An investigation into the effects of smoke water and GR24 on the growth of *Nicotiana benthamiana* seedlings

by
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Thesis presented in partial fulfilment of the requirements for the degree
Master of Science in Plant Biotechnology
at the University of Stellenbosch

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December 2010

Declaration

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Date: October 2010

Summary

Plant growth promotion is a complex process and is often poorly understood. The demand for plant-derived biomass is increasing, whether to be used for animal and human consumption or for biofuel production. Biomass accumulation is closely linked to primary metabolism; any perturbation to this system often results in strong detrimental effects. Consequently, metabolism is tightly governed by regulatory control mechanisms. The screening and characterisation the effects of bioactive substances has therefore proven a useful alternative tool to investigate plant growth promotion.

Novel plant growth regulating substances (PGRs) are emerging as a useful tool to investigate important growth traits in plants. This study reports on growth promotion pathways leading to enhanced biomass accumulation in two PGRs sharing a common α, β-unsaturated furanone moiety. Growth promotion by GR24, a synthetic strigolactone, and an aqueous smoke solution (including the active compound, KAR_1) in physiologically normal seedlings was characterized by enhanced biomass accumulation and higher seedling vigour. Root architecture (lateral root number and root length) and shoot size (fresh and dry shoot weight and leaf area) were also dramatically improved following GR24 and smoke/KAR_1 treatment. Despite these apparent similarities, parallel transcript and phytohormone profiling identified only a limited number of overlapping entities. Four common up-regulated and nineteen down-regulated mRNA transcripts were identified; whilst amongst the phytohormones that were analyzed, only ABA and JA levels were commonly increased between the treatments. This suggests that, whilst the phenotypic end response(s) was similar, it was attained via distinct pathways. The limited number of co-expressed transcripts between these treatments, as well as repressed biomass accumulation when combining GR24 and aqueous smoke in a

single treatment suggests, however, that a certain degree of cross-talk in either signal perception/transduction and/or biomass regulation could not be ruled out.

In light of the structural similarity between the strigolactone and KAR_1 molecules and the degree of redundancy between these treatments, it is possible that these two molecules might share a common receptor/perception pathway. Two silencing vectors were constructed, specifically aimed at silencing *Nicotiana benthamiana* genes *MAX4* and *MAX2* which are known to function in the strigolactone biosynthesis pathway and signal transduction pathway, respectively. Transgenes designed to express single- or double-stranded-self- complementary hairpin RNA have a post translational gene silencing effect. The *pHELLSGATE2* plasmid a binary vector that incorporates GATEWAY cloning technology which makes use of λ-phage-based site specific recombination, rather than restriction endonucleases and ligation, was used to construct these gene silencing vectors. These constructs can in future be used to produce *Nicotiana* plants with impaired strigolactone production and perception abilities and may provide evidence as to whether the signaling cascade of KAR_1 and strigolactone share a degree of crosstalk.

Opsomming

Aanvraag na plantmateriaal is besig om toe te neem, hetsy vir gebruik as mens- en diervoeding of vir die produksie van biobrandstof. Om aan hierdie behoefte te voldoen, word verskeie pogings geloods wat fokus op die optimisering van plantproduksiestelsels.

Om plantgroei te stimuleer/verbeter, is ’n ingewikkelde proses en is oor die algemeen moeilik om te begryp. Die produksie van plantbiomassa is nou gekoppel aan primêre metabolisme en enige verandering in hierdie biochemiese padweë kan lei tot ongewenste newe-effekte. Gevolglik word primêre metabolisme streng beheer deur reguleringsmeganismes. ’n Nuttige alternatief tot metaboliese wysiging is deur bio-aktiewe agente te karakteriseer op grond van die veranderinge aan plantgroei wat waargeneem word.

Nuwe stowwe met biologiese aktiwiteite in plantontwikkeling word elke dag ontdek en speel ’n belangrike rol in die studie van plantgroei en -ontwikkeling. Hier word verslag gelewer van twee plantgroei-stimulerende stowwe wat albei lei tot die aktivering van verbeterde plantbiomassa-akkumulasie-padweë. Swaarder plantjies met ’n verhoogde oorlewingsvermoë is waargeneem in fisiologies normale saailinge wat met ’n sintetiese strigolaktoon (GR24) of met rookwater (met aktiewe bestanddeel, KAR_1) behandel is. Behandeling met hierdie twee stowwe het gelei tot soortgelyke plantbiomassa-akkummulasie- vermoë. Hierdie twee stowwe (GR24 en KAR_1) deel ’n ooreenstemmende molekulêre struktuur in die vorm van ’n α, β-onversadigde furanone-moieteit.

Ten spyte van die groeiverbeteringsooreenkomste, gesien in saalinge behandel met GR24 en rook/KAR_1, dui verskille in transkripsie- en hormoonprofiel op twee verskillende groeistimuleringspadweë. Saailinge wat gelyktydig behandel is met ’n kombinasie van die

twee stowwe het egter ’n stremming in groei getoon in vergelyking met die kontroleplantjies. Dit is egter waargeneem dat daar wel ’n mate van oorvleueling in die aantal transkripte was tussen die drie behandelinge, wat daarop dui dat die groei-regulerende padweë nie in totale onafhanklikheid funksioneer nie, maar wel sekere stappe deel.

Na aanleiding van die strukturele ooreenkomste tussen die strigolaktoon (GR24) en KAR_1 molekules en die mate van molekulêre kommunikasieoorvleueling word gepostuleer dat hierdie twee molekules dalk aan dieselfde reseptormodule kan bind of stimuleer. Om hierdie rede is twee geendempingsvektors geskep wat daarop gemik is om twee gene, *MAX2* en *MAX4,* in *Nicotiana benthamiana* uit te doof. Die *MAX2* geenproduk is betrokke in die kommunikasie en waarneming van die strigolaktoon en die *MAX4* geenproduk is betrokke by die vervaardiging van die hormoon.

Oordraagbare geen-kostruksies wat daarop gemik is om enkel- en dubbelstring selfkomplimentêre haarnaald-RNS te vorm, besit die vermoë om getranskribeerde geenprodukte te vernietig. Die *pHELLSGATE2* plasmied is ’n binêre vektor wat GATEWAY kloneringstegnologie gebruik, waar λ-faag gebaseerde setelspesifieke rekombinasie eerder as die tradisionele ligeringsreaksie gebruik word. Hierdie konstrukte kan gebruik word om transgeniese plantjies te skep waar die vermoë om strigolaktoon te maak of waar te neem, verloor of onderdruk is. Hierdie transgeniese plantjies kan gebruik word om te bepaal of die plantgroei-stimulerende vermoë van GR24 en rook/KAR_1 wel dieselfde padweë gebruik.

Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisor Dr Paul Hills, He has supported me throughout all the hours of work that has gone into this thesis with a lot of patience and knowledge with aside of smileys. I attribute the quality of my Masters thesis to his encouragement and support.

In the laboratory, I have been blessed with a many friends and a cheerful group of fellow students. Thank you to all Institute of Plant Biotechnology (IPB) students and staff who made the laboratory environment one of serious work and serious gossip.

I am very grateful to Prof Jens Kossmann (co-supervisor), and Dr Marna van der Merwe for their guidance and research inputs. Also, I am very grateful for the resources made available to my study by Prof Johannes van Staden, Prof Uwe Sonnewald and Mr Stephan Ferreira

The work presented in this thesis would not be possible without the support and love from my family, especially my mother, father and my huge little brother. You are my inspiration and motivation everyday. Mama, Pappa… ons het dit gemaak!

To the glue that kept me together, Carl, thank you for every special day. We made this journey together. Thank you for all the love, help and support through this endeavour. You have a special place on every page of this thesis. Tannie Lizette en oom Anton, Dankie vir al die ondersteuning en liefde, aanmoeding en mooi woorde wanneer dit die nodigste was.

I would like to thank the National Research Foundation (NRF) and Prof Jens Kossmann for funding this venture.

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List of Abbreviations

˚C	Degrees celsius
µg	Micrograms
µl	Microlitre
µM	Micromolar (10^{-6})
ABA	Abscisic acid
bp	Base pairs
BR	Brassinosteroid
cDNA	Complementary DNA
Ck	Cytokinin
CTAB	Cetyltrimethylammonium bromide
dH_20	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetic acid
EST	Expressed seqence tag
g	Gram
GA	Gibberellins
HCA	Hierarchical cluster analysis
IAA	Indole acetic acid
iP	Isopentenyladenine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
KAR_1	Karrikinolide $_1$
l	Litre
LRR	Leucine-rich repeat
m/v	Mass/volume
mg	Milligrams
min	Minute
MS	Murashige and Skoog nutrient medium
OD	Optical density
PCA	Principal component analysis

PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
s	Seconds
SA	Salicylic acid
TMS	Trimethylsilyl
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
v/v	Volume/volume
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
Z	Zeatin

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Chapter 1

General introduction

"For plants, the struggle for light, is the struggle for life"

(Epstein 1977)

1.1. Background

Plants are anchored by their roots, and so must adapt to a harsh and ever-changing environment. Extreme temperature fluctuations, mineral availability and soil moisture content make up only a small part of the challenges that plants have to face every day. Their ability to grow depends on their own photosynthetic and metabolic ability. The biomass accumulation capacity of a plant in the vegetative growth phase can therefore be regarded as a direct expression of its metabolic performance (Meyer *et al.*, 2007). Plants function as integrated systems, in which metabolic and developmental processes draw on common resource pools. The allocation of resources to plant developmental pathways, pathogen defence and storage compounds have to be very tightly regulated. The drain of metabolites into cellular components has to adjust to the capacity of the available resources to provide these metabolites without having detrimental effects on other systems such as pathogen response. This can be demonstrated by numerous observations of growth depression (Dietrich *et al.*, 2005) and reduction of primary metabolism (Gibon *et al.*, 2004) when the plant finds itself in less than favourable conditions. Thus, growth rate of the plant has to be adjusted to accommodate the current metabolic status of the plant and hence allocate resources accordingly. The metabolic status of the plant is closely linked to biomass and growth. The development of tools to alter, analyze and predict intrinsic metabolism will prove imperative

to future endeavours to develop bio-engineered crops with increased product quality and/or improved biomass production.

1.1.1 Developmental regulation of plant biomass production

In general, efforts aimed at plant growth promotion have three goals: to attempt to increase plant biomass, attempt to improve the quality of the plant product and to alter plant architecture to form a valuable phenotype. Plant biomass has been considered as a renewable source of energy for the production of fuel. Starch and sucrose from sugarcane are currently the main sources of monosaccharides for biofuel production (Somerville, 2007). Crops such as maize, rice, sorghum and sugarcane are also being considered as sources for the production of cellulosic biofuels because of their high biomass yield with low input of resources (Carroll and Somerville, 2009). Plant biomass also serves as one of the main energy sources for humans and animals. These two consumers create competition between resources that would inevitably cause controversy between whether biomass should be channelled towards the production of biofuel or for human and animal nutrition. Scientists therefore have to come up with new and innovative ways to improve plant biomass accumulation.

One approach to solving this dilemma could come from investigating the molecular and genetic mechanisms that underlie plant biomass accumulation and subsequently apply this information in the genetic engineering of plant species to produce a greater yield. The complex nature of plant biomass production has proven a worthy adversary in the search for intrinsic mechanisms to boost plant product quality and yield. To date, there have been many genes described that, when alternatively expressed, can increase plant biomass production and yield (Gonzalez *et al*., 2009 Van Camp, 2005). Transcriptional regulation has proven to

be an effective tool in the search for mechanisms to improve plant biomass accumulation. Van der Knaap *et al.* (2000) identified a *GROWTH REGULATING FACTOR* (*GRF*) gene family in rice which appears to encode novel transcription factors that have regulatory roles in stem elongation. However, in *Arabidopsis*, over-expression of *AtGRF1* and *AtGRF2* resulted in the production of larger cotyledons and leaves. Increased biomass was attributed to an increased cell size and indicated that the *AtGRF*s probably have a cell-expansion regulation function (Kim *et al.*, 2003).

The *Arabidopsis* regulatory gene *AINTEGUMENTA* (*ANT*) was shown to enhance organ size by maintaining meristem competence and therefore increasing plant organ cell number (Mizukami and Fischer, 2000). Loss of *ANT* function reduced the size of all lateral shoot organs by decreasing cell number. Conversely, gain of *ANT* function enlarged embryonic and all shoot organs, without affecting the external morphology of the cells, by increasing cell number in both *Arabidopsis* and tobacco plant shoots (Mizukami and Fisher, 2000.

The plant-specific NAC protein family (a class of transcription factors) also increases biomass production. Plants with increased *NAC1* expression levels produced more lateral roots (Xie *et al.*, 2000). Over-expression of *ATAF2*, another NAC transcription factor, also leads to an increased biomass. However, over-expression of *ATAF2* caused yellowing of the leaves and a higher susceptibility to the soil-borne fungal pathogen *Fusarium oxysporum* (Delessert *et al.*, 2005).

Another gene from *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*), delayed flowering by up to 36 days when expressed in *Nicotiana tobaccum* (Demura and Ye 2010; Salehi *et al.*, 2005).

These transgenic lines displayed increased leaf size and biomass yield and reduced height at flowering time.

TOR (TARGET OF RAPAMYCIN) kinase regulates numerous biological processes, including translation of ribosomal components (Deprost *et al.*, 2007). Reduction or increase in the levels of TOR kinase results in a dose-dependent decrease or increase, respectively, in cell and organ size, resistance to osmotic stress and seed production (Deprost *et al.*, 2007). When over- expressed, *AtTOR* from *Arabidopsis* resulted in enhanced root and shoot growth, with the increase in mass being attributed to an increase in cell size (Deprost *et al.*, 2007). These plant growth promoting genes are involved in various metabolic processes and our insight into the molecular changes that occur in these plants is still very limited (Van Camp, 2005). Systems-level studies are urgently needed to combine the presently fragmented data into one developmental framework in order to engineer crops with superior biomass accumulation.

1.1.2. Plant biomass production through bioactive growth promoting substances

Systems biology-driven approaches have revealed thus far that biomass accumulation is closely linked to primary central metabolism (Meyer *et al.*, 2007; Sulpice *et al.*, 2009). However, any perturbation to primary metabolism often results in strong detrimental effects (Trethewey *et al.*, 1998; Veljovic-Jovanovic *et al.*, 2001). Consequently, primary metabolism is tightly governed by regulatory control mechanisms (Hofmeyr and Cornish-Bowden, 2000). Screening and characterisation of bioactive substances have therefore proven a useful alternative tool to investigate plant growth promotion and, to date, a number of metabolites (or metabolite classes) to facilitate this have been identified. These include exudates from microorganisms, such as acetoin, 2,3-butanediol (Ryu *et al.*, 2003), opines (Piper *et al.*, 1993)

and lumichrome (Phillips *et al.,* 1999); exudates from plants, such as alkamides (Ramírez-Chávez *et al.,* 2004) and sphingolipids (Worrall *et al.,* 2003), as well as biologically-associated materials such as humic and fulvic acid (Dobbss *et al.,* 2007), and compounds from plant-derived smoke (Chiwocha *et al.,* 2009; Light *et al.,* 2010).

1.2. Motivation

The goal of this project was to expand on our existing knowledge of plant growth promotion through a molecular and physiological investigation into three plant growth promoting substances; smoke water, KAR_1 and the strigolactone analogue, GR24.

1.3. Layout and aims of the Chapters

This thesis is laid out as follows as a compilation of five chapters.

CHAPTER 2: Literature Review

Chapter 2 focuses on current knowledge of plant growth regulation through hormonal interactions and the recent developments in hormone perception and signalling. Secondly, strigolactones, a newly identified group of plant signalling chemicals, are discussed in the context of molecular studies describing the function that these chemicals have on plant growth and development. Thirdly, aqueous smoke is reviewed as a potent plant growth stimulator, of which the active constituent was identified as KAR_1.

CHAPTER 3: Strigolactone and aqueous smoke promote biomass accumulation via different signalling pathways

The plant growth promoting properties of strigolactone, smoke and KAR_1 are investigated, with focus on their physiological and metabolic effects on the model plant *Nicotiana benthamiana* in light of the structural similarities existing between these chemicals.

CHAPTER 4: Construction of *MAX2* and *MAX4* gene silencing vectors through RNAi to characterize growth response to strigolactone treatment

DNA vector based RNAi-technology was implemented in the construction of *pHG2-MAX2* and *pHG2-MAX4* silencing vectors. These vectors were constructed to specifically target the *MAX2* and *MAX4* homologues in *Nicotiana benthamiana* for future loss-of-function analyses to determine if the plant growth promoting functions of GR24 and smoke/KAR_1 channel their growth effects through the *MAX* pathway.

CHAPTER 5: General discussion

Aim: Through the use of current literature, observations and discussion from the previous chapters are examined. Plant growth promotion via strigolactone, smoke and KAR_1 treatment is discussed in the context of the available literature. Limitations of this study are discussed and recommendations for further studies are made.

Chapter 2

Plant growth promotion is a complex process and incorporates various physiological systems

"Ohne Wuchsstoff, kein Wachstum"

Translated, "Without growth substances no growth"

F.W. Went, early 1900

2.1. Introduction

The demand for more plant-derived product is increasing. The growing human population gives rise to increased consumption of animal products, which in turn require more feed. The demand for cellulose for the production of bio-fuels is finding itself competing against animal and human feed resources and this puts even more strain on already failing economies. This creates an opportunity for biotechnology to try to boost intrinsic yield and biomass production with the minimum input of fertilizer, water and agrochemicals. To achieve this goal, an understanding of normal plant growth and development will prove essential in order to optimize plant metabolism.

2.2. Plant growth and development is regulated by plant hormones

Work by Julias von Sachs, the father of plant physiology in the 19th century, demonstrated that small chemicals in the plant can move around from one part to another and influence physiological processes (Sachs, 1880). Von Sachs was the first to postulate that organ forming chemicals move through the plant in response to environmental signals such as gravity and light. This was said in a time where most scientists believed that nutritional

factors rather than plant growth substances were responsible for plant growth and development (Kraus and Kraybill, 1918; Arteca, 1996). Fitting (1910) first introduced the term *hormone* into plant physiology and the term has remained in use ever since, describing naturally occurring organic substances that have regulatory roles in plant development (Fitting 1910). Almost a century later, the definition of a phytohormone (or plant hormone) has been refined to describe a chemical present at very low concentrations which can act at or near the site of synthesis or can be transported to elicit a response in distant tissues (Davies, 2010; van Overbeek, 1954). Since their discovery, the pantheon of plant hormones has been steadily expanding (Figure 2.1) and now includes absisic acid (ABA), auxins, cytokinins, ethylene, salicylic acid (SA), gibberellins (GA), jasmonic acid (JA) and brassinosteroids (BR) (Davies, 1995; Browse, 2005; Vert *et al*., 2005; Loake and Grant, 2007).

Indole-3-acetic acid (IAA), the most important naturally occurring auxin, is synthesized from tryptophan or indole in leaf primordia, young leaves and developing seeds (Normanly *et al*., 2005). It is transported from apical meristems toward the roots in a polar manner. Levels of IAA vary dramatically throughout the plant body and life span, forming gradients which are crucial to its action (Benfey, 2002; Berleth *et al* 2000; Doerner, 2000; Hamann, 2001; Muday, 2001). Some of the functions of auxins include: cell expansion and stem growth, cell division, apical dominance, flowering and the delay of leaf senescence (Normanly *et al*., 2005). In the roots, auxins have an inhibitory role on root growth due to an interaction with ethylene (Normanly *et al*., 2005).

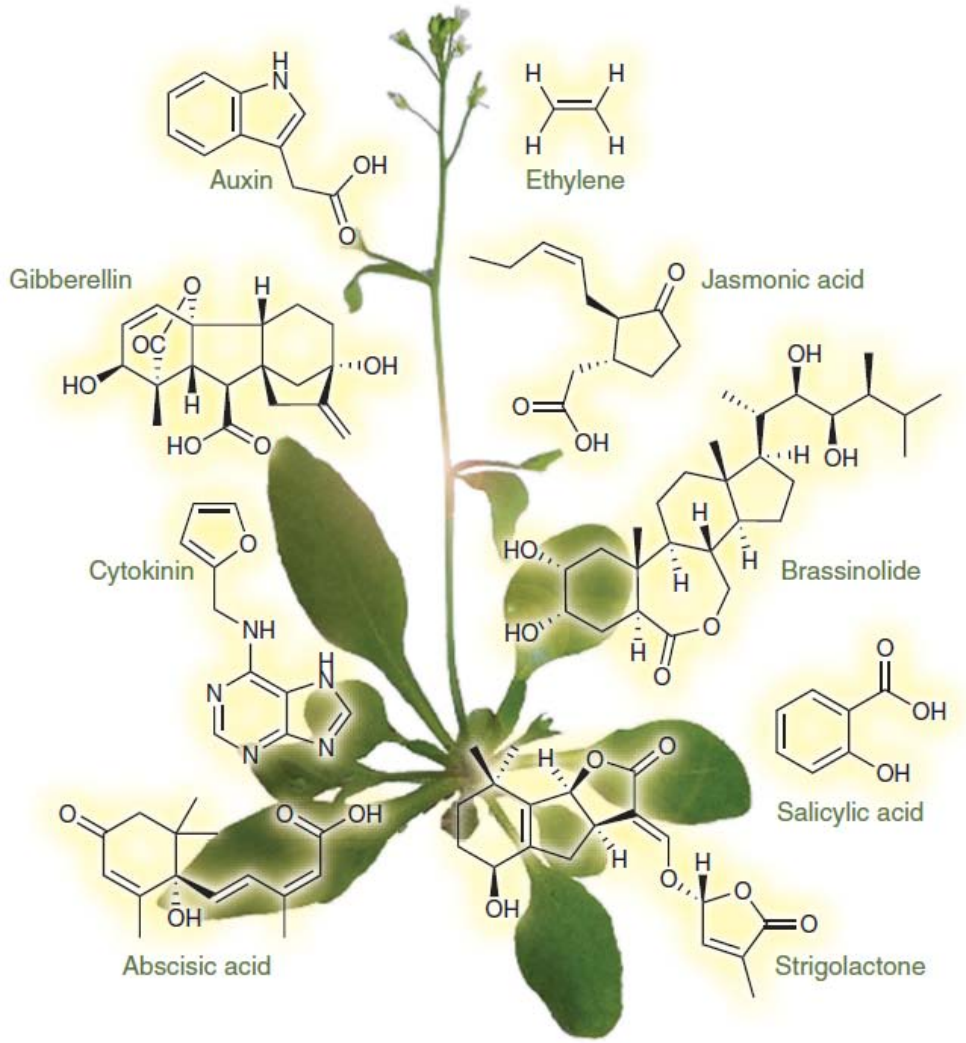


Figure 2.1. Chemical structures of some of the basic plant growth regulators or phytohormones: Ethylene, jasmonic acid (JA), brassinosteroids (BR), salicylic acid (SA) strigolactones (SL) absisic acid (ABA), cytokinins, gibberellins (GA) and auxins. There is considerable cross-talk between phytohormones, which also influence each others pivotal regulatory modules and in a dose-dependant manner regulate growth responses involved almost every aspect of plant development (Figure from Santner *et al*., 2009).

Cytokinins are adenine derivatives and in the presence of auxin induce cell division in tissue culture (Klämbt, 1992; Letham, 1963; 1983; Letham *et al.,* 1994). Cytokinins induce the growth of lateral buds and promote shoot initiation (Sakakibara, 2004). They have also been demonstrated to be involved in the delay of leaf senescence and have a role in chloroplast development, where cytokinin was demonstrated to promote the conversion of etioplasts in to chloroplasts (Parthier, 2004).

GAs are diterpenoids and are synthesized from glyceraldehyde-3-phosphate via isopentenyl-diphosphate in young tissues in the shoot and in developing seeds (Sponsel and Hedden, 2004). Effects include: stem growth through cell division and elongation, induction of seed germination, fruit setting and growth (Sponsel and Hedden, 2004).

Ethylene gas is synthesized from methionine in response to stress and is the hormone responsible for fruit ripening and senescence (Pech *et al*., 2004; Fluhr and Maltoo, 1996). Functions include: maintenance of the apical hook in seedlings, release from dormancy, adventitious root formation, leaf and fruit abscission and flower opening (Pech *et al*., 2004).
Abscisic acid (ABA) is synthesized in roots and mature leaves from glyceraldehyde-3-phosphate via isopentenyl diphosphate and carotenoids (Schwartz and Zeevaart, 2004; Seo and Koshiba, 2002). This hormone functions in stomatal closure, inhibition of shoot growth, induction of storage protein synthesis in seeds and also has roles in the induction and maintenance of seed dormancy (Schwartz and Zeevaart, 2004; Seo and Koshiba, 2002).
Brassinosteroids are a group of steroidal compounds that were first isolated from *Brassica* pollen (Choe, 2004; Mandava, 1988). This hormone has functions in cell division and elongation, vascular differentiation, fertility, inhibition of root growth and development. Brassinosteroids also promote ethylene biosynthesis and epinasty (Choe, 2004).

