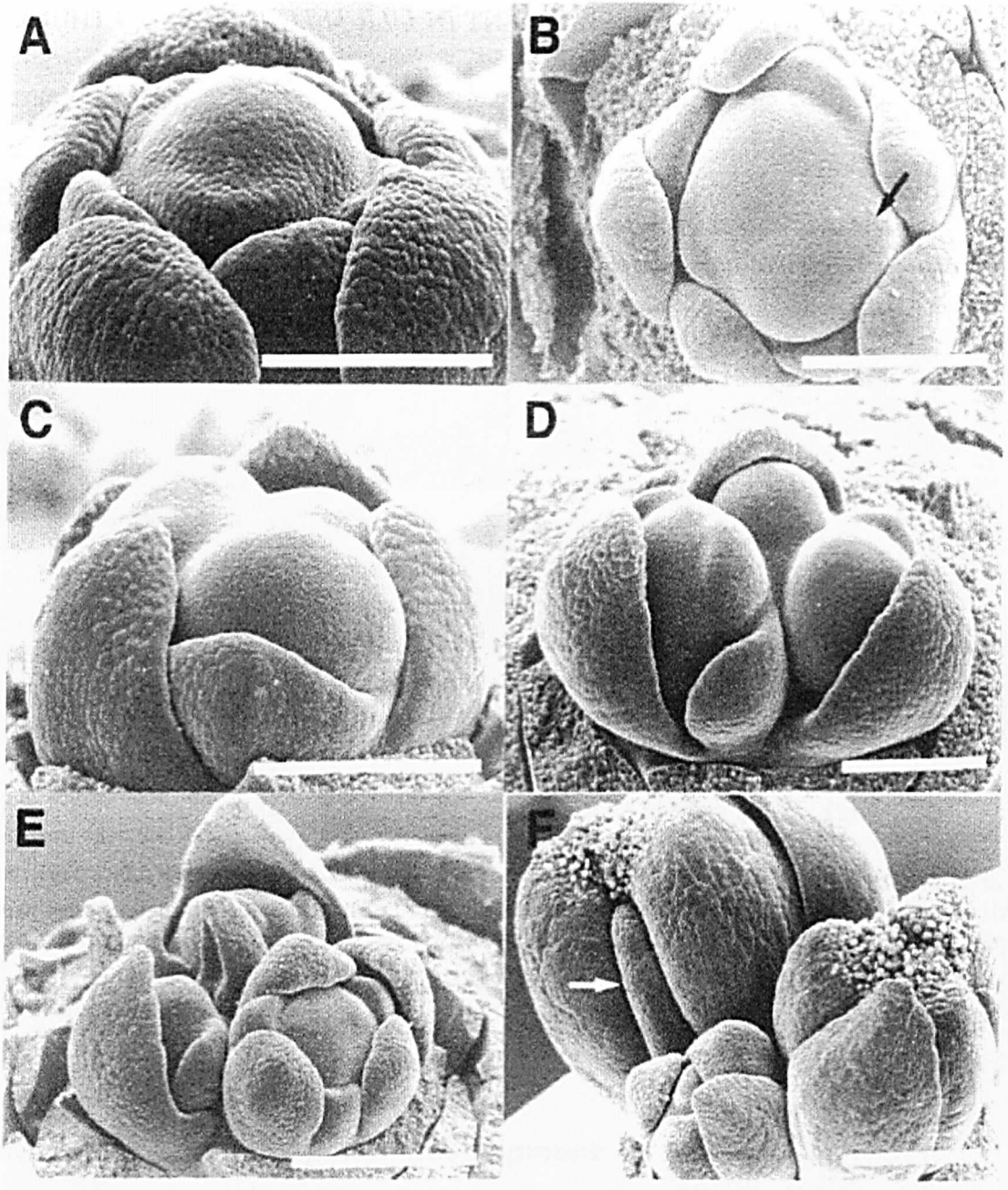

Figure 1.4 Flower initiation and inflorescence development in *Lilium*. (A) Vegetative apex. Bar=500 µm. (B) Arrow indicates swollen axillary bud, first signal of floral initiation. Bar=500 µm. (C) All axillary buds swollen. Bar=500 µm. (D) Shoot apex of main stem disappeared. Bar=500 µm. (E) Each floret developes acropetally. Bar=l mm. (F) Inflorescence composed of three flower buds. Bar= 1 mm. Arrow indicates mid rib of inner perianth which fuse with the margin of outer perianth (Fukai and Goi, 2001)

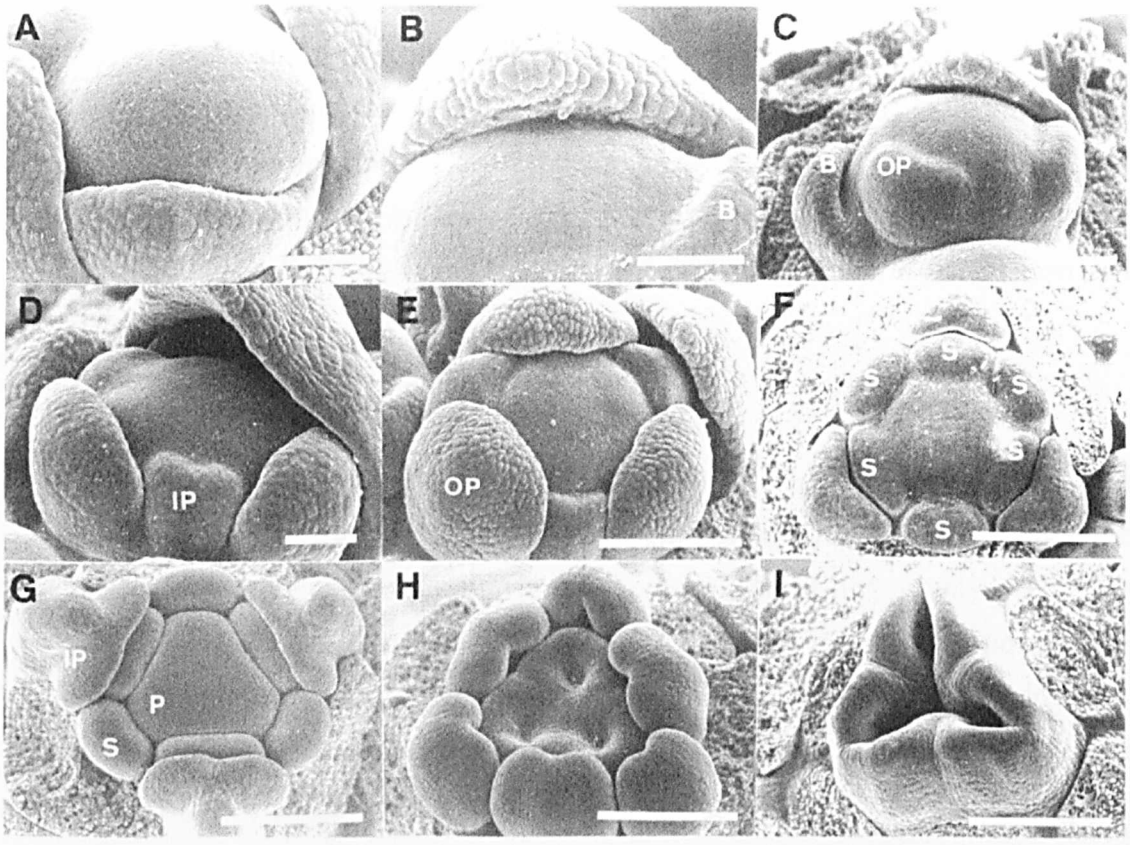

Figure 1.5 Floret development in *Lilium*. (A) Swollen apex. Bar=200 µm. (B) B, bract. Bar=200 µm. (C) OP, outer perianth. Bar=200 µm. (D) IP, inner perianth. Bar=200 µm. (E) OP forming a broad leaf-like structure. Bar=500 µm. (F) S, stamen. Bar=500 µm. (G) P, pistil; Abaxial mid rib developed on inner perianthes. Bar=500 µm. (H) Three concave points in pistil primordium. Bar=500 µm. (I) Pistil growing upward. Bar=500 µm. (Fukai and Goi, 2001).

1.3.2.3 Anther and pollen development

Anther and pollen development represent a critical stage in the plant's life cycle. It is vital for pollen formation and dehiscence and therefore for sexual reproduction and breeding (Yang *et al.*, 2001). In recent years considerable information has been discovered about the molecular genetic control of anther and pollen development in several model systems. Genetic screens have isolated many mutants showing defects in anther and pollen development, and pollen release and delivery.

Recent research has demonstrated that genetic manipulation of the production of pollen can be achieved by the overexpression of a number of genes, for example the *AtMS1* gene (Yang *et al.,* 2007a) in *Arabidopsis*, the mutated melon ethylene receptor gene *Cm-ERS1/H70A* in lettuce (Takada *et al.,* 2007), tobacco (Takada *et al.,* 2006) and chrysanthemum (Shynoyama *et al.,* 2012) and by the silencing of *TAZ1* and *MEZ1* gene in Petunia.

Pollen release from the anthers can also be manipulated using the *AtMYB26* gene (Yang *et al.,* 2007b). Such advances offer excellent opportunities for the development of new varieties in lily. This is particularly the case since lily pollen can trigger allergic responses in humans (Bright *et al.,* 2009), has been linked to kidney failure in pets (particularly in cats) and poses a problem for the cut flower market due to the staining of pollen. These genetic approaches may therefore represent possible opportunities for product development for the ornamental industry.

1.3.2.3.1 Anther development

Anther development has been studied extensively in a number of plants including tobacco, *Arabidopsis* and rice (Scott *et al.,* 2004; Yang *et al.,* 2007a; Zhang and Wilson 2009). However anther development in *Arabidopsis* has been used as a good model for other systems. Anther development has been divided into 14 stages (Ma, 2005); stages 1-8 comprise phase 1 and stages 9-14 constitute phase 2 (Figs. 1.6 and 1.7).

The anther consists of a bilateral structure of locule, wall, connective and vascular regions, which is established within the developing anther primordia during stages 1 to 4 by several cell division events. Archesporial cells in the floral meristem divide periclinally to give rise to distinct 1°

parietal and 1° sporogenous cell lineages that will form the endothecium, middle layer, tapetum, and microspore mother cells (Sanders *et al.*, 1999).

Meiosis of the microspore mother cells occurs between stages 5 and 7 generating tetrads of haploid microspores. Microspores are then released from the tetrads at stage 8 and differentiate into three-celled pollen grains between stages 9 and 12. At stages 11 and 12, degeneration of the septum results in a bilocular anther. This is followed by stomium cell breakage and anther opening and pollen release during stages 12 and 13. Following dehiscence the anther senesces and falls off the plant at stage 14 (Sanders *et al.*, 1999).

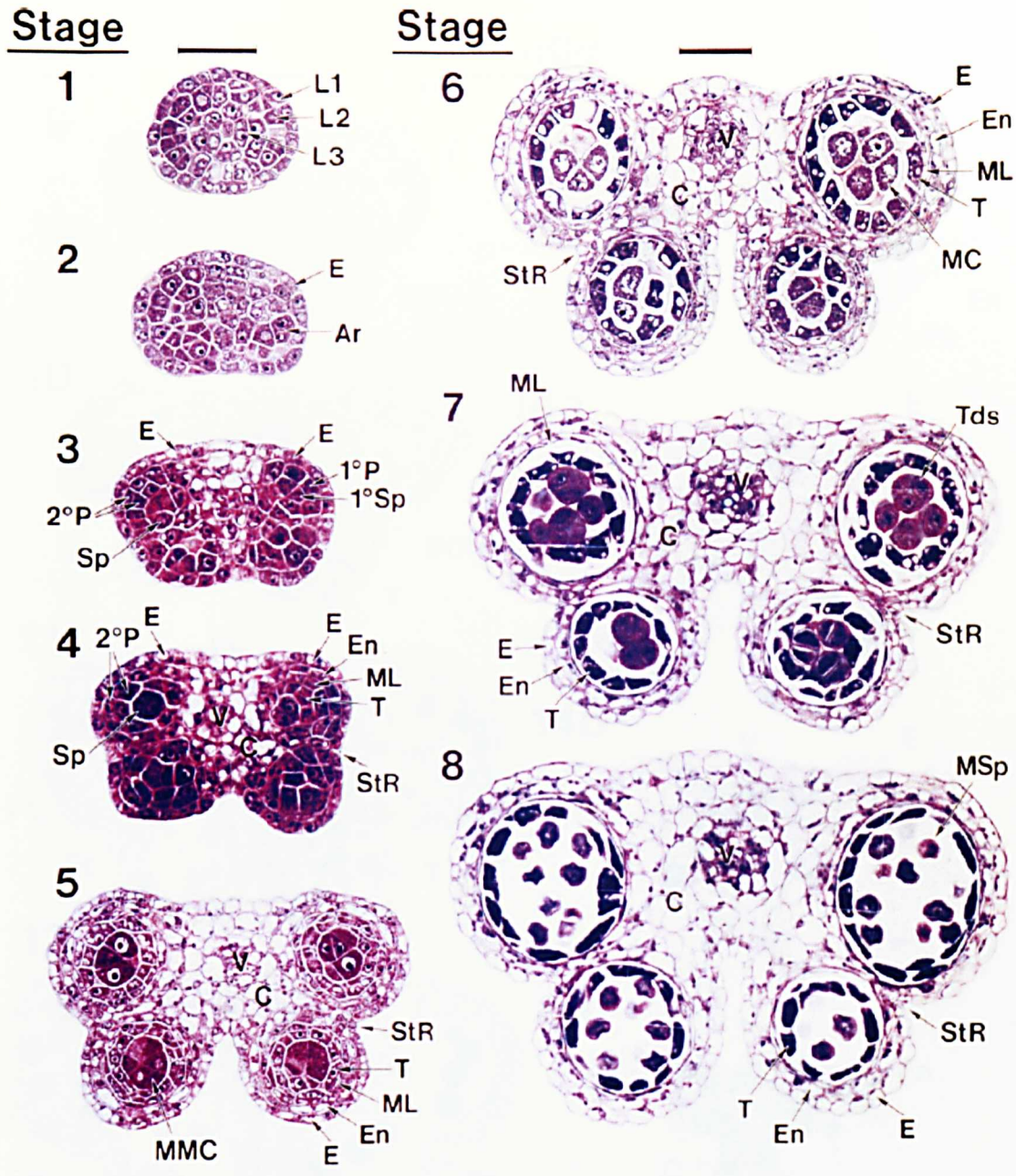

Figure 1.6 Anther development in *Arabidopsis* phase 1. Ar, archesporial cell; C, connective; E, epidermis; En, endothecium; L1, L2, and L3, the three cell-layers in stamen primordia; MC, meiotic cell; ML, middle layer; MMC, microspore mother cells; MSp, microspores; 1°P, primary parietal layer; 2°P, secondary parietal cell layers; 1°Sp, primary sporogenous layer; Sp, sporogenous cells; StR, stomium region; T, tapetum; Tds, tetrads; V, vascular region. For stages 1-4 Bar over stage 1=25 µm. For stages 5-8 Bar over stage 6=25 µm. (Sanders *et al.,* 1999).

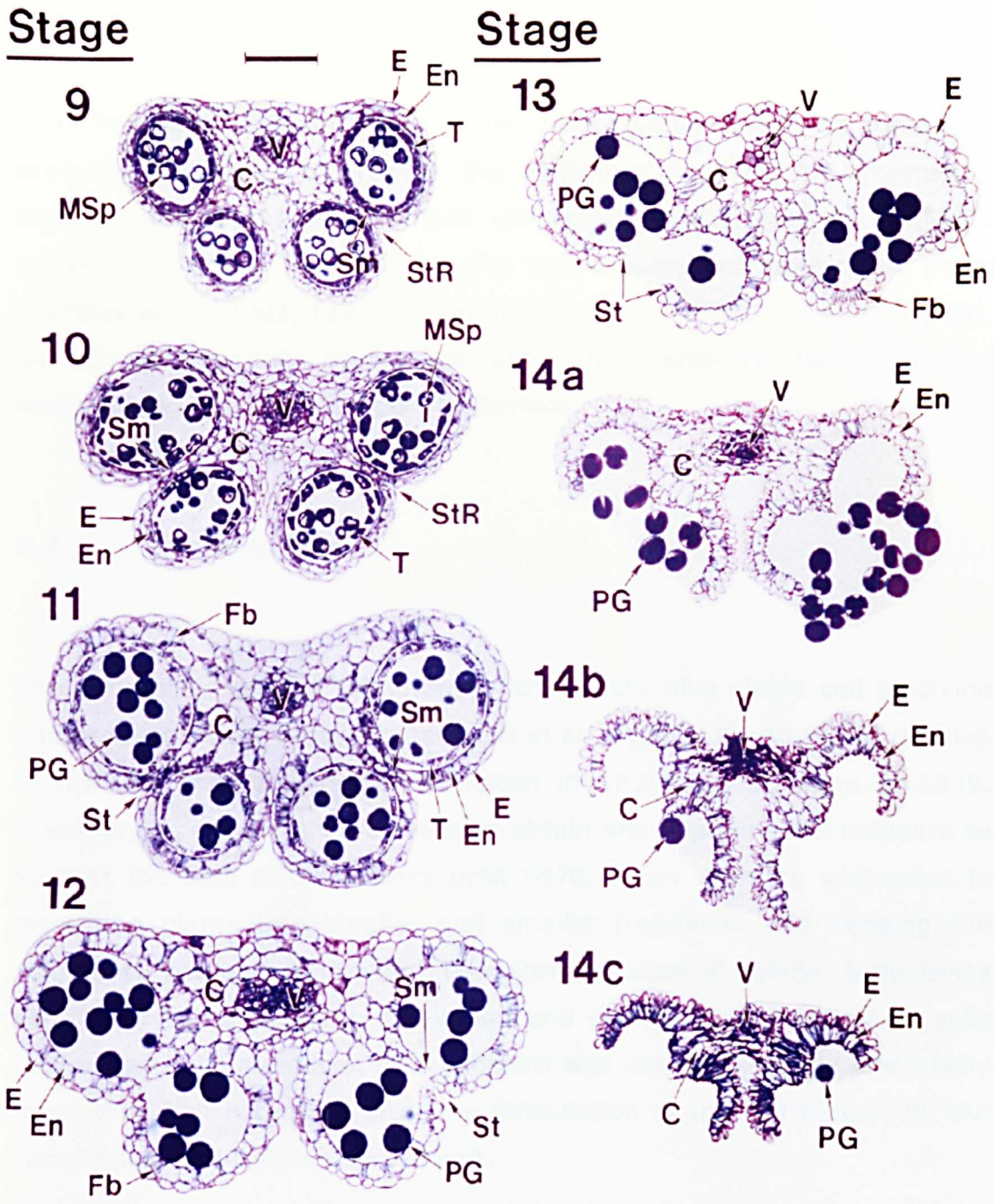

Figure 1.7 Anther development in *Arabidopsis* phase 2. C, connective; E, epidermis; En, endothecium; Fb, fibrous bands; MSp, microspores; PG, pollen grains; Sm, septum; St, stomium; StR, stomium region; T, tapetum; V, vascular region. Bar=50 µm.

1.4 Genetic manipulation of ornamental species.

Plant breeders have combined tissue culture approaches with traditional breeding techniques to broaden the gene pool available for ornamental improvement, but these methods still require backcrosses to eliminate undesired traits in the offspring and this process can take many years (Deroles *et* al., 2002; Robinson and Firoozabady, 1993; Mol *et al.*, 1995). Genetic engineering overcomes both the necessity for successive backcrossing and the gene pool limitations.

1.4.1 Tissue culture.

The concept of cellular totipotency, "the ability of a single cell to divide and produce all the differentiated cells in an organism", started to develop in the Cell Theory works of Schleiden in 1838 and Schwann in 1839. However, no attempts were made to obtain any experimental evidence to support the idea of totipotency until 1878, when Vöchting succeeded in dissecting plants into smaller and smaller fragments and keeping the fragments viable and growing. The ultimate proof of cellular totipotency could only come from the cultivation and growth of isolated single cells into a complete organism. This problem was set forth clearly and boldly more than half a century after the formulation of the cell theory, by the famous botanist Gottlieb Haberlandt.

In 1902 Haberlandt made the first experiments to culture plant cells from *Lamium purpureum*, *Utrica dioica* and *Tradescantia virginiana*, among others, but he failed because of cell and plant selection, and principally due to the growing medium used, which lacked hormones, vitamins and other substances now known to be needed for tissue culture.

The first successful culture of excised roots for indefinite periods of time was achieved by White in 1934. In 1939, Gautheret in Paris, Nobécourt in Grenoble, and White in Princeton, independently succeeded in cultivating cambial tissues of carrot and tobacco. From then to the present era tissue and cell culture has become more and more important to foster genetic variability and facilitate efficient clonal propagation in plants, due to its major development in growth media and plant tissue isolation techniques (Arditti and Ernst, 1993).

Different techniques can be used for breeding purposes, including protoplast fusion, somaclonal variation, *Agrobacterium*-mediated transformation and Biolistics (Sahijram *et al.*, 2003). Somaclonal variation among plants regenerated *in vitro* is often common. These differences can be influenced by several parameters such as the type of explant used since highly differentiated tissues can give more variation. The subculture number can also increase variation since rapid multiplication and *in vitro* conditions can affect the genetic stability and increase the mutation rate (Sahijram *et al.*, 2003).

Other important parameters are the use of hormones and growth regulators in culture medium, the genotype fidelity and flexibility, and genomic status of donor plants. Somaclonal variation involves all forms of variation obtained among regenerated plants from tissue culture, such as physical and morphological changes, differences in the ability to organize and form organs *in vitro* and chromosomal changes (Rout *et al.*, 2006). As a general rule, somaclonal variation is not very desirable, but in some cases it can be advantageous for plant breeding since it can increase the genetic variability in crops. These approaches can also be used in combination with appropriate selection procedures to target key traits, for example to generate disease resistant plants.

The technique of protoplast fusion has been very helpful to overcome reproductive barriers such as self-incompatibility or interspecific crossing. It has also been used to fuse nucleated protoplasts and enucleated cytoplasts to generate hybrid cells with different combinations of cytoplasmic and nuclear genes (Gürel *et al.*, 2002). These fusions can be achieved by either electrical pulses, or chemical treatments, including sodium nitrate, polyethylene glycol, high pH, high Ca^{+2} or a combination of the last two (Navrátilová 2004).

1.4.2 Plant transformation technologies.

Transformation systems require an efficient DNA delivery method whereby a transgene can be transferred into a single plant cell, followed by stable incorporation into the host genome under selective conditions, combined with the subsequent ability to recover transgenic plants from these transformed cells by an established propagation system (Hansen and Wright, 1999; Deroles *et al.*, 2002).

Theoretically any gene can be isolated for transformation and this procedure avoids backcrossing as the gene of interest is directly transferred into the target species (Deroles *et al.*, 2002). Several ornamental species have been transformed with the aim of introducing novel horticultural traits such as colour in rose (Katsumoto *et al.*, 2007), *Antirrhinum* (Ono *et al.*, 2006), petunia (Tanaka *et al.*, 2005) and *Torenia fournieri* (Aida *et al*, 2001); scent in petunia (Tanaka *et al.*, 2005); architectural modification in *Solanum nigrum* (Dijkstra *et al.*, 2008); and extended vase life in carnation (Savin *et al.*, 1995) and *Torenia fournieri* (Aida *et al.*, 1998).

For ornamental species, genetic transformation is useful in reducing breeding programme time (Deroles *et al.*, 2002). Transgenic carnations

(*Dianthus caryophyllus*) with modified colour have been successful developed by the introduction of a F3'5H' gene (Shimada *et al.*, 1999; Okinaka *et al.*, 2003) together with a Petunia *DFR* gene into a DFR-deficient white carnation (Mol *et al.*, 1999). This technology allowed the production of delphinidum-based pigments in the petals producing coloured flowers that were variegated mauve and purple. This research has been commercially exploited by Florigene Ltd. in the USA, Australia and Japan. Transgenic violet carnations are marketed under the name of Florigene Moondust™, Florigene Moonshadow™, Florigene Moonvista™, Florigene Moonacqua™ and Florigene Moonshade™. The success of Florigene in the ornamental market has also allowed development of blue roses that produce Delphinidin in their petals (Bhattacharya *et al.*, 2010).

Different techniques have been developed to transfer foreign DNA into plant cells, such as *Agrobacterium*-mediated transformation, biolistics-mediated transformation, direct introduction of DNA into protoplasts (direct gene transfer), and direct injection of DNA into a single cell (microinjection) (Zuker *et al.*, 1998), whisker-mediated transformation (Wang *et al.*, 1994).

1.4.2.1 *Agrobacterium*-mediated transformation.

The original studies to transform and express foreign genes in plants used the natural ability of causal agents of crown disease and hairy roots, *Agrobacterium tumefaciens* and *A. rhizogenes* respectively, to transfer a defined DNA fragment (T-DNA) of the tumour inducing (Ti) or rhizogenic (Ri) plasmid into plant cells (Gelvin, 2003; Zuker *et al.*, 1998). T-DNA contains the oncogenic genes encoding the enzymes involved in the synthesis of auxins and cytokinins and tumour formation, and genes encoding for opine biosynthesis (de la Riva *et al.*, 1998). The T-regions are flanked by 25 bp. direct repeats (T-DNA left and right borders) and the genetic information contained inside these borders is transferred to

the plant genome. T-DNA integrates into the host genome mediated by a 30-40 kb virulence (*vir*) region of the Ti plasmid which stimulates plant cell division, causing the tumour (Zupan *et al.*, 2000). The *vir* region encodes genes for the production of proteins specific for the growth of the bacteria and genes that regulate the T-DNA.

For molecular cloning, two approaches to create *Agrobacterium* DNA vectors for plant transformation have been developed, co-integrate and T-DNA binary vectors. The first consists of cloning the gene(s) of interest, which is contained between the T-DNA left and right borders of a small vector plasmid, by single recombination with *Agrobacterium* Ti plasmid lacking the genes for tumour induction (Ti disarmed plasmid). The selection of recombinant cells is based on the resistance genes of the plasmid backbone, as only these will be expressed (Gelvin, 2003).

To create T-DNA binary vectors it is necessary to introduce a ColE1 replicon into the T-region of the Ti plasmid and the next step is to integrate the T-region into a disarmed vector, which carries the *vir* region and the resistance marker gene for selection (Gelvin, 2003). Using *Agrobacterium* co-integrated vectors or T-DNA binary vectors for plant transformation have the advantage that both insert low copy numbers of the transgene into the host genome.

For ornamental species, transformation of leaf disks mediated by *Agrobacterium* transformation is the most widely used method. This approach was developed by Horsch *et al.* (1985) to transform leaf explants of Petunia, tobacco and tomato with the kanamycin resistance gene, *npt*II; the gene was integrated and transgenic plants were recovered.

1.4.2.2 Particle Bombardment transformation.

Biolistics is a microprojectile-mediated delivery system where DNA-coated particles of tungsten or gold (1-4 μm diameter) are mechanically shot directly into plant tissues, using a high pressure gas (usually helium), electrical discharge or gun powder (Zhang *et al.,* 2007).

The biolistic system has the advantage, compared with *Agrobacterium*-mediated transformation, that it does not have specificity for the host (species, cultivar or tissue-type). A disadvantage is the uncontrolled integration of the transgene into the host. The equipment and materials such as gold particles are also expensive compared with other transformation technologies (Zuker *et* al., 1998; Marchant *et al.*, 1998).

The original particle delivery system was designed by Sanford (1987). Later, DuPont developed the Helios™ gene gun (Helenius *et al*, 2000) and Biolistic PDS-100 device (Kikkert, 1993); this is the most commonly used system in particle bombardment research. Both pieces of equipment use pressurised helium to operate, but the PDS-100 needs a vacuum, restricting its use to laboratory conditions while the Helios™ gene gun can be used in the field. These systems provide a commercial device to deliver DNA into plant tissue with almost no damage to the tissue and they provide uniform particle delivery; this allows standardisation between laboratories (Kikkert *et al.,* 2004).

Biolistics delivery transforms many cells at the time of bombardment, as the microprojectiles are delivered as a fine spray. The microprojectiles penetrate the cuticle, cell wall and membrane of the plant cells and then move in conjunction with the cytoplasmic stream, they are then transported to the nucleus, chloroplasts or mitochondria (Sanford *et al.*, 1987). Transient expression mediated by biolistics delivery can be

affected by inefficient incorporation of the transgene, loss of the transgene at the time of DNA replication, natural DNA repair mechanisms or methylation (Southgate *et al.*, 1995).

Despite these issues, successful biolistic transformation of ornamental plants has been achieved. The optimisation of the biolistics delivery includes the osmotic treatment of the tissue, or incubating of the tissue on medium containing an osmotic agent after bombardment. Plasmolysis treatment was shown to reduce cell damage by preventing leakage of the protoplasm (Southgate *et al.*, 1995). Also the modification of gas pressure, which controls the acceleration of particles, using different sizes of microprojectiles and modifying the distance that the microprojectiles travel to reach the target tissue all resulted in variation in DNA delivery efficiency (Hansen and Wright, 1999; Marchant *et al.*, 1998).

1.5 Aims of the research project.

The aims of the work were to develop techniques for high frequency transformation of *Lilium* and to transfer genes associated with key breeding traits into this species. The desired traits that were targeted in these plants were resistance to pathogens and the manipulation of pollen production and release from the anthers. The development of a transformation protocol with a higher efficiency and faster results was of key importance for the success of the project.

1.5.1 Objectives of the project.

1. The development of an efficient transformation protocol for Lily.

Based on novel reported research, literature reviews and knowledge of the species, a faster and more efficient DNA delivery protocol was developed (Chapter 3) and extended to more species and cultivars of the genus *Lilium* (Chapter 4).

2. To generate transgenic *Lilium* plants with enhanced resistance to plant disease by insertion of genes *RCH10* from rice (Os03g0418000) and *CHIT2* from wheat leaf rust (Entrez accession number AY267184).

PCR and RT-PCR was used to confirm the insertion and expression of the gene in the plants. Positive lines were used for biochemical and molecular analyses to determine the effects of altered gene expression. *Botrytis cinerea* was used to determine the level of resistance in these transgenic plants (Chapter 5).

3. To control production and/or release of pollen.

On-going research in the Wilson laboratory (UoN) has involved studying the process of pollen development and the control of male fertility. As part of this work, transgenes have been produced which can silence targeted gene expression in model plant species (*Arabidopsis*) to control the production and release of pollen. This work was extended to *Lilium* to develop strategies for the control of pollen production or release in ornamentally important material. The selected gene for these

transformations was A*tMYB26*. Expression analysis and developmental studies were carried out on *Lilium* flowers to establish the patterns of expression for key genes from the same species and putative orthologs of *Arabidopsis* genes (Chapter 6).

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Tissue culture and maintenance of plant material.

Bulbs from cultivar Star Gazer were purchased from R.V. Roger Ltd nursery (North Yorkshire, UK). Stem explants and bulb scales from mature plants were used to induce bulblets *in vitro*. Explants were washed under running tap water to eliminate the excess soil, any rotten or necrotic parts were removed with a scalpel and the cleaned tissues were then sterilized by immersion in a 2% (v/v) Trigene solution (Medichem International, Kent, UK.) for a period of 10 minutes followed by immersion in a 15% (v/v) Domestos solution (Unilever UK Ltd, United Kingdom) for 35 minutes. Then they were rinsed three times with sterile double distilled water. Stems were cut transversely into segments (0.2-0.4 cm long) and were then placed horizontally onto MS-SI (MS-Shoot Induction) medium in Petri dishes. Each dish contained 25 ml of MS-SI medium composed of MS basal salts supplemented with 2.0 mg/L picloram, 1.0 mg/L BAP, 30 g/L sucrose, 2.5 g/L activated charcoal and 2.6 g/L phytagel as gelling agent.

Adventitious bulblets that developed from the explants after 4 to 6 weeks were then transferred individually into 175 ml glass jars containing 45 ml of MS-SI medium. For routine maintenance, subculture of these bulblets was carried over every 4 to 5 weeks. Cultures were maintained in the dark growth room at 23 ± 1°C until needed.

Callus induction was needed for genetic transformation. Calli that were used for these experiments were generated following the procedures of Horita *et al.* (2002); axenic bulblet scales were cut into several pieces (3-4mm) and transferred, epidermal side down, onto MS-CIH medium (MS-Callus Induction Horita) comprised of MS medium, 12.3 µM picloram, 30

g/L sucrose, and 0.25% (w/v) agar. An average of 16 explants were used per 9 cm Petri dish. These were incubated in the dark at 23 ± 1°C. White morphogenic callus started to form from the explants in 3-4 weeks. The explants and the formed callus were subcultured on the same media on a biweekly basis. Compact friable calli was selected for future transformation.

Lilium tissues were shown to be very sensitive to oxidization, and calli on MS-CIH medium only lasted up to 2 months before they turned brown and died, a new callus induction medium was developed. The composition of the MS-CIN (Callus Induction Núñez) was 2.0 mg/L picloram, 1.5 mg/L zeatin, 1.5 mg/L kinetin, 50 mM lipoic acid, 30 g/L sucrose, 2.5 g/L activated charcoal and 2.6 g/L of phytagel as gelling agent. In this medium the calli could be maintained indefinitely under the same incubation conditions described previously.

2.2 Nucleic acid extraction.

2.2.1 DNA extraction.

Four DNA extraction methods were tested to generate material for the identification of putative transgenic lines. Two commercial kits Extract 'n' Amp Plant PCR Kit (Sigma-Aldrich) and Gen-Elute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) were used following manufacturer's instructions. These were subsequently compared with two other protocols described (sections 2.2.1.1 and 2.2.1.2).

2.2.1.1 Modified protocol of Edwards *et al.* (1991).

A small piece of leaf tissue (approximately 100 mg) was removed from each plant and transferred into a 1.5 ml microfuge tube and was snap frozen in liquid nitrogen. Tissue was then ground thoroughly using a micro pestle until a fine powder was obtained. 600 µl of DNA extraction buffer (Appendix 1) was added to the tube and vortexed until no tissue clumps were visible. The sample was incubated at 45°C for 15 minutes and after that centrifuged at 9500 x *g* for 7 minutes.

The supernatant was then transferred into a new microfuge tube and ice-cold isopropanol was added in a ratio of 1:1. The sample was incubated on ice for at least 5 minutes and then centrifuged at 9500 x *g* for 7 minutes. The supernatant was then discarded and the pellet retained and washed with 200µl of 70% (v/v) ethanol centrifuging at 9500 x *g* for 2 minutes. Ethanol was discarded carefully not to disturb the pellet and the tube was left to air-dry. The pellet was finally resuspended in 40µl of Nuclease free water.

2.2.1.2 Modified protocol of Porcar *et al.* (2007).

A small piece of leaf tissue (approximately 100 mg) was excised from each plant and transferred into a 1.5 ml microfuge tube. Tissue was ground with a micro pestle, after adding to the tube 40µl of NaOH 0.5 M, until no large pieces of tissue were visible. The mixture was incubated at 95 °C for 2 minutes and then centrifuged at 9500 x *g* for 3 minutes. 12µl of the supernatant were transferred into a fresh 1.5 ml tube and 4µl of sodium acetate 3M (pH 5.2) added and mixed thoroughly by pipetting. The sample was then centrifuged at 9500 x *g* for 1 minute and the supernatant transferred into a fresh microfuge tube.

2.2.2 RNA extraction.

Total RNA extraction was prepared from approximately 100 mg of plant tissue (leaf or anther). It was harvested into a 1.5 ml microfuge tube and snap frozen in liquid nitrogen, and stored at -70 °C until needed. Tissues were then ground thoroughly using a micro pestle until a fine powder was obtained and processed using the Qiagen RNeasy Mini Kit following manufacturer's instructions. In general an average of 250 ng/µl of total RNA from 100mg of plant material was obtained using this kit. Yield and quality was analysed using a NanoDrop® ND-1000 UV-Vis Spectophotometer (NanoDrop Technologies, Wilmington, Delaware, USA).

2.2.2.1 RNA extraction from bulb scales.

During the early stages of development, *Lilium* bulblets lacked sufficient leaf tissue to perform a standard RNA extraction. The only material available was the small bulblet scales. This type of tissue is rich in polysaccharides and proteins which interfere with many enzymes, thus making any further molecular analysis impossible.

The CTAB protocol from Li *et al.* (2011) with modifications was therefore used for RNA extraction from bulb or bulblet scales. Scales were excised and chopped before transferring into an Eppendorf tube and immediately snap frozen in liquid nitrogen. 700 µl of pre-warmed extraction buffer (Appendix 1) and 1.5% of β-Mercaptoethanol were added to each sample and vortexed to mix completely.

Samples were incubated at 65°C for 30 minutes and vortexed every 10 minutes. After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly. Tubes were then centrifuged at

9000 x *g* at 4°C for 10 minutes. After centrifugation, the upper aqueous phase was carefully transferred into a new Eppendorf tube and an equal volume of chloroform-isoamyl alcohol was added, mixed and centrifuged as previously described. This step was repeated 3 times.

The upper aqueous phase was then transferred into a new Eppendorf tube and mixed with 1/3 volume of 8M lithium chloride. The RNA was precipitated overnight in the freezer at -20°C and pelleted by centrifugation at 9000 x *g* at 4°C for 20 minutes. The supernatant was carefully discarded and the pellet was dissolved with 400 µl DEPC-treated water. The solution was extracted once with an equal volume of chloroform-isoamyl alcohol. The supernatant was then transferred into a new tube and RNA was precipitated with 1/10 volume of 3M sodium acetate and 3 volumes of 100% ethanol at -20°C for 45 minutes and centrifuged at 9000 x *g* at 4°C for 20 minutes.

The supernatant was carefully removed and the pellet washed with 200 µl of 70% (v/v) ethanol. After centrifugation for 5 minutes at room temperature, the supernatant was discarded and the pellet air dried and resuspended in 25 µl of DEPC-treated water.

2.2.3 DNA purification from gels.

DNA was purified from agarose gels by membrane electrodialysis (Gobel *et al.*, 1987) or by QIAquick PCR Purification Kit.

For the electrodialysis method, PCR products were carefully excised from the gel avoiding a long exposure to UV light. Dialysis membrane (Sigma D9777) was previously boiled and stored in 0.5x TBE buffer (Appendix 1) at 4°C. The membrane was cut to size, depending of the amount of

excised gel, and washed with 0.5x TBE buffer five times. Then, one of the ends was clipped and 2 ml of 0.5x TBE buffer poured into the membrane along with the gel pieces. Excess of buffer and air bubbles were removed before closing the other side with a clip.

The dialysis membrane was then placed into an electrophoresis tank in line with the electrodes at 90 volts for 45 minutes. After that, the membrane was inverted for 1 minute and DNA checked under a UV lamp. At this point the DNA had moved to the opposite site from the gel pieces. Using a 1 ml pipette, the buffer was mixed with the DNA within the membrane and transferred to a 1.5 ml microfuge tube.

Butanol concentration was conducted by adding an equal volume of butanol to the sample, shaking briefly and centrifuging for 3 minutes at 9500 x *g*. The supernatant was then discarded and an equivalent amount of fresh butanol was added. This step was repeated until the supernatant was 50-60 µl. The DNA was then precipitated by adding 0.5 volumes of sodium acetate 3M and 10 volumes of 100% ethanol and incubation at -20°C for one hour.

Samples were centrifuged for 15 minutes at 9500 x *g*; the supernatant was discarded being careful not to disrupt the pellet. Samples were then washed in 150 µl 70% (v/v) ethanol by centrifugation for 15 minutes at 9500 x *g*, the supernatant was discarded and the pellet was air dried. DNA was resuspended in 12-15 µl distilled water depending on the desired concentration.

2.2.4 Plasmid DNA extraction.

A single bacterial colony was inoculated into liquid LB medium (Appendix 1) supplemented with the appropriate antibiotics and transferred into the shaking incubator at 150 rpm at 27 °C for *Agrobacterium* and 37 °C for *E. coli* and incubated overnight. The next day, 2 ml of the bacterial culture were pelleted in a 2 ml Eppendorf tube by centrifugation at 1400 x *g* for 5 minutes, the supernatant was discarded and another 2 ml were added to the tube and centrifuged again, this to increase the number of cells and therefore the yield of plasmid DNA.

After discarding the supernatant the pellet was thoroughly resuspended in 200 µl of ice-cold solution I (Appendix 1). Following resuspension 400 µl of solution II (Appendix 1) was added to the sample and mixed by inversion 5 or 6 times. The tube was left at room temperature for 5 minutes. After this time 300 µl of ice-cold solution III (Appendix 1) was added to the Eppendorf and it was mixed gently by inversion and incubated on ice for 15 minutes.

The tube was then centrifuged for 5 minutes at 9500 x *g* to pellet debris and the supernatant was carefully transferred into a new tube. Ice-cold isopropanol was added to the supernatant in a ratio of 1:1 and left at room temperature for 3 minutes. After this the tube was centrifuged for 5 minutes at 9500 x *g* and supernatant was discarded carefully to avoid disturbing the pellet which then was washed by adding 300 µl of 70 % (v/v) ethanol and centrifuged for 2 minutes. The ethanol was discarded and the tube was left to air-dry. The pellet was finally resuspended in 50 µl of RNase free water. For biolistics transformation or cloning, plasmid DNA extraction of a higher purity was needed; therefore Gen-Elute Plasmid Miniprep Kit (Sigma-Aldrich, USA), following manufacturer's instructions, was used. A fresh 100 ml overnight culture of the bacteria was used each time.

2.3 Molecular analyses.

For the confirmation of the correct insertion and expression of the desired genes into the lily genome, as well as the identification of putative gene orthologs from other species into lily, molecular analysis such as PCR and RT-PCR were performed.

2.3.1 Polymerase Chain Reaction analysis.

PCR analysis was used to screen transgenic plants (Wassenegger, 2001) and to amplify DNA fragments for cloning applications. The PCR mixture used to screen for transgene insertion is described in Table 2.1. For the amplification of DNA for further cloning, Phusion High Fidelity DNA polymerase (New England Biolabs, USA) was used; the reaction conditions are described in Table 2.2.

All the primers used were manufactured by MWG Biotech (Ebersberg, Le Dome, Germany) and a stock solution of 100 pmol/µl of primer was prepared with sterile purified water and stored at -20°C. The working solution of primers in the PCR reactions was 20 pmol/µl. A detailed list of primers and specific thermal cycles is described in each corresponding chapter.

PCR REACTION MIXTURE FOR TRANSGENE ANALYSIS	
Sigma RED Taq Ready Mix	7µl
Gene specific forward primer (20 pmol/µl)	0.3µl
Gene specific reverse primer (20 pmol/µl)	0.3µl
Genomic DNA (30-40 ng/µl)	2µl
Betaine 1M	3µl
PCR reaction water	2.4µl
FINAL VOLUME	**15µl**

Table 2.1 Standard PCR reaction mixture and volumes.

PHUSION PCR REACTION MIXTURE FOR CLONING	
Phusion High-Fidelity polymerase (NEB)	0.2µl
Gene specific forward primer (20 pmol/µl)	0.5µl
Gene specific reverse primer (20 pmol/µl)	0.5µl
DNA sample (30-40 ng/µl)	2µl
Betaine 1M	4µl
High Fidelity Buffer (NEB)	4µl
dNTP's (10mM)	0.4µl
DMSO	0.6µl
PCR reaction water	7.8µl
FINAL VOLUME	**20µl**

Table 2.2 Phusion High-Fidelity polymerase PCR reaction mixture and volumes.

The PCR reaction mixture was pipetted into individual thin-walled 0.5 ml PCR Eppendorf tubes, and subsequently transferred to the PCR machine (DNA Thermal Cycler 480; Perkin Elmer Applied Biosystems Division, Warrington, UK) for amplification.

The appropriate plasmid DNA (1µl ≈ 50ng) was used as a positive control in all cases in the PCR reactions. All reactions included DNA from a non-transgenic control plant and one water sample as negative controls to validate the PCR. Thermal cycles for each set of primers used are described in the appropriate section of each chapter.

PCR products were separated by gel electrophoresis. Gels were prepared using 1.0% (w/v) Seakem LE Agarose (Cambrex, Rockland, USA) in 1 x TAE buffer (Appendix 1) containing ethidium bromide (0.05µl/ml). The agarose gel was run in an electrophoresis tank (Fisher Scientific) with 1 x TAE buffer for 40 minutes at 95 V. Depending on the expected size of the products Hyperladder I, IV or V (Bioline, London, UK.) were used as DNA markers. Gel images were taken with a UV transilluminator with a camera to visualise the bands and size of the PCR products. Genesnap software (Syngene, Loughborough, UK) was used to capture and save the images.

2.3.2 Colony PCR.

Each of the single bacterial colonies from *E. coli* and *Agrobacterium* to be analysed was first subcultured in 2 ml of liquid LB medium supplemented with the appropriate antibiotics and incubated for 2 hours on a rotary shaker at 37 °C or 27 °C respectively.

A routine PCR reaction using RED Taq polymerase was then performed as described in section 2.3.1 but only 1 µl of the liquid culture was used as template. For the thermal cycle an initial denaturation of 6 minutes was used.

2.3.3 Reverse Transcriptase-PCR analysis.

For RT-PCR analysis, cDNA was synthesized from total RNA extracted as described in section 2.2.2, using the Superscript III kit (Invitrogen) following manufacturer's instructions. For each sample 1 µg of total RNA was added into a microcentrifuge tube, the reaction comprised 1 µl of oligo$(dT)_{12-18}$, 1 µl of 10 mM dNTP Mix (dATP, dGTP, dCTP and dTTP at neutral pH), and the volume was topped up to 13 µl with RNase free water. The mixture was heated at 65°C for 5 minutes and immediately incubated on ice for 2 minutes.

The tubes were briefly centrifuged and 4 µl of 5X First-Strand Buffer, 1 µl of 0.1M DTT, 1 µl of RNaseOUT and 1 µl of Super Script III RT were added, everything was gently mixed by pipetting and incubated at 50°C for 60 minutes. Immediately after this, the reaction was inactivated by heating at 70°C for 15 minutes. Synthesized cDNA was ready to use for PCR reaction. The remaining cDNA was stored at -70°C until needed, cDNA used was stored for no longer than 2 months.

2.3.4 Quantitative Reverse Transcriptase-PCR analysis.

Primers for QRT-PCR were designed using PRIMER 3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and ordered from Eurofins. SYBR Green qPCR master mix 2x (Fermentas) was used for all the experiments according to manufacturer's instructions; the reaction mixture is described in Table 2.3. Samples were analysed in triplicate on a Light Cycler 480 apparatus (Roche Diagnostics, West Sussex, UK) and the resulting data was analysed using Microsoft Office Excel (Microsoft Corporation, Redmond, USA).

QRT-PCR REACTION MIXTURE	
SYBR Green qPCR Master Mix 2x	4.5µl
Gene specific forward primer (10 µM)	0.1µl
Gene specific reverse primer (10 µM)	0.1µl
cDNA (50 ng/µl)	0.4µl
PCR reaction water	4.0µl
FINAL VOLUME	**9.1µl**

Table 2.3 QRT- PCR reaction mixture and volumes.

2.4 Cloning of genomic sequences into plasmids.

Topoisomerase based cloning technology allows ligation of DNA with compatible ends. PCR products were amplified using Phusion polymerase as described in section 2.3.1. Purified products were then cloned into pCR®8/GW/TOPO, pDONR207 or pCR®-BluntII-TOPO using the protocol supplied by the manufacturer (Invitrogen, USA). Cloning was performed overnight and is described in Table 2.4.

Cloning reaction	
Purified PCR product (20 ng/µl)	4µl
Salt Solution (1.2 M NaCl + 0.06 M $MgCl_2$)	0.5µl
PCR Reaction water	1.2µl
Plasmid Vector (10 ng/µl)	0.3µl
FINAL VOLUME	**6µl**

Table 2.4 Cloning reaction mixture and volumes.

The cloning reaction was then transformed into chemically-competent DH5α *E. coli* cells by adding 4 µl from the TOPO cloning reaction into a 1.5 ml vial of competent cells (~60µl). After 30 minutes of incubation on ice, the cells were heat shocked at 42°C for 1 minute and 30 seconds, then, immediately, transferred to ice for 5 minutes and after that, 250 µl of SOC medium (Appendix 1) was added. The sample was incubated for 1.5 hours in a rotary 37°C shaker at 150 rpm, and then volumes of 50 µl, 100 µl and 150 µl were spread onto semisolid LB medium plates supplemented with the appropriate antibiotic depending of the plasmid used.

Plates were left overnight at 37°C and colonies were screened using primers flanking the cloning sites to amplify the target fragment plus part of the vector sequence between the two primers. For the pCR®8/GW/TOPO and pCR®-BluntII-TOPO plasmids the primers used were M13F (5'-TGTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') adding around 170bp to the expected band size and for the pDNOR207 the primers pD207F (5'-TCG CGT TAA CGC TAG CAT GGA TCT C-3') and pD207R (5'-GTA ACA TCA GAG ATT TTG AGA CAC-3') adding around 210bp to the expected band size.

2.4.1 Overhanging of blunt-end PCR products.

DNA amplification by Phusion polymerase gives blunt PCR products therefore there was a need to add an overhanging adenine base before they could be cloned into the TOPO vector pCR8®/GW/TOPO. The reaction mix for the overhanging is described in Table 2.5. The mixture was incubated at 72°C for 20 minutes and then immediately used for cloning.

Overhang reaction	
Purified PCR product (20 ng/µl)	13µl
dATP (10 mM)	1µl
PCR Buffer with Mg (10x)	5µl
Taq DNA Polymerase (5 U/µl)	0.2µl
PCR reaction water	0.8µl
FINAL VOLUME	**20µl**

Table 2.5 Overhang reaction mixture and volumes.

2.4.2 Sequencing

Sequencing reactions were performed using 100-200 ng template DNA and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosynthesis, USA) following the manufacturer's instruction. The reaction and thermal cycles used are described in Table 2.6 and 2.7 respectively. After the Big dye reaction, the sample was transferred into a 500 µl Eppendorf tube and precipitated by adding 10 µl of PCR water, 5 µl of 3M NaOAc and 50 µl of 100% ethanol and centrifuging at room temperature for 20 minutes at 9500 x *g*. The pellet was washed with 150 µl of 70% (v/v) ethanol and centrifuged for 5 minutes. The supernatant was discarded and the pellet air dried. Samples were sent to the Genomics Facility at Queen's Medical Centre of the University of Nottingham where they were read on an ABI 3130 genetic analyser (Applied Biosystems). Results were checked using Bioedit and Vector NTI software.

Big Dye reaction	
DNA template (50 ng/µl)	2µl
Big dye terminator V1.1	0.8µl
Primer (10 pmol)	0.5µl
Better Buffer 5X	2µl
PCR reaction water	2.7µl
FINAL VOLUME	**8µl**

Table 2.6 Big Dye sequencing reaction mixture and volumes.

Thermal Cycle for Big Dye			
Initial denaturation	96°C	1:30 min	1 step
Denaturation	96°C	0:30 min	60 cycles
Annealing	50°C	0:30 min	
Extension	60°C	4:00 min	
Final Extension	28°C	1:00 min	1 step

Table 2.7 Thermal Cycle for Big Dye sequencing reactions.

2.5 PREPARATION OF COMPETENT CELLS.

2.5.1 Modified protocol from Inoue *et al.* (1990) for *E. coli* chemically ultra-competent cells.

A single colony of *E. coli* from a fresh LB plate was inoculated in a sterilized 250 ml Erlenmeyer flask with 100 ml of SOB media (Appendix 1)

without antibiotics as a starter culture. The flask was transferred to the 37°C shaker at 120 rpm and left overnight. The next day 2 Erlenmeyer flasks each with 100 ml of SOB media without antibiotics and 1 ml of the starter bacterial culture were incubated until the optical density (OD_{600}) reached 0.9 - 1.0; cell cultures were placed on ice for 10 minutes and then split into four pre-chilled 50 ml falcon tubes and centrifuged at 1000 x *g* for 15 minutes at 4°C. The supernatants were discarded and cells were gently resuspended in 20ml ice-cold TB (Appendix 1). In this step, the four falcon tubes were combined into two tubes and were kept on ice for 15 minutes. Cells were then centrifuged at 1000 x *g* for 15 minutes at 4°C. Carefully the supernatant was removed and the pellet was finally resuspended in 5 ml ice-cold TB, 0.4 ml pre-chilled DMSO were then added to the suspension and mixed. Aliquots of 50 µl were prepared for immediate use in transformations, the remaining aliquots were snap frozen in liquid nitrogen and further kept in the -70°C freezer until needed.

2.5.2 *E. coli* electro competent cells.

A single *E. coli* colony from a fresh LB plate was inoculated in a sterilized 250 ml Erlenmeyer flask with 100 ml of LB media without antibiotics as a starter culture. The flask was transferred to the 37°C shaker at 120 rpm and left overnight. The next day 2 Erlenmeyer flasks were incubated each with 100 ml of LB media without antibiotics and 1 ml of starter bacterial culture. When the OD_{600} was 0.9 - 1.0 cell cultures were placed on ice for 15 minutes and then split into four pre-chilled 50 ml falcon tubes and centrifuged at 1400 x *g* for 25 minutes at 4°C.

Tubes were kept on ice and the supernatant quickly, but carefully not to disturb the pellet, removed. Each tube was then resuspended in 50 ml of ice-cold sterilized water and centrifuged again at 1400 x *g* for 25 minutes at 4°C. The supernatants were removed and the pellet was resuspended

in 25 ml of ice-cold sterilized water, at this point the four falcon tubes were combined into two tubes and centrifuged at 1400 x *g* for 25 minutes at 4°C.

The supernatant was removed and pellet was resuspended in 20 ml ice-cold 10% (w/v) sterilized glycerol and centrifuged at 1400 x *g* for 10 minutes at 4°C. The supernatant was discarded and the pellet was finally resuspended in 3 ml of ice-cold 10% (w/v) sterilized glycerol. Cell suspensions were kept on ice and 50 µl were aliquoted in 1.5 ml microcentrifuge tube and snap frozen in liquid nitrogen. Competent cells were kept at -70°C until required.

2.5.3 *Agrobacterium* electro competent cells.

A single *Agrobacterium* colony from a fresh LB plate was inoculated in a sterilized 250 ml Erlenmeyer flask with 100 ml of LB media with the appropriate antibiotics as a starter culture. The flask was transferred to the 27°C shaker at 150 rpm and incubated overnight. Late the next day 2 Erlenmeyer flasks were incubated each with 100 ml of LB media with antibiotics and 1 ml of starter bacterial culture and incubated overnight. On the following morning cell cultures reached an OD_{600} between 1.0 and 1.2 and immediately were placed on ice for 15 minutes to stop growth. Cultures were split into four pre-chilled 50 ml falcon tubes and centrifuged at 1000 x *g* for 20 minutes at 4°C. The supernatant was then discarded and the pellet was thoroughly resuspended in 30 ml ice-cold sterilized water and centrifuged at 1000 x *g* for 15 minutes at 4°C.

The supernatant was removed and pellet was resuspended in 15 ml of ice-cold sterilized water, at this point the four falcon tubes were combined into two and centrifuged at 1000 x *g* for 15 minutes at 4°C. The supernatant was carefully removed and the pellet was resuspended again

in 15 ml of ice-cold sterilized water and centrifuged at 1000 x *g* for 10 minutes at 4°C. This step was repeated one more time. Finally after discarding the supernatant, the pellet was completely resuspended in 4 ml of 15% (w/v) ice-cold glycerol. Aliquots of 50 µl were dispensed and snap frozen in liquid nitrogen. Competent cells were kept at -70°C for further use.

