Tools for strigolactone research: Towards a strigolactone-responsive promoter and a strigolactone-activity inhibitor

By

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Declaration

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Abstract

Strigolactones are a novel group of phytohormones reported to control branching in plants. Strigolactones also plays a pivotal role in the establishment of symbiotic relationships between plants and symbiotic fungi. Furthermore, its presence in the soil is responsible for the germination of the seeds of devastating plant parasitic plants known as broomrapes and witchweeds. They have also been implicated in playing roles in root development and architecture, secondary growth, adventitious root formation and leaf senescence. These phytohormones are derived from the carotenoid synthetic pathway, with β-carotene as the precursor. Several genes and the proteins (enzymes) they code for have been identified by reverse and forward genetics. A few components of the perception and signalling mechanism have been identified, but most of the pathway remains unknown. This highlights the need for more innovative scientific tools in order to fully elucidate the role of strigolactones in higher plants. A specific inhibitor of strigolactone signalling and strigolactone-responsive reporter are two tools that could aid in the further characterization of this poorly defined pathway.

In this study, the effects of furanone-derivatives, with the main focus being on trimethylbutenolide (TMB), on the growth of *Arabidopsis thaliana* were investigated. The aim of this study was to determine if TMB is a competitive inhibitor of strigolactone signalling, because currently there are no known inhibitors of strigolactone signalling available. Having such an inhibitor would enable researchers to study the effects of strigolactones in non-model plant species where no mutants are available. Such an inhibitor would also aid in the further elucidation of the strigolactone signalling pathway. For the second part of this study, an attempt was made to create a strigolactone-response reporter construct in *A. thaliana* that is activated only in the presence of exogenously applied strigolactone. Having such a reporter-construct in *A. thaliana* would be valuable, as strigolactones are difficult to detect and quantify in these plants due to them being bioactive at picomolar concentrations in the plant. Such a reporter-construct would also aid in the further elucidation of the strigolactone pathway, the discovery of more functions and any interactions with other phytohormones and biochemical processes in the plant.

During this study, it was found that the presence of TMB induced an increase in lateral root formation in wild-type *A. thaliana* seedlings, suggesting that it may act as a competitive inhibitor of strigolactone signalling, at least in terms of lateral rooting. It was also found that TMB affects the expression of a small group of strigolactone-responsive genes in an opposite way than GR24, a racemic mixture of strigolactone analogues. This effect on gene expression was observed after prolonged treatment of *A. thaliana* seedlings with TMB. For the second part of the project, we were unable to create a strigolactone-specific reporter construct,

although the results suggest that the 990 bp region immediately upstream of *AtBRC1* does respond more strongly to the presence of GR24 than the full, native promoter. It was also found that the 1480 bp and 990 bp regions immediately upstream of *AtBRC1* is already severely deregulated version of the native *AtBRC1* promoter. From these results, it was concluded that DNA binding motif/s for SMAX-like repressor proteins probably lie upstream of the 1480 bp promoter region of *AtBRC1*, while possible strigolactone-responsive motif/s lie downstream of the 990bp promoter region of *AtBRC1*.

Opsomming

Strigolaktone is ‘n nuwe groep fitohormone waaroor daar berig is dat hul vertakking in plante reguleer en beheer. Strigolaktone speel ook ‘n deurslaggewende rol in die aanknoping van simbiotiese verhoudings tussen plante en arbuskulêre mikorisaie swamme. Verder is die teenwoordigheid van strigolaktone in die grond ook verantwoordellik vir die ontkieming van die saadjies van verwoestende parasitiese plante beter bekend as besemrape en kopseerblomme. Daar word geïmpliseer dat hulle rolle speel in wortelontwikkeling en argitektuur, sekondêre groei, onverwagte wortelformasie en blaarveroudering. Hierdie fitohormone word afgelei van die karotenoïed sintese padweg, met beta-karoteen as die voorloper molekule. Verskeie gene en die proteine (ensieme) waarvoor hulle kodeer is reeds geidentifiseer met behulp van keer en stuur genetika. ‘n Paar komponente van die persepsie en sein meganismes is reeds geïdentifiseer, maar meeste van die padweg bly grootliks onbekend. Dit beklemtoon die behoefte vir meer innoverende wetenskaplike gereedskap om die volledige rol van strigolactone in hoër plante te ontrafel. ‘n Strigolaktoon sein-inhibeerder en strigolaktoon-spesifieke verklikker is twee instrumente wat kan help met die verdere karakterisering van hierdie swak gedefinieërde padweg.

Deur die loop van hierdie studie is die effek van furanoon-afgeleides, met trimetielbutenoliet (TMB) as die hoof fokus, op die groei van *Arabidopsis thaliana* ondersoek. Die doel van hierdie studie was om te bepaal of TMB ‘n kompeterende inhibeerder van strigolaktoon-seine is, want tans is daar geen bekende inhibeerder van strigolaktoon-seine beskikbaar nie. Met so ‘n inhibeerder sal wetenskaplikes in staat wees om die effek van strigolaktone in nie-model plant spesies waar geen mutante beskikbaar is nie te ondersoek. So ‘n inhibeerder sal ook help met die verdure ontrafeling van die strigolaktoon sein padweg. Vir die tweede gedeel van hierdie studie is ‘n poging aangewend om ‘n strigolaktoon-reagerende verklikkerkonstruk te skep in *A. thaliana* wat slegs geaktiveer word in die teenwoordigheid van eksogene toegevoegde strigolaktone. So ‘n verklikker-konstruk in *A. thaliana* sal waardevol wees, want strigolaktone is moeilik om te bepaal en te kwantifiseer in hierdie plante, te danke aan die feit dat hul aktief is by piko-molêre konsentrasies in die plant. So ‘n verklikker-konstruk sou ook help met die verdure ontrafeling van volledige strigolaktoon-padweg, die ontdekking van meer funksies en enige interaksies met ander fitohormone and biochemiese prosesse in die plant.

Tydens hierdie studie is daar bevind dat die teenwordigheid van TMB ‘n toename in laterale wortelvorming in wilde-tipe *A. thaliana* saailinge veroorsaak, wat daarop dui dat dit kan dien as ‘n kompeterende inhibeerder van strigolaktoon-seine, ten minste in terme van laterale wortels. Dit is ook bevind dat TMB ‘n invloed het op ‘n klein groupie van strigolaktoon-reagerende gene in ‘n teenoorgestelde manier as GR24, ‘n rasemiese mengsel van

strigolaktoon-analoë. Hierdie effek op die uitdrukking van gene was waargeneem na langdurige behandeling van *A. thaliana* saailinge met TMB. Vir die tweede gedeelte van hierdie projek, was ons nie in staat gewees om ‘m strigolaktoon-spesifieke verklikker-konstruk te skep nie, alhoewel die resultate daarop dui dat die 900 bp streek onmiddellik stroomop van *AtBRC1* sterker reageer in die teenwordigheid van GR24 as die volle, inheemse promotor. Daar is ook bevind dat die 1480 bp en 990 bp streke onmiddellik stroomop van *AtBRC1* reeds ‘n dereguleerde weergawe van die inheemse *AtBRC1* promotor is. Vanaf hierdie resultate is die gevolgtrekking gemaak dat DNS-bindende motief/motiewe vir SMAX-agtige onderdrukker proteïne waarskynlik verder stroomop van die 1480 bp promotor streek van *AtBRC1* lê, terwyl moontlike strigolaktoon-reagerende motief/motiewe stroomaf van die 990 bp promoter streek geleë is.