Jasmonates, particularly jasmonic acid, are synthesized from linolenic acid (Howe, 2004) and play an important role in plant defence against insect feeding. This hormone is also important in the onset of senescence, tuber formation, abscission, fruit ripening and pigment formation. Jasmonic acid has been demonstrated to have an important role in male reproductive development in *Arabidopsis* (Howe, 2004).

Lastly, SA, or salicylic acid, has a major role in plant pathogen response. It is involved in the Systemic Acquired Resistance (SAR) response in which pathogen attack on older leaves causes resistance in younger leaves. Salicylic acid has been reported to enhance flower longevity, inhibit ethylene biosynthesis and can also reverse the effects of ABA (Delaney, 2004).

It is clear that hormone levels are highly regulated and responsive to the changing environment (Santner *et al*., 2009; Vieten *et al*., 2007). Our understanding of hormone responses has increased dramatically over the last 15 years and includes the identification and characterization of several receptors from some of the major hormones and has lead to the emergence of several common signalling themes: Firstly, plant hormone receptors are diverse and distinct from those in animals (Spartz and Gray, 2008). In addition, regulated protein degradation is essential in hormone signalling and in the ubiquitin-protein conjugation pathway the hormone receptors may themselves be enzymes that target proteins for degradation by poly-ubiquitination (Arite *et al*., 2009; Smalle and Vierstra, 2004). Also, in hormone signalling, the levels of the downstream metabolites are regulated by ubiquitin dependant degradation. Hormone signalling leads to major changes in transcription levels (Santner and Estelle, 2009). Lastly, the synergistic behaviour of plant hormones regulates various growth and defence processes in the plant (Davies, 1995) (Figure 2.1).

2.3. The ubiquitin-proteasome system is a common theme in phytohormone signalling

All aspects of a plant's life are regulated by the synthesis of new polypeptides and the degradation of pre-existing ones. It is via this protein cycle that the plant maintains its supply of amino acids for new protein construction, removes redundant or abnormal proteins and dismantles existing regulatory networks (Hellmann and Estelle, 2002; Vierstra, 2003). By use of these tools, the plant can fine-tune its internal homeostasis to adapt to new environmental cues and conditions in order to direct growth and development to its ever-changing surroundings (Hellmann and Estelle, 2002; Vierstra, 2003). Many short-lived proteins are degraded in a ubiquitin (Ub)-dependant manner, shortly after polyubiquitination through the 26S proteasome, a 2 MDa protease complex (Smalle and Vierstra, 2004). These post-translational modifications to proteins are important processes used by plants to rapidly respond to environmental and intercellular signals. The (Ub)-26S proteasome pathway of protein degradation is most likely the dominant proteolitic system in plants (Smalle and Vierstra, 2004). This pathway entails the addition of a ubiquitin protein to a specific target protein. Ubiquitin is covalently attached to the target protein through the sequential action of three enzyme families, E1 (Ub activating enzyme), E2 (Ub conjugating enzyme) and E3 (Ub ligase) (Smalle and Vierstra, 2004; Somers and Fujiwara, 2009). Polyubiquitinated proteins (four or more Ub units) are recognized and then degraded by the 26S proteasome (Smalle and Vierstra, 2004). Ubiquitin is activated by E1 and conjugated to E2 in an ATP dependant manner. E1 has no function in substrate specificity, whereas E2 bound to the appropriate E3 presumably assists in targeting specific proteins to be degraded (Smalle and Vierstra, 2004).

The SCF ubiquitin-ligase complex is an E3 enzyme and functions as the scaffolds that bring together the activated Ub-E2 and the specific target protein without forming an E3-Ub intermediate (Smalle and Vierstra, 2004). The SCF ubiquitin-ligase complex is composed of

four polypeptides: CULLIN (CUL1), SUPRESSOR OF KINETOCHORE PROTEIN1 (SKP1), RING BOX1 (RBX1) and an F-box protein (Deshaies, 1999). CUL1 functions as the structure and backbone of the complex, binding RBX1 on the carboxyl terminus and SKP1 on the N terminus. The F-box protein interacts with the N terminus of SKP1 through an F-box motive on the protein (Figure 2.2).

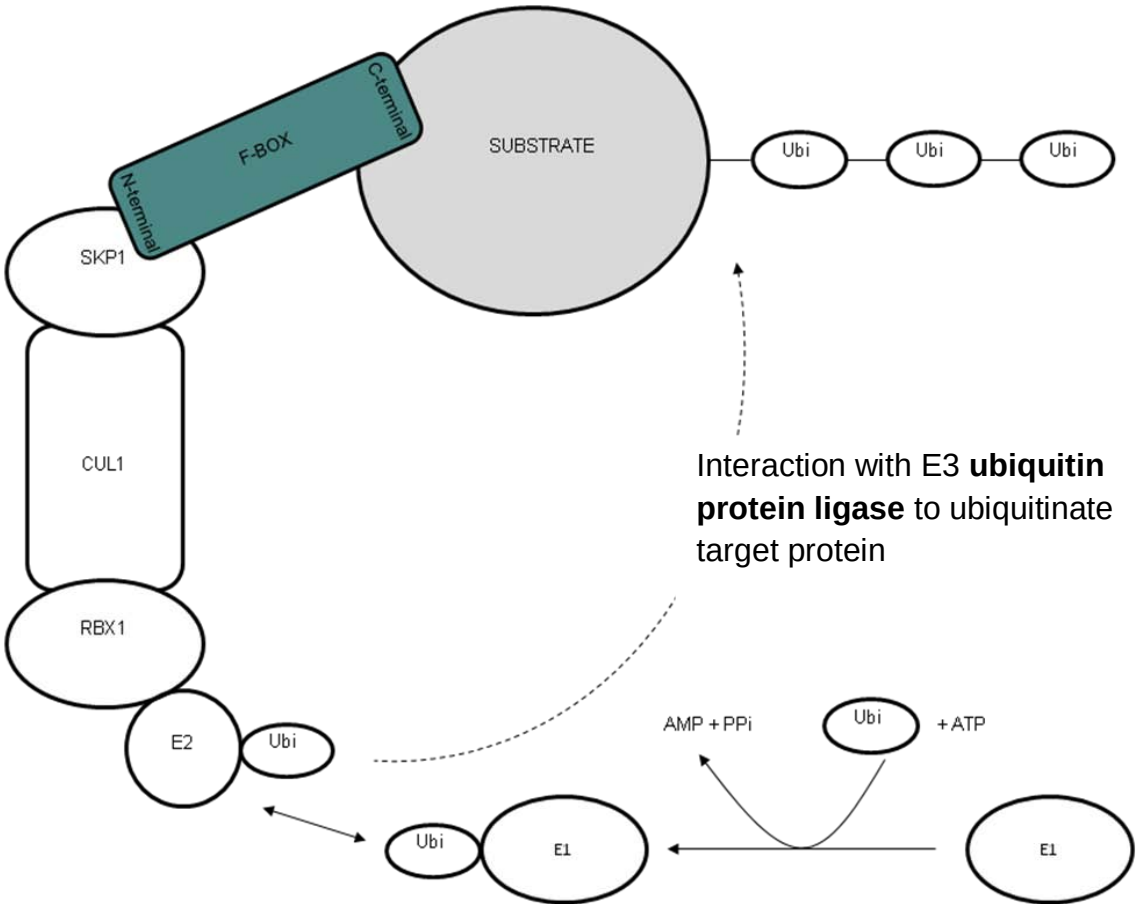


Figure 2.2. The SCF ubiquitin ligase-complex. This complex bring together all the SCF protein components in the correct formation. This complex recognizes and poly-ubiquitinates target substrate proteins to for degradation by the 26S proteasome.

The substrate specificity is conferred by a carboxyl terminal protein interaction motive that consists of kelch repeats, leucine-rich repeats (LLRs) or a WD40 domain. From the over 700 F-box proteins identified in *Arabidopsis*, it is evident that SCF complexes are used to identify a wide variety of substrate proteins (Gagne *et al*., 2002). It is clear from the number of genes dedicated to proteasome-dependant protein degradation that the regulation of proteolysis in plants bears great importance in physiology and development, especially during hormone perception (Hellmann and Estelle, 2002; Vierstra, 2003, Gagne *et al.*, 2002).

F-box proteins have been shown to be involved in many physiological responses (Table 2.1), ranging from hormone responses to the circadian clock, flowering time and pathogen defence (Smalle and Vierstra, 2004). Ubiquitin/26S proteasome-dependant protein degradation is directly and indirectly implicated in the signalling cascades of most major plant hormones (Hellmann and Estelle, 2002; Vierstra, 2003).

Molecular and genetic studies have helped bring about the acceptance of four more phytohormones into the traditional set of five plant hormones (auxins, gibberellins, cytokinin, ethylene, abscisic-acid). Brassinosteroids, jasmonic acid, salicylic acid and very recently, strigolactones have been shown to regulate plant development at very low concentrations and to act in a variety of plant tissues. Throughout these nine classes of growth regulators, biosynthetic pathways and molecular structures vary considerably. However, at least six of these share a common mechanism of regulation through the proteasome-dependant proteolytic pathway. These include, but are not limited to, auxins, gibberellins, abscisic acid ethylene, jasmonic acid and strigolactones (Smalle and Vierstra, 2004; Stirnberg *et al*., 2007).

Table 2.1. SCF E3 type F-box proteins and their individual substrates targeted for degradation by the Ub/26S proteasome pathway. These F-boxes are involved in various aspects of plant growth such as cell cycle, hormone regulation, responses to the biotic and abiotic environment and development. Adapted from Smalle and Vierstra, 2004.

	F-box protein	*Target protein(s)*	*References*
Cell cycle			
G1/S (Rb pathway)	SKP2	E2Fc	del Ponzo *et al*., 2002
Hormone regulation			
Auxin	TIR1	AUX/IAA family	Grey *et al*. 2001; Zenzer *et al*. 2001
Ethylene	EBF1 and 2	EIN3	Potuschak *et al*. 2003; Gagne *et al*. 2004
Gibberellins	SLY1	RGA	McGinnes *et al*. 2003
Gibberellins	GID2	SLR1	Sasaki *et al*. 2003
Jasmonic acid	COI1	RPD3b	Devoto *et al*. 2002
Responses to the abiotic environment (light)			
Red/far red	EID1	unkownn	Dietrele *et al*. 2001
Red/far red	AFR	unkownn	Harmon and Kay 2003
Blue (circadian)	FKF1, LKP2	unkownn	Nelson *et al*. 2000; Schultz *et al*. 2001
Blue (circadian)	ZTL	TOC1	Mas *et al*. 2003; Somers *et al*. 2000
Responses to the biotic environment			
NIM1 pathway	SON1	unkownn	Kim and Delaney 2002
Self-incompatibility	SFB	unkownn	Ushijima *et al*. 2003
Development			
Flower development	UFO/FIM/STP	unkownn	Samach *et al*. 1999; Zhao *et al*. 2001
Senescence/ branching	ORE9/MAX2	unkownn	Woo *et al*. 2001; Stirnberg *et al*. 2002

Genetic screens in *Arabidopsis* and in rice have identified GA-insensitive mutants resulting from mutations in genes encoding F-box proteins (McGinnis *et al*., 2003; Sasaki *et al.*, 2003). Gibberellins regulate the abundance of transcription repressors by promoting their ubiquitination via the SCF-type E3 ligases. In the case of GA signalling, nuclear-localized DELLA transcription factors function as repressors of GA-induced gene transcription (Schwechheimer, 2008). F-box proteins related to GA signalling have been identified in *Arabidopsis* as SLEEPY1 (SLY1) (McGinnis *et al.*, 2003) and in rice as GID2 (*GIBBERELLIN INSENSITIVE DWARF2*) (Sasaki *et al.*, 2003). Their associated SCF

complexes, SCF^{SLY1} and SCF^{GID2}, promote the degradation of the DELLA proteins such as REPRESSOR OF GA1-3 (RGA) and SLENDER RICE 1 (SLR1) respectively by the 26S proteasome. Rice GID2 and *Arabidopsis* SLY1 F-box proteins are closely related (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003 and Fu *et al.*, 2004). In response to GA, the $SCF^{GID2\backslash SLY1}$ targets the DELLA proteins for poly-ubiquitination and subsequent degradation by the 26S proteasome (Sasaki *et al.*, 2003).

GID1, an α/β hydrolase protein, is nuclear localized and was found to bind bioactive GAs in vitro, providing compelling evidence that GID1 is the GA receptor (Ueguch- Tanaka *et al.*, 2005). The GID1 receptor enhances the interaction between DELLA proteins and the F-box protein GID2 (Griffiths *et al.*, 2006). DELLA proteins are able to better interact with SCF^{GID2} while in complex with gibberellin-bound GID1 (Griffiths *et al.*, 2006). For gibberellin signalling, it is not the F-box protein that functions as a hormone receptor but the α-β hydrolase, GID1. GID1 belongs to the hydrolase super family, which shares similarity to hormone sensitive lipases (Ueguch- Tanaka *et al.*, 2005; Arite *et al.*, 2009).

One example of the F-box protein itself functioning as a hormone receptor can be seen in the auxin-induced degradation of AUX\IAA proteins. Auxin responses are primarily controlled by a family of short-lived nuclear localized repressor proteins, the AUX\IAA proteins, which block the auxin response transcription factors (ARFs), a DNA-binding protein family of transcription activators (Kepinski and Leyser, 2005). Auxin induces gene transcription by targeting these AUX/IAA proteins for degradation by the 26S proteasome. The *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) gene encodes an F-box protein that contains 16 degenerate leucine-rich repeats (LLRs) (Ruegger *et al.*, 1998). Auxin binds to the SCF^{TIR1} complex directly and promotes SCF^{TIR1} complex-AUX\IAA interaction (Kepinski and Leyser 2004).

The auxin-bound SCF^{TIR1} complex LRRs specifically recognize the conserved, proline rich Domain II in the AUX\IAA proteins. The poly-ubiquitinated AUX\IAA proteins are then degraded, permitting ARF-dependant transcription. It is thus clear that for auxins, the F-box protein *TIRI* serves as the auxin receptor.

Jasmonic acid signalling has only recently been connected to the Ub/26S proteasome pathway through the discovery of an essential F-box protein CORONATINE-INSENSITIVE1 (COI1) (Xu *et al.*, 2002). Jasmonic acid and its metabolites regulate a variety of biotic and abiotic stress responses as well as developmental processes such as senescence and reproductive development (Devoto and Turner, 2003). Jasmonic acid signalling responses are mediated in a remarkably similar way to auxin signalling. COI1 is closely related to TIR1 F-box protein and has been shown to assemble in an SCF^{COI1} complex (Xu *et al.*, 2002). Jasmonate ZIM-domain (JAZ) proteins are transcription factors that regulate the JA-mediated transcription (Chini *et al.*, 2007; Thines *et al.*, 2007 Yan *et al.*, 2007).

ABA controls many aspects of seedling development and mainly functions to arrest growth during adverse conditions such as drought or salt stress. In *Arabidopsis,* a key regulator of this post-germination growth arrest is the ABI5 protein, a bZIP transcription factor (Lopez-Molina *et al.*, 2001). The abundance of the transcription factor is increased by ABA signalling at both transcriptional and post-transcriptional levels (Lopez-Molina *et al.*, 2001), which inhibits its ubiquitination and turnover by the 26S proteasome. ABA possibly achieves this function by changing the phosphorylation status of the ABI5 protein (Lopez-Molina *et al.*, 2001).

2.4. Strigolactones regulate plant architecture through the MAX/RMS/DAD pathway

The pattern of shoot branching is one of the important determinants of plant aerial architecture. The regulation of plant architecture is of great importance to the plant's ability to adapt to its environment. The fate of axillary buds is determined by a complex interplay between environmental and endogenous signals. After germination, it is the primary meristems that give rise to the entire root and shoot systems (Natesh and Rau, 1984). The tissues that the primary meristems establish can give rise to secondary meristems which, if activated, can produce an entirely new axis of growth with the same developmental potential as the primary meristems from which they were derived (Natesh and Rau, 1984). The plant shoot system is formed from the primary shoot apical meristem early in development. It is this apical meristem that first initiates leaf formation at the node and the subsequent elongation of the stem (Natesh and Rau, 1984). The apical meristem produces repeating units of a node and a secondary meristem (Ongaro and Leyser, 2007). At the base of each leaf petiole, in the axils of the leaf, one or more secondary axillary meristems can develop. However, axillary meristems often form dormant buds after they have produced only a few leaves. These buds can later reactivate to produce lateral branches (Evans and Barton, 1997). In the presence of an intact shoot apex, lateral bud outgrowth is inhibited. Removal of the primary shoot apex allows the dormant axillary buds to activate and form lateral branches (Evans and Barton, 1997). With respect to shoot branching, it is the phytohormones that play a principal role in regulating secondary shoot meristem activity. Hence the plant body is continually being determined by the environment in a process regulated by the phytohormones.

The term apical dominance is used to describe the control of the shoot tip over axillary bud outgrowth (Cline, 1997). This process is best demonstrated by removal of the shoot tip

(decapitation) and has for years been used to study bud outgrowth. IAA, the most abundant type of auxin, is synthesized in the shoot apex (Thimann and Skoog, 1933; 1934; Ljung *et al*., 2001) and is transported basipetally, down the shoot, in a polar manner by active transport in the polar transport stream in the vascular parenchyma (Blakeslee *et al*., 2005) to inhibit bud outgrowth. Auxin transport is facilitated by at least three protein families. AUXIN INFLUX CARRIER PROTEIN1 (AUX1)\ LIKE-AUX1(LAX) proteins (Parry *et al*., 2004), the p-glycoprotein auxin efflux carriers (PGP), and the PIN-FORMED auxin efflux carriers (PIN) (Paponov *et al.*, 2005).

However, auxin, moving downward in the polar transport stream, does not enter the bud (Parsad *et al*., 1993; Booker *et al*., 2003) and auxin applied directly onto the bud does not inhibit bud outgrowth (Brown *et al.*, 1979; Cline, 1996; Leyser, 2003). *In vivo*, mutations in the *AUXIN RESISTANT1* (*AXR1*) gene renders *Arabidopsis* defective in auxin-regulated transcription and results in an increased-branching phenotype and buds that are resistant to apically applied auxin (Lincoln *et al.*, 1990; Booker *et al*., 2003). Taken together, these findings suggest that auxin acts indirectly to inhibit bud outgrowth. This indirect mode of action for auxin to inhibit bud outgrowth has led to the hypothesis that a secondary messenger carries the auxin signal into the bud and several candidates have been proposed (for a review, see Cline, 1991 and Napoli *et al*., 1999), of which the strongest contender was cytokinin.

Cytokinin also plays a role in shoot branching and has been shown to directly promote bud outgrowth (Cline, 1991). Cytokinin levels rise in activated buds (Turnbull *et al.*, 1997) and exogenously-applied cytokinin has been shown to activate axillary buds (Sachs and Thimann, 1967; Miguel *et al*., 1998) when directly applied to them, even in the presence of an intact

apex or apically-applied auxin (Sachs and Thimann, 1967). Cytokinins are synthesized in both roots and shoots (Chen *et al*., 1985; Nordström *et al.,* 2004) and move acropetally through the xylem to the bud (Emery *et al*., 1998). Auxin has been shown to be able to regulate the synthesis and export of cytokinin from the root (Bangerth, 1994; Li *et al*., 1995) and regulate its biosynthesis locally in the nodal stem (Nordström *et al*., 2004 ; Tanaka *et al*., 2006).

The classical apical dominance phenotype is typical of wild varieties of garden pea (*Pisum sativum),* where total apical dominance is observed during vegetative growth (Cline, 1997). Removal of the shoot apex by decapitation alleviates inhibition on lateral axillary buds, with resulting outgrowth of those buds. Decapitation reduces the levels of indole-3-acetic acid (IAA), which is produced in the shoot apex (Thimann and Skoog, 1933, 1934; Van Overbeek, 1938; Morris *et al*., 2005). The classical hypothesis further states that auxin content regulates shoot branching by influencing the levels, transport capacity and actions of secondary messengers such as cytokinin (Sachs and Thimann, 1967) to inhibit bud outgrowth (Hall and Hillmann, 1975; Morris, 1977; Bangerth, 1989).

Towards the end of the 20th century, outdated theories based largely on decapitation studies were pushed aside by many interesting discoveries made in mutants displaying highly branched phenotypes. It became obvious that in this particular class of branching mutants, displaying specific increases in bud outgrowth, IAA and cytokinin were not solely responsible for the increased branching (Beveridge *et al*., 1997). These mutants were identified in *Arabidopsis thaliana* as *more axillary branching* (*max*) (Stirnberg *et al.,* 2002; Turnbull *et al.,* 2002; Sorefan *et al.,* 2003), in petunia (*Petunia hybrida*) as *decreased apical dominance* (*dad*) (Napoli 1996; Napoli and Ruehle 1996; Napoli *et al.,* 1999), in pea (*Pisum*

sativum) as *ramosus* (*rms*) (Beveridge 2000; Morris *et al.*, 2001; Rameau *et al.*, 2002) and in rice (*Oryza stativa*) as *dwarf* (*d*) (Ishikawa *et al.*, 2005) (Table 2.2). Mutations in the above-mentioned genes result in auxin-resistant bud outgrowth and a subsequently branched phenotype.

Table 2.2. Mutations identified in the strigolactone signalling pathway in *Arabidopsis*, pea, petunia and rice plants

	Strigolactone biosynthesis			Signalling	
	Carotenoid cleavage dioxygenase 7 (CCD7)	Carotenoid cleavage dioxygenase 8 (CCD8)	Cytochrome p450	F-Box protein	Unknown
Arabidopsis	*MAX 3*	*MAX 4*	*MAX 1*	*MAX 2*	
Pea	*RMS 5*	*RMS 1*		*RMS 4*	*RMS3; RMS2*
Petunia		*DAD 1*			*DAD2; DAD3*
Rice		*D10*		*D3*	*D14; D27*

Extensive physiological examination and grafting experiments of these mutants revealed that a graft-transmissible, branch-inhibiting novel hormone was involved in the control of shoot branching. Analysis of these mutants indicated that they were all deficient in either the biosynthesis mechanisms or in the perception of the novel signal. The genes *MAX1*, *MAX3* and *MAX4* encode proteins that function as enzymes involved in the biosynthesis of signal. *MAX2* encodes a member of an F-box protein family that is often involved in phytohormone signalling and plays a role during the perception or signalling of the hormone (Stirnberg *et al.*, 2002; 2007). Equally, in pea, *RMS1* and *RMS5* are involved in the biosynthesis of the hormone while *RMS3* and *RMS4* have functions in the signal transduction pathway of the hormone (Morris *et al.*, 2001; Beveridge, 2000).

RMS1, *RMS4* and *RMS5* were shown to be orthologous to *MAX4, MAX2* and *MAX3* respectively (Sorefan *et al.,* 2003; Foo *et al.,* 2005; Johnson *et al.,* 2006). In petunia, *DAD1* was shown to be an orthologue to *MAX4 /RMS1* (Snowden *et al.,* 2005), implying that the branching regulatory pathway genes are well conserved across species. In rice, the tillering dwarf mutants, *dwarf3* (*d3*), *d10, d14, d17,d27* and *high tillering dwarf1* (*htd1*) were also identified as part of the branching mutant arsenal (Ishikawa *et al.,* 2005). Importantly, *D3* has been identified as an orthologue of *MAX2*/*RMS4*, and *D10* as an orthologue of *MAX4*/*RMS1*/*DAD1*. Further more, *htd1* and *d17* were identified to be mutant alleles of the rice orthologue to *MAX3/RMS5* (Zou *et al.,* 2006; Umehara *et al.,* 2008)(Table 2.2).

MAX3, RMS5 and *HTD1/D17* encode a carotenoid cleavage deoxigenase (CCD), CCD7 (Johnson *et al*., 2006; Zou *et al*., 2006; Booker *et al*., 2004; Umehara *et al*., 2008). *MAX4, RMS1, D10* and *DAD1* encode a second class of CCD, referred to as CCD8 (Sorefan *et al*., 2003; Snowden *et al*., 2005; Arite *et al*., 2007). It is believed that CCD7 and CCD8 catalyse sequential carotenoid cleavage reactions to produce the branching inhibition signal. *MAX1* encodes a cytochrome P450 protein and has been shown to act downstream of *MAX3/MAX4* to produce the signal compound (Booker *et al*., 2005). When mutants of these genes are grafted onto wild type root stocks, the branching phenotype reverts to a wild-type branching pattern.

The branched phenotype of the mutants *max2*, *rms4*, *dad2* and *d3* is can not be rescued by grafting onto wild type (WT) rootstock, which suggests that they do not perceive the branching inhibition signal. The *DAD2* gene, from petunia, is not orthologues to *MAX2, RMS4* and *D3* and has yet to be identified. *MAX2, RMS4* and *D3,* being orthologous F-box leucine-rich-repeat (LRR) proteins act as the substrate recognition subunit of a *SCF*

ubiquitin- E3 ligase which targets the substrate for proteolysis by the proteasome (Stirnberg *et al*., 2007; Lechner *et al*., 2006). The chemical identity of the branching signal was recently revealed, by Umehara *et al*. (2008) and Gomez-Roldan *et al*. (2008), to be a strigolactone. These authors demonstrated that the application of strigolactone to mutants rescued the phenotypes (revert back to the wild type phenotype) of the biosynthesis mutants, *d10*/*rms1/max4* and *d17*/*rms5/max3* but did not restore the signalling mutants (*d3*/*rms4/max2*) to a wild type branching pattern (Umehara *et al*., 2008; Gomez-Roldan *et al*., 2008). It was confirmed that endogenous strigolactone levels were reduced to undetectable levels in *d10* and *d17* mutants, in contrast to elevated levels of strigolactone in the *d3* and *max2* mutant root exudates. The elevated levels of strigolactone in the root exudates of the *max2* mutants were attributed to feedback regulation (Umehara *et al*., 2008). The Mutant max2, not being able to respond to its own strigolactone, produced elevated levels of strigolactones in order to compensate for its inability to sense it.