Regardless of the experiment, all competent cells used in this research were not more than 6 months old.

2.5.3.1 Transformation of *Agrobacterium* electroccompetent cells by electroporation.

For transformation, a vial of *Agrobacterium* electro-competent cells was used. 1 µl of plasmid DNA (~5 ηg) was added and mixed by gentle swirling. The mixture was transferred into a prechilled electroporation cuvette. An electric pulse (2.5 V, 25µFD, 400 Ω) was applied to the cuvette using a Bio-Rad MicroPulser (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), immediately 500 µl of LB medium were added and the mix transferred into a 2 ml microfuge tube. The tube was incubated at 28°C in a rotary shaker for 4 hours. After 4 hours the cultures were spread on Petri dishes containing semisolid LB medium supplemented with the appropriate antibiotics.

2.6 GUS assay of transformed tissues.

Histochemical analysis of *GUS* gene expression was performed based on the method of Jefferson (1987), but lacking potassium ferricyanide and potassium ferrocyanide. Tissues were scored with a scalpel before being incubated for 24 to 48 h at 37°C in 100 mM sodium phosphate (pH 7.0)

containing 0.1% (v/v) Triton X-100 and 1mM X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid; Melford, Ipswich, UK). The reaction was terminated by transferring explants into 100% ethanol, which also removed chlorophyll from the tissues, facilitating visualization of GUS activity. Samples were transferred into the fridge and ethanol was changed daily until all the chlorophyll was eliminated from the tissues. After this, the analysis of GUS expression was made on a stereomicroscope.

CHAPTER 3: GENETIC TRANSFORMATION OF *LILIUM*

3.1 Introduction.

Genetic transformation of lily represents an alternative approach to traditional breeding. Production of novel cultivars can potentially be achieved in a shorter period of time by introducing specific desirable traits directly into lily's genome (Mori *et al.,* 2005). This can also represent a solution for limitations, such as incompatibility between several species and/or cultivars.

For lily, successful *Agrobacterium*-mediated transformation protocols have been reported in different tissues. High efficiency (90-95%) pollen transformation for transient expression has been reported by Kim *et al.* (2007) and Dong *et al.* (2007). Stable transformation and regeneration of complete plants has been reported using callus (Lipsky *et al.,* 2002; Mercuri *et al.,* 2003; Hoshi *et al.,* 2004; Ogaki *et al.,* 2008) with efficiencies ranging from 1.0% to 1.5%, thus achieving slightly better results compared with reported efficiencies of 0.7% using stems (Langeveld *et al.,* 1995) or 0.8% to 1.0 % with bulblet scales (Cohen and Meredith, 1992; Tsuchiya *et al.,* 1996; Qiu Hua *et al.,* 2008). However, none of these methods have been reported to be used by another author in further research, which suggests that reproducibility is low.

A successful and efficient transformation protocol for any species has to be based on a quick and efficient method of *in vitro* regeneration of tissues. The selection of starting material is crucial for this objective. In *Lilium* several authors have reported successful *in vitro* regeneration from a variety of tissues for species like *L. x formolongi* hort. (Godo *et al.,* 1998), oriental hybrids Casa Blanca, Siberia and Acapulco (Horita *et al.,* 2002), oriental hybrid Star Gazer and asiatic hybrid Elite (Bachetta *et al.,* 2003), *L. longiflorum* (Nhut *et al.,* 2001; Nhut 2003) *L. auratum, L.*

lancifolium, L. formosanum and L. rubellum (Mori et *al.,* 2005). These successful regeneration reports for lily provide the possibility of improvement that can lead to a more efficient transformation protocol.

Although the transformation efficiencies are very low, the fact that transgenic lily plants have been obtained by *Agrobacterium* mediated protocols indicate that such transformations are possible and therefore provide opportunities to further improve this technique. Parameters like strain, co-culture period and medium composition have still not been optimized. Higher transformation efficiencies in other species using callus shows that tissues with high number of undifferentiated cells and high cellular division are the desirable starting material to use for transformation (Cohen *et al.,* 2004).

Particle bombardment protocols in *Lilium* have had the best results reported so far with transformation efficiencies ranging from 1.0% to 2.6% (Nishihara *et al.,* 1993; Watad *et al.,* 1998; Ahn *et al.,* 2004; Benedito *et al.,* 2005; Kamo and Han, 2008), with the highest to date of 2.6% (Irifune *et al.,* 2003; Cohen *et al.,* 2004). This is almost twice the transformation efficiency compared with *Agrobacterium* mediated protocols; therefore biolistics was initially adopted as the main transformation procedure used for this research.

A key requirement for the genetic manipulation of lily, either by biolistics or *Agrobacterium* approaches, is the selection of transgenic tissues. So far it has taken a very long time for the death of non-transgenic tissues to occur (up to 5 months) and the large amount of surviving escapes (non-transgenic plants showing resistance to the selection agent) make protocols inefficient, time consuming and unreliable. The use of liquid medium (Cohen *et al.,* 2004) and combinations of different medium and selection agents are modifications that could provide improvements to the

selection procedure making the genetic manipulation of this species easier.

3.2 Materials and Methods.

3.2.1 Regeneration protocol.

3.2.1.1 Callus induction.

Several combinations of auxins, for example picloram, NAA, dicamba and 2,4-D, combined with cytokinins such as zeatin, kinetin, thidiazuron and BAP were tested for the production of callus from different tissues of lily. Evaluation was made on several parameters including percentage of explants producing callus, size, life span, colour and type of callus.

3.2.1.2 Effect of antioxidants on callus development.

After the analysis on the composition of the callus induction medium it was realized that even though rapid generation of callus occurred, prolonged maintenance of it was not possible. The main reason for this was the high sensitivity of lily tissues to stress and mechanic manipulation resulting in the production of high levels of phenolic compounds. Therefore several antioxidant compounds such as ascorbic acid, silver nitrate, thiamine, activated charcoal and lipoic acid, were tested in the callus induction medium.

3.2.1.3 Selection agent assay.

Once the optimal growing medium was established for the explants it was necessary to establish the best selection agent to use for the transformation experiments. *Lilium* bulblets were previously transformed by Ribas (2007) using the particle bombardment approach. Five transgenic lines carrying the *neomycin phosphotransferase* II gene, conferring resistance kanamycin, were obtained. This material was used to determine which selection agent was best suited for *Lilium* transformation. Since kanamycin and hygromycin belong to the same antibiotic family they were used to perform the antibiotic resistance assay.

Bulblet scales from *in vitro* grown material were excised from wild type and transgenic plants and placed on 9 cm Petri dishes containing MS-SC, composed of MS basal salts, 30 g/l sucrose, 8 g/L agar, 1 mg/L ascorbic acid, 1 mg/L citric acid and 1 mg/L of 2,4-D and supplemented with 100, 150, 200, 250 mg/L of kanamycin or with 50, 100, 125, 150 mg/L of hygromycin. Explants were transferred into the dark for 6 weeks with subculturing every two weeks. After this time measurement of callus growth and presence of any shoots was recorded.

3.2.2 Biolistics-mediated transformation.

White-yellow, compact morphogenic callus and adventitious bulblet scales of *Lilium* cv. Star Gazer were produced from tissue cultures based upon the procedures of Horita *et al.* (2002) as described in chapter 2 section 2.1 and maintained either in MS-SC medium for the scales, or MS-CI4 (Appendix 1) for the calli. These explants were used as target material in all biolistics transformation experiments. Young white-green scales showed a higher growth rate, therefore these were presumed to be better suited for transformation experiments. The target tissue was arranged in

the centre of the Petri dishes in a 2 cm diameter circle. Depending on the size of the adventitious bulblets and/or callus, 6 to 10 explants were placed in the central circle.

3.2.2.1 Plasmid DNA isolation.

For all the biolistics transformation experiments plasmid pBI121, kindly provided by Dr. Karmesware Naiken from the University of Nottingham, harbouring the *NPTII* gene driven by the nos promoter and the *GUS* gene driven by the CaMV35S promoter was used. Plasmid DNA was isolated using DNA mini preparation extraction kit (Sigma-Aldrich, USA) according to the manufacturer instructions. Plasmid DNA was eluted in 50 µl nuclease-free water, or if a higher concentration was needed, into 25 µl. The extracted DNA was then quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Plasmid DNA with a concentration > 500 ng/µl was used for biolistics. The extracted DNA was maintained at -20°C until needed.

3.2.2.2 Preparation of microprojectiles.

Gold particles of 0.4-1.2 µm in diameter (Alfa Aesar, Lancaster Avocado, UK) were used as microprojectiles. A stock suspension of 60 mg/ml was prepared by placing 60 mg of gold particles in a microfuge tube. One ml of absolute ethanol was added and then mixed by vortexing for 2 minutes. The gold particles were pelleted by centrifugation at 9500 x *g* for 1 minute and the supernatant removed. This step was repeated 3 more times. After discarding the supernatant following the final centrifugation, the gold particles were resuspended in 1 ml of sterile purified water. The gold particles were stored at 4°C until needed.

3.2.2.3 Coating of microprojectiles.

After preparation described in section 3.2.2.2 the microprojectiles were coated with plasmid DNA shortly before each bombardment. This protocol produced enough plasmid DNA coated microprojectiles to prepare 6 macrocarriers (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The gold particle stock suspension was vortexed for 5 minutes and 50µl transferred into a microfuge tube. With continuous vortexing, 10 µl of plasmid DNA (1 µg/µl) was added followed by 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine (free-base; Sigma-Aldrich). Protamine (Sigma-Aldrich) has been reported to increase the efficiency of transformation by biolistics due to a better coating and release of DNA from microparticles (Sivamani *et al.,* 2009). Based on this report tissues were also bombarded with the microparticles which were coated using an equivalent amount of protamine rather than spermidine. This mixture was vortexed for an additional 3 minutes and then immediately centrifuged at 9500 x *g* for 20 seconds and the supernatant removed. The particles were then resuspended in 250 µl absolute ethanol and mixed by vortexing for 1 minute. Gold particles were pelleted by centrifugation at 9500 x *g* for 20 seconds and the supernatant discarded. Finally the gold particles were resuspended in 75 µl absolute ethanol and mixed by vortexing. An aliquot of 10 µl of coated gold particles was pipetted onto the centre of a sterile macrocarrier (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), which was assembled in the steel macrocarrier holder, and left in a laminar flow cabinet to air dry for 15-20 minutes.

3.2.2.4 Microprojectile bombardment.

Bombardments were performed using the Biolistics® PDS-1000/He device (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) installed inside a laminar flow cabinet. The laminar flow cabinet and the chamber of the Biolistics® PDS-1000/He instrument were sprayed with 70% (v/v) ethanol

for sterilization and left to dry. The macrocarriers, macrocarrier holders and the stopping screens (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) were sterilised by autoclaving at 120°C for 16 minutes and rupture discs were sterilised by soaking them in 70% (v/v) isopropanol prior to assembling the retaining cap.

Prior to each bombardment, the explants were pre-cultured on Petri dishes filled with MS-CI (Appendix 1) for a period of 24 hours in the dark. The bombardment parameters used in all the experiments were a 30 mm distance between rupture disc and macrocarrier, 10 mm between macrocarrier and stopping screen, a vacuum of 26 inches of Hg. The distance between target tissue and the stopping screen was 7cm. The rupture discs used for the experiments were of 1550 psi. For both plasmids, the total number of shots to the tissues was two. When a second bombardment was carried out on adventitious bulblets, the explants were turned around using sterile forceps prior to the second bombardment. This is to allow more particles to penetrate into the thick tissue of the explants.

3.2.2.5 Growth of tissues post bombardment.

Bombarded adventitious bulblets and calli were transferred into Petri dishes containing 30 ml of MS-CI4 without antibiotics for 48 hours in the dark. After that period half of the bombarded tissues were transferred into Petri dishes containing MS-CI4 supplemented with 5 mg/L of hygromycin which had been previously reported as a good selection antibiotic (Kamo and Han, 2008) and kept in the dark. The other half of the explants were transferred into 10 µl of MS-CI4 liquid medium supplemented with 5 mg/L of hygromycin into 100 ml Erlenmeyer flasks. The flasks were transferred into a rotary shaker at 110 rpm in the dark. Both batches of explants were then subcultured on a weekly basis for the slow selection protocol. In each

experiment, a minimum of 10 explants were grown under the same parameters without being bombarded as a control.

3.2.3 *Agrobacterium*-mediated transformation.

3.2.3.1 *Agrobacterium* strains and plasmids.

Five *Agrobacterium tumefaciens* strains were selected in order to test their pathogenicity on *Lilium* tissues by transient GUS expression. Strains C58 and GV3101 were provided by Dr Caiyun Yang and Dr Hongying Li respectively, strains LBA4404 and EHA105 were provided by Dr Paul Anthony, University of Nottingham, UK. Finally, strain AGL1 was provided by Mark Smedley, John Innes Centre, Norwich, UK. Table 3.1 provides more information about the strains used in this research. Plasmid pBI121 (Fig. 3.1) harbouring the intron-containing β-glucuronidase (*GUS*) gene fused to the CaMV35S promoter and the neomycin phosphotransferase (*NPTII*) gene under the control of the NOS promoter was kindly provided by Dr. Karmesware Naiken, University of Nottingham, UK.

Strain	Opine	Ti Plasmid
LBA4404	Octopine	pAL4404
C58	Nopaline	-
GV3101	Nopaline	-
EHA105	Succinamopine	pTiBo542ΔT-DNA
AGL1	Succinamopine	pTiBo542ΔT-DNA

Table 3.1 *Agrobacterium* strains used in this research. Modified from Hellens *et al.*, 2000.

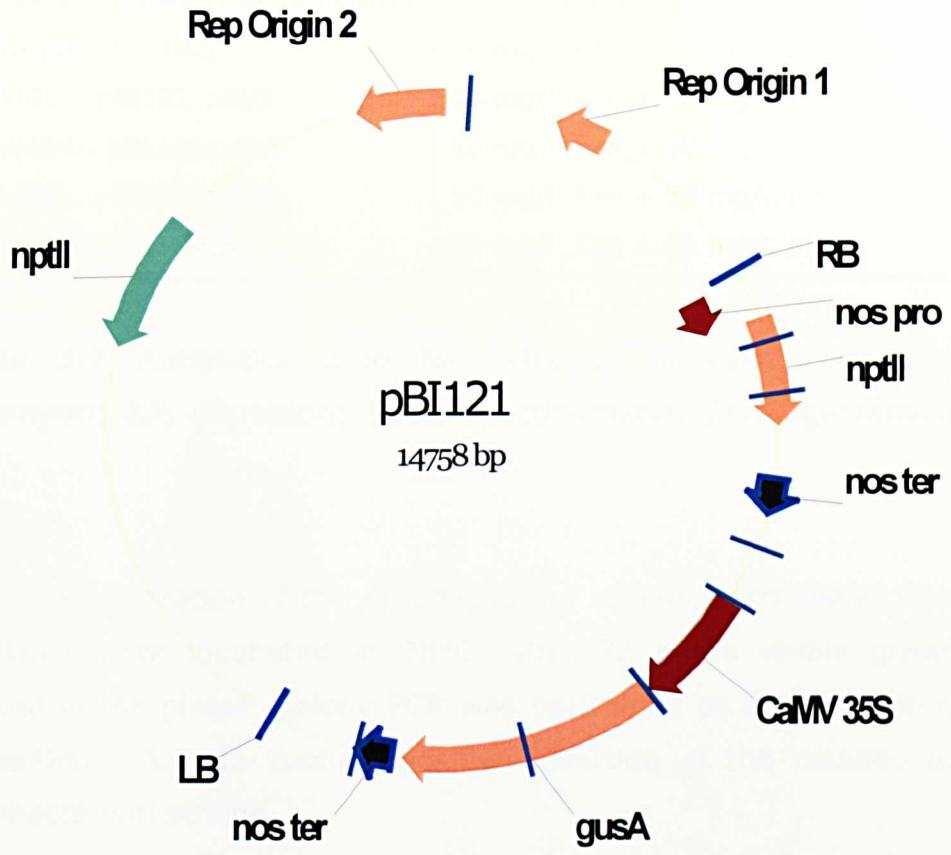

Figure 3.1 Plasmid pBI121 harbouring the intron-containing *β*-glucuronidase (*GUS*) gene fused to the CaMV35S promoter and the neomycin phosphotransferase (*NPTII*) gene under the control of the NOS promoter.

3.2.3.1.1 *Agrobacterium* strains transformation and maintenance.

Plasmid pBI121, described in section 3.2.3.1, was transformed into each of the *Agrobacterium* strains from Table 3.1, resulting in five different vectors. Transformation of the plasmid into *Agrobacterium* was done by electroporation as described in chapter 2, section 2.5.3.1. Table 3.2 describes the appropriate antibiotic combinations used in the subculture medium for each construct.

Agrobacterium/CONSTRUCT	SUBCULTURE MEDIUM
C58:: pBI121::*GUS*	50 mg/L Kan + 30 mg/L Rif
GV3101:: pBI121::*GUS*	50 mg/L Kan + 30 mg/L Gent + 5 mg/L Tet
LBA4404:: pBI121::*GUS*	50 mg/L Kan + 30 mg/L Rif
EHA105:: pBI121::*GUS*	50 mg/L Kan + 50 mg/L Rif
AGL1:: pBI121::*GUS*	50 mg/L Kan + 50 mg/L Rif

Table 3.2 Antibiotics used for selection of each construct. Kan, kanamycin; Rif, rifampicin; Spec, spectinomycin; Gent, gentamicin; Tet,

After electroporation of the *Agrobacterium* strains, Petri dishes from each construct were incubated at 28°C; after 72 hours visible growth was noticed in the plates. Colony PCR was performed as described in chapter 2, section 2.3.2, to confirm positive insertion of the plasmid into the *Agrobacterium* strains.

One positive colony of each construct was then subcultured into LB liquid medium supplemented with the correspondent antibiotics and incubated into the rotary shaker at 28°C in the dark. If needed this bacterial suspension was subcultured every 7 days into LB liquid medium to maintain it for experiments. Glycerol stocks were prepared of each construct from an overnight culture with a final concentration of 80 % bacterial suspension and 20% (w/v) sterile glycerol. Stocks were kept at -70°C until needed.

3.2.3.2 Standard *Agrobacterium*-mediated transformation.

Using the five developed constructs, *Agrobacterium*-mediated transformation was attempted for lily using *in-vitro* grown bulblet scales as explants. To act as a positive control for these transformation

experiments, *Solanum nigrum* leaf explants were used due to its high susceptibility to *Agrobacterium* infection (Djikstra *et al.,* 2008). Prior to each transformation, young leaves were excised from the glasshouse plants and then sterilized by immersion in a 15% (v/v) Domestos solution (Unilever UK Ltd, United Kingdom) for a period of 20 minutes. Then they were rinsed three times with sterile purified water and scored with a sterile scalpel on each side.

For the *Lilium* transformation, the five constructs carrying the *GUS* gene were grown overnight before each transformation in LB liquid medium supplemented with the appropriate antibiotics. The overnight culture with an OD_{600} (optical density) of 0.8-1.2 was then diluted 1:5 (v:v) with MS0 liquid medium. The tissues were immersed in a 15ml diluted bacterial culture in a 9cm Petri dish for 10 minutes for the C58 construct and for 3 minutes for the remaining constructs. Explants were then placed into Petri dishes containing 20 ml of semi-solid MS-SC medium (5-10 explants per dish). The plates were sealed with Nescofilm (Bando Chemical Ind. Ltd, Kobe, Japan), and explants were left to incubate for a period of 3 days.

After this time, explants were transferred onto MS-SC medium supplemented with antibiotics (further denominated MS-SL) to give final concentrations of 500mg/L carbenicillin, 100mg/L cefotaxime and 150mg/L kanamycin. Whenever overgrowth of *Agrobacterium* was observed, explants were transferred to a new plate containing semi-solid MS-SL medium after blotting the explants on filter paper to remove excess bacteria. Similarly, 25 un-inoculated explants were placed in five 9cm Petri dishes containing 20ml of the MS-SC media without antibiotics as positive controls. A second batch of 25 explants was placed in another set of five Petri dishes with MS-SL which acts as negative controls.

For the *S. nigrum* transformation, the procedure was the same, but the medium used was MSZ with or without antibiotics (MS basal salts + 30 mg/L sucrose + 1.0mg/L zeatin, 0.8% (w/v) agar) instead of MS-SC.

Non-transgenic tissue of both species were raised alongside putatively transgenic plants to validate that the results obtained from the transformation experiments were due to the presence of transgenes and not due to phenotypic variation induced by the tissue culture process.

3.2.3.3 *Agrobacterium*-mediated transformation protocol modified from Qiu Hua *et al.* (2008).

A different protocol was also used for lily transformation, based on the reports by Qiu-Hua *et al.* (2008) and the lack of results from the previous protocol; some of the parameters were modified to attempt the production of transgenic plants. This protocol was applied only to *Lilium* explants since the constructs successfully transformed the *Solanum nigrum* leaf discs using the previous protocol, thus confirming that they could transfer the transgene effectively.

A single colony of *Agrobacterium*, carrying the target gene, was inoculated in LB liquid medium supplied with the appropriate antibiotics and cultured overnight on a rotary shaker at 28°C and 200 rpm. Four to six hours before shaking stopped, 20 mg/L of acetosyringone (AS) was added. The *Agrobacterium* was used with an OD_{600} between 0.8 and 1.2. Bulblet scales excised from *in-vitro* grown plants were scored on both sides immediately before inoculation.

The explants were immersed in a 20ml bacterial culture supplemented with 0.1% Tween 20, to enhance penetration of the bacterial suspension

into the wounded tissues, in a 9cm Petri dish. Inoculation time for all the constructs was 5 minutes. After infiltration, the explants were transferred on to MS-CC medium (MS-SC supplemented with 20 mg/L AS + 0.1% Tween 20) and cultured in the dark for a period of 7 days. At the end of co-cultivation, half of the explants were transferred on to MS-SL Petri dishes and the other half into liquid MS-SL to analyse which selection method was better. The explants were subcultured on a weekly basis.

3.2.3.4 *Agrobacterium*-mediated transformation protocol for lily.

Due to the failure to transform lilies with the previous protocols, a different procedure was developed based on the regeneration protocol used in the lab and some new information on the media composition during inoculation and co-culture (Ogaki *et al.*, 2008; Azadi *et al.*, 2010).

3.2.3.4.1 Pre-culture of basal plate discs.

Basal plate discs of 3-6 mm in diameter and 0.5-1.5 mm of thickness were excised and pre-cultured for 1h on callus induction medium composed of MS basal salts supplemented with 2.0 mg/L picloram, 1 mg/L zeatin, 1 mg/L kinetin, 30 g/L sucrose, 50 mM lipoic acid and semi-solidified with 2.6 g/L Phytagel.

3.2.3.4.2 Inoculation of explants.

A single colony of *Agrobacterium,* strain AGL1, was cultured overnight on a rotary shaker at 28°C in 50 ml of liquid Luria Broth (Bertani, 1951) at pH 7.0 containing 50 mg/L kanamycin and 50 mg/L rifampicin. Five hours prior to inoculation of plant material, acetosyringone was added to the

bacterial suspension to a final concentration of 100 µM. The bacterial culture was centrifuged at 1400 x *g* for 20 minutes and resuspended in inoculation medium composed of MS salts, but lacking KH_2PO_4, NH_4NO_3, KNO_3 and $CaCl_2$, supplemented with 100 µM acetosyringone and 30 g/L sucrose (Azadi *et al.*, 2010). The formulation of Azadi *et al.* (2010) was modified to contain 2.0 mg/L picloram, 1 mg/L zeatin, 1 mg/L kinetin, 3.9 g/L 2-(N-morpholino)ethanesulfonic acid (MES) and 0.2% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, USA). The resuspended bacterial culture used for inoculation had an OD_{600} = 0.8. Twenty µl of bacterial suspension were pipetted onto each explant and left for 5 minutes.

3.2.3.4.3 Co-Culture of inoculated explants.

Immediately after inoculation, the explants were transferred onto co-culture medium of similar composition to the medium used for inoculation, but lacking Silwet and acetosyringone, with the addition of 50 mM lipoic acid and semi-solidified with 2.6 g/L Phytagel. Explants were cultured in the dark at 23°C for 2 days.

3.2.3.4.4 Transition stage of inoculated tissues.

After 2 days co-culture, explants were washed twice with sterile reverse osmosis water containing 200 mg/L timentin and 400 mg/L cefotaxime to remove *Agrobacterium* overgrowth, before rinsing with sterile reverse osmosis water. Explants were blotted with a sterile filter paper before transfer onto transition medium. The latter was composed of MS salts with 2.0 mg/L picloram, 1 mg/L zeatin, 1 mg/L kinetin, 30 g/L sucrose, 2.5 g/L activated charcoal, 50 mM lipoic acid, 150 mg/L kanamycin, 150 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. Cultures were incubated in the dark at 23°C for 14 days.

3.2.3.4.5 Selection of transformed tissues.

Following the transition period, explants were transferred onto selection medium composed of MS salts with 2.0 mg/L picloram, 1 mg/L BAP, 30 g/L sucrose, 50 mM lipoic acid, 200 mg/L kanamycin, 150 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. Cultures were maintained in the dark at 23°C.

3.2.3.4.6 Rooting of regenerated, transformed shoots.

After 28 – 42 days on selection medium, shoots, each 1 - 2 cm in height, were excised from kanamycin-resistant tissues and transferred into 60 ml capacity screw-capped Powder Round glass jars (Beatson Clark Co. Ltd., Rotherham, UK), each containing 25 ml of rooting medium. The latter was composed of MS salts with 2.0 mg/L picloram, 5 mg/L BAP, 2.5 g/L activated charcoal, 30 g/L sucrose, 50 mM lipoic acid, 100 mg/L kanamycin, 150 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. The cultures were incubated at 23°C with a 16 h photoperiod (180 µMol m^{-2} sec^{-1}, Daylight fluorescent tubes, TLD/58W 35V; Phillips, Guildford, UK).

After 21 days in rooting medium, or when they had 4 or 5 well-developed leaves, regenerated shoots were transferred into 13 cm pots containing Levington M3 compost (Scotts Professional, Ipswich, UK). Potted plants were covered with 27 x 15 cm plastic sleeves and transferred to the glasshouse under natural light conditions. The top corners of the sleeves were removed after 10 days and the sleeves were removed completely after 20 days to help gradual acclimatization of the bulbs.

3.2.4 PCR analysis of putative transgenic lines.

To confirm transgene insertion into the putative lines, PCR analysis was conducted as described in chapter 2, section 2.3.1. Analysis was done for three genes and the primers used are described in Table 3.3. The thermal cycles for each reaction are described in Table 3.4.

Gene	Primers	Sequence (5'-3')	Expected Band size
NPTII	nptII_For	AGACAATCGGCTGCTCTGAT	261 bp.
	nptII_Rev	ATACTTTCTCGGCAGGAGCA	
GUS	GUS F	AGTGTACGTATCACCGTTTGTGTGAAC	1026 bp.
	GUS R	ATCGCCGCTTTGGACATACCATCCGTA	
Polyubiquitin (housekeeping gene)	Polyb F	GAAGCAGCTGGAAGATGGAC	196 bp.
	Polyb R	GATCCCTTCCTTGTCGTGAA	

Table 3.3 Primers used for transgene detection.

Thermal cycle for transgene detection				
NPTII	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:35 min	35 cycles
	Annealing	57°C	0:35 min	
	Extension	72°C	0:35 min	
	Final Extension	72°C	5:00 min	1 step
GUS	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:45 min	33 cycles
	Annealing	58°C	0:45 min	
	Extension	72°C	1:00 min	
	Final Extension	72°C	8:00 min	1 step
Polyubiquitin	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:30 min	30 cycles
	Annealing	58.4°C	0:45 min	
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step

Table 3.4 Thermal cycles used for PCR reactions.

3.2.5 Reverse Transcriptase-PCR of transgenic lines.

After confirming transgene insertion by PCR, the expression of the gene was checked by RT-PCR following the procedure described in section 2.3.3. The same primers and thermal cycles listed in Tables 3.3 and 3.4 were used for the analysis.

3.2.6 GUS assay.

To further confirm transformation of tissues, histochemical analysis of *GUS* gene expression was performed as described in chapter 2, section 2.6. Tissues were checked 4 days after inoculation for transient expression and 11 weeks after inoculation for stable transformation.

3.3 Results.

3.3.1 Regeneration protocol.

3.3.1.1 Callus induction.

After several experiments a fast and reliable method for callus induction from different tissues of lily was established. The best results were obtained using MS basal salts supplemented with 2.0 mg/L picloram, 30 g/L sucrose, 2.5 g/L activated charcoal, 1.5 mg/L zeatin, 1.5 mg/L kinetin and 2.6 g/L phytagel. Comparison of the tissues used for callus induction (Fig. 3.2) showed that optimal results were obtained using the basal plate disc as a starting material therefore it was selected for further

transformation experiments. Table 3.5 summarizes the results obtained for the different media and explants.

Medium	Type of explant	Number of explants	% of developed callus	Callus average size (cm)	Callus avg. life span (weeks)	Avg. time to callus formation	Type and colour of callus
MS-SC	BS	200	4	0.48	12	16	NH, DG
	SD	50	0	-	-	-	-
UM	BS	100	0	-	-	-	-
CI1	BS	300	87	0.52	3	5	NS,PG
	SD	50	10	0.25	6	8	NS,PG
CI2	BS	100	0	-	-	-	-
	SD	50	0	-	-	-	-
CI3	BS	300	93	1	15	4	NS, YG
	SD	50	7	0.27	6	8	NS,PG
	BPD	200	96	1.4	10	3	F,YG
CI4	BS	300	97	1.3	16	4	NS,YG
	SD	50	11	0.31	6	9	NS,PG
	BPD	200	99	1.7	12	3	F,YG
CI5	BS	300	95	1	15	4	NS,YG
	SD	50	6	0.24	6	8	NS,PG
	BPD	200	97	1.2	10	4	F,YG
CI6	BS	100	10	0.9	4	5	NS,PG
CI7	BPD	100	3	0.2	3	5	F,YG
CI8	BS	100	50	0.4	5	6	NS,PG

Table 3.5 Results obtained from the callus induction experiments. Type of explant: BS (Bulblet Scale), SD (Stem Disc), BPD (Basal Plate Disc). Type and colour of callus: NH (Nodular Hard), NS (Nodular Soft), F (Friable), DG (Dark Green), PG (Pale Green), YG (Yellow Green). Composition of each medium is described in Appendix 1.

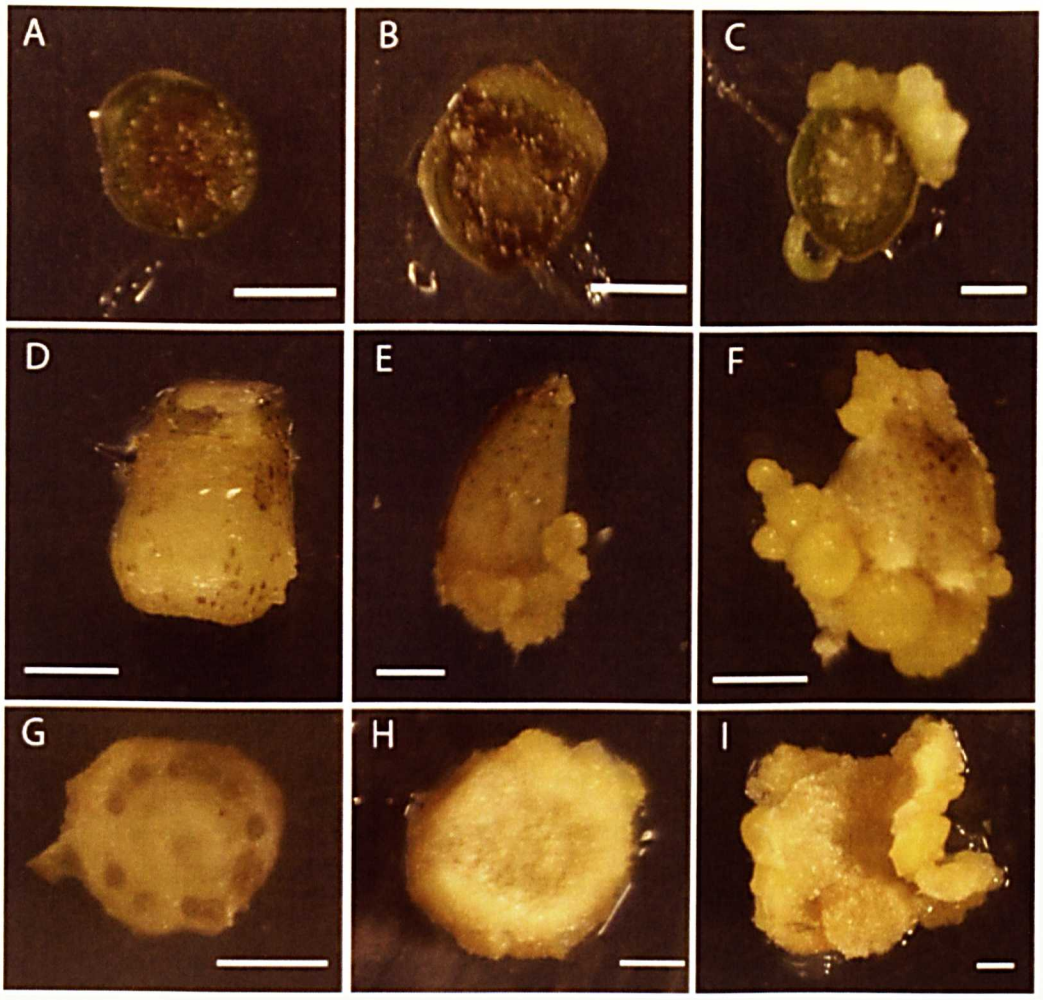

Figure 3.2 Comparison of callus development in cv. Star Gazer using different wild type tissues as explant in callus induction (CI4) medium. (A) Stem disc after 1 week; (B) Stem disc after 6 weeks; (C) Stem Disc after 10 weeks; (D) Bulblet scale after 1 week; (E) Bulblet scale after 4 weeks; (F) Bulblet scale after 7 weeks; (G) Basal plate disc after 1 week; (H) Basal Plate disc after 3 weeks; (I) Basal plate disc after 5 weeks. Bar = 2.5 mm.

3.3.1.2 Effect of antioxidants on callus development.

The chemicals evaluated for their antioxidant properties in this study were ascorbic acid, citric acid, glutathione, thiamine, silver nitrate, activated charcoal and lipoic acid; these were evaluated individually or in combination. The medium MS-CI4 was used in all experiments because it showed optimal callus induction during initial tests. The explants used

were calli established on MS-CI4 medium and then transferred onto the antioxidant supplemented medium.

Silver nitrate (17μM) resulted in significant problems, with the explants starting to brown and die within 1 week of treatment. Thiamine (1mg/L) and glutathione extended the life of the callus for 4 weeks but eventually browning occurred and the calli died. A combination of ascorbic acid and citric acid (50mg/L) kept the callus for 3 weeks until browning. Activated charcoal and lipoic acid gave better results with no significant browning of the callus observed for 6 to 8 weeks. However a combination of both potentiated their antioxidant properties limiting the oxidization of the tissues for up to three months (Fig. 3.3). It is worth mentioning that the addition of activated charcoal into the callus induction medium also promoted a faster growth rate and in some cases differentiation of cells into shoots or roots. Table 3.6 summarizes the results from the antioxidant experiments performed in this study.

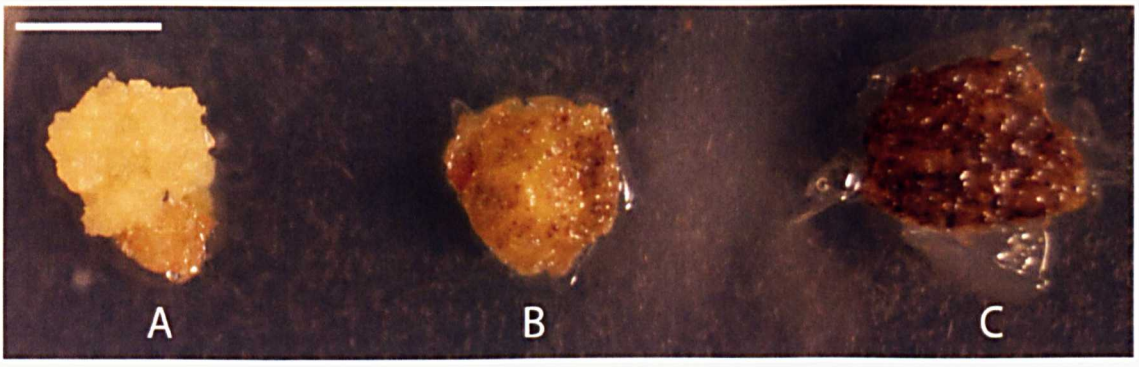

Figure 3.3 Effect of antioxidant agents on lily callus showing the extent of phenolic production after 10 weeks. (A) Callus grown in MS-CI4 medium supplemented with 2.5 g/L activated charcoal and 50 mM lipoic acid. (B) Callus grown in MS-CI4 medium supplemented with 2.5 g/L activated charcoal. (C) Callus grown in MS-CI4 medium without any antioxidant. Bar = 1cm.

Medium	Number of explants	Type of explants	Type and colour of callus	Avg. time to browning (weeks)	% of browning
CI4 + Ascorbic acid and Citric acid (50mg/L each)	20	Callus	F, YG	3	95
CI4 + Glutathione (0.1mM)	20	Callus	F, YG	4	90
CI4 + Thiamine (1mg/L)	20	Callus	F, YG	4	95
CI4 + Activated charcoal (2.5g/L)	20	Callus	F, YG	6	60
CI4 + Silver nitrate (17μM)	20	Callus	F, YG	<1	100
CI4 + Lipoic acid (50mM)	20	Callus	F, YG	8	50
CI4 + all of the above	20	Callus	F, YG	5	80
CI4 + Activated charcoal and Lipoic acid	20	Callus	F, YG	12	20

Table 3.6 Analysis of antioxidant treatments on the growth and phenolic production from lily callus. MS-CI4 was used for all experiments. Type and colour of callus: F (Friable), YG (Yellow Green).

3.3.1.3 Antibiotic resistance assay.

To increase the effectiveness of the selection process and limit the growth of non-transformed cells, an antibiotic resistance assay was performed. Four different concentrations of either kanamycin, or hygromycin were evaluated. Bulblet scales from wild type plants and from a transgenic line expressing the *NPTII* gene, previously obtained by Ribas (2007), were used for all experiments. Hygromycin caused significant damage to both

wild type and transgenic tissues, since no growth was visible and chlorosis of all the explants occurred at all concentrations tested (Fig. 3.4).

Kanamycin proved to be less aggressive in its effect on the explants. Concentrations of 100 and 150 mg/L allowed growth of transgenic tissues as well as wild type ones, although the difference between growth rates was noticeable. High concentrations of kanamycin (200 – 250 mg/L) limited the growth of wild type explants, but transgenic ones grew relatively quickly thus reducing the possibility of obtaining non-transformed shoots in future transformation experiments (Fig. 3.5). Table 3.7 summarizes the results of the antibiotic resistance experiments.

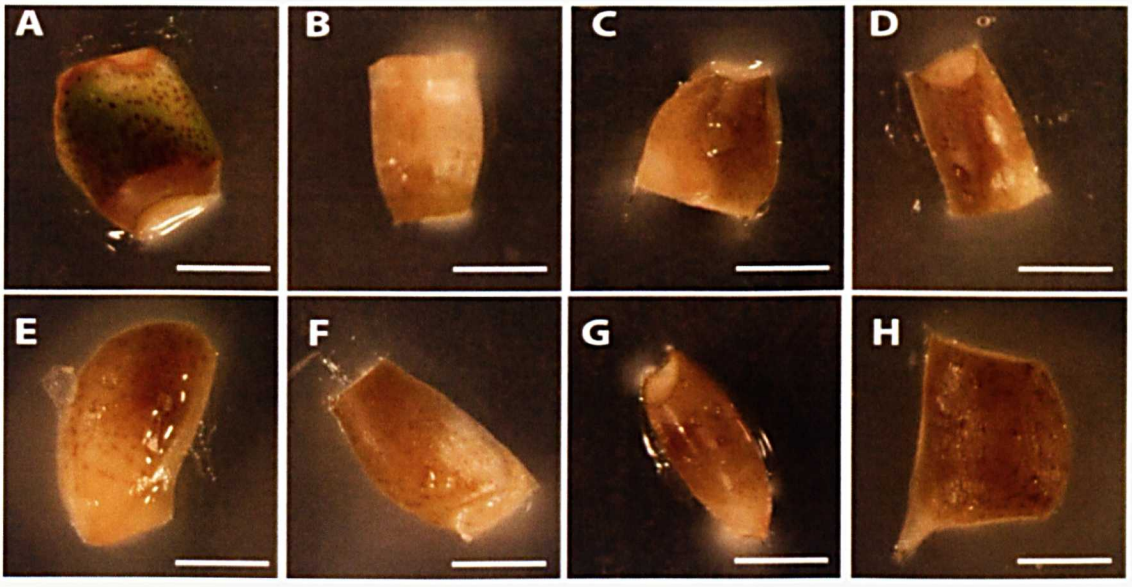

Figure 3.4 Bulblet scales growing on MS medium supplemented with different concentrations of hygromycin. Upper row shows wild type material and lower row transgenic explants. (A, E) 50 mg/L; (B, F) 100 mg/L; (C, G) 125 mg/L; (D, H) 150 mg/L. Bar = 3.5 mm.

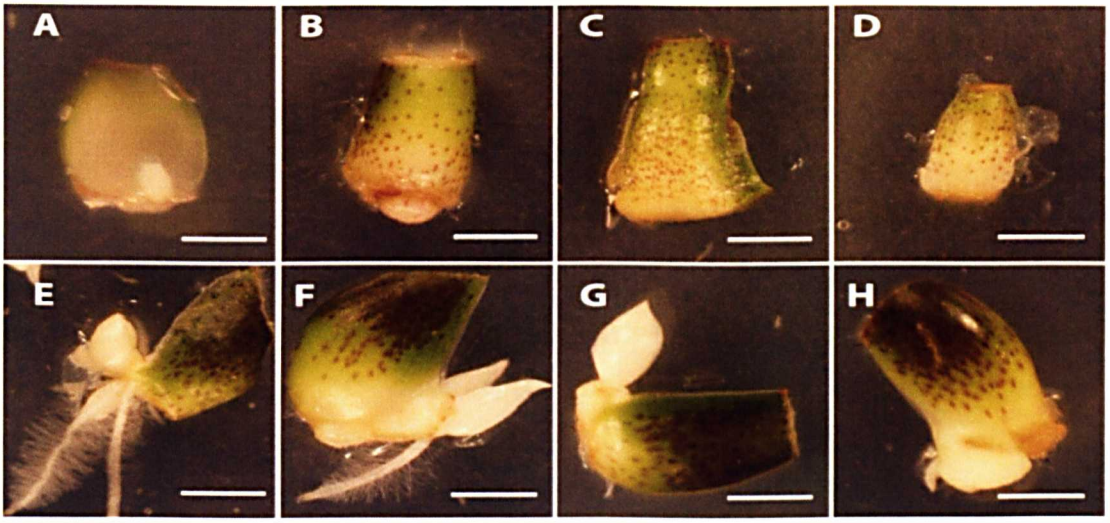

Figure 3.5 Bulblet scales growing on MS medium supplemented with several concentrations of kanamycin. Upper row shows wild type material and lower row transgenic explants. (A, E) 100 mg/L; (B, F) 150 mg/L; (C, G) 200 mg/L; (D, H) 250 mg/L. Bar = 3 mm.

Type of explant	Antibiotic	Number of explants	Concentration (mg/L)	Explants growing (%)	Average time for visible growth (weeks)
Wild Type Bulblet Scale	Kanamycin	25	100	40	6
		25	150	20	6
		25	200	20	8
		25	250	0	-
	Hygromycin	25	50	0	-
		25	100	0	-
		25	125	0	-
		25	150	0	-
Transgenic for *NPTII* Bulblet scale	Kanamycin	25	100	80	3
		25	150	100	3
		25	200	90	4
		25	250	70	4
	Hygromycin	25	50	0	-
		25	100	0	-
		25	125	0	-
		25	150	0	-

Table 3.7 Effects of antibiotics on lily explant growth for wild type and transgenic materials. Plant growth regulator-free MS medium was used for all the experiments.

3.3.2 Biolistics-mediated transformation.

A total of 1,130 callus explants were used for biolistics-mediated transformation with the plasmid pBI121 described in section 3.2.2.1. Of these explants, 590 were bombarded using protamine and 540 using spermidine to coat the microprojectiles with DNA. GUS assays were made on bombarded calli 10 days after being transferred onto selection medium, as described in chapter 2 section 2.5. Only one callus obtained from all the bombardments tested showed limited GUS expression (Fig. 3.6), this callus was derived from the protamine experiments. Calli were able to develop shoots on selection medium but after 7 to 9 weeks all died (Fig. 3.7). Eleven plants were recovered from all of the bombardment experiments but after performing PCR analysis on them none were positive for insertion of the *NPTII* or *GUS* genes (Fig. 3.8).

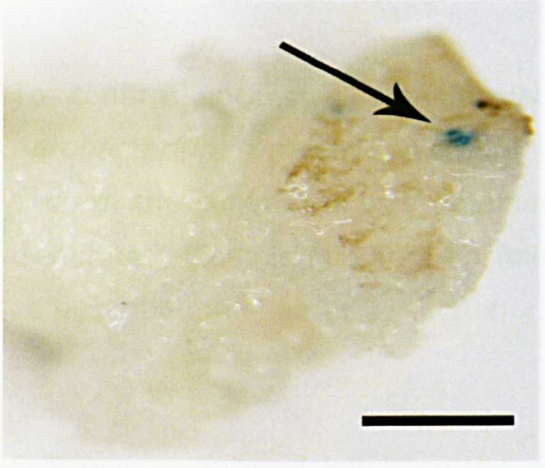

Figure 3.6 GUS expression in callus derived from particle bombardment experiments 10 days after bombardment. This callus was transformed with plasmid pBI121; protamine was used instead of spermidine for coating the microprojectiles with DNA. Arrow indicates the blue region confirming

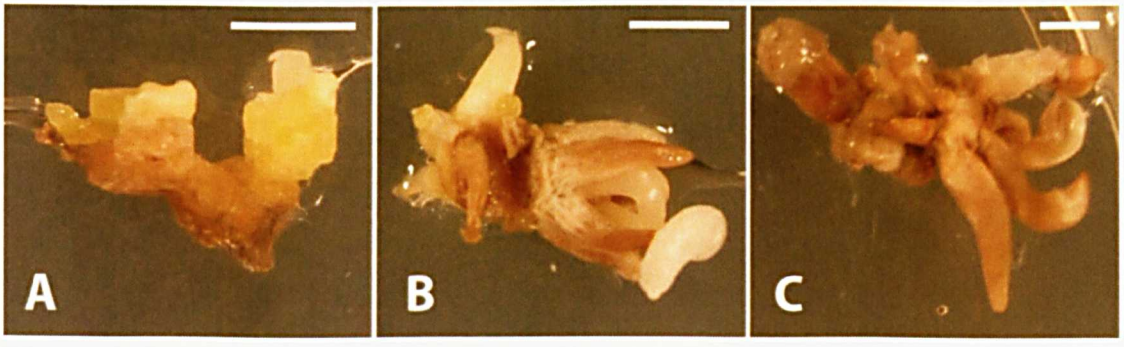

Figure 3.7 Development of lily callus after particle bombardment with plasmid pBI121. Callus growing in selection medium; (A) 2 weeks after bombardment; (B) 7 weeks after bombardment; (C) 9 weeks after bombardment. Bar = 3 mm.

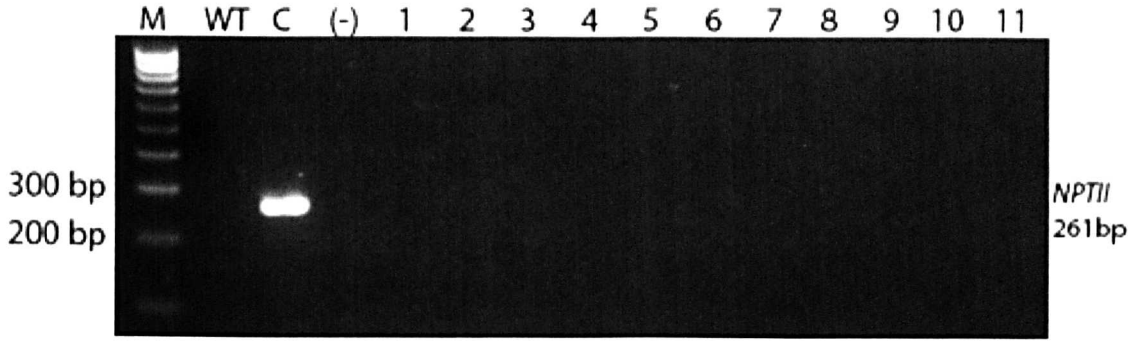

Figure 3.8 PCR for biolistics-mediated transformation derived lines of *Lilium* cv. Star Gazer. Lanes: (M) DNA marker; (WT) wild type DNA as negative control; (C) plasmid DNA as positive control; (-) H_2O as negative control; (1-11) putative transgenic lines all negative for the *NPTII* gene.

3.3.2 *Agrobacterium*-mediated transformation.

3.3.2.1 *Agrobacterium* strains transformation.

To confirm successful transformation of the pBI121 plasmid into the *Agrobacterium tumefaciens* strains PCR analysis was performed. DNA from each of the constructs was obtained from a fresh overnight culture using GenElute™ Plasmid Miniprep Kit following manufacturer's recommendation as described in chapter 2, section 2.2.4.

PCR was performed following the protocol from chapter 2, section 2.3.1; after gel electrophoresis the four constructs were positive for the *NPTII* and *GUS* genes (Figure 3.9).

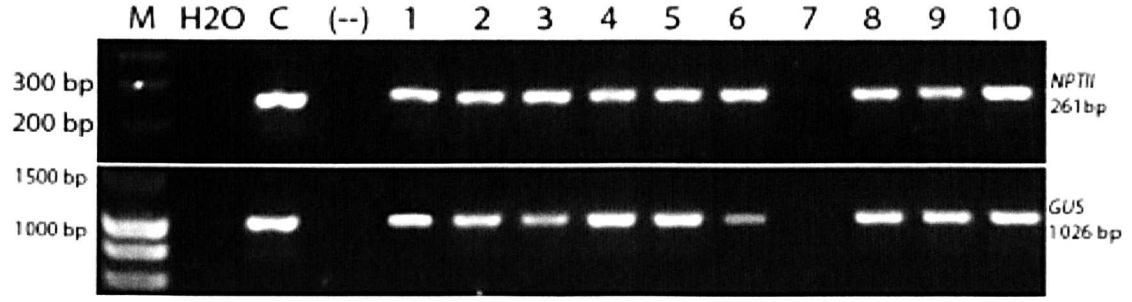

Figure 3.9 PCR of the four *Agrobacterium* strains to confirm the presence of the plasmid pBI121. Lanes: (M) DNA marker; (H_2O) water as negative control; (C) pBI121 plasmid DNA as positive control; (-) empty AGL1 as negative control; (1,2) colonies of strain C58; (3,4) colonies of strain GV3101; (5,6) colonies of strain EHA105; (7,8) colonies of strain LBA4404; (9,10) colonies of strain AGL1.

3.3.2.2 Standard *Agrobacterium*-mediated transformation.

Solanum nigrum explants were used to validate and confirm that the previously developed constructs were capable of transforming plant cells. Explants were transformed as previously described in section 3.2.3.2.

Agrobacterium tumefaciens strains C58 and LBA4404 produced excessive bacterial overgrowth on the inoculated tissues which was very difficult to control and killed the inoculated explants within 3 weeks without regenerating a single putative transgenic shoot. Explants inoculated with strains GV3101 and EHA105 regenerated putative transgenic plantlets but bacterial overgrowth was a major problem as with the previous two strains and these lines died after 10 weeks. A total of 20 putative transgenic plantlets were recovered form inoculations with strain AGL1 which did not showed bacterial overgrowth on the explants or plantlets. Twelve of these regenerated plantlets were analysed by PCR (Fig. 3.10) and RT-PCR (Fig. 3.11) to confirm successful insertion and expression of *NPTII* gene.

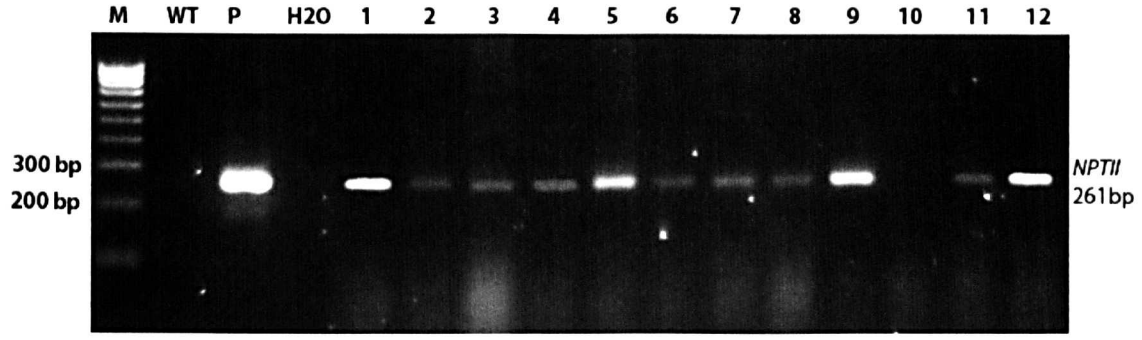

Figure 3.10 PCR analysis of putative transgenic lines of *Solanum nigrum* transformed with *A. tumefaciens* strain AGL1 carrying the plasmid pBI121. Lanes: (M) DNA marker; (WT) *S. nigrum* wild type DNA as negative control; (P) pBI121 plasmid DNA as positive control; (H_2O) water as negative control; (1-9,11,12) transgenic lines showing the insertion of the *NPTII* gene; (10) line showing no insertion of the *NPTII* gene.

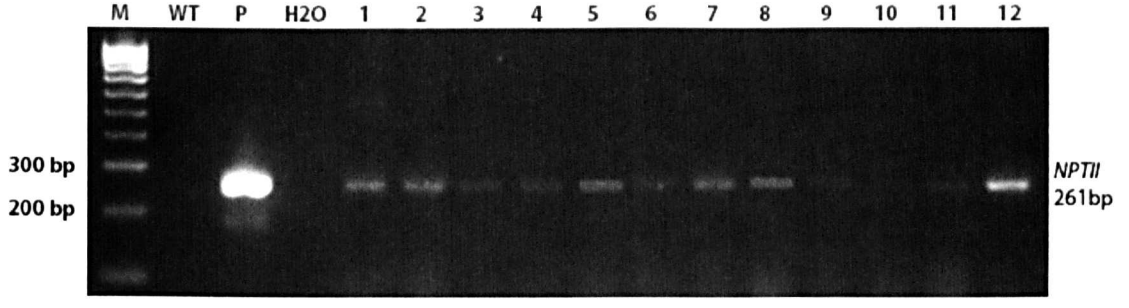

Figure 3.11 RT-PCR analysis of *Solanum nigrum* transgenic lines which had previously been confirmed as carrying the plasmid pBI121 (Fig.3.10). Lanes: (M) DNA marker; (WT) *S. nigrum* wild type DNA as negative control; (P) plasmid DNA as positive control; (H_2O) water as negative control; (1-9,11,12) transgenic lines expressing the *NPTII* gene; (10) putative line not expressing the *NPTII* gene.