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''*If you're the smartest one in the room, you're in the wrong room*.'' – Richard Tirendi

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Dedication

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List of Abbreviations

ºC	degrees Celsius
µg	micro-gram
µL	micro-litre
µM	micromolar (10^{-6})
AM	arbuscular mycchorhizal (fungi)
ANOVA	analysis of variance
BAP	6-benzylaminopurine
bp	base pairs
cDNA	complementary deoxyribonucleic acid
Col-0	Columbia-0
CTAB	cetyl trimethylammonium bromide
ddH_20	deionised distilled water
DAG	days after germination
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
FAA	formaldehyde-acetic acid-ethanol
gDNA	genomic deoxyribonucleic acid
GM	genetic modification
GMO	genetically modified organism
GUS	β-glucuronidase
KAR_1	karrikinolide
kPa	kilopascals
KOH	potassium hydroxide
L	litre
LB	Luria Broth
LRE	light responsive element
M	molar
m/v	mass/volume
mg	milligram
mM	millimolar
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
ng	nanogram

pg	picogram
pM	picomolar
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
s	seconds
SOC	super optimal broth with catabolite repression
T-DNA	transfer deoxyribonucleic acid
TMB	trimethylbutenolide
U	units
V	volt
v/v	volume per volume
WT	wild type
YEP	yeast extract phosphate
x*g*	times gravitational force
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Chapter 1: General Introduction

''To avoid criticism, say nothing, do nothing, be nothing'' – Fred Shero

1.1 Background

Strigolactones, molecules consisting of a tricyclic lactone connected to an α,β-unsaturated furanone moeiety *via* an enol ether bridge (Humphrey and Beale, 2006), were first determined to stimulate the germination of broomrapes and witchweeds (Cook *et al*., 1966), both well-known root hemiparasites belonging to the Orobanchaceae family. Later it was discovered that strigolactones are also the signal responsible for the establishment of a symbiotic relationship with arbuscular mycorrhizal fungi (Akiyama *et al*., 2005) and that they play a role in the inhibition of shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). These ground-breaking discoveries in plant biology paved the way for a booming increase in strigolactone research. Since then, functions other than those described above have been connected to strigolactones. Some of these functions include increased resistance to biotic (Ha *et al*., 2014) and abiotic stress, root architecture and development (Koltai, 2011; Ruyter-Spira *et al*., 2011), adventitious root formation (Rasmussen *et al*., 2012a; Rasmussen *et al*., 2012b), secondary growth through increased cambium activity (Agusti *et al*., 2011), senescence (Yamada *et al*., 2014; Ueda and Kusaba, 2015), flowering (Niwa *et al*., 2013), branching of moss protonema (Proust *et al*., 2011) and light-regulated development (Tsuchiya *et al*., 2010).

This rapid accumulation of new information on strigolactones has laid an important groundwork for further discoveries to be made, in order to fully elucidate the biosynthesis and signalling pathway of strigolactones and its subsequent physiological responses. However, studying a phytohormone such as strigolactones is challenging owing to it acting at sub-picomolar (pM) concentrations in plants. In *Gossypium hirsutum* (cotton) cotton, an average of 15 pg strigol are produced each day (Sato *et al*., 2005). Research tools, such as forward and reverse genetics, have been critical in elucidating the components of this unique metabolic pathway, but many aspects of the research and components of the biosynthesis and signalling pathways remain unknown, highlighting the need for new and/or improved scientific tools.

1.2 Strigolactones and molecules with similar structure and/or activity

The functions of strigolactones, as described above, effectively defined it as a phytohormone involved in growth and development, which resulted in studies being directed towards finding analogues of strigolactones. Not only would these analogues be an important asset to strigolactone research, but they could also hold potential to be used in agriculture. In a previous study, such analogues provided important information on branching and hormonal activity in *Pisum sativum* (Boyer *et al*., 2012; Fukui *et al*., 2013). The two chemicals of interest for the first part of this project are furanone (Fig. 1.1A) and trimethylbutenolide (TMB) (Fig. 1.1B). Initially, TMB was known as 3,4,5-trimethylbutenolide-2(*5H*)-one and was also purchased as such, but in literature it is referred to as trimethylbutenolide (TMB). The molecular structure of furanone is identical to the structure of the D-ring of naturally-occurring strigolactones (Fig. 1.1C) and GR24 (Fig. 1.1D), a synthetic strigolactone molecule mixture often used in strigolactone research. Trimethylbutenolide (TMB), isolated from smoke, is of interest because it is a trimethylated version of furanone and is known to inhibit seed germination of *Lactuca sativa* (Soós *et al*., 2012). A recent study provided evidence that TMB also has the ability to inhibit the germination of certain weed species (Papenfus *et al*., 2015a). The inhibitory characteristics of TMB is due to its antagonistic effect to karrikins *(*Ghebrehiwot *et al*., 2013a), also isolated from smoke, which are known to positively stimulate germination on several plant species, with the most potent germination karrikin being KAR_1 (Flematti *et al*., 2004; Stevens *et al*., 2007) (Fig. 1.1E).

Strigolactones are also known to stimulate germination of parasitic weed species (Cook *et al*., 1966). Strigolactones and karrikins share a component in their signalling pathways known as *MAX2* (Nelson *et al*., 2011; Waters *et al*., 2012a). Strigolactones are perceived by an enzyme/receptor known as D14, while karrikin are perceived a paralogue of D14, known as KAI2 (Guo *et al*., 2013) With the signalling pathways of these two groups of chemicals being intertwined, TMB might also have an antagonistic effect on strigolactone signalling.