Strigolactones have traditionally been described as sesquiterpene lactones. The structural backbone of the molecules is a tricyclic lactone ring structure and is connected by an enol-ether bridge to an α, β-unsaturated furanone moiety referred to as the D-ring (see Figure 3.1). The first natural biologically-active strigolactone to be identified, Strigol, was isolated from cotton root exudates (Cook *et al.,* 1966). Strigolactones were initially discovered on account of their role as an important germination cue for parasitic weed species like *Striga* spp. and *Orobanche* spp. (Butler, 1995). Parasitic weeds are a serious problem to agriculture in many parts of the world and are responsible for huge crop losses (Joël, 2000; Press *et al*., 2001; Shen *et al*., 2006). The seeds of these plants lie dormant in the ground until they are triggered to germinate by a chemical signal exuded from the roots of their host plants (Press, 1995; Butler 1995). These seeds only carry a small amount of stored reserves and need to establish

a xylem connection with the host plant within a few days or the seed will perish (Parker and Riches, 1993; Press and Gurney, 2000; Musselman, 1987). The interaction between host and parasite begins when a mixture of secondary metabolites is exuded from the roots of the host plant, thereby triggering the germination of the parasite seeds (Hirsch *et al*., 2003; Bouwmeester *et al*., 2003). Several germination-stimulating compounds have been identified from root exudates and are collectively referred to as strigolactones. Strigol was the first to be identified as a *Striga* spp. germination cue in the false-host cotton, *Gossypium hirsutum* (Cook *et al*., 1966. Strigolactones were later identified in maize (Siame *et al*., 1993), in sorghum (Hauck *et al*., 1992, Siame *et al*., 1993) and in millet (Siame *et al*., 1993). Sorgolactone is also a member of the strigolactone family and was first identified in sorghum (Hauck *et al*., 1992). Germination stimulants have been identified for *Orobanche* spp. and these compounds are also classified as being strigolactones. Orobanchol and alectrol, germination stimulants for *O. minor,* were isolated from red clover roots (Yokota *et al*., 1998). All of these compounds share a structural similarity, even though they were isolated from a wide variety of host (crop) sources, and are obviously derived from the same biosynthetic pathway (Bouwmeester *et al*., 2003).

2.5. Plant derived smoke water promotes plant growth

Fire shapes ecosystems. It is a major environmental selective force in many plant communities and in particular in the Fynbos biome of South Africa. Plant-derived smoke and smoke water have for decades been used as a seed primer for agricultural crops such as maize (Modi, 2002; 2004). Smoke has demonstrated to stimulate various plant species over many different taxa from all continents (excluding Antarctica) over the world, ranging from Mediterranean-type vegetation to desert, alpine and wetland ecosystems (Crosti *et al.*, 2006; Pierce *et al.*, 1995; Marsden-Smedley *et al*., 1997; Roche *et al*., 1997). Smoke is now widely

recognized as a germination stimulator in seed not only from fire-prone environments, but also from non-fire dependant ecosystems (Jäger *et al.*, 1996; Light *et al.*, 2005; Light and van Staden, 2004). Smoke has also been demonstrated to stimulate germination in commercially important crop plants such as rice (Kulkarni *et al*., 2006), maize (Soós *et al*., 2009; Sparg *et al*., 2006), tomatoes, bean and okra (Van Staden *et al*., 2006) and a variety of South African medicinal plants (Sparg *et al*., 2005). In addition to the germination-stimulating abilities of smoke, it has also been demonstrated that smoke has a post-germination effect and can increase seedling viability and vigour. In a study conducted by Baxter and van Staden (1994), on the perennial grass species, *Themeda triandra* (Red grass), seedling vigour was stimulated without any morphological abnormalities. A similar effect was reported for *Erica* and Asteraceae species (Brown *et al.*, 2003). Smoke water has also been shown to stimulate flowering (Keeley, 1993), rooting (Taylor and van Staden, 1996) and somatic embryogenesis (Senaratna *et al.*, 1999). More recently, Sparg *et al*. (2006) has shown that in addition to its germination function in maize, smoke can also enhance seedling vigour, making the seedling taller with enhanced root development.

The major active ingredient in smoke, Karrikinolide (KAR_1), was identified by two groups in 2004 as a butenolide (3-methyl-2H-furo[2,3-c]pyran-2-one) (Flematti *et al.*, 2004, Van Staden *et al.*, 2004). In addition, a synthetic KAR_1 was produced which demonstrated potent germination activity at concentrations as low as 10^{-9}M (Flematti *et al*., 2005), providing definitive proof of structure. Karrikins represent a new class of bioactive plant growth regulating compounds which are structurally related to the naturally occurring butenolides. A range of karrikins have been produced to date, however, responses to treatment varies across species (Flematti *et al.*, 2007; Goddard-Borger *et al.*, 2007; Sun *et a*l., 2008).

2.6. General themes investigated in this thesis

Strigolactone and smoke/KAR_1 treatments have been previously demonstrated to be powerful germination stimulants for a wide range of species. For smoke/KAR_1, in addition to having a germination stimulating ability, a broad range of other functions in young seedlings have also been identified. Given the data above, it would be interesting to elucidate a function for strigolactone in the early developmental stages of plant growth. Could the strigolactone also promote post-germination in the same way as KAR_1? These two chemicals share structural identity and may induce similar plant growth responses in seedlings. This study aims to elucidate the physiological and molecular response of *Nicotiana benthamiana* seedlings to smoke/KAR_1 and GR24 treatment.

Also, as second objective, this thesis reports on the construction of silencing vectors containing transgenes of the *MAX2* and *MAX4* gene sequences. These constructs could be implemented to further characterize the *MAX* pathway in *Nicotiana benthamiana*, in order to determine if the plant growth promotion through GR24 and smoke\KAR_1 treatment works through the *MAX* strigolactone signalling pathway or not. Given the structural similarity between GR24 and KAR_1, it is hypothesized that the plant growth enhancement signalling cascades may be brought on by both chemicals acting on the same receptor entity.

Chapter 3

Strigolactone and smoke promote plant growth through different signalling pathways

"Every great advance in natural knowledge has involved the absolute rejection of authority."

Thomas Henry Huxley, biologist (1825-1895)

3.1. Introduction

Smoke from burning vegetation (aerosol smoke or aqueous smoke extract) has proven to be a widely-recognized germination cue for several plant species from both fire-prone and non-fire-prone environments (De Lange and Boucher, 1990; Brown *et al*., 2003), presumably through the stimulatory effect of the butenolide compound, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (Flematti *et al.*, 2004, Van Staden *et al.*, 2004). The identification of the active compound has led to the discovery of a family of structurally-related compounds, the karrikins, in which most exhibit plant growth regulatory actions (Flematti *et al*., 2007). The active compounds in smoke are heat stable, water soluble and long lasting in water and in soil (Van Staden *et al.,* 2004. These signalling molecules may have a profound significance in angiosperms which was not previously anticipated (Nelson *et al.,* 2009). Their effects and activity have been demonstrated in a wide range of plant species. Smoke has demonstrated to enhance germination of over 1200 plant species from more than 80 genera world-wide (Dixon *et al.,* 2009).

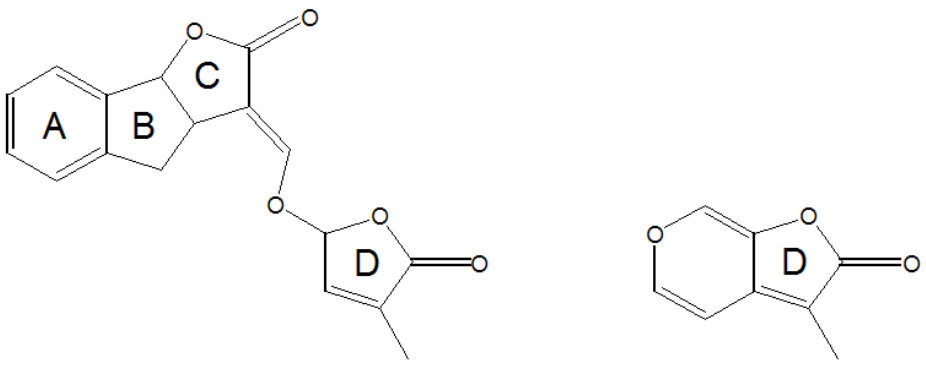
In addition to enhanced germination effects, both smoke and KAR_1 have been shown to enhance seedling growth in several plant species (Baxter and Van Staden, 1994; Blank and Young, 1998; Sparg *et al.*, 2005; Sparg *et al.*, 2006; Jain and Van Staden, 2006; Kulkarni *et al.*, 2006; Van Staden *et al.*, 2006; Kulkarni *et al*., 2007). KAR_1-treated tomato, okra, bean

and maize seedlings, for example, showed an increase in leaf number and shoot height compared with untreated controls (Van Staden *et al.*, 2006).

Strigolactone root exudates, on the other hand, are also known to induce the germination of seeds of the parasitic weeds, *Striga* and *Orobanche spp* (Cook *et al.*, 1966; Hauck *et al.*, 1992; Müller *et al.*, 1992; Yokota *et al*., 1998). While several studies have focused on mutant analysis to elucidate strigolactone biosynthesis pathways and/or signal transduction (Brewer *et al.*, 2009; Hayward *et al.*, 2009), the use of the synthetic strigolactone GR24 (Johnson *et al.*, 1976; Mangnus *et al.*,1992) has also gained a great deal of attention (e.g., Besserer *et al.*, 2008). The structural backbone of all known natural strigolactones share three cyclic lactone rings (designated A, B and C rings) connected by an enol-ether bridge to an α,β- unsaturated furanone moiety (designated the D-ring) .

The bioactiphore in the strigolactone structure resides in the D-ring and enol-ether bridge (situated between the C-and D rigs of the molecule) (Mangnus and Zwanenburg, 1992). In addition, a methyl substituent at the C-4'of the D-ring is essential for bioactivity and is retained in all natural strigolactones (Mangnus and Zwanenburg, 1992). Interestingly, one of the two rings of KAR_1 is identical to the D-ring of strigolactones (Figure 3.1) and could potentially interact with a strigolactone receptor in the model proposed by Zwanenburg *et al.* (2009). However, the identification and characterisation of a strigolactone receptor remains elusive. Interestingly, strigol and a synthetic strigolactone analogue (GR24) have been shown to stimulate the germination of highly KAR_1-sensitive species, namely Grand Rapids lettuce seeds in the dark (Bradow *et al.*, 1988). Furthermore, smoke solutions have been demonstrated to activate seed germination in the strigol-responsive parasitic species *Orobanche aegyptiaca* (Nun and Mayer, 2005).

These data, as set out above, elicited the question of whether strigolactones in the form of the synthetic molecule, GR24, can effect the same plant growth responses as seen following smoke and KAR_1 treatment. Since smoke/KAR_1 not only promotes seed germination but also has a plant growth promoting effect in early seedlings, it would be interesting to determine whether strigolactones play a role in the early developmental stages of plant growth. Would the strigolactone also promote plant growth after germination? If so, given the structural similarity between the KAR_1 and GR24 molecules, would treatment with the strigolactone provoke the same molecular mechanisms leading to plant growth promotion as seen for KAR_1 treatment?



GR24 KAR_1

Figure 3.1. Chemical structures of the synthetic strigolactone GR24 and the main active butenolide compound identified in smoke water, namely 3-methyl-2H-furo[2,3-c]pyran-2-one (karrikinolide, KAR_1). These two compounds share the same furanone ring (D) structure.

3.2. Results

3.2.1. Germination rate and efficiency of GR24- and smoke-treated *N. benthamiana* seedlings

Due to the germination stimulating effect previously reported for strigolactones and smoke/KAR_1 on parasitic weeds and other species (Cook *et al.*, 1966; De Lange and Boucher, 1990; Hauck *et al.*, 1992; Müller *et al.*, 1992; Yokota *et al*., 1998; Brown *et al*., 2003), the germination rate of *N. benthamiana* seedlings treated with GR24, KAR_1 or smoke-water were determined. Whilst all seeds germinated synchronously, none of the treatments resulted in significant differences in either germination rate or efficiency compared with untreated seeds (Table 3.1), suggesting that any alterations observed following seedling growth experiments were due to a post-germination effect.

Table 3.1. Germination rate and efficiency of *N. benthamiana* seeds treated with GR24, smoke-water and KAR_1. Values represent the mean ± SE of pooled seeds from three independent experimental trials. Different letters indicate values that were determined by ANOVA to be significantly different ($P < 0.05$) from each other.

Treatment:		Germination percentage (%) ± SE		
		24 hours	36 hours	48 hours
Continuous dark				
	Control	00.00± 0.00	00.00± 0.00	00.00± 0.00
	GR24	00.00± 0.00	00.00± 0.00	00.00± 0.00
	Smoke	00.00± 0.00	00.00± 0.00	00.00± 0.00
	KAR_1	00.00± 0.00	00.00± 0.00	00.00± 0.00
16/8 light dark				
	Control	00.00± 0.00	20.00± 4.47(a)	98.00± 1.22(a)
	GR24	00.00± 0.00	22.00± 5.83(a)	96.00± 1.00(a)
	Smoke	00.00± 0.00	28.00± 4.06(a)	96.00± 1.00(a)
	KAR_1	00.00± 0.00	23.00±4.10(a)	95.00± 1.00(a)

3.2.2. Phenotypic characterisation of *N. benthamiana* seedlings treated with GR24 and smoke-water

While the growth stimulatory effect for smoke-water/KAR_1 treatment has been previously reported (Baxter and Van Staden, 1994; Blank and Young, 1998; Sparg *et al.*, 2005; Sparg *et al.*, 2006; Jain and Van Staden, 2006; Kulkarni *et al.*, 2006; Van Staden *et al.*, 2006; Kulkarni *et al.*, 2007), no study to date has investigated biomass accumulation following strigolactone treatment. Since smoke is known to be a complex mixture of around 4800 compounds (Andreoli *et al.*, 2003), KAR_1 was included to determine whether diluted smoke-water can be used as a substitute for purified KAR_1. The growth promoting effects of KAR_1 at 10^{-7}M have been previously reported in tomato (Jain and Van Staden, 2006). Subsequently, these findings were confirmed in *N. benthamiana* when KAR_1 was investigated for its plant growth promoting properties at 10^{-7}M (Figure 3.2) and KAR_1 was consequently used at that concentration for all following experiments. In order to investigate the biomass accumulation with strigolactone treatment, a concentration range from 10^{-6} to10^{-8} M for GR24, in parallel to a dilution series ranging from 1:100 to 1:5000 for smoke-water, were supplied to *N. benthamiana* seedlings (Figure 3.3). These concentrations and dilution ranges have been previously reported to enhance germination (Wigchert *et al.*, 1999*;* Humphrey and Beale, 2006; Daws *et al.*, 2008) in the case of strigolactone. or to promote biomass accumulation such as in the case of smoke and KAR_1 (Jain and Van Staden, 2006; Daws *et al.*, 2007) In this study, both GR24 and smoke-water treatments resulted in a significant increase in biomass accumulation under all concentrations or dilutions tested (Figure 3.3).

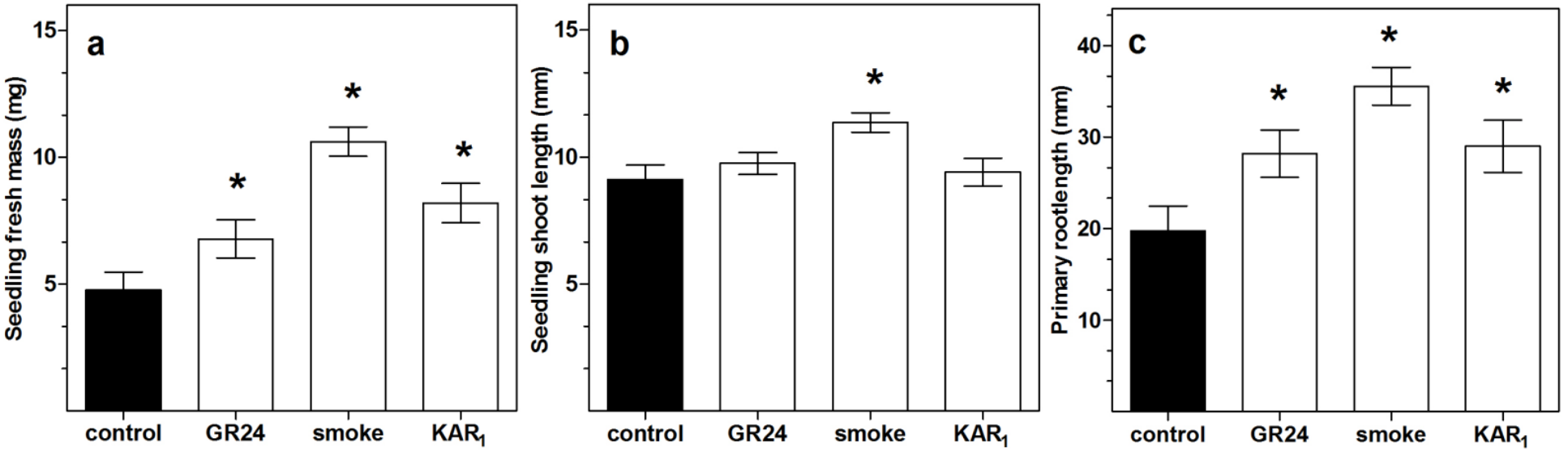


Figure 3.2. Growth of three week old *N. benthamiana* seedlings treated with GR24, smoke-water and KAR1. (**a**) Seedling fresh mass (mg), (**b**) Seedling shoot length (mm), (**c**) Primary root length (mm). Values represent the mean ± SE (n = 25). An asterisk indicates a value that was determined by one-way ANOVA to be significantly different ($P < 0.05$) from the control.

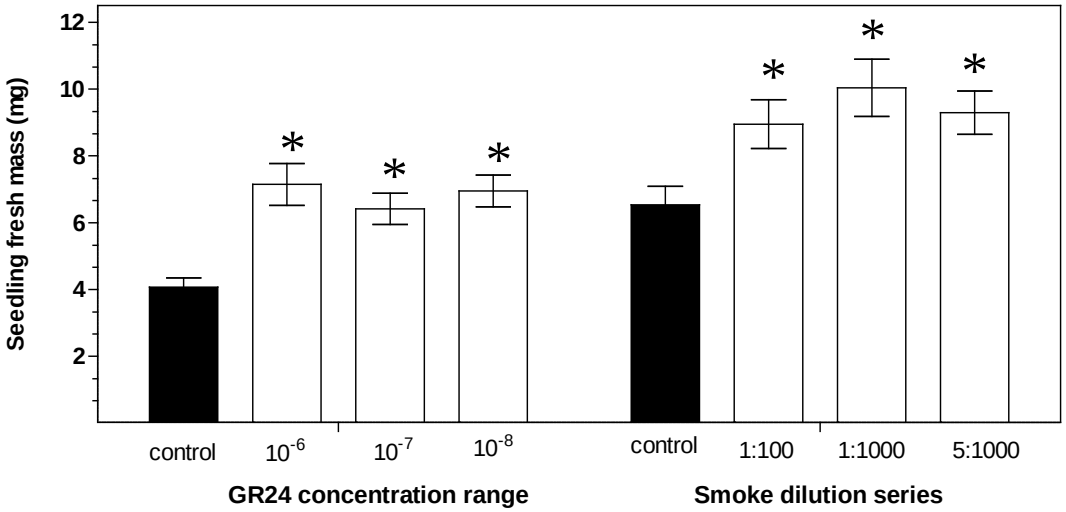


Figure 3.3. Seedling fresh mass of *N. benthamiana* seedlings treated with a range of concentrations of GR24 or a dilution range of smoke. Values represent the mean ± SE (n = 25). An asterisk indicates a value that was determined by one-way ANOVA to be significantly different ($P < 0.05$) from the control.

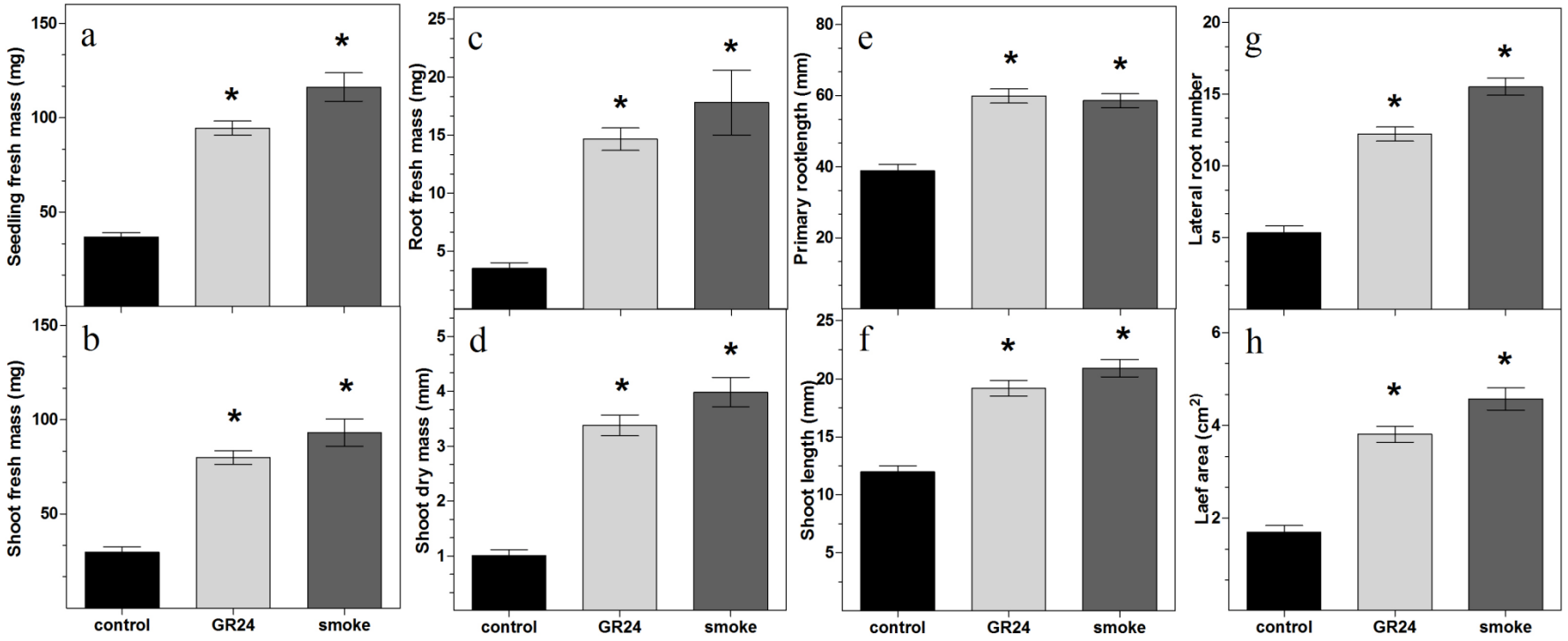


Figure 3.4. Growth of *N. benthamiana* seedlings treated with the synthetic strigolactone GR24 or smoke-water. (a) Seedling fresh mass, (b) Shoot fresh mass (c) Root fresh mass, (d) Shoot dry mass, (e) Primary root length, (f) shoot length , (g) lateral root number, and (h) leaf area of three-week-old seedlings treated with 10-7M GR24 and 1:1000 smoke dilution. Values represent the mean ± SE (n = 25) from three independent experimental trials. An asterisk indicates a value that was determined by one-way ANOVA to be significantly different ($P < 0.05$) from the control.

In order to further evaluate plant architecture, a 1:1000 dilution of smoke-water and 10^{-7} M GR24 were selected to monitor additional growth parameters. The plant growth promoting effect of KAR_1 has previously been verified in this study (Figure 3.2). Physiological measurements were not made for KAR_1 treated plants in this experiment because of the time sensitive nature of the plant material and the limited amount of chemical available for use in growth experiments (Refer to *Chapter 5.6*).

The 10^{-7} M GR24 concentration was specifically selected in order to match the working concentration for KAR_1 as previously demonstrated to be effective by Jain and Van Staden, (2006). Apart from the total seedling fresh mass accumulation (Figure 3.4 a), both root fresh mass and shoot fresh and dry mass increased (Figure 3.4 b, c, d). Root architecture was also affected in both GR24 and smoke-water treatments, characterised by an increase in primary root length (Figure 3.4 e) and an increase in the number of lateral roots (Figure 3.4 g). Seedling shoot length (Figure 3.4 f) and total leaf area (Figure 3.4 h) were also significantly increased in both treatments. Root dry mass proved too small to be accurately assessed on available balances.