Callus, leaf and bulblet scales from *Lilium* were inoculated following this protocol using the same *A. tumefaciens* strains previously mentioned. However no shoots were regenerated in any of the inoculation experiments.

Based on the results obtained by the *S. nigrum* transformations, *A. tumefaciens* strain AGL1 carrying the plasmid pBI121 was used for all further inoculations.

3.3.2.3 *Agrobacterium*-mediated transformation protocol modified from Qiu Hua *et al.* (2008).

After failing to regenerate transgenic *Lilium* plants with the previous protocol, transformation experiments were done using the protocol published by Qiu Hua *et al.,* (2008) with modifications as described in section 3.2.3.3. A total of three hundred bulblet scales and three hundred calli of cv. Star Gazer were inoculated in three independent transformation experiments.

Explants showed *Agrobacterium* overgrowth after three days in co-culture and after seven days 90% of them were dead. The ones surviving after co-culture were transferred to selection medium but started to show browning and necrosis within 3 days and eventually died after 7 days. For this reason, no shoots were regenerated in any of the transformation experiments using this method.

3.3.2.4 *Agrobacterium*-mediated transformation protocol for lily.

Since the regeneration of transgenic material using biolistics and two *Agrobacterium*-mediated protocols was unsuccessful, a new procedure was attempted. Basal plate discs of *Lilium* cv. Star Gazer were transformed with *A. tumefaciens* strain AGL1 carrying the plasmid pBI121 (section 3.3.2.1) as described in section 3.2.3.4.

A total of 700 explants were inoculated in three different transformation experiments. More than 150 putative transgenic lines were recovered from all experiments. DNA was extracted from bulblet scales following the procedure described in chapter 2, section 2.1.1; PCR analysis of these lines confirmed the presence of *NPTII* and *GUS* genes (Fig. 3.12).

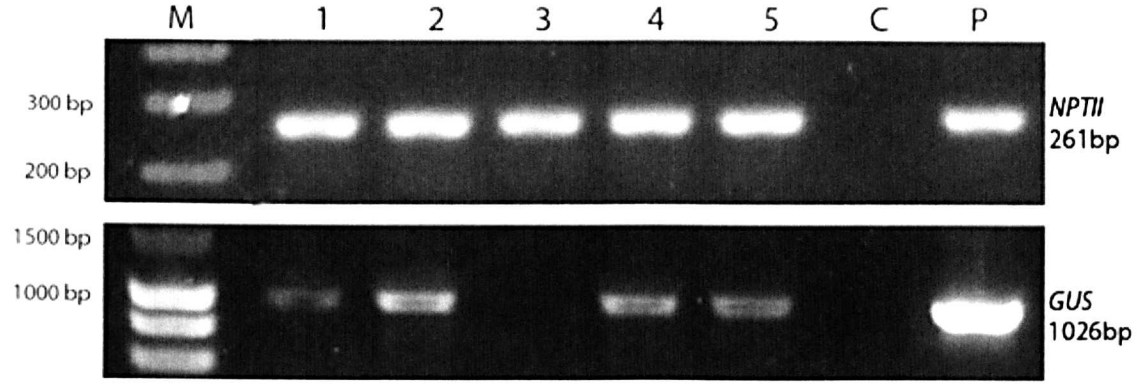

Figure 3.12 PCR analyses for the *NPTII* and *GUS* genes in putative transgenic plants of *Lilium*. Lanes: (M) DNA marker; (1, 2, 4, 5) transformed plants showing the presence of both genes; (3) transgenic plant showing the presence of the *NPTII* gene, but not of the *GUS* gene (this plant was also negative for GUS histochemical staining, whilst the *GUS*-PCR positive plants showed GUS staining); (C) a non-transformed wild-type plant; (P) pBI121 plasmid DNA used as positive control.

After confirming that these lines were transgenic, total RNA was extracted from bulblet scales as described in chapter 2, section 2.2.2.1 and RT-PCR analysis was performed as described in chapter 2, section 2.3.3. Expression levels of the *NPTII* gene were similar to those of an endogenous polyubiquitin control gene following RT-PCR screening (Fig. 3.13).

Histochemical analysis of the expression of the *GUS* gene was performed to further analyse the stable transformation of these transgenic plants. The analysis revealed blue regions of GUS activity confirming the expression of the *GUS* gene in transgenic tissues at different stages of development (Fig. 3.14).

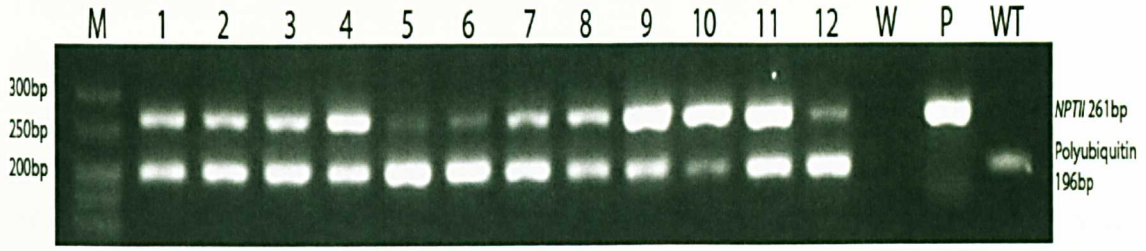

Figure 3.13 RT-PCR analysis of transgenic plants of *Lilium* to confirm expression of the *NPTII* gene, and comparison of its expression level against an endogenous polyubiquitin control gene. Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control for *NPTII* gene; (WT) wild type *Lilium* DNA; (1 - 4, 7 – 11) transformed plants showing high expression of *NPTII*; (5, 6, 12) transgenic plants showing low expression of *NPTII* compared to the expression of the polyubiquitin gene.

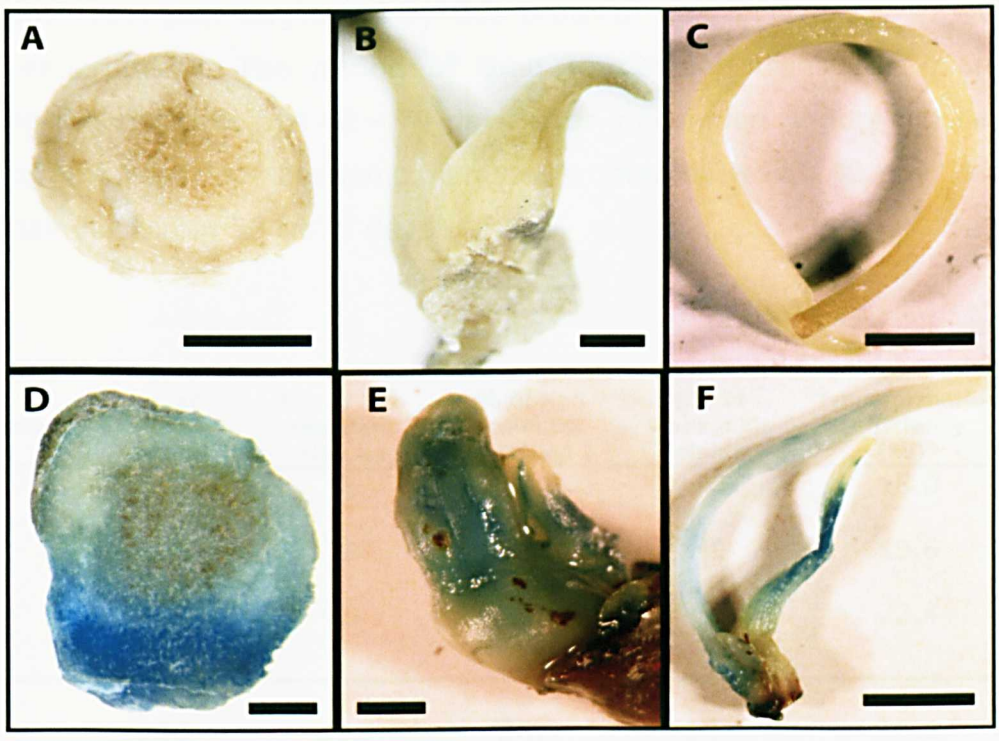

Figure 3.14 GUS expression in transgenic *Lilium* explants and plantlets. Upper row shows wild type tissues, lower row shows transgenic tissues. (A,D) Basal Plate discs four days after inoculation. Bar A= 3mm, Bar D= 1mm. (B,E) Bulblets 6 weeks after inoculation. Bar = 4mm. (C,E) Plantlets 11 weeks after inoculation. Bar = 1.5cm.

Transformation efficiency obtained by this protocol was recorded. The analysis revealed that the number kanamycin resistant tissues was in excess of 27% in all experiments, however not all of these transformed tissues regenerated putative transgenic plants. Consequently, the number of PCR positive plants compared to the number of explants inoculated was a more accurate estimate of the transformation frequency. Table 16 summarizes the number of transformed tissues and transgenic plants generated compared to the number of basal plate disc explants inoculated in three independent experiments.

The percentage of kanamycin resistant plants that were not transformed (false positives) that originated by this protocol was on average 12% in the 3 experiments. The period of time to develop transgenic plants following this protocol was 42 - 70 days after inoculation. Eighty per cent of the total number of transgenic plants was obtained within 42 days with the remainder within 56 to 70 days.

Experiment No.	No. of inoculated tissues	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
1	200	56	28.0	36	18.0
2	250	73	29.2	49	19.6
3	250	69	27.6	56	22.4
Total	**700**	**198**	**28.3**	**141**	**20.1**

Table 3.8 Results obtained in three independent *Agrobacterium*-mediated transformation experiments of *Lilium* cv. Star Gazer. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

3.4 Discussion.

Genetic transformation in lily represents an alternative approach to traditional breeding. Production of novel cultivars can potentially be achieved in a shorter period of time by introducing specific desirable traits directly into lily's genome (Mori *et al.,* 2005). A successful and efficient transformation protocol for any species has to be based on a quick and efficient method of *in vitro* regeneration of tissues. The selection of starting material and the optimal conditions for selection are key factors for the success of such protocols.

Successful transformation in *Lilium* has been reported (Cohen and Meredith, 1992; Langeveld *et al.,* 1995; Tsuchiya *et al.,* 1996; Watad *et al.,* 1998; Lipsky *et al.,* 2002; Irifune *et al.,* 2003; Mercuri *et al.,* 2003; Hoshi *et al.,* 2004; Ahn *et al.,* 2004; Cohen *et al.,* 2004; Benedito *et al.,* 2005; Kim *et al.* 2007; Dong *et al.* 2007; Kamo and Han, 2008; Ogaki *et al.,* 2008; Qiu Hua *et al.,* 2008; Azadi *et al.,* 2010a). However, transformation efficiencies were very low, with the highest reported as 4.8% (Ogaki *et al.,* 2008). Although Azadi *et al.,* (2010) reported an efficiency of 25.4%, this was the percentage of kanamycin resistance tissues and they did not mention how many transgenic plants their protocol was able to regenerate, thus the apparent efficiency of the procedure is highly questionable.

In addition these protocols are laborious and time consuming with an average of 40 weeks to obtain just a few transgenic bulblets. This makes them very hard to reproduce. Another factor to mention is that all the reports have been tested on one, or just very few cultivars, which suggest that they might be cultivar dependant. That might explain the unsuccessful attempt to generate transgenic plants from cv. Star Gazer based on the *Agrobacterium*-mediated protocol from Qiu Hua *et al.,*

(2008) and the biolistics-mediated protocol from Watad *et al.,* (1998) in this research.

In this research a rapid and highly efficient *Agrobacterium*-mediated transformation has been developed. In three independent transformation experiments the average transformation efficiency was 20.1%, almost five times higher than the highest previously reported (Ogaki *et al.,* 2008). In addition the average time to obtain PCR positive plantlets using this protocol was 12 weeks, almost a quarter of the time reported by other researchers (Mercuri *et al.,* 2003; Cohen *et al.,* 2004; ; Qiu Hua *et al.,* 2008; Azadi *et al.*, 2010a).

The success of the present transformation protocol relies on the use of basal plate discs as the starting material. Cells in these discs were found to regenerate adventitious shoots rapidly and reproducibly in preliminary assessments, compared to bulblet scales and embryogenic calli. This tissue also provides a greater surface area of wounded cells for the *Agrobacterium* to infect.

Another key factor in this protocol was its division into several stages in which the explants were grown. One of the most important of these was the transition phase that was found to be crucial. All explants inoculated with *Agrobacterium* that were not subjected to a transition stage in preliminary tests died within 14 days of bacterial inoculation. The use of a lower concentration of antibiotic at this stage allowed the explants to recover from the stress of mechanical manipulation during inoculation and the infection of *Agrobacterium* but at the same time limiting the development of non-transformed cells.

The optimal conditions provided by the different growing medium in each stage also contributed to the success of the protocol. The use of

antioxidants prevented the production of phenolic compounds by the tissues reducing the stress and allowing better and faster regeneration. Several antioxidants were tested in this research. All of them reduced the production of phenolics but only to a certain extent. A combination of 2.5 g/L of activated charcoal and 50 mM of lipoic acid proved to be the most effective, not only extended the life of the explants more than twice compared to the other antioxidants tested, but in some cases also promoted root and shoot formation.

Selection is another very important factor for a transformation protocol, since this limits the regeneration of non-transformed cells whilst allowing the development of transgenic tissues. For this purpose an antibiotic resistance assay was performed. Results showed that hygromycin caused significant damage to lily explants compared to kanamycin, thus the latter was used in further experiments. In addition, results showed that with a concentration of 250 mg/L of kanamycin no growth was seen in wild type tissues. The use of only half of that concentration in the transition stage helped to reduce the stress to the inoculated explants, but still applied selection pressure to avoid the development of false positives.

This rapid and efficient transformation procedure has the potential to be exploited to introduce agronomically important traits into *Lilium*, such as those for pathogen resistance, extended post-harvest life, and the manipulation of floral pigmentation or pollen development. Its simplicity will enable it to be extended to other research laboratories. Future research was therefore aimed at testing the robustness and reproducibility of this protocol with other cultivars/species of *Lilium* (chapter 4).

CHAPTER 4: *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF DIFFERENT SPECIES/CULTIVARS OF *LILIUM*.

4.1 Introduction.

Agrobacterium tumefaciens naturally infects dicotyledonous plants; monocotyledonous plants remained recalcitrant to genetic manipulation until Chan *et al.* in 1993 reported the first stable transformation of rice using immature embryos. However, in these early studies the number of reported transformed plants showing integration of the target genes was very low thus making the efficiency of the method equally low and hardly reproducible.

Since then, several factors influencing *Agrobacterium*-mediated transformation of monocotyledonous plants have been investigated and elucidated (Ashok and Horst, 2006). These factors include the binary vector used for gene transfer, *Agrobacterium* strains and infection of the host tissues due to chemotaxis, attachment, Ti plasmid mediated T-DNA transfer and integration, wound response and differences in cellular structures (Fig 4.1); inoculation and co-culture conditions, and tissue culture and regeneration of the explants; selectable marker gene and promoter; and screening for the most responsive genotype and explant (Sood *et al.*, 2011). Of all these factors, genotype and explant are considered to be the major limitation in *Agrobacterium*-mediated transformation of monocots, especially in extending the host range to commercial cultivated or elite cultivars. To date, rice appears to be the least genotype dependent, as more than 40 genotypes of japonica, indica and javonica have been successfully transformed but for other important monocots, mostly cereals, the reports of successful transformations are limited to a few cultivars or species (Ashok and Horst, 2006).

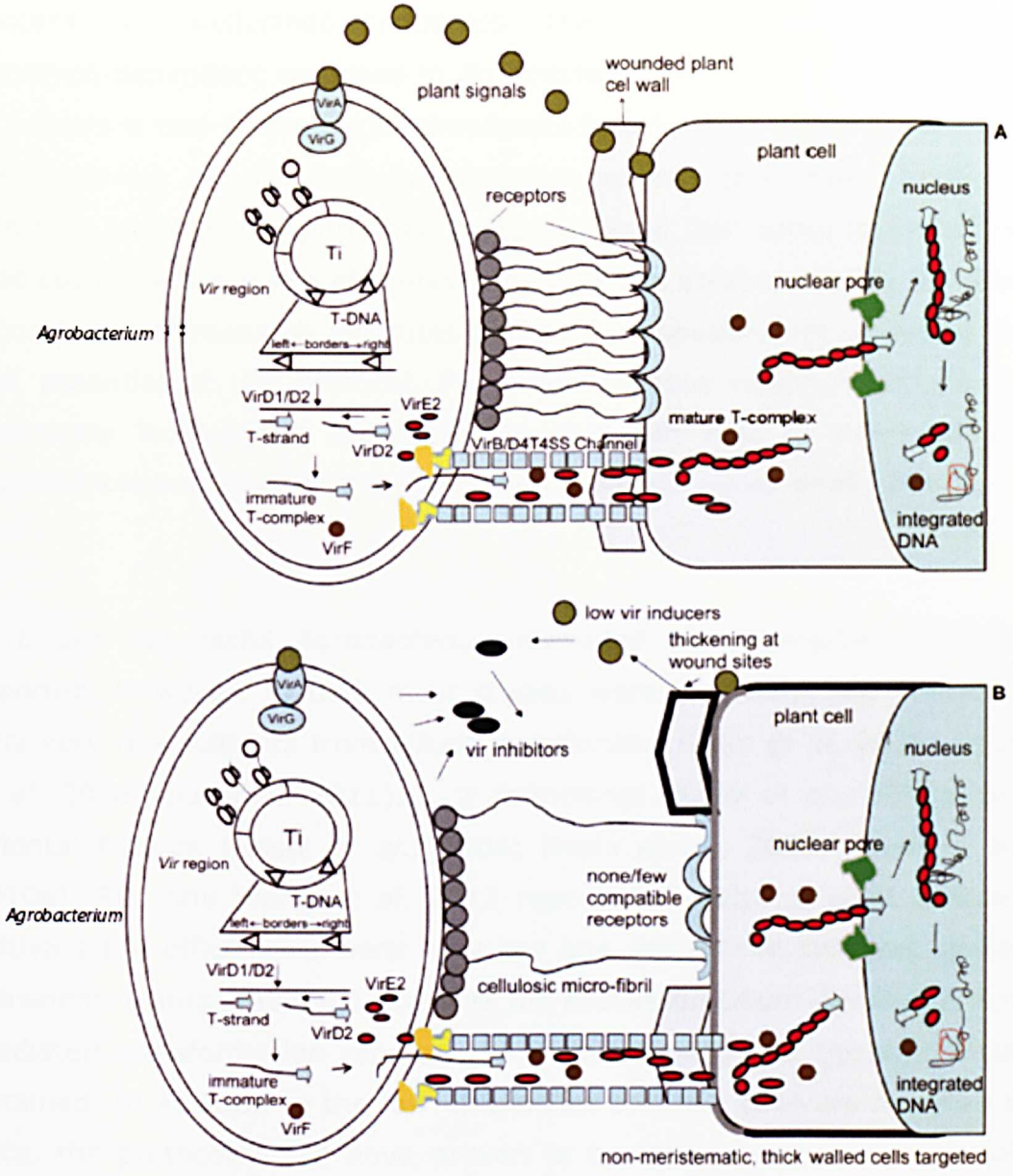

Figure 4.1 (A) Diagram showing *Agrobacterium* infection of a dicot cell, optimal amounts of inducers secreted from wound sites promote chemotaxis, T-DNA transfer and integration followed by extensive cell division of transformed cells/sectors. (B) Diagram showing *Agrobacterium* infection of a monocot cell, very few or no inducers along with inhibitors are secreted from wound sites thereby, inhibiting chemotaxis, T-DNA transfer and integration. Sclerification of wound sites prevent further cell division and cordons off the transformed sector thus the transformed cells can't multiply (Modified from Sood *et al.*, 2011).

In maize the cultivar A188, or its hybrids, has been the one used for successful transformation (Ishida *et al.*, 1996; Zhao *et al.*, 2001), cultivar Bobwhite in wheat (Khanna and Daggard 2002; Wu *et al.*, 2003) and

Golden Promise in barley (Bartlett *et al.*, 2008) have been the only successfully transformed genotypes. These studies indicate that the genotype-dependent response in *Agrobacterium*-mediated transformation of cereals is one of the major drawbacks in extending these protocols to elite cultivars of economically important cereals (Harwood, 2012). In addition, some of the published protocols could lack some minor details that could lead to lower efficiencies making the implementation on other laboratories or research institutes difficult and possibly not reflecting the real potential of the protocol. For these reasons research groups are constantly looking for improvements that can lead to more robust, reproducible and efficient transformation protocols (Sood *et al.*, 2011).

In *Lilium*, successful *Agrobacterium*-mediated transformation has been reported. However, to date most studies were inefficient and limited to only very few cultivars from *Lilium longiflorum* (Hoshi *et al.* 2005; Ogaki *et al.* 2008; Liu *et al.* 2011). *L. x formolongi* (Azadi *et al.*, 2010a) and Oriental hybrids (Hoshi *et al.*, 2004; Hoshi *et al.*, 2005; Azadi *et al.*, 2010a). Recently Wang *et al.* 2012 reported a study of eight different cultivars but efficiencies were very low and not all the cultivars yielded transgenic plants. Table 4.1 presents the successful *Lilium Agrobacterium*-mediated transformation reports, the cultivars used and the efficiencies obtained. In addition to the low efficiencies and few cultivars reported to date, the protocols used have proven to be laborious, not reproducible and most of all time consuming, most of them taking between 8 to 12 months to produce a few transgenic lines. The development in this research of a transformation protocol which is highly efficient, reaching over 20% and mostly very rapid, obtaining PCR positive transgenic plants in about 3 months (chapter 3, section 3.2.3.4), opens the possibility to explore its robustness with other cultivars not previously reported as successfully transformed. This could lead to its use as a standard transformation procedure for the genus *Lilium.*

Section and Hybrids	Cultivar	Number of explants	Efficiency %	Author
Lilium longiflorum	Snow Queen	215	0	Wang *et al.*, 2012
	Nellie White	N/M	0	Cohen and Meredith 1992
	Georgia	280	1.78	Hoshi *et al.*, 2005
	Tiepao	100	76*	Liu *et al.*, 2011
Lilium x formolongi	Akasu	700	4.28	Ogaki *et al.*, 2008
	Akasu	N/M	25.4**	Azadi *et al.*, 2010a
	Akasu	400	1.25	Azadi *et al.*, 2010b
L. longiflorum x L. formosanum	Leishan No. 1	162	1.2	Qiu Hua *et al.*, 2008
Oriental Hybrids	Barbados	540	0.18	Wang *et al.*, 2012
	Gracia	670	0	Wang *et al.*, 2012
	Lake Carey	445	0.67	Wang *et al.*, 2012
	Santander	355	1.40	Wang *et al.*, 2012
	Sheila	420	0.71	Wang *et al.*, 2012
	Sorbone	510	0.39	Wang *et al.*, 2012
	Acapulco	200	3	Hoshi *et al.*, 2004
	Acapulco	N/M	23**	Azadi *et al.*, 2010a
	Casa Blanca	N/M	0	Azadi *et al.*, 2010a
	Red Ruby	N/M	0	Azadi *et al.*, 2010a
	Star Gazer	700	20.1	Núñez de Caceres *et al.*, 2011
OT Hybrids	Yelloween	740	0.13	Wang *et al.*, 2012
Asiatic hybrids	Harmony	N/M	0	Langeveld *et al.*, 1995

Table 4.1 Reported cultivars/species used for *Agrobacterium*-mediated transformation and efficiencies obtained.* Transformation efficiency was reported as the percentage of PCR positive plants from the total screened lines. ** Transformation efficiency was reported as the percentage of hygromycin resistance calli, and not the number of transgenic plants. (N/M) Not mentioned in the report; (OT) Oriental-Trumpet.

4.2 Materials and Methods.

4.2.1 Cultivars/Species

All the cultivar/species used for the experiments were purchased from R.V. Roger Ltd nursery (North Yorkshire, UK). The six cultivars/species selected for the experiments were Beverly´s Dream (OT hybrid), Acapulco (Oriental hybrid), *Lilium leichtlinii,* Night Flyer (Asiatic hybrid), Snow Queen (*Lilium longiflorum*), Sweet Surrender (Asiatic hybrid) and *Lilium henryii* (Fig 4.2).

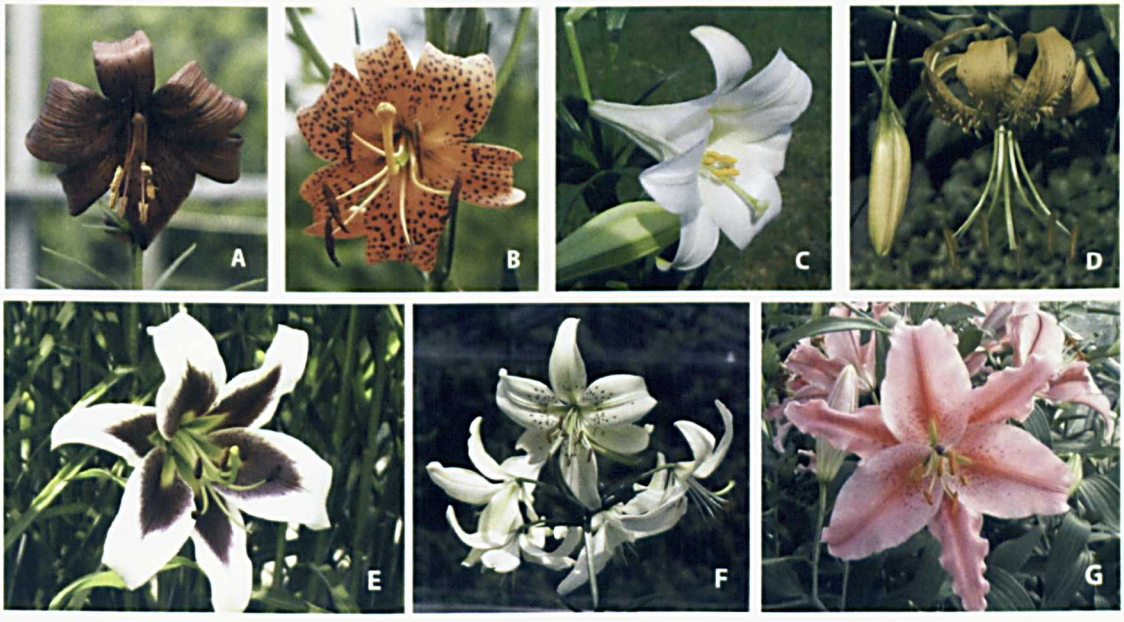

Figure 4.2 Selected cultivars/species for *Agrobacterium*-mediated transformation. (A) Night flyer (asiatic hybrid); (B) *L. leichtlinii*; (C) Snow Queen (*L. longiflorum*); (D) *L. henryii*; (E) Beverly's Dream (OT hybrid); (F) Sweet Surrender (asiatic hybrid); (G) Acapulco (oriental hybrid).

4.2.2 Regeneration protocol.

4.2.2.1 Sterilization.

Bulb scales from mature plants of each cultivar/species were used to induce *in vitro* shoots. Explants were washed with running tap water to eliminate the excess soil, all the rotten or necrotic parts were eliminated with a scalpel and the cleaned tissues were then sterilized by immersion in a 2% (v/v) Trigene solution for a period of 10 minutes, followed by immersion in a 15% (v/v) Domestos solution for 45 minutes and finally an immersion in a 50% (v/v) ethanol solution for 45 seconds. Then they were rinsed three times with sterile, purified water.

4.2.2.2 Shoot induction.

After sterilization all the edges and 1 cm of the top of the scales were removed and then placed onto Petri dishes containing 25 ml of MS-SIN (MS-Shoot Induction Nuñez) medium composed of MS basal salts supplemented with 2.0 mg/L picloram, 1.0 mg/L BAP, 60 g/L sucrose, 2.5 g/L activated charcoal and 2.6 g/L of phytagel as gelling agent and grown in the dark at 23 ± 1°C. Each Petri dish contained an average of 6 bulblets scales. The scales of two bulbs were used from each cultivar/species to induce *in vitro* shoots.

Explants were subcultured every week discarding the ones showing any sign of browning or contamination. Shoots for all cultivars/species were obtained after an average of 5 weeks. Adventitious bulblets that developed from the explants were then transferred to 175 ml glass jars containing 45 ml of MS-SIN medium. Each jar contained an average of 7 bulblets. For routine maintenance, these bulblets were subcultured every

4 to 5 weeks. Cultures were maintained in the dark growth room at 23 ± 1°C.

4.2.2.3 Selection agent assay.

It was necessary to establish the optimal concentration of selection agent to use for the transformation experiments due to the differences between cultivars/species. Previously kanamycin was found to be the best option as selection agent compared to hygromycin (chapter 3, section 3.3.1.3) therefore kanamycin was used for all the experiments. Bulblet scales from *in vitro* grown material were excised and placed on 9 cm Petri dishes containing MS-SIN supplemented with 50, 100, 150 mg/L of kanamycin. Explants were transferred into the dark for 8 weeks with subculturing every two weeks onto fresh medium containing antibiotics of the appropriate concentration. Measurement of growth and shoots, if any, was recorded on a weekly basis after the third week. Three replicates were used per cultivar/species to validate the results.

4.2.3 *Agrobacterium*-mediated transformation.

4.2.3.1 *Agrobacterium* strain and plasmid.

Agrobacterium tumefaciens strain AGL1 carrying plasmid pBI121, described in chapter 3, section 3.3.3.1, was used for all the inoculations. This was incubated on a rotary shaker at 28°C in LB liquid medium supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin overnight prior to use.

4.2.3.2 *Agrobacterium*-mediated transformation.

All transformation experiments were done following the protocol described in chapter 3 section 3.2.3.4 with the following modifications:

4.2.3.2.1 Pre-culture of basal plate discs.

Due to an apparent higher sensitivity to phenolic compounds and mechanical manipulation shown by these cultivars/species, in preliminary tissue culture experiments, compared to that previously used, cv. Star Gazer, the amount of Lipoic Acid used in the different medium compositions was raised from 50 mM to 100 mM in all cases and all stages.

4.2.3.2.2 Inoculation of explants.

In a similar way assuming that the explants from these cultivars/species were more sensitive than cv. Star Gazer, to avoid stressing the tissues too much the *Agrobacterium* suspension was pipetted onto the explants and left only for 3 minutes instead of 5 minutes as described previously. This was based in preliminary findings in which the dipping of 5 minutes produced much more *Agrobacterium* overgrowth at later stages than the dipping of 3 minutes.

4.2.3.2.3 Transition of inoculated tissues.

After 2 days co-culture, explants were washed twice with sterile reverse osmosis water containing 200 mg/L timentin and 400 mg/L cefotaxime to

remove *Agrobacterium* overgrowth, before rinsing with sterile reverse osmosis water. Explants were blotted with a sterile filter paper before transfer onto transition medium. The latter was composed of MS salts with 2.0 mg/L picloram, 1 mg/L zeatin, 1 mg/L kinetin, 60 g/L sucrose, 2.5 g/L activated charcoal, 100 mM lipoic acid, 50 mg/L kanamycin, 300 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. Cultures were incubated in the dark at 23°C for 21d.

4.2.3.2.4 Selection of transformed tissues.

Following the transition period, explants were transferred onto modified selection medium composed of MS salts with 2.0 mg/L picloram, 1 mg/L BAP, 60 g/L sucrose, 100 mM lipoic acid, 2.5 g/L activated charcoal , 150 mg/L kanamycin, 300 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. Cultures were maintained in the dark at 23°C.

4.2.3.2.5 Rooting of regenerated transformed shoots.

After 30 – 50 days on selection medium, shoots each 1 - 2 cm in height were excised from kanamycin-resistant tissues and transferred into 60 ml capacity screw-capped Powder Round glass jars, each containing 25 ml of modified rooting medium. The latter was composed of MS salts with 2.0 mg/L picloram, 1 mg/L BAP, 2.5 g/L activated charcoal, 60 g/L sucrose, 100 mM lipoic acid, 100 mg/L kanamycin, 150 mg/L timentin and semi-solidified with 2.6 g/L Phytagel. The cultures were incubated at 23°C ± 1°C with a 16 h photoperiod. After 21 days in rooting medium, or when they had 3 or 4 well-developed leaves, regenerated shoots were transferred into paper bags containing wet Levington M3 compost, perlite and vermiculite in a ratio of 4:2:1 and transferred into the cold room for 8 weeks for dormancy break.

4.2.4 PCR analysis of putative transgenic lines.

To confirm transgene insertion into the putative lines, PCR analysis was conducted as described in chapter 2, section 2.3.1. Analysis was done for two genes and the primers used are described in Table 4.2. The thermal cycles for each reaction are described in Table 4.3.

Gene	Primers	Sequence (5'-3')	Expected Band size
NPTII	nptII_For	AGACAATCGGCTGCTCTGAT	261 bp.
	nptII_Rev	ATACTTTCTCGGCAGGAGCA	
LP59 Actin (housekeeping gene)	Lactin_2 F	TGGTGTGATGGTTGGTATGG	222 bp.
	Lactin_2 R	TTTGCCTTAGGGTTGAGTGG	

Table 4.2 Primers used for transgene detection in all the putative transgenic lines obtained from all cultivars/species.

Thermal cycle for transgene detection				
nptII	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:35 min	35 cycles
	Annealing	57°C	0:35 min	
	Extension	72°C	0:35 min	
	Final Extension	72°C	5:00 min	1 step
LP59 Actin	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:30 min	33 cycles
	Annealing	57.5°C	0:30 min	
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step

Table 4.3 Thermal cycles used for PCR.

4.2.5 Reverse Transcriptase-PCR of transgenic lines.

After confirming transgene insertion by PCR, the expression of the gene was checked by RT-PCR following the procedure described in chapter 2 section 2.3.3. The same primers and thermal cycles mentioned in tables 4.2 and 4.3 were used for the analysis. Total RNA was extracted from small bulblets following the procedure described in chapter 2, section 2.2.2.1.

4.2.6 GUS assay.

To further confirm transformation of tissues, histochemical analysis of *GUS* gene expression was performed as described in chapter 2, section 2.6. Tissues were checked 4 days after inoculation for transient expression and if possible 12 weeks after inoculation for stable transformation.

4.3 Results.

4.3.1 Regeneration protocol.

4.3.1.1 Shoot induction.

Although differences in average time for shoot development and shoots produced per explant were observed among all cultivars/species used (Table 4.4), after several subcultures and selection of clean explants, all of them were able to develop healthy adventitious shoots (Fig 4.3). Thus a fast and reliable method for shoot induction from different cultivars of lily was established. In general, all the cultivars showed good production of

shoots both in number and size except *L. henryii* and *L. leichtlinii.* This could have been due to the size of the adult bulbs in these two species, which were approximately two or three times the size of the other cultivars' bulbs, implying that the stage of the material could have led to the low response. Table 4.4 summarizes the results obtained for each of them.

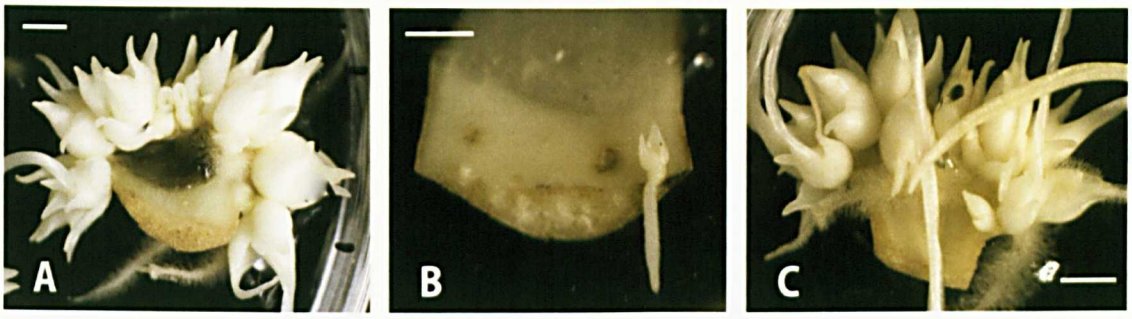

Figure 4.3 Differences in the amount and size of adventitious shoots developed in some of the cultivars. All the pictures were taken after 7 weeks on shoot induction medium. A) Bulblets of cv. Acapulco; B) Bulblet of *L. henryii;* C) Bulblets of cv. Sweet Surrender. Bar = 0.6 mm.

Cultivar/Species	Average time to produce shoots (± 3 days)	Average shoots per explant after 7 weeks. (± 1 shoot)	Average shoot size after 7 weeks. (± 0.2 mm)
Beverly's Dream	28 days	12	0.8 mm
Sweet Surrender	28 days	14	0.6 mm
Night Flyer	35 days	9	0.8 mm
L. leichtlinii	42 days	3	0.5 mm
Acapulco	28 days	10	0.8 mm
Snow Queen	35 days	6	0.6 mm
L. henryii	49 days	2	0.4 mm

Table 4.4 Regeneration of adventitious bulblets from scales of the cultivars/species on shoot induction medium.

4.3.1.2 Antibiotic resistance assay.

To increase the effectiveness of the selection process in limiting the growth of non-transformed cells, whilst avoiding the risk of killing transformed cells, an antibiotic resistance assay was performed for all the cultivars/species. Three different concentrations of kanamycin were evaluated 50, 100 and 150 mg/L. Figures 4.4 and 4.5 show the explants of all the cultivars under the three kanamycin concentrations.

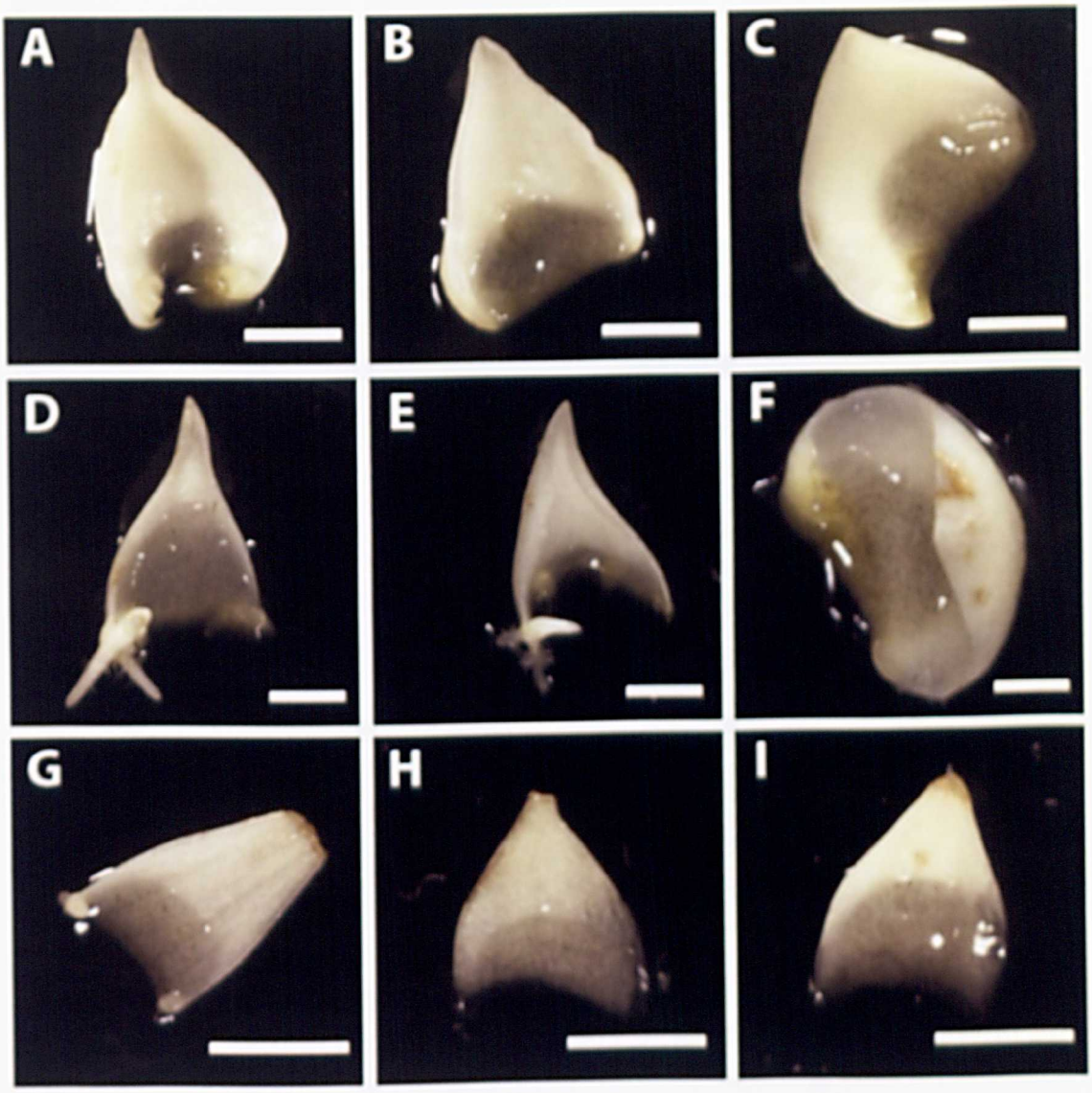

Figure 4.4 Bulblet scales growing for six weeks on MS-SIN medium supplemented with several concentrations of kanamycin; first column 50 mg/L , second column 100 mg/L, third column 150 mg/L. A-C) Cultivar Night Flyer, D-F) Cultivar Beverly's Dream, G-I) Cultivar Sweet Surrender. Bar= 2mm.

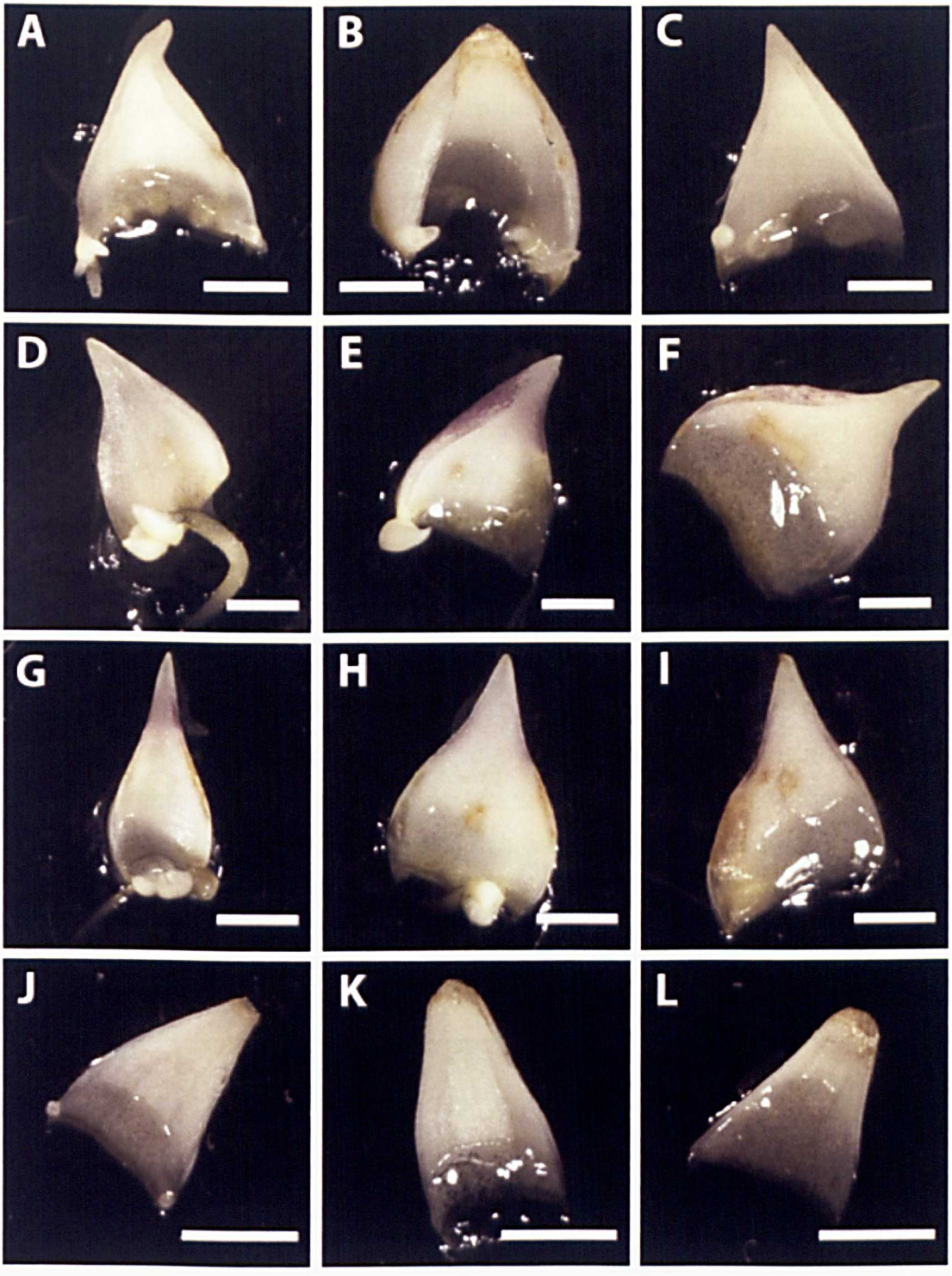

Figure 4.5 Bulblet scales growing for six weeks on MS-SIN medium supplemented with several concentrations of kanamycin; first column 50 mg/L, second column 100 mg/L, third column 150 mg/L. A-C) Cultivar Acapulco, D-F) *Lilium henryii*, G-I) Cultivar Snow Queen, J-L) *Lilium leichtlinii*. Bar= 2mm.

There were differences observed in the time to shoot development, using different concentrations of kanamycin, between cultivars (Table 4.5). However this might have been due to genotype and not to the kanamycin treatment, since differences in shoot development had been previously observed between the different cultivars/species (Table 4.4). In general all the cultivars showed similar sensitivity compared to each other to the three different antibiotic treatments (Table 4.5). Concentrations of 50 mg/L and 100 mg/L allowed growth of all cultivars/species after 6 weeks, although a difference between growth rates and number of shoots was noticeable. The highest concentration of kanamycin 150 mg/L limited considerably the growth of all cultivars except Acapulco, thus providing a suitable level of antibiotic to reduce the possibility of obtaining false positive shoots in future transformation experiments. Table 4.5 summarizes the results of the antibiotic resistance experiments. After examining the results for the resistance assay it was decided that 50 mg/L and 150 mg/L of kanamycin was the optimal concentration to use in the transition and selection stages respectively to minimise the development of non-transformed shoots, but at the same time allow the transformed cells to grow with adequate and not excessive selection pressure.

Cultivar/Species	Number of explants	Average shoots per explant after 6 weeks (± 1 shoot)		
		50 mg/L	100 mg/L	150 mg/L
Beverly's Dream	45	3	1	1
Sweet Surrender	45	2	1	0
Night Flyer	45	2	0	0
L. leichtlinii	45	2	0	0
Acapulco	45	5	3	1
Snow Queen	45	4	2	1
L. henryii	45	3	1	0

Table 4.5 Results obtained for the antibiotic resistance assay for all the cultivars/species. MS-SIN medium was used for all experiments.

4.3.2 *Agrobacterium*-mediated transformation.

A fast and highly efficient *Agrobacterium*-mediated transformation protocol for cv. Star Gazer has been developed in this research (chapter 3). To analyse its reproducibility and efficiency among the genus *Lilium*, basal plate discs of several *Lilium* cultivars/species were used for transformation. Three, four or five replicates were done per cultivar, depending on the availability of plant material, to validate results. All cultivars/species used for these experiments were successfully transformed by the protocol previously developed in this research.

Sensitivity to mechanical manipulation and phenolic compounds is a major problem in the *in vitro* culture of *Lilium.* After analysing the growth of all the cultivars in the antibiotic assay it was noted that Asiatic hybrids were more prone to suffer browning of the tissues. The increase in concentration of lipoic acid and the addition of activated charcoal into the selection medium helped to speed up the recovery of transgenic bulblets, reducing the average time to get PCR positive plants from 12 weeks to 9 weeks compared to the previous protocol (chapter 3, section 3.2.3.4). In general all the cultivars showed the same growth rate after inoculation, with the fastest being cv. Acapulco and the slowest *L. leichtlinii*; these results were similar to the ones obtained in the shoot induction experiments (Table 4.4).

Endogenous contamination present in the starting material of *Lilium henryii* and cv. Snow Queen limited the amount of material available for transformation. Even after several selection processes bulblets and explants that looked to be sterile subsequently developed a white bacterial contamination around them. Explants that were thought to be clean were inoculated, but after a couple of weeks later they developed the same contamination which was not *Agrobacterium* overgrowth.

Several explants regenerated putative transgenic shoots but they could never be cleaned and died approximately four months after inoculation.

It has to be noted that *L. leichtlinii* presented similar endogenous contamination problems than *L. henryii* and cv. Snow Queen but not as severe, this might be one of the reasons of the slower growth rate compared to the other cultivars and the low transformation efficiency obtained in the first experiment.

4.3.3 PCR analysis of putative transformed lines.

To confirm transgene insertion into the regenerated putative transgenic lines PCR screening was performed. Figures 4.6-4.10 show the results obtained for this molecular analysis on a randomly selected group for each cultivar/species.

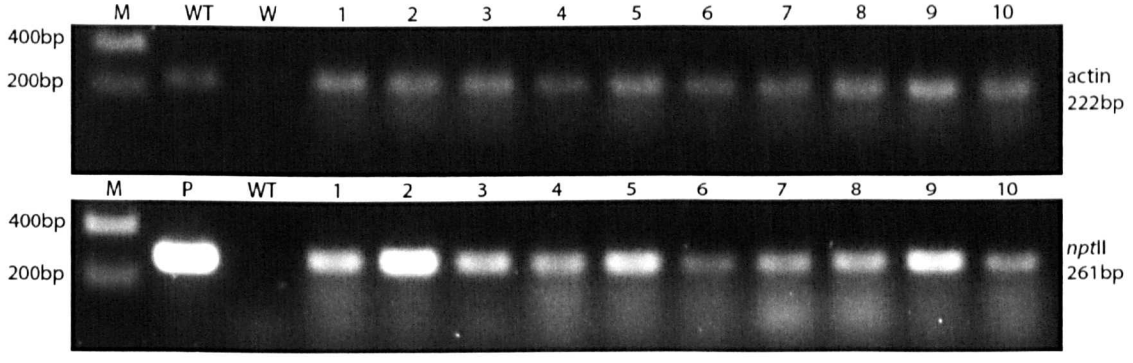

Figure 4.6 PCR for putative transgenic lines of *Lilium* cv. Beverly's Dream. Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (WT) Beverly's Dream wild type DNA as positive control for actin and negative control for *NPTII*; (1) Line BD2-11; (2) Line BD1-1; (3) Line BD5-6; (4) Line BD1-10; (5) Line BD3-2; (6) Line BD2-4; (7) Line BD4-9; (8) Line BD4-6; (9) Line BD1-6; (10) Line BD2-7. All of the putative transgenic lines were positive for the *NPTII* gene.

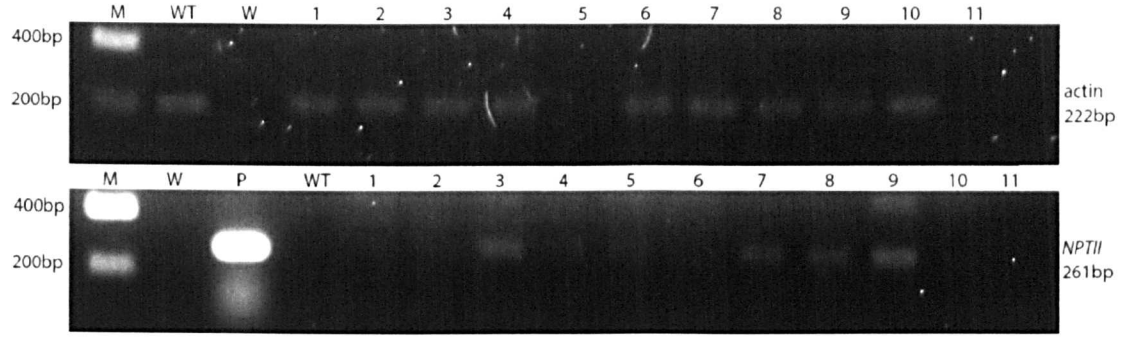

Figure 4.7 PCR for putative transgenic lines of *Lilium* cv. Night Flyer. Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (WT) Night Flyer wild type DNA as positive control for actin and negative control for *NPTII*; (1) Line NF2-1; (2) Line NF2-2; (3) Line NF2-3; (4) Line NF1-1; (5) Line NF1-7; (6) Line NF2-8; (7) Line NF2-10; (8) Line NF1-8; (9) Line NF2-11; (10) Line NF1-6; (11) Line NF1-4. Lanes 3, 5-10 were positive for the *NPTII* gene.

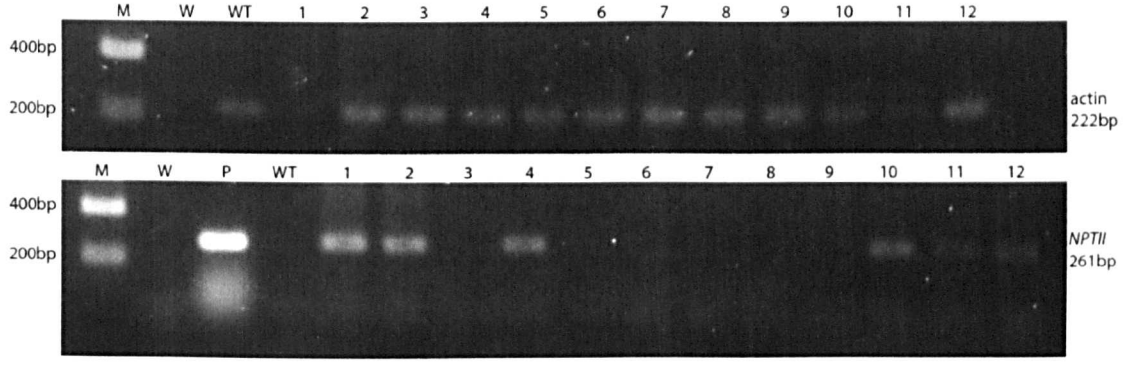

Figure 4.8 PCR for putative transgenic lines of *Lilium* cv. Sweet Surrender. Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (WT) Sweet surrender wild type DNA as positive control for actin and negative control for *NPTII*; (1) Line SS1-10; (2) Line SS1-11; (3) Line SS2-1; (4) Line SS2-3; (5) Line SS2-4; (6) Line SS2-5; (7) Line SS2-6; (8) Line SS2-7; (9) Line SS2-8; (10) Line SS2-9; (11) Line SS2-10; (12) Line SS2-12. Lanes 1, 2, 4, 9-12 were positive for the *NPTII* gene.