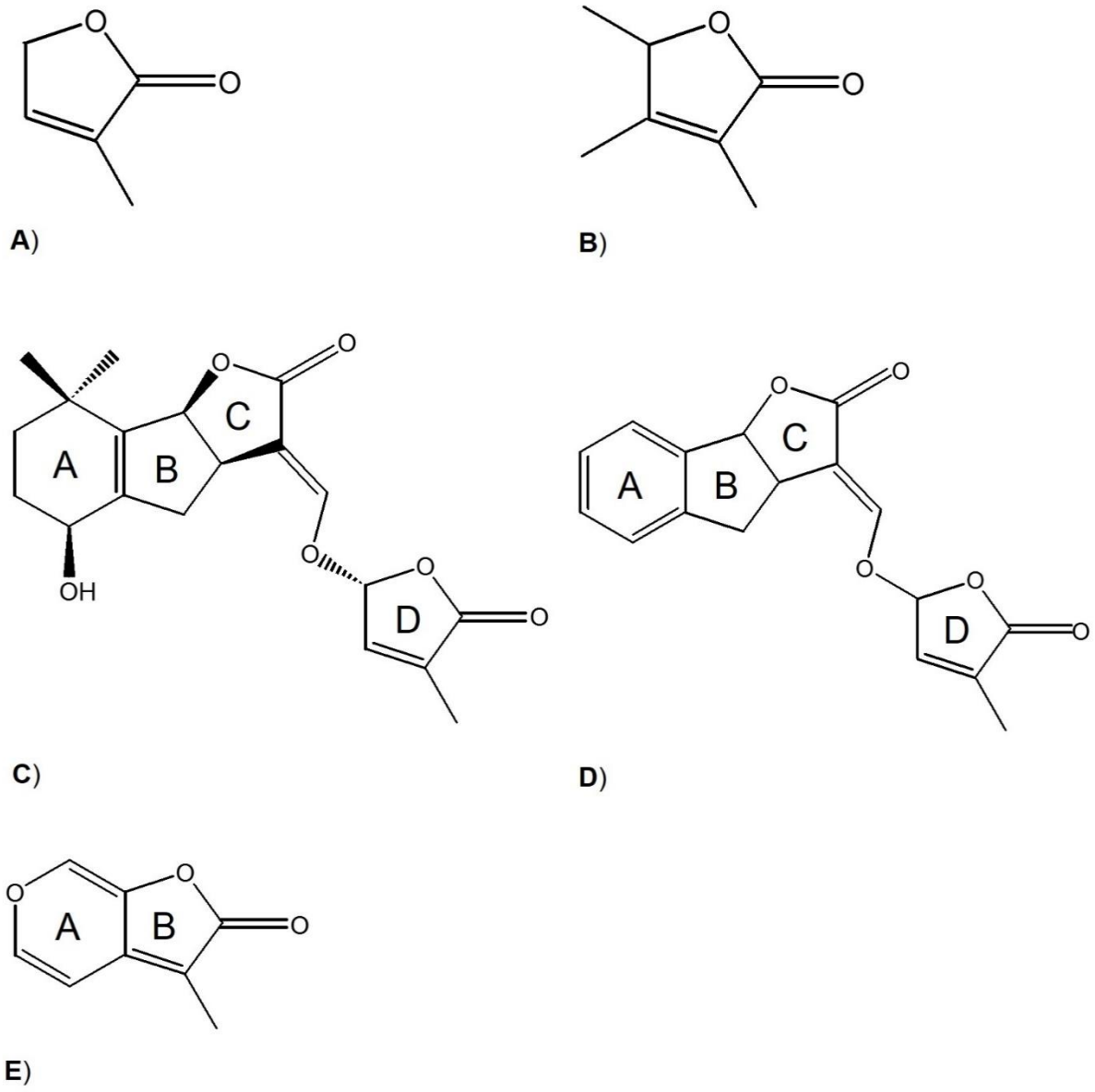

Figure 1.1: Molecular structures of **(A)** furanone, **(B)** trimethylbutenolide (TMB), naturally occurring strigolactone **(C)** (+)-strigol, **(D)** GR24 and **(E)** KAR_1 (3-methyl-2H-furo[2,3-c]pyran-2-one).

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Biosynthesis inhibitors for strigolactones have been identified and used in strigolactone research, including TIS108 (Ito *et al*., 2010) and TIS13 (2,2-dimethyl-7-phenoxy-4-1*H*-1,2,4-triazol-1-yl)heptan-3-ol) (Ito *et al*., 2011). Fluridone, a carotenoid biosynthetic inhibitor has also been used in research aimed at discovering more strigolactone functions and elucidating the *MAX*-pathway (Matusova *et al*., 2005; Jamil *et al*., 2010; López-Ráez *et al*., 2011; Rasmussen *et al*., 2012a). Pleiotropic effects were observed when plants were treated with these chemicals, which can be explained by the fact that the carotenoid pathway provides precursors

for several downstream metabolic pathways, many of which are still unknown. There are currently no known inhibitors of strigolactone signalling. A strigolactone-signalling inhibitor would be beneficial, specifically to determine more functions of strigolactones and aid in furthering the elucidation of strigolactone signalling. Studying the possible resulting feedback mechanisms would also provide valuable insight into strigolactone biosynthesis. Both TMB and furanone could also hold great agricultural potential by altering the growth of crops in a non-genetically modified (non-GM) manner, by using them as agro-chemicals.

Agro-chemicals in general refer to herbicides, insecticides and fertilizers, but growth-promoting agro-chemicals that stimulate plant growth or alter plant architecture in novel ways are modern means for growth-promotion that excludes any form of genetic modification. Genetically modified organisms (GMO's) and their use remain a heavily debated subject. This non-GMO strategy of altered shoot architecture that could potentially lead to a yield-increase by using a compound like TMB or furanone would be a valuable asset to the agricultural and food production industries.

In theory, the use of strigolactones as agro-chemicals does have some potential, as evidenced by the fact that plants treated with GR24, are generally sturdier than untreated plants owing to an increase in secondary growth (Agusti *et al*., 2011), with a reduction in shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). The exudation of strigolactones in the soil can often be detrimental, because it stimulates the germination of certain parasitic plants (Cook *et al*., 1966). In an effort to address this problem, both GR24 and Nijmegen-1, another synthetic analogue of strigolactones, have been used in a study where soil was treated with these analogues to stimulate a process known as suicidal germination of *Striga* species (Zwanenburg *et al*., 2009; Kgosi *et al*., 2012). After germination, these plant parasites need to attach themselves to a host plant in order to survive. If the soil is treated with these analogues before sowing, it reduces the presence of these parasites in the soil.Although GR24 is more stable than naturally occurring strigolactone, it is not a viable agrochemical owing to its short half-life of approximately ten days (Akiyama *et a*l., 2010). In a more recent study it was determined that GR24 actually has a much shorter half-life, ranging from 22-26h, than previously described, especially at higher temperatures and at a higher pH (Rasmussen *et al*., 2013). If a molecule that mimics the functions of strigolactones were to be found and remains relatively stable, it could be used as an agro-chemical to condition the soil before sowing or improve the growth of crops that have shown to have an increase in yield when a strong apical dominance phenotype is observed Similarly, if a molecule that has an antagonistic effect on strigolactone activity were to be found, it could also be used as an agro-chemical.

After studying the structures of the different strigolactone-molecules, it was determined that the presence of the D-ring of strigolactone molecules (Fig. 1.1C,D) is needed for biological activity (Yoneyama *et al*., 2009; Zwanenburg *et al*., 2009; Xie *et al*., 2010; Yoneyama *et al*., 2010). The structure of the D-ring of bio-active strigolactone molecules is identical to the structure of furanone Furanone, also known as 3-methyl-2(*5H)*-furanone, is more stable than both GR24 and Nijmegen-1. The B-ring of KAR_1 (Fig. 1.1E) is also identical to the D-ring and furanone. A study in *Oryza sativa* (rice) plants revealed that high concentrations of this purified D-ring (50 µM) inhibited the outgrowth of the second tiller bud when applied directly to the bud (Nakamura *et al*., 2013). GR24 are active at concentrations of 0.1 µM. Another molecule with similar structure is TMB, which has two additional methyl groups in this ring (Fig. 1.1B). Strigolactones and KAR_1 both stimulate germination in a MAX2-dependant manner (Nelson *et al*., 2011), but strigolactones are perceived by a receptor known as D14, while KAR_1 is perceived by the KAI2 receptor. Both strigolactones and karrikins are known to stimulate germination, while TMB is an inhibitor of germination. This raises the question of whether TMB inhibits germination by binding to either the D14 or the KAI2 receptor, or via a novel mechanism not involving either of these two receptors. Since TMB inhibits germination, presumably through interference with karrikin signalling, it is possible that it may also inhibit strigolactone signalling, either by competitive binding to the D14 receptor or via another, unknown mechanism.