3.2.3. Transcript profiling of GR24, smoke-water and KAR_1 treatments of *N. benthamiana* seedlings

In an attempt to elucidate the molecular change(s) induced by the treatments, the gene expression profiles of seedlings treated with GR24, smoke-water and KAR_1 were compared. Comparative microarray analyses identified only a few differentially expressed (normalized to the control) transcripts common to all three treatments (Table 3.2).

Table 3.2. Co-expressed transcripts (up- and down-regulated) in GR24, smoke and KAR_1-treated three-week-old *N. benthamiana* seedlings normalized to the control.

			log2-fold change		
Array ID	*Nicotiana* Accession	Putative annotation	GR24	Smoke	KAR_1
P162792	EH621771	Serine-threonine protein kinase	2.56	2.84	2.86
P304283	FG196119	Trehalose-6-phosphate phosphatase	1.3	3.46	2.33
P021776	EB439530	Acyl carrier protein	2.4	2.72	2.45
P065135	BP136250	Kelch repeat	2.68	2.87	2.23
P128102	EB427647	Ubiquitin protein	-3.7	-2.8	-2.68
P093243	BP532187	Polyubiquitin containing 7 ubiquitin monomers	-2.44	-1.2	-3.21
P010236	FG187570	Monoterpene synthase 2	-3.32	-2.96	-2.5
P042636	BP130409	Proteasome activator subunit 4-like	-4.07	-3.6	-3.11
P023416	BP131349	Hypothetical protein from *Vitis vinifera*	-4.69	-2.17	-3.54
P114832	CV021775	Metallocarboxypeptidase inhibitor precursor	-5.36	-2.11	-4.44
P316243	FG187377	Calcium-binding protein annexin	-3.72	-3.26	-3.21
P405121	BP131055	None	-3.2	-2.69	-2.47
P048181	BP131841	None	-3.22	-2.78	-2.16
P050746	BP132473	Unkown	-6.47	-2.19	-4.99
P051846	BP132760	None	-3.24	-2.92	-2.45
P082680	BP528567	Predicted protein *Populus trichocarpara*	-3.17	-2.64	-2.44
P090713	BP531066	None	-3.06	-2.88	-2.46
P096518	AM780932	None	-2.58	-2.24	-2.12
P109087	CV019144	None	-2.71	-2.69	-2.23
P111432	CV020195	Poly-binding protein	-3.24	-2.52	-2.06
P129772	EB429725	None	-3.8	-3.15	-2.56
P218407	EH61927	Predicted protein *Populus trichocarpara*	-5.3	-6.71	-5.94
P232349	BP531659	None	-4.35	-3.83	-3.35
P299368	FG172886	Cockayne syndrome 1 homolog	-3.5	-2.71	-2.95

Whilst no clear biochemical pathway or gene ontology could be deduced from this selection, it was, however, interesting to note that most of the transcripts are predicted to be plastid-localised (Table 3.2) (for full accession list of significantly altered transcripts, see Addendum A Table 3.3 a, b, c).

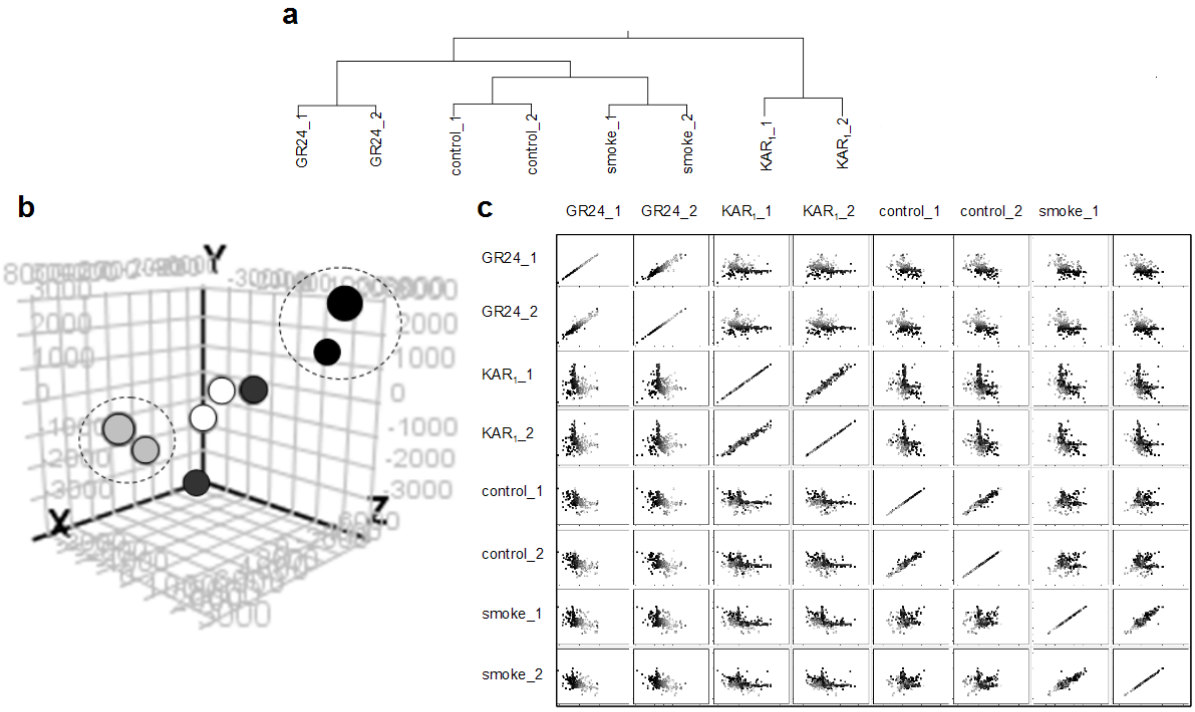


Figure 3.5 Evaluation of transcript variance in three-week-old *N. benthamiana* seedlings treated with GR24, smoke-water and KAR_1 (a) Hierarchical cluster analysis (average Euclidian distance). (b) Principal component analysis (control: black, GR24: light grey, smoke: white, and KAR_1: dark grey; principal component (PC) loadings: PC1: 69.3%, PC2: 1.3%, PC3: 1.09%) (c) Correlation matrix plot (Pearson correlations). Transcripts were identified via GeneSpring GX software (Agilent technologies, www.agilent.com) and analysis was based on gene expression profiles of significantly altered transcripts (see text for details) by a log2 value of 2 or more.

Since these replicates originated from independent experimental trials, hierarchical cluster analysis (HCA) on the complete transcript data set indicated that biological noise clustered the individual experimental trials rather than the replicate treatments together (data not shown). However, when filtering the dataset in order to obtain only biological significant transcripts (one-way ANOVA, $P < 0.05$), and subjecting again to HCA analysis (GeneSpringGX, Agilent, www.agilent.com), biological replicates of each treatment clustered together (Figure 3.5 a). Unless stated otherwise, this subset of genes was exclusively used to analyse the molecular effect of GR24, smoke-water and KAR_1 treatments on the three-week-old *N. benthamiana* seedlings. In addition, the same subset of filtered genes was evaluated for maximum variability to separate the treatments via principal component analysis (PCA) (Figure 3.5 b). This PCA analysis suggested that GR24 could be clearly separated from the control and smoke/ KAR_1 treatment (Figure 3.5 c). In addition, smoke/KAR_1 could also be distinguished from the control, although smoke and KAR_1 were not as clearly distinguishable in the PCA compared with the HCA analysis (compare Figure 3.5 a, b). Furthermore, correlation matrix evaluation of this subset indicated that, whilst a strong linear correlation could be observed for replicates within each treatment, this was not observed when comparing the individual replicates of the different treatments against each other (Figure 3.5 c). Thus, with the exception of the limited number of overlapping transcripts, the various treatments evoked very different changes in gene expression profiles in the seedlings that were independent from biological variability originating from the separate experimental trials (Figure 3.5 c).

Genes commonly up-regulated due to treatment with GR24, KAR_1 or smoke were selected for further analysis. Gene ontology could only be ascribed for four commonly up-regulated

and 9 down-regulated transcripts (Table 3.2). Genes up- regulated in all three treatments showed strong homology to a protein serine/threonine kinase, a kelch repeat protein, an acyl carrier and a trehalose-6-phosphate phosphatase. Down-regulated genes include ubiquitination proteins, monoterpene synthase, metallocarboxypeptidase inhibitor, a Calcium-binding protein annexin, Proteasome activator subunit 4-like protein, a hypothetical protein from *Vitis vinifera* and a poly-ubiquitin containing protein.Validation of the microarray data was conducted using semi quantitative RT-PCR (Figure 3.6). To confirm the reliability of the microarray results, differential expression was corroborated for three genes, whilst the expression of *NbMAX2* and *NbMAX4* was also analyzed (For details of the identification of the MAX2 and MAX4 sequences see *Chapter 4*.)

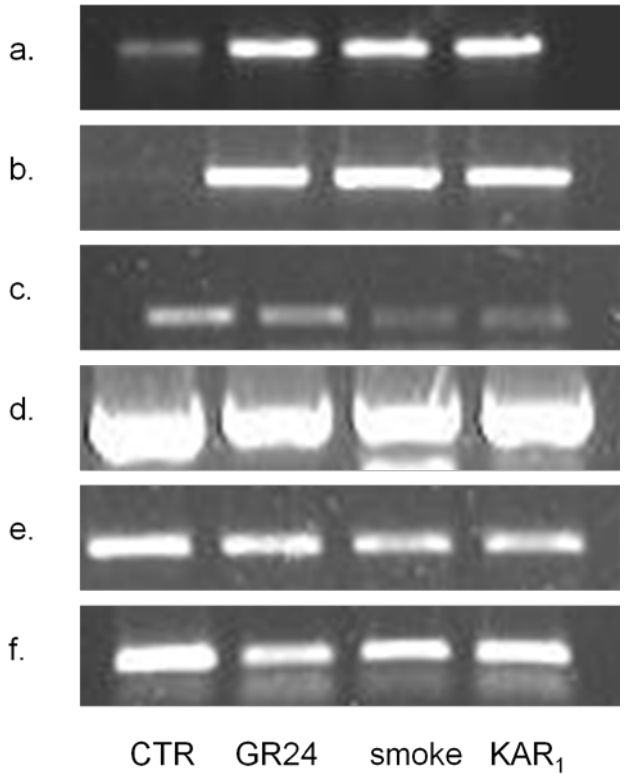


Figure 3.6. Semi-quantitative PR-PCR analysis of (a.) Acyl carrier protein (EB439530), (b.) Serine-threonine protein kinase (EH621771), (c.) Proteasome activator subunit 4-like (BP130409), (d.) *NbMAX2*, (e.) *NbMAX4*, (f) Actin. Samples were loaded onto an 0.8% agorose gel containing ethidium bromide in the following sequence: Control (CTR), GR24, smoke-water, KAR_1.

Up-regulation of the Acyl carrier protein and serine-threonine protein kinase, and down-regulation of proteasome activator subunit, was evident relative to the expression levels in the control (Figure 3.6).

3.2.4 Phytohormone levels of GR24-, smoke-water and KAR_1-treated *N .benthamiana* seedlings

Since both GR24 (via auxin) and smoke-water (via ABA, cytokinins and gibberellins) have been shown or proposed to interact with phytohormones, these different classes of phytohormones were profiled (Table 3.4). Results indicated that the GR24 treatment resulted in a four-fold increase in both indole 3-acetic acid (IAA) and jasmonic acid (JA) levels compared with the untreated control (Table 3.4). No significant changes in phytohormone contents were observed following smoke-water treatment compared with the untreated control. However, multi-way ANOVA analysis indicated that the hormone levels of smoke-water-treated seedlings were also not significantly different from those of KAR_1-treated seedlings as far as increased levels of isopentenyladenine (iP), zeatin (Z), dihydrozeatin ([diH]Z) and zeatin-9-riboside ([9R]Z) were concerned (Table 3.4). In addition, an unknown compound identified in the profile, with similar elution index and fragmentation pattern to kinetin, also increased eighteen-fold in both smoke and KAR_1 treatments (Table 3.4). Lastly, it was also observed that KAR_1 treatment caused a significant increase in ABA and JA levels compared with the control treatment (Table 3.4).

Table 3.4. Phytohormone levels of control, GR24, smoke-water- and KAR_1-treated *N. benthamiana* seedlings determined by GC MS. Values represent the mean±SE of pooled seedlings from three independent experimental trials. Bold values or different letters denominations indicate values that were determined by Students *t-test* (bold) or multi-way ANOVA (letters) to be significantly different ($P < 0.05$) from either the control (*ttest*) or each other (ANOVA). Abbreviations: IAA: indole 3-acetic acid, iP: isopentenyladenine, [diH]Z: dihydrozeatin, Z: zeatin, [9G]Z: zeatin 9-glucoside, [9R]Z: zeatin 9-riboside, [9R-5'P]Z: zeatin 9-riboside 5'-monophosphate, GA: gibberellin, ABA: abscisic acid, JA: jasmonic acid, SA: salicylic acid.

	Treatment											
	control			GR24			smoke			KAR_1		
	pmol.mg^{-1} FW											
IAA	7.8	±	3.4a	**25.9**	**±**	**3.3b**	2.9	±	1.3a	3.6	±	0.4a
iP	110.0	±	5.4a	152.0	±	40.5ab	195.1	±	37.8ab	**261.8**	**±**	**39.0b**
(diH)Z	334.6	±	136.3a	550.1	±	113.4a	523.1	±	203.7a	**970.7**	**±**	**148.7a**
Z	517.2	±	47.5a	567.8	±	122.1a	475.4	±	185.6a	**950.9**	**±**	**148.7a**
[9G]Z	14.4	±	1.0a	38.2	±	19.2a	46.7	±	19.0a	56.9	±	24.4a
[9R]Z	8.7	±	5.7a	25.5	±	10.2ab	14.1	±	5.8ab	**42.9**	**±**	**9.0b**
[9R-5'P]Z	27.3	±	7.3a	79.9	±	37.9a	74.8	±	49.8a	129.1	±	52.7a
GA3	0.5	±	0.1a	2.9	±	1.2a	2.0	±	0.9a	3.6	±	1.3a
GA8	43.3	±	12.9ab	46.5	±	9.7ab	39.6	±	13.7a	**84.9**	**±**	**7.2b**
GA9	43.7	±	21.0a	82.4	±	20.6ab	69.2	±	25.6a	**181.7**	**±**	**36.0b**
GA25	19.1	±	15.6a	20.1	±	11.6a	9.8	±	3.9a	17.6	±	3.2a
ABA	59.5	±	6.9a	76.1	±	17.1ab	71.1	±	12.3ab	**117.1**	**±**	**15.8b**
JA	0.2	±	0.01a	**1.8**	**±**	**0.1c**	0.7	±	0.4ab	**1.7**	**±**	**0.4bc**
SA	4.8	±	1.4a	2.6	±	1.5a	0.07	±	0.03a	5.3	±	2.9a
unknown	18.0	±	7.3a	312.3	±	170.6ab	**389.7**	**±**	**100.9b**	**544.5**	**±**	**152.2b**

3.2.5. Carbohydrate metabolism in GR24-, smoke-water- and KAR_1-treated seedlings

To determine whether biomass accumulation in GR24-, smoke- and KAR_1-treated plants were related to changes in carbohydrate metabolism, starch and soluble sugar content of the *N. benthamiana* seedlings were determined. KAR_1 treatment resulted in an increased glucose content, while GR24 induced an increase in sucrose level (Figure 3.7a). No statistically relevant changes occurred in starch content in GR24-, smoke- and KAR_1 treated seedlings when compared with the untreated control (Figure 3.7 b).

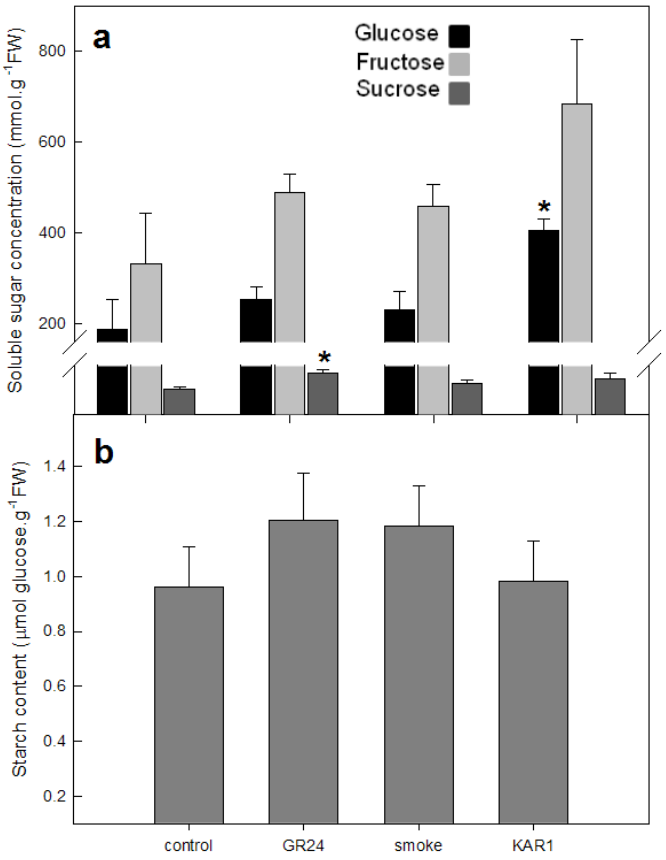


Figure 3.7. Carbohydrate content of *N. .benthamiana* seedlings treated with GR24-, smoke-water and KAR_1 (**a**) Soluble sugar, and (**b**) starch measurements were performed according to Müller-Röber *et al*. (1992). Values represent the mean ± SE (n = 25) from three independent experimental trials. An asterisk indicates values that were determined by one way ANOVA to be significantly different ($P < 0.05$) from the control.

3.2.6. Effect of simultaneous GR24 and smoke application on *N. benthamiana* seedling growth

The data presented above suggests that GR24 and smoke-water promote plant growth via alternative mechanisms. In order to determine whether these pathways operate independently or whether they display a degree of redundancy, *N. benthamiana* seedlings were treated with GR24 and smoke, either individually or in combination (Figure 3.8). When half of the usual amounts of GR24 or smoke were applied separately, growth was still significantly enhanced in comparison with the untreated control seedlings. However, when these amounts of smoke and GR24 were combined in a single treatment, total seedling and shoot fresh mass were significantly reduced (Figure 3.8 a, b), although root fresh mass and shoot length did not differ from the untreated control (Figure 3.8 c, d).

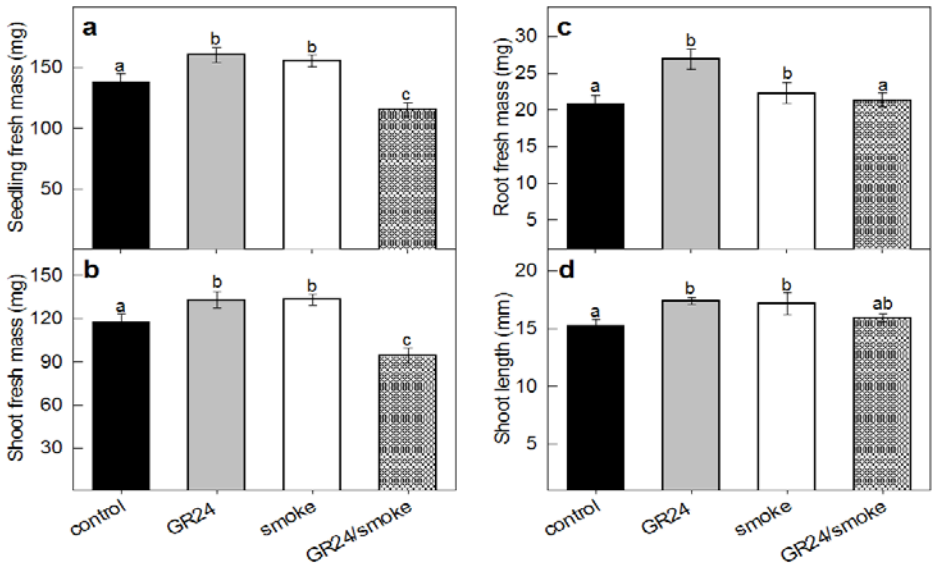


Figure 3.8. Growth responses following treatment with GR24 and smoke-water either individually or in combination. (**a**) Seedling fresh mass, (**b**) shoot fresh mass, (**c**) root fresh mass, and (**d**) shoot length in three-week-old *N. .benthamiana* seedlings. The amount of GR24 and smoke-water were halved for this experiment. Values represent the mean ± SE of pooled seedlings (75 plants per pool) from three independent experimental trials. Different letters indicate values that were determined by ANOVA to be significantly different ($P < 0.05$) from each other.

3.3. Discussion

The accumulation of plant biomass depends upon a finely-balanced interplay between carbon accumulation, respiration and resource allocation, on the one hand, and plant architecture and opportunism on the other. Considering the complexity of these interactions, it is not surprising that unravelling the mechanisms behind this interplay is somewhat difficult. The presence of a number of low molecular weight bioactive compounds in the environment which promote plant growth offers a "chemical genetics" approach towards obtaining a deeper understanding of this process.

Recently, strigolactones have gained considerable attention, primarily for their role in regulating branching patterns in both plant (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008) and microbial (Akiyama *et al*., 2005) structures. Smoke, on the other hand, has long-proven an attractive stimulant for subsistence farmers to enhance crop productivity, both in terms of germination success and improved seedling vigour (Modi, 2002; 2004). Both KAR_1, the main active ingredient in smoke, and strigolactones have an identical α,β-unsaturated furanone moiety (Figure 3.1). Removal of either this furanone D-ring or modification of the enol-ether bridge (Mangnus and Zwanenburg, 1992; Zwanenburg *et al*., 2009) results in a loss of bioactivity in the strigolactones. This structural similarity suggests that both molecules may elicit some similar responses in plants. Indeed, such has been shown to be the case in the parasitic species *Striga* and *Orobanche*, where KAR_1 is able to substitute for strigolactones in inducing seed germination (Daws *et al*., 2008; Nun and Meyer, 2005). Since the ability of both smoke and KAR_1 to increase the vigour and growth of seedlings in the post-germination phase has been well documented, it would be of interest to determine whether strigolactones might trigger a similar response.

Evaluation of architecture and biomass accumulation in *N. benthamiana* indicated that smoke-water, KAR_1 and a synthetic strigolactone, GR24, caused the seedlings to increase in physical size and to accumulate more biomass (fresh mass) than the control seedlings (Figure 3.2). Germination tests indicated that neither GR24, KAR_1 nor smoke treatment affected germination either positively or negatively (Table 3.1). Consequently, all of the observed changes in growth may be attributed to post-germination effects of the treatments. The overall phenotypic effects of smoke and GR24 treatments were highly similar (increased root length, longer shoots, more lateral roots and greater leaf area). Although promotion of plant growth in response to smoke/KAR_1 has been widely reported, this is the first known report of a similar observation for exogenously-applied strigolactones. Strigolactones were recently identified as a novel class of phytohormones with a role in the repression of lateral shoot development. The results observed here suggest that strigolactones are likely to have a much wider role in plants than has thus far been reported. It is possible, however, that the growth promoting effects observed are only evident during seedling establishment and this will be tested further in the future.

Both smoke/KAR_1 (Gardner *et al.*, 2001; Nelson *et al.*, 2009) and GR24 (Brewer *et al.*, 2009; Hayward *et al.*, 2009) have been shown or suggested to interact with various known phytohormones. In this study, a four-fold increase in IAA levels was observed in the GR24-treated plants (Table 3.4). Auxins are known to regulate strigolactone levels *in vivo* via an AXR1-dependent (AUXIN RESISTANT1) mechanism which regulates the *MAX3* and *MAX4* (*MORE AXILLARY BRANCHING3* and *4*) genes involved in strigolactone synthesis in *Arabidopsis* (Hayward *et al.*, 2009) and their orthologues in other species (Sorefan *et al.*, 2003; Bainbridge *et al.*, 2005; Foo *et al.*, 2005; Johnson *et al.*, 2006). Increased auxin levels

in the stems of *Arabidopsis* (Bennett *et al*., 2006) and rice (Arite *et al*., 2007) strigolactone mutants have previously been observed, probably as a result of perturbations in auxin transport. This, however, is the first report that indicates that exogenously-applied strigolactones can enhance endogenous auxin levels in plants.

In addition to increased IAA levels, a nine-fold increase was also observed in free JA levels upon GR24 treatment (Table 3.4). There is a significant amount of cross-talk between the auxin and JA signalling pathways (Chandler, 2009). In tobacco thin cell layer cultures, adventitious root formation is regulated by interplay between jasmonates and auxins (Fattorini *et al*., 2009). Taken together, these results suggest that, at least in part, the growth promotion effect observed in *N. benthamiana* may be mediated through signalling cross-talk and cross-regulation between strigolactones, auxins and jasmonic acid.