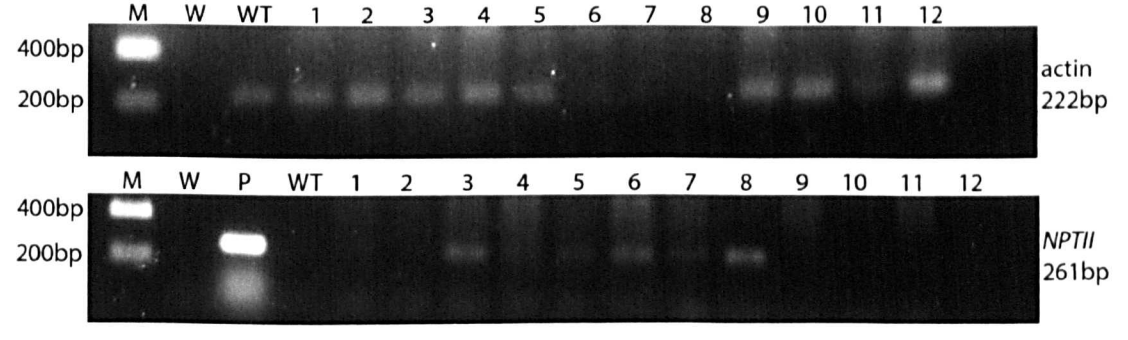

Figure 4.9 PCR for putative transgenic lines of *Lilium* cv. Acapulco. Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (WT) Acapulco wild type DNA as positive control for actin and negative control for *NPTII*; (1) Line AC1-6; (2) Line AC2-1; (3) Line AC1-7; (4) Line AC1-11; (5) Line AC2-3; (6) Line AC2-5; (7) Line AC2-6; (8) Line AC2-7; (9) Line AC2-8; (10) Line AC2-9; (11) Line AC2-10; (12) Line AC2-12. Lanes 3-8 were positive for the *NPTII* gene.

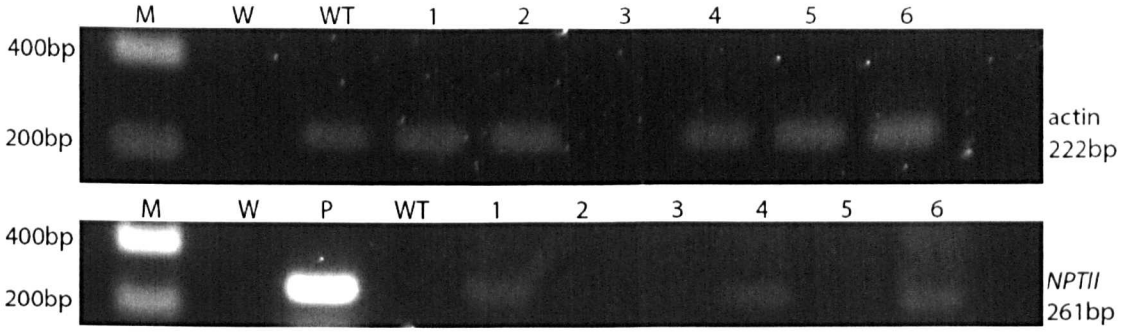

Figure 4.10 PCR for putative transgenic lines of *Lilium leichtlinii.* Lanes: (M) DNA marker; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (WT) *L. leichtlinii* wild type DNA as positive control for actin and negative control for *NPTII*; (1) Line LL1-2; (2) Line LL1-4; (3) Line LL2-1; (4) Line LL2-3; (5) Line LL3-2; (6) Line LL3-1; Lanes 1, 4, 6 were positive for the *NPTII* gene.

4.3.4 RT-PCR analysis of transgenic lines.

After confirming the insertion of the transgene, RT-PCR analysis was conducted to check the expression of the *NPTII* gene in the transgenic lines from all cultivar/species. Figures 4.11-4.15 show the results obtained from the RT-PCR analysis in the regenerated transgenic lines.

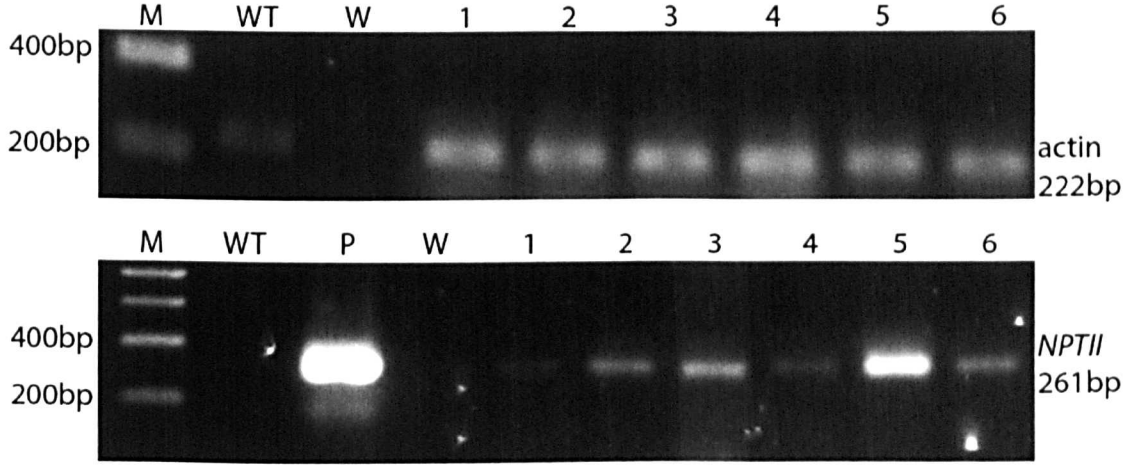

Figure 4.11 RT-PCR for transgenic lines of *Lilium* cv. Beverly's Dream. Lanes: (M) DNA marker; (WT) Beverly's Dream wild type cDNA as positive control for actin and negative control for *NPTII*; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (1) Line BD4-6; (2) Line BD1-6; (3) Line BD4-9; (4) Line BD2-7; (5) Line BD3-2; (6) Line BD5-6. All transgenic lines showed *NPTII* gene expression.

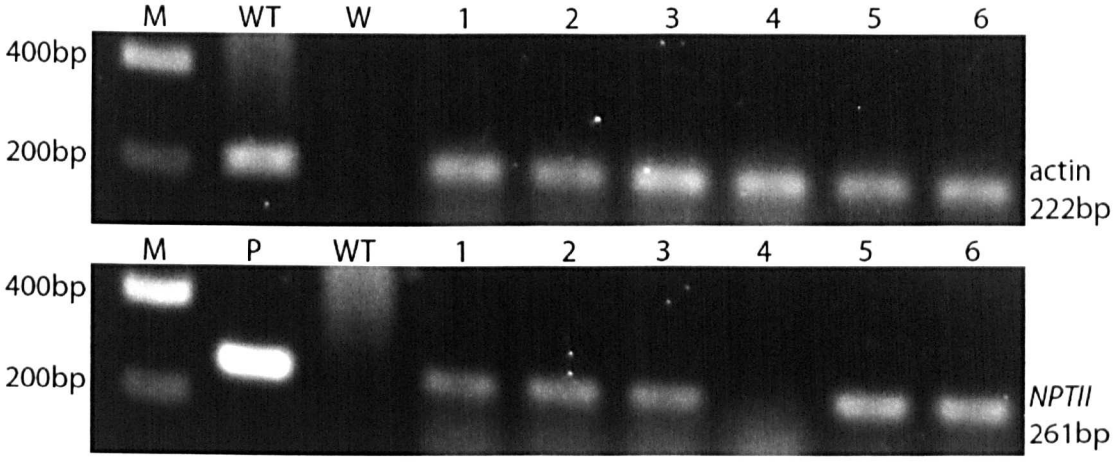

Figure 4.12 RT-PCR for transgenic lines of *Lilium* cv. Night Flyer. Lanes: (M) DNA marker; (WT) Night Flyer wild type cDNA as positive control for actin and negative control for *NPTII*; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (1) Line NF2-1; (2) Line NF2-2; (3) Line NF2-3; (4) Line NF1-1; (5) Line NF1-7; (6) Line NF2-8. All the transgenic lines except (4) showed *NPTII* gene expression.

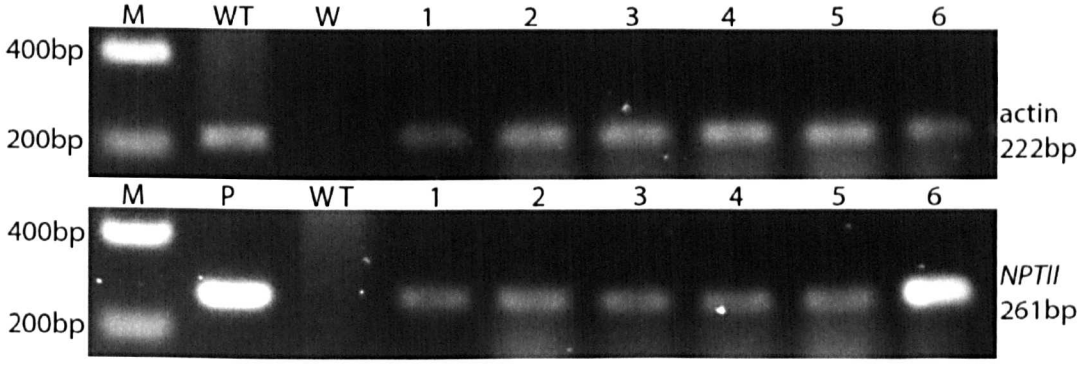

Figure 4.13 RT-PCR for transgenic lines of *Lilium* cv. Sweet Surrender. Lanes: (M) DNA marker; (WT) Sweet Surrender wild type cDNA as positive control for actin and negative control for *NPTII*; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (1) Line SS2-9; (2) Line SS1-2; (3) Line SS1-10; (4) Line SS1-11; (5) Line SS2-11; (6) Line SS2-12. All the transgenic lines showed *NPTII* gene expression.

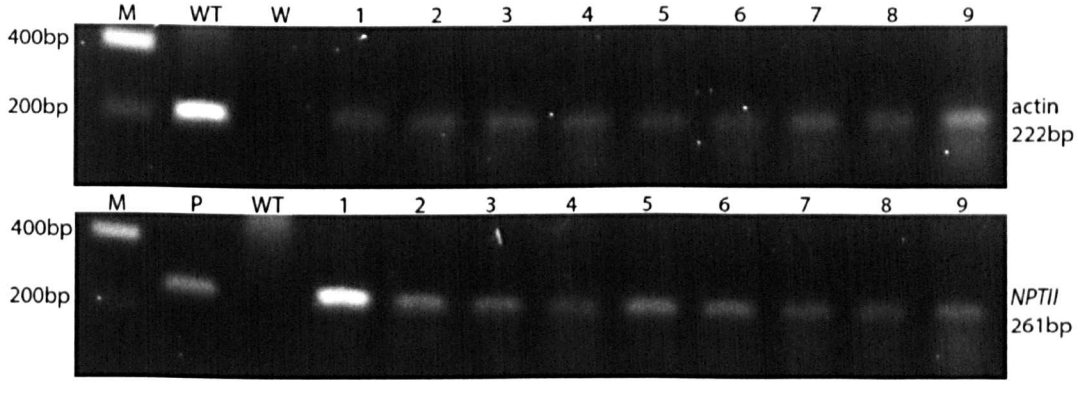

Figure 4.14 RT-PCR for transgenic lines of *Lilium* cv. Acapulco. Lanes: (M) DNA marker; (WT) Acapulco wild type cDNA as positive control for actin and negative control for *NPTII*; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (1) Line AC2-6; (2) Line AC1-2; (3) Line AC1-7; (4) Line AC2-3; (5) Line AC2-5; (6) Line AC1-11; (7) Line AC2-7; (8) Line AC2-13; (9) Line AC2-17. All the transgenic lines showed *NPTII* gene expression.

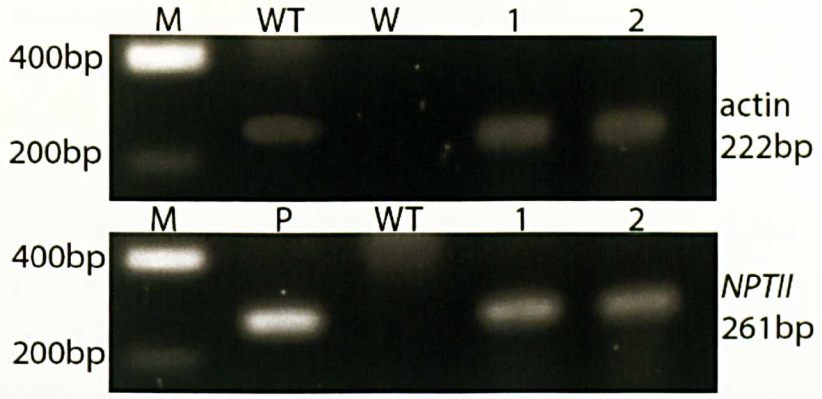

Figure 4.15 RT-PCR for transgenic lines of *Lilium leichtlinii*. Lanes: (M) DNA marker; (WT) *L. leichtlinii* wild type cDNA as positive control for actin and negative control for *NPTII*; (W) water as negative control; (P) pBI121 plasmid DNA as positive control; (1) Line LE3-1; (2) Line LE2-3. Both transgenic lines showed *NPTII* gene expression.

4.3.5 GUS assay.

To confirm that explants of all cultivars/species were being transformed and to localise marker expression, histochemical analysis of the *GUS* gene was performed 4 days after inoculation. Results obtained for all cultivars/species are presented in Fig 4.16.

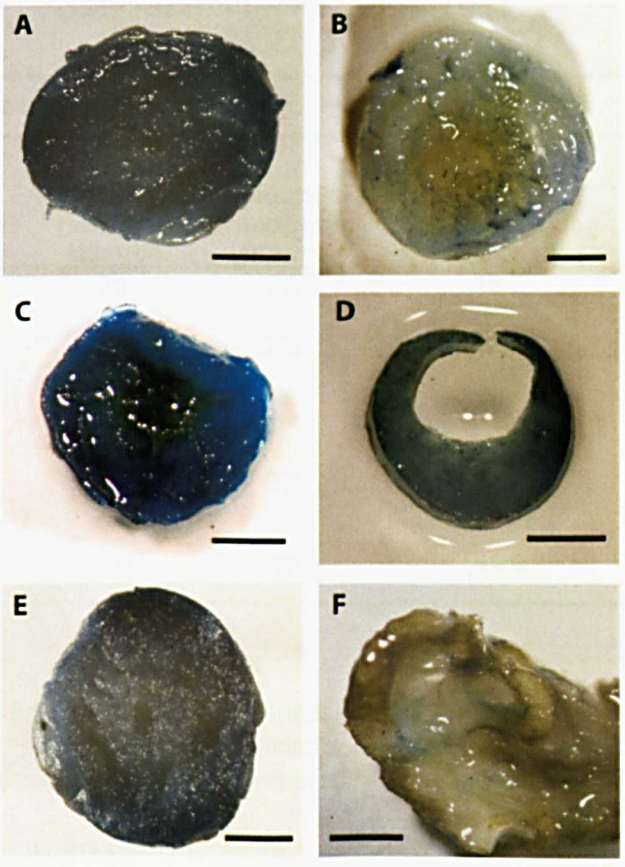

Figure 4.16 Transient GUS expression in basal plate discs 4 days after inoculation. (A) Cultivar Beverly's Dream. Bar=2mm; (B) Cultivar Night Flyer. Bar=1mm; (C) Cultivar Sweet Surrender. Bar=0.5mm; (D) Cultivar Acapulco. Bar=2mm; (E) *Lilium leichtlinii.* Bar=1mm; (F) Cultivar Snow Queen. Bar=1.5mm. All the explants showed high level of expression of the *GUS* gene except Snow Queen which had only a few localised blue spots.

4.3.6 Summary of transformation of different *Lilium* species/cultivars

In this experiment the transformation efficiency of the previously developed *Lilium* transformation protocol (chapter 3, section 3.2.3.4) was analysed on 7 cultivars/species of *Lilium*. Due to the different nature of each cultivar, in terms of size, growth habit or sensitivity to *in vitro* culture and manipulation, some modifications to the original protocol had to be made to adapt it to this broader spectrum of genotypes. Results showed that efficiency on five of the seven cultivars was similar to the one obtained for cv. Star Gazer (chapter 3) or even higher. Only two cultivars showed very low transformation efficiency, but this was because of endogenous contamination of the starting material, which meant that the explants failed to develop properly and regenerate shoots even in non-inoculated control explants. Tables 4.6-4.13 summarize the number of transformed tissues and transgenic plants generated compared to the number of basal plate disc explants inoculated.

CULTIVAR BEVERLY'S DREAM					
Experiment No.	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)***
1	100	71	71	36	36.0
2	70	52	74	23	32.8
3	100	69	69	31	31.0
4	80	65	81	27	33.7
5	60	42	70	19	31.6
Total	**410**	**299**	**72.9**	**136**	**33.17**

Table 4.6 Results obtained for cv. Beverly's Dream in five independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

CULTIVAR NIGHT FLYER					
Experiment No.	No. of explants inoculated	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
1	60	14	23.3	11	18.3
2	30	12	40	9	30.0
3	33	19	57.5	12	36.3
Total	**123**	**45**	**36.5**	**32**	**26**

Table 4.7 Results obtained for cv. Night Flyer in four independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

CULTIVAR SWEET SURRENDER					
Experiment No.	No. of explants inoculated	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
1	60	22	36.6	11	18.3
2	33	13	39.3	9	27.7
3	52	39	75	18	34.6
Total	**145**	**74**	**51**	**38**	**26.2**

Table 4.8 Results obtained for cv. Sweet Surrender in four independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

CULTIVAR ACAPULCO					
Experiment No.	No. of explants inoculated	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
1	76	23	30.2	18	23.6
2	33	8	24.2	6	18.1
3	45	21	46.6	17	37.7
4	72	39	50.6	20	27.7
Total	**226**	**91**	**40.2**	**61**	**26.9**

Table 4.9 Results obtained for cv. Acapulco in four independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

LILIUM HENRYII					
Experiment No.	No. of explants inoculated	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
1	60	0	0	0	0
2	30	0	0	0	0
3	35	5	14	3	8.5
Total	**125**	**5**	**4**	**3**	**2.4**

Table 4.10 Results obtained for *Lilium henryii* in three independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

CULTIVAR SNOW QUEEN					
Experiment No.	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)***
1	20	0	0	0	0
2	40	0	0	0	0
3	13	11	84	1	7.9
Total	**73**	**11**	**15**	**1**	**1.3**

Table 4.11 Results obtained for cv. Snow Queen in three independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

LILIUM LEICHTLINII					
Experiment No.	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)***
1	40	8	20	6	15
3	22	5	22.7	4	18.1
4	28	7	25	7	25
Total		**20**	**22.2**	**17**	**18.8**

Table 4.12 Results obtained for *Lilium leichtlinii* in four independent *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

SUMMARY FOR ALL CULTIVARS/SPECIES					
Cultivar/ Species	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)**
Beverly's Dream	410	299	72.9	136	**33.17**
Night Flyer	123	45	36.5	32	**26**
Sweet Surrender	145	74	51	38	**26.2**
Acapulco	226	91	40.2	61	**26.9**
Lilium henryii	125	5	4	3	**2.4**
Snow Queen	73	11	15	1	**1.3**
Lilium leichtlinii	90	20	22.2	17	**18.8**

Table 4.13 Summary of results obtained for each cultivar/species in all *Agrobacterium*-mediated transformation experiments. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

4.4 Discussion.

Previous research in this thesis (chapter 3, section 3.2.3.4) resulted in the development of a rapid and efficient *Agrobacterium*-mediated transformation of *Lilium* cultivar Star Gazer (Núñez de Caceres *et al.*, 2011). This method was highly efficient, reaching 20% compared to the highest previous report made by Ogaki *et al.* (2008) of 4.8%, and very rapid, regenerating PCR positive transgenic plants in an average of 12 weeks. Azadi *et al.* (2010a) reported efficiencies of around 25%, but these were calculated based upon the number of hygromycin resistant tissues and not for the number of transgenic plants regenerated, therefore the overall efficiency of their method remains unknown. Liu *et al.* (2011) reported an efficiency of 76% but that number reflects the percentage of positive plants from the total plants screened by PCR, in addition the type

of explant and methodology suggests that a high number of the regenerated transgenic plants could be clones and not independent lines. This opened the possibility to test its robustness with other cultivars and species of *Lilium*.

In this experiment the transformation efficiency of the developed protocol was analysed on seven cultivars or species of *Lilium*. Due to the different nature of each cultivar, in terms of size, growth habit or sensitivity to *in vitro* culture and manipulation, some modifications to the original protocol had to be made to adapt it to this broader spectrum of cultivars. One of the most important stages of a transformation protocol is the selection of the transformed tissues *in vitro*. The use of the optimal concentration of selection agent in the medium that reduces the stress on the wounded inoculated tissues and at the same time applies enough selective pressure to minimize the amount of false positives, but also allows the fast development of the transformed tissues is critical. Every genotype had different endogenous resistance to kanamycin but all were within the same range showed previously by oriental cultivar Star Gazer.

After adapting the growing medium based upon the results of the shoot induction and antibiotics assay for all the cultivars, transformation experiments were made with oriental-trumpet (OT) cultivar Beverly's Dream, asiatic cultivars Night Flyer and Sweet Surrender, oriental cultivar Acapulco, *Lilium longiflorum* cv. Snow Queen, *Lilium henryii* and *Lilium leichtlinii*. All the cultivars showed transient *GUS* expression 4 days after inoculation with the exception of *Lilium henryii*. Average transformation efficiency was higher than that reported for cv. Star Gazer for the cultivars Beverly's Dream (33.1%), Night Flyer (26.0%), Sweet Surrender (26.2%) and Acapulco (26.9%). For *Lilium leichtlinii* the average transformation efficiency was just slightly lower at 18.8%. *Lilium henryii* and cv. Snow Queen had low transformation efficiency of 2.4% and 1.3% respectively; this was due mainly to the endogenous bacterial contamination which was recurrent throughout the whole culture and

transformation process and therefore reduced the survival rate of the explants and thus the regeneration of putative transgenic shoots. Even after several selection processes, inoculated tissues that seem to be sterile developed bacterial contamination (not *Agrobacterium*) after a couple of weeks under *in vitro* culture; the same contamination was present in the non-inoculated control explants. Inoculated explants and the few recovered transgenic plants could never be cleaned of this contamination and died approximately 4 months after inoculation.

This results obtained in this research demonstrate that the developed protocol is robust and highly reproducible, and most important, highly efficient and fast. The modifications to the original protocol developed in this research (chapter 3, section 3.2.3.4) also reduced the time to obtain PCR positive plants from an average of 12 weeks to an average of 10 weeks. This is significantly faster than previous reports of *Lilium* transformation, which required an average of 34 weeks to generate transgenic plants (Hoshi *et al.*, 2004; Ogaki *et al.* 2008; Qiu Hua *et al.*, 2008; Azadi *et al.*, 2010a; Liu *et al.* 2011).

The establishment and success of this protocol represents a major advance in the future of *Lilium* research. Although the general acceptance of GMO's worldwide is still low, this also opens the possibility for a future commercial implementation to manipulate agronomically important traits in a faster and more efficient way compared to traditional breeding programs.

A major challenge however is the quality of the starting material. Future research should be aimed at improving the sterilization process to obtain the best starting material since high levels of endogenous contamination, bacterial and fungal (not symbiotic to the plants), were observed; overcoming this could further increase the efficiency of this protocol. Also the genetic manipulation of agronomically important traits on *Lilium* like

pathogen resistance, colour pattern or pollen development could be conducted since to date all the transformation reports, including the experiments described here, have been done using the *NPTII* or *GUS* genes.

CHAPTER 5: INDUCED PATHOGEN RESISTANCE IN *LILIUM*

5.1 Introduction.

Fungal, bacterial or viral diseases are the cause of reduced yields and marketable quality of cultivated plants including ornamental species. This results in significant losses of income and at the same time increases the production costs as a direct result of disease prevention or control (Zuker *et al.,* 1998; Inglis and Kawchuk, 2002; Tanaka *et al.,* 2005). It is important to identify the range of potential pathogens for each crop as well as the pathogen symptoms and vectors. This will help to decide the best approach to prevent the disease and/or control it (Wang *et al.,* 2003).

The fungal pathogen *Botrytis* causes huge economic losses to a very wide range of host crop species as the disease can infect several tissues like flowers, leaves, fruits and stems. Disease symptoms range from blossom blight, fruit rots, leaf spots and bulb rots under both field and storage conditions (Staats *et al.,* 2005).

The genus *Botrytis* was one of the first genera of pathogenic fungi, to be described and was named from the Greek word meaning "bunch of grape berries" (Jarvis, 1980). It comprises one generalist, *B. cinerea*, infecting over 200 eudicot hosts. All other species are considered specialists infecting only one or a few closely related species within the same plant genus (Staats *et al.,* 2005). The exception is *B. fabae*, which infects species of the genera *Vicia, Lens, Pisum,* and *Phaseolus.* Table 5.1 shows the species of *Botrytis* and their hosts for ornamental crops.

B. cinerea is derived from Greek words meaning "grapes like ashes" referring to the bunching of fungal spores and to the greyish (ash-like) colour of the conidia (Laboh, 2009). It causes the grey mould, a common disease of flowers and one of the most important in *Lilium* (Hou and Chen, 2003). It is most destructive on mature or senescent tissues, but it usually gains entry to such tissues at a much earlier stage in crop development and remains dormant before rapidly rotting tissues when the environment is conducive and the host physiology changes (Williamson *et al.*, 2007; Munafo and Gianfagna, 2011). Manifestation of the pathogen occurs when there is condensation and temperatures are between 2°C and 24°C; symptoms appear as reddish brown spots and later lesions become elliptical with a tan colour in the centre and a yellow halo around it. (Lawson and Hsu, 1996).

Species	Disease Name	Host/Tissue	Host Species
B. cinerea Pers. / *B. fuckeliana*	Gray mould	Fallen leaves, fruits, flowers, stems.	>235 plant species
B. calthae		Stem of marsh-marigold	*Caltha palustris*
B. ranunculi		Buttercup (whole plant)	*Ranunculus spp. L.*
B. ficariarum		Buttercup (whole plant)	*Ficaria verna*
B. pelargonii		Leaves of geranium	*Pelargonium spp. L.*
B. paeoniae	Peony blight	Stems of peonies	*Paeonia spp. L.*
B. hyacinthi	Hyacinth fire	Leaves of hyacinth	*Hyacinthus spp. L.*
B. tulipae	Tulip fire	Leaves, stems and flowers of tulips	*Tulipa spp. L.*
B. elliptica	Lily fire	Leaves, stems and flowers of lilies	*Lilium spp. L.*
B. narcissicola	Smoulder mould	Bulbs of narcissus	*Narcissus spp. L.*
B. polyblastis	Narcissus fire	Leaves of narcissus	*Narcissus spp. L.*
B. galanthina	Blight	Snowdrop	*Ghalanthus spp. L.*
B. convoluta	*Botrytis* rhizome rot	Rhizomes of iris	*Iris spp. L.*
B. croci	Crocus blight	Leaves of cultivated crocus	*Crocus spp. L.*
B. gladiolorum	Gladiolus blight	Stems of gladiolus	*Gladiolus spp. L.*

Table 5.1 Species of *Botrytis* and their host ornamental plants. Modified from Staats *et al.*, 2005

Genetic manipulation techniques have become a very powerful tool to improve a wide variety of crops. Such techniques have allowed the introduction of agronomically important traits which would be difficult to achieve by conventional breeding programmes. Traditional approaches to enhance pathogen resistance in crops are laborious and time consuming, taking up to 15 to 20 years to obtain a resistant cultivar; hence the development of resistant cultivars by transgenic approaches has become an important target for plant breeding (Rommens and Kishore, 2000). Among the different strategies that are being adopted the introduction of genes encoding proteins that inhibit fungal growth appears to be one of the most promising approaches.

Introducing genes encoding potential Antifungal Proteins (AFPs) such as chitinase and/or β-1,3 glucanase has been used for the control of several fungal pathogens (Pierpoint *et al.,* 1996). Other approaches have utilised genes present in wild relatives that can be identified by molecular techniques such as Random Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP). For example, the level of induced resistance as well as the disease spectrum can be variable depending on the gene and level of expression and need further evaluation (Rommens and Kishore, 2000).

The antifungal activity of plant chitinases makes them an ideal target to induce enhanced resistance to fungal pathogens, with chitin as the main component of their cell wall, in ornamental species (Wang *et al.,* 2003). Chitinases belong to family 3 of the tobacco pathogenesis related proteins and their substrate is chitin (Palli and Retnakaran, 1999). Chitinases break bonds between the C1 and C4 of two consecutive N-acetylglucosamines of chitin, which is a main component of the cell wall in fungi. Plant chitinases are classified into seven classes (I–VII) based on their primary structures and have been found in many species of higher plants exhibiting complex developmental and hormonal regulation (Shin *et al.,* 2008). Chitinases are

involved in the hypersensitive resistance response to microbial attack. Purified plant chitinase attacks and partially digests isolated cell walls of several pathogenic fungi such as ascomycetes, basidiomycetes and deuteromycetes (Zhu and Lamb, 1990).

Several reports of successful transgene-induced pathogen resistance in monocotyledonous and dicotyledonous plants have been published (Roby *et al.,* 1990; Lorito *et al.,* 1998; Carstens *et al.,* 2003). Zhu *et al.* (1994) generated tobacco plants with a greater protection against Frogeye disease (*Cercospora nicotianae*) by crossing transgenic parental lines exhibiting strong constitutive expression of CaMV 35S enhancer/RCH10 rice chitinase gene encoding a basic chitinase, and a CaMv35S double promoter/AGLU1 acidic β-1,3-glucanase from alfalfa.

Marchant *et al.* (1998) introduced the *RCH10* chitinase gene from rice into rose (*Rosa hybrida*) cv. Glad Tidings by particle bombardment. They showed that there was a correlation between chitinase activity from their transgenic lines and the level of resistance to Blackspot disease (*Diplocarpon rosae*). Overexpression of the rice chitinase *RCC2* in transgenic cucumber demonstrated that the level of induced resistance to Grey mould disease (*Botrytis cinerea*) was directly related to the expression level of the RCC2 protein (Kishimoto *et al.,* 2002). Takatsu *et al.* (1999) transformed the rice chitinase gene *RCC2* into spray-type chrysanthemum using *Agrobacterium tumefaciens*. The results also showed that among the different transgenic lines, the level of resistance to Grey mould (*Botrytis cinerea*) was directly linked to the level of the RCC2 protein.

Rohini and Sankara Rao (2001) transformed peanut embryos (*Arachis hypogaea*), with *Agrobacterium* carrying the binary vector pBI121-pBTex harbouring a 0.89kb class I tobacco chitinase gene driven by the CaMV35S promoter. The results showed that the observed enhanced

resistance to *Cercospora arachidicola* was correlated with the increase of chitinase activity. *Gossypium hirsutum*'s (cotton) hypocotyls were transformed with three different bean chitinase genes by *Agrobacterium*-mediated transformation (Tohidfar *et al.,* 2005). These transgenic plants were then used to obtain extracts that showed growth inhibition activity against *Verticillium dahliae* on some *in vitro* cultures

Plants of strawberry cultivar Pájaro were transformed by *Agrobacterium tumefaciens* with three defence related genes, alone or combined. The *ch5B* gene from *Phaseolus vulgaris* which encodes for a chitinase, the *gln2* and *ap24* genes from *Nicotiana tabacum* encoding for a glucanase and a thaumatin-like protein respectively. Results showed that two transgenic lines expressing only the *ch5B* gene displayed high levels of resistance to grey mould disease (*Botrytis cinerea*). Such resistance was correlated with the presence and amount of the CH5B protein in the leaves (Vellicce *et al.,* 2006).

More recently, transgenic lemon plants expressing the *chit42* gene from *Trichoderma harzianum* showed enhanced resistance to *Botrytis cinerea* compared to the wild type plants, the level of resistance was also correlated with the level of expression of the transgene (Distefano *et al.,* 2008). Transgenic wheat expressing a barley class II chitinase gene showed enhanced resistance in the greenhouse and in field conditions against Fusarium head blight disease (*Fusarium graminearum*) and the level of resistance was related to the level of expression of the transgene (Shin *et al.,* 2008).

To date, there are no reports of *Lilium* cultivars with enhanced fungal resistance. Thus the aim of this work was to evaluate the level of induced resistance to *B. cinerea* of transgenic lines from cv. Star Gazer generated by *Agrobacterium*-mediated transformation expressing either a rice chitinase (*RCH10*) or a wheat leaf rust chitinase (*CHIT2*) gene.

5.2 Materials and Methods.

5.2.1 *Botrytis* strain and maintenance.

Botrytis cinerea was used for all the experiments. An initial mycelial culture was kindly provided by Louis Cheetham from University of Nottingham. An inoculum of mycelia was cultured in the centre of a Petri dish containing 25 ml of sterile Potato Dextrose Agar medium (PDA, Appendix 1). Cultures were maintained by subculturing every two months onto fresh PDA medium using a sterile disposable inoculation loop under sterile conditions using a 0.5 cm^2 plug and incubated at 18 °C ± 1°C in the dark. Ten days old cultures were used for spore solution preparations.

5.2.2 Plant material

For all the transformation experiments, basal plate discs from *in vitro* derived bulblets of cultivar Star Gazer were used, as previously described in chapter 3, section 3.2.3.4.1.

Putative transgenic plants regenerated from inoculated explants were grown in jars until they reached an average weight of 600 mg. They were then transferred to individual containers with wet soil composed of peat, perlite and vermiculite in a ratio of 4:2:1 and kept for 8 weeks in the dark at 5 °C for dormancy break. After this period, bulbs were transferred to pots filled with the same soil composition and maintained in the growth room at a 16/8 h photoperiod with temperatures of 22 °C in the day and 16 °C at night.

After growing for around 16 weeks, the aerial parts of plants with bulbs that weighed on average 4 grams were cut back and the bulbs kept at 5 °C for 8-10 weeks for floral induction. After this vernalization period, bulbs were replaced back in the growth room and cultured at a 16/8 h photoperiod with temperatures of 22 °C ± 1°C in the day and 16 °C ± 1°C at night.

5.2.3 *Agrobacterium*-mediated transformation.

5.2.3.1 *Agrobacterium* strain and constructs.

Agrobacterium tumefaciens strain AGL1 was used in all experiments. Plasmid pBI101 carrying either the rice chitinase gene *RCH10* (Zhu and Lamb, 1990), or the wheat leaf rust (*Puccinia triticina*) chitinase gene (Zhang *et al.*, 2003) driven by the CaMV35S promoter and the neomycin phosphotransferase (*NPTII*) gene under the control of the *nos* promoter (Fig 5.1) were kindly provided by Dr Gracia Ribas Vargas from the University of Nottingham and transformed into AGL1 by electroporation as described in chapter 3, section 3.2.3.1.1.

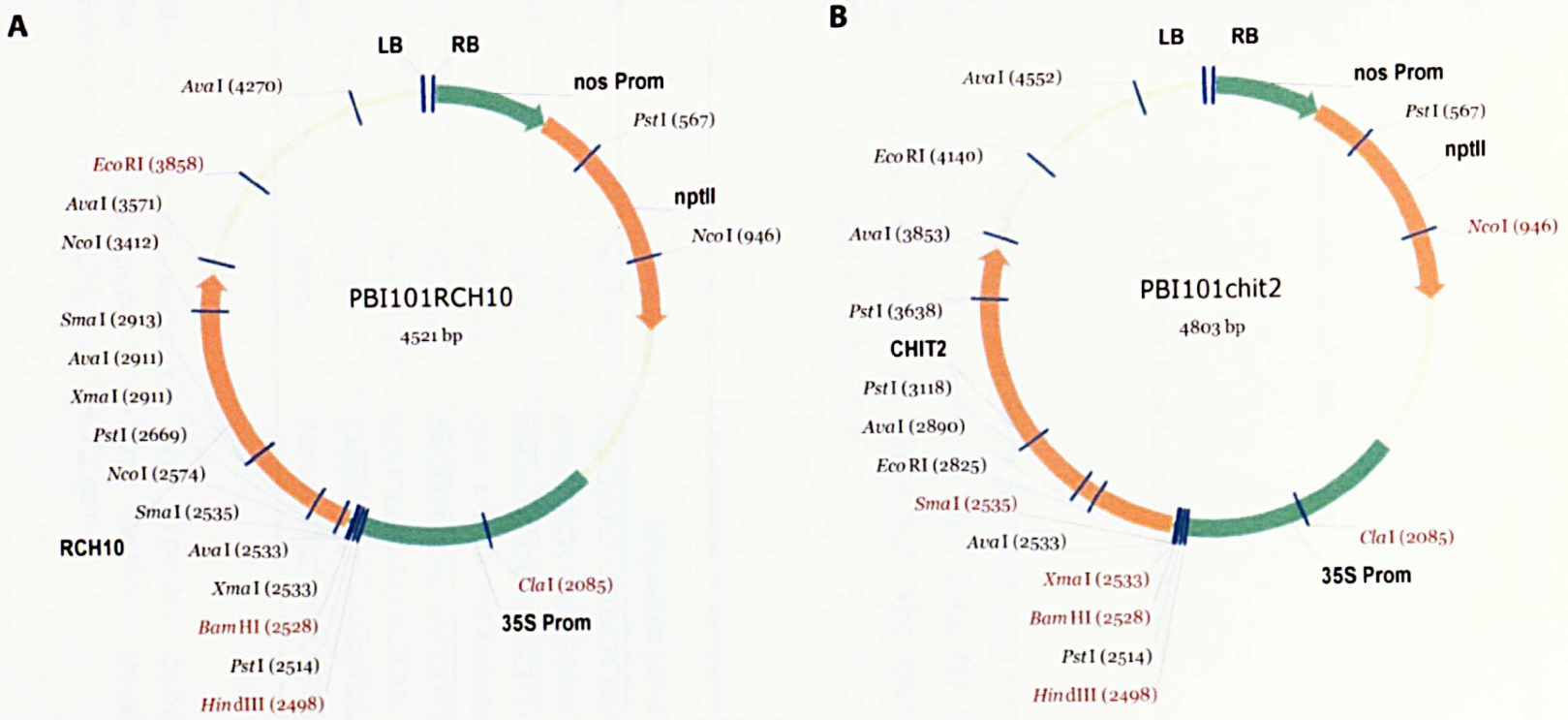

Figure 5.1 Constructs used to induce enhanced pathogen resistance in *Lilium.* A) pBI101 carrying the rice chitinase *RCH10* gene; B) pBI101 carrying the wheat leaf rust chitinase *CHIT2* gene.

5.2.3.2 *Agrobacterium*-mediated transformation

Transformation experiments were performed as described in chapter 3, section 3.2.3.4.

5.2.3.3 Molecular analysis.

5.2.3.3.1 PCR analysis of putative transgenic lines.

To confirm transgene insertion, PCR analysis was conducted as described in chapter 2 section 2.3.1. Analysis was done for three genes and the primers used are described in Table 5.2. The thermal cycles for each reaction are described in Table 5.3.

Gene	Primers	Sequence (5'-3')	Expected Band size
NPTII	nptII_For	AGACAATCGGCTGCTCTGAT	261 bp.
	nptII_Rev	ATACTTTCTCGGCAGGAGCA	
RCH10	35S_For	CACAATCCCACTATCCTTCGCAAGAC	352 bp.
	ChitVer2	CAGCATCTGGTCGAAGAGC	
CHIT2	35S_For	CACAATCCCACTATCCTTCGCAAGAC	543 bp.
	RustChitR2	CCATTGGGGTATTCCCAA	
Polyubiquitin (housekeeping gene)	Polyb F	GAAGCAGCTGGAAGATGGAC	196 bp.
	Polyb R	GATCCCTTCCTTGTCGTGAA	

Table 5.2 Primers, sequence and expected band size used for PCR screening of each gene from the obtained putative transgenic lines carrying either the *RCH10* or *CHIT2* gene.

Thermal cycle for transgene detection				
	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:35 min	
NPTII	Annealing	57°C	0:35 min	35 cycles
	Extension	72°C	0:35 min	
	Final Extension	72°C	5:00 min	1 step
	Initial denaturation	94°C	4:00 min	1 step
	Denaturation	94°C	0:30 min	
RCH10	Annealing	58.2°C	0:45 min	33 cycles
	Extension	72°C	0:45 min	
	Final Extension	72°C	5:00 min	1 step
	Initial denaturation	94°C	4:00 min	1 step
	Denaturation	94°C	0:45 min	
CHIT2	Annealing	56°C	0:45 min	35 cycles
	Extension	72°C	0:45 min	
	Final Extension	72°C	8:00 min	1 step
	Initial denaturation	94°C	5:00 min	1 step
	Denaturation	94°C	0:45 min	
Polyubiquitin	Annealing	58.4°C	0:45 min	30 cycles
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step

Table 5.3 Thermal cycles used for PCR.

5.2.3.3.2 Reverse Transcriptase-PCR of transgenic lines.

After confirming transgene insertion by PCR, the expression of the gene was checked by RT-PCR following the procedure described in chapter 2, section 2.3.3. Table 5.4 describes the primers used for RT-PCR and table 5.5 their respective thermal cycles. Total RNA was extracted from leaf material following the procedure described in chapter 2, section 2.2.2.

Gene	Primers	Sequence (5'-3')	Expected Band size
NPTII	nptII_For	AGACAATCGGCTGCTCTGAT	261 bp.
	nptII_Rev	ATACTTTCTCGGCAGGAGCA	
RCH10	RCH10_F	GCCTTCTGGTTCTGGATGAC	247 bp.
	RCH10_R	AATCCAAGTTGGCGTCGTAG	
CHIT2	CHIT2_F	CCCGCAAGTACAAGGTCAAT	240 bp.
	CHIT2_R	CCTTGACGAGTAAGCCGAAG	
Polyubiquitin (housekeeping gene)	Polyb F	GAAGCAGCTGGAAGATGGAC	196 bp.
	Polyb R	GATCCCTTCCTTGTCGTGAA	

Table 5.4 Primers, sequence and expected band size used for RT-PCR analysis.

	Thermal cycle for transgene detection			
NPTII	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:35 min	35 cycles
	Annealing	57°C	0:35 min	
	Extension	72°C	0:35 min	
	Final Extension	72°C	5:00 min	1 step
RCH10	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:30 min	33 cycles
	Annealing	56°C	0:30 min	
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step
CHIT2	Initial denaturation	94°C	3:00 min	1 step
	Denaturation	94°C	0:30 min	35 cycles
	Annealing	57.5°C	0:30 min	
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step
Polyubiquitin	Initial denaturation	94°C	5:00 min	1 step
	Denaturation	94°C	0:45 min	30 cycles
	Annealing	58.4°C	0:45 min	
	Extension	72°C	0:30 min	
	Final Extension	72°C	5:00 min	1 step

Table 5.5 Thermal cycles used for RT-PCR analysis.

5.2.3.3.3 Quantitative Reverse Transcriptase-PCR (QRT-PCR).

For a more accurate measurement of the level of expression of the transgenes, QRT-PCR was performed on 22 transgenic lines carrying the *RCH10* gene and 6 transgenic lines carrying the *CHIT2* gene; wild type plants and water were used as negative controls. QRT-PCR was performed as described in chapter 2, section 2.3.4. Samples were analysed in triplicate on a Light Cycler 480 apparatus (Roche Diagnostics, West Sussex, UK), Table 5.6 shows the primers used for the QRT-PCR analysis and Table 5.7 the amplification conditions.

Gene	Primers	Sequence (5'-3')	Expected Band size
RCH10	RCH10_F	GCCTTCTGGTTCTGGATGAC	247 bp.
	RCH10_R	AATCCAAGTTGGCGTCGTAG	
CHIT2	CHIT2_F	CCCGCAAGTACAAGGTCAAT	240 bp.
	CHIT2_R	CCTTGACGAGTAAGCCGAAG	
LP59 Actin (housekeeping gene)	Lactin_2 F	TGGTGTGATGGTTGGTATGG	222 bp.
	Lactin_2 R	TTTGCCTTAGGGTTGAGTGG	

Table 5.6 Primers, sequence and expected band size used for QRT-PCR analysis.

Thermal cycle for QRT-PCR				
RCH10/CHIT2	Initial denaturation	95°C	5:00 min	1 step
	Denaturation	95°C	0:10 min	45 cycles
	Annealing	56.5°C	0:30 min	
	Extension	72°C	0:30 min	

Table 5.7 Amplification conditions for the QRT-PCR.

5.2.4 Resistance assay.

5.2.4.1 Experimental design.

To analyse the induced resistance of the generated transgenic lines against the fungal pathogen *Botrytis cinerea*, an *in vitro* assay was performed. Leaves from the middle of the stem were excised from 16 month old plants in the adult flowering stage growing in the growth room and briefly cleaned with a paper tissue soaked with 70% (v/v) ethanol to eliminate any soil particles or other contaminants.

Explants were then transferred abaxially to square Petri dishes containing 50 ml of sterile medium composed of water and 10% (w/v) phytagel, previously autoclaved. Three replicates of each transgenic line and several wild type plants as negative controls were used and three different time points were measured 7, 10 and 14 days after inoculation. Measurement of the wound size and extent of visible sporulation were used to assess whether the explants were showing any resistance to the fungal pathogen.

5.2.4.2 Spore solution preparation and inoculation procedure.

Ten days before each inoculation experiment a plug of *Botrytis cinerea* of about 0.5 cm^2 was subcultured into a new Petri dish containing fresh PDA medium and maintained at 23 °C in the dark.

Spore solutions were then prepared by a modified method from Fiddaman *et al.* (2000) by flooding the Petri dishes with 10 ml of spore nutrition solution composed of double distilled water supplemented with 0.1 g/L of glucose, 0.1 g/L of KH_2PO_4 and 0.5% (v/v) of Tween 20. Plates were scraped to dislodge the conidia and the suspensions were then filtered through four-fold sterile muslin. Spore solutions were diluted with a water/phytagel solution previously autoclaved to obtain a 3.5% final concentration of the gelling agent. Spore number was counted with a haemocytometer and adjusted to a final concentration of 5×10^5 spores/ml. Spore solutions were freshly prepared on the day of each inoculation and discarded afterwards. Concentration of spores for all the experiments was 5×10^5 spores/ml.

Two inoculation sites were made on each explant by pipetting 10 µl of spore solution on each site. Explants were kept in the light at 23 °C ± 2°C. Pictures and measurements of lesions were taken at 7, 10 and 14 days after inoculation.

5.3 Results.

5.3.1 Transformation.

After *Agrobacterium* transformation of explants from cv. Star Gazer, 212 putative transgenic plants were recovered from 750 explants inoculated with the *RCH10* construct, and 64 from 300 explants inoculated with the *CHIT2* construct.

5.3.1.1 PCR analysis.

PCR analysis of randomly selected plants for each gene of interest was performed as previously described. Plants were from different independent transformation experiments. Analysis of the presence of *NPTII* and polyubiquitin genes was also performed. All the plants were positive for the *NPTII* gene, but not all of them were positive for the *RCH10* gene (Fig. 5.2) and none were positive for the *CHIT2* gene (Fig 5.5). PCR for the polyubiquitin gene was performed to confirm that the DNA sample was of sufficient quality for molecular analysis. Figures 5.2-5.7 show the results obtained for some of the analysed plants.

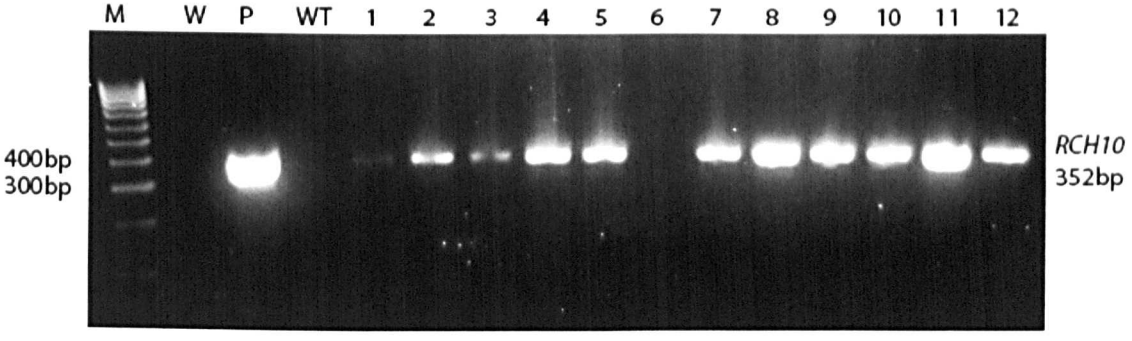

Figure 5.2 PCR analysis of the *RCH10* gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R108; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line R47; (11) Line R666; (12) Line R165. Lanes 1-5, 7-12 were positive for the *RCH10* gene.

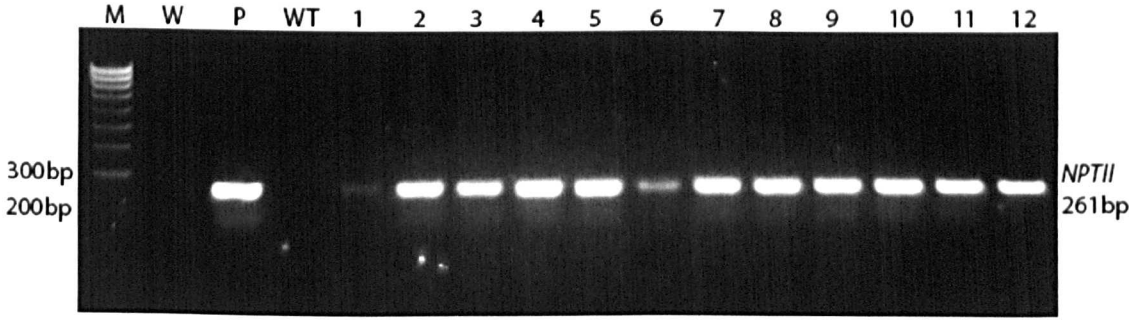

Figure 5.3 PCR analysis of the *NPTII* gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R108; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line R47; (11) Line R666; (12) Line R165. All putative transgenic lines were positive for *NPTII* gene.

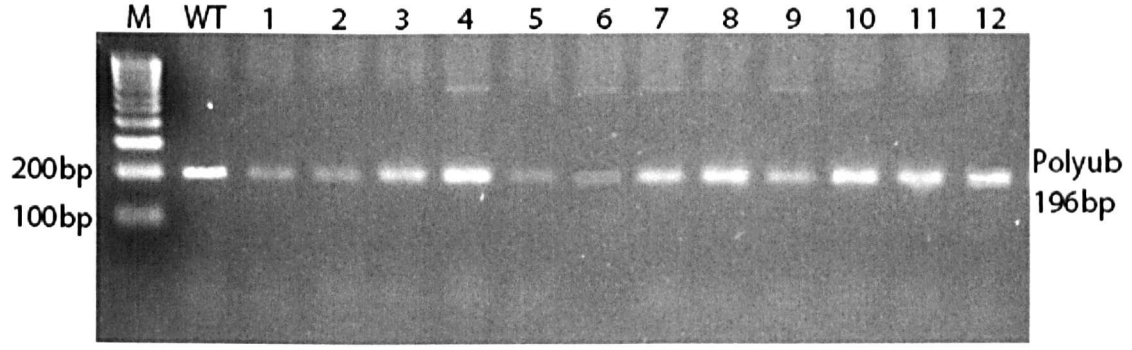

Figure 5.4 PCR analysis of the polyubiquitin gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (WT) wild type DNA as positive control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R108; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line R47; (11) Line R666; (12) Line R165. All lines were positive for the polyubiquitin gene.

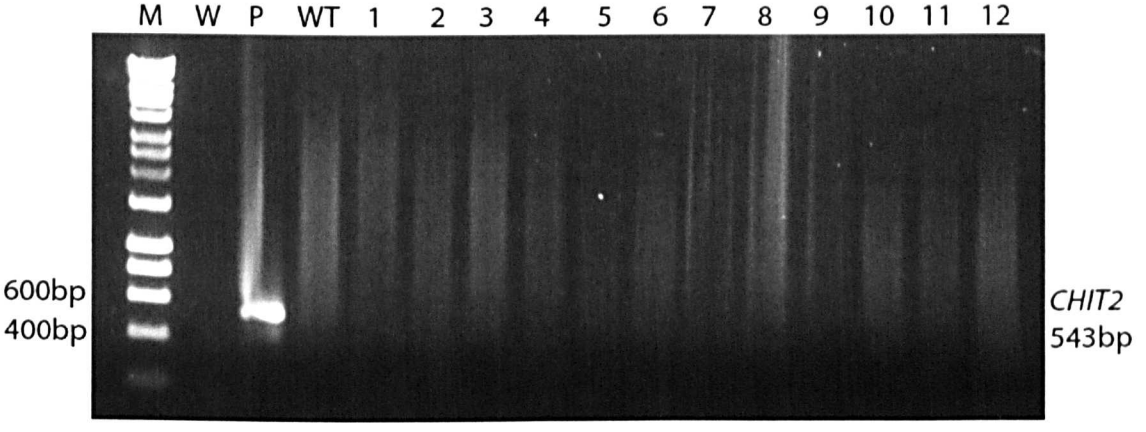

Figure 5.5 PCR analysis of the *CHIT2* gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line C681; (2) Line C10; (3) Line C675; (4) Line C664; (5) Line C668; (6) Line C663; (7) Line C661; (8) Line C679; (9) Line C678; (10) Line C682; (11) Line C671; (12) Line C677. All lines were negative for the *CHIT2* gene.

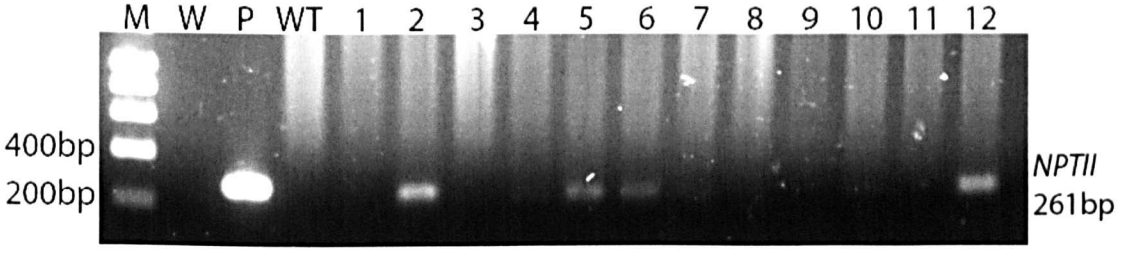

Figure 5.6 PCR analysis of the *NPTII* gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line C681; (2) Line C10; (3) Line C675; (4) Line C664; (5) Line C668; (6) Line C663; (7) Line C661; (8) Line C679; (9) Line C678; (10) Line C682; (11) Line C671; (12) Line C677. Lanes 2, 4-7, 10 and 12 were positive for *NPTII* gene.