Shoot branching is a very important agricultural trait, which has been selected for in crops such as *Zea mays* (maize) and rice since their domestication by humans, where increased branching has led to an increase in crop yield. If TMB increases branching owing to an antagonistic effect to strigolactones, it could have potential to be used as an agro-chemical to be sprayed on crops.

1.3 Detection and quantification of strigolactones in *Arabidopsis thaliana*

Phytohormones are difficult to detect and quantify, owing to being present in low concentrations in plants (Spartz and Gray, 2008). Strigolactones are produced and synthesized at even lower concentrations than other phytohormones, such as auxins and cytokinins, which makes them difficult to detect using the currently available technology. Natural strigolactones have been isolated from sorghum (Xie *et al*., 2009), rice and cotton (Sato *et al*., 2005) root exudates, but not from *A. thaliana*. The likely explanation for this is that plants which form mycorrhizal associations exude large amounts of strigolactones from their roots to attract the mycorrhizal fungal partner, but contain only minute amounts of strigolactones within the plant that perform the hormonal function. *Arabidopsis thaliana* does

not exude strigolactones owing to the fact that it is a non-mycotrophic plant, and thus contains only very small amounts of strigolactones which are difficult to accurately quantify (Ruyter-Spira *et al*., 2011; Seto *et al*., 2014). For a model organism, this poses a problem and restricts the further characterization of this pathway within *A. thaliana*. One possible means of detecting the presence of a phytohormone other than investigating a genetic response or isolating and quantifying the metabolite, is via the use of a reporter-construct. A widely used example is *DR5*:*GUS*, a reporter-construct that is activated in plants in the presence of auxins (Ulmasov *et al*., 1997). This reporter construct consists of a seven-time repeat of a known auxin-responsive motif fused to the coding sequence of β-glucuronidase (GUS). This reporter construct has been useful in the elucidation of auxin-responses not only in *Arabidopsis*, but also other to study auxin accumulation in the organs of *Pisum sativum* (DeMason and Polowick, 2009), auxin distribution in *Physcomitrella patens* (Bierfreund *et al*., 2003) and the presence of auxin in the leaves, stem and roots of *Populus* species (Chen *et al.*, 2013). If a similar reporter could be developed for the detection of strigolactones, it would greatly aid in the elucidation of strigolactone function in a variety of plant species.

1.4 Importance of project

Although there are several strigolactone biosynthesis and -signalling mutants available in *A. thaliana*, rice, *Pisum sativa* (pea) and *Petunia hybrida* (petunia), it is generally accepted that many components of both the biosynthesis and signalling pathways of strigolactones have yet to be discovered and characterized. Furthermore, in other plants and trees where no mutants are available, it is difficult to study strigolactones and their effects. Developing tools to study these effects would not only be agriculturally and industrially, but also academically valuable. Having a strigolactone-responsive reporter would be beneficial as it can be used to determine the presence of strigolactones without isolating the metabolites. Having an inhibitor of strigolactone signalling would be valuable as it would enable simpler strigolactone research in species for which no genetic mutants are available. This project has the potential to aid in closing the gaps between strigolactone biosynthesis, signalling and the physiological response.

Chapter 2: Literature review

"Everything must be made as simple as possible. But not simpler." – Albert Einstein

2.1 Introduction

All plants are rooted to the soil, with only a few exceptions such as bryophytes, epiphytes and seaweeds. Mosses and liverworts are bryophytes and root themselves using rhizoids, while epiphytes grow harmlessly on other plants for physical support. Seaweed can take up nutrients through their entire body, a useful adaptation because of their complete submergence. Nevertheless, plants are sessile organisms. This physical limitation in terms of movement poses a challenge to these eukaryotes, because the water, nutrients and light they require to survive and thrive have to be obtained from their immediate environment. The conditions of this immediate environment can change. In contrast to animals, plants do not have the option of rapidly relocating themselves to more favourable growth conditions. They cannot run towards more nutritious soil or increased light, away from an insect, fungal infection or any other biotic or abiotic factor which may be hazardous to their survival.

As early as 1880, Charles Darwin and his brother observed that plants were able to 'sense' their environment, and change certain aspects of their growth in response to different environmental conditions in order to grow and develop as optimally as possible. One such aspect is known as positive phototropism, described as growing towards the light when exposed to a lateral light source by Darwin in the 1880's. More than a century later we are still only beginning to understand some of the mechanisms a plant has to rely on to compensate for its sessility.

2.1.1 Phytohormones

All processes and major events during the lifetime of a plant, such as germination, root growth and development, shoot elongation and branching, flowering and seed dispersal, are directly or indirectly subjected to endogenous hormonal signals. Hormones excreted by neighbouring plants and microorganisms in the soil can also affect plants in the same way and alter their growth. In plants, these aforementioned signals that regulate growth and development are known as phytohormones, defined as a collection of small organic molecules shown to be derived from essential primary metabolic pathways (Rohilla *et al*., 2011). These small organic molecules are biosynthesized and perceived by plants at low concentrations and regulate

important processes such as cell division, differentiation, division and programmed cell death (Spartz and Gray, 2008).

Auxins are a well-researched group of phytohormones. An example and key auxin in plants is indole-3-acetic acid (IAA) (Went and Thimann, 1937). It was first described by Dutch scientist Frits Warmolt Went and subsequently isolated by Kenneth Thimann. Auxin is well characterized, because it was the first phytohormone discovered and isolated. It is implicated in almost all aspects of plant growth and development (Woodward and Bartel, 2005; Vanneste and Friml, 2009; Overvoorde *et al*., 2010), ranging from root growth to shoot elongation to leaf expansion. The discovery of other phytohormones, such as cytokinins, gibberellins, ethylene and abscisic acid, soon followed.

Although each phytohormone is generally associated with a specific function, research has indicated that there is definite interaction and cross-talk between the different groups of phytohormones. Together, they act in a complex network to direct and redirect physiological and developmental processes during the entire lifetime of the plant in the form of cell division stimulation, lateral bud outgrowth, induction of germination, fruit ripening and shoot growth inhibition (reviewed by Herold, 2009). Environmental stresses, both biotic and abiotic, significantly affect the amounts and composition of the phytohormones by promoting or suppressing their biosynthesis and/or efflux (Xie *et al*., 2010), confirming the stimulatory effect of the environment on phytohormones. In this way, plants detect environmental stimuli through hormone signals, and the response is transduced through changes in gene expression, pre- and posttranscriptional changes and protein degradation. Such responses can also be halted quickly if the external stimulus is not prolonged (Krasensky and Jonak, 2012).