Smoke-water treatment resulted in a significant amount of biological noise with no significant changes in phytohormone levels relative to the control (Table 3.4). However, multi-way analyses indicated that levels of iP, [diH]Z, [9G]Z, ABA and JA in the smoke were also not significantly different from those of the KAR_1-treated seedlings (Table 3.4), which can be partially attributed to the chemical complexity within the smoke solution (see below for further discussion). KAR_1 treatment, on the other hand, resulted in clear increases in levels of cytokinins (iP, [diH]Z, Z and [9R]Z), gibberellins (GA_8 and GA_9), and the stress-associated hormones ABA and JA.

Recently, it was suggested that smoke-treatment during seedling establishment results in improved growth and seedling vigour via an ABA/stress-priming response (Soós *et al*.,

2009). ABA is recognized as a stress hormone which coordinates cell stress responses in the plant cell and is an essential mediator aimed at triggering plant stress responses to non-favourable environmental conditions (Chandler and Robertson, 1994). Exogenously-applied ABA has been demonstrated to increase the plants adaptive responses to varying environmental conditions (Smith-Espinoza *et al.,* 2005). This means that the priming of young seedlings may promote vegetative growth which could potentially lead to improved seedling yield and biomass accumulation. The three treatments used here (GR24, smoke-water and KAR_1) all demonstrated elevated levels of ABA and may be exhibiting improved seedling vigour through a high ABA-mediated priming response. Studies have indicated that seedlings can, after a short latency period, respond to exogenously applied ABA with increased vegetative growth (Hall and McWha, 1981). There have also been reports of studies where improved growth was an aspect of a stress response mediated by ABA treatment (De Smet *et al*., 2006; Aguilar *et al.*, 2000).

Interestingly, KAR_1-treated seedlings showed a significant increase in glucose content (Figure 3.7). Studies in *Arabidopsis* have indicated a link between carbohydrate status and ABA signalling (Arenas-Huertero *et al*., 2000), whereby glucose was shown to regulate ABA levels via the modulation of GIN5 (GLUCOSE INSENSITIVE 5) activity and to affect ABA signalling mediated by a GIN6/ABI4 (ABA INSENSITIVE 4) response. Although some alterations in carbohydrate levels might be expected in response to changes in plant growth, the only other alteration in carbohydrate status across all of the treatments was a small increase in sucrose levels in GR24-treated seedlings.

KAR_1 has previously been demonstrated to exhibit auxin- and cytokinin-like activities (Jain *et al*., 2008) and is believed to promote seed germination via interactions with the GA

biosynthetic pathway (Nelson *et al*., 2009). Both smoke (Drewes *et al*., 1995) and KAR_1 (Merritt *et al*., 2006) have been shown to substitute for red light during germination in a similar manner to GAs. In this study, KAR_1-treated seedlings had significant increases in levels of GA_8 and GA_9 (Table 3.4). Unfortunately, changes in endogenous levels of GAs in embryos during germination and early seedling growth are not well understood for any plant species (Ayele *et al*., 2006), which makes interpretation of these specific results very difficult. However, GA_8 is known to be a biologically inactive GA, arising from conversion of other active GAs. The observed increase suggests that levels of active GAs might have been reduced from a previously high level (for example, during seed germination). This is corroborated by the finding that GA_{20} is converted, via GA_1, to GA_8 during seed imbibition in *Pisum sativum* (Ayele *et al*., 2006). GA_9 has also been shown to be extremely active in promoting stem elongation in seedlings of *Thlaspi arvense* (Metzger, 1990), which may provide a partial explanation for the greater shoot length observed in KAR_1-treated seedlings.

Transcript profiling via microarray analysis, following GR24, smoke-water and KAR_1 treatment, indicated alterations in the expression of a number of genes compared with the controls. However, most of these were unique to the different treatments (Addendum A,Table 3.3 a, b, and c). Only a limited number (4 up-regulated and 19 down-regulated) of transcripts were identified which were common to all three treatments. Whilst no clear pathway could be deduced from these, it appeared that plastid/organelle metabolism was significantly affected across all three treatments (Table 3.3 a, b, and c). Genes conferring stress-tolerance functions were also identified.

One of the common up- regulated transcripts (Table 3.2), a serine-threonine protein kinase, showed homology to *PBS1* (avrPphB Susceptible) gene. This domain was identified in

Arabidopsis thaliana (Swiderski and Innes 2001) and *Populus trichocarpa* (Tuskan *et al*., 2006) and plays a role during recognition of pathogen avirulence proteins.

Jasmonic acid signalling is known to be important for plant stress responses to wounding, pathogen interaction, insect attack, and ultraviolet light (Weber *et al*., 2002). An up-regulation of acyl carrier proteins in the three treatments along with elevated levels of jasmonic acid suggest that an increase in fatty acid signalling may also account for a heightened stress-tolerance response in the treated plants.

Interestingly, a *trehalose-6-phosphate phosphatase* (*TPP*) gene (up-regulated in all three treatments) from rice (*Oryza sativa*) *OsTPP1* (*Oryza sativa trehalose-6-phosphate phosphatase*) confers stress tolerance and results in the activation of stress-tolerance responsive genes (Ge *et al*., 2008). Trehalose is a disaccharide widely utilized by bacteria, fungi, plants and invertebrate animals (Ge *et al*., 2008). The role of this disaccharide in higher plants is not very well understood. However, in yeast and microbes, trehalose is a metabolic regulator, serves as a sugar storage molecule and protects against abiotic stress (Strom and Kaasen, 1993; Wiemken, 1990). *TPP* and *trehalose -6-phosphate synthase* (*TPS*) genes are widely found in higher plant genomes in large gene families (Leyman *et al*., 2001; Schluepmann *et al*., 2003) inferring the importance of these genes. Many of these genes are regulated by environmental stresses (Chao *et al*., 2005; Rabbani *et al*., 2003; Wang *et al*., 2003; Pramanik and Imai, 2005), suggesting that a plant-environmental interaction function is conferred by these genes. Trehalose has also been demonstrated to function during carbohydrate metabolism and plant development (Eastmond *et al*., 2002; Gomez *et al*., 2006; Satoh-Nagasawa *et al*., 2006; Schluepmann *et al*., 2003). In rice, over-expression of a bi-functional fusion of bacterial *TPP* and *TPS* increased the trehalose content of the plant and

improved tolerance to abiotic stress with no phenotypical alterations to the plant (Garg *et al*., 2002; Jang *et al*., 2003).

Both HCA and PCA analyses on all significantly-altered transcripts indicated that the three treatments (GR24, smoke-water and KAR_1) separated from the control, grouping to their respective biological replicates (Figure 3.5). It is interesting to note that smoke and KAR_1 still have a degree of separation (less in the PCA than in the HCA), which was also reiterated by the carbohydrate (Figure 3. a) and phytohormone datasets (Table 3.4). Since smoke is a complex mixture containing thousands of different chemicals, several of which may have growth promoting or growth retarding activities, this result is only to be expected. Indeed, there is evidence suggesting that KAR_1 is not the only bioactive ingredient in smoke that promotes germination and/or plant growth. More recently, other related butenolide compounds have been isolated (Flematti *et al.*, 2009), in addition to the isolation of a butenolide with inhibitory activity (Light *et al.,* 2010). Furthermore, Correlation matrix plots indicated that, while the biological replicates of the various treatments exhibited a similar linear expression pattern, none of the treatments mirrored each other's behaviour (Figure 3.5 c), suggesting that the three treatments did not result in the same transcriptional response. Chiwocha *et al.* (2009) recently commented that, with the exception of enhanced *Striga* and *Orobanche* spp. germination, strigolactone treatment did not cause enhanced germination in other species (as reported several times for smoke/KAR_1). In addition, GR24 treatment did not induce changes in GA levels and would thus be unable to stimulate germination via a GA-dependent mechanism as seem to be the case for smoke/KAR_1 (Nelson *et al.,* 2009). This study clearly indicates that, even at a post-germination phase, GR24 and smoke/KAR_1 did not induce the same pathways. However, given that a degree of overlap in phenotypic responses

and transcript profiles does exist, it is likely that there may be some cross-talk or overlap between the various growth-promoting pathways that are induced.

Combination experiments involving GR24 and smoke-water resulted in seedlings with repressed shoot biomass accumulation (Figure 3.8). The repressed growth provides evidence that these chemicals (GR24 and smoke) are not promoting plant growth via completely independent pathways and that the plant growth promoting pathways share some degree of overlap. The underlying cause of the repressions is still not understood but the possibility that these to pathways may be competing for common recourses may explain the growth repression. In order to understand the connection shared between these plant growth promoting pathways the receptor(s) for these chemicals need to be isolated and characterized. The receptor(s) for KAR_1 and strigolactones have yet to be identified, though such knowledge may help to explain this observation.

In conclusion, it was demonstrated here that both GR24 and smoke-water could enhance biomass accumulation in three-week-old *N. benthamiana* seedlings, possibly mediated via plastidial alterations and interactions with a variety of endogenous phytohormones. These pathways seem to operate largely independently from each other, although there are hints that there may be some degree of cross-talk or cross-regulation between them.

3.4. Materials and Methods

3.4.1. Chemicals

All auxiliary enzymes, cofactors and substrates used for metabolite determinations and/or transcript profiling were purchased from either Sigma Aldrich Fluka (SAF) Chemical Company (St. Louis, MO, USA) or Roche Diagnostics (Basel, Switzerland), unless stated otherwise. Smoke-water was prepared by burning fynbos material, essentially as described by Baxter *et al*. (1994). KAR_1 (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, 99% purity) was isolated from plant-derived smoke according to the method outlined in Van Staden *et al.* (2004). GR24 was purchased from Professor Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, Netherlands). Both smoke and KAR_1 solutions were kindly donated by Prof Johannes Van Staden (Research Centre for Plant Growth and Development, University of KwaZulu-Natal, Pietermaritzburg).

3.4.2. Plant material, growth conditions and phenotypic assessment

Nicotiana benthamiana seeds were surface decontaminated (washed in 1.75% sodium hypochlorite for 5 min, followed by wash in 75% ethanol for 5 min followed by wash in dH_2O five-times for 2 min each) and allowed to germinate on half-strength Murashige and Skoog (MS) nutrient medium (pH 5.9) solidified with 8 g l^{-1} bacteriological agar containing the relevant growth promoting substances. All test compounds were added to the media prior to autoclaving. Growth experiments were conducted with both autoclaved and filter-sterilized GR24 in order to determine if the sterilization process altered the activity of the compound (See Figure 3.9). It was determined that the GR24 remained active after autoclaving. Whether seedlings were treated with autoclaved or filter-sterilized GR24, the growth parameters were

consistent between the GR24 treatments. All further experiments were conducted with autoclaved GR24.

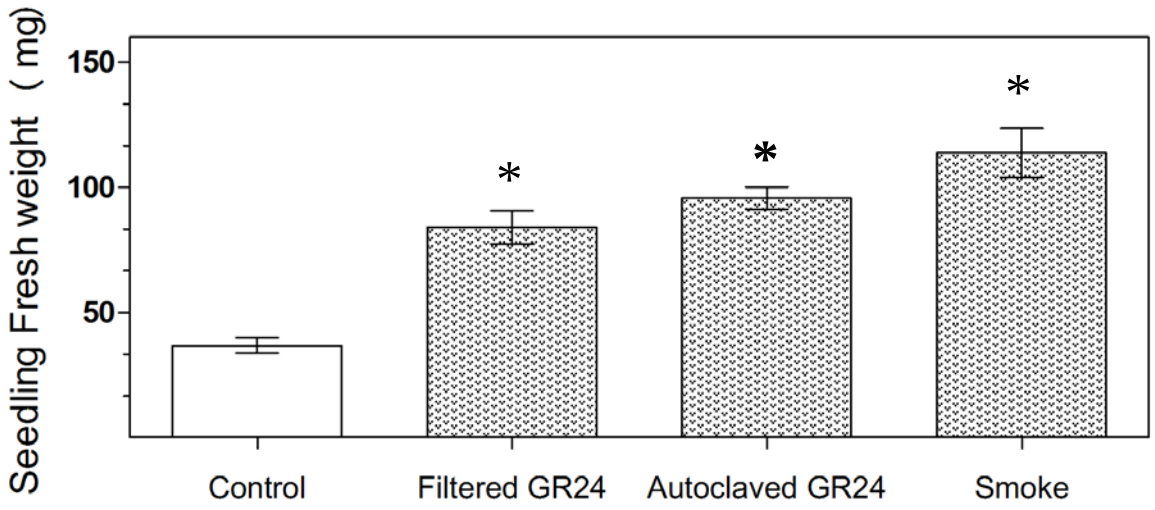


Figure 3.9. Seedling fresh mass determined following treatment with autoclaved GR24, filter-sterilized GR24 and smoke-water in order to determine if the sterilization process altered the activity of the GR24 compound. An asterisk (*) indicates a value that was determined by one-way ANOVA to be significantly different ($P < 0.05$) from the control.

Plates were grown under cool fluorescent light (Osram L 58V/740) in a growth room using a 16/8 h light/dark regime with a photosynthetic photon flux density of 50 µmol photons $m^{-2}s^{-1}$. All data were collected three weeks post germination, in three independent experimental trials, unless stated otherwise, and phenotypically assessed for fresh and dry mass (dried at 65°C for 2 days), primary root length, shoot length and lateral root number. Leaf area was digitally obtained and calculated by comparison with a 100 mm^2 reference marker processed within each image.

3.4.3. Germination studies

For seed germination trials, four replicates of 25 seeds each were placed on two layers of Whatman No. 1 filter paper and placed in 90-mm Petri dishes. The filter paper was moistened with 5 ml dH_2O (control), 1:1000 (v/v) diluted smoke-water, 10^{-7} M GR24 or 10^{-7} M KAR_1 solution. Petri dishes were sealed in plastic bags and incubated in a growth room under 16/8 h light/dark conditions as above (for the light germination experiments) or in a dark, sealed cupboard (for the dark germination experiments) at 25°C. Germination was examined at 12 h intervals for 4 days and again after 7-8 days. Dark germination experiments were examined under green safe-light conditions (490-560 nm, 0.3 μmol photons m^{-2} s^{-1}). Two independent experiment trials were performed for each treatment.

3.4.4. RNA isolation, microarray analysis and processing

Total RNA was extracted according to a method modified from Chang *et al.* (1993). Homogenised plant material (200 mg) was extracted with 1 ml CTAB buffer (2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g l^{-1} spermidine). Samples were vortexed immediately and incubated for 15 min at 65°C. Thereafter, they were centrifuged at 13000 xg for 10 min after which the supernatant was transferred to a new tube. Samples were extracted twice with chloroform:isoamyl alcohol (24:1, v.v) and the supernatant again transferred to a new tube. Total RNA was precipitated overnight at 4°C by adding 8 M LiCl to a final concentration of 2 M LiCl per sample. RNA was collected by centrifugation at 13000 xg for 60 min at 4°C. RNA was washed twice with ice cold 70% ethanol and once with 100% ethanol. RNA was resuspended in 50 μl dH_2O and kept at -80°C until use.

The microarray analysis was performed at University of Erlangen-Nürnberg. For microarray analysis, the RNA purity was measured using a ND-1000 spectrophotometer (NanoDrop Technologies). To check for RNA degradation 2 µg of total RNA were separated on a 1.5% formaldehyde gel. Total RNA was purified using RNeasy Mini Spin Columns (QIAGEN, Valencia, CA; www.qiagen.com). Thereafter, RNA quality and quantity were tested using the Agilent 2100 BioAnalyzer (vB.02.03 BSI307) as recommended by the manufacturer. (Agilent RNA 6000 Nano Assay Protocol2). Synthesis of cDNA was performed as described in the one-colour microarray-based gene expression analysis protocol provided by Agilent including the one-color RNA spike-in kit (v5.0.1, 2006; Agilent Technologies, Santa Clara, www.agilent.com). Cy3-labelled samples were loaded on the array (44K *Nicotiana tabacum* microarray, Agilent, www.agilent.com) and hybridised overnight (17 h at 65°C). Slides were washed as recommended by manufacturer's protocol and scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5 µm). Data sets were extracted by use of the feature extraction software (v9.5.3.1/Agilent Technologies) using a standard protocol. Microarray data obtained in this study can be accessed at www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20520.

Each hybridised Agilent array was normalised and analysed via the embedded R scripts in GeneSpringGX (Version 10.0, Agilent Technologies Inc., Santa Clara, California, USA). In brief, global normalization and scaling was achieved by normalization to the median of all probe sets for all the arrays using the MAS5.0 algorithm and absolute calls for each probe set were obtained using a lower critical cut-off value of 0.05 and higher critical cut-off value of 0.065. Gene selections based on an asymptotic one-way ANOVA were made to filter those genes whose residuals between the compared sample pairs are significantly higher than the measured noise level and considered to be differentially expressed. The residual *P*-value

obtained by the one-way ANOVA was corrected for multiple testing by the Benjamini and Hochberg (1995) algorithm and these false discovery rate (FDR) corrected *P*-values ($P < 0.05$) and an associated 2.0-fold change cut-off value in at least one of the gene pairs normalised to the control treatment were considered significant.

Gene annotation based on the *Nicotiana* accession number and/or oligosequence information yielded only cursory information in NCBI BLAST search. Consequently, the *Nicotiana* accession number and probed oligosequences, respectively, were analyzed in Blast2GO (www.blast2go.de) according to the method described by Conesa *et al*., (2005).

Nicotiana accession and oligosequence were used to design primers specifically aimed at amplifying genes commonly expressed between the three treatments (GR24, smoke-water and KAR_1) in order to corroborate microarray results. PCR was used to amplify regions of interest (See Table 3.5). PCR conditions for EB439539, BP130409, CK286340 and DW004809 were as follows: 94˚C for 3 min followed by 40 cycles of 94˚C for 30 s, 60˚C for 30 s and 72˚C for 30 s. PCR conditions for EH621771 were as follows: 94˚C for 3 min followed by 40 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 30 s. PCR products were separated using a constant 90V current (1h) during electrophoresis on a 0.8% agarose gel.

3.4.5. Starch and sugar measurements

Starch and soluble sugar extraction and measurements were performed via an enzymatic assay, according to Müller-Röber *et al*. (1992)**.**

Table 3.5. Primer sequences used for gene amplification during RT-PCR.

Gene annotation	*Nicotiana* accession number		Primer sequences	Product size
Acyl carrier protein	EB439530	*Forward*	5'- GAGCCCTGAAGTCCACTTTG-3'	
		Reverse	5'- CAGCTCAAGATCCAGCATCA-3'	516 bp
Receptor serine-threonine protein	EH621771	*Forward*	5'- CTTGACGAACAGGTTGTTGC-3'	
		Reverse	5'- CTTGAAGCGTTGGATCTGC-3'	639 bp
Proteasome activator subunit 4-like	BP130409	*Forward*	5'- GGCCGCGAATAAATACCG-3'	
		Reverse	5'- GTGGGCTAAACACGTAGCAA-3'	341bp
MAX2	CK286340	*Forward*	5'-GGCCATG-GCAATGGATATAG-3'	
		Reverse	5'-CTATGGAAGCACTGGATACGG-3'	675bp
MAX4	DW004809	*Forward*	5'- AAGGCTGAGCCCA-CACCACTT-3'	
		Reverse	5'-CAAGGCCTCTTAGCACCACAAGCA-3'	449 bp

3.4.6. Phytohormone profiling

Phytohormones were extracted according to Edlund *et al.* (1995). In brief, 500 µl of a 0.05 M Na-phosphate buffer (pH 7.0) was added in a 10:1 ratio to homogenised tissue, and incubated for 1 h in the dark with continuous shaking at 4°C. After extraction, the pH was adjusted to 2.6, the sample enriched with ca. 35 mg Amberlite XAD-7 (Serva, Heidelberg, Germany) and further incubated for 1 h in the dark with continuous shaking at 4°C. After centrifugation, the XAD-7 was washed twice with 500 µl 1% (v.v) acetic acid before elution with 500 µl dichloromethane for 30 min and elution repeated once more. The combined dichloromethane fractions were reduced under vacuum till dryness. Derivitisation of the sample (modified from Schmelz *et al.*, 2003) was achieved by adding 50 µl of 2 M trimethylsilyl (TMS) diazomethane in hexane (Sigma Co., www.sigma.com) and 10 µl methanol, followed by incubation at room temperature for 30 min. Excess TMS diazomethane was destroyed by adding 50 µl 1% acetic acid. An *n*-Alkane standard was added to each sample prior to reducing the sample to dryness under vacuum. Samples were resuspended in 50 µl heptane and injected splitless into a GCT Premier™ benchtop orthogonal acceleration time-of-flight (oa-TOF) MS (Waters, www.waters.com). Running conditions were exactly as described by Edlund *et al.*, (1995), and phytohormone identification and quantification was done by means of linear calibration curves of authentic standards.

3.4.7. Statistical analyses

Unless otherwise specified, statistical analyses were performed using the one-way ANOVA embedded in Statistica (Statistica 8.0, www.statsoft.com) and Duncan's *post-hoc* test was performed to statistically group the treatments according to effect size. Only the return of a *P*-value < 0.05 was designated significant.

Chapter 4

Construction of *MAX2* and *MAX4* gene silencing vectors to characterize growth response to strigolactone treatment through RNAi

"An important scientific innovation rarely makes its way by gradually winning over and converting its opponents: What does happen is that the opponents gradually die out."

Max Planck

4.1. Introduction

4.1.1. KAR_1 and GR24 promote plant growth through different signalling cascades

KAR_1 and the synthetic strigolactone, GR24, have both shown to be powerful plant growth regulators (*Chapter 3*). The basic structural unit of strigolactones is composed of a tricyclic lactone (A-, B- and C-ring) connected to a butyrolactone (butenolide) D-ring (Mangnus and Zwanenburg, 1992). Bioactive strigolactones all possess at least one methyl substituent on the A-ring and variable hydroxylation around the A- and B-rings (Mangnus *et al.*, 1992; Mangnus and Zwanenburg, 1992). The bioactivity of strigolactone resides in the C-D part of the molecule and its germination stimulating ability is critically linked to the enol-ether bridge and the methyl substituted- α-β furane moiety, the D-ring, which it shares with KAR_1, the active germination stimulant identified from smoke water (Chiwocha *et al.*, 2009; Mangnus *et al.*, 1992; Mangnus and Zwanenburg, 1992). Treatment of *Nicotiana benthamiana* with GR24 and smoke/KAR_1 was characterised by similar plant growth promoting abilities, but molecular probing indicated that the plant growth promoting abilities of these chemicals triggered different signalling cascades (*Chapter 3*). However, a degree of overlap in the growth responses was observed when the two treatments were combined. It is

possible that these chemicals trigger overlapping pathways through which they are regulating plant growth.

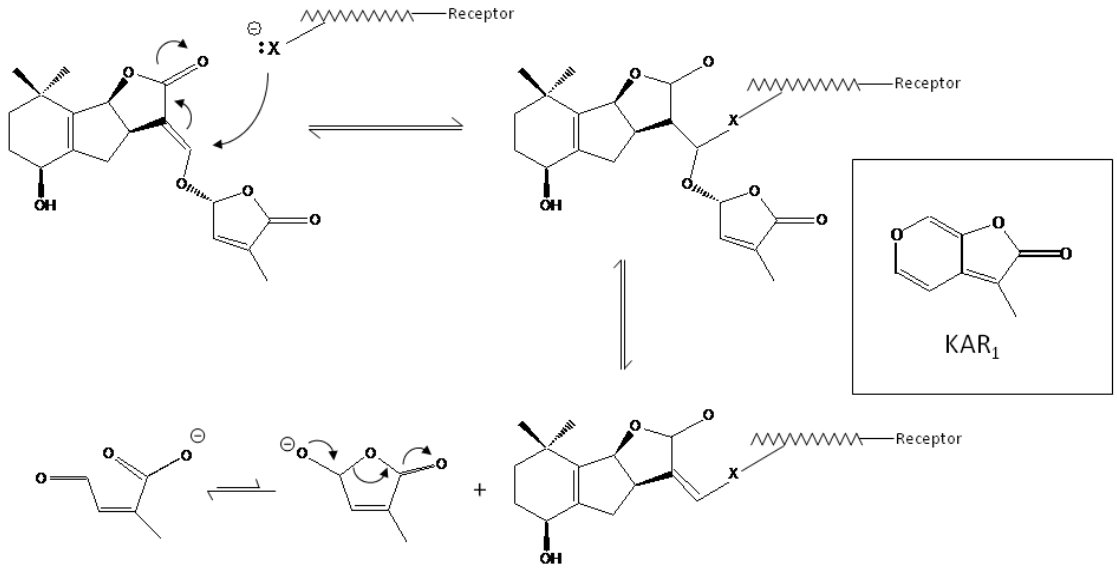


Figure 4.1. Strigolactone and KAR_1 potential receptor interaction as proposed by Zwanenburg *et al*. (2009).

In light of the structural similarity between the molecules (GR24 and KAR_1), a common interception point of the signalling cascades might be at the receptor site. Studies aimed at the identification of a possible strigolactone receptor have been unsuccessful, thus far (Thuring *et al.*, 1997). However, Mangnus and Zwanenburg, (1992) and Zwanenburg *et al*. (2009) have proposed a chemical molecular mechanism for receptor interaction during which KAR_1 and GR24 both may potentially interact at the same receptor site. This mechanism proposes that, during germination stimulation, a nucleophilic species present at a putative receptor site is added to the enol-ether carbon double bond in a Michael fashion, followed by elimination of the D-ring from the rest of the strigolactone structure (ABC-rings) (Figure 4.1).