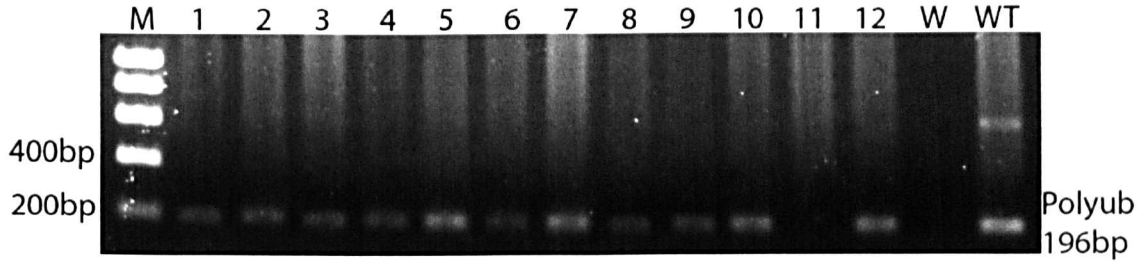

Figure 5.7 PCR analysis of the polyubiquitin gene in putative transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line C681; (2) Line C10; (3) Line C675; (4) Line C664; (5) Line C668; (6) Line C663; (7) Line C661; (8) Line C679; (9) Line C678; (10) Line C682; (11) Line C671; (12) Line C677. All lines except line C671 were positive for the polyubiquitin gene.

5.3.1.2 RT-PCR analysis.

After confirmation by PCR of transgene insertion, gene expression was analysed by Reverse Transcriptase PCR. The results showed that for the *RCH10* gene the majority of lines were expressing the transgene (Fig 5.8) and all of them were expressing the *NPTII* gene (Fig 5.9). RT-PCR analysis of a polyubiquitin gene confirmed that the quality and quantity of the cDNA samples used was adequate for the analysis (Fig 5.10).

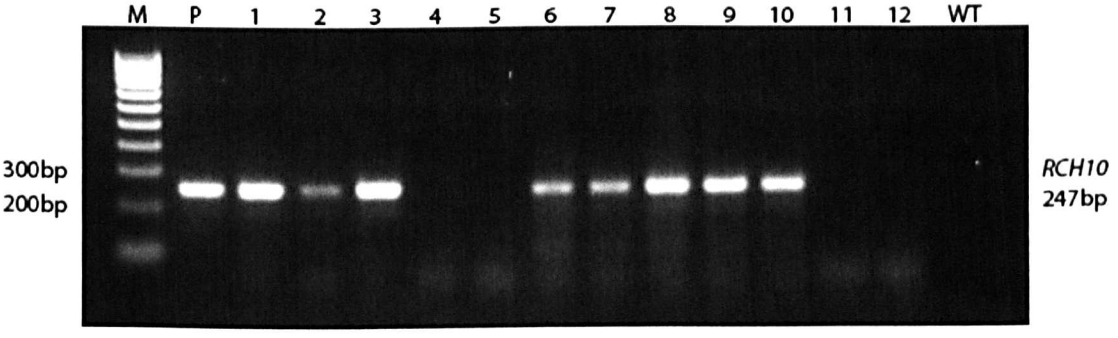

Figure 5.8 RT-PCR analysis of the *RCH10* gene in transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (P) plasmid DNA as positive control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line 666; (11) Line R108; (12) Line R47; (WT) wild type DNA as negative control. Lanes 1-3 and 6-10 show transgenic plants with expression of the *RCH10* gene.

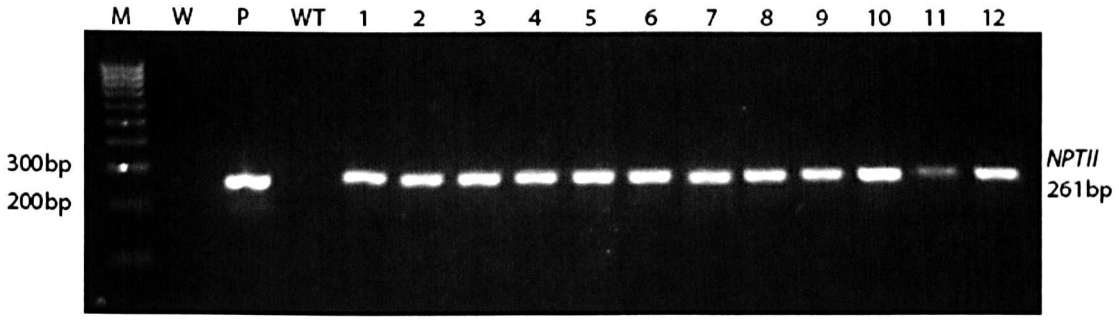

Figure 5.9 RT-PCR analysis of the *NPTII* gene in transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (W) water as a negative control; (P) plasmid DNA as positive control; (WT) wild type DNA as negative control; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line 666; (11) Line R108; (12) Line R47. All plant samples showed expression of the *NPTII* gene.

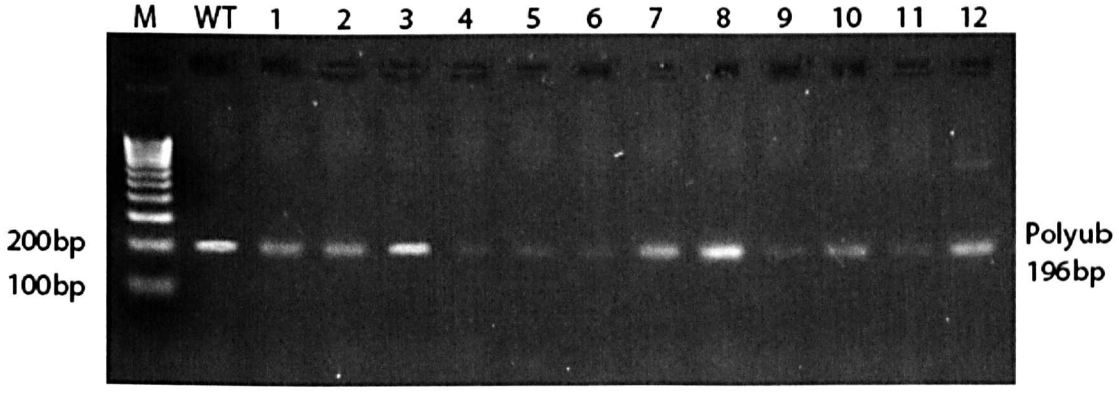

Figure 5.10 RT-PCR analysis of the polyubiquitin gene in transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (WT) wild type DNA as positive control ; (1) Line R169; (2) Line R660; (3) Line R679; (4) Line R29; (5) Line R400; (6) Line R165; (7) Line R206; (8) Line R673; (9) Line R504; (10) Line 666; (11) Line R108; (12) Line R47. All transgenic plants showed expression of the polyubiquitin gene.

Results of the RT-PCR performed with the *CHIT2* transgenic lines showed no expression of this transgene in any of the plants, as expected after the PCR analysis (Fig 5.11). The analysis of the *NPTII* gene showed expression in all but three of the lines (Fig 5.12). LB59 Actin expression analysis demonstrated that the cDNA samples were of sufficient quality for RT-PCR analysis (Fig 5.13). These results suggest that there could be a problem in the construction of the vector that leads to the lack of expression in transgenic plants.

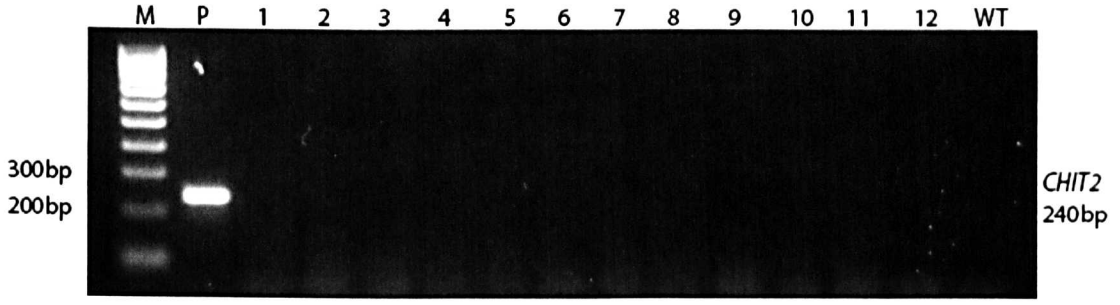

Figure 5.11 RT-PCR analysis of the *CHIT2* gene in transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (P) plasmid DNA as positive control; (1) Line C10; (2) Line C664; (3) Line C668; (4) Line C663; (5) Line C681; (6) Line C661; (7) Line C675; (8) Line C682; (9) Line C677; (10) Line C689; (11) Line C71; (12) Line C79; (WT) wild type DNA as negative control. None of the transgenic plants showed expression of the *CHIT2* gene.

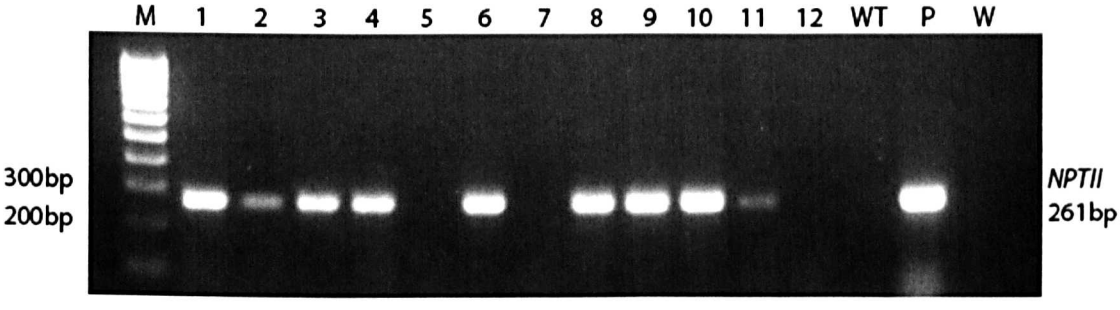

Figure 5.12 RT-PCR analysis of the *NPTII* gene in transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (1) Line C10; (2) Line C664; (3) Line C668; (4) Line C663; (5) Line C681; (6) Line C661; (7) Line C675; (8) Line C682; (9) Line C677; (10) Line C689; (11) Line C71; (12) Line C679; (WT) wild type DNA as negative control; (P) plasmid DNA as positive control; (W) Water as negative control. Lanes 1-4, 6 and 8-11 showed expression of the *NPTII* gene.

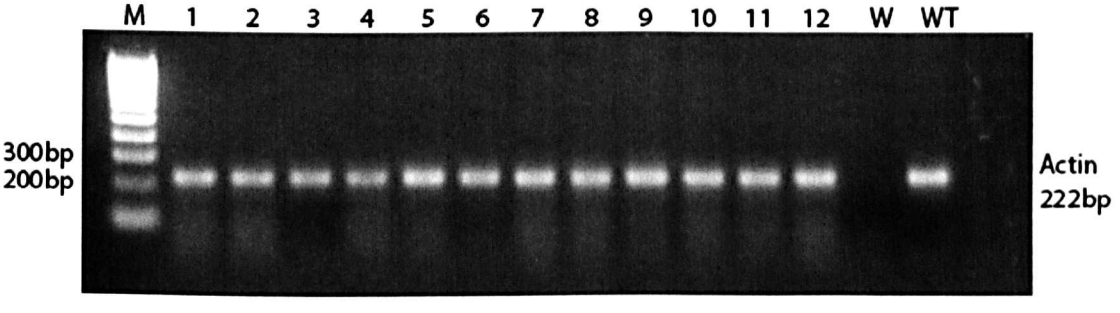

Figure 5.13 RT-PCR for transgenic lines of *Lilium* cv. Star Gazer Lanes: (M) DNA marker; (1) Line C10; (2) Line C664; (3) Line C668; (4) Line C663; (5) Line C681; (6) Line C661; (7) Line C675; (8) Line C682; (9) Line C677; (10) Line C689; (11) Line C71; (12) Line C679; (W) Water as negative control; (WT) wild type DNA as positive control. All plants showed expression of the actin gene.

5.3.1.3 QRT-PCR.

To test whether the level of chitinase expression was correlated to the level of resistance between the different transgenic lines a QRT-PCR was performed as described in section 5.2.2.3.3.

Results showed that the relative expression of the *RCH10* gene, in terms of fold change, varied between the different transgenic lines (Fig 5.14). Levels of transgene expression were subsequently compared against the wound size and sporulation level data to determine if resistance to *Botrytis cinerea* was observed and whether it was correlated to the level of chitinase expression.

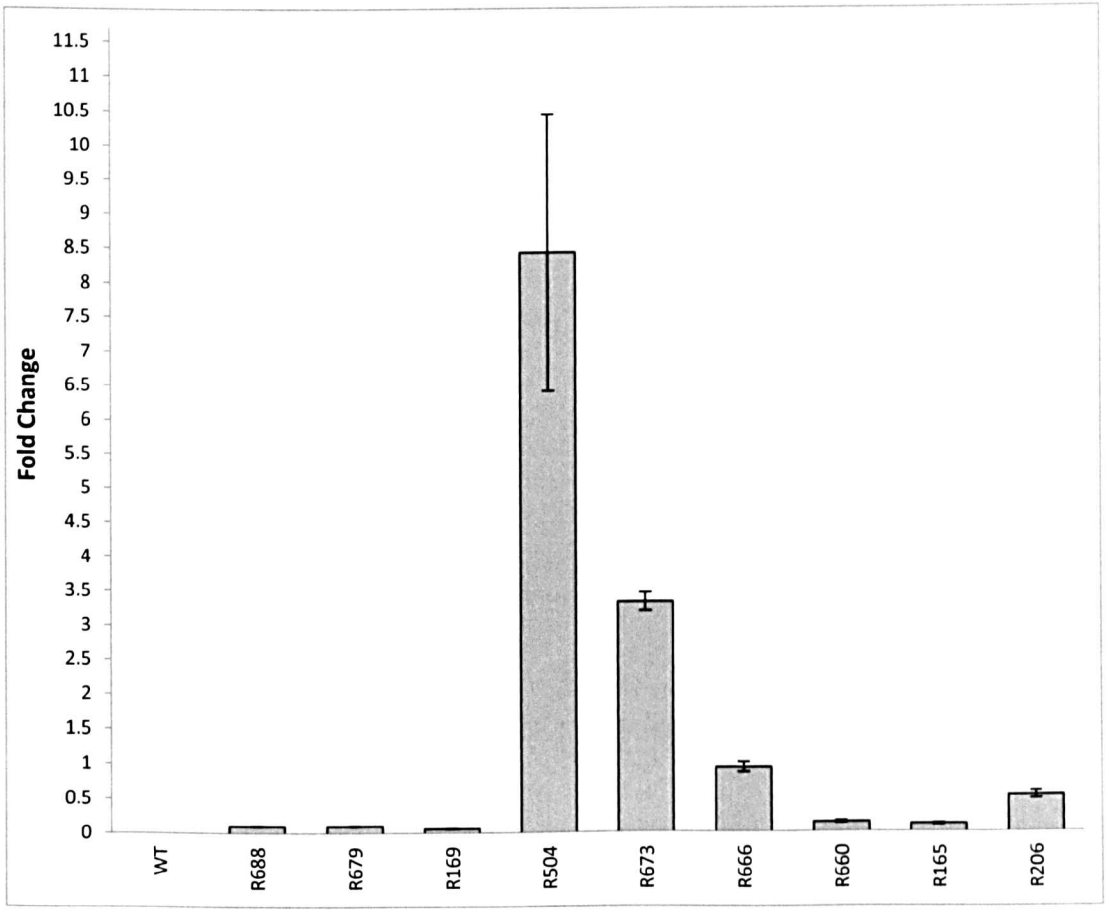

Figure 5.14 qRT-PCR Analysis of the *RCH10* transgene. Relative expression of the *RCH10* gene from different transgenic lines was compared to wild type after normalization to the LB59 actin gene (see Table 5.6 for primer information).

5.3.2 Infection on leaf explants.

5.3.2.1 Wound size.

A common way to evaluate the level of infection in a resistance assay is to measure the size of the wound left by the pathogen after several days post inoculation. Leaf explants from plants expressing the *RCH10* gene and wild type plants were excised and transferred into Petri dishes with water agar and inoculated with 10 µl of spore solution in two points (Fig 5.15). Wound size diameter was measured at 7, 10 and 14 days after inoculation from three biological replicates in three different experiments. As the average measurements showed (Table 5.7), the wound size was very variable amongst all samples either transgenic or wild type (Fig 5.16). A right sided Dunnett´s statistical test (Appendix 3) showed that the difference of wound size between the wild type and the transgenic lines was not significant in any of the time points.

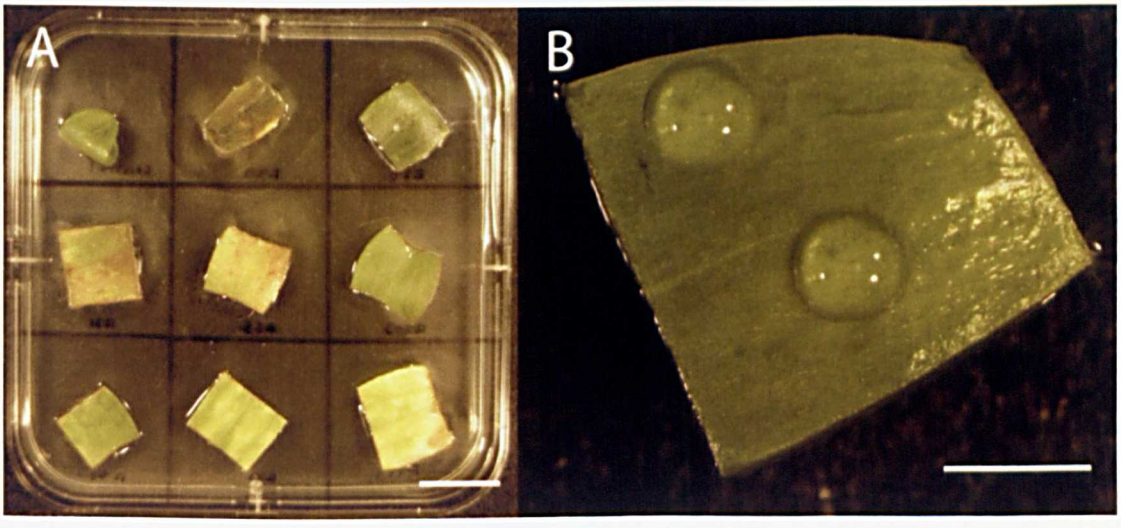

Figure 5.15 Pathogen resistance assay. A) Leaf explants were placed into square Petri dishes; three explants were used per transgenic line to take measurements at 7, 10 and 14 days post inoculation. Bar=3cm; B) Two inoculation points of 10 µl of spore solution were made on each explant. Bar=1cm.

Line	Wound Size in mm		
	7 days	10 days	14 days
R504	5.58	6	6.64
R169	7.02	7.2	7.04
R666	7.2	7.6	8
R673	5.4	5	5.2
R660	7.02	7.2	8.8
R165	5.58	5.8	6.24
R688	6.3	6.4	6.8
R206	4.86	5.4	6
R679	5.58	5.6	6.8
Wild Type	5.04	5.8	5.8

Table 5.7 Average wound size in millimeters on inoculated leaf explants from transgenic plants and wild type. Dunnett´s test showed that the difference in wound size between the control wild type and the transgenic lines was not significant with a confidence interval of 95% (Appendix 3).

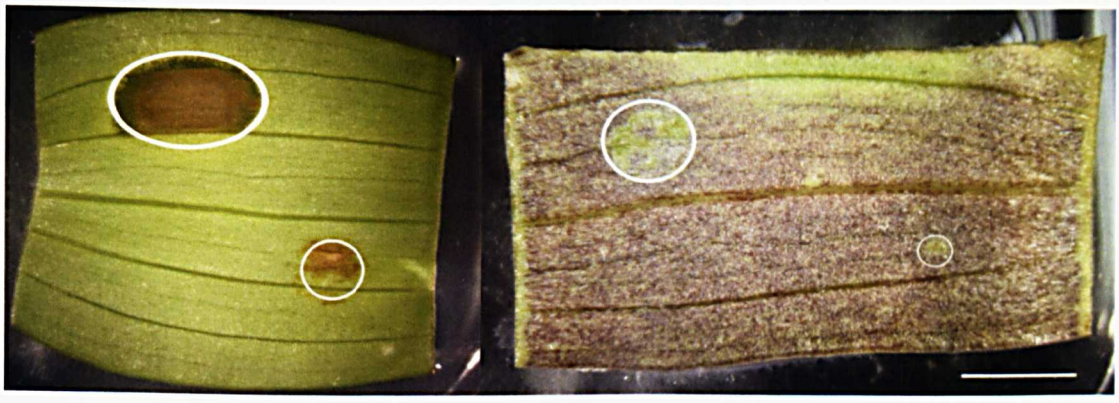

Figure 5.16 Lesions generated 10 days after inoculation with *Botrytis cinerea*. Wound size measurements were in general very variable with major differences seen in the size of lesions within the same explant. Two leaf explants showing very different wound sizes are highlighted by the white circles; left explant wild type, right explant transgenic for *RCH10* gene. Bar=0.5cm.

5.3.2.2 Sporulation.

To further analyse whether there was any enhanced resistance in the transgenic lines showing expression of chitinase, a sporulation count was performed. After lesion measuring at 7, 10 and 14 days after inoculation, the explants were individually transferred into single 7 ml universal tubes filled with 1 ml of sterile water supplemented with 0.5 % (w/v) of Tween 20. Average spore counts were made based on the average of the three replicates for each line at each time point (Table 5.8). These results showed that *Botrytis* sporulation on the wild type explants was higher compared with that from explants from the transgenic lines expressing the *RCH10* gene. Dunnett's test was used to determine if there was any statistically significant difference between sporulation levels on the wild type and transgenic lines (Appendix 3). Results showed that at all of the time points the difference in sporulation level between the transgenic lines compared with wild type were statistically significant with a confidence interval of 95%. This suggests that expression of *RCH10* in the transgenic lily lines is conferring enhanced resistance to *Botrytis cinerea* infection.

Line	Chitinase relative expression	Sporulation (Spores/ml)		
		7 days	10 days	14 days
R504	Very high	2	11	60
R169	Low	30	49	142
R666	Medium	7	35	139
R673	High	6	14	73
R660	Low	9	23	99
R165	Low	15	74	194
R688	Very low	16	82	264
R206	Medium	7	28	105
R679	Very low	22	69	212
Wild Type	None	311	795	3568

Table 5.8 Sporulation measurements on inoculated leaf explants from transgenic and wild type plants.

To analyse the correlation between the QRT-PCR expression analysis and the sporulation counts a Spearman's correlation test was performed (Appendix 3). A correlation coefficient (*rs*) of -0.997 was obtained at 7 days after inoculation, -0.842 at 10 days after inoculation and of -0.867 at 14 days after inoculation. These results indicate that there is a very strong negative correlation between the relative expression of the *RCH10* gene and the sporulation level at any time point (Fig 5.17).

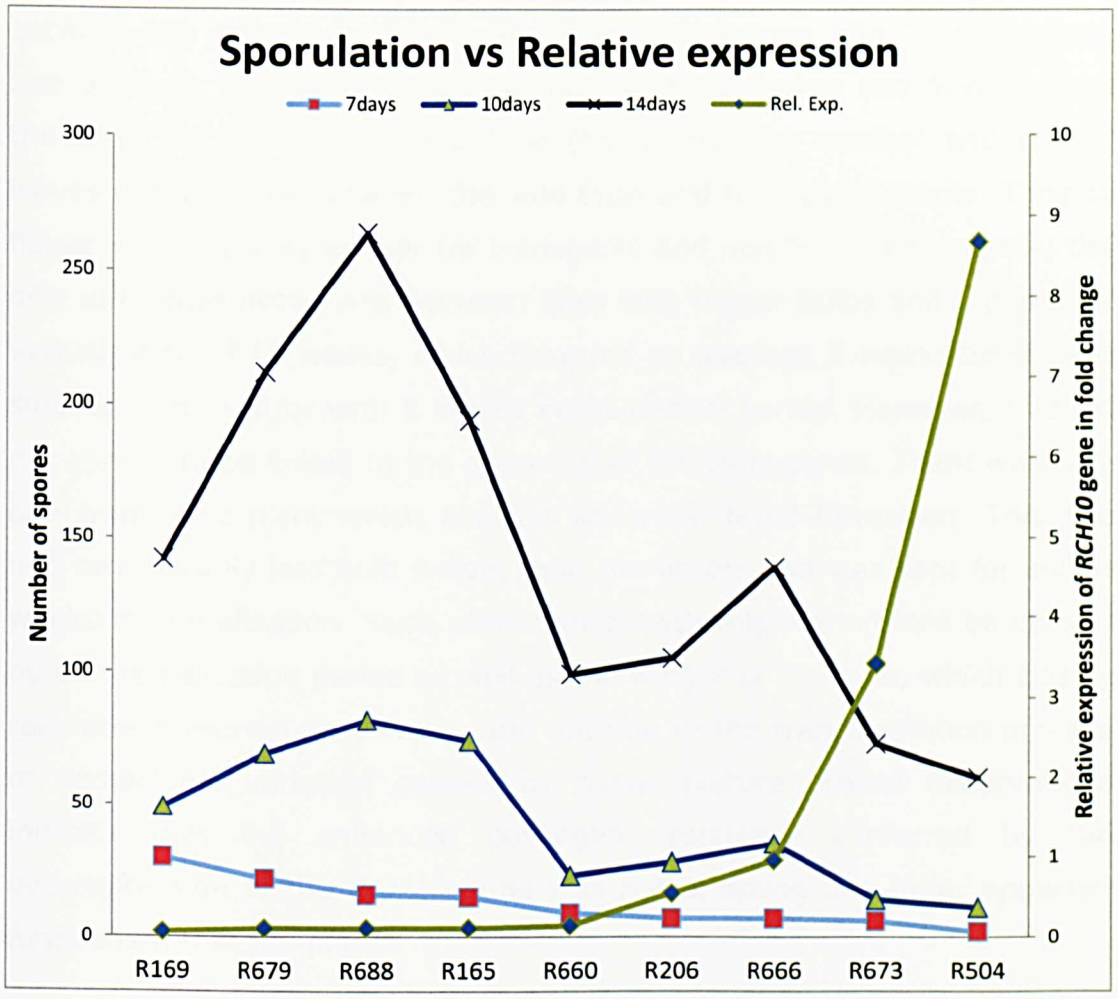

Figure 5.17 Sporulation levels versus relative expression of RCH10 gene. Sporulation counts for transgenic lines are shown at 7, 10 and 14 days after inoculation and plotted against their relative level of expression of RCH10 gene. The graph shows a negative correlation between expression and sporulation.

5.3.3 Phenotypic analysis

Transgenic lines were grown until flowering to determine if the transformation process or the expression of the transgene would affect the phenotypic characteristics of the cultivar. Wild type plants were grown under the same conditions of tissue culture as the transgenic lines for comparison. No significant reproducible differences were observed between the transgenic plants and the control ones (Fig 5.18). Height, size of flower and floral organs, colour of stem, leaves and flowers were the same as seen in the wild type (Fig 5.19); the number and size of leaves did not vary between the wild type and transgenic plants. Time to flower was also very similar for transgenic and non-transgenic plants; the only difference noted was between lines with bigger bulbs and a period of vernalization of 10 weeks, which flowered on average 2 weeks before the smaller bulbs and/or with 8 weeks vernalization period. However, this did not appear to be linked to the presence of the transgenes. There was only one transgenic plant which showed abnormal tepal formation. This line had considerably less bulb weight than the others and was kept for only 8 weeks in vernalization. Such abnormal growth might therefore be caused by the vernalization period as well as the weight of the bulb, which closely regulates flowering time in lily, and not due to the transformation process or somaclonal variation caused by tissue culture. These observations indicate that the enhanced pathogen resistance conferred by the overexpression of the *RCH10* gene was not affecting any other apparent functions within the plants.

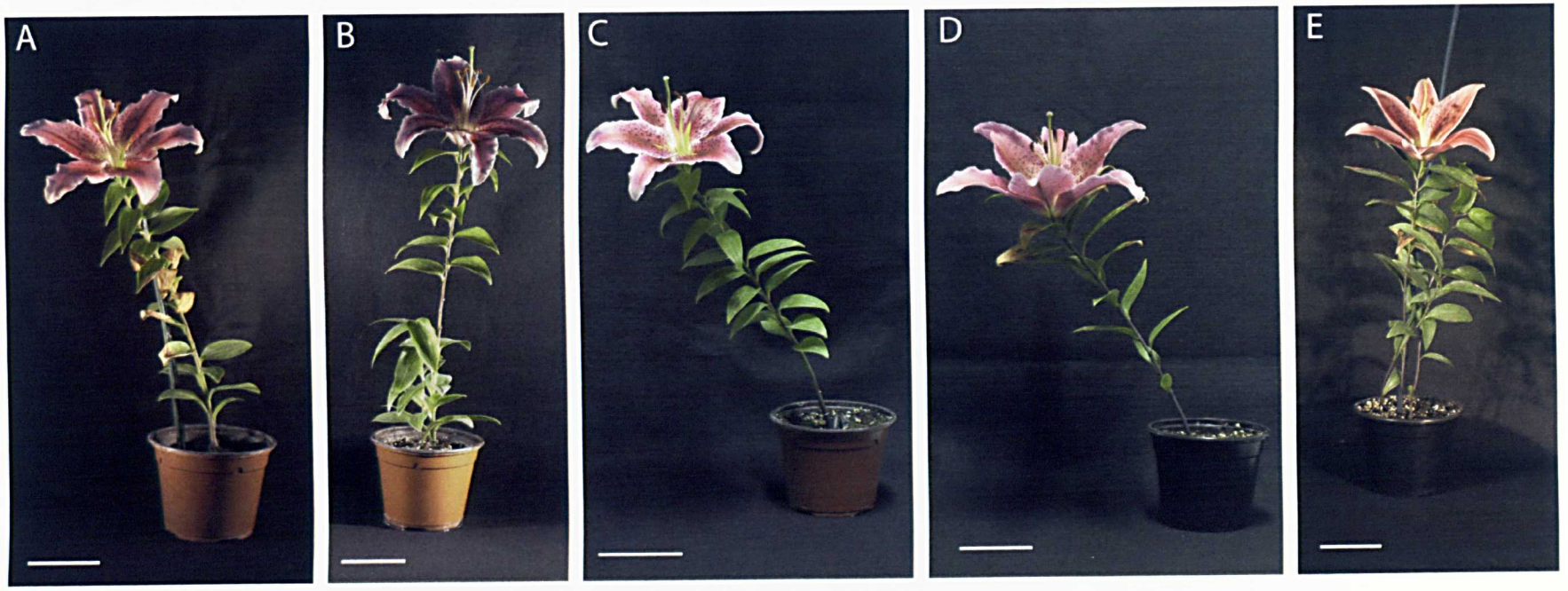

Figure 5.18 Examples of the phenotypic comparison between transgenic plants showing expression of the *RCH10* gene against the wild type. All the flowering plants showed approximately the same size, colour, shape and number of flowers as the wild type, all of them were fertile and appeared to produce the same amount of pollen. All flowers lasted for approximately the same time before senescence, which was around 3 weeks. (A) Wild type; (B) Line R29; (C) Line R504; (D) Line R2; (E) Line R673. Bar = 9 cm.

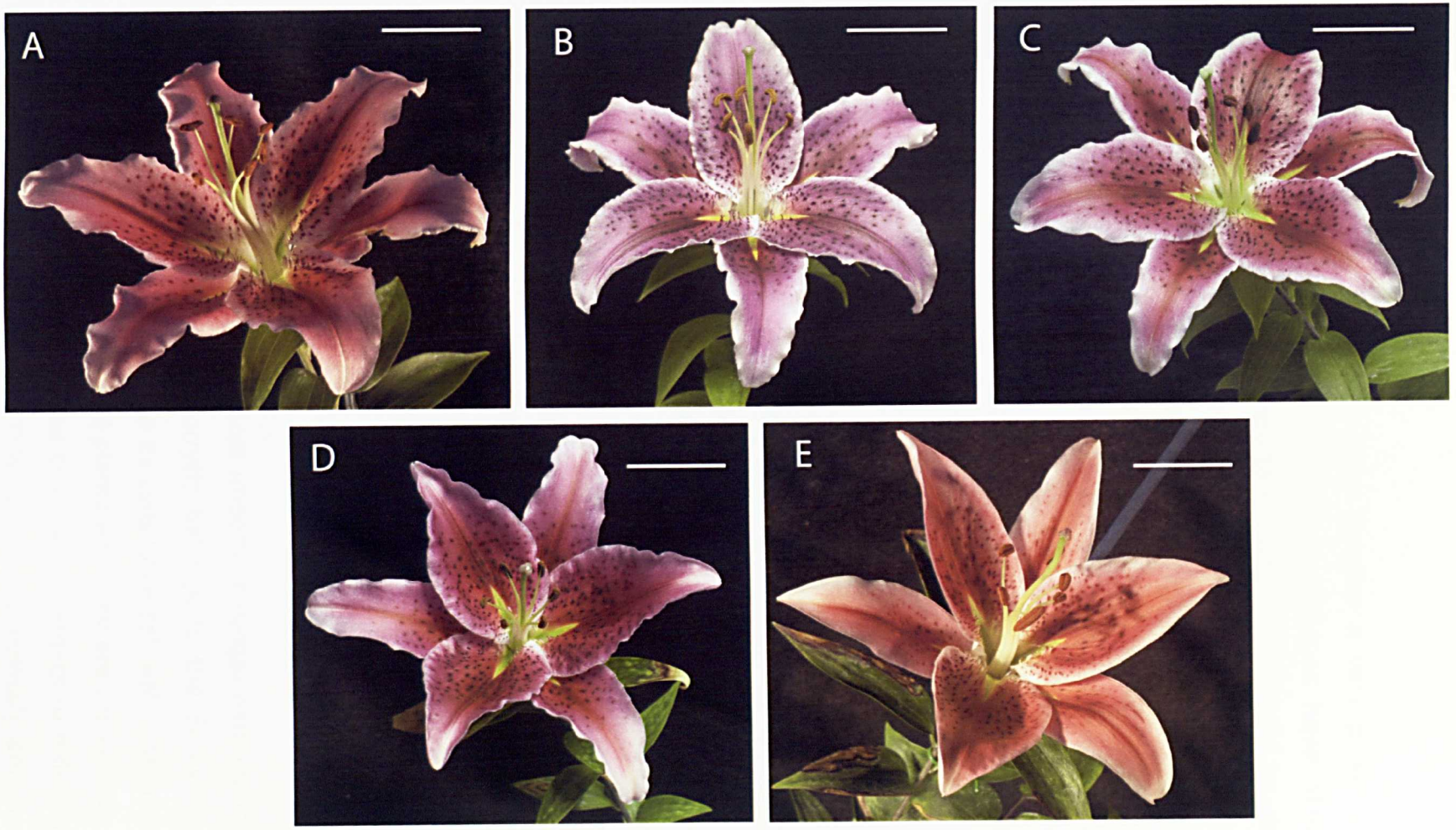

Figure 5.19 Examples of the phenotypic comparison between transgenic plants showing expression of the *RCH10* gene against the wild type. All the flowers showed the same shape, colour pattern and tone. All of them had the same size and arrangement of floral organs as in wild type. The amount of pollen produced by each flower was very similar between all of them. (A) Wild type; (B) Line R29; (C) Line R504; (D) Line R2; (E) Line R673. Bar = 5 cm.

5.4 Discussion

Genetic manipulation techniques have become a very powerful tool to improve a wide variety of crops. Such techniques have allowed the introduction of agronomically important traits which would be difficult to achieve by conventional breeding, for example the development of roses with resistance to Black spot disease (Marchant *et al.,* 1998), carnations with modified flower colour and pattern (Tanaka *et al.,* 2009), pollenless petunias (Kapoor and Takatsuji 2006) or chrysanthemum with non-dehiscent anthers (Shinoyama *et al.,* 2012). The development of an efficient transformation protocol for *Lilium* in this research (chapter 3), has provided the opportunity to manipulate such traits, for example resistance to pathogens.

Traditional approaches to enhance pathogen resistance in crops are laborious and time consuming, taking up to 15 to 20 years to obtain a resistant cultivar. With a transgenic approach this can be done in a considerably reduced time and in a more targeted and efficient way as previously reported in tobacco (Zhu *et al.,* 1994), rose (Marchant *et al.,* 1998), strawberry (Vellicce *et al.,* 2006), lemon (Distefano *et al.,* 2008) and wheat (Shin *et al.,* 2008). Although in *Lilium* there are some cultivars with some degree of native resistance to fungal pathogens these cultivars are not those used commercially and the spectrum of pathogens to which they are resistant is limited.

Botrytis cinerea is one of the most important fungal pathogens in *Lilium* causing grey mould disease. *Botrytis* belongs to the Eumycetes and is considered a true fungus because its cells have cell walls containing chitin. Chitinase genes are present in all plants and they are responsible for plant defence mechanisms, which make them ideal targets to induce enhanced resistance to fungal pathogens. This has been previously demonstrated in other ornamentals such as rose (Marchant *et al.,* 1998) and

chrysanthemum (Takatsu *et al.*, 1999), and also in wheat (Shin *et al.*, 2008). These examples indicated that the extent of induced resistance was directly correlated to the level of chitinase expression.

PCR analysis confirmed insertion of the *RCH10* and *NPTII* genes in the putative transgenic lines and RT-PCR and qRT-PCR analysis confirmed that both genes were being expressed. In general, transformations with this construct were highly efficient by recovering 212 putative transgenic plants from 750 explants inoculated, giving an efficiency of 28.6%. RT-PCR showed that 79% of the analysed plants were expressing the *RCH10* gene.

However PCR analysis of the putative transgenic plants regenerated from explants transformed with the wheat leaf rust chitinase construct showed the successful insertion of the *NPTII* gene, but not the *CHIT2* gene. RT-PCR analysis of these lines confirmed the expression of the *NPTII* gene.

Further screening by PCR of more than a hundred putative transgenic lines for the *CHIT2* chitinase gene construct, indicated that all of the transgenic plants showed only insertion and expression of the *NPTII* gene. Nonetheless the transformation efficiency obtained from transformations with this construct was 21.3%, very similar to that obtained with the *RCH10* gene construct and with the one reported in chapter 3 (20.1%). This suggests that there might be a problem with the construction of this vector; based on this, no more transformations were conducted using this construct and the transgenic plants obtained previously were not further analysed.

QRT-PCR analysis of the transgenic plants indicated that the levels of *RCH10* expression varied between the different transgenic lines. These lines were assessed for any associated enhanced disease resistance by

testing for infection with *Botrytis cinerea.* Initially this was conducted by measuring lesion size, however this was found to be highly variable and not a reliable measure of infection. Measurement of the wound size gave highly variable results both in wild type and between all the transgenic lines, and also showed high variation between wound sizes on the same leaf explant. Dunnett's test confirmed that there was no statistical difference in wound size between the wild type and the transgenic lines. The high level of variation could be due to microscopic wounds present on different parts of the surface of the leaf increasing the infection rate in one inoculation site rather than the other. Infection was therefore monitored by spore counts over time after inoculation.

Inoculation was an important part of the assay because the density of spores in the solution needed to be homogeneous and the inoculum solid enough for an accurate measurement of infection development. Cole *et al.* (1996) found that *Botrytis* needed carbon and phosphate for germination and penetration; other authors found that the addition of glucose and KH_2PO_4 increased the development of *Botrytis* infection (Benito *et al.,* 1998; De Meyer *et al.,* 1998; Fiddaman *et al.,* 2000; Guetsky *et al.,* 2001), therefore conidial suspensions were supplemented with 0.1 g/L of glucose, 0.1 g/L of KH_2PO_4 and 0.5% of tween 20. First attempts to inoculate the spore solution were unsuccessful due to the dispersion of the drop throughout the surface of the leaf. The use of 5% gelatine in the spore solution (Fiddaman *et al.,* 2000) was also unsuccessful due to the formation of clumps and bubbles. Finally the addition of 3.5% phytagel to the conidial suspension allowed a homogenous distribution of spores in the solution, whilst the inoculum drop was solid enough to remain on the inoculation point.

To get a more accurate measurement of the level of infection on the leaf explants the number of spores on the leaf surface were counted. Spore density increased overtime but was significantly reduced in transgenic lines expressing the chitinase gene. QRT-PCR analysis indicated that the

transgenic plants with higher relative expression of *RCH10* were the ones showing lower levels of sporulation on the *in vitro* assay, thus showing higher fungal resistance. These results are consistent with those reported in other crops (Marchant *et al.,* 1998; Takatsu *et al.,* 1999; Rohini and Sankara Rao 2001; Kishimoto *et al.,* 2002; Vellicce *et al.,* 2006; Distefano *et al.,* 2008; Shin *et al.,* 2008) in which the level of the chitinase protein was correlated to the level of resistance showed by the transgenic plant.

This research has been successful in producing several transgenic plants showing different levels of resistance to the fungal pathogen *Botrytis cinerea.* This can have high implications in the breeding of new elite cultivars and also on the pest impact and control on the field in the future. This is the first report of a *Lilium* plant with resistance to *Botrytis cinerea* by a transgenic approach. This is also the first report of a stable transformation with a transgene different than *GUS* and *NPTII* in any cultivar or species of *Lilium.*

Future research should focus on analysing the progeny of these transgenic plants to check the levels of resistance inherited. Also an *in vivo* resistance assay could be carried out to have a more accurate result of the level of resistance in the whole plant. This work has demonstrated the utility of using a chitinase gene to induce pathogen resistance, transformations with other *R* (pathogen resistance related) genes may also provide opportunities to induce resistance to a wider spectrum of pathogens in *Lilium.*

CHAPTER 6: CHARACTERIZATION OF POLLEN DEVELOPMENT GENES IN *LILIUM*.

6.1 Introduction

The genome sequence of any species is a valuable tool to help answer key questions of its biology and molecular mechanisms that underpin trait formation. DNA sequences can be used as the central elements to develop a breeding program to maximize the full potential of natural genetic variation for future crop improvement. Such approaches involve the application of the knowledge of gene function in other species and capitalisation of the similarity that can exist between genomes. Unfortunately the genome of several crops including *Lilium*, have not been sequenced due to limitations of the size and complexity and the high associated cost (Mayer *et al.*, 2011).

Although genomes vary greatly in size and complexity between species, mainly because of the expansion of retroelement repeats, there is generally an underlying conserved gene order (Wiker and Keller, 2007). Comparative sequence analysis is a tool to study genes sharing common ancestry, named homologues, and also to define or predict conserved gene function between orthologues, which are homologous genes from different species (Mohavedi *et al.*, 2012).

The principle of comparative sequence analysis is that the DNA sequences encoding proteins and RNAs responsible for functions that are conserved from the last common ancestor, should be preserved in contemporary genome sequences. In the same way, the DNA sequences controlling expression of genes that are regulated in a similar pattern in two related species are likely to be conserved (Hardison, 2003). On the other hand,

sequences that encode proteins and RNAs responsible for differences between species are more likely to be divergent, thus giving important clues about evolutionary history and species adaptation (Tirosh *et al.*, 2007). Comparison between two species can therefore help to detect and characterize functional elements that might not be known in another related species in which the genome is partially or not yet sequenced. This can then be used to identify genes and characterize their function in those plant species (Mohavedi *et al.*, 2011).

Regulation of gene expression at the level of transcription controls many of the biological processes in organisms, such as metabolic and physiological balance, cell cycle or response to environmental factors (Xiong *et al.*, 2005). Transcription factors act as switches of regulatory cascades controlling these biological processes (Riechmann *et al.*, 2000). Therefore there is a high likelihood that there will be conservation of these regulatory networks between species.

Pollen development and anther dehiscence are two processes regulated by a complex network of genes triggered by transcription factors (Wilson *et al.*, 2001; Ito and Shinozaki, 2002; Ito *et al.*, 2007; Yang *et al.*, 2007a; Yang *et al.*, 2007b; Wilson and Zhang, 2009; Xu *et al.*, 2010; Phan *et al.*, 2011; Wilson *et al.*, 2011; Li *et al.*, 2011). *Arabidopsis thaliana MALE STERILITY1* (*MS1*) is a transcription factor which is nuclear localized and expressed in the tapetum between late tetraspore and microspore release, it is critical for viable pollen development (Wilson *et al.*, 2001; Ito and Shinozaki, 2002; Vizcay-Barrena and Wilson, 2006; Ito *et al.*, 2007; Yang *et al.*, 2007a). *AtMS1* encodes a 672 amino acid protein, containing a putative Leucine Zipper and a Plant Homeodomain (PHD) finger motif which are essential for its function (Wilson *et al.*, 2001). Several putative *AtMS1* orthologues have been identified in other species, such as poplar (Ito and Shinozaki, 2002; Ito *et al.*, 2007). Recently, the rice *PERSISTENT TAPETAL CELL1* (*OsPTC1*) gene has been identified as a functional orthologue of the *Arabidopsis MALE STERILITY1* (*AtMS1*) (Li *et al.*, 2011);

this contains a highly conserved PHD domain in the C-terminal region and appears to have a similar function to *AtMS1*.

Arabidopsis thaliana MYB26/MALE STERILE 35 (*MS35*) is a transcription factor critical for the secondary thickening in the anther's endothecium and subsequent dehiscence (Yang *et al.*, 2007b; Wilson *et al.*, 2011). It is nuclear localized and expressed early during endothecial expansion, with maximal expression during pollen mitosis I and bicellular stages, indicating a regulatory role in specifying early endothecial cell development (Yang *et al.*, 2007b). *AtMYB26* encodes a 358 amino acid protein, containing a putative SANT DNA-binding domain, a Plant Homeodomain-like and a Myb DNA-binding domain. To date no reports of any identified and characterized orthologues in other species have been published.

The vast quantities of pollen that *Lilium* produces which can be allergenic, toxic to pets and stain clothes is a big problem commercially. Breeders have already produced pollenless/antherless lilies, in which the anthers do not fully develop (http://www.lilybreeding.com/lilies/); this has solved the problem of the vast amount of pollen, but the flower is less appealing without anthers, or with anthers that are deformed. A key biotechnological target for *Lilium* is therefore manipulation of pollen development, or dehiscence. Comparative genomic analysis was therefore used to identify the putative orthologues of *AtMS1* and *AtMYB26* in *Lilium.*

Multiple sequence alignments were used as tools to identify conserved regions to designs primers for this purpose. A transgenic approach was subsequently used for these two transcription factors to try to develop a lily with modified pollen development and/or release.

6.2 Materials and Methods.

6.2.1 Bioinformatics analysis.

Bioinformatics tools were used to identify the putative orthologue of the *MS1* and *MYB26* genes. TAIR database (www.arabidopsis.org) was used to obtain the CDS sequence from *Arabidopsis.* A Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) analysis using Gramene and the National Center for Biotechnology Information (NCBI) databases (www.gramene.org and www.ncbi.nlm.nih.gov) was performed to identify putative orthologues.

Multiple sequence alignment was done using sequences that shared significant similarity over most of their length from maize, sorghum, wheat, rice and barley. These alignments were used to recognize conserved regions between the different genes for primer design.

Additionally, all the sequences from *Lilium* amplified in this research were aligned against gene sequences of the putative orthologues from other species in order to compare and establish the level of identity. Sequence alignments and editing were done using Vector NTI software (Invitrogen).

6.2.2 Primer design.

Primer design was done by initially identifying the conserved regions between the putative orthologues from other cereals, whilst avoiding conserved family domains that are shared with other non-target genes. Web platform Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to analyse the selected

sequences and design. The sets of primers obtained with Primer3 Plus were subsequently analysed using Vector NTI and BLAST tool. Primer details are described in Tables 6.1 and 6.2.

Gene	Primers	Sequence (5'-3')	Expected Band size
LsgMYB26	LM610F	CAACAAGCAGAAGGTGAGGAG	325 bp.
	LM610R	GCTTCTTCTTGATGGTGGAGTT	
	LM611F	AGCCTCCACTCCGGTAACAT	261 bp.
	LM611R	GGCACAGCCTGATGAACTCC	
	LM612F	AGTGCCCTCCATCTTCAGTTC	284 bp.
	LM612R	CGTCCATGAAGTCCACCACC	
	LM613F	GCTGCAACAAGCAGAAGGTGA	516 bp.
	LM613R	GCCGAGTTGTGAACTGAAGAT	
	LM614F	AACTCCACCATCAAGAAGAAGC	467 bp.
	LM614R	GATGGCGTCCATGAAGTCCA	

Table 6.1 Primers, sequence and expected band size used for amplification of the putative orthologue of *AtMYB26* in *Lilium* cv. Star Gazer. Annealing temperatures are described in section 6.3.2.

Gene	Primers	Sequence (5'-3')	Expected Band size
LsgMS1	LM110F	TTCCGGTTCGATTCCTTCT	285 bp.
	LM110R	CACGAAGTGAAACCTCTTG	
	LM111F	CCACCAGATCATGGACCTC	230 bp.
	LM111R	AGAGCGGTATGGACTGGAG	
	LM112F	GAGGAAAGGGCACATGGTG	228 bp.
	LM112R	CTGATGGCCTGGTACTTGG	
	LM113F	TGCGATTCCATCTCACCAG	209 bp.
	LM113R	GTACACATGCCCGACTACG	
	LM114F	CTTGGACATCAAACACTTCG	441 bp.
	LM114R	CCACATACGCAGTCTACGA	
	LM115F	CATATGCTCAGCCTTACAC	849 bp.
	LM115R	GTCCAAGATCATTCTTACC	
	LM116F	ATATGCAGCAAGAGGTTTC	1091 bp.
	LM116R	ATGTCCAAGATCATTCTTA	

Table 6.2 Primers, sequence and expected band size used for amplification of the putative orthologue of *AtMS1* in *Lilium* cv. Star Gazer. Annealing temperatures are described in section 6.3.2.

6.2.3 Plant material.

For total RNA extraction, anthers of *Lilium* cv. Star Gazer were excised from floral buds of different lengths, transferred into Eppendorf tubes and snap frozen in liquid nitrogen. Anthers were then kept at -70 °C until needed. For the staging of the pollen and anther development, fresh anthers were collected and immediately used for microscopy examination as described in section 6.2.3.1.

6.2.3.1 Anther staging in *Lilium* cv. Star Gazer.

Since *AtMS1* and *AtMYB26* genes are expressed at specific stages in pollen and anther development of *Arabidopsis*, *Lilium* anther material was staged so appropriate samples could be selected for analysis; the pollen development staging published by Nakamura (1979) on *Lilium longiflorum* was used as a reference. To confirm that the collected material was at the correct developmental stage a microscopy analysis was done using fresh anthers from floral buds of cv. Star Gazer ranging from 1.0 cm to 5.0cm. Sections of anthers were hand cut with a scalpel, immediately placed onto a microscopy slide and stained for 5 minutes with a droplet of Alexander's stain (Alexander, 1969). The slices were then covered with a coverslip and examined by microscopy.

6.2.3.2 RNA extraction and cDNA synthesis.

Total RNA was extracted from approximate 100 mg of anther material using the method described in chapter 2, section 2.2.2. The cDNA was synthesized following the procedure detailed in chapter 2, section 2.3.3. This cDNA was used for primer testing and amplification.

6.2.4 RT-PCR.

Primers (Tables 6.1 and 6.2) were tested on cDNA material from anther length sizes 1 cm to 5 cm. RT-PCR analysis was performed as described in chapter 2 section 2.3.3 using RedTaq Ready Mix (Sigma). PCR products were run on a 1% (w/v) agarose gel and the bands of the appropriate expected size were excised and sent for sequencing. PCR reaction conditions and primers used are described later in this chapter in section 6.3.1.

6.2.5 Sequencing.

The amplified products of the expected size were purified from agarose gels by membrane electrodialysis as described in chapter 2, section 2.2.3. The DNA was resuspended and cloned into the pCR®II-TOPO® vector as described in chapter 2, section 2.4 and transformed into chemically competent *E.coli* cells.

Colony PCR was performed (chapter 2, section 2.3.2) using the M13 Reverse primer (5'- CAGGAAACAGCTATGAC-3') and the M13 Forward primer (5'- TGTAAAACGACGGCCAG-3') with an annealing temperature of 55°C. Plasmid DNA was extracted from positive colonies as described in chapter 2, section 2.2.4. Plasmid DNA was then used for Big Dye sequencing reactions as described in chapter 2 section 2.4.2. Samples were sent to the Genomics Facility at Queen's Medical Centre, The University of Nottingham where they were read on an ABI 3130 genetic analyser. Results were analysed using Bioedit and Vector NTI software.

6.2.6 Rapid Amplification of cDNA Ends PCR.

RACE-PCR was conducted to obtain the full length sequence of the putative *LsgMS1* and *LsgMYB26* genes. 5′ and 3′ RACE-PCR were conducted using the GeneRacerTM kit (Invitrogen) according to the manufacturer's instructions.

The mRNA was reverse-transcribed using the GeneRacer TM oligoTM primer (Invitrogen). 1 µg of total RNA was first treated with 1 µl calf intestinal phosphatase (CIP, 10u/µl), 1 µl of CIP Buffer (10X), 1 µl of RNaseOut (40u/µl) and then DEPC water to a volume of 10 µl. Samples were incubated for 1 hour at 50°C to remove the 5′ phosphates and eliminate truncated mRNA and non-mRNA from the subsequent ligation with the GeneRacer RNA oligo. RNA was purified with 90 µl DEPC water and 100 µl of (1:1) phenol:chloroform, samples were centrifuged at 9500 x *g* for 5 minutes and the top aqueous phase transferred into a new Eppendorf; 2 µl 10 mg/ml mussel glycogen, 10 µl 3 M sodium acetate and 220 µl of 100% ethanol were added to the tube and stored at -20°C for one hour. RNA was pelleted by centrifuging at 9000 x *g* for 20 minutes at 4°C, washed with 500 µl 70% (v/v) ethanol and centrifuged at 9000 x *g* for 2 minutes at 4°C. The pellet was air dried and then resuspended in 7 µl DEPC water.

The dephosphorylated RNA was then treated with 1 µl of tobacco acid pyrophosphatase (TAP) buffer (10X), 1 µl RNaseOut (40u/µl) and 1 µl TAP, (0.5u/µl) for 1 hour at 37°C to remove the 5′ cap structure from intact, full length mRNA. RNA was then precipitated as in the previous step and resuspended in 7 µl of DEPC water.

Finally the GeneRacer RNA Oligo (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) was

ligated to the 5′ end of the mRNA by adding to the sample 1 µl of Ligase buffer (10X), 1 µl of ATP (10mM), 1 µl of RNaseOut (40u/µl) and 1 µl of T4 RNA ligase (5u/µl). Reaction was incubated at 37°C for 1 hour; the RNA was then precipitated as previously described and resuspended in 11 µl of DEPC water. The GeneRacer RNA Oligo was used to provide a known priming site for GeneRacer amplification.

SuperScript III RT and the GeneRacer Oligo dT Primer (5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$-3′) were used to generate RACE-ready first-strand cDNA with known primers sites at the 5′ and 3′ ends. To obtain the 5′end, first-strand cDNA was amplified using a reverse gene specific primer and the Gene Racer 5′primer which binds within the GeneRacer RNA oligo. A nested PCR was performed using this PCR product as a template. Details of the primers used are described in section 6.3.4.

To obtain the 3′end, the first strand cDNA was amplified using the forward gene specific primer and the GeneRacer 3′Primer. This PCR product was subsequently used as a template for a further Nested PCR using the Gene Racer nested 3′ primer and the gene specific nested primer. Primers used are described in section 6.3.4.