2.1.2 Other phytohormones – newly discovered role-players

Apart from the classical phytohormones described in the previous section, five other chemicals have also been discovered and determined to be phytohormones. These are brassinosteroids (Grove *et al*., 1979), salicylic acid, jasmonates, oligosaccharides (Creelman and Mullet, 1997) and strigolactones (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Although phytohormones and their functions during plant growth and development have been extensively studied and described, many components, especially in signalling, remain poorly understood. Taking this into account, as well as evidence that there is a definite interaction in terms of feedback regulation between phytohormones; the scientific community is still far from understanding the complete picture of phytohormone control and all its effects on plant growth.

2.2 Strigolactones

The basic structure of a strigolactone molecule consists of a tricyclic lactone (ABC ring) connected to an α,β-unsaturated furanone moiety *via* an enol ether bridge, also known as a methylbutenolide ring (D ring) (Fig. 1.1B) (Humphrey and Beale, 2006). Strigolactones are mainly synthesized in the roots and some parts of the stem and move upwards towards the shoot apex (Foo *et al*., 2001; Brewer *et al*., 2009; Ferguson and Beveridge, 2009; Kapulnik *et al*., 2011b). Strigol (Fig. 1.1C) was the first strigolactone-molecule isolated and it was determined that it is responsible for stimulating the germination of *Striga lutea*, commonly known as witchweed (Cook *et al*., 1966). More than fifteen different types of strigolactones have been isolated from a wide variety of mono- and dicotyledonous plant species (Yoneyama *et al*., 2009; Rameau, 2010) and characterized to date. A synthetic strigolactone analogue, GR24 (Fig 1.1D), has been synthesized and used in strigolactone-research.

2.2.1 First known function of strigolactones

Strigolactones obtained their name from *Striga lutea*, but are also known to stimulate the germination of another parasitic weed named *Orobanche* (broomrape). There are two main classes of strigolactones, namely strigol and orobanchol type strigolactones. Strigol, the first germination stimulant isolated from *Gossypium hirsutum* (Cook *et al*., 1972), is a known stimulant of *Striga* spp, while orobanchol type strigolactones stimulate the germination of *Orobanche* spp. In the presence of strigolactones, the small seeds of these parasites are stimulated to germinate and develop an organ known as the haustorium. Strigolactones are only needed for initial establishment of the parasitic relationship with *S. hermonthica* and play no further role in the infection process (Umehara *et al*., 2008). The fact that strigolactone-detection by the parasites occurs at picomolar (pM) concentrations (Sato *et al*., 2005) and that the infection is irreversible means that it is almost impossible for the plant to avoid this unwanted relationship. The seeds of the parasites contain very little in terms of storage reserves, which means that the presence of a host is essential for the survival of the parasites after germination. The interaction of roots with parasites has a negative effect on the plant, because the parasite deprives its host of water and nutrients, effectively suppressing growth and development (Joel, 2000) and leading to a major decrease in harvest yields.

This phenomenon has a high occurrence in soil with low concentrations of nitrogen and phosphates, which is a direct result of farmers being unable to supplement the soil with nutrients. This effect worsens when the soil is over-used and not given enough recovery time after the harvest (Humphrey *et al*., 2006). *Striga* is found in up to two-thirds of sub-Saharan

Africa, representing a big challenge to food security in this region (Humphrey *et al*., 2006). *Striga* spp. affect maize, millet, rice and sorghum in Asia, sub-Saharan Africa and the Middle East, while *Orobanche* species target dicotyledonous species including tomato, carrot, cucumber and sunflower (Yoneyama *et al*., 2010). Farmers often lose all or most of their harvest when the soil is heavily infested with these parasites.

A proposed method to counteract this devastating effect is known as suicidal germination (Kgosi *et al*., 2012), where the soil is treated with strigolactones or strigolactone-analogues which result in the germination of large numbers of parasitic seeds from the soil seedbank, which then cannot survive without immediate attachment to a host. The result will therefore be the death of these parasites. Thereafter, the desired seeds can be sowed with reduced risk of being infected.

2.2.2 A new role for strigolactones

For several decades after the discovery by Cook *et al*. (1966), strigolactones were solely defined as being the signal for root parasite infestation. However, they are also synthesized and exuded by plants that are not hosts of *Striga* and *Orobanche* spp. Since the discovery by Cook *et al*. (1966) many scientists have been puzzled as to why plants would synthesize and exude strigolactones if they only have a negative and very often fatal effect on the survival of the plant. If the parasitic plants evolved to detect strigolactones, there had to be a reason why the plants would synthesize and exude them into the soil.

Three natural strigolactones, exuded by the roots of *Lotus japonicus*, stimulated the extensive branching of the hyphae of arbuscular mycorrhizial (AM) fungi (Akiyama *et al*., 2005). A synthetic analogue of strigolactones, GR24, had the same effect. This was the first publication implicating strigolactones in another function other than providing the detrimental signal for root parasitic infestation. This symbiotic relationship was not a new discovery, but Akiyama *et al*. (2005) determined that the establishment of this mutually beneficial relationship is dependent on the presence of strigolactones.

The mechanism of root colonisation by AM fungi occurs by means of an organ known as the arbuscules (Parniske, 2008), similar to the haustorium organ produced by *Striga* and *Orobanche* to attach to the roots of their hosts (Estabrook and Yoder, 1998; Joel, 2000). Up to 80% of all plant species have been shown to be involved in such symbiotic relationships (Humphrey *et al*., 2006), which have proven to be essential for the growth of both parties. The plant benefits from this relationship because the AM fungi aids in the acquisition of phosphates

(P) and nitrogen (N), while improving pathogen resistance and drought tolerance (Humphrey *et al*., 2006). The fungi, which rely on the critical developmental step of hyphal branching (Akiyama *et al*., 2005), is provided with carbohydrates derived from photosynthesis. In tomato plants, AM symbiosis induces a decrease in strigolactone production, which decreases the plants susceptibility to parasitic infestation (López-Ráez *et al*., 2011).

2.2.3 A novel class of phytohormones

The detection of strigolactones in the root exudates of non-mycotrophic plant species was an interesting discovery, indicating that these chemicals possibly had functions other than stimulating the germination of parasitic plants and establishing a symbiotic relationship with AM fungi. *A. thaliana*, belonging to the non-mycotrophic family Brassicacea, exude strigolactones at a lower concentration in comparison to mycotrophs (Akiyama *et al*., 2005). The first suggestion that strigolactones act as phytohormones was made after observing hyper-branched pea mutants which could not be explained by a response to, or altered levels of, known phytohormones (Beveridge *et al*., 1997). An unknown signal responsible for this phenotype was subsequently suggested.

It was only in 2008 that two independent research groups reported that strigolactones inhibit the outgrowth of axillary buds in *A. thaliana* (Gomez-Roldan *et al*., 2008) and *Oryza sativa* (Umehara *et al*., 2008). The outgrowth of axillary buds leads to more a branched phenotype. This novel function of strigolactones is different and independent to those previously established, because strigolactones here act as an endogenous hormone. The mutants used in the two studies are classified as either biosynthetic or signalling mutants. Biosynthetic mutants are able to resume a wild-type phenotype when treated with exogenous strigolactones, while signalling mutants are unaffected by the same treatment. The conclusion that was made is that strigolactones also serve as a long-distance signal to suppress branching.