Molecular probing (*Chapter3*) indicated that the pathways leading to increased growth operate largely independently from each other, but that a degree of crosstalk could not be ruled out. These results are not surprising when considering the fact that the molecular architecture of the strigolactone molecule (containing 4 stereogenic centres) is much more complex when compared with the structurally related- karrikinolide molecule (no stereogenic centers) (Flematti *et al.*, 2005). These molecules could potentially be perceived by the same receptor entity, but the signal may be differentially interpreted by the plant due to structural divergence between these molecules. The binding of these chemicals to the receptor could lead to the initiation of separate signalling cascades of which improved plant biomass accumulation is a common end result.

4.1.2. KAR_1 and strigolactone may potentially promote plant growth through the *MAX* strigolactone signalling pathway

The *Arabidopsis* mutants, *max1, max2, max3 and max4* (Stirnberg *et al.,* 2004, Turnbull *et al.,* 2002, Sorefan *et al.,* 2003; Booker *et al*., 2003) and rice *d* mutant have all been described previously in *Chapter 2*. The Phenotypes of these various mutants appear very similar and include reduced stature, increased branching, and length reductions in the leaf blades and petioles. The *MAX* loci (*MAX1–4*) are all required for the production or perception of an upwardly-mobile branch inhibition signal, strigolactone (Stirnberg *et al.,* 2004, Turnbull *et al.,* 2002, Sorefan *et al.,* 2003; Booker 2003; Umehara *et al*., 2008). The *MAX* genes are therefore proposed to all act in the same signalling pathway, with *MAX1*,*MAX3* and *MAX4* functioning in the biosynthesis process of the strigolactone signal and *MAX2* acting in the perception process.

A common strategy during hormone signalling in plants involves the removal of key transcription factors by use of F-box-containing ubiquitin E3 ligases in a hormone concentration-dependant manner. The transcription factors may be activators of gene expression such as in the case of transcriptional regulator *ETHYLENE INSENSITIVE3* (*EIN3*), where *EIN3* degradation is significantly attenuated by the presence of ethylene hormone in the cell. In contrast to activators, transcriptional factors acting as repressors of gene transcription are more common, such as the DELLA proteins involved in GA signalling (McGinnes *et al*., 2003), AUX/IAA proteins in auxin signalling (Kepinski and Leyser, 2002; Gray *et al*., 2001) and the JAZ proteins functioning during jasmonic acid signalling (Xu *et al*., 2002).

MAX2 has been shown to encode an F-box protein, which can interact with ASK1 and SKP1 to form an SCF-complex (Stirnberg *et al.*, 2007). F-box proteins have been demonstrated to function as phytohormone receptors which directly or indirectly perceive signals and facilitate specific target-protein degradation to regulate downstream pathways (Smalle and Vierstra, 2004). F-box proteins functions as the substrate recognition module for the multi-subunit Skp1-Cullin1-F-box (SCF) complex to specifically target their dedicated substrates for poly-ubiquitylation (Smalle and Vierstra, 2004). Poly-ubiquitinated proteins are recognized and degraded by 26S the proteasome (Smalle and Vierstra, 2004). Given the chemical and molecular identity of the *MAX2* protein, it is possible that *MAX2* may be the long sought-after strigolactone receptor.

The plant growth promoting properties of the synthetic strigolactone GR24, as described in *Chapter 3*, may be mediated, after binding to the strigolactone receptor, through the *MAX* strigolactone signalling pathway. It is possible that the KAR_1 molecule may also stimulate the

strigolactone receptor and may also be eliciting its growth promoting properties at least partially through the *MAX* strigolactone signalling pathway. Given the structural similarity between the KAR_1 compound and GR24 it would not be surprising if these two compounds bind to the same receptor. Given that some F-box proteins act as hormone receptors (e.g. TIR1 in auxin signalling), it is possible that MAX2 itself may act as the strigolactone receptor. Alternatively, it is possible that *MAX2* functions immediately downstream of the strigolactone receptor. A strigolactone-insensitive mutant in rice, *d14*, exhibits the extreme branched phenotype also seen in the *MAX* mutants in *Arabidopsis*. Interestingly, the *D14* protein encodes an α-β-hydrolase protein from the same protein family as the GID1 GA receptor protein from rice (Refer to *Chapter 2*). This mutant also exhibits a highly reduced sensitivity to strigolactone and accumulates elevated levels of endogenous strigolactone. This provides evidence that the D14 product functions downstream of strigolactone biosynthesis. Evidence exists that the α-β-hydrolase family acts as a high affinity binding protein of small molecules such as receptors but does not participate in a hydrolytic reaction itself (Arite *et al.*, 2009). It is thus possible that this α-β-hydrolase, D14, may be the strigolactone receptor in rice, with MAX2/D3 functioning downstream in a manner similar to the GA receptor, GID1, acting upstream of the F-box protein GID2/SLY1.

4.1.3. RNAi and the production of plant gene silencing constructs

Fire *et al.*, (1998) demonstrated that when injected into or ingested by nematodes, double stranded RNA (dsRNA) triggers sequence-specific RNA degradation in a process known as RNA interference (RNAi). This process facilitates targeted post-transcriptional gene silencing (PTGS) and was recently implemented to study over 4000 genes in *Ceanorhabditis elegans* (Fraser *et al.*, 2000). Transgenes designed to express single- or double-stranded, self-

complimentary, hairpin RNAs (hpRNA) have similar post-transcriptional silencing effects in plants (Wang and Waterhouse, 2000; Wang *et al.,* 1998; Wesley *et al.,* 2001). Inclusion of a functional intron spacer between the arms of hpRNA constructs have been shown to massively increase the gene silencing effect in plants. It is possible, through the use of RNAi transgene technology, to target specific genes for silencing in order to determine the function of selected gene.

pHELLSGATE2 is a plant transformation vector system which uses Gateway cloning technology to easily generate an intron-separated hpRNA construct (Wesley *et al.,* 2001). The inversely-orientated, gene-specific arms can be cloned into the vector in one step, avoiding the use of restriction enzymes and greatly streamlining the construction procedure (Wesley *et al.,* 2001). The concept of the *pHELLSGATE* vectors is based on the *Lambda* phage recombination strategy. PCR products are generated with flanking *attb1* and *attb2* sites and, when incubated with a plasmid containing two *attP1* and *attP2* casettes separated by an intron sequence, these PCR products would be inserted by a BP clonase^{TM} (Invitrogen, Carlsbad, CA) enzyme in an inverted repeat with arms separated by a hairpin-forming intron (Wesley *et al.,* 2001).

This study hypothesized that KAR_1 promotes plant growth through a branch of the *MAX* signalling pathway. KAR_1 shares the active constituent (D ring) of the synthetic strigolactone (GR24) molecule. *MAX2,* being a possible strigolactone receptor, may potentially also interact with KAR_1. It is clear that strigolactones have more than one function in seedlings as plant growth was shown in *Chapter 3* to be another function added to the list of functions already described for strigolactones.

In order to determine whether the plant growth promotion resulting from GR24 treatment is actually being propagated through the same MAX strigolactone signalling pathway as operates in the regulation of lateral branching, this study aimed to construct two gene silencing vectors to silence expression of two genes (*MAX2* and *MAX*4) in the strigolactone signalling pathway. This study further hypothesizes that KAR_1 may also promote plant growth through a branch of the *MAX* signalling pathway, since KAR_1 shares the active constituent (D ring) of the synthetic strigolactone (GR24) molecule. These constructs can, in future, be used to create transformed seedlings with reduced/silenced *MAX2* and *MAX4* transcription levels to further investigate these hypotheses.

4.2. Results

4.2.1. Construct design and RNAi for efficient gene silencing in *Nicotiana benthamiana*

The presence of double-stranded RNA (dsRNA) in a cell can trigger specific RNA degradation in a process known as RNA interference (Fire *et al.*, 1998; Wang and Waterhouse, 2000; Waterhouse *et al.*, 1998). Therefore, silencing vectors encoding self-complimentary hairpin RNA of sequences from the *MAX2* and *MAX4* genes were constructed in an attempt to silence the expression of these genes in *Nicotiana benthamiana*. Sequences encoding the *Arabidopsis MAX2* (*AtMAX2*, Genbank accession nr: NM129823.2) and *Arabidopsis MAX4* (*AtMAX4*, Genbank accession nr: AT4G32810) genes identified from the NCBI database were used in BLAST (blastn and blastx) searches of the Expressed Sequence Tags (EST) database (Genbank, NCBI) using *Nicotiana* (taxid:4085) as an additional search criterion (See Addendum C for BLAST results). *AtMAX2* showed significant homology with *Nicotiana* EST, CK286340 and *AtMAX4* with *Nicotiana* EST DW004809. These sequences were aligned using a web-based sequence alignment tool, ClustalW

(http://www.ebi.ac.uk/Tools/clustalw2/index.html) (See Addendum B Figure i, ii for complete alignment), and primers were designed for PCR amplification of a 675 bp *Nicotiana MAX2* (*NbMAX2*) fragment and a 449bp *Nicotiana MAX4* (*NbMAX4*) fragment (See Figure 4.2). After PCR cleanup, PCR fragments from *NbMAX2* and *NbMAX4* were successfully cloned into the pGEM®-T Easy cloning vector and transformed into *Escherichia coli* (*E. coli*) DH5α (Figure 4.2).

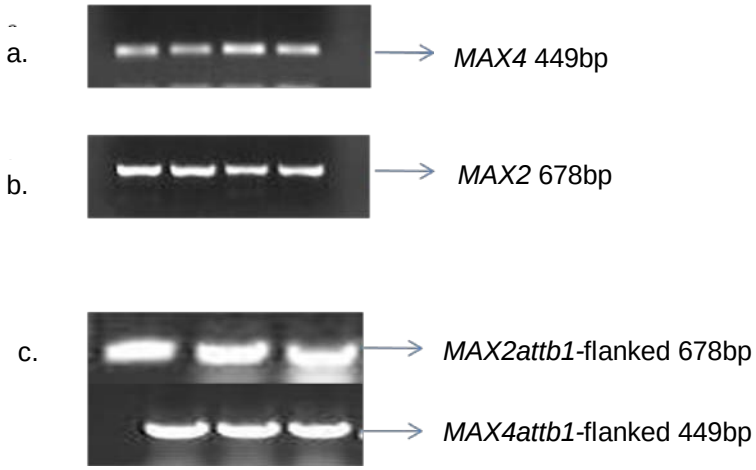


Figure 4.2. PCR amplification of (a) *NbMAX4*, (b) *NbMAX2* gene fragments from *N. benthamiana*. (c) PCR amplification of *NbMAX4* and *NbMAX2* fragments from pGEM®-T Easy vector using SP6*attb1* and T7*attb1* primers

4.2.2. Construction of the gene silencing (RNAi) vector *pHELLSGATE2* high-throughput hpRNA vector

SP6-*attb2* and T7-*attb2* primers were designed to amplify the *NbMAX2* and *NbMAX4* inserts with *attb*2 sites at the 5' and 3' ends from the polycloning site in the pGEM®-T Easy vector. The silencing constructs were generated in PB clonase enzyme reactions and ample transformed colonies were retrieved. Plasmids were designated *pHG2-MAX2* and *pHG2-MAX4.* Colony PCR was performed on the transformed cells and the resulting PCR products were sequenced to confirm the identity of the insert in the vector. Sequence data was aligned using ClustalW (Figure 4.3 a, b), indicating that the *NbMAX2* and *NbMAX4* gene sequences had been successfully inserted into the *pHELLSGATE2* vector (Figure 4.4).

```
A31T7          TGGGACCCTTTGTACAAGAAAGCTGGGTCTATTTAGGTGACACTATAGAATACTCAAAGC 60
A31SP6         ----------------------------------------------------AACTCAA-GC 9
MAX2           -------------------------------------------CATCAATAACCAAAGC 16
                                                           *  *** **

A31T7          TATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTC 120
A31SP6         TATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTC 69
MAX2           ACAAACCTTATTGCATATAAAGAGATCTGA-AAATTTTATACATATTCTGACAAAAGAAA 75
                         *  ** *    **   **  * *   *  *     *  * * *   *

A31T7          ACTAGTGATTTGGAAGCACTGGATACGGTAACATTATGTGAGCATTGTATTCCTTGCATC 180
A31SP6         ACTAGTGATTTGGAAGCACTGGATACGGTAACATTATGTGAGCATTGTATTCCTTGCATC 129
MAX2           ACTA------TGGAAGCACTGGATACGGTAACATTATGTGAGCATTGTATTCCTTGCATC 129
               ****      **************************************************

A31T7          AATCGGATATTGGGCGCCTGTTTAGGGCAGCTTCAAAGCGGCTCAAGGAGTCGGCTCTCA 240
A31SP6         AATCGGATATTGGGCGCCTGTTTAGGGCAGCTTCAAAGCGGCTCAAGGAGTCGGCTCTCA 189
MAX2           AATCGGATATTGGGCGCCTGTTTAGGGCAGCTTCAAAGCGGCTCAAGGAGTCGGCTCTCA 189
               ************************************************************

A31T7          TCTCTGTACTCATGTCATTCTCTGGTGCTGGATAGTAATCCTCTCTCAGTTGTACATCTC 300
A31SP6         TCTCTGTACTCATGTCATTCTCTGGTGCTGGATAGTAATCCTCTCTCAGTTGTACATCTC 249
MAX2           TCTCTGTACTCATGTCATTCTCTGGTGCTGGATAGTAATCCTCTCTCAGTTGTACATCTC 249
               ************************************************************

A31T7          TTAAGTTTGGGATTCTAAGAAGGAACATCATGAAATGTTCATGCGCTGTTCCATGGATGA 360
A31SP6         TTAAGTTTGGGATTCTAAGAAGGAACATCATGAAATGTTCATGCGCTGTTCCATGGATGA 309
MAX2           TTAAGTTTGGGATTCTAAAAAGGAACATCATGAAATGTTCATGCGCTGTTCCATGGATGA 309
               ****************** ****************************************

A31T7          ACAGTTTTCTGAGTGTGACGCATTCTTGTAGCAGCCCAGCTGCTGGTAGGGATAGACACC 420
A31SP6         ACAGTTTTCTGAGTGTGACGCATTCTTGTAGCAGCCCAGCTGCTGGTAGGGATAGACACC 369
MAX2           ACAGTTTTCTGAGTGTGACGCATTCTTGTAGCAGCCCAGCTGCTGGTAGGGATAGACACC 369
               ************************************************************
```

```
A31T7           TTTGGTTAACGTCCCTATCTTGTGGTGGCCAGTAATCTAGTTCAGTAAGGCTCAAATTTC 480
A31SP6          TTTGGTTAACGTCCCTATCTTGTGGTGGCCAGTAATCTAGTTCAGTAAGGCTCAAATTTC 429
MAX2            TTTGGTTAACGTCCCTATCTTGGGGTGGCCAGTAATCTAGTTCAGTAAGGCTCAAATTTC 429
                ********************* ************************************

A31T7           CAATCCCAAACAGATAAAACCTTTCCCACAAGCTCAAATCCATCTGCCCTGATGGAGCAG 540
A31SP6          CAATCCCAAACAGATAAAACCTTTCCCACAAGCTCAAATCCATCTGCCCTGATGGAGCAG 489
MAX2            CAATCCCAAACAGATAAAACCTTTCCCACAAGCTCAAATCCATCTGCCCTGATGGAGCAG 489
                ************************************************************

A31T7           TGTGTGCATAACCTATGATATCTCCACAATCCAAATGCATCTTGGATAGCTTAGGATAGA 600
A31SP6          TGTGTGCATAACCTATGATATCTCCACAATCCAAATGCATCTTGGATAGCTTAGGATAGA 549
MAX2            TGTGTGCATAACCTATGATATCTCCACAATCCAAATGCATCTTGGATAGCTTAGGATAGA 549
                ************************************************************

A31T7           GAAGAAGGGTGCTCAGTCCAAATGCCCGCTCCGAAGGTTTTGACCATAGCCTGCAATCTC 660
A31SP6          GAAGAAGGGTGCTCAGTCCAAATGCCCGCTCCGAAGGTTTTGACCATAGCCTGCAATCTC 609
MAX2            GAAGAAGGGTGCTCAGTCCAAATGCCCGCTCCGAAGGTTTTGACCATAGCCTGCAATCTC 609
                ************************************************************

A31T7           CTTCCACCCTAATCTTGATCTCCTCTAAATTTGGACAGTCTTCAAGACCTGCAGCTGTTA 720
A31SP6          CTTCCACCCTAATCTTGATCTCCTCTAAATTTGGACAGTCTTCAAGACCTGCAGCTGTTA 669
MAX2            CTTCCACCCTAATCTTGATCTCCTCTAAATTTGGACAGTCTTCAAGACCTGCAGCTGTTA 669
                ************************************************************

A31T7           AAGGAGTCAAAAGCTCACCAACACCAATCCAAAGAGAGAGGCATTGCAGCCGATCCCATG 780
A31SP6          AAGGAGTCAAAAGCTCACCAACACCAATCCAAAGAGAGAGGCATTGCAGCCGATCCCATG 729
MAX2            AAGGAGTCAAAAGCTCACCAACACCAATCCAAAGAGAGAGGCATTGCAGCCGATCCCATG 729
                ************************************************************

A31T7           ATCGTCCACTATATCCATTGCCATGGCCAATCGAATTCCCGCGGCCGCCATGGCGGCCGG 840
A31SP6          ATCGTCCACTATATCCATTGCCATGGCCAATCGAATTCCCGCGGCCGCCATGGCGGCCGG 789
MAX2            ATCGTCCACTATATCCATTGCCATGGCCATTGACTTCCTCATACAAACTATTAAGATCA- 788
                ***************************** *    * * *       * **   *  *

A31T7           GAGCATGCGACGTCGGGCCCAATTCGT-------------------------------- 867
A31SP6          GAGCATGCGACGTCGGGCCCAATTYGCCCTATAGTGAGTCGTATTACAGCCTGCTTTTTT 849
MAX2            ---CAGGTGAACTTGCATCTTTTCTT--------------------------------- 811
                   ** * **  * *   *   *
```

Figure 4.3.a. Alignment of *Nicotiana* EST CK286340 (labelled *MAX2*) from NCBI with sequenced product *NbMAX2* (A31SP6; A31T7) amplified from p*HG2-MAX2* vector with *attb1*-**SP6** and *attb1*-**T7** primers. Sequences were aligned using ClustalW.

```
A13T7           ------------------------------------------------------------

A13SP6          ------------------------------------------------------------
MAX4            AATACTACATTAGCTATTCAAGAGGTGGAAAATGTAATATCTTATTATATGACTTGTAAG 60

A13T7           ------------------------------------------------------------
A13SP6          ------------------------------------------------------------
MAX4            GTGTATTTGGCTATCATATAACACTGTCTAGGATGTGGGGGGTGGTTCTATTTCTTTGGA 120

A13T7           ------------------------------------------------------------
A13SP6          ------------------------------------------------------------
MAX4            ACCCAACAACCATGCAGTCCATAGGGAAGACCATAAGGAAACTTTGCTCGTGCAATTTCT 180
```

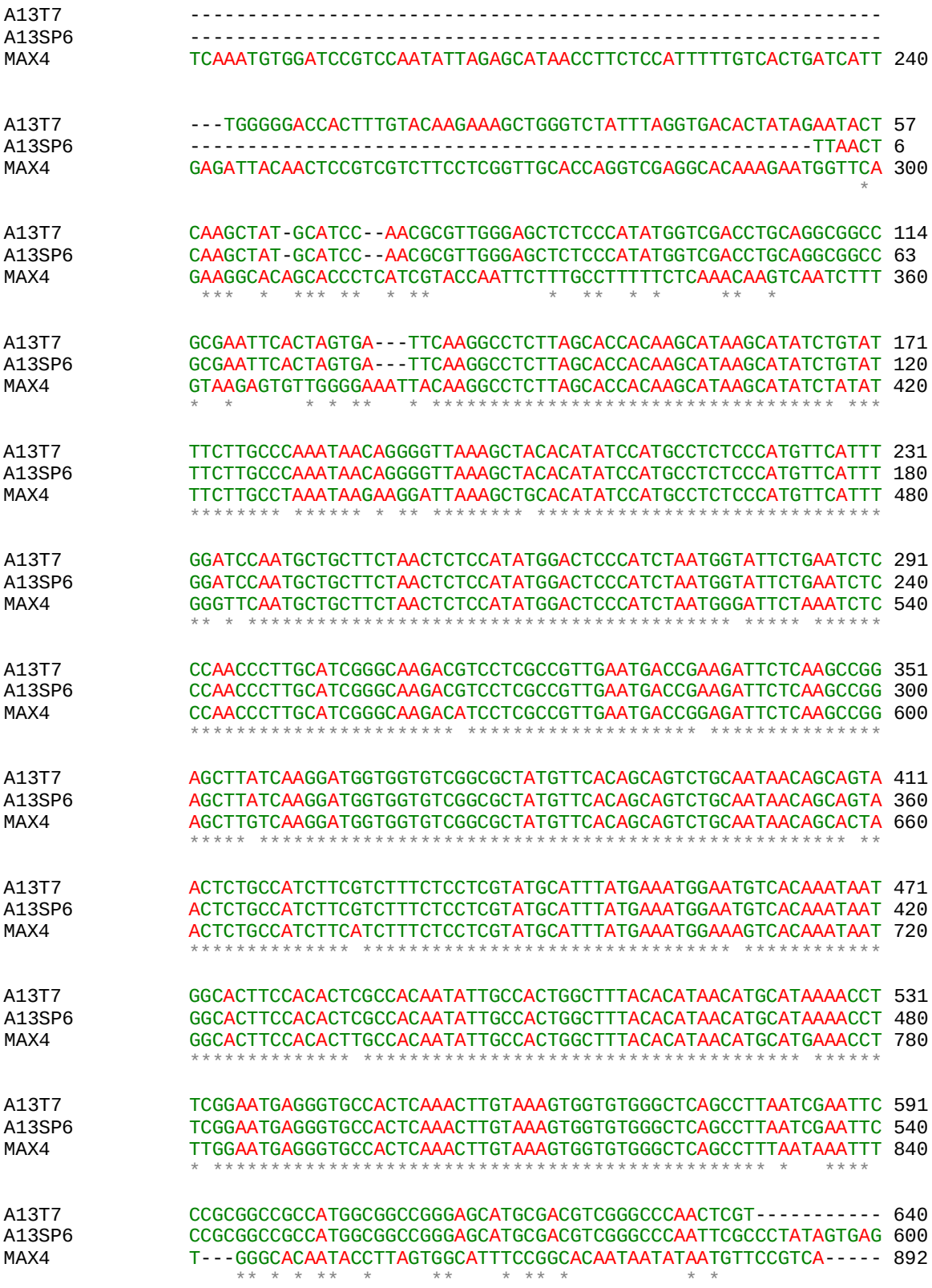
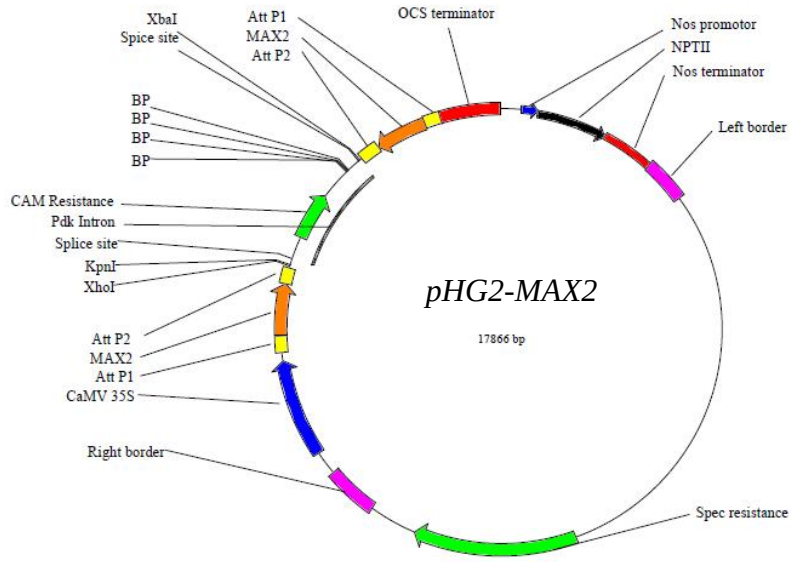


Figure 4.3.b. Alignment of *Nicotiana* EST DW004809 (labelled *MAX4*) from NCBI with sequenced product *NbMAX4* (A13SP6; A13T7) amplified from *pHG2-MAX4* vector with *attb1*-**SP6** and *attb1*-**T7** primers. Sequences were aligned using ClustalW



.