Extension was carried out using Phusion High-Fidelity DNA Polymerase and 1 µl of cDNA as described in chapter 2, section 2.3.1. Touchdown PCR (Don *et al.*, 1991; Roux, 1995), as recommended by the Gene Racer manual, was used to increase the specificity of the amplification. Touchdown PCR increases specificity and reduces background amplification by exploiting the high annealing temperatures of the GeneRacer™ primers to selectively amplify gene-specific cDNAs that are tagged with the GeneRacer™ RNA Oligo. By starting at a high annealing temperature in the first 5 PCR cycles, only gene-specific or GeneRacer™-tagged cDNA is amplified, allowing the desired product to accumulate; the

decrease of the annealing temperature in the remaining cycles permits efficient amplification of tagged, gene-specific template. Tables 6.3 and 6.4 detail the primers designed for RACE PCR according to manufacturer's recommendations. Table 6.5 describes the thermal cycles used for Touchdown and Nested PCR as recommended by the manufacturer and adjusted to the designed primers.

Gene	Primers	Sequence (5'-3')	Expected Band size
LsgMYB26 Touchdown	LMYB26_3T RACE 3'	AAGTACATCACCGCGCATGG GCTGTCAACGATACGCTACGTAACG	~830 bp.
	LMYB26_5T RACE 5'	GCCTTCTTGAGCCAAGAGGTTG CGACTGGAGCACGAGGACACTGA	~810 bp.
LsgMYB26 Nested	LMYB26_3N RACE 3' Nested	CACTCCGGTAACATCCCTTCCT CGCTACGTAACGGCATGACAGTG	~533 bp.
	LMYB26_5N RACE 5' Nested	AGAAGCTCCCCCTCTTGAGGTC GGACACTGACATGGACTGAAGGAGTA	~237 bp.

Table 6.3 Primers, sequence and expected band size designed for RACE-PCR to amplify the putative orthologue of *AtMYB26* in *Lilium* cv. Star Gazer.

Gene	Primers	Sequence (5'-3')	Expected Band size
LsgMS1 Touchdown	R3T RACE 3'	GCTGCACGGCATCGTGCACCTCAAC GCTGTCAACGATACGCTACGTAACG	~1586 bp.
	R5T RACE 5'	GCCCTTTCCTCGCCGTGTCCACAAG CGACTGGAGCACGAGGACACTGA	~646 bp.
LsgMS1 Nested	R3N RACE 3' Nested	AGCCTTGTGGACACGGCGAGGAAAG CGCTACGTAACGGCATGACAGTG	~1441 bp.
	R5N RACE 5' Nested	GGCCGTAGCCGTTGAGGTGCACGAT GGACACTGACATGGACTGAAGGAGTA	~510 bp.

Table 6.4 Primers, sequence and expected band size designed for RACE-PCR to amplify the putative orthologue of *AtMS1* in *Lilium* cv. Star Gazer.

Thermal cycle for RACE PCR				
Touchdown PCR	Initial denaturation	98°C	1:00 min	1 step
	Denaturation	98°C	0:30 min	5 cycles
	Anneal/Extension	72°C	1:15 min	
	Denaturation	98°C	0:30 min	5 cycles
	Anneal/Extension	70°C	1:15 min	
	Denaturation	98°C	0:30 min	25 cycles
	Annealing	67°C	0:30 min	
	Extension	72°C	1:15 min	
	Final Extension	72°C	9:00 min	1 step
Nested PCR	Initial denaturation	98°C	1:00 min	1 step
	Denaturation	94°C	0:20 min	35 cycles
	Annealing	58°C	0:30 min	
	Extension	72°C	1:15 min	
	Final Extension	72°C	9:00 min	1 step

6.2.7 *Lilium* transformation.

Transformation of cv. Star Gazer with the *MYB26* gene from *Arabidopsis,* both by overexpression and RNAi, was performed with the aim of regenerating transgenic lines with modified anther dehiscence phenotype. Plasmid pK7GWIWG2 with a fragment of 510 bp. of *AtMYB26* was used for RNAi (Fig 6.1) and plasmid pGWB5 with the full length *AtMYB26* driven by the CaMV35S promoter was used for overexpression. Both plasmids were kindly provided by Dr. Caiyun Yang from the University of Nottingham. The plasmids were transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation as described in chapter 2, section 2.5.3.1. *Agrobacterium*-mediated transformation was performed as described in chapter 4 section 4.2.3.2.

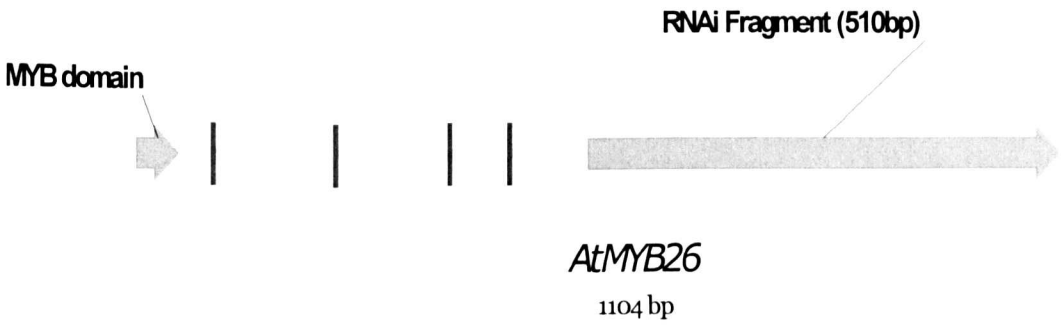

Figure 6.1 *Arabidopsis thaliana MYB26* full length CDS used for overexpression experiments in lily and the 510bp fragment used for RNAi transformation experiments. Blue lines are restriction enzyme sites.

PCR and RT-PCR analysis was performed on the regenerated lines to check for transgene insertion and expression respectively. Table 6.6 details the primers used for PCR and RT-PCR. Thermal cycles used are detailed in Table 6.7.

Gene	Primers	Sequence (5'-3')	Band size
AtMYB26 RNAi PCR	RNAi MS35 L	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATCACTCCAAGCCATCTCTCTC	510 bp.
	RNAi MS35 R	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTGAAAGTTGTACGTGGTGGGAAG	
AtMYB26-Oexp PCR/RT-PCR	RNAi MS35 L	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATCACTCCAAGCCATCTCTCTC	1400 bp.
	PGWB8 R	TGAACGATCGGGGAAATTCG	
NPTII	nptII_For	AGACAATCGGCTGCTCTGAT	261 bp.
	nptII_Rev	ATACTTTCTCGGCAGGAGCA	
Polyubiquitin	Polyb F	GAAGCAGCTGGAAGATGGAC	196 bp.
	Polyb R	GATCCCTTCCTTGTCGTGAA	

Table 6.6 Primers, sequence and expected band size used for PCR and RT-PCR analysis.

Thermal cycle for PCR and RT-PCR				
AtMYB26 Oexp. *NPTII* Polyubiquitin	Initial denaturation	94°C	5:00 min	1 step
	Denaturation	94°C	0:30 min	35 cycles
	Annealing	57°C	0:30 min	
	Extension	72°C	1:15 min	
	Final Extension	72°C	9:00 min	1 step
AtMYB26 RNAi	Initial denaturation	94°C	5:00 min	1 step
	Denaturation	94°C	0:30 min	35 cycles
	Annealing	57°C	0:30 min	
	Extension	72°C	0:35 min	
	Final Extension	72°C	6:00 min	1 step

Table 6.7 Thermal cycles used for PCR and RT-PCR on putative transgenic lily plants.

After identification of partial *Lilium* sequences of the putative orthologues of *AtMYB26* and *AtMS1* in lily, RNAi transformation was used to test the function of these putative transgenes. Transformation of cultivars Star Gazer, Night Flyer, Acapulco and Sweet Surrender was conducted to analyse whether the partial sequences obtained had the same function as those reported in *Arabidopsis* (Yang *et al.*, 2007a; Yang *et al.*, 2007b).

Plasmid pK7GWIWG2, kindly provided by Tsuyoshi Nakagawa (Shimane University), was used for RNAi vector construction; it confers kanamycin resistance to plants and both fragments are driven by the CaMV35S promoter. The plasmids were transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation as described in chapter 2, section 2.5.3.1. *Agrobacterium*-mediated transformation was then performed as described in chapter 4 section 4.2.3.2.

6.3 Results.

6.3.1 BLAST analysis.

For the *AtMS1* orthologue, blast analysis was conducted using the *OsPTC1* full length gene sequence (Os09g0449000) against the NCBI database. It showed high identity at the nucleotide level to the *Sorghum bicolor* gene Sb02g026200 (91%) and *Hordeum vulgare* gene with accession number AK373836.1 (92%); all of them had three exons and two introns and a PHD Zinc-finger domain near the C-terminal end. For the *AtMYB26* orthologue, since there are no reports of characterized putative orthologues of this gene in other species a search on the Gramene database was performed using the full length gene sequence. This database provided a list of putative orthologues based on protein and cDNA alignments (Table 6.8).

Species	Gene name	Alignment	Target %id	Query %id
Brachypodium distachyon	BRADI2G47887	Protein	38	31
Oryza brachyantha	OB01G37410	Protein	45	30
Oryza glaberrima	ORGLA01G0234600	Protein	38	32
Oryza indica	BGIOSGA004306	Protein	38	32
Oryza sativa	LOC_Os01g51260	Protein	38	32
Sorghum bicolor	Sb03g032600	Protein	37	32

Table 6.8 Putative orthologues of *AtMYB26* from Gramene.org database, based on protein alignment.

A further BLAST analysis of Sb03g032600 from *Sorghum bicolor* against the NCBI database showed high similarity with the genes LOC100842650 from *Brachypodium distachyon,* LOC100281948 (MYB9) from *Zea mays* and AK372926.1 from *Hordeum vulgare.* In addition the gene from barley showed expression in adult flowers, which is similar to the expression pattern of the *AtMYB26* gene.

Multiple sequence alignment using the coding sequence (CDS) from the previously mentioned genes was done with Vector NTI to identify conserved regions for primer design (Appendix 2). Tables 6.1 and 6.2 from section 6.2.2 describe the primers designed for amplification of the putative orthologue of *MYB26* and *MS1* in lily respectively. Figures 6.2 and 6.3 show the sites were the primers were located against the multiple sequence alignment. Figures 6.4 and 6.5 show a phylogenetic tree of the aligned CDS sequences.

Primers designed to conserved sequences in maize, sorghum, barley and *Brachypodium* were tested first by a gradient PCR with four different annealing temperatures, 51°C, 54°C, 57.5°C and 61°C. The cDNA template used was a pool from anthers ranging from 2 cm to 4.7 cm long in all reactions corresponding to Sanders' *Arabidopsis* stage 5 (Four defined locules established, all anther cells present and pattern of anther defined; pollen mother cells appear) to stage 11 (Pollen mitotic division occurs, tapetum degenerates, expansion of the endothecial layer and secondary thickening appear in endothecium and connective cells, Septum degeneration starts, stomium differentiation begins) (Sanders *et al.*, 1999).

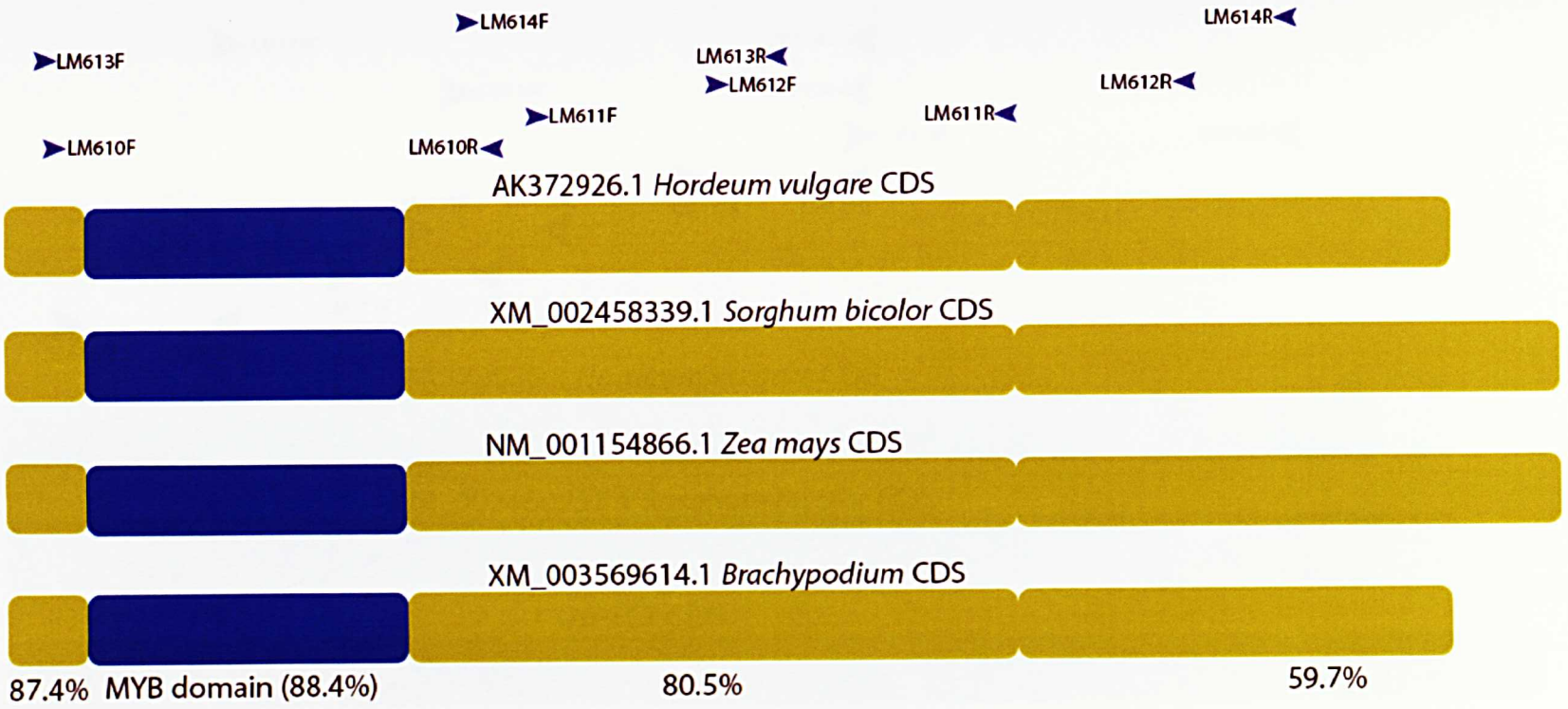

Fig 6.2 Multiple sequence alignment using the CDS (coding sequence) for the putative orthologues of *AtMYB26* from Barley, Sorghum, Maize and *Brachypodium.* The percentage indicates the level of homology between all of the genes in four different regions of the coding sequences. Arrows indicate the sites where primers were designed to attempt the amplification of the *AtMYB26* orthologue in *Lilium* cv. "Star Gazer".

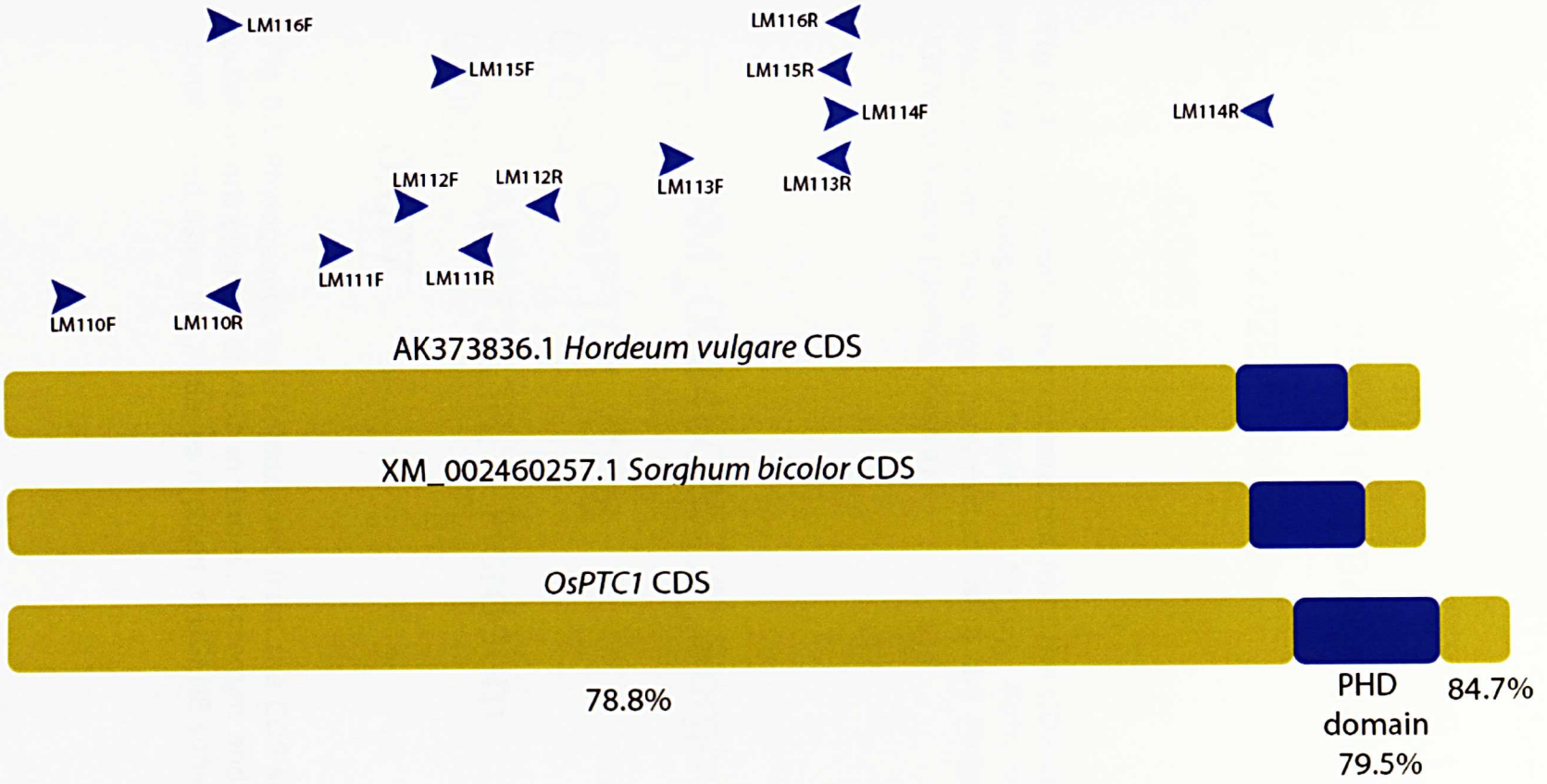

Fig 6.3 Multiple sequence alignment using the CDS (coding sequence) of the putative orthologues of *AtMS1* from Rice, Sorghum and Barley. The percentage indicates the level of homology between all of the genes in three different regions of the coding sequences. Arrows indicate the sites where primers were designed to attempt the amplification of the *AtMS1* orthologue in *Lilium* cv. Star Gazer.

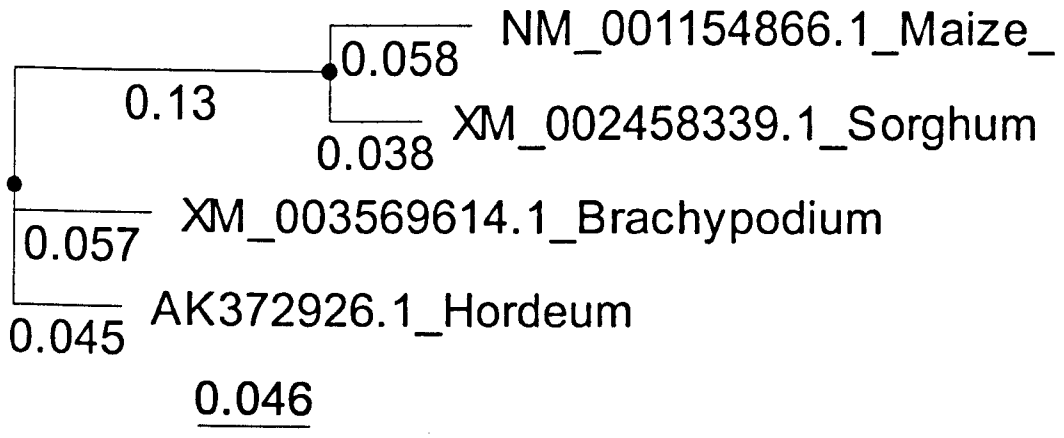

Fig 6.4 Phylogenetic tree constructed from the CDS sequences of the putative orthologues of *MYB26* in Barley, Sorghum, Maize and *Brachypodium*. Tree was constructed using the MrBayes method in UGENE software (UniPro, Russia).

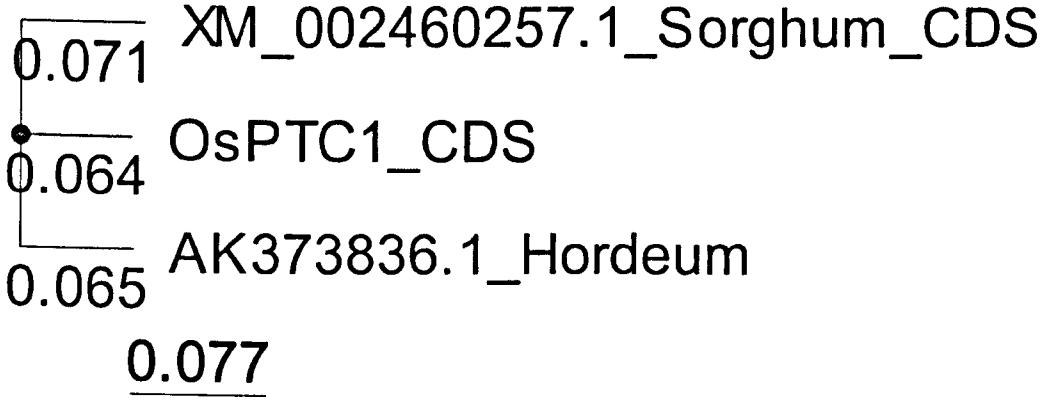

Fig 6.5 Phylogenetic tree constructed from the CDS sequences of the putative orthologues of *MS1* in Barley, Sorghum and Rice. Tree was constructed using the MrBayes method in UGENE software.

6.3.2 Anther staging.

To confirm that the collected material was in the correct developmental stage, anther sections of different flower bud sizes were analysed under the microscope. Figure 6.6 shows the results obtained. Anther sections were hand cut as described in section 6.2.3.1.

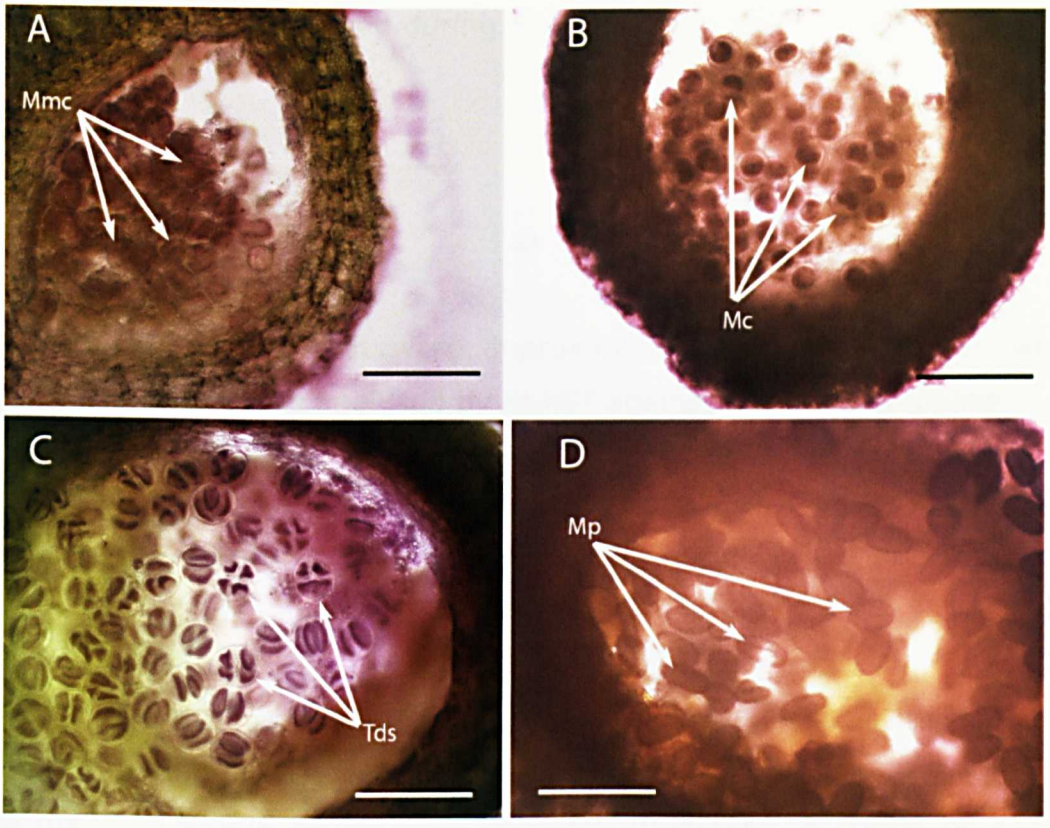

Fig 6.6 Anther development staging on cv. Star Gazer based on Sanders' scale (Sanders *et al.,* 1999).; (A) Anther from 2.7cm flower buds, (Sanders' stage 5; Four clearly defined locules established, all anther cell types present and pattern of anther defined, microspore mother cells appear) Bar=0.5µm; (B) Anther from 3.0cm flower buds, (Sanders' stage 6; Microspore mother cells enter meiosis, middle is crushed and degenerates, tapetum becomes vacuolated and the anther increases in size) Bar=0.5µm; (C) Anther from 3.9cm flower buds, (Sanders' stage 7; Meiosis completed, tetrads of microspores free within each locule) Bar=0.5µm; (D) Anther from 4.7cm flower buds, (Sanders' stage 11; Pollen mitotic divisions occur, tapetum degenerates, expansion of endothecial layer, septum cell degeneration initiated. Stomium differentiation begins) Bar=0.5µm. Mmc, Microspore Mother Cells; Mc, Meiotic Cell; Tds, Tetrads; Mp, Mature Pollen.

6.3.3 RT-PCR analysis.

After testing the sets of primers, and optimizing reaction conditions, a new RT-PCR reaction was performed with the appropriate annealing temperature which was 54.5°C. However not all the designed primers amplified a band. Figures 6.7-6.9 show the results obtained for the RT-PCR reaction with different primer combinations. The template cDNA used was from a pool of anthers ranging from 2 cm to 4.7 cm long since the targets should be expressed during these stages.

6.3.4 Sequencing.

The amplified bands that were approximately the expected size were sequenced and results analysed by BLAST against the NCBI database.

6.3.4.1 *Lilium MYB26* putative orthologue analysis

For the *AtMYB26* orthologue analysis, bands amplified using the primers LM613 F+R and LM614 F+R (Fig 6.7) resulted in a high identity match to the putative *MYB26* orthologues used for alignment. A contig of 723bp was constructed with both fragments. Figure 6.8 shows the multiple sequence alignment for this contig against the putative orthologues in Barley, Sorghum, Maize and *Brachypodium*. The results of these alignments showed that the putative *AtMYB26 Lilium* orthologue shares a very high sequence identity with the putative *MYB26* barley sequence, being around approximately 99.6%. In addition the obtained fragment had the expected conserved MYB domain region. Figure 6.9 shows a phylogenetic tree constructed with the contig, the *AtMYB26* gene and the putative orthologues in Barley, Sorghum, Maize and *Brachypodium*. Table 6.9 describes the homology between each of the compared sequences.

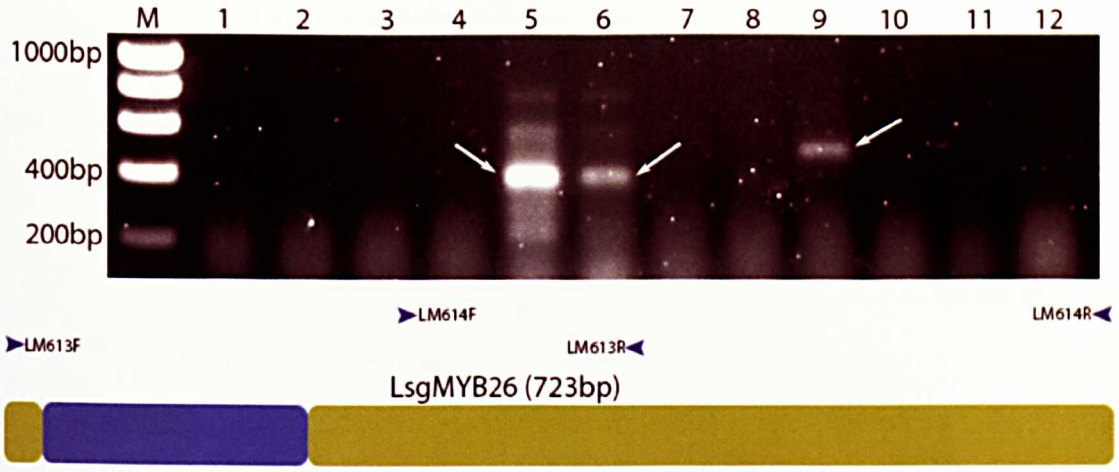

Fig 6.7 RT-PCR using *Lilium* cv. Star Gazer cDNA pool from anthers of 2.0 to 4.7 cm with primers designed based on multiple sequence alignment for the putative *MYB26* gene. (1, 2) LM610F+R; (3, 4) LM611F+R; (5, 6) LM614F+R; (7, 8) LM612F+R; (9) LM613F+R; (10) LM610F + LM613R (11) LM610F + LM611R; (12) LM610F + LM612R. Lanes (5-6) and (9) amplified a band of approximately 460 and 510 bp respectively. Map shows the location of the primers used to amplify the bands on the putative orthologue of *MYB26* in *Lilium*.

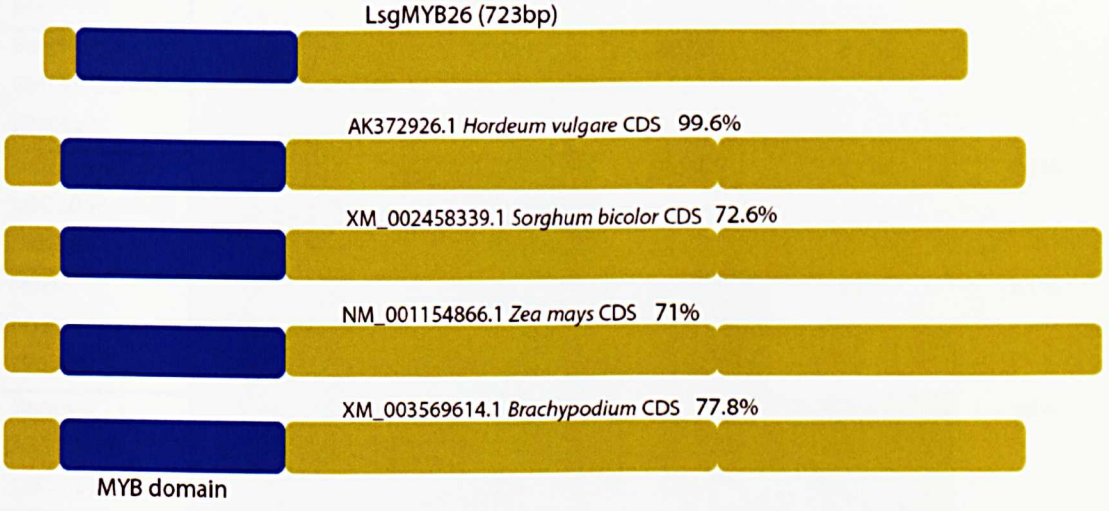

Fig 6.8 Multiple sequence alignment for the putative *MYB26* orthologues in Barley, Sorghum, Maize and *Brachypodium*, including the contig obtained for *Lilium*. The percentage identity is shown between the *Lilium* partial sequence (top) and each of the other CDS sequences from the other species.

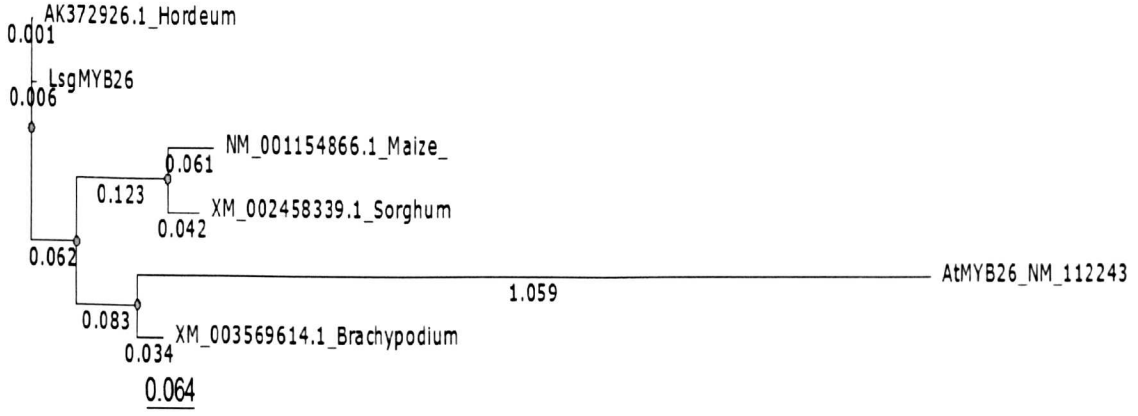

Fig 6.9 Phylogenetic tree constructed from the CDS sequences of the *Arabidopsis MYB26* gene and the putative orthologues in Barley, Sorghum, Maize, *Brachypodium* and the partial sequence in *Lilium* (*LsgMYB26*). Tree was constructed using the MrBayes method on UGENE software.

	Sorghum Sb03g032600	*Brachypodium* LOC100842650	Maize *MYB9*	Barley AK372926	*Lilium LsgMYB26*
A. thaliana MYB26 (1104bp)	52%	55%	50%	53%	51%
Sorghum Sb03g032600 (960bp)		80%	91%	84%	83%
Brachypodium LOC100842650 (882bp)			80%	87%	84%
Maize *MYB9* (963bp)				82%	81%
Barley AK372926 (882bp)					99%

Table 6.9 Percentage of homology between *Arabidopsis thaliana MYB26* coding sequence and the CDS of the putative orthologues in Sorghum, *Brachypodium,* Maize, Barley and the partial sequence obtained from the putative *LsgMYB26*.

6.3.4.2 *Lilium MS1* putative orthologue analysis

For the *AtMS1* orthologue analysis, only one of the amplified bands, the one obtained using the primers LM111 F and LM112 R (Fig 6.10) matched with the putative orthologues from other species. The highest identity (100%) was obtained when aligned against the barley putative orthologue; this was very similar with the results obtained for the putative orthologue of *AtMYB26* in terms of identity with barley (99.3%).

Figure 6.11 shows the location site of the amplified fragment and the percentage of identity relative to the other putative *MS1* orthologues in the other species. Figure 6.12 shows a phylogenetic tree constructed based upon sequence data from the amplified fragment from *Lilium*, the *AtMS1* gene and the putative orthologues in Barley, Sorghum and Rice. Table 6.10 describes the homology between each of the compared sequences.

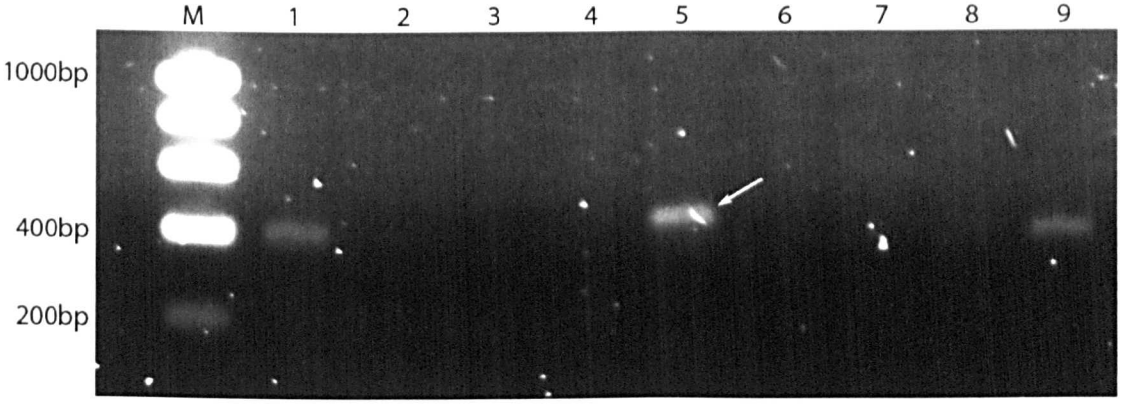

Fig 6.10 RT-PCR using *Lilium* cv. Star Gazer cDNA pool from anthers of 2.0 to 4.7 cm with primers designed based on multiple sequence alignment for the putative *MS1* gene. (1) LM110F+R; (2) LM111F+R; (3) LM112F+R; (4) LM113F+R; (5) LM114F+R; (6) LM115F+R; (7) LM116F+R; (8) LM110F + LM114R; (9) LM111F + LM112R. (5) Amplified a band of the expected size

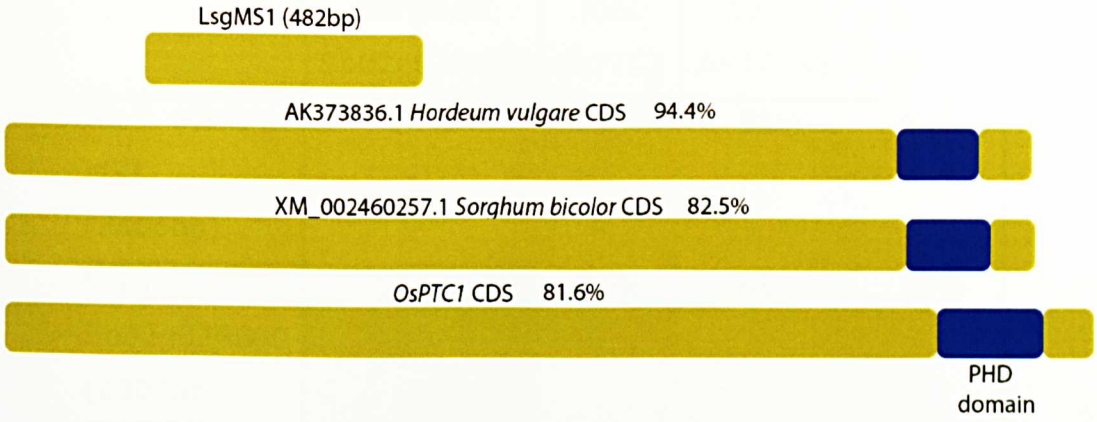

Fig 6.11 Multiple sequence alignment for the putative *MS1* orthologues in Barley, Sorghum and Rice, including the amplified fragment obtained for *Lilium*. The percentage of identity is between the *Lilium* sequence (top) and each of the other CDS sequences from other species.

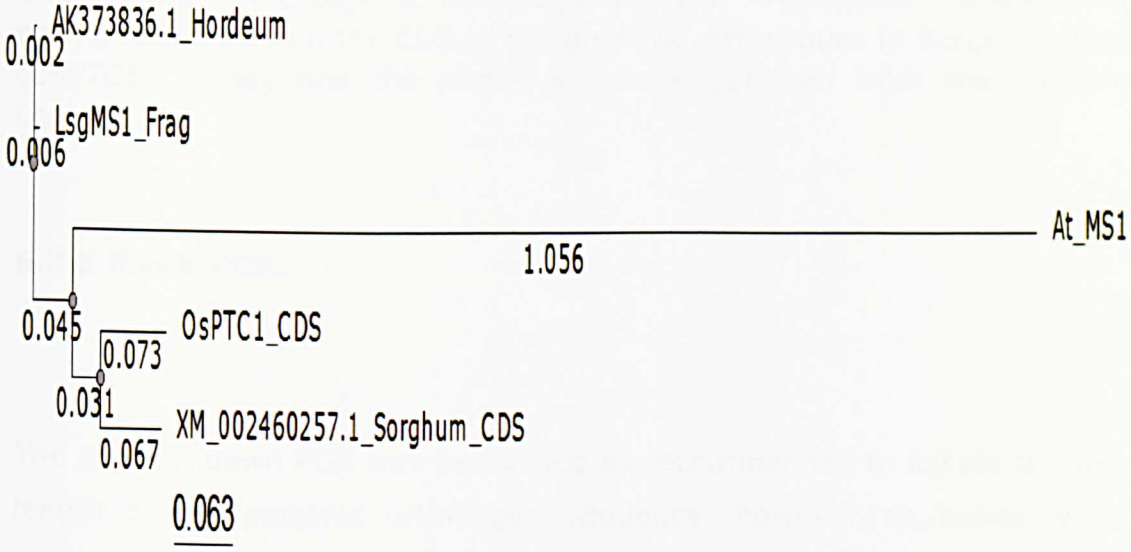

Fig 6.12 Phylogenetic tree constructed from the CDS sequences of the *Arabidopsis MS1* gene and the putative orthologues in Barley, Sorghum, Rice and the partial sequence in *Lilium* (*LsgMS1*). Tree was constructed using the MrBayes method on UGENE software.

	Sorghum Sb02g026200	Rice *OsPTC1*	Barley AK373836	*Lilium LsgMS1*
A. thaliana MS1 (2486bp)	52%	51%	51%	63%
Sorghum Sb02g026200 (2007bp)		51%	88%	88%
Rice *OsPTC1* (2040bp)			88%	90%
Barley AK373836 (2007bp)				100%

Table 6.10 Percentage of homology between *Arabidopsis thaliana MS1* coding sequence and the CDS of the putative orthologues in Sorghum, Rice (*OsPTC1*), Barley and the partial sequence obtained from the putative *LsgMS1*.

6.3.5 RACE-PCR.

The 3' Touchdown PCR was performed as recommended to isolate the full length of the putative orthologue sequence, however no bands were amplified. The same PCR product was then used to perform nested PCR and multiple amplified bands were observed (Fig 6.13); the bands of the expected size or an approximate size, were excised and cloned for sequencing. Unfortunately after several attempts to obtain the 3' end of the putative *MS1* and *MYB26* genes in *Lilium*, no sequences were generated that matched the putative orthologues from the other species. Several templates from anthers obtained from floral buds ranging from 2.0 cm to 6.0 were used and amplified bands of up to ±200 bp of the expected size were analysed but the results were similar and

unsuccessful. Most of the sequence obtained matched partial fragments of plasmids; five of the sequenced bands matched to genes from barley and sorghum, but with low identity and did not correspond to the expected sequences.

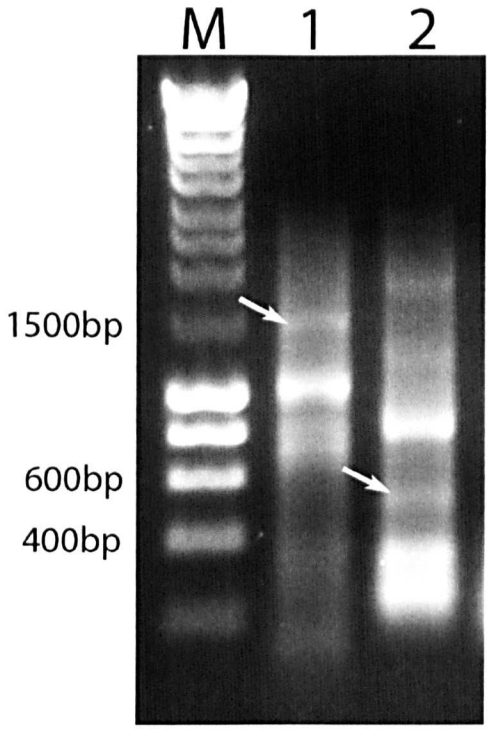

Figure 6.13 Nested PCR results for the amplification of the 3' end of the putative *LsgMS1* and *LsgMYB26* using as template the 3' RACE Touchdown PCR products. (M) DNA marker; (1) Sample for *LsgMS1* primers, a band of approximately 1500 bp. (arrow) was amplified, excised, cloned and sent for sequencing; (2) Sample for *LsgMYB26* primers, a band of approximately 500 bp. (arrow) was amplified, excised, cloned and sent for sequencing.

In the case of the 5' end the same approach was followed by using several anther templates from a range of developmental stages based upon bud lengths. Touchdown PCR failed to amplify any band for the putative *LsgMS1* gene, or the putative *LsgMYB26*. This PCR product was used to perform nested PCR; however no bands were amplified after the nested PCR either. Therefore unfortunately no amplification of any bands were achieved for the 5' Race PCR. Thus the 5' and 3' ends of both genes remain currently unknown.

6.3.6 *Lilium* transformation.

Tables 6.11 and 6.12 describe the results obtained for the transformation experiments with the overexpression and RNAi constructs of *AtMYB26* respectively. Plants that were PCR positive were grown until flowering; this process took an average of 80 weeks. However not all of the plants flowered. This was true for both transgenic and wild type plants, and was mainly because the cold incubation for dormancy break and vernalization were insufficient to induce flowering in some of them, and more specifically in the bulbs with a weight below 3 grams. Bulblets recovered from transformations with the RNAi constructs for the putative *LsgMYB26* and *LsgMS1* are still developing and currently too small for further analysis, therefore their analysis will be made in the future.

Transformations with Overexpression construct of *AtMYB26*					
Cultivar	**No. of explants inoculated**	**No. of Kan resistant tissues**	**% of Kan resistant tissues**	**No. of transgenic plants**	**Transformation efficiency (%)***
Star Gazer	350	75	21.4	16	4.5
Star Gazer	200	58	29.0	11	5.5
Total	**550**	**133**	**24.1**	**27**	**4.9**

Table 6.11 Results obtained for cv. Star Gazer in two independent *Agrobacterium*-mediated transformation experiments with plasmid pGWB5 for overexpression of *AtMYB26* gene. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

Transformations with RNAi construct of *AtMYB26*					
Cultivar	No. of explants inoculated	No. of Kan resistant tissues	% of Kan resistant tissues	No. of transgenic plants	Transformation efficiency (%)*
Star Gazer	250	61	24.4	58	23.2
Star Gazer	200	49	24.5	51	25.5
Star Gazer	200	54	27.0	48	24.0
Total	**650**	**164**	**25.2**	**157**	**24.1**

Table 6.12 Results obtained for cv. Star Gazer in three independent *Agrobacterium*-mediated transformation experiments with plasmid pK7GWIWG2 for RNAi of *AtMYB26* gene. (*) Transformation efficiency was calculated as the number of PCR-positive regenerated transgenic plants compared with the number of explants inoculated.

Transformations using the overexpression construct yielded a very low efficiency of transformation compared to the transformations using the RNAi construct and the previous transformation experiments in this research (chapters 3, 4 and 5). As seen in tables 6.11 and 6.12, the percentage of kanamycin resistance tissues were very similar between both, and the difference was only the amount of transgenic plants regenerated.

6.3.6.1 PCR analysis of putative transgenic plants.

PCR analysis of the putative transgenic plants from the *AtMYB26* overexpression construct were positive for the *NPTII* gene, but not all of them were positive for the *AtMYB26* gene (Fig 6.14). Analysis of the putative transgenic lines regenerated from transformation experiments using the RNAi construct of *AtMYB26* showed positive insertion of the *NPTII* gene and the RNAi fragments (Fig 6.15). Primers used for each PCR reaction are described in Table 6.7, section 6.2.7.

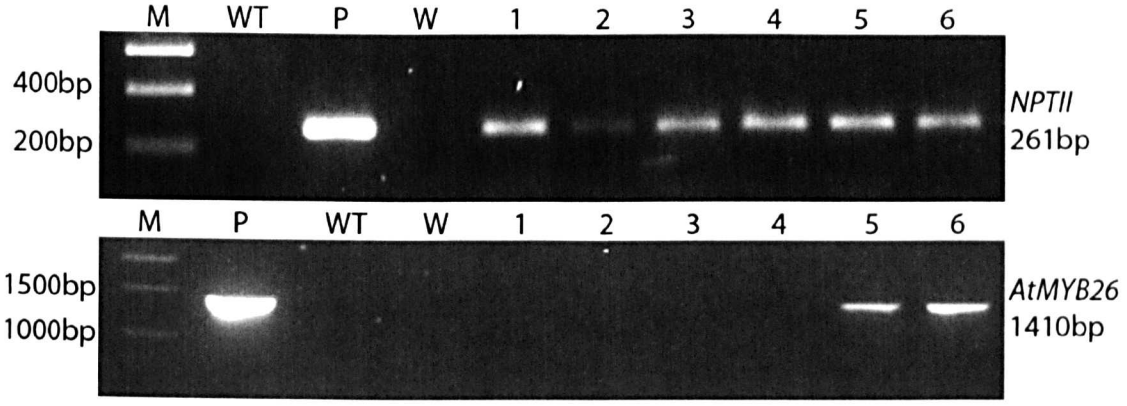

Figure 6.14 PCR screening for the *NPTII* and *AtMYB26* transgenes in putative transgenic lines of cv. Star Gazer overexpressing the *AtMYB26* gene. Lanes: (M) DNA marker; (WT) wild type DNA as negative control; (W) water as negative control; (P) plasmid DNA as positive control; (1) Line O-19; (2) Line O-35; (3) Line O-22; (4) Line O-3; (5) Line O-20; (6) Line PG-16. All lines were positive for the *NPTII* gene. Only two lines, 5 and 6, showed insertion of the *AtMYB26* gene.

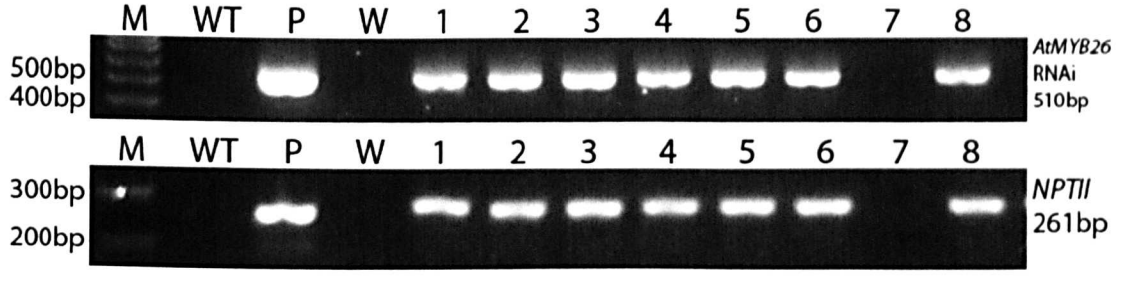

Figure 6.15 PCR screening for the *NPTII* gene and the forward fragment of *AtMYB26* for RNAi in putative transgenic lines of *Lilium* cv. Star Gazer. Lanes: (M) DNA marker; (WT) wild type DNA as negative control; (W) water as negative control; (P) plasmid DNA as positive control; (1) Line K-1; (2) Line K-5; (3) Line K-7; (4) Line K-18; (5) Line K-32; (6) Line K-34; (7) Line K-42; Line K-51. All lines except K-42 were positive for the *NPTII* gene and for the RNAi forward fragment of *AtMYB26*.

6.3.6.2 RT-PCR analysis of transgenic *AtMYB26* overexpression lines.

RT-PCR analysis from the transgenic lines overexpressing the *AtMYB26* gene was carried out as described in chapter 2, section 2.3.3. The primers used and the PCR conditions are described in Tables 6.6 and 6.7 respectively. The results showed that compared to an endogenous polyubiquitin gene, the relative expression of the *AtMYB26* gene was low (Fig. 6.16).

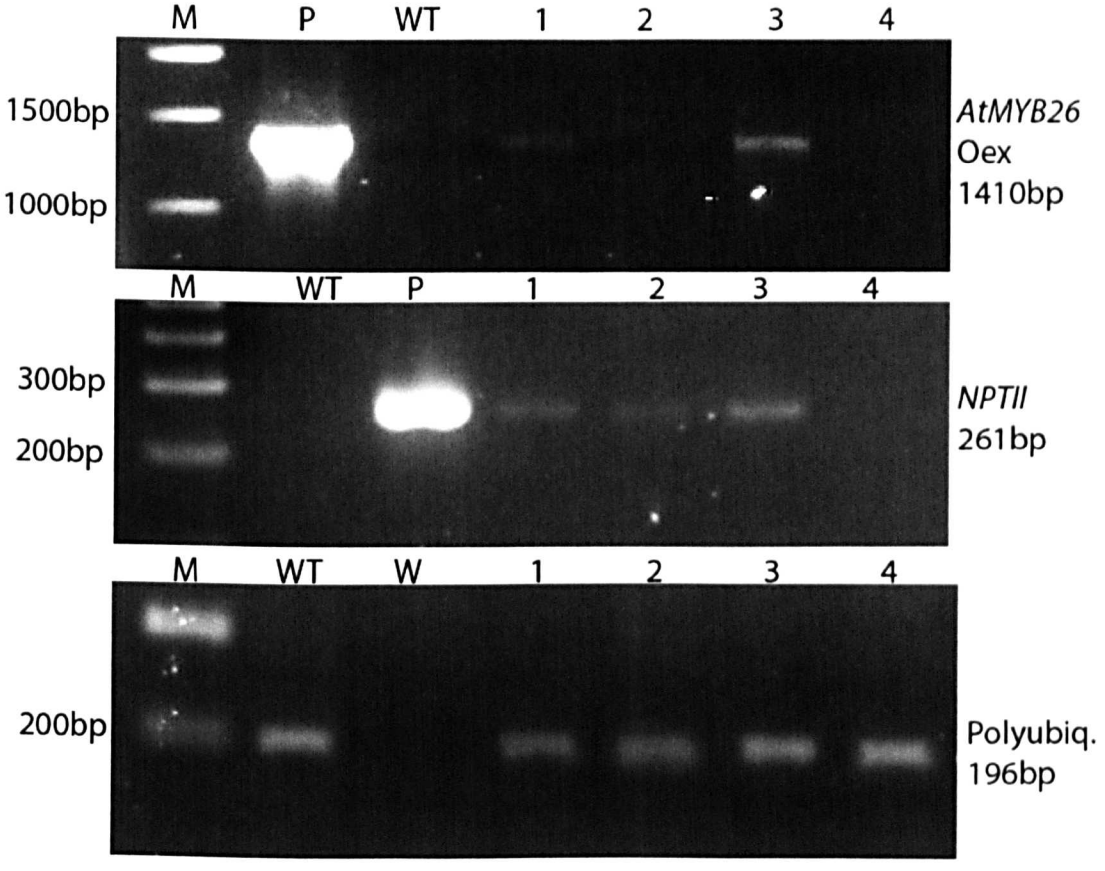

Figure 6.16 RT-PCR analyses for the *NPTII, AtMYB26* and polyubiquitin genes in transgenic lines of *Lilium* cv. Star Gazer. Lanes: (M) DNA marker; (WT) wild type DNA as negative control for *NPTII* and *AtMYB26* genes and positive control for polyubiquitin gene; (W) water as negative control; (P) plasmid DNA as positive control; (1) Line O-606; (2) Line O-35; (3) Line O-676; (4) Line O-23; (5). Lanes 1 and 2 were positive for *AtMYB26*; lanes 1-3 were positive for the *NPTII* gene. Polyubiquitin expression confirmed that the cDNA templates were of sufficient quality and equal amounts for the analysis.