Although the specific mode of action has not yet been fully elucidated, strigolactones may inhibit the outgrowth of axillary buds by negatively regulating cell division, leading to the inhibition of mesocotyl elongation (Hu *et al*., 2010; Yamaguchi and Kyozuka, 2010). The down-regulation of *AtBRC1* (At3g18550) in response to the absence of strigolactone leads to an increase in branching in *A. thaliana* (Aguilar-Martínez *et al*., 2007). The *AtBRC1* gene codes for a TCP transcription factor (TCP18) which prevents the outgrowth of axillary buds by arresting growth in the organ. Using a yeast one-hybrid screening, it was determined that the BRC1 protein specifically recognizes the DNA sequence TGGGC(C/T) (Giraud *et al*., 2010).

This binding sequence can be found in *Arabidopsis* ribosomal genes and cyclin B (reviewed by Giraud *et al*., 2010).

2.2.4 Strigolactone biosynthesis

The pathway responsible for shoot branching is commonly known as the *MAX/RMS/D* pathway (Beveridge and Kyozuka, 2010), as it is described in *A*. *thaliana*, garden pea and petunia. The *MAX* acronym stands for *more axillary growth*, descriptive of the phenotype observed in plants where any of the *MAX* genes are non-functional. The most likely initial precursor for strigolactone synthesis was proposed to be β-carotene (Schwartz *et al*., 2004), which was confirmed a few years later (Alder *et al*., 2012). Using carotenoid biosynthetic mutants, plants that are unable to synthesize carotenoid due to the silencing or knockout of one gene, and inhibitors of isoprenoid pathways on different plant species, it was determined that strigolactones are mainly synthesized in roots (Matusova *et al*., 2005), which is also the site of synthesis of abscisic acid and cytokinins. Hypothetical biochemical pathways have been proposed by a number of research groups, with general consensus on the key steps (Fig. 2.1).

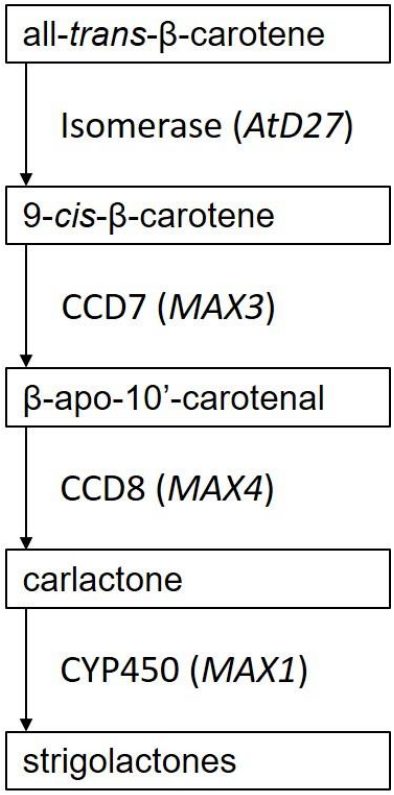

Figure 2.1: Proposed strigolactone biosynthesis pathway as defined and characterized in *Arabidopis thaliana* (adapted from a figure by de Saint Germain *et al*., 2013).

The strigolactone biosynthesis pathway, characterized to date, is conserved across *Pisum sativum*, *Oryza sativa*, *Petunia hybrida* and *Arabidopsis thaliana* (de Saint Germain *et al*. 2013). The first enzyme that acts within this pathway is a carotene isomerase, encoded in *Arabidopsis* by *AtD27* (At1g03055). The gene was first identified as *D27* (Os08g02210) in rice (*O. sativa*). This carotene isomerase reversibly converts all-*trans*-β-carotene into 9-*cis*-β-carotene (Alder *et al*., 2012). The next two enzymes of the pathway, carotenoid cleavage dioxygenase 7 (CCD7) and CCD8, were also discovered in rice and are encoded by the genes *D17* (Ps04g0550600) and *D10* (Os01g54270) respectively (Umehara *et al*., 2008). In *A. thaliana*, they are known as *MAX3* (At2g44990) and *MAX4* (At4g32810). These two CCD enzymes are involved in a series of cleavage reactions using 9-*cis*-β-carotene as substrate and eventually yielding carlactone. They are non-heme iron enzymes that cleave C-C bonds by incorporating a dioxygen (Alder *et al*., 2012). The intermediate produced by the cleavage reaction of β-carotene is then oxidized, a process mediated by a cytochrome P450 (CYP450), encoded by *MAX1* (At2g26170) (Matusova *et al*., 2005; Kohlen *et al*., 2011). To form the entire ABC-skeleton of the strigolactones probably involves a series of enzymatic oxidations, which have yet to be discovered and studied (Humphrey *et al*., 2006). An enzyme known as NCED1 (9-*cis*-epoxycarotenoid dioxygenase), is also suggested to be involved in the biosynthesis of strigolactones in tomato plants (López-Ráez and Bouwmeester, 2008). This enzyme has cleavage activity, known to catalyse the first step of abscisic-acid biosynthesis from carotenoids in response to water stress in *Phaseolus vulgaris* (Qin and Zeevaart, 1999). It remains unclear if NCED1 affects strigolactone biosynthesis directly or through its effect on ABA production (López-Ráez *et al*., 2008).

As mentioned, there is general consensus that the aforementioned components do not include all of the enzymes that act within the strigolactone biosynthetic pathway, mostly since the currently known enzymes are unable to explain the wide variety of strigolactone structures that have been isolated from plants thus far. Methylation and acetylation are also two enzymatic reactions that have not yet been accounted for and the enzymes responsible for these reactions have not yet been identified.

2.2.5 Regulation, perception and signalling

The deciphering of the mechanism by which strigolactones transmits the signal to inhibit shoot branching is one of the central unanswered questions in modern plant biology (Kagiyama *et al*., 2013). In an effort to start answering this question, Kagiyama and his team provided the first evidence for the direct binding of strigolactones to a receptor known as DWARF14 (D14).

Only a few components of the proposed strigolactone perception/signalling pathway have been identified yet.

The D14 protein, encoded by *D14 (At3g03990)*, was also first discovered in rice. This protein, believed to be the receptor of strigolactone molecules, has both enzymatic and receptor activities (de Saint Germain *et al*., 2013). The D14 protein is an α/β hydrolase enzyme. In a knockout study, a *d14* mutant was created in rice. This mutant had enhanced tillering outgrowth, a bushy phenotype that can be seen in any of the biosynthetic mutants. However, the *d14* mutant was insensitive to exogenous application of GR24, which did not rescue the *d14* phenotype (Arite *et al*., 2009; Goulet and Klee, 2010). This implicates D14 as part of the signalling pathway and not biosynthesis as previously thought.