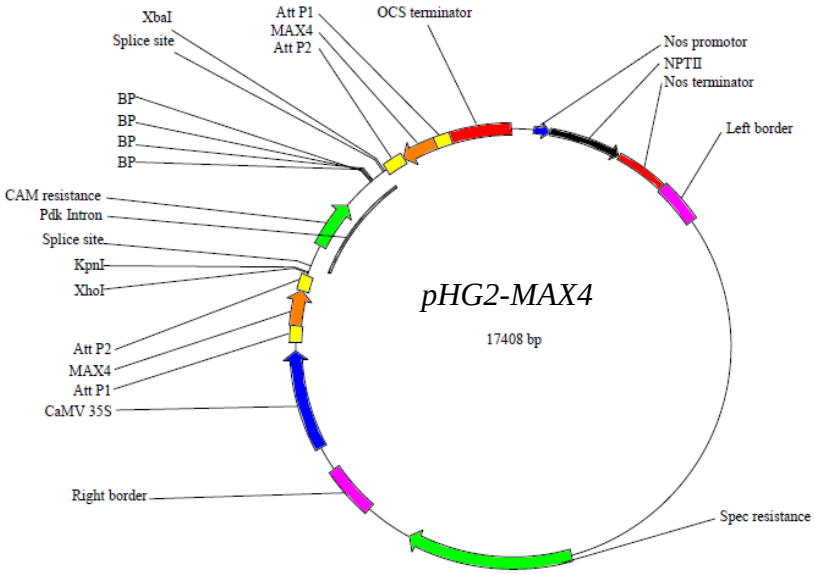


Figure 4.4. Plasmid maps of constructed silencing vectors, *pHG2-MAX2* (top) and *pHG2-MAX4* (bottom). Plasmids were constructed using a BP clonase recombination enzyme reaction in which *NbMAX2* and *NbMAX4* gene sequences were inserted between attP1and attP2 sites on the pHELLSGATE2 binary vector in a reversed-orientation.

4.3. Discussion

In this study two gene silencing vectors were constructed, *pHG2-MAX2* and *pHG2-MAX4*. Fragments of the *NbMAX2* and *NbMAX4* genes were successfully cloned into the *pHELLSGATE2* plant transformation vector. The main aim envisaged in the construction of these vectors was to create a novel and effective way to silence these genes *in planta*. This was done in order to characterize the involvement of the *MAX* signalling pathway during plant growth stimulation resulting from GR24 and KAR_1 treatment.

These two constructs will be used to transform *Nicotiana benthamiana*. Following plantlet regeneration, putative transformants will be selected and subjected to GR24 and KAR_1 treatments. Further phenotypic and molecular characterization will hopefully provide answers into the signal transduction mechanisms via which GR24 and smoke/KAR_1 elicit their plant growth promoting effects.

Seedlings impaired in strigolactone production and signalling would be evaluated for their ability to respond to exogenously-applied strigolactone and KAR_1. Although the transformants in which *MAX4* is silenced should be unable to produce strigolactones, these plants should be able to respond to exogenously-applied strigolactones. Thus, it is expected that seedling transformed with the *pHG2-MAX4* (*nbmax4*) should respond positively to GR24 treatment in comparison to an untreated *nbmax4* control. These plants are expected to also be able to respond positively to KAR_1 treatment as well.

On the other hand, seedling impaired in strigolactone signal transduction would not be able to perceive exogenously-applied strigolactones. Plants transformed with the *pHG2-MAX2* would be able to produce strigolactone but unable to respond to it. Thus, seedlings

transformed with *pHG2-MAX2* should not be able to respond to GR24 treatment because these transformants would be strigolactone-, and hence GR24, insensitive. If these plants (*nbmax2*) however respond positively to KAR_1 treatment compared with an untreated control, it would be a clear indication that the plant growth promoting pathways initiated by KAR_1 do not function through the strigolactone (*MAX*) signalling pathway, but rather through an independent cascade. This would indicate that KAR_1 and GR24 do not stimulate the same receptor and the plant growth promoting pathway would be assumed to be independent of KAR_1 signalling.

4.4. Materials and Methods

4.4.1. RNA extraction and cDNA synthesis

Total RNA was extracted as described in *Chapter 3.4.4*. First-strand cDNA was generated from ~5 µg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions.

4.4.2. Construction of suppressor vectors

The *NbMAX2 and NbMAX4* fragments were amplified from cDNA using the MAX2 and MAX4 forward and reverse primers respectively (Table 4.1). Conditions for PCR were as follows: 94˚C for 3 min followed by 40 cycles of 94˚C for 30 seconds, 60˚C for 30seconds and 72˚C for 30 seconds.

The *NbMAX2* and *NbMAX4* PCR products were cloned into the pGEM®-T Easy Bacterial Transformation Vector (Promega) as per manufacturer's specifications. Briefly, a dilution

series of the PCR products was established and incubated overnight at 4˚C with the pGEM®-T Easy vector, T4 DNA Ligase and 2X Rapid Ligation Buffer. The *E. coli* DH5α cells were transformed with the pGEM®-T Easy-*NbMAX2* and pGEM®-T Easy-*NbMAX4* recombinant construct vector by electroporation (Bio-Rad Gene Pulser Xcell™ electroporation system) according to manufacturers guidelines and incubated for 1 h in LB medium (1% [m/v] bacto-tryptone, 0.5% [m/v] yeast extract, 1% [m/v] NaCl, 0.1% [m/v] glucose, pH7.5) media at 37˚C. The transformed bacterial cultures were then plated out on LB media containing 50 mg/µL ampicillin, 100 µM IPTG and 50 mg/ml X-gal for selection of transformed colonies. Non-staining (white) colonies were selected and colony PCR was performed with the SP6 and T7 primers (Table 4.1) to confirm the integrity of the insert. Plasmid extraction was performed using the GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's specifications. SP6*attb2* and T7-*attb2* (Table 4.1) primers were then used, following the same PCR conditions as used previously, to amplify the *NbMAX2* and *NbMAX4* sequences from pGEM®-T Easy-*MAX2* and pGEM®-T Easy-*MAX4* vectors.

The *attb2*-flanked *NbMAX2* and *NbMAX4* sequences were then cloned into the *pHELLSGATE2* binary vector in a BP Clonase™ II enzyme reaction as follows: Equal amounts of 150ng *Attb2*-PCR product (10ng/µl to a final amount ~15-150 ng; 1-7 µl) and *pHELLSGATE2* (150ng/µl; 1 µl) vector were added to a 1.5 ml microcentrifuge tube. TE buffer (0.025MTris-HCl EDTA, pH 8.0) was added to a final volume of 8 µl. To this mix, 2 µl of BP Clonase™ II enzyme was added and the reaction was incubated at 25˚C for 1 h. After incubation, Proteinase K solution was added to each sample and the reactions incubated at 37˚C for 10 min to terminate the reaction. Electro-competent *E. coli* DH5α cells were transformed with the *pHG2-MAX2* and *pHG2-MAX4* vectors and grown on LB plates solidified with 1.5% (m/v) bacteriological-agar (LBA) containing 50µg/ml spectinomycin.

Table 4.1. Primer sequences used for gene amplification during the construction of the silencing vectors *pHG2-MAX2* and *pHG2-MAX4*.

Gene annotation	*Nicotiana* accession number		Primer sequences	Product size
MAX2	CK286340	*Forward*	5'-GGCCATG-GCAATGGATATAG-3'	
		Reverse	5'-CTATGGAAGCACTGGATACGG-3'	675bp
MAX4	DW004809	*Forward*	5'- AAGGCTGAGCCCA-CACCACTT-3'	
		Reverse	5'-CAAGGCCTCTTAGCACCACAAGCA-3'	449 bp
Attb2 flanked SP6 and T7 primers		*SP6attb2*	5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TAT TTA GGT GAC ACT ATA GAA-3'	
		T7attb2	5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG TAA TAC GAC TCA CTA TAG GGC-3'	
pGEM-T Easy Specific primers		*SP6*	5'-AT TTA GGT GAC ACT ATA GAA-3'	
		T7	5'-TAA TACGA CTCAC TATAG GGC-3'	

Chapter 5

General discussion and conclusions

5.1. Introduction

The main aim of the work presented in this thesis was to investigate the plant growth promoting abilities of the synthetic strigolactone, GR24, in comparison to plant-derived smoke-water and KAR_1, in light of the structural similarities shared between GR24 and KAR_1. This chapter will focus on highlighting the main concepts developed during the course of this study.

5.2. Plant growth promotion is a complex process that is subject to the interplay of genetic and environmental influences

Plant biomass yield is determined by a number of factors, such as the efficiency in the capture of solar energy and the conversion of the captured solar energy into vegetative tissues that constitute the bulk of plant biomass. Plant growth is a highly complex process, subject to an intricate combination of genetic and environmental influences. There exists a tremendous interest in the potential of generating biofuels from plant material and subsequently the elucidation of the molecular mechanisms underlying the regulation of enhanced plant biomass production to improve biomass yield. Sadly, these mechanisms remain elusive and considerably more work has to be done in order to further our understanding of the processes involved in plant biomass production before it can be possible start to engineer plant species with enhanced biomass production on a commercial basis.

5.3. Strigolactones have proven to be powerful plant growth regulators with a wide range of functions in plants

Reports of parasitic plant seed germination stimulated by external cues from plant roots have been around since the 1960s when Cook *et al.* (1966) reported the isolation of (+)strigol from root exudates from cotton (*Gossypium hirsutum*). (+)Strigol is a highly potent germination stimulator of witchweed seeds. It is possible that the parasitic plant species such as *Striga* exploit a symbiotic communication system that was already active in ancient plant-AM fungi associations (Parniske, 2008). Parasitic plants take a heavy toll on crop plants such as corn (*Zea mays*), grain sorghum (*Sorghum vulgare*), sugarcane (*Saccharum officinarum* L.) and other cereal crops (Siame *et al.,* 1993) in Africa and other developing countries.

In a completely separate line of research that started over a decade ago, a novel mobile signal from Pea (*Pisum sativum*) and *Petunia* was identified that was responsible for branching inhibition. Grafting experiments identified a mobile signal coming from the roots that functions to inhibit lateral bud outgrowth. Following the grafting experiments, the focus shifted to a set of mutants identified in these species as well as in rice (*Oryza stativa*) and *Arabidopsis*. The genes involved were identified as *MAX3/RMS5/HTD1/D17* and *MAX4/RMS1/D10/DAD1* and were shown to code for two different carotenoid cleavage dioxygenase (CCD) enzymes, CCD7 and CCD8, respectively (Schwartz and Zeevaart, 2004; Sorefan *et al*., 2003). The phenotypes of mutants for these genes exhibit extreme bushiness. Two recent studies from Umehara *et al*. (2008) and Gomez-Roldan *et al.* (2008) then linked the branching mutants with a deficiency in strigolactones. They demonstrated that strigolactone levels were strongly reduced in the CCD mutants and that exogenously-applied, synthetic strigolactone (GR24) could rescue the extreme branching defects.

From the same shoot branching screen that brought about the characterization of the *MAX1* gene as encoding a cytochrome P450, came the most intriguing mutant, *MAX2* (Booker *et al.*, 2004; 2005). *MAX2* encodes an LRR-containing F-box protein related to TIR1 from *Arabidopsis* auxin signalling (Ruegger *et al.*, 1998) and COI1 from jasmonate signalling (Xu *et al.*, 2002) The MAX2 protein was shown to participate in the formation of an SCF complex, SCF^{MAX2} (Stirnberg *et al.*, 2007). Exogenously-applied GR24 can rescue the branched phenotypes of *max1*, *max3* and *max4* which were classified as being part of the strigolactone biosynthesis pathway. The *max2* phenotype, on the other hand, cannot be restored to the normal branching phenotype with strigolactone (GR24) application, and through genetic evidence was classified as functioning downstream of the strigolactone biosynthesis pathway (Stirnberg *et al.*, 2007).

Interestingly, *MAX2* has also been recovered previously as *ORESARA9* (*ORE9*) in a screen for senescence-delayed *Arabidopsis* mutants. The cloning of *MAX2* revealed its identity to *ORE9*, which had been isolated earlier as a positive regulator of *Arabidopsis* leaf senescence that limits the longevity of the leaf (Woo *et al.*, 2001). Yet another screen for mutants, this time for light signalling mutants, revealed that *MAX2* is also identical to *PLEIOTROPIC PHOTOSIGNALLING* (*PPS*), which functions as positive regulator of photomorphogenisis in *Arabidopsis* (Shen *et al.*, 2007).

Taken together, it is clear that strigolactones have more than just a branch inhibition function in plant systems. Three separate isolations of the same F-box protein known as MAX2, ORE9 or PPS indicate a wide range of functions for this LRR-containing F-box protein and strigolactones. Given the relation of auxin, gibberellins and jasmonate to their respective LRR-containing F-box proteins, a strong hormone perception function for *MAX2* becomes

likely. It is possible that F-Box protein MAX2 may be the elusive strigolactone receptor or it may function downstream of the receptor, similar to the F-box protein GID2/SLY1 acting downstream of the receptor, the α, β-hydrolase, GID1 in GA signalling. It would be of special importance to identify the transcription factors associated with the MAX2 F-box in an effort to identify more genes that connect the numerous roles of MAX2 in relation to the *MAX* signalling pathways. Of special interest would be the identification of genes related to the plant growth promotion function seen when seedlings are treated with strigolactone.

This study has added one more function to the list of functions for the novel hormone. Strigolactones, in the form of the synthetic analogue GR24, were demonstrated to promote plant growth in *Nicotiana benthamiana* considerably in a statistically significant manner. Not only did the GR24 improve whole plant biomass accumulation, but root length and number of the lateral roots were also increased in the treated seedlings. An increased shoot length, leaf area and dry mass were also observed upon GR24 treatment. This suggests that strigolactones may also be important regulators of plant growth and development, at least in young seedlings.

5.4. Smoke is a powerful plant growth regulator with agronomic importance

Numerous plant species have demonstrated a sensitivity to smoke derived from burning plant material. The germination promoting abilities of plant-derived smoke have been mainly attributed to the butenolide compound, KAR_1. To date, most studies have focused on the germination stimulating abilities of plant-derived smoke and KAR_1 (Sparg *et al.,* 2005). Smoke has also been demonstrated to have a range of plant growth regulating effects of which germination stimulation is only one. Plants from fire-prone and non-fire-prone

environments have shown varying responses to plant-derived smoke. This was attributed to the fact that smoke is a mixture of over 4000 compounds of which some are positive and some are negative regulators of germination. In some plant species, smoke treatment may not have a germination stimulating effect. It may, however, play a role during seedling development after germination has taken place, i.e. improved seedling vigour. It is for this reason that experiments need to be extended past a germination phase to evaluate the seedling development upon treatment in addition to characterizing the germination response.

It is possible that an interplay between some of the many chemicals present in smoke is responsible for the plant growth regulation. The environmental aspects present in the post-fire habitats may influence the reactions that plants have to the germination stimulating ability of smoke and the karrikins dramatically. Factors such as light, temperature and soil consistency all create intricate environmental signals after a fire has spread through the habitat. Rain may dilute out chemicals present in the soil after a fire that inhibit germination and in this way cue the seed bank in the soil to germinate when conditions are optimal. Plants have shown to respond differently to different karrikins. Nelson *et al*., 2009 have identified four different karrikins which stimulate germination to varying degrees of efficiency.

Seedling vigour is an important agronomic trait. It is important that seedlings can overcome adverse conditions encountered under field environments. Smoke treatment may help to overcome these conditions during seedling establishment which would lead to higher plant biomass yields. There has been little documented work on commercial crop plants with regards to the post germination plant growth promoting abilities of smoke treatment to enhance seedling vigour. Maize (*Zea mays* L.) was shown to exhibit enhanced germination as well as improved seedling vigour upon smoke treatment (Soós *et al.,* 2009, Sparg *et al.,*

2006), as well as tomato (Jain and van Staden 2006; Kulkarni *et al.*, 2007), okra (Kulkarni *et al*., 2007) bean (Van Staden *et al.*, 2006) and rice (Kulkarni *et al*., 2005).

This study has demonstrated that *Nicotiana benthamiana*, although not an agronomically important species, also exhibit improved seedling vigour (improved biomass accumulation) upon smoke treatment. *Nicotiana benthamiana*, being a model species, may lead to an enhanced understanding of the molecular processes present in commercially-important crop species. . *Nicotiana benthamiana* was specifically chosen for this study because this species functions well in the chosen tissue-culture system. *N. benthamiana* seedlings are fast growing and deliver reproducible results in this system. However, the *N. benthamiana* genome has not been fully annotated which makes identification of gene ontology extremely difficult and time consuming. Future work would commence on commercially-important *Nicotiana tobaccum* plants to determine whether improved seedling vigour would also result from smoke treatment.

5.5. Strigolactone and smoke promote plant growth through different signalling pathways

Strigolactone perception is critically linked to the enol-ether bridge and the D-ring, the latter of which is shared by the active karrikin in smoke, KAR_1. The receptor interaction described by Zwanenburg *et al.* (2008) opens the door to the possibility that these two chemicals might be perceived by the same receptor. However, some structural divergence exists between these chemicals' molecular structures that may contribute to alternative signalling cascades being elicited on perception, as described in Chapter 3.

This study hypothesized that strigolactones, in the form of the synthetic analogue (GR24), being chemically related to the karrikins (in particular, KAR_1), may potentially promote plant growth in the same way as previously described for smoke/KAR_1 treatment. A physiological investigation into the plant growth promoting properties of GR24 and smoke/KAR_1 revealed a similar physiological response to the treatments. GR24 and smoke-water treatments resulted in significant increases in both root mass and shoot mass on all tested dilutions and concentrations of the chemicals.

However, upon molecular investigation, different signalling cascades seem to be elicited on treatment with GR24 and smoke/KAR_1, with a common end result being improved plant biomass production and heightened seedling vigour. Hormonal and transcriptional data revealed that the plant growth promoting properties of the compounds evoked divergent metabolic processes leading to plant growth, but a combination experiment revealed that some degree of overlap between pathways cannot be ruled out. ABA-mediated stress priming seems to be a common element shared between the treatments but further investigation is needed to confirm this hypothesis.

To investigate whether the plant growth promotion function of strigolactones and smoke/KAR_1 is mediated by the *MAX* signalling pathway, two gene silencing vectors have been constructed. A gene in the strigolactone biosynthetic pathway, *MAX4*, encodes a CCD8 enzyme needed to cleave a carotenoid product, yielding an apocarotenoid that, possibly after further modification, produces the strigolactone hormone. The incorporation of the *MAX4* gene into a gene silencing vector, *pHELLSGATE2*, can be used to create *N. benthamiana* plants with impaired strigolactone production.

The *MAX2* gene, which codes for the proposed perception protein gene, incorporated in the *pHELLSGATE2* silencing vector, can be used to create *N. benthamiana* plants unable to respond to exogenously-applied strigolactone. Through the use of these silencing vectors we can develop experimental procedures to answer some of the questions related to the plant growth promoting abilities of GR24 and smoke/KAR_1 treatment: Is the *MAX* pathway involved in the GR24-promoted growth and if so, does this pathway mediate the KAR_1 plant growth stimulating response as well?

5.6. Limitations of this study

The aim of this study was not to characterize the plant growth promotion in response to KAR_1 treatment. The KAR_1 compound is difficult and very expensive to isolate or synthesize. It is for this reason that only a limited number of physiological parameters were acquired for the KAR_1 treatment data set, as the limited amount of material available had to be handled in a focussed and time appropriate manner.

It was, however, verified in this study that KAR_1 also has potent plant growth promoting properties in *N. benthamiana*. Smoke contains a variety of germination stimulators but also inhibitors. For this reason KAR_1 was included in this study purely for comparative purposes. The physiological growth response of KAR_1 has extensively been studied. The aim of this study was to compare the plant growth promoting abilities of smoke (as it contains KAR_1) and the synthetic strigolactone (GR24).

Once an appropriate amount of KAR_1 is obtained for this project, an effort to completely characterize the plant growth promoting properties of GR24 and KAR_1 would be made by use of the *Nicotiana* plants containing the silencing constructs.

5.7. Final conclusions

The structural similarity shared between the synthetic strigolactone GR24 and smoke-derived KAR_1 molecules seemed too big to be coincidental. For this reason, the plant growth regulating properties of strigolactone were evaluated. This study clearly demonstrated a novel function for strigolactone treatment in the form of a plant growth promoting ability. It was the first to report seedlings with increased biomass accumulation, increased root- and shoot development and a greater leaf area upon treatment with exogenously-applied strigolactones. It was also demonstrated following microarray analysis and phytohormone- and carbohydrate profiling that treatment with these two chemicals (GR24 and KAR_1) elicit largely different, but not entirely independent, signalling cascades in seedlings where increased plant biomass is a common end result. A combination experiment demonstrated that a degree of overlap is shared between the growth promotion signalling cascades and a limited number of transcripts identified in this study suggest that an ABA-mediated priming response is shared between the three treatments. It was hypothesized that these two chemicals (GR24 and KAR_1) may stimulate the same receptor entity. The apparently different signalling cascades elicited by treatment with these chemicals may be attributed to the structural divergence between the GR24 and KAR_1 molecule.

In order to further characterize the plant growth response pathways, stimulated by GR24 and smoke/KAR_1 treatment, two silencing vectors (*pHG2-MAX2* and *pHG2-MAX4*) have been

constructed to silence genes in the *MAX* strigolactone signalling pathway. These constructs can in future be used to produce *N. benthamiana* seedlings with impaired strigolactone production or signalling abilities. These seedlings could be used to determine if GR24 and KAR_1 stimulates plant growth responses through the same signalling pathways by binding to the same receptor entity.

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Addendum A

Table 3.3.a. GR24-responsive genes in 24 day old *N. benthaniana* seedlings

Array ID (A_95_)	*Nicotiana* accession number	Fold change	Putative annotation
P162792	EH621771	2.56	Receptor serine-threonine protein
P094993	BP532966	2.57	None
P310658	FG638908	2.58	None
P092903	BP532039	2.59	None
P270561	FG173998	2.60	Aberrant large forked
P007821	AJ633017	2.61	None
P104717	CV017143	2.62	Aim1 protein
P304283	FG196119	1.3	Trehalose-6-phosphate phosphatase
P021776	EB439530	2.4	Acyl carrier protein
P065135	BP136250	2.68	Kelch repeat
P128102	EB427647	-3.70	Ubiquitin protein
P232349	BP531659	-4.35	None
P218407	EH619427	-5.31	Predicted protein from *Populus trichocarpa*
P109087	CV019144	-2.71	None
P220227	BP529913	-6.51	None
P258871	FG165916	-2.86	6b-interacting protein 2
P105827	CV017631	-3.52	Ribulose bisphosphate carboxylase
P283233	AM812256	-3.31	Unnamed protein product from *Vitis vinifera*
P283673	FG621838	-4.00	60s ribosomal protein
P093243	BP532187	-2.44	Polyubiquitin containing 7 ubiquitin monomers
P069925	BP192475	-4. 90	60s ribosomal protein l32
P299368	FG172886	-3.50	Cockayne syndrome 1 homolog
P316243	FG187377	-3.72	Calcium-binding protein annexin
P169401	EH664669	-2.58	None
P010236	FG187570	-3.32	Monoterpene synthase 2
P023416	BP131349	-4.69	Hypothetical protein from *Vitis vinifera*
P062585	BP135552	-2.59	GTP- binding
P285923	FG177054	-2.15	Phospholipase D
P129772	EB429725	-3.80	None
P050746	BP132473	-6.47	None
P082680	BP528567	-3.17	Predicted protein from *Populus trichocarpa*
P215617	EB678672	-2.57	None
P042636	BP130409	-4.07	Proteasome activator subunit 4-like
P085555	BP529314	-2.34	None
P114832	CV021775	-5.36	Metallocarboxypeptidase inhibitor iia precursor
P090713	BP531066	-3.06	None
P111432	CV020195	-3.25	Poly binding protein
P260806	AM809585	-2.71	Retrotransposon ty3-gypsy sub-class
P107142	FS376179	-4.145	Adenylate kinase 1
P094308	BP532663	-31.86	None

Table 3.3.b. Smoke-responsive genes in 24 day old *N. benthaniana*seedlings

Array ID (A_95_)	*Nicotiana* accession number	Fold change	Putative annotation
P304283	FG196119	3.47	Trehalose-6-phosphate phosphatase
P021776	EB439530	2.72	Acyl carrier protein
P124382	DW004219	2.22	Binding protein
P065135	BP136250	2.86	Kelch repeat
P310658	FG137257	2.67	Nuclear RNA binding
P162792	EH621771	2.66	Receptor serine-threonine protein
P303313	FG177485	2.61	Amino acid
P094993	BP532966	3.20	None
P242312	DW000058	2.56	None
P187057	DV159114	3.94	60s Ribosomal protein l13a
P284298	EB683736	3.47	None
P128102	EB427647	-2.85	Ubiquitin protein
P048181	BP131841	-2.78	None
P232349	BP531659	-3.84	None
P218407	EH619427	-6.71	Predicted protein from *Populus trichocarpa*
P051846	BP132760	-2.93	None
P045121	BP131055	-2.69	None
P221817	EH621081	-2.42	Chlorophyllase 2
P109087	CV019144	-2.70	None
P299368	FG172886	-2.71	Cockayne syndrome 1 homolog
P316243	FG187377	-3.26	Calcium-binding protein annexin
P169401	EH664669	-2.31	None
P010236	FG187570	-2.96	Monoterpene synthase 2
P023416	BP131349	-2.17	Hypothetical protein [*Vitis vinifera*]
P062585	BP135552	-2.30	GTP binding
P129772	EB429725	-3.15	None
P082680	BP528567	-2.64	Predicted protein [*Populus trichocarpa*]
P042636	BP130409	-3.59	Proteasome activator subunit 4-like
P114832	CV021775	-2.11	Metallocarboxypeptidase inhibitor iia precursor
P090713	BP531066	-2.88	None
P260561	BP528681	-2.26	None
P096518	AM780932	-2.24	None
P111432	CV020195	-2.52	Poly binding protein