6.3.7 Phenotypic analysis of the *AtMYB26* RNAi and overexpression transgenic plants.

Transgenic lines were grown until flowering to determine if the transformation process or the expression of the transgene was affecting the phenotypic characteristics of the cultivar. For comparison, wild type plants were grown under the same conditions of tissue culture, but without antibiotics, as the transgenic lines. Bulbs from transgenic and wild type plants flowered after an average of 90 weeks.

On the early stages of *in vitro* development after inoculation, no significant differences were noted between the transgenic plants carrying the *AtMYB26* fragments and the wild type. Both had very similar growth and shoot regeneration rates. After being transferred to pots, the similar growth rate was maintained. At adult flowering stage, in general the height, size of flower, colour of stem, leaves and flowers were as seen in the wild type (Fig. 6.17 and 6.18). However there was a difference in the anthers between the wild type and transgenic lines, with an apparent delay in dehiscence in the RNAi plants compared to the wild type (Fig 6.19). Due to transgenic flowers not opening at the same time as wild type ones, the staging of lily flower opening reported by Bieleski *et al.*, (2000) was used as a reference to analyse this possible delay in dehiscence (Table 6.13). At stage 1, in which tepals begin to separate (cracking bud), the wild type flowers showed partially opened anthers, whilst in the RNAi lines the anthers were still closed; at stage 2, in which the petals start to separate, the wild type plants showed fully opened anthers but on the RNAi lines were still closed; at stage 3, in which all tepals were fully separated and straight, the anthers of the RNAi lines started to open; At stage 4; in which the flower is considered to be fully opened when all the tepals started to curl, the anthers of RNAi lines were fully opened. The timescale between stage 1 and stage 4 was 8 hours. These phenotypes were observed on 4 wild type flowers and on 7 RNAi lines.

Stage/Description	Wild Type (4 flowers)	RNAi Lines (7 flowers)
Stage 1- Tepals begin to separate (cracking bud).	Partially opened anthers	Closed anthers
Stage 2- Petals start to separate.	Fully opened anthers	Closed anthers
Stage 3- All tepals are fully separated and straight.	Fully opened anthers	Anthers started to open
Stage 4- The flower is considered to be fully opened and all the tepals start to curl	Fully opened anther	Fully opened anthers

Table 6.13 Phenotypic description of anther dehiscence delay showed in the *AtMYB26* RNAi transgenic lines compared to the wild type. Using as a reference the staging of lily flower opening reported by Bieleski et al. (2000)

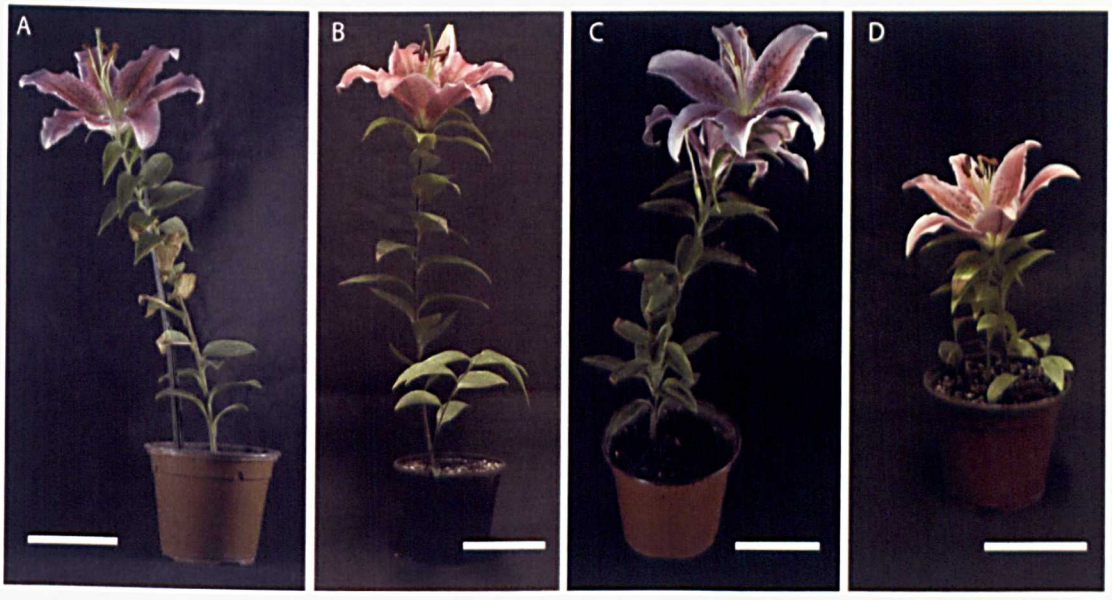

Figure 6.17 Phenotypic comparison of the transgenic *AtMYB26* RNAi plants compared to wild type. All the flowering plants showed relatively the same size, colour, shape and number of flowers as the wild type, all of them were fertile and set seed. All flowers lasted approximately 3 weeks before senescence. (A) Wild type; (B) Line K56; (C) Line K38; (D) Line K30, this line had almost half of the bulb weight of the other transgenic plants and wild type hence the reduced height at flowering stage. Bar = 9 cm.

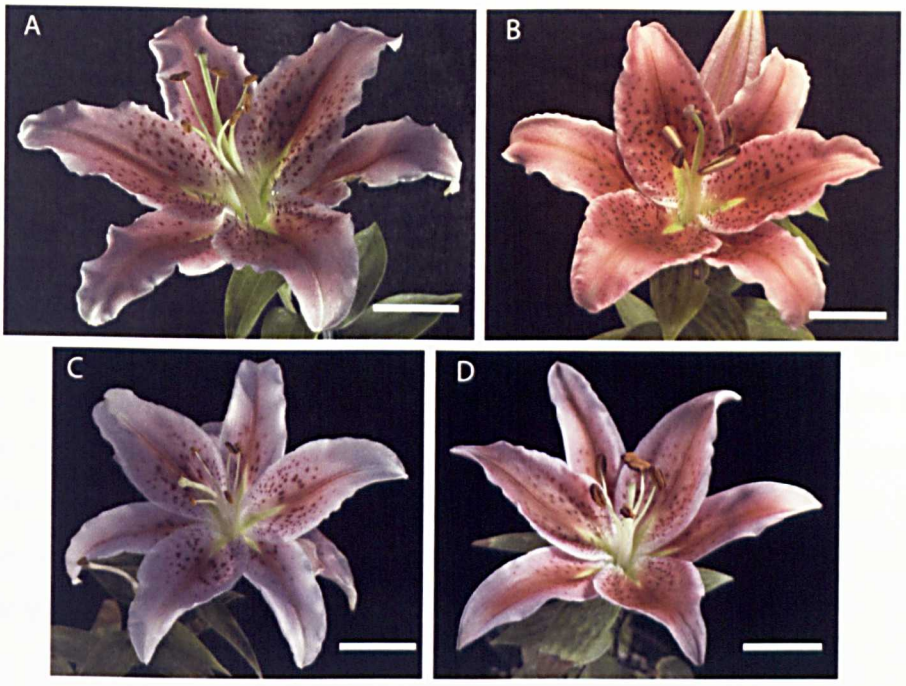

Figure 6.18 Phenotypic comparison of the transgenic *AtMYB26* RNAi flowers against the wild type. All the flowers showed the same shape, colour pattern and tone. All had a similar size and normal arrangement of floral organs (anthers, tepals and stigma). Transgenic flowers showed delayed dehiscence and uneven anther shape. Pictures were taken 8 hours after stage 1 of flower opening (Bieleski *et al.,* 2000). (A) Wild type with normal anthers; (B) Line K56 showing the greatest delay in anther dehiscence of all the lines (Fig. 6.18); (C) Line K38; (D) Line K30. Bar = 4cm.

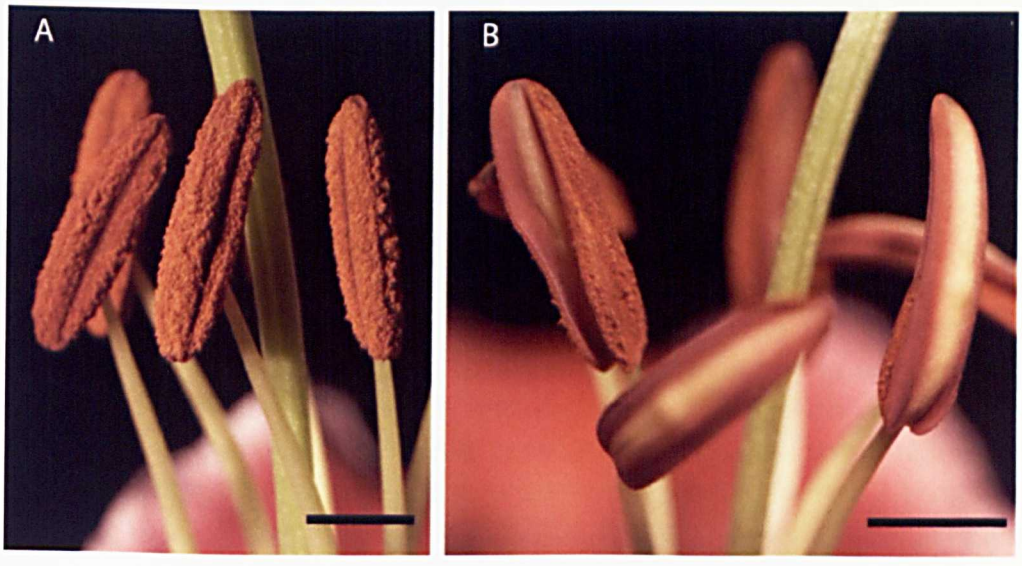

Figure 6.19 Phenotypic comparison of the transgenic *AtMYB26* RNAi anthers compared to the wild type. In general the anthers from the *AtMYB26* lines showed delayed dehiscence, with some lines more severe than others. Pictures were taken 8 hours after stage 1 of flower opening (Staging based upon Bieleski *et al.,* 2000). (A) Wild Type with normal fully opened anthers. Bar = 7mm; (B) Line K56 showing partial anther opening and delayed anther dehiscence. Bar = 4mm.

Anthers in the wild type opened longitudinally, the stomium broke evenly across the whole anther forming a straight oval shape. However, anthers from the RNAi plants did not showed the same pattern of opening. Although they opened longitudinally, the stomium either broke unevenly across the anther, or the anther walls retracted back unevenly which resulted in a wavy shape to the anther walls, instead of the straight line seen in the wild type (Fig 6.20 A,B); in some cases this edges gave the entire anther a wavy shape instead of a straight oval one as seen in the wild type (Fig 6.20 C,D). In addition, anthers from RNAi lines appeared to dehydrate and senesce faster in approximately 7 days compared to the wild type, which senesced in approximately 21 days after the flower was completely open.

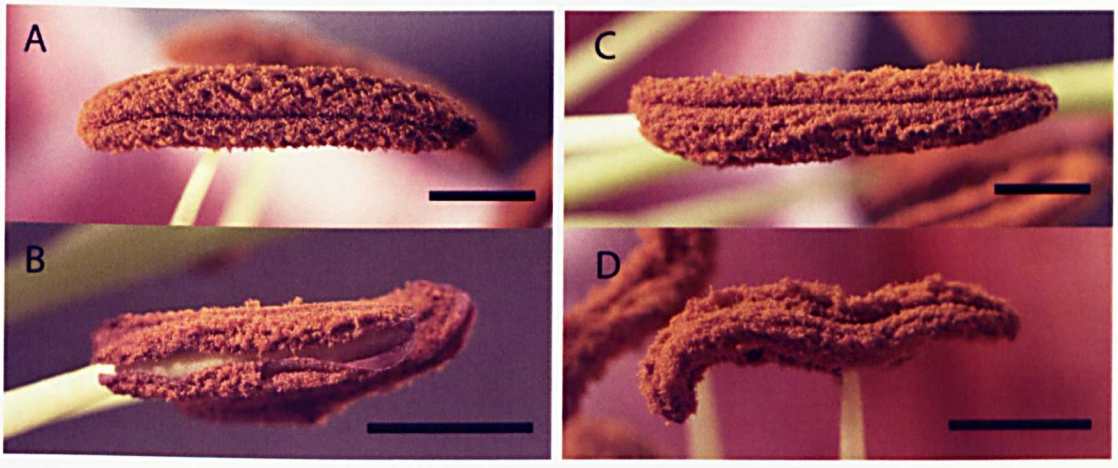

Figure 6.20 Phenotypic comparison between open anthers from transgenic *AtMYB26* RNAi and wild type plants. In most of the transgenic lines the anthers showed a wavy appearance at opening compared to a straight line of the wild type; in addition the anthers from the RNAi lines were smaller in size and had a wavy shape compared to the straight, oval shape of the wild type. (A) Wild type with normal straight anther; (B) Line K24 with wavy pattern on the edges of anther wall; (C) Wild type anther with a straight line shape; (D) line K24 with smaller size anthers and wavy shape. Bar = 3.5mm.

Although no analysis was done to check for altered secondary thickening, regenerated *Lilium* tissues looked deformed and/or dwarf (Fig 6.21 B,C); the appearance of the bulb scales from transgenic lines also looked thicker than those of the wild type (Fig. 6.21 D)

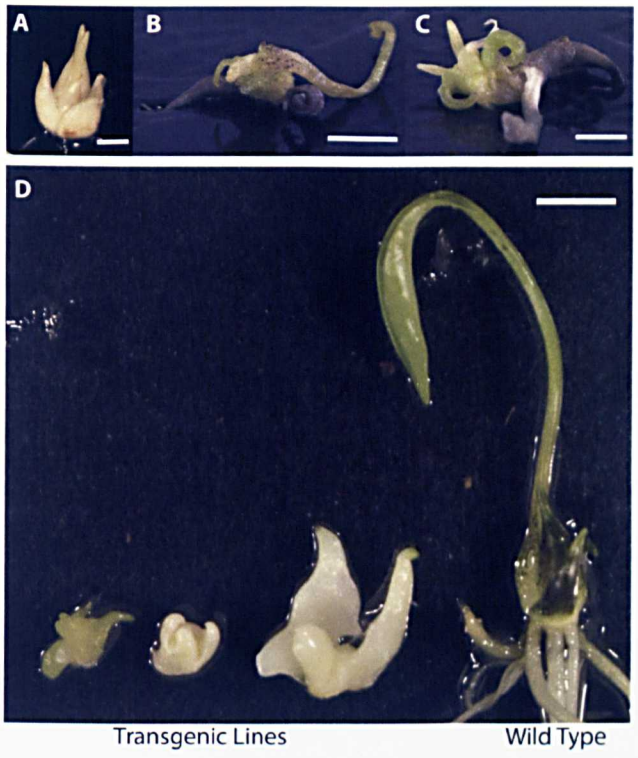

Figure 6.21 *In vitro* development of putative transgenic lines overexpressing the *AtMYB26* gene. (A) Seven weeks old wild type shoot; (B and C) Deformed bulblet-like tissues from putative transgenic lines seven weeks after inoculation. A, B and C Bar=1.5mm. (D) Regenerated transgenic bulblets remained dwarf and development was very slow compared to wild type after 12 weeks. Bar = 8mm.

Two transgenic lines that showed low relative levels of expression of the *AtMYB26* gene on the RT-PCR analysis (Fig. 6.16) and developed normally with no defects noted throughout the *in vitro* culture stage were transferred to soil and grown until flowering to determine if the transformation process or the expression of the transgene was affecting the phenotypic characteristics of the cultivar. In general no differences were noted between the transgenic plants overexpressing the *AtMYB26* gene. Growth rates *in vitro* and in soil were similar. At the flowering stage, the height, size of flower, colour of stem and leaves, number and

arrangement of leaves, and time to flowering were as seen in the wild type (Fig. 6.22).

Flowers showed a similar appearance between wild type and transgenic lines (colour pattern and tone) size and arrangement of floral organs was similar too (Fig. 6.23). Anther size and shape was not affected on the transgenic plants (Fig 6.24). Dehiscence and flower senescence were approximately equal in the transgenic lines compared to the wild type. Although it was expected that the overexpression lines would have a modified phenotype, no alterations in phenotype were seen. The results suggest that this may be because the low levels of expression of the *AtMYB26* might not be sufficient to alter secondary thickening in those lines. This phenotypic analysis also confirms that the transformation process does not affect the external characteristics of the cultivar.

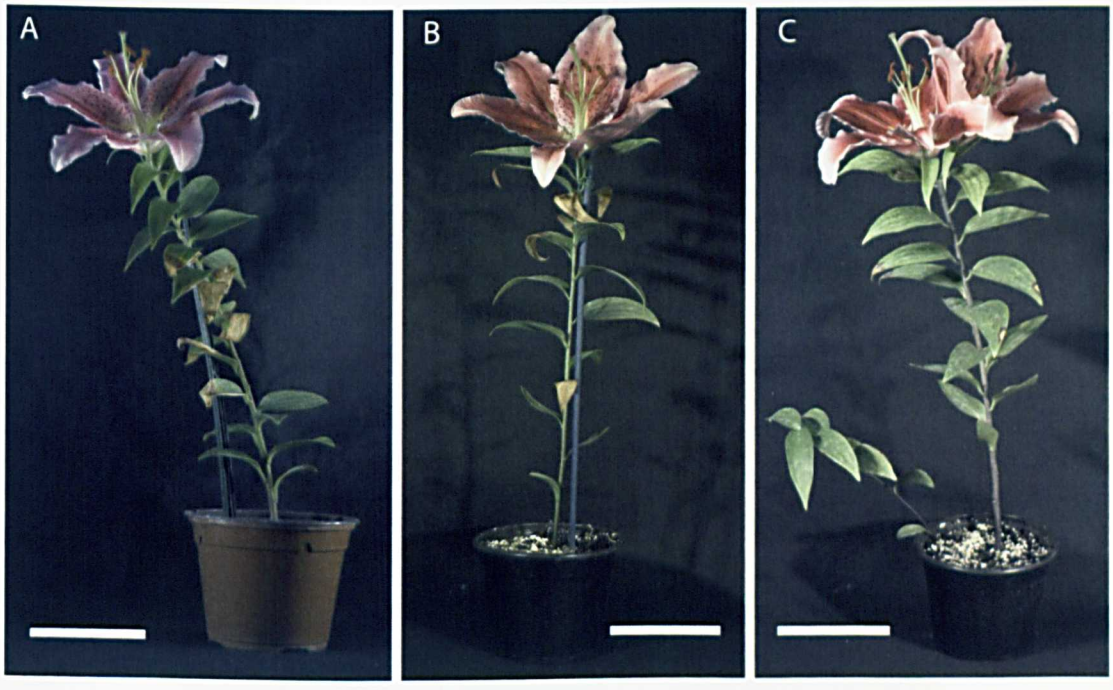

Figure 6.22 Phenotypic comparison of the transgenic plants overexpressing the *AtMYB26* gene compared to wild type. All the flowering plants showed approximately the same size, colour, shape and number of flowers as the wild type, all of them were fertile and set seed. All flowers lasted approximately the same time before senescence which was around 3 weeks. (A) Wild type; (B) Line O676; (C) Line O606. Bar = 9 cm.

Figure 6.23 Phenotypic comparison of flowers from the *AtMYB26* overexpression transgenic plants compared to the wild type. All the flowers showed the same shape, colour pattern and tone. All of them were of approximately the same size in a normal arrangement of floral organs (anthers, tepals and stigma). The amount of pollen produced by each flower also appeared to be similar. (A) Wild type; (B) Line O676; (C) Line O606. Bar= 4 cm.

Figure 6.24 Phenotypic comparison of anthers between transgenic plants overexpressing the *AtMYB26* gene compared to the wild type. No difference was noted in shape, size, pollen production or dehiscence between transgenic and wild type anthers. (A) Wild type; (B) Line O19. Bar = 4mm.

6.4 Discussion.

The production of vast amount of pollen by *Lilium* flowers can be an undesirable commercial trait. Therefore one of the major breeding objectives in *Lilium* is to obtain cultivars with modified characteristics of pollen development and release. The development of systems for inducible male sterility without any modification of the floral architecture have been previously reported in tobacco (Takada *et al.,* 2006), lettuce (Takada *et al.,* 2007), petunia (Kapoor *et al.,* 2002; Kapoor and Takatsuji, 2006) and recently in chrysanthemum (Shinoyama *et al.,* 2012).

The development of a transformation protocol on this research served as a starting point for targeting specific traits for lily improvement. Pollen development and release from the model plant *Arabidopsis* has been extensively studied by the Wilson's Lab at the University of Nottingham, this work has been extended to monocot crops such as barley (Fernandez, 2011) and rice (Wilson and Zhang, 2009).

Analysis of *Arabidopsis* male sterile mutants has provided a greater knowledge of the gene regulatory networks controlling maternal development of the anther and the resultant sporophytes (Wilson and Zhang, 2009). The increased availability of genome sequences of crops such as barley (Matsumoto *et al.*, 2011), wheat (Eversole *et al.,* 2009) and tomato (The Tomato Genome Consortium, 2012), as well as the development of tools for the analysis of gene function, have made it possible that this knowledge base can now be extended into other crops.

Arabidopsis MYB26/MS35 transcription factor regulates secondary thickening of the endothecium cell wall; previous work showed that overexpression resulted in ectopic secondary thickening in tobacco and *Arabidopsis* with anthers failing to dehisce (Yang *et al.,* 2007b). The *A.*

thaliana ms35 mutant also showed male sterility due to failure of the anthers to dehisce due to a lack of force during anther opening; however pollen produced by the mutant was viable (Dawson *et al.*, 1999; Yang *et al.*, 2007b; Nelson *et al.*, 2012).This reported phenotype may be able to overcome the problem for the excessive release of pollen by lily anthers, whilst still producing viable pollen for breeding purposes. *Agrobacterium*-mediated transformations in *Lilium* were carried out using constructs for overexpression and RNAi of the *AtMYB26* gene. Transformation with the overexpression construct resulted in minimum recovery of putative transgenic plants (Table 6.11, section 6.3.5). This might have been caused by the function of the gene in secondary thickening. Although no analysis was done for secondary thickening, the inoculated explants showed deformed clumps of cells, or bulblet scale-like structures with apparent ectopic secondary thickening compared to the wild type tissues, which limited, or in most of the cases, interrupted its development.

PCR analysis of the few recovered putative transgenic lines confirmed the insertion of the *AtMYB26* gene; however RT-PCR analysis showed that only a small number of those lines were expressing the gene and that the relative expression level was low compared to an endogenous polyubiquitin gene, which might explain why these plants managed to grow with a normal phenotype compared to the other tissues originating from these transformations. Transgenic plants expressing the *AtMYB26* gene were grown until flowering to analyse any effect of the transgene on the phenotype of the plants, especially on the anthers. Transgenic and wild type control plants flowered after approximately 80 to 90 weeks in the growth room. No difference was noted in height, bulb size, time to flowering, colour pattern in flowers, number and colour of leaves, size of flowers or flower organs arrangement between the wild type plants and the overexpression lines. Dehiscence was also very similar for both transgenic and wild type. These transgenic plants were fertile and set seed.

These results showed that neither the overexpression of the *AtMYB26* nor the transformation process had any effect on the phenotype of the cultivar. However, the location of the MYB26 transcript and levels compared to native MYB26 in *Arabidopsis* is unknown. Future research should be aimed at analysing how the level of expression of the transgene is affected in the progeny.

Another approach to solve the problem of secondary thickening in early stages of development could be to use inducible constructs that would allow the expression of the transgene at a specific time; this approach has been successful to induce male sterility in tobacco (Kriete *et al.,* 1996), *Arabidopsis* (Phan *et al.,* 2011) and eggplant (Toppino *et al.,* 2011). In addition, the use of tissue specific promoters to direct the expression of the transgene only into the selected tissues can represent another approach to obtain the desired phenotype. This approach has been used to induce male sterility in tobacco (Yang *et al.* 2011; Viswanathan *et al.,* 2011), eggplant (Toppino *et al.,* 2011) and rapeseed (Engelke *et al.,* 2011).

In the transformation experiments with the RNAi construct more than 150 transgenic plants were obtained. PCR analysis confirmed the insertion of both fragments into the plants. These transgenic lines, alongside wild type control plants, were grown for approximately 80-90 weeks until flowering to analyse any effect of the transgene and/or the transformation and tissue culture process. No difference was noted in height, bulb size, time to flowering, colour pattern in flowers, number and colour of leaves, size of flowers or flower organs arrangement between the wild type plants and the *AtMYB26* RNAi lines. However, differences in the anthers were noted for the transgenic lines. The shape of the anther was affected, in most of the transgenic lines the anthers showed a wavy appearance upon opening compared to the straight line presented by the wild type anthers.

An apparent delay in dehiscence was noted in the anthers from the transgenic RNAi lines compared to dehiscence in the wild type flowers. Wild type anthers were fully opened at stage 2 of flower opening from Bieleski's scale (Bieleski *et al.,* 2000) compared to stage 4 in the RNAi lines. In addition the anthers from transgenic lines showed an uneven wavy pattern on the edge possibly due to abnormal secondary thickening. Cell walls consist of cellulose microfibrils, embedded within a pectin-hemicellulose matrix (Cosgrove, 2005); several studies have shown that if cellulose orientation or amount varies between different cell layers, changes in turgor can lead to unequal shrinkage of the cell layers which can cause the tissue to bend (Nelson *et al.,* 2012) which might explain the wavy pattern seen in these transgenic lines.

Although the expected phenotype of non-dehiscent anthers was not achieved by overexpression or the RNAi approach, the delayed dehiscence and uneven surface and shape of anthers from the gene silencing experiments might be due to a possible low level of silencing of the putative orthologue of *AtMYB26* in lily. Given that *Arabidopsis* and *Lilium* are dicot and monocot respectively; the identity between genomes is expected to be low, and may explain why a low level of silencing was observed in these transgenic lines, but at the same time might be giving an insight of the conservation of gene function between the species.

With the objective of analysing this synteny between *Lilium* and *Arabidopsis*, an attempt to find and sequence the putative orthologue of *AtMYB26* and *AtMS1* was attempted. Due to the lack of genomic sequences of *Lilium*, a bioinformatics analysis was used to try to find putative orthologues of both genes in other species by searching in several genome databases.

Interspecies comparisons can be used to analyse conservation between species that can lead to identification and further characterization of

functional elements. Recently, the increase availability of functional genomics data has transformed comparative approaches from basic sequence analysis to detailed studies of functional attributes (Mohavedy *et al.*, 2012). The regulatory networks of pollen development in the monocot crop rice have shown high levels of similarity between those of *Arabidopsis* (Wilson and Zhang, 2009; Li *et al.*, 2011) and barley (Fernandez, 2011), therefore this can be used as an important tool to identify and understand this developmental pathway in other plants such as *Lilium*.

Multiple sequence alignment using the putative orthologues identified in monocot species such as rice, sorghum, maize, barley and *Brachypodium* was performed to identify conserved regions of high identity between them. Primers were designed based on these regions and used for amplification of *Lilium* cDNA from cv. Star Gazer. For the putative orthologue of the *AtMS1* gene, one partial sequence was obtained in *Lilium*. Alignment of this sequence against the other putative orthologues showed that the identity between them was high, at 81% with the *OsPTC1* gene from rice already reported as the putative *AtMS1* orthologue gene in rice (Li *et al.*, 2011) and 100% with the putative orthologue in barley (Fernandez, 2011).

For the putative orthologue of the *AtMYB26* gene, two partial sequences were obtained in *Lilium*. A contig from these two sequences was generated and aligned against the other putative orthologues. Results showed that the identity between them was very high reaching 99.6% with the barley putative orthologue. The partial sequence from lily covers approximately 88% of the gene from barley and has also a MYB domain in the same region as the other putative orthologues.

To obtain the full length of both genes, primers were designed based upon the partial sequences and a Rapid Amplification of cDNA Ends was

conducted. Unfortunately after several attempts the amplified and sequenced bands obtained did not matched any of the putative orthologues of other species or the partial sequences from lily. However, with the partial sequences of both the putative *LsgMS1* and *LsgMYB26* RNAi vectors were constructed for gene silencing. Transformation of cultivars Star Gazer, Sweet Surrender, Acapulco and Night Flyer was performed. Since the recovered putative transgenic lines are still too small they will be analysed in future research.

Although the regeneration of non-dehiscent *Lilium* plants was not achieved, the delayed dehiscent lines obtained suggest that gene function is conserved between *Arabidopsis* and *Lilium*. Future research should be aimed at analysing the transgenic plants obtained with the RNAi constructs for both the putative *LsgMS1* and *LsgMYB26*, which might have a stronger phenotype than those showed with the RNAi lines of *AtMYB26* as well as the analysis of the next generation of the latter transgenic lines.

The full length of both genes should be obtained so they can be characterized and confirm if they are the true orthologues of both genes in *Lilium* by functional analysis. This can be attempted by the use of Targeted rapid amplification of cDNA ends (T-RACE) that has shown higher specificity in the amplification of cDNA ends from difficult templates by using an oligo-dT adapter incorporating a dUTP-containing PCR primer and asymmetric PCR followed by degradation of the non-target transcripts containing dUTP degraded by Uracil DNA glycosylase, leaving only those transcripts produced during the asymmetric PCR (Bower and Johnston, 2010). The delayed dehiscent RNAi plants represent the first report of an altered phenotype in any cultivar of *Lilium* by transgenic approach.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION.

7.1 Introduction.

The main objective of this research was to genetically modify traits of agronomic importance in one of the most important ornamental crops in the world, *Lilium*. The reports of transformation protocols in *Lilium* were scarce. All of them were time consuming, complicated, with problems of reproducibility and most of the transformation efficiencies reported were very low. Thus the need for a faster, more efficient and reproducible transformation protocol was evident. After the development of an efficient transformation protocol the focus of the project was then on the manipulation of important traits, such as pathogen resistance and pollen development. These would provide an alternative to traditional breeding, which is less efficient and can take up to 20 years to obtain a new cultivar with the desired trait.

In addition, the identification and characterization of putative orthologues in *Lilium* of well characterized genes involved in pollen development and release (Yang *et al.*, 2007a; Yang *et al.*, 2007b) was carried out. Such resources will provide opportunities for manipulation of male reproduction in *Lilium*, whilst generating valuable information on the conservation of pollen development pathways.

7.1.1 GMOs and commercialization perspectives.

Research in genetic manipulation has increased over the last decade. Genetically manipulated crops like maize, soybean, cotton, canola and papaya have been commercialized and produced in the last 16 years on 29 countries with over 148 million hectares of land used for its production

(James 2010). However a relatively small market for ornamental crops, compared to edible crops, makes many of these strategies less profitable for within the floricultural industry. The very high cost of the licenses for patented methods and gene applications combined with the pricey and time consuming approval and registration procedures of GM plants hold back the use of transgenic technology by such companies (Lütken *et al.*, 2012).

However, the biggest limiting factor for the production and commercialization of ornamental GMOs is the same as for any other edible transgenic crop, the acceptance of this type of organisms by the public and then by the retailers. Despite these concerns, the commercialization of a low number of transgenic ornamentals, mainly blue carnations produced by Florigen Ltd., have been done for more than 15 years, opening the possibilities for future crops to be introduced to the market (Lütken *et al.*, 2012). Thus, if the costs of regulatory approval can be reduced, there could be numerous opportunities for GM ornamentals in the marketplace.

Although this might not be reached in the near future due to the current legislation around GMOs, it would help to accelerate the commercialisation process if all countries could allow regulation based on phenotype and not on the process (Chandler and Sanchez 2012). In summary, the regulatory costs could be lowered by a global regulation, more flexibility for risk assessment and reduced requirements for molecular characterization. In the European Union the regulation is even stricter compared to other parts of the world such as North America making the development and commercialisation of genetically modified ornamentals even slower. This is a very important hurdle to overcome for the success commercialisation of ornamental GMOs, since Europe remains the largest market for this industry and it is the home of some of the biggest flower and pot plant breeders (Chandler and Sanchez 2012).

7.2 *Agrobacterium*-mediated transformation of *Lilium*.

Several previously reported transformation protocols were tested in this research; both biolistics mediated (Watad *et al.*, 1998; Cohen *et al.*, 2004; Benedito *et al.*, 2005; Kamo and Han, 2008), or *Agrobacterium*-mediated (Mercuri *et al.*, 2003; Hoshi *et al.*, 2004; Ogaki *et al.*, 2008) had been previously reported, but unfortunately in this research none of these methods yielded any transgenic plants. These methods were also time consuming, laborious, complicated and even from the original reports had very low transformation efficiency, and as demonstrated were not reproducible.

Agrobacterium transformation can offer more advantages than particle bombardment, for instance it doesn't require expensive equipment, it is high throughput and there is less mechanical manipulation of the tissues compared to biolistics. The risk of contamination is also higher in biolistics than in *Agrobacterium* transformation and the parameters that can be modified to try to increase the frequency of gene insertion are more complex. However *Agrobacterium* typically infects dicot species and although monocot transformations have been achieved these have proven more difficult than for dicots (Ashok and Horst 2006; Sood *et al.*, 2011; Harwood 2012). Other direct methods for transformation have been successfully used in monocot crops such as whiskers in rice (Takahashi *et al.*, 2003), maize (Petolino *et al.*, 2000) or wheat (Brisibe *et al.*, 2000) in which silicon carbide fibres are mixed in a vortex with a suspension of tissue and DNA allowing its introduction by abrasion, it is a cheap method that can be applied to several species but the damage to the cells can affect regeneration and the reported efficiencies are very low (Rivera *et al.*, 2012); the use of micro or macro injection has also been reported in crops such as barley (Holm *et al.*, 2000) and wheat (Mu *et al.*, 1999) with high efficiencies but these methods are extremely expensive due to the need of especial machinery, very slow and laborious. However no reports of using direct DNA delivery other than biolistics have been done in *Lilium*.

The first key factor in any transformation protocol is to have an efficient *in vitro* regeneration method, so that the transformed cells can develop quickly into a new transgenic plant. With this in mind the selection of the best starting material becomes a key consideration, it needs to show a high regeneration rate, a high number of undifferentiated cells and for the purpose of *Agrobacterium* transformation, a high number of open wounds for the bacteria to infect. Lastly the *Agrobacterium* should be provided with the optimal conditions to transfer DNA into the host cells. Based on observations of how different *Lilium* tissues develop *in vitro* and understanding of the action of how *Agrobacterium* infects plant cells, a transformation protocol was developed and tested.

An average of 20.1% transformation efficiency was obtained; this is almost five times higher than the previous highest efficiency reported of 4.6% by Ogaki et al. (2008). Another major improvement was the rapidity of the method, taking on average 12 weeks to obtain PCR positive transgenic plants compared to an average of 34 weeks by other protocols (Ogaki *et al.,* 2008; Azadi *et al.,* 2010a). In general these features make this newly developed protocol better compared to the previous reports by using the optimal starting material for transformation, as well as providing the optimal conditions for infection of the *Agrobacterium* and the optimal conditions for the tissues to grow after inoculation; hence the fast and efficient recovery of transgenic plants.

This new protocol was developed for an oriental hybrid, cultivar Star Gazer. Transformation reports frequently describe the use of only one, or a very limited number of cultivars or species from *Lilium,* this raises the issue of reproducibility in other more important elite and/or commercial cultivars. Transformation efficiency protocols have been found to be cultivar dependent in crops like barley and wheat (Harwood 2011). The Lily transformation protocol was therefore tested on seven different cultivars/species. Two Asiatic hybrids, cultivars Night Flyer and Sweet Surrender; an oriental-trumpet (OT) hybrid, cultivar Beverly's Dream; an

oriental hybrid, cultivar Acapulco; *Lilium longiflorum* cultivar Snow Queen; *Lilium henryii* and *Lilium leichtlinii*.

After at least three independent experiments, the transformation efficiencies obtained for the different species/cultivars were similar, or even higher, than the ones from the original protocol used for cv. Star Gazer (chapter 3, section 3.2.3.4). Efficiencies ranging from 18.8 % (*L. leichtlinii*) to 33.1% (cv. Beverly's Dream) were obtained in these experiments. In addition the use of higher concentration of lipoic acid (100mM) and the use of activated charcoal (2.5g/L) in the selection medium increased the growth rate of all cultivars reducing the time to obtain PCR positive plants from 12 weeks (Núñez de Cáceres *et al.,* 2011) to an average of 10 weeks.

These results indicate that the *Agrobacterium*-mediated transformation protocol developed in this research is highly efficient, rapid and most important of all robust enough to be used with other cultivars/species of the genus *Lilium*. Such characteristics, makes it easy to adapt and implement for future breeding programs as well as a tool for research for several agronomically important traits such as plant architecture and flowering time.

The understanding of the molecular basis of plant architecture offers the prospect of developing new desirable varieties differing in height, flower structure, altered phyllotaxis and/or number of flowering branches (Meng *et al.,* 2009). In transgenic chrysanthemum, the overexpression of the gene isopentyl transferase (*ipt*) resulted in shorter plants with more flowers (Khodakovskaya et al. 2009). Flowering time in ornamentals has a very significant impact on production costs as well as in commercialization price (Chandler and Brugliera 2009). Control of flowering by manipulation of photoperiod and/or use of plant growth regulators can be achieved with limited success and increased cost, thus

the possibility of using a transgenic approach to develop plants which will grow under day-neutral climates or can be induced to flower is one possible use of genetic modification technology (Flachowsky et al. 2009). Transformation of ornamentals such as chrysanthemum (Shulga et al. 2009) and orchids (Thiruvengadam and Yang 2009) with flower induction related regulatory genes has been shown to significantly speed time to flowering. Due to the similarities between *Lilium* and other important bulbous ornamental crops such as tulip, narcissus or gladiolus, this transformation protocol could also represent a good starting point for mass propagation and a highly efficient transformation protocol for the future in those species.

7.3 Pathogen Resistance

Traditional approaches to enhance pathogen resistance in crops are laborious and time consuming, taking up to 15 to 20 years to obtain a resistant cultivar. With a transgenic approach this can be done in a considerably reduced time and in a more targeted and efficient way. Although in *Lilium* there are some cultivars with some degree of resistance to fungal pathogens, especially the asiatic cultivars such as Connecticut King and Orlito which present high resistance to *Fusarium oxysporum*, these cultivars are not the most commercial and the spectrum of pathogens to which they are resistant is limited (Shahin *et al.*, 2009; Shahin *et al.*, 2011).

The use of plant chitinases to induce pathogen resistance has been well documented in several crops. Tobacco and potato plants overexpressing the *CHIT42 and ThEn42* chitinase genes from *Trichoderma harzianum* were highly tolerant, or completely resistant to *Alternaria, Botrytis and Rhizoctonia* (Lorito *et al.,* 1998). Eleven chrysanthemum plants overexpressing the *RCC2* chitinase gene from rice showed enhanced resistance to *B. cinerea* (Takatsu *et al.,* 1999); transgenic cucumber

plants overexpressing the *RCC2* chitinase gene from rice also had varying levels of resistance to *B. cinerea* depending on the level of expression of the transgene (Kishimoto *et al.,* 2002). In strawberry overexpression of the *Ch5b* chitinase gene from *Phaseoulus vulgaris* resulted in enhanced levels of resistance to *B. cinerea* (Vellicce *et al.,* 2006), transgenic wheat expressing a barley class II chitinase gene showed enhanced resistance in the greenhouse and in field conditions against Fusarium head blight disease (*Fusarium graminearum*), with the level of resistance related to the level of expression of the transgene (Shin *et al.,* 2008). However, there are no commercial cultivars available currently using transgenic approaches (www.gmo-compass.org).

Overexpression of the rice chitinase *RCH10* gene was attempted in lily. More than 150 transgenic plants overexpressing the *RCH10* gene were obtained from transformation experiments with cultivar Star Gazer. Results of molecular analyses showed the correct insertion and expression of the transgene (chapter 5, section 5.3.1). Further analysis by QRT-PCR confirmed that the level of relative expression of the *RCH10* gene varied between the transgenic lines and that these levels were directly correlated with the resistance shown by the transgenic plants in terms of sporulation levels (chapter 5, section 5.3.2.2). In addition there were no obvious differences noted between the wild type plants and the transgenic ones. These results are very similar to those obtained in other crops (Marchant *et al.,* 1998; Takatsu *et al.,* 1999; Rohini and Sankara Rao 2001; Kishimoto *et al.,* 2002; Vellicce *et al.,* 2006; Distefano *et al.,* 2008; Shin *et al.,* 2008) in which the level of induced resistance to the fungal pathogen was higher or lower depending on the level of expression of the chitinase gene.

A phenotypic analysis was done on these transgenic plants to confirm that the transgene, the *in vitro* culture or the transformation process did not have any apparent effect on the phenotype of the cultivar. Several plants were compared to non-inoculated control plants and no differences were

noted in any characteristic of the adult plant. Height, number and colour of leaves, flower size and colour, floral organs, pollen production and viability, flower organ arrangement and fertility of the plants were very similar between the control plants and the transgenic ones (chapter 5, section 5.3.3). This confirms that no adverse effects to the overall appearance of the cultivar were seen as a consequence of the transformation procedure.

Future research should be aimed at analysing the level of resistance of this transgenic material in an *in vivo* assay, as several authors have demonstrated that transgenic plants showing enhanced resistance to fungal pathogens *in vitro* did not showed the same level of resistance in field tests (Rommens and Kishore 2000; Balconi *et al.,* 2007; Shin *et al.,* 2008) This might be cause by the interaction between several factors such as micro flora on the surface of the leaves, pH, temperature and humidity levels in the environment. In addition an *in vitro* and *in vivo* resistance assay of these transgenic lines should be done to analyse the induced resistance to other pathogens such as *F. oxysporum*. . Analysis of the progeny of these lines should be carried out to test if there is any increase or lost in resistance compared to the parental lines.

This is the first report of a *Lilium* cultivar with enhanced resistance to *Botrytis cinerea* by a transgenic approach. The results obtained in this research will encourage the use of genetic manipulation to obtain new cultivars with enhanced resistance to other pathogens with a different number of other genes. The use of other genes such as β-1,3-glucanases (Balasubramanian *et al.,* 2012), polygalacturonase-inhibiting proteins (Ferrari *et al.,* 2012; Hassan *et al.,* 2012), or a combination of them have shown to enhance resistance against a broader spectrum of pathogens, this approach could also be attempted in *Lilium* to enhance broad spectrum resistance

7.4 Manipulation of pollen development in *Lilium*.

The vast production of pollen is an undesirable trait in *Lilium* flowers. This pollen can be allergenic to people, toxic to pets (Stokes and Forrester 2004; Fitzgerald 2010; Thompson 2012) and easily stains clothes and the flower itself. Recently the antherless phenotype (*lal*, *Lilium* antherless mutation) has been reported to be a single gene based trait (Shahin *et al.*, 2011) and commercially antherless lily cultivars have been successfully developed by traditional breeding (www.lilybreeding.com/lilies/), however the appearance of the anthers are important features for the overall appeal of the flowers. Genetic manipulation by transgenic approaches may therefore be an alternative method to overcome this problem.

The development of systems for inducible male sterility without any modification of the floral architecture have been previously reported in other systems for example, by the anther-specific expression of the *Cm-ERS1/H70A* gene from melon in tobacco (Takada *et al.*, 2006) and the overexpression of the same gene in lettuce (Takada *et al.*, 2007). In petunia, silencing of the anther-specific *MEZ1* gene and the tapetum specific *TAZ1* gene has been used to induce sterility (Kapoor *et al.*, 2002; Kapoor and Takatsuji 2006) and recently overexpression of the *Cm-ETR1/H69A* gene from melon has been used in chrysanthemum (Shinoyama *et al.*, 2012).

Pollen development and release related genes such as *MS1* and *MYB26* from *Arabidopsis thaliana* (Yang *et al.*, 2007a; Yang *et al.*, 2007b) were used to attempt to modify these characteristics in *Lilium* plants. Both overexpression and gene silencing by RNAi of these genes was investigated in several *Lilium* cultivars, but principally in cv. Star Gazer. Although the desired phenotype of non-dehiscent anthers in lily flowers was not achieved either by overexpression or gene silencing, the apparent

delayed dehiscence showed by the RNAi lines suggests possible conserved gene function between *Lilium* and *Arabidopsis*. This conservation of gene function has previously been described for the *MS1* transcription factor from *Arabidopsis* and the orthologue gene in rice *OsPTC1* (Li *et al.*, 2011), and the *HvMS1* in barley (Fernandez 2011). However the alteration in anther formation/opening in this thesis is the first report in any *Lilium* cultivar or species of manipulation of anther development by a transgenic approach.

7.4.1 Conservation of pollen development pathways in *Lilium*.

With the objective of analysing the levels of synteny between *Lilium* and *Arabidopsis* pollen pathways, an attempt to find and sequence the putative orthologues of *AtMYB26* and *AtMS1* was conducted. Due to the lack of genome sequence for *Lilium*, a bioinformatics analysis to try to find putative orthologues of both genes in other species was initially conducted using several genome databases.

For the putative orthologue of the *AtMS1* gene, one partial sequence of approximately 500 bp was amplified in *Lilium* using primers designed based upon conserved regions from multiple sequence alignments. Blast analysis against the NCBI's *Lilium* nucleotide collection database did not show any matches. However, alignment of this partial sequence against other putative orthologues in other species showed that the identity between them was high. An identity of 81% with the *OsPTC1* gene from rice already reported as the *AtMS1* orthologue gene (Li *et al.*, 2011) and 100% with the putative orthologue in barley (AK372926) was found.

For the putative orthologue of the *AtMYB26* gene, two partial sequences were amplified in *Lilium* using primers designed using conserved regions from multiple sequence alignments. A contig from these two sequences

was constructed and aligned against the putative orthologues from other species. The results showed that the identity between them was very high reaching 99.6% with the barley putative orthologue. The partial sequence from lily covers approximately 88% of the gene from barley and has also a MYB domain in the same region as the other putative orthologues. This similarity suggests that there is a high probability that this partial sequence is the putative orthologue of the *AtMYB26* gene in *Lilium*. To obtain the full length sequence of both genes a RACE-PCR was performed. Unfortunately after several attempts the amplified and sequenced bands obtained did not match any of the putative orthologues of other species.

Future research should be aimed at analysing the transgenic plants obtained with the RNAi constructs for both the putative *LsgMS1* and *LsgMYB26* for a possible stronger phenotype than the one already obtained. The full length of both genes should be obtained so they can be characterized further and confirm whether they are the true orthologues of both genes in *Lilium*. One approach could be to use new primers designed on the already identified sequence from lily and pair them with primers designed in conserved regions from the multiple sequence alignments of the other putative orthologues with high genome identity such as barley. Another possibility could be to perform Targeted rapid amplification of cDNA ends (T-RACE) that has shown higher specificity in the amplification of cDNA ends from difficult templates. This utilises an oligo-dT adapter incorporating a dUTP-containing PCR primer and asymmetric PCR, followed by degradation of the non-target transcripts containing dUTP degraded by Uracil DNA glycosylase, leaving only those transcripts produced during the asymmetric PCR (Bower and Johnston 2010).

The use of next generation sequencing (NGS) could be a good approach to generate more info on the *Lilium* genome as several NGS methods recently developed allows larger-scale DNA sequencing. Currently, five NGS platforms are commercially available, including the Roche GS-FLX

454 Genome Sequencer (originally 454 sequencing), the Illumina Genome Analyser (originally Solexa technology), the ABI SOLiD analyser, Polonator G.007 and the Helicos HeliScope platforms. These new technologies have increased the speed and throughput capacities of DNA sequencing and this has dramatically reduced the overall costs of sequencing (Zhang *et al.*, 2011).

7.5 Conclusions

In conclusion, this work has developed and robust, rapid, highly efficient and reproducible system for the transformation of a range of lilium species and cultivars, which has utilised been utilised for the manipulation of traits of economic importance for *Lilium.* This transformation system and the transgenic germplasm that has been generated provide exciting opportunities for future improvements to *Lilium* and the horticultural industry.

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APPENDICES

APPENDIX 1 - Chemicals, reagents and growing medium.

1.1 Callus induction medium.

MS-CI1 (Per litre)		MS-CI2 (Per litre)		MS-CI3 (Per litre)		MS-CI4 (Per litre)	
4.4 g	MS Basal Salts	4.4 g	MS Basal Salts	4.4 g	MS Basal Salts	4.4 g	MS Basal Salts
30 g	Sucrose	30 g	Sucrose	30 g	Sucrose	30 g	Sucrose
12.3 µM	Picloram	1 mg	NAA	8.3 µM	Picloram	8.3 µM	Picloram
8g	Agar	0.1 mg	BAP	2.5 mg	Zeatin	1.25 mg	Zeatin
		8g	Agar	8 g	Agar	1.25 mg	Kinetin
						2.6 g	Phytagel

MS-CI5 (Per litre)		MS-CI6 (Per litre)		MS-CI7 (Per litre)		MS-CI8 (Per litre)	
4.4 g	MS Basal Salts	4.4 g	MS Basal Salts	4.4 g	MS Basal Salts	4.4 g	MS Basal Salts
30 g	Sucrose	30 g	Sucrose	30 g	Sucrose	45 g	Maltose
12.3 µM	Picloram	8.3 µM	Picloram	8.3 µM	Picloram	2.5 mg	Dicamba
2.5 mg	Zeatin	1.25 mg	Zeatin	1.25 mg	Zeatin	2.5 mg	Zeatin
2.6 g	Phytagel	1.25 mg	Kinetin	1.25 mg	Kinetin	2.6 g	Phytagel
		50 mg	Ascorbic acid	50 mg	Ascorbic acid		
		2.6	Phytagel	50 mg	Citric acid		
				2.6 g	Phytagel		

1.2 SOB liquid medium.

For 1 litre of Medium	
Yeast Extract	0.5%
Tryptone	2%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
ddH_2O	Complete to 1lt

The medium was then sterilised by autoclaving at 120°C for 16 minutes.

1.3 LB (Luria Broth).

For 1 litre of Medium	
Yeast Extract	0.5%
Tryptone	1%
NaCl	10 g
Bacto-agar	1.5%
ddH_2O	Complete to 1lt

Before being sterilized by autoclaving at 120°C for 16 minutes pH was adjusted to 7.0.

1.4 Semi-solid PDA (Potato dextrose agar) medium.

For 1 litre of Medium	
Potato extract	4 g
Dextrose	20 g
Agar	15 g
ddH_2O	Complete to 1lt

Before being sterilized by autoclaving at 120°C for 16 minutes pH was adjusted to 6.5.

1.5 DNA extraction buffer (Edwards *et al.* 1991).

For 1 litre of Medium	
Tris-HCl	200 ml
4M NaCl	62.5 ml
0.5M EDTA (pH 8)	50 ml
SDS 10%	50 ml
ddH_2O	Complete to 1lt

1.6 CTAB RNA extraction buffer (*Li et al.*, 2011).

Component	Concentration
CTAB	2% w/v
Tris-HCL pH 8.0	100 mM
EDTA pH8.0	20 mM
PVP	2% w/v
DEPC treated water	As necessary

1.7 Plasmid DNA extraction solution I, II and III.

Solution I (Per litre)		Solution II (Per litre)		Solution III (Per litre)	
50% Glucose	18 ml	10% SDS	100 ml	5M K Acetate	600 ml
1M Tris-HCl (pH 8.0)	25 ml	1N NaOH	200 ml	Glacial Acetic acid	115 ml
0.5 M EDTA (pH 8.0)	20 ml	ddH_2O	Complete to 1 lt	ddH_2O	Complete to 1 lt
ddH_2O	Complete to 1 lt				

1.8 TAE buffer.

For 1 litre of 50x Buffer	
Tris Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA (pH 8)	100 ml
ddH_2O	Complete to 1lt

1.9 TB buffer

For any volume of buffer	
PIPES	10 mM
$CaCl_2$	15 mM
KCl	250 mM

Adjust the pH to 6.7 and then add $MnCl_2$ to 55 mM, complete to final volume with ddH_2O , sterilize by filtration and store at 4 °

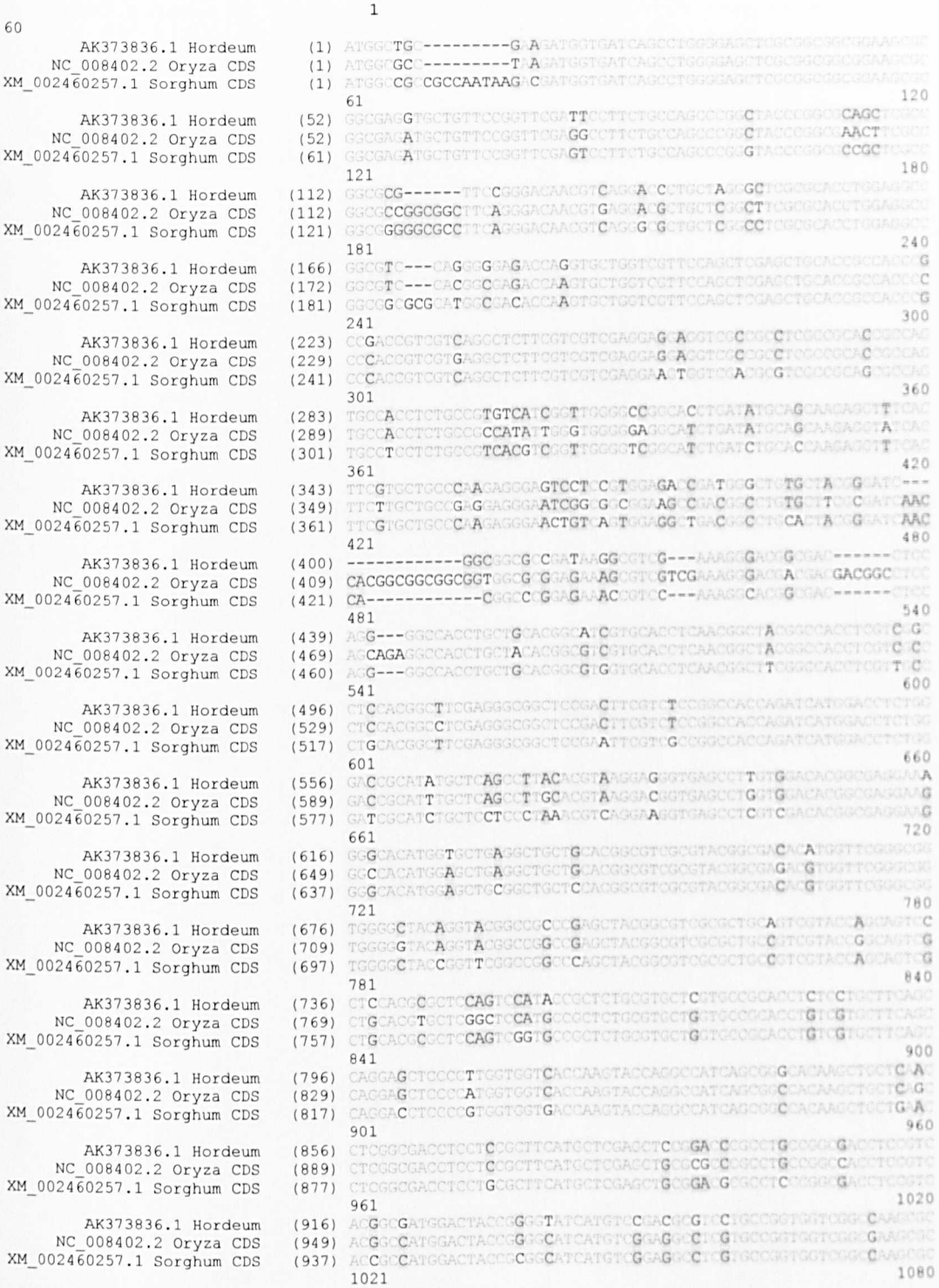
1.10 TBE buffer

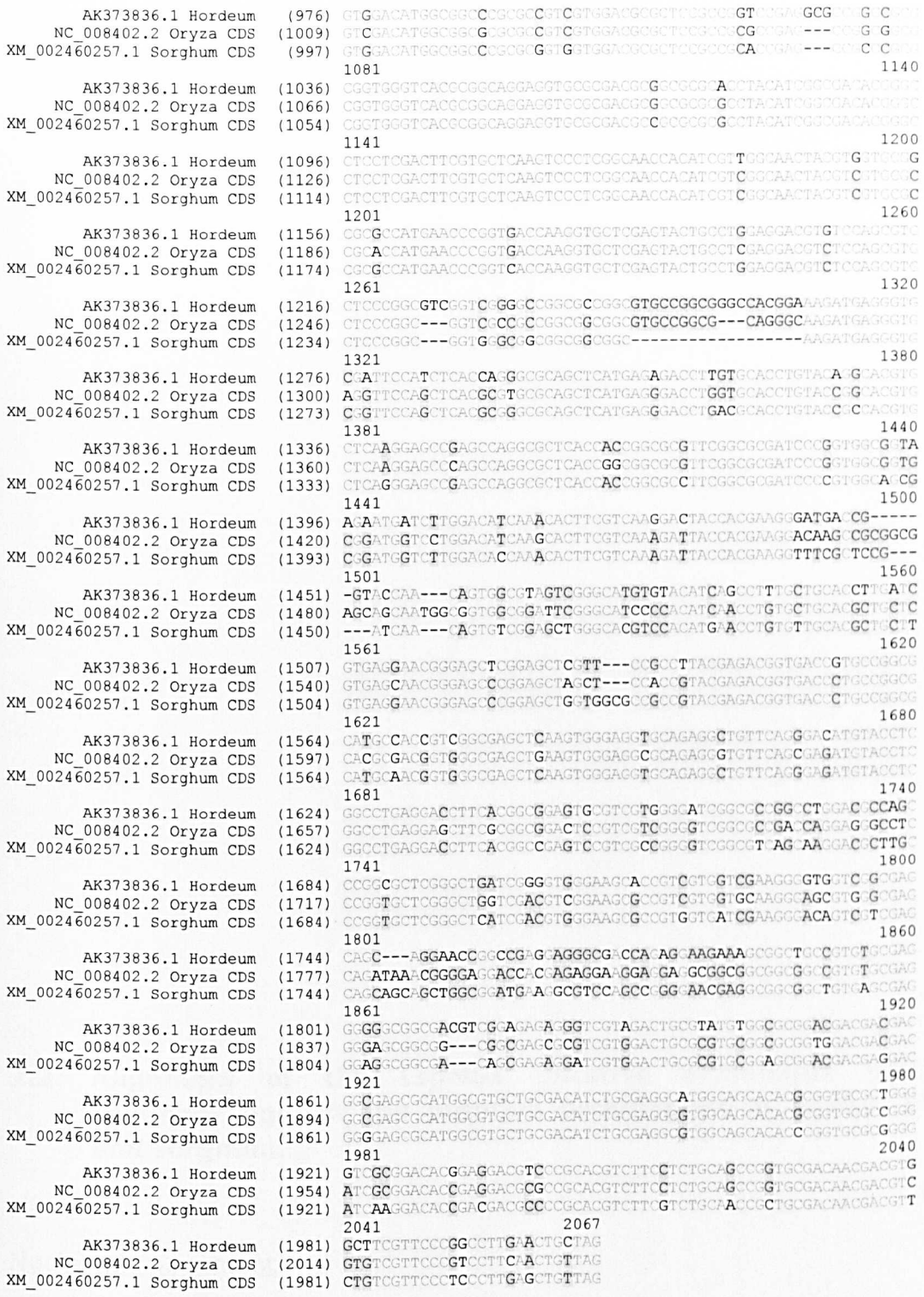
For 1 litre of 10x Buffer	
Tris base	108 g
Boric acid	55 g
Na_2EDTA 0.5M (pH 8.0)	40 ml
ddH_2O	Complete to 1lt

APPENDIX 2 - Multiple sequence alignments.