The next component, MAX2, is one of the best characterized components of the strigolactone signalling pathway. The MAX2 protein, encoded by the *MAX2* (At2g42620) gene in *Arabidopsis*, acts downstream of the biosynthesis pathway (Fig. 2.1) and *D14*. This protein is an F-box leucine-rich repeat protein (Stirnberg *et al*., 2002; Stirnberg *et al*., 2007), generally accepted to be recognized by D14 (de Saint Germain *et al*., 2013). D14 can only recognize MAX2 once a strigolactone molecule has bound to it, suggesting that a conformational change of D14 induced by the binding of the strigolactone molecule enables it to recognize MAX2 and bind to it. The F-box protein MAX2 forms part of the SCF ubiquitin E3 ligase complex that recognizes substrates for proteolysis by the proteasome (Stirnberg *et al*., 2002; Johnson *et al*., 2006). Target proteins of MAX2 and other F-box proteins are typically poly-ubiquitinated and subsequently recognized and degraded by the 26S proteasome.

The target protein for MAX2 appears to be a repressor protein known as SMAX1 (Fig. 2.2), encoded by *SMAX1* (At1g07200), initially discovered as D53, encoded by *D53* (Os11g01330) in rice, and its degradation is essential for strigolactone signalling (Zhou *et al*., 2013). The *smax1* mutant has a similar phenotype to wild-type *A. thaliana* plants treated with karrikins/strigolactones (Stanga *et al*., 2013a), suggesting an opposite role to *D14* and *MAX2*. Another phenotype observed in *smax1* is the lack of seed dormancy and long hypocotyl phenotypes displayed by the *max2* mutant, but both *smax1* and *max2* display the same bushy phenotype (Smith and Li, 2014a). However, SMAX1 does not replace the requirement for MAX2 in responses to karrikins and strigolactones, which suggests that SMAX1 acts downstream of *MAX2* in the karrikin/strigolactone signalling pathway (Smith and Li, 2014b).

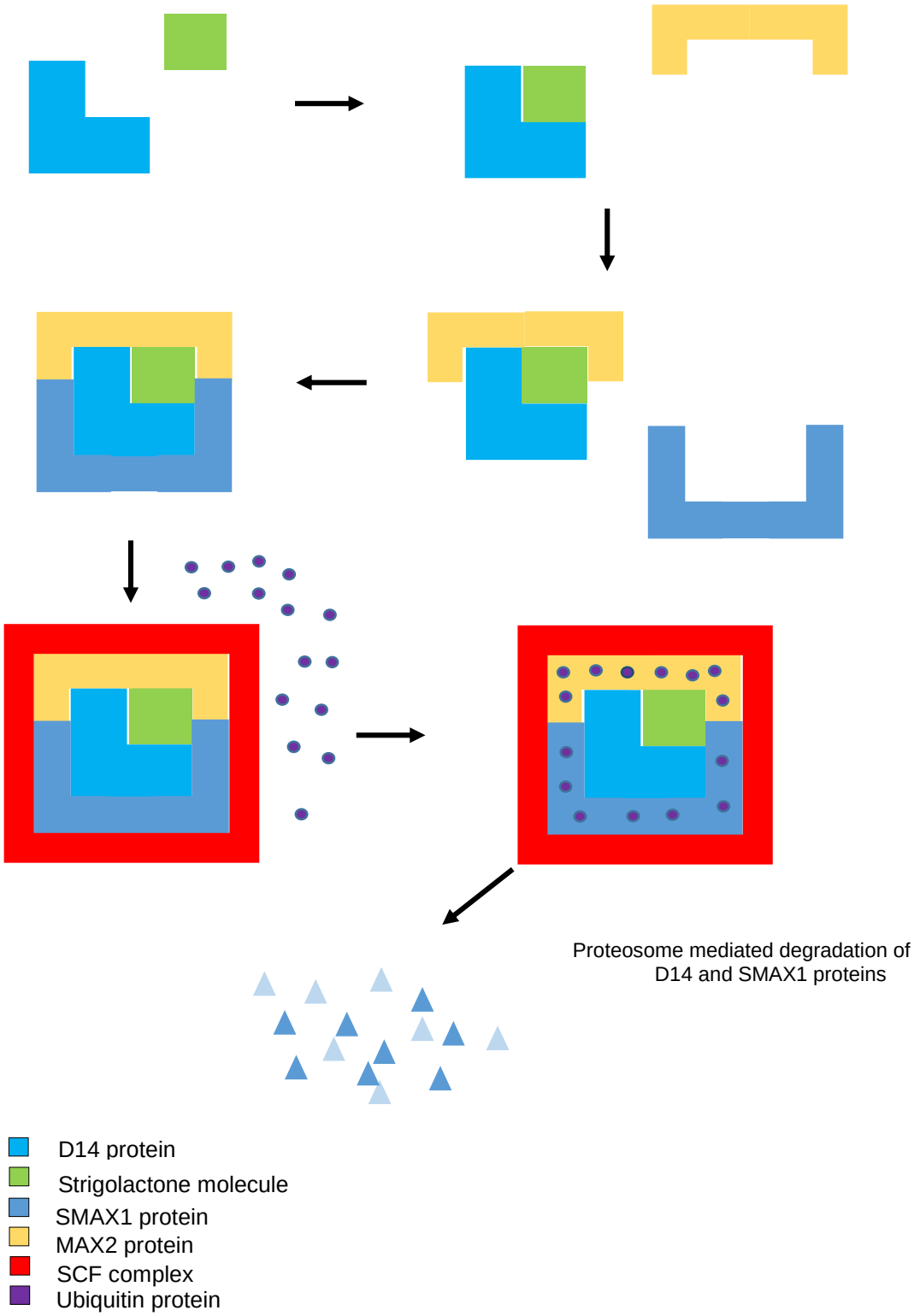

Figure 2.2*:* Hypothetical model of strigolactone signalling, starting with the binding of a strigolactone molecule binding, followed by the binding of the MAX2 protein and subsequently the SMAX1 protein. This is recognized by the SCF complex and ubiquitination and proteasome-mediated degradation of D14 and SMAX1 proteins occurs. An unknown mechanism downstream of this cascade then results in strigolactone activity (Zhou *et al*., 2013).

2.3 Strigolactones, smoke, karrikins and TMB

Fires and the resulting smoke have been a part of plant growth, development and evolution since before the existence of mankind. With the high oxygen levels, spontaneous fires were frequent and the assumption is that this imposed selective pressures on plant genotypes to adapt to fire and the resulting smoke. For a full review of this phenomenon, refer to Nelson *et al*. (2012). The effect of smoke on plant growth and development have been largely limited to germination studies (Brown, 1993; Calder *et al*., 2010; Light *et al*., 2010; Ghebrehiwot *et al*., 2013b). A famous example is the effect of fire and the resulting smoke on Fynbos seed germination. The heat generated by the fires fracture the hard seed coats and stimulate seed embryos, while ethylene, ammonia and karrikins (unknown at the time) stimulate germination (Brown, 1993). Without the fires, the seeds would most likely be unable to germinate. Apart from focussing on the effect of smoke on seed germination, numeral studies have been conducted to determine the component/s that are responsible for germination stimulation. The breakthrough came when a group of molecules known as karrikins were discovered (Flematti *et al*., 2004) and the structure of these molecules described (van Staden *et al*., 2004). The primary germination stimulant is known as 3-methyl-2H-furo[2,3-c]pyran-2-one (KAR_1) (Nelson *et al.,* 2009). Karrikins, specifically KAR_1 (Fig. 1.1E), are perceived by KAI2, an α/β-hydrolase that is a paralogue of D14 (Brewer *et al*., 2013; Guo *et al*., 2013). The most common correlation between strigolactones and karrikins is that both are stimulators of seed germination, which is regulated in a MAX2-dependant manner. The second correlation is the D-ring that forms part of all bioactive strigolactones (Fig. 1.1C) and which also forms part of the molecular structure of KAR_1 (Fig. 1.1E). Apart from the similarities, there are also some clear differences between KAI2 and D14 and the roles they play in karrikin signalling and strigolactone signalling. The D14 protein is destabilized by the presence of strigolactones and subsequently hydrolysed/degraded (Fig. 2.2), whilst KAI2 is not degraded following recognition of KAR_1. The catalytic site of KAI2 is also smaller than the catalytic site of D14 (Bythell-Douglas *et al*., 2013).