Table 3.3c KAR_1-responsive genes in 24 day old *N. benthaniana* seedlings

Array ID (A_95_)	*Nicotiana* accession number	Fold change	Putative annotation
P239709	EB447137	5.96	Glycosyltransferase [*Nicotiana tabacum*]
P241300	FG640798	3.30	Deoxyuridine 5 -triphosphate nucleotidohydrolase
P038621	BP129318	2.66	None
P304283	FG196119	2.33	Trehalose-6-phosphate phosphatase
P021776	EB439530	2.46	Acyl carrier protein
P124382	DW004219	2.81	Protein binding
P065135	BP136250	2.23	Kelch repeat
P162792	EH621771	3.15	Receptor serine-threonine protein
P189337	AM792283	3.01	Coproporphyrinogen oxidase
P077815	BP527350	3.98	None
P130432	AM788877	2.93	None

P289818	DV161070	16.24	Aim1 protein
P046331	BP131369	3.89	None
P297938	FG151843	4.01	mRNA decapping enzyme 2
P111027	CV020010	2.76	None
P285923	FG177054	3.49	phospholipase D
P310233	FG135657	5.11	Proline-rich protein precursor
P297958	FG142422	3.26	5-enolpyruvylshikimate-3-phosphate synthase
P068620	BP137174	3.44	None
P108102	CV018670	6.73	None
P050676	BP132455	4.99	None
P214477	EB444046	4.59	None
P042496	BP130373	7.35	Serine-threonine kinase receptor-associated
P293133	FG149991	65.18	Zinc finger
P108732	CV018979	2.69	None
P278068	AM805703	6.60	None
P276878	AM788901	4.73	Hypothetical protein *Vitis vinifera*
P020341	BP526294	5.49	Potassium efflux antiporter
P274583	AM836037	18.25	None
P201077	DW003667	23.78	Protein
P075940	BP526854	5.30	None
P159872	EH618403	12.87	Hydroxycinnamoyl quinate transferase
P262836	AM787512	4.19	None
P183572	CV018718	10.05	NADH-ubiquinone oxidoreductase b18 subunit
P065350	BP136305	4.05	unknown protein Prunus dulcis
P036763	BP128817	3.82	None
P316108	FG184485	21.78	Predicted protein [*Populus trichocarpa*]
P020106	FG624797	5.48	HT protein
P159942	EH618516	3.91	Sterol-regulatory element binding protein site 2 protease
P115852	DV158236	2.91	None
P057156	BP134141	2.70	Potassium transporter
P211557	EH622534	6.40	None
P312443	FG149790	5.33	Transport protein
P231819	FG149554	14.44	GTB binding protein
P085895	BP529406	2.89	predicted protein *Populus trichocarpa*
P046996	BP131545	2.84	None
P054331	BP133409	23.29	None
P270561	FG173998	3.53	Aberrant large forked
P007821	AJ633017	2.51	None
P104717	CV017143	3.62	Aim1 protein
P128102	EB427647	-2.68	Ubiquitin protein
P232349	BP531659	-3.35	None
P218407	EH619427	-5.94	Predicted protein [*Populus trichocarpa*]
P045121	BP131055	-2.47	None
P221817	EH621081	-2.72	Chlorophyllase 2
P109087	CV019144	-2.23	None
P220227	BP529913	-4.14	None
P258871	FG165916	-2.12	6b-interacting protein 2
P105827	CV017631	-2.55	Ribulose bisphosphate carboxylase
P283673	FG621838	-4.55	60s ribosomal protein
P093243	BP532187	-3.21	Polyubiquitin containing 7 ubiquitin monomers
P069925	BP192475	-6.42	60s ribosomal protein l32
P299368	FG172886	-2.95	Cockayne syndrome 1 homolog
P316243	FG187377	-2.84	Calcium-binding protein annexin
P010236	FG187570	-2.5	Monoterpene synthase 2

P023416	BP131349	-3.54	Hypothetical protein [*Vitis vinifera*]
P129772	EB429725	-2.56	None
P082680	BP528567	-2.44	predicted protein *Populus trichocarpa*
P042636	BP130409	-3.11	Proteasome activator subunit 4-like
P094703	AM846413	-4.03	Lipid binding
P085555	BP529314	-2.8	None
P114832	CV021775	-4.44	Metallocarboxypeptidase inhibitor iia precursor
P090713	BP531066	-2.46	None
P096518	AM780932	-2.12	None
P111432	CV020195	-2.06	Poly binding protein
P260806	AM809585	-2.06	Retrotransposon ty3-gypsy sub-class
P107142	FS376179	-2.69	Adenylate kinase 1
P168471	EH664436	-3.09	None
P028206	BP137321	-9.82	at5g40670 mnf13_190

Addendum B

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Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      ATCTCCTCTCTTGTAGATTTGCCGCTTCTCATGGCTTCCACTACTCTCTCCGACCTCCCT 60

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GACGTCATCTTATCCACCATTTCCTCTCTCGTATCCGATTCCCGAGCTCGCAACTCTCTC 120

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      TCCCTCGTCTCTCACAAATTCCTCGCTCTCGAACGATCCACTCGCTCTCACCTCACTATC 180

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      CGTGGCAACGCTCGTGATCTCTCCCTCGTCCCCGACTGTTTCCGATCAATCTCACATCTC 240

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GATCTCTCTTTCCTCTCCCCATGGGGTCACACTCTTCTCGCTTCTCTCCCAATCGATCAC 300

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      CAGAACCTTCTCGCTCTCCGTCTCAAATTCTGTTTCCCTTTCGTCGAGTCTCTAAACGTC 360

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      TACACACGATCTCCGAGCTCTCTCGAGCTTCTACTTCCTCAATGGCCGAGAATTCGCCAC 420

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      ATCAAGCTCCTCCGATGGCATCAACGAGCTTCTCAGATCCCTACCGGTGGCGATTTTGTT 480

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      CCTATTTTTGAACACTGTGGTGGTTTCCTTGAGTCTTTAGATCTCTCCAACTTCTATCAC 540

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      TGGACTGAAGACTTACCTCCTGTGCTTCTCCGCTATGCTGACGTGGCGGCGAGGCTTACA 600

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      CGGTTAGATCTCTTGACGGCGTCGTTCACCGAGGGATACAAATCAAGCGAAATCGTTAGT 660

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      ATCACCAAATCTTGCCCTAATTTGAAGACTTTTCGTGTAGCTTGTACGTTTGATCCGAGA 720

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      TACTTTGAATTCGTCGGAGACGAGACTCTCTCCGCCGTAGCTACCAGTTCCCCTAAGTTA 780

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      ACGCTTCTACACATGGTGGACACAGCTTCGTTGGCGAATCCTAGAGCTATTCCAGGTACG 840

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GAAGCTGGAGATTCAGCTGTCACGGCGGGGACGCTAATTGAAGTTTTCTCAGGTTTACCG 900

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      AATCTAGAGGAGCTGGTTCTTGACGTAGGAAAGGATGTGAAGCATAGTGGTGTAGCTTTA 960

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GAGGCATTGAATTCTAAATGCAAGAAGTTAAGAGTATTGAAGCTAGGACAGTTCCAAGGT 1020
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Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GTTTGCTCTGCTACAGAATGGAGGAGGCTCGACGGTGTGGCTTTATGTGGAGGATTGCAG 1080

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      TCGTTGTCGATTAAGAATTCCGGCGATTTGACTGATATGGGTTTGGTGGCTATAGGGAGA 1140

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      GGATGTTGTAAGTTGACTACGTTTGAGATTCAAGGGTGTGAGAATGTAACAGTGGATGGA 1200

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      CTAAGAACAATGGTTAGTCTTCGGAGTAAGACTTTGACTGATGTGAGAATCTCTTGCTGC 1260

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      AAGAATCTTGACACAGCTGCTTCTTTAAAGGCAATTGAGCCGATTTGTGATCGGATCAAG 1320

Nicotiana_MAX2        ------------------------------------------------------------
Arabidopsis_MAX2      AGACTGCATATAGACTGTGTGTGGTCTGGTTCAGAGGACGAGGAGGTAGAAGGAAGAGTG 1380

Nicotiana_MAX2        ------------------------------------------------------AAGAAAAGA 9
Arabidopsis_MAX2      GAAACTAGTGAGGCTGACCACGAAGAGGAGGATGATGGTTACGAGAGGAGCCAGAAGAGG 1440
                                                                       **** **

Nicotiana_MAX2        TGCAAGT--TCACCTGTGATCTTA-ATAGTTTGTATGAGGAAGTCAATGGCCATGGCAAT 66
Arabidopsis_MAX2      TGCAAGTATTCATTCGAGGAAGAACACTGCTCAACTAGTGATGTGAATGGATTCTGTTCT 1500
                      *******  ***   * *     * *  * *    *   ** ** *****     *   *

Nicotiana_MAX2        GGATATAGTGGACGATCATGGGATCGGCTGCAATGCCTCTCTCTTTGGATTGGTGTTGGT 126
Arabidopsis_MAX2      GAAGATAGAG------TATGGGAGAAACTGGAGTATCTATCTTTATGGATCAATGTTGGA 1554
                      * * **** *       ******    *** * *  ** *** * *****   ******

Nicotiana_MAX2        GAGCTTTTGACTCCTTTAACAGCTGCAGGTCTTGAAGACTGTCCAAATTTAGAGGAGATC 186
Arabidopsis_MAX2      GAATTTTTGACGCCATTACCTATGACAGGACTAGATGACTGTCCGAATTTGGAAGAGATT 1614
                      **  ******* ** *** *     **** ** ** ******** ***** ** *****

Nicotiana_MAX2        AAGATTAGGGTGGAAGGAGATTGCAGGCTATGGTCAAAACCTTCGGAGCGGGCATTTGGA 246
Arabidopsis_MAX2      AGGATCAAGATAGAAGGAGATTGCAGAGGTAAACGCAGGCCAGCCGAGCCAGAGTTTGGG 1674
                      * *** * * * **************          *  **  * ****  *  *****

Nicotiana_MAX2        CTGAGCACCCTTCTTCTCTATCCTAAGCTATCCAAGATGCATTTGGATTGTGGAGATATC 306
Arabidopsis_MAX2      TTAAGTTGTCTCGCTCTCTACCCAAAGCTCTCAAAGATGCAGTTAGATTGCGGGGACACA 1734
                       * **    **   ****** ** ***** ** ******** ** ***** ** ** *

Nicotiana_MAX2        ATAGGTTATGCACACACTGCTCCATCAGGGCAGATGGATTTGAGCTTGTGGGAAAGGTTT 366
Arabidopsis_MAX2      ATCGGTTTCGCACTGACCGCACCGCCAATGCAGATGGATTTGAGTTTATGGGAAAGATTC 1794
                      ** ****  ****  ** ** **  **  *************** ** ******** **

Nicotiana_MAX2        TATCTGTTTGGGATTGGAAATTTGAGCCTTACTGAACTAGATTACTGGCCACCCCAAGAT 426
Arabidopsis_MAX2      TTCTTGACCGGAATTGGAAGCTTGAGCTTGAGCGAGCTTGATTATTGGCCACCACAGGAT 1854
                      *   **   ** *******  ****** * *  ** ** ***** ******** ** ***

Nicotiana_MAX2        AGGGACGTTAACCAAAGGTGTCTATCCCTACCAGCAGCTGGGCTGCTACAAGAATGCGTC 486
Arabidopsis_MAX2      AGAGATGTTAACCAGAGGAGTCTCTCGCTTCCTGGAGCAGGTCTGTTACAAGAGTGCCTG 1914
                      ** ** ******** *** **** ** ** ** * *** ** *** ******* *** *

Nicotiana_MAX2        ACACTCAGAAAACTGTTCATCCATGGAACAGCGCATGAACATTTCATGATGTTCCTTTTT 546
Arabidopsis_MAX2      ACTTTGAGGAAGCTGTTCATCCATGGAACAGCTCATGAGCATTTCATGAACTTTTTGTTG 1974
                      **  * ** ** ******************** ***** **********  **  * **

Nicotiana_MAX2        AGAATCCCAAACTTAAGAGATGTACAACTGAGAGAGGATTACTATCCAGCACCAGAGAAT 606
Arabidopsis_MAX2      AGAATCCCAAACTTAAGGGATGTACAGCTTAGAGCAGACTATTATCCGGCGCCGGAGAAC 2034
                      ***************** ******** ** ****  ** ** ***** ** ** *****

Nicotiana_MAX2        GACATGAGTACAGAGATGAGAGCCGACTCCTTGAGCCGCTTTGAAGCTGCCCTAAACAGG 666
Arabidopsis_MAX2      GATATGAGCACAGAGATGAGAGTTGGTTCGTGTAGCCGATTCGAGGACCAATTGAACAGC 2094
                      ** ***** *************  *  ** *  ***** ** ** *      * *****

Nicotiana_MAX2        CGCCCAATATCCGATTGATGC--AAGGAATACAATGCTCACATAATGTTACC-GTATCCA 723
Arabidopsis_MAX2      CGCAACATCATTGACTGAAACTTGAAGAGTGAGTTACCTACACTATTATATCTGTATTGA 2154
                      ***   **    ** ***  *   * ** *    * *  ***  **  ** * ****  *
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Nicotiana_MAX2          GTGCTTCCATAGTTTTCTTTTGTCAGAATATGTATAAAATTTTCAGATCTCTTTATATGC 783
Arabidopsis_MAX2        CTTCAGAAACTGGTCCATTTTATTTGTATGGTCAAGAGTGTTTTGTATATGTTTGTAAGA 2214
                         * *    *  * *   **** *  * **    *  *   ***   ** * *** ** *

Nicotiana_MAX2          AATAAGGTTTGTG-CTTTGGTTATTGATG------------------------------ 811
Arabidopsis_MAX2        GGAAAGGACAAAGACTATAATTTGCGATGATTAATGATATCATAAAACAATAATCCATTT 2274
                           ****     * ** *  **   ****
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Addendum Figure i. Alignment of *AtMAX2* (Genbank accession nr: NM 129823.2) with *NbMAX2(* Genbank accession nr: CK286340). Sequences were aligned using ClustalW.

```
Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      ATTCACTAAAACTTCATTTACTTTCTTGAACTTGTCTTGTGCCCTCTTTCCAATACACTG 60

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      AAATCCACTTCAAAATATTTATTTAATAAAAAGAAAAGAAAAGAAAAAAACTCTGCCAGG 120

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      ATGGCTTCTTTGATCACAACCAAAGCAATGATGAGTCATCATCATGTTTTGTCGTCAACT 180

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      AGAATCACTACTCTTTATTCCGACAATTCCATCGGCGATCAACAAATAAAAACAAAACCT 240

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      CAAGTCCCTCACCGGTTATTTGCTCGGAGGATCTTCGGTGTAACCAGAGCTGTAATTAAT 300

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      TCAGCGGCACCGTCTCCGTTGCCGGAGAAAGAGAAGGTGGAAGGTGAGAGACGGTGTCAT 360

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GTTGCGTGGACAAGTGTACAACAAGAGAATTGGGAGGGTGAACTTACTGTCCAAGGAAAG 420

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      ATACCCACTTGGCTGAATGGTACGTACCTAAGAAACGGTCCTGGTCTATGGAACATTGGA 480

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GACCACGATTTCCGGCATCTCTTCGACGGCTACTCCACACTCGTCAAGCTTCAATTCGAT 540

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GGCGGTCGTATATTCGCCGCCCACCGTCTCCTTGAATCCGACGCTTACAAAGCCGCCAAG 600

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      AAACACAATAGGCTTTGTTACCGTGAATTCTCCGAGACTCCAAAATCGGTGATCATAAAC 660

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      AAAAACCCTTTCTCCGGGATCGGAGAAATCGTCAGGCTTTTCTCCGGAGAGTCTTTAACG 720

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GACAACGCCAACACCGGAGTGATCAAACTCGGTGACGGGCGGGTCATGTGTCTGACGGAG 780

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      ACTCAAAAAGGATCGATTTTAGTCGACCATGAGACGCTAGAGACGATCGGGAAATTTGAG 840

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      TACGACGACGTATTGTCCGATCATATGATCCAATCAGCGCATCCGATAGTGACGGAGACG 900

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GAGATGTGGACGTTGATACCGGATTTGGTTAAACCGGGTTATCGGGTCGTGAGGATGGAA 960

Nicotiana_MAX4        ------------------------------------------------------------
Arabidopsis_MAX4      GCCGGGTCGAATAAAAGAGAGGTTGTGGGGCGGGTGAGGTGTCGAAGTGGGTCGTGGGGA 1020

Nicotiana_MAX4        ------------------------TGACGGAACATTATATTATTGTGCCGGAAATGCCA 35
Arabidopsis_MAX4      CCCGGTTGGGTCCATTCGTTTGCGGTGACGGAGAATTATGTTGTAATACCGGAAATGCCC 1080
                                              *******  ***** ** *  * ***********

Nicotiana_MAX4        CTAAGGTATTGTGCCCAAAATTTATTAAAGGCTGAGCCCACACCACTTTACAAGTTTGAG 95
Arabidopsis_MAX4      CTGAGATATTCGGTGAAGAATCTTCTTAGAGCTGAGCCGACGCCACTTTACAAGTTCGAG 1140
                      ** ** ****  *   * *** *  * *  ******** ** ************** ***
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Nicotiana_MAX4      TGGCACCCTCATTCCAAAGGTTTCATGCATGTTATGTGTAAAGCCAGTGGCAATATTGTG 155
Arabidopsis_MAX4    TGGTGTCCCCAAGACGGAGCTTTTATTCATGTCATGTCCAAACTCACCGGAGAAGTCGTG 1200
                    ***   ** **   *  ** *** ** ***** ****  ***  **  **  *  * ***

Nicotiana_MAX4      GCAAGTGTGGAAGTGCCATTATTTGTGACTTTCCATTTCATAAATGCATACGAGGAG--- 212
Arabidopsis_MAX4    GCTAGCGTGGAGGTTCCAGCATACGTAACGTTTCACTTCATAAACGCGTATGAAGAAGAT 1260
                    ** ** ***** ** ***  **  ** ** ** ** ******** ** ** ** **

Nicotiana_MAX4      AAAGATGAAGATGGCAGAGTTAGTGCTGTTATTGCAGACTGCTGTGAACATAGCGCCGAC 272
Arabidopsis_MAX4    AAAAATGGCGATGGAAAAGCGACGGTCATCATTGCAGATTGTTGTGAACACAACGCCGAT 1320
                    *** ***  ***** * **  *  *   * ******** ** ******** * ******

Nicotiana_MAX4      ACCACCATCCTTGACAAGCTCCGGCTTGAGAATCTCCGGTCATTCAACGGCGAGGATGTC 332
Arabidopsis_MAX4    ACTCGGATACTCGATATGCTCCGTCTCGATACCCTACGTTCTTCCCATGGTCACGACGTT 1380
                    **    ** ** ** * ****** ** ** *  ** ** ** * * * **  * ** **

Nicotiana_MAX4      TTGCCCGATGCAAGGGTTGGGAGATTTAGAATCCCATTAGATGGGAGTCCATATGGAGAG 392
Arabidopsis_MAX4    TTACCCGATGCTAGGATCGGGAGATTCAGGATACCATTGGACGGGAGCAAATACGGGAAA 1440
                    ** ******** *** * ******** ** ** ***** ** *****   *** **  *

Nicotiana_MAX4      TTAGAAGCAGCATTGAACCCAAATGAACATGGGAGAGGCATGGATATGTGCAGCTTTAAT 452
Arabidopsis_MAX4    CTAGAGACAGCCGTGGAGGCAGAGAAGCATGGGAGAGCGATGGATATGTGCAGCATCAAT 1500
                     ****  ****  ** *  ** *  * **********  *************** * ***

Nicotiana_MAX4      CCTTCTTATTTAGGCAAGAAATATAGATATGCTTATGCTTGTGGTGCTAAGAGGCCTTGT 512
Arabidopsis_MAX4    CCTTTGTATTTGGGTCAAAAATACCGTTACGTTTATGCATGCGGTGCTCAACGACCTTGT 1560
                    ****  ***** **  * *****  * ** * ****** ** ****** *  * ******

Nicotiana_MAX4      AATTTCCCCAACACTCTTACAAAGATTGACTTGTTTGAGAAAAAGGCAAAGAATTGGTAC 572
Arabidopsis_MAX4    AACTTCCCCAATGCTCTCTCCAAGGTTGATATTGTGGAGAAGAAAGTGAAGAACTGGCAC 1620
                    ** ********  ****  * *** ****  *  * ***** ** *  ***** *** **

Nicotiana_MAX4      GATGAGGGTGCTGTGCCTTCTGAACCATTCTTTGTGCCTCGACCTGGTGCAACCGAGGAA 632
Arabidopsis_MAX4    GAGCATGGTATGATACCATCTGAACCATTCTTCGTGCCTCGACCCGGTGCAACCCATGAG 1680
                    **  * ***    * ** ************** *********** ********* * **

Nicotiana_MAX4      GACGACGGAGTTGTAATCTCAATGATCAGTGACAAAAATGGAGAAGGTTATGCTCTAATA 692
Arabidopsis_MAX4    GATGATGGAGTGGTGATATCGATAGTAAGTGAAGAAAATGGAGGAAGCTTTGCAATCTTG 1740
                    ** ** ***** ** ** ** **  * *****  ********* * * * ***  *  *

Nicotiana_MAX4      TTGGACGGATCCACATTTGAAGAAATTGCACGAGCAAAGTTTCCTTATGGTCTTCCCTAT 752
Arabidopsis_MAX4    CTTGATGGGAGCTCCTTTGAAGAAATAGCAAGAGCCAAGTTTCCCTATGGCCTTCCTTAT 1800
                     * ** **   * * ********** *** **** ******** ***** ***** ***

Nicotiana_MAX4      GGACTGCATGGTTGTTGGGTTCC-AAAGA----------------AATAGAACCACCCCC 795
Arabidopsis_MAX4    GGCTTGCATGGTTGCTGGATCCCCAAAGATTAACTACAAAGTCTCAACAAAGATACCTTC 1860
                    **  ********** *** * ** *****                 ** * *   ***  *

Nicotiana_MAX4      CACATCCTAGACAGTGTTATATG-ATAGCCAA-ATACACCTTACAAGTCATATAATAAGA 853
Arabidopsis_MAX4    ATTATACAAAACA-CAACATATGTATAATTAATACCCTCTGTGCAGGTTTTGTAAATTGT 1919
                       ** * * ***     ***** ***   ** *  * *  * ** **  * ***   *

Nicotiana_MAX4      TATTACATTT-TCCACCTCTTGAATAGCTAATGTAGTATT------------------- 892
Arabidopsis_MAX4    TGTCCCTTATATATGCTTTTTGTCTATATA-TGTGATGTACAAAACCAAAATAAAAGGAA 1978
                    * *  * * * *   * * ***  **  ** ***  * *

Nicotiana_MAX4      ----------------------------------------
Arabidopsis_MAX4    CGGATTGTGGTGGATACAGTTATTAAAAATTGCACTAGACC 2019
```

Addendum Figure ii. Alignment of *AtMAX4*(Genbank accession nr: AT4G32810) with *NbMAX4* (Genbank accession nr: DW004809). Sequences were aligned using ClustalW.

Addendum C

Table 4.1. *N. Benthamiana* sequences producing significant alignments with *AtMAX2* (NM129823) and *AtMAX4* (AT4G32810)

	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
MAX2	**CK286340.1**	EST749062 *Nicotiana benthamiana* mixed tissue cDNA library,	313	313	22%	3.00E-83	73%
	CK281837.1	EST744559 *Nicotiana benthamiana* mixed tissue cDNA library,	297	297	22%	2.00E-78	72%
	CK281835.1	EST744557 *Nicotiana benthamiana* mixed tissue cDNA library	297	297	22%	2.00E-78	72%
	CK281834.1	EST744556 *Nicotiana benthamiana* mixed tissue cDNA library	297	297	22%	2.00E-78	72%
	AM831903.1	AM831903 COL, cold overnight library *Nicotiana tabacum* cDNA clone	291	291	16%	8.00E-77	76%
	CK295395.1	EST758109 *Nicotiana benthamiana* mixed tissue cDNA library	282	282	20%	4.00E-74	73%
MAX4	**DW004809.1**	KR3B.109M13F.051111T7 KR3B *Nicotiana tabacum* cDNA clone KR3B.109M13	369	369	45%	3.00E-100	70%
	FG163219.1	AGN_RNC015xh09r1.ab1 AGN_RNC *Nicotiana tabacum* cDNA 3'	340	340	42%	1.00E-91	70%
	FG163141.1	AGN_RNC015xh09f1.ab1 AGN_RNC *Nicotiana tabacum* cDNA 5'	118	118	15%	8.00E-25	70%