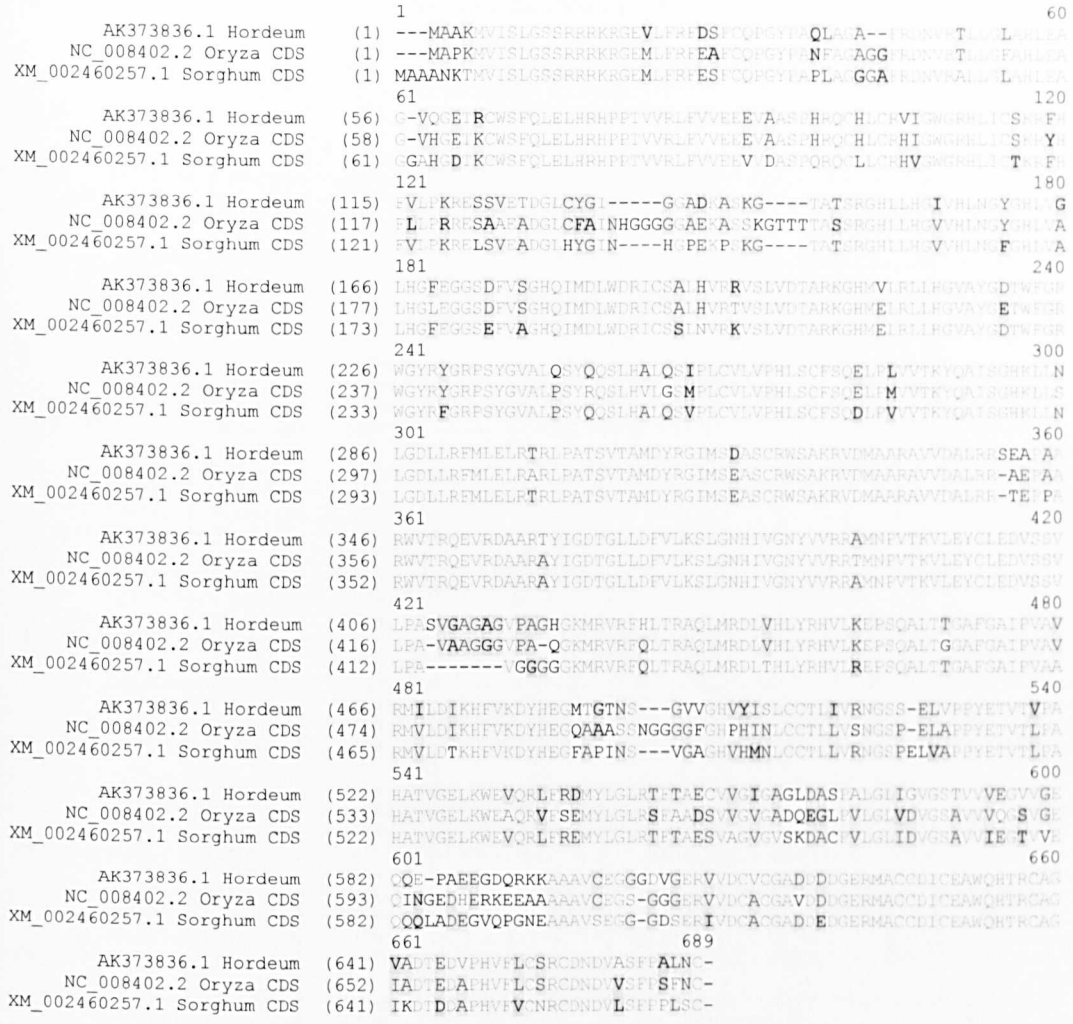
2.1 Alignment of the putative orthologues of *AtMS1* in barley, rice and sorghum to identify conserved regions.

Nucleotide alignment



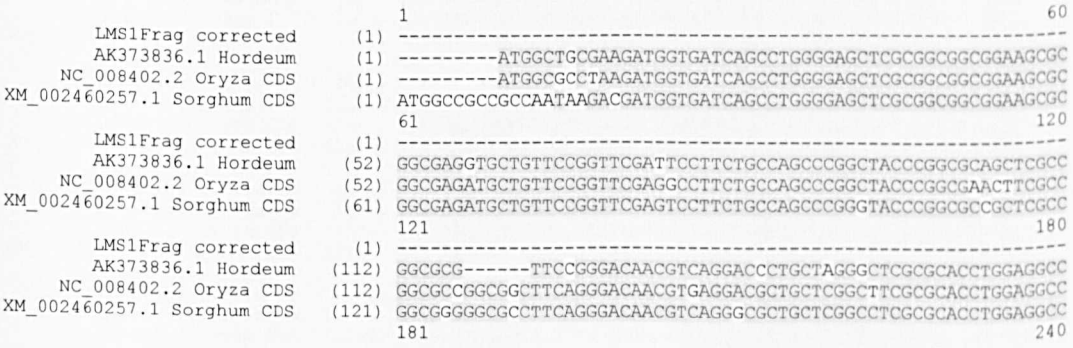


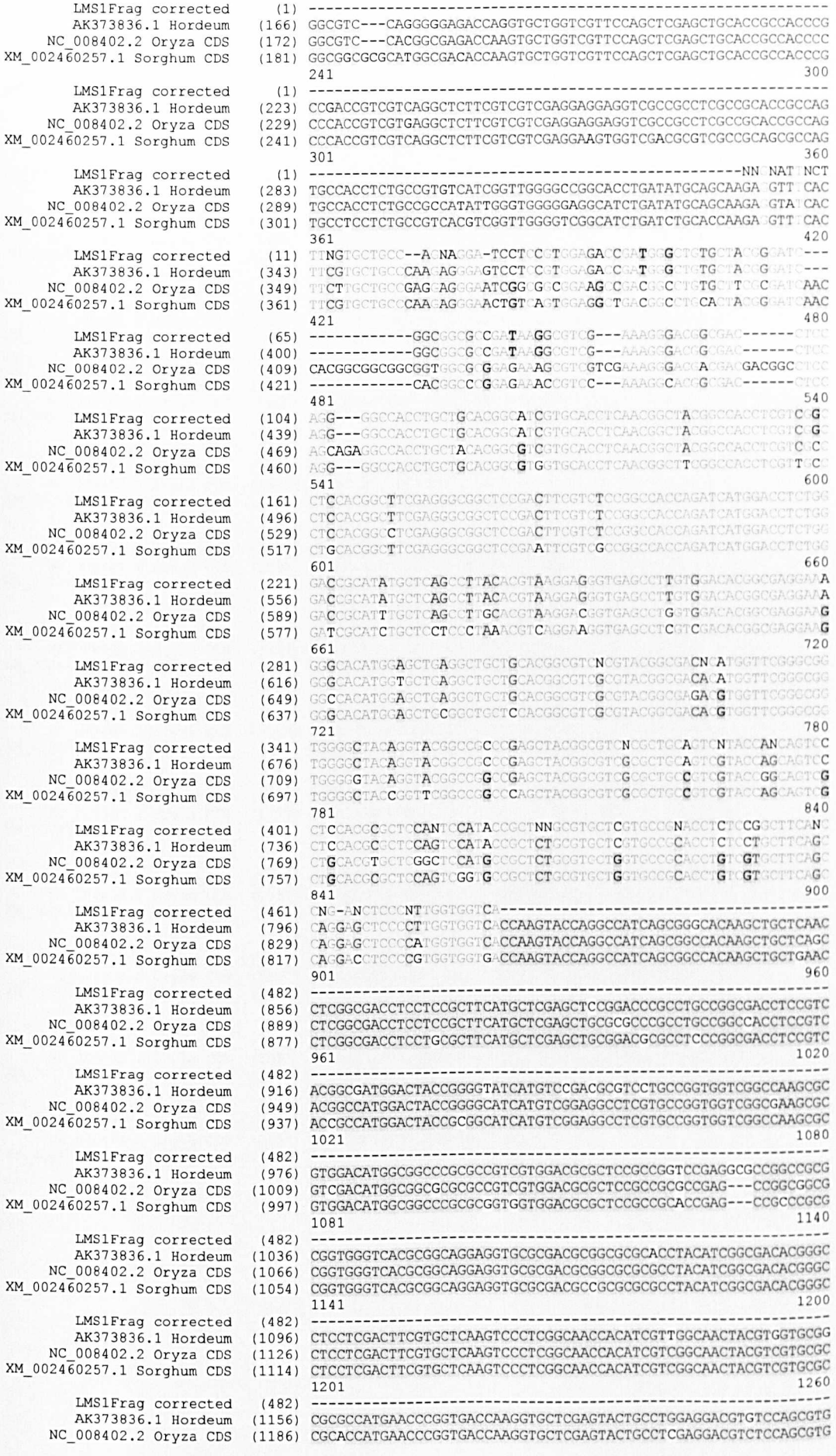
Protein alignment



2.2 Alignment of the *LsgMS1* putative orthologue partial sequence against the putative orthologues in barley, rice and sorghum.

Nucleotide alignment

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NC_008402.2 Oryza CDS
XM_002460257.1 Sorghum CDS

```
XM_002460257.1 Sorghum CDS  (1174) CGCGCCATGAACCCGGTCACCAAGGTGCTCGAGTACTGCCTGGAGGACGTCTCCAGCGTG
                                    1261                                                1320
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         AK373836.1 Hordeum  (1216) CTCCCGGCGTCGGTCGGGGCCGGCGCCGGCGTGCCGGCGGGCCACGGAAAGATGAGGGTG
      NC_008402.2 Oryza CDS  (1246) CTCCCGGC---GGTCGCCGCCGGCGGCGGCGTGCCGGCG---CAGGGCAAGATGAGGGTG
XM_002460257.1 Sorghum CDS  (1234) CTCCCGGC---GGTGGGCGGCGGCGGCGGC----------------AAGATGAGGGTG
                                    1321                                                1380
       LMS1Frag corrected    (482) ------------------------------------------------------------
         AK373836.1 Hordeum  (1276) CGATTCCATCTCACCAGGGCGCAGCTCATGAGAGACCTTGTGCACCTGTACAGGCACGTG
      NC_008402.2 Oryza CDS  (1300) AGGTTCCAGCTCACGCGTGCGCAGCTCATGAGGGACCTGGTGCACCTGTACCGGCACGTG
XM_002460257.1 Sorghum CDS  (1273) CGGTTCCAGCTCACGCGGGCGCAGCTCATGAGGGACCTGACGCACCTGTACCGCCACGTG
                                    1381                                                1440
       LMS1Frag corrected    (482) ------------------------------------------------------------
         AK373836.1 Hordeum  (1336) CTCAAGGAGCCGAGCCAGGCGCTCACCACCGGCGCGTTCGGCGCGATCCCGGTGGCGGTA
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XM_002460257.1 Sorghum CDS  (1333) CTCAGGGAGCCGAGCCAGGCGCTCACCACCGGCGCCTTCGGCGCGATCCCCGTGGCAGCG
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      NC_008402.2 Oryza CDS  (1540) GTGAGCAACGGGAGCCCGGAGCTAG---CTCCACCGTACGAGACGGTGACCCTGCCGGCG
XM_002460257.1 Sorghum CDS  (1504) GTGAGGAACGGGAGCCCGGAGCTGGTGGCGCCGCCGTACGAGACGGTGACCCTGCCGGCG
                                    1621                                                1680
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      NC_008402.2 Oryza CDS  (1597) CACGCGACGGTGGGCGAGCTGAAGTGGGAGGCGCAGAGGGTGTTCAGCGAGATGTACCTC
XM_002460257.1 Sorghum CDS  (1564) CATGCAACGGTGGGCGAGCTCAAGTGGGAGGTGCAGAGGCTGTTCAGGGAGATGTACCTC
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XM_002460257.1 Sorghum CDS  (1624) GGCCTGAGGACCTTCACGGCCGAGTCCGTCGCCGGGGTCGGCGTCAGCAAGGACGCTTGC
                                    1741                                                1800
       LMS1Frag corrected    (482) ------------------------------------------------------------
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      NC_008402.2 Oryza CDS  (1717) CCGGTGCTCGGGCTGGTCGACGTCGGAAGCGCCGTCGTGGTGCAAGGGAGCGTGGGCGAG
XM_002460257.1 Sorghum CDS  (1684) CCGGTGCTCGGGCTCATCGACGTGGGAAGCGCCGTGGTGATCGAAGGGACAGTCGTCGAG
                                    1801                                                1860
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XM_002460257.1 Sorghum CDS  (1744) CAGCAGCAGCTGGCGGATGAAGGCGTCCAGCCGGGGAACGAGGCGGCGGCTGTGAGCGAG
                                    1861                                                1920
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XM_002460257.1 Sorghum CDS  (1804) GGAGGCGGCGACAGCGAGAGGATCGTGGACTGCGCGTGCGGAGCGGACGACGAGGACGGG
                                    1921                                                1980
       LMS1Frag corrected    (482) ------------------------------------------------------------
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      NC_008402.2 Oryza CDS  (1897) GAGCGCATGGCGTGCTGCGACATCTGCGAGGCGTGGCAGCACACGCGGTGCGCCGGGATC
XM_002460257.1 Sorghum CDS  (1864) GAGCGCATGGCGTGCTGCGACATCTGCGAGGCGTGGCAGCACACCCGGTGCGCGGGGATC
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       LMS1Frag corrected    (482) ------------------------------------------------------------
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      NC_008402.2 Oryza CDS  (1957) GCGGACACCGAGGACGCGCCGCACGTCTTCCTCTGCAGCCGGTGCGACAACGACGTCGTG
XM_002460257.1 Sorghum CDS  (1924) AAGGACACCGACGACGCCCCGCACGTCTTCGTCTGCAACCGCTGCGACAACGACGTTCTG
                                    2041              2064
       LMS1Frag corrected    (482) ----------------------
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      NC_008402.2 Oryza CDS  (2017) TCGTTCCCGTCCTTCAACTGTTAG
XM_002460257.1 Sorghum CDS  (1984) TCGTTCCCTCCCTTGAGCTGTTAG
```
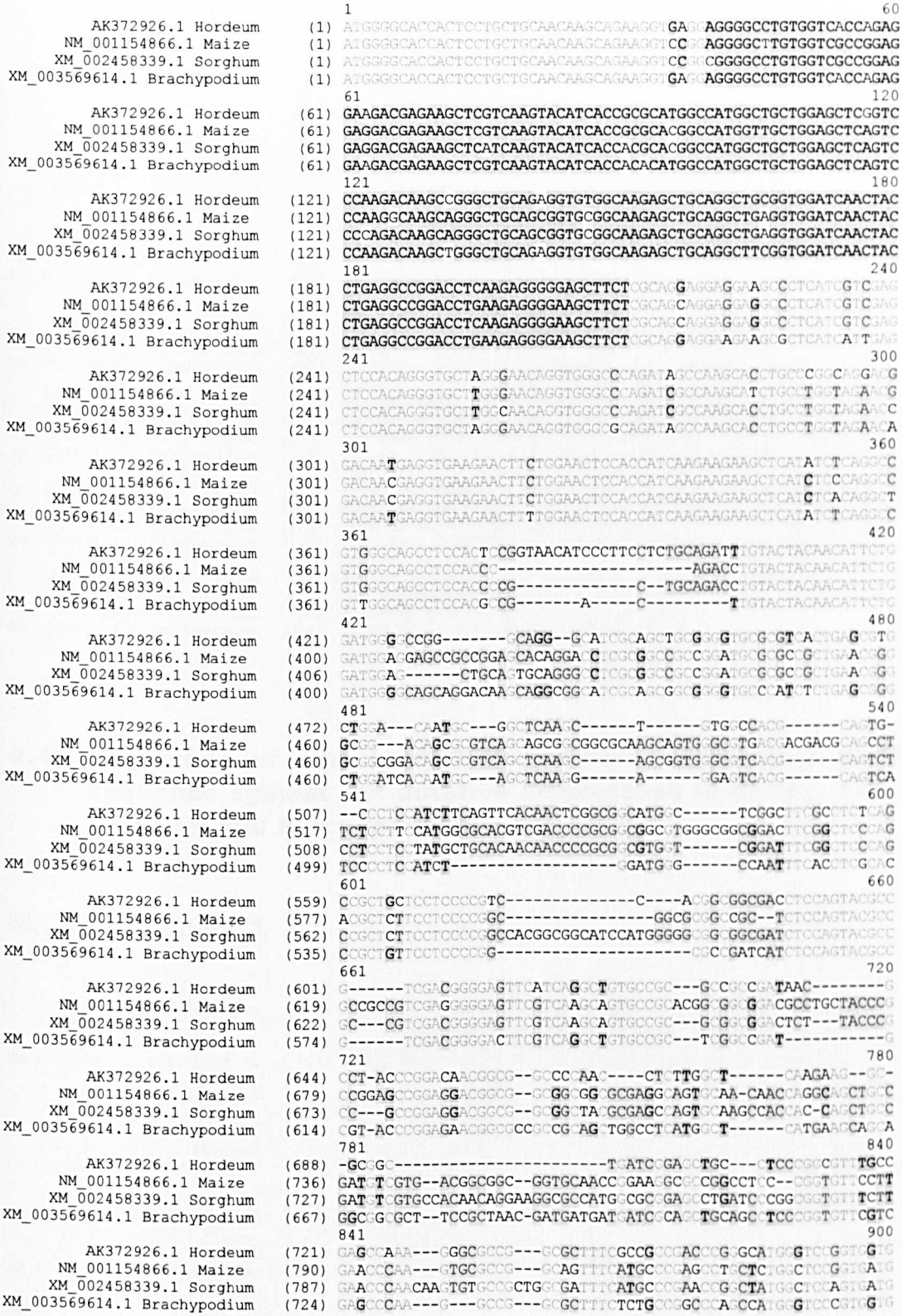
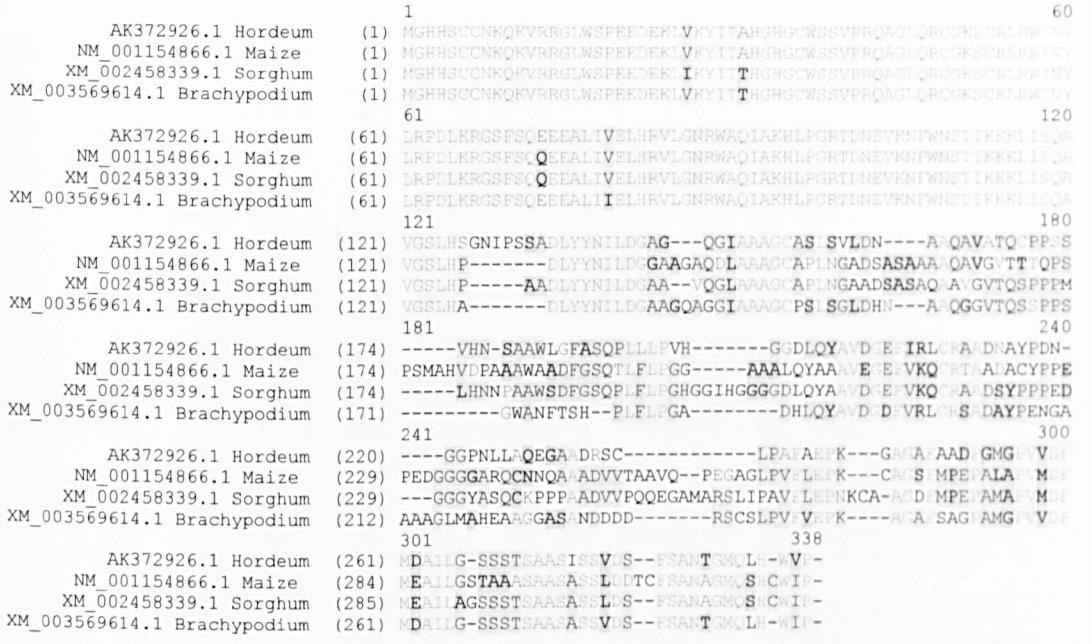

2.3 Alignment of the putative orthologues of *AtMYB26* in barley, maize, sorghum and *Brachypodium* to identify conserved regions.

Nucleotide alignment

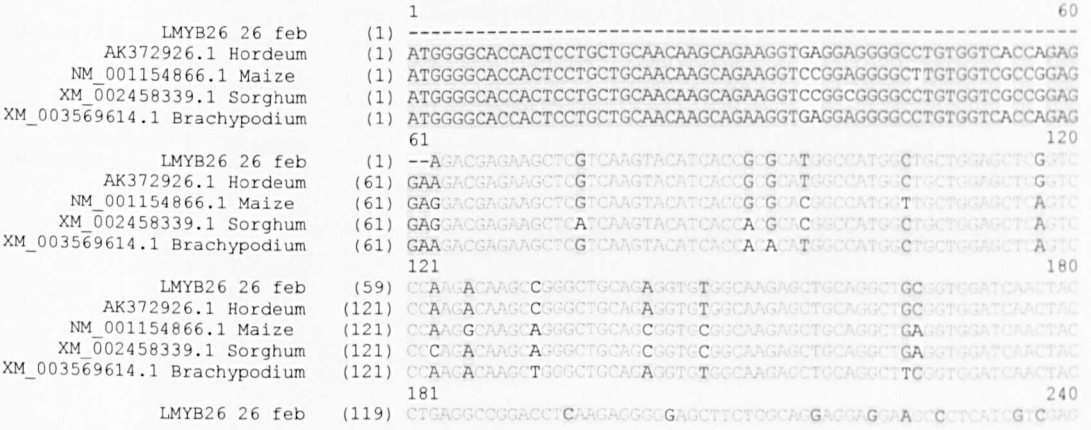


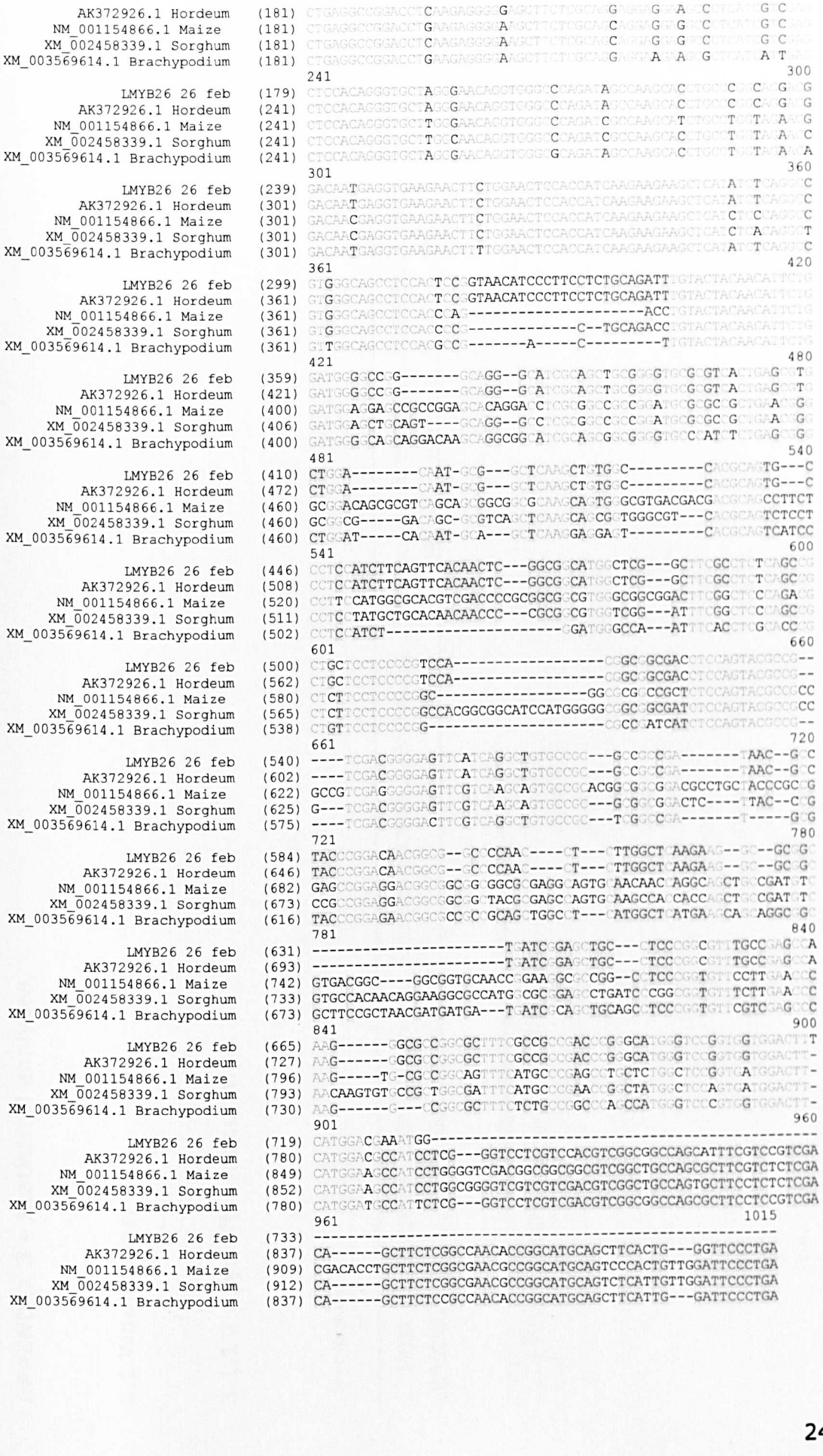
Protein alignment



2.4 Alignment of the *LsgMYB26* putative orthologue partial sequence against the putative orthologues in barley, maize, sorghum and *Brachypodium*.

Nucleotide alignment




AK372926.1 Hordeum
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XM_002458339.1 Sorghum
XM_003569614.1 Brachypodium
LMYB26 26 feb

3.1 Wound size.

3.1.1 7 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Wound size	30	0	30	4.420	7.620	5.961	0.910

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Wound size:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	0.848
Adjusted R^2	0.779
MSE	0.183
RMSE	0.427
MAPE	4.988
DW	2.942
Cp	10.000
AIC	-43.174
SBC	-29.162
PC	0.304

Analysis of variance:

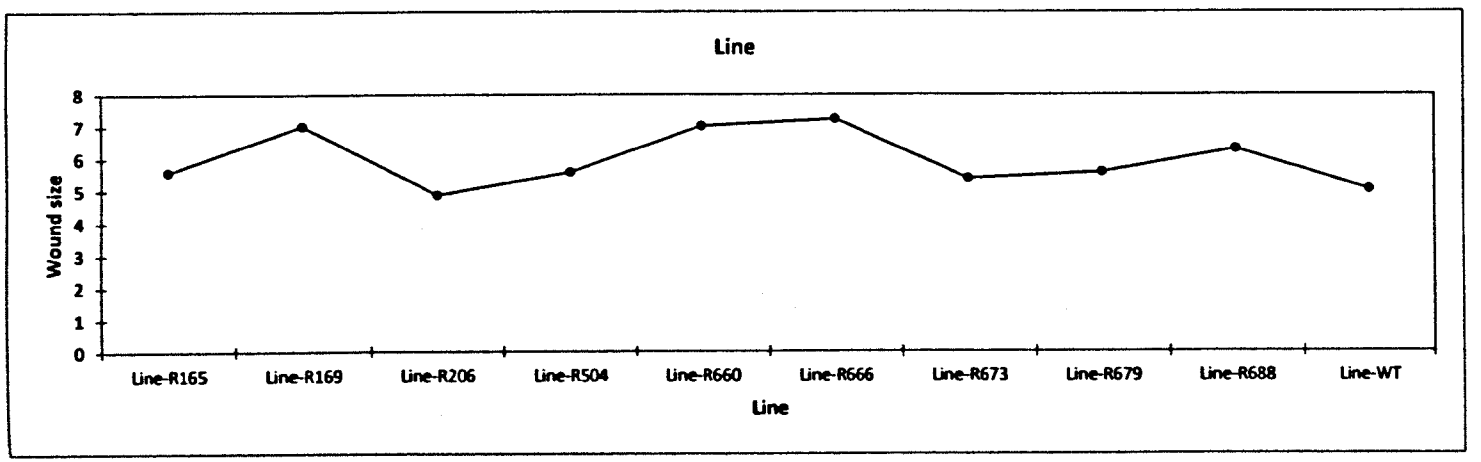
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	20.363	2.263	12.389	< 0.0001
Error	20	3.652	0.183		
Corrected Total	29	24.015			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	5.580	0.247	22.616	< 0.0001	5.065	6.095
Line-R165	0.000	0.000				
Line-R169	1.440	0.349	4.127	0.001	0.712	2.168
Line-R206	-0.717	0.349	-2.054	0.053	-1.445	0.011
Line-R504	0.003	0.349	0.010	0.992	-0.725	0.731
Line-R660	1.437	0.349	4.117	0.001	0.709	2.165
Line-R666	1.653	0.349	4.738	0.000	0.925	2.381
Line-R673	-0.193	0.349	-0.554	0.586	-0.921	0.535
Line-R679	0.003	0.349	0.010	0.992	-0.725	0.731
Line-R688	0.727	0.349	2.083	0.050	-0.001	1.455
Line-WT	-0.540	0.349	-1.548	0.137	-1.268	0.188

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R666	-2.193	-6.286	2.601	0.907	1.000	No
WT vs R169	-1.980	-5.675	2.601	0.907	1.000	No
WT vs R660	-1.977	-5.665	2.601	0.907	1.000	No
WT vs R688	-1.267	-3.630	2.601	0.907	1.000	No
WT vs R679	-0.543	-1.557	2.601	0.907	0.999	No
WT vs R504	-0.543	-1.557	2.601	0.907	0.999	No
WT vs R165	-0.540	-1.548	2.601	0.907	0.999	No
WT vs R673	-0.347	-0.994	2.601	0.907	0.993	No
WT vs R206	0.177	0.506	2.601	0.907	0.746	No

3.1.2 10 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Wound size	30	0	30	4.750	7.960	6.232	0.918

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Wound size:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	0.821
Adjusted R^2	0.741
MSE	0.218
RMSE	0.467
MAPE	4.992
DW	2.314
Cp	10.000
AIC	-37.810
SBC	-23.798
PC	0.357

Analysis of variance:

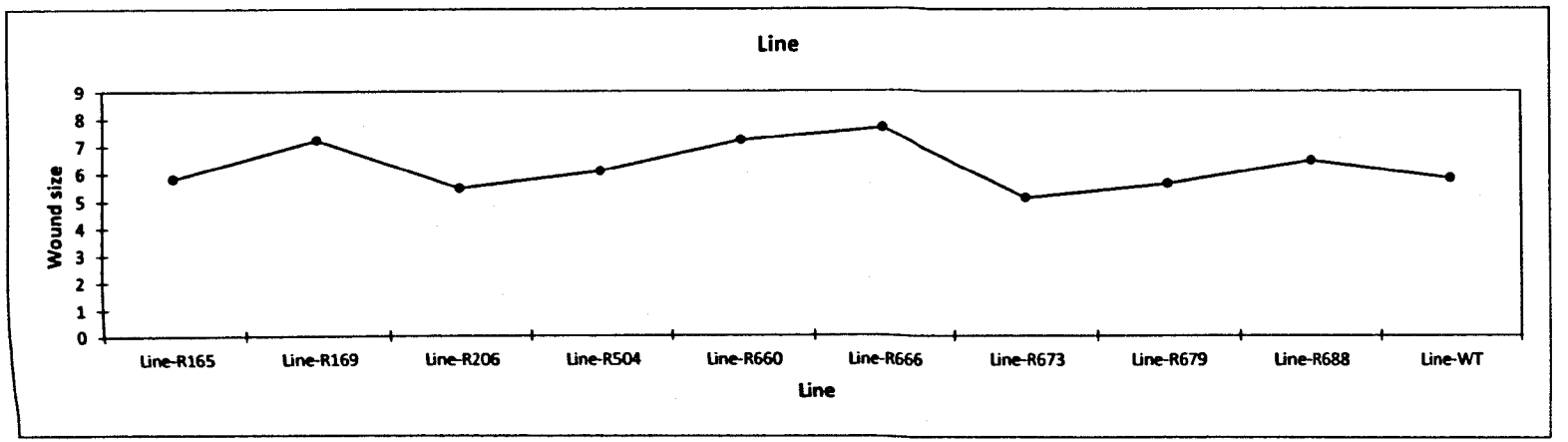
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	20.068	2.230	10.210	< 0.0001
Error	20	4.368	0.218		
Corrected Total	29	24.435			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	5.807	0.270	21.522	< 0.0001	5.244	6.369
Line-R165	0.000	0.000				
Line-R169	1.400	0.382	3.669	0.002	0.604	2.196
Line-R206	-0.363	0.382	-0.952	0.352	-1.159	0.433
Line-R504	0.260	0.382	0.681	0.503	-0.536	1.056
Line-R660	1.390	0.382	3.643	0.002	0.594	2.186
Line-R666	1.853	0.382	4.857	< 0.0001	1.057	2.649
Line-R673	-0.717	0.382	-1.878	0.075	-1.513	0.079
Line-R679	-0.207	0.382	-0.542	0.594	-1.003	0.589
Line-R688	0.647	0.382	1.695	0.106	-0.149	1.443
Line-WT	-0.007	0.382	-0.017	0.986	-0.803	0.789

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R666	-1.860	-4.875	2.601	0.992	1.000	No
WT vs R169	-1.407	-3.687	2.601	0.992	1.000	No
WT vs R660	-1.397	-3.660	2.601	0.992	1.000	No
WT vs R688	-0.653	-1.712	2.601	0.992	0.999	No
WT vs R504	-0.267	-0.699	2.601	0.992	0.983	No
WT vs R165	-0.007	-0.017	2.601	0.992	0.904	No
WT vs R673	0.710	1.861	2.601	0.992	0.183	No
WT vs R206	0.357	0.935	2.601	0.992	0.557	No
WT vs R679	0.200	0.524	2.601	0.992	0.739	No

3.1.3 14 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Wound size	30	0	30	4.920	9.250	6.743	1.091

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Wound size:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	0.867
Adjusted R^2	0.808
MSE	0.229
RMSE	0.479
MAPE	4.901
DW	2.208
Cp	10.000
AIC	-36.374
SBC	-22.362
PC	0.265

Analysis of variance:

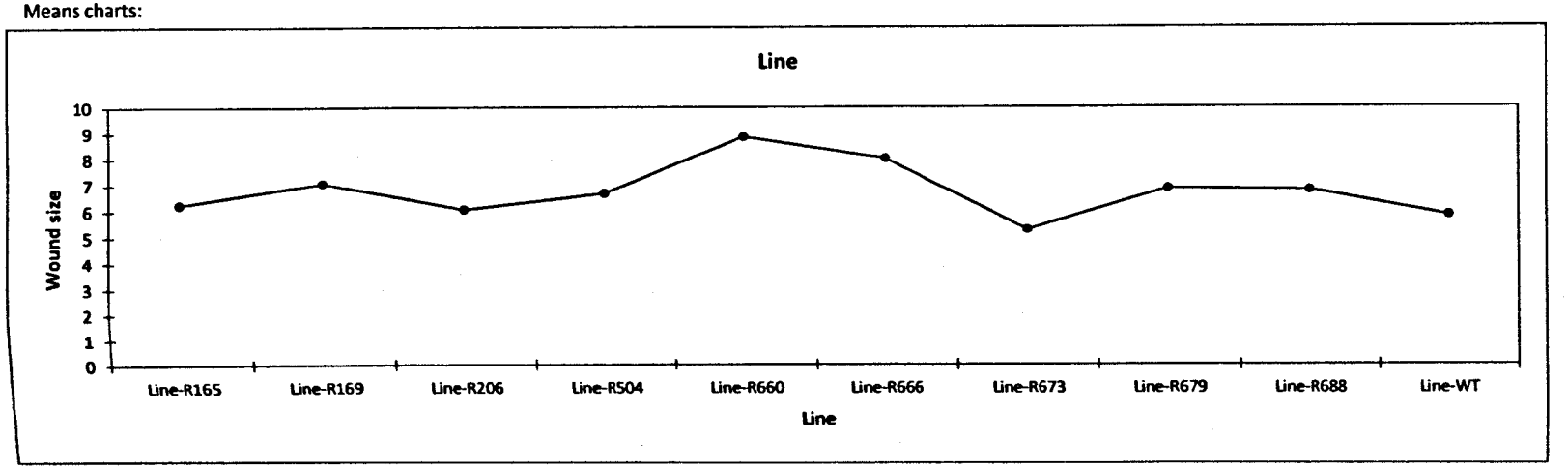
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	29.946	3.327	14.525	< 0.0001
Error	20	4.582	0.229		
Corrected Total	29	34.528			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	6.237	0.276	22.569	< 0.0001	5.660	6.813
Line-R165	0.000	0.000				
Line-R169	0.807	0.391	2.064	0.052	-0.009	1.622
Line-R206	-0.203	0.391	-0.520	0.609	-1.019	0.612
Line-R504	0.407	0.391	1.041	0.310	-0.409	1.222
Line-R660	2.603	0.391	6.662	< 0.0001	1.788	3.419
Line-R666	1.760	0.391	4.504	0.000	0.945	2.575
Line-R673	-0.993	0.391	-2.542	0.019	-1.809	-0.178
Line-R679	0.593	0.391	1.518	0.145	-0.222	1.409
Line-R688	0.533	0.391	1.365	0.187	-0.282	1.349
Line-WT	-0.440	0.391	-1.126	0.274	-1.255	0.375

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R660	-3.043	-7.788	2.601	1.016	1.000	No
WT vs R666	-2.200	-5.630	2.601	1.016	1.000	No
WT vs R169	-1.247	-3.190	2.601	1.016	1.000	No
WT vs R679	-1.033	-2.644	2.601	1.016	1.000	No
WT vs R688	-0.973	-2.491	2.601	1.016	1.000	No
WT vs R504	-0.847	-2.167	2.601	1.016	1.000	No
WT vs R165	-0.440	-1.126	2.601	1.016	0.995	No
WT vs R206	-0.237	-0.606	2.601	1.016	0.978	No
WT vs R673	0.553	1.416	2.601	1.016	0.339	No

3.2 Sporulation.

3.2.1 7 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Spores	30	0	30	1.000	321.000	42.500	91.444

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Spores:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	0.999
Adjusted R^2	0.998
MSE	14.733
RMSE	3.838
MAPE	20.113
DW	2.260
Cp	10.000
AIC	88.539
SBC	102.551
PC	0.002

Analysis of variance:

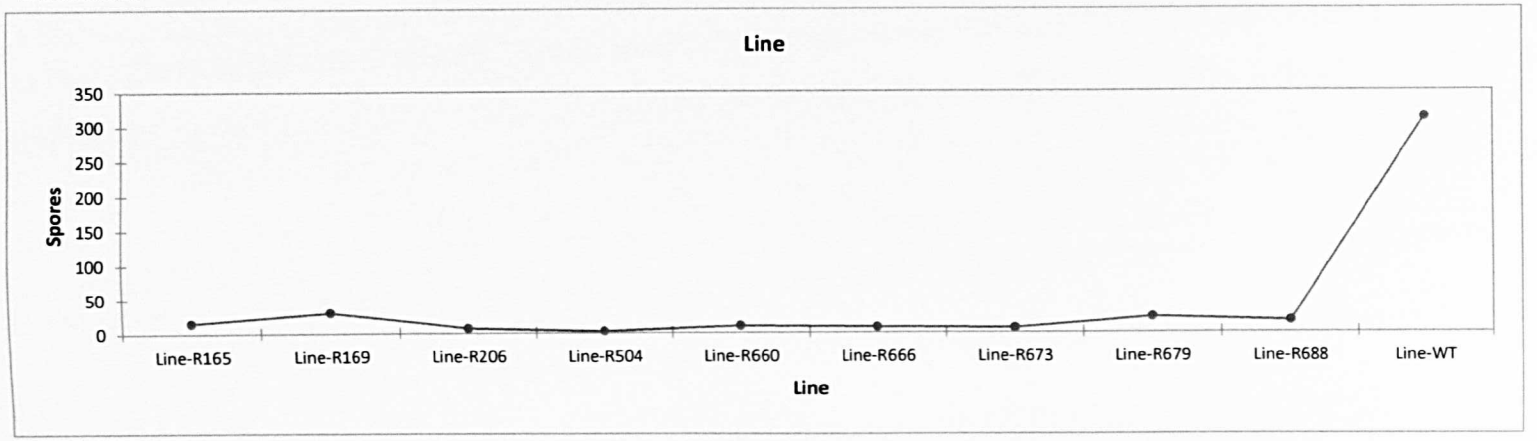
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	242202.833	26911.426	1826.567	< 0.0001
Error	20	294.667	14.733		
Corrected Total	29	242497.500			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	15.000	2.216	6.769	< 0.0001	10.377	19.623
Line-R165	0.000	0.000				
Line-R169	15.000	3.134	4.786	0.000	8.463	21.537
Line-R206	-8.333	3.134	-2.659	0.015	-14.871	-1.796
Line-R504	-13.000	3.134	-4.148	0.000	-19.537	-6.463
Line-R660	-5.667	3.134	-1.808	0.086	-12.204	0.871
Line-R666	-7.667	3.134	-2.446	0.024	-14.204	-1.129
Line-R673	-9.000	3.134	-2.872	0.009	-15.537	-2.463
Line-R679	6.667	3.134	2.127	0.046	0.129	13.204
Line-R688	1.000	3.134	0.319	0.753	-5.537	7.537
Line-WT	296.000	3.134	94.447	< 0.0001	289.463	302.537

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R504	309.000	98.595	2.601	8.151	0.000	Yes
WT vs R673	305.000	97.318	2.601	8.151	0.000	Yes
WT vs R206	304.333	97.106	2.601	8.151	0.000	Yes
WT vs R666	303.667	96.893	2.601	8.151	0.000	Yes
WT vs R660	301.667	96.255	2.601	8.151	0.000	Yes
WT vs R165	296.000	94.447	2.601	8.151	0.000	Yes
WT vs R688	295.000	94.128	2.601	8.151	0.000	Yes
WT vs R679	289.333	92.320	2.601	8.151	0.000	Yes
WT vs R169	281.000	89.661	2.601	8.151	0.000	Yes

3.2.2 10 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Spores	30	0	30	8.000	800.000	118.067	230.943

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Spores:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	1.000
Adjusted R^2	0.999
MSE	27.400
RMSE	5.235
MAPE	9.932
DW	2.582
Cp	10.000
AIC	107.152
SBC	121.164
PC	0.001

Analysis of variance:

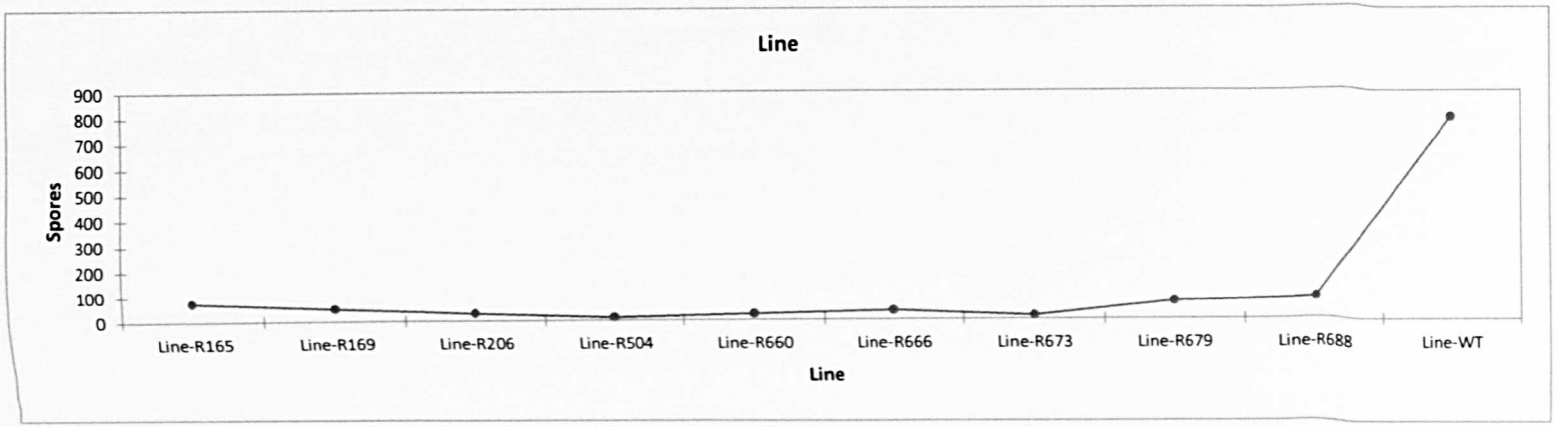
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	1546159.867	171795.541	6269.910	< 0.0001
Error	20	548.000	27.400		
Corrected Total	29	1546707.867			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	74.333	3.022	24.596	< 0.0001	68.029	80.637
Line-R165	0.000	0.000				
Line-R169	-25.333	4.274	-5.927	< 0.0001	-34.249	-16.418
Line-R206	-46.000	4.274	-10.763	< 0.0001	-54.915	-37.085
Line-R504	-63.000	4.274	-14.740	< 0.0001	-71.915	-54.085
Line-R660	-51.333	4.274	-12.011	< 0.0001	-60.249	-42.418
Line-R666	-39.333	4.274	-9.203	< 0.0001	-48.249	-30.418
Line-R673	-60.667	4.274	-14.195	< 0.0001	-69.582	-51.751
Line-R679	-5.333	4.274	-1.248	0.226	-14.249	3.582
Line-R688	7.333	4.274	1.716	0.102	-1.582	16.249
Line-WT	721.000	4.274	168.696	< 0.0001	712.085	729.915

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R504	784.000	183.437	2.601	11.116	0.000	Yes
WT vs R673	781.667	182.891	2.601	11.116	0.000	Yes
WT vs R660	772.333	180.707	2.601	11.116	0.000	Yes
WT vs R206	767.000	179.459	2.601	11.116	0.000	Yes
WT vs R666	760.333	177.899	2.601	11.116	0.000	Yes
WT vs R169	746.333	174.624	2.601	11.116	0.000	Yes
WT vs R679	726.333	169.944	2.601	11.116	0.000	Yes
WT vs R165	721.000	168.696	2.601	11.116	0.000	Yes
WT vs R688	713.667	166.981	2.601	11.116	0.000	Yes

3.2.3 14 days after inoculation.

Summary statistics:

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Spores	30	0	30	59.000	3645.000	485.800	1047.184

Variable	Categories	Frequencies	%
Line	R165	3	10.000
	R169	3	10.000
	R206	3	10.000
	R504	3	10.000
	R660	3	10.000
	R666	3	10.000
	R673	3	10.000
	R679	3	10.000
	R688	3	10.000
	WT	3	10.000

Regression of variable Spores:

Goodness of fit statistics:

Observations	30.000
Sum of weights	30.000
DF	20.000
R^2	0.999
Adjusted R^2	0.999
MSE	827.667
RMSE	28.769
MAPE	2.878
DW	2.816
Cp	10.000
AIC	209.394
SBC	223.406
PC	0.001

Analysis of variance:

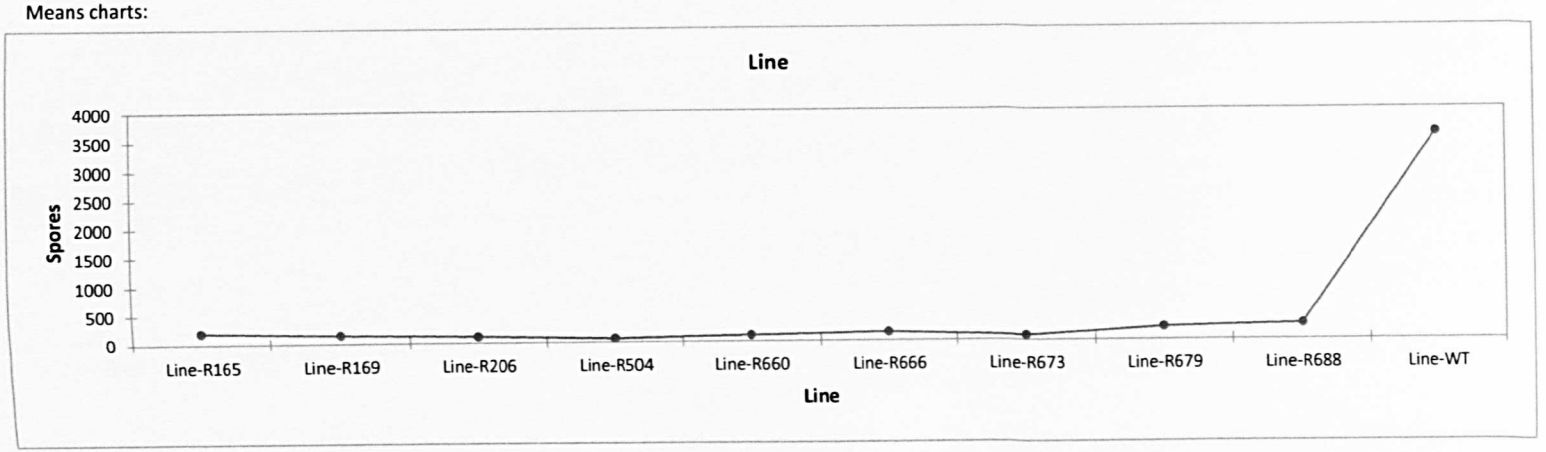
Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	9	31784661.467	3531629.052	4266.970	< 0.0001
Error	20	16553.333	827.667		
Corrected Total	29	31801214.800			

Computed against model Y=Mean(Y)

Model parameters:

Source	Value	Standard error	t	Pr > |t|	Lower bound (95%)	Upper bound (95%)
Intercept	193.667	16.610	11.660	< 0.0001	159.019	228.314
Line-R165	0.000	0.000				
Line-R169	-51.333	23.490	-2.185	0.041	-100.333	-2.334
Line-R206	-88.333	23.490	-3.760	0.001	-137.333	-39.334
Line-R504	-133.667	23.490	-5.690	< 0.0001	-182.666	-84.667
Line-R660	-94.333	23.490	-4.016	0.001	-143.333	-45.334
Line-R666	-54.333	23.490	-2.313	0.031	-103.333	-5.334
Line-R673	-120.333	23.490	-5.123	< 0.0001	-169.333	-71.334
Line-R679	18.667	23.490	0.795	0.436	-30.333	67.666
Line-R688	70.333	23.490	2.994	0.007	21.334	119.333
Line-WT	3374.667	23.490	143.664	< 0.0001	3325.667	3423.666

Means charts:



Line / Dunnett (right sided) / Analysis of the differences between the control category Line-WT and the other categories with a confidence interval of 95%:

Category	Difference	Standardized difference	Critical value	Critical difference	Pr > Diff	Significant
WT vs R504	3508.333	149.355	2.601	61.093	0.000	Yes
WT vs R673	3495.000	148.787	2.601	61.093	0.000	Yes
WT vs R660	3469.000	147.680	2.601	61.093	0.000	Yes
WT vs R206	3463.000	147.425	2.601	61.093	0.000	Yes
WT vs R666	3429.000	145.977	2.601	61.093	0.000	Yes
WT vs R169	3426.000	145.850	2.601	61.093	0.000	Yes
WT vs R165	3374.667	143.664	2.601	61.093	0.000	Yes
WT vs R679	3356.000	142.870	2.601	61.093	0.000	Yes
WT vs R688	3304.333	140.670	2.601	61.093	0.000	Yes

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