Smoke water is formed when it rains after bush fires. This smoke water solution then gets integrated into the soil. Interestingly, high concentrations of smoke water inhibit seed germination, while diluted smoke water stimulates germination (Light *et al*., 2002). The reason for this is the presence of another molecule known as trimethylbutenolide (TMB, Figure 1B), which is now known to inhibit the seed germination of *Lactuca sativa* (Soós *et al*., 2012) by inhibiting the stimulatory effect of karrikins (Light *et al*., 2010). It also inhibits the germination of certain weed species (Papenfus *et al*., 2015a). In smoke, TMB concentrations are considerably higher than those of karrikins (Ghebrehiwot *et al*., 2013a). After rainfall, the high concentrations of TMB are greatly diluted or washed away and the seeds can subsequently

germinate (reviewed by Ghebrehiwot *et al*., 2013a). It has been reported that TMB inhibits certain metabolic processes by down-regulating genes that are involved in plant metabolism, while up-regulating genes associated with maturation and abscisic acid (Soós *et al*., 2012) Very recently it has been shown that this chemical not only inhibits germination, but also affects the development rate index of *Ansellia africana* seeds (Papenfus *et al*., 2015b).

It is clear that TMB has an antagonistic effect to karrikins, which leaves the possibility that it might have the same effect on strigolactones. There is, however, no verified model that describes the interaction between karrikins and TMB. No model of the effect of TMB on strigolactones have also been reported.

2.4 Strigolactones – More than just branching inhibitors

The major studies relating to the functions of strigolactones defines them as being germination stimulators of parasitic plants (Cook *et al*., 1966), initiators of symbiotic relationships with arbuscular mycorrhizal fungi (Akiyama *et al*., 2005) and inhibitors of shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). However, several other studies have shown that strigolactones play a very diverse role in plant growth and development. Strigolactones are implicated in functions ranging from leaf senescence to root hair elongation.

2.4.1 Root growth and architecture

Similarly to controlling shoot architecture by inhibiting shoot branching, recent studies have indicated that strigolactones also controls root-architecture and growth in several ways. The first evidence suggesting that strigolactones are involved in root-growth was the observation that *max3* and *max4* biosynthetic mutants and the *max2* signalling mutant had more lateral roots than wild-type plants under the same conditions (Kapulnik *et al*., 2011a), while GR24 increased the primary root length (Ruyter-Spira *et al*., 2011) and root hair length in wild-type and *max3 Arabidopsis* (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011). The same mutants also struggle to increase root hair density when subjected to low concentrations of phosphates after germination (Mayzlish-Gati *et al*., 2012). Lateral root formation is inhibited by GR24 when sufficient phosphates are available (Kapulnik *et al*., 2011a; Koltai, 2011; Ruyter-Spira *et al*., 2011). Low concentrations of GR24 have been shown to increase the primary root length of *Arabidopsis* plants *in vitro* grown in nutrient-sufficient conditions, while higher concentrations had an opposite effect. When carbohydrates were limited and primary root length subsequently reduced (Jain *et al*., 2007), both low and high concentrations of GR24 led to an

increase of primary root length. It appears that strigolactones are vital integrators of root growth and abiotic conditions, because under nutrient-deficient conditions (especially low phosphates), the root architecture of plants are modified in response to strigolactones.

2.4.2 Adventitious rooting

The initiation and formation of roots from non-root tissue types like the stem or leaves is known as adventitious rooting (Rasmussen *et al*., 2012b), and is induced by environmental factors like light and wounding. This complex process is controlled by a variety of phytohormones, of which auxins play a central role. Auxin over-producing mutants *supperroot1*, *supperroot2* and *yucca* all have an increase in the production of adventitious roots. In contrast to auxin, light inhibits adventitious root formation in *Eucalyptus saligna* (Fett-Neto *et al*., 2001; Mckhann and Garcion, 2005), a common hardwood tree found in Australia.

Inhibition of strigolactone biosynthesis by fluridone in *Arabidopsis* and pea plants led to an increase in the formation of adventitious roots (Rasmussen *et al*., 2012b). This result suggests that strigolactones inhibit the formation of adventitious roots, at least in the early stages. However, fluridone inhibits the biosynthesis of carotenoids, upstream of the first step of strigolactones biosynthesis, leading to pleiotropic effects which made it difficult to explain the exact mechanism of inhibition. In rice, adventitious root formation is positively regulated by strigolactones via the *D3* (Os06g0154200) (*MAX2*) pathway (Sun *et al*., 2015). Dark grown *Psccd7* (Abd67496.1) and *Psccd8* (Aas66906.1) pea mutants had significantly reduced adventitious root formation versus the wild-type (Urquhart *et al*., 2015).

2.4.3 Responses to biotic and abiotic stress conditions

The symbiotic relationship between plants and arbuscular mycorrhizal fungi increases the plants' resistance to a variety of stresses. Logically, the increased nutrient uptake facilitated by this symbiotic relationship would make for stronger plants that are better able to fend off fungi, viruses and insects just by natural defence responses. More specifically, it is proposed that plant tissue is conditioned after mycorrhizal establishment to more quickly activate jasmonic-acid-dependent defence responses that lead to enhanced resistance to wounding and necrotrophs (Pozo and Azcón-Aguilar, 2007).

Drought and salinity stress are two of the biggest abiotic stresses leading to major crop losses owing to a decrease in plant growth and development (Yamaguchi and Blumwald, 2005;

Farooq *et al*., 2009; Shahbaz and Ashraf, 2013; Shrivastava and Kumar, 2015). In *A. thaliana*, *max2*, *max3* and *max4* are significantly more sensitive to drought and salt stress in comparison to their wild-type counterparts (Ha *et al*., 2014). In that study the susceptibility to these stresses was reversed by exogenous application of 5 µM GR24 in *max3* and *max4*, but not *max2*. In another experiment in the same study that complemented these results, wild-type plants treated with GR24 had a 100% survival rate against drought conditions in comparison to wild-type plants only treated with water. Together, these results suggest that strigolactones positively regulate drought and high salinity responses. In correlation with these findings, osmotic stress-induction of *LjNCED2* transcription was prevented by GR24 treatment in *Lotus japonicas* (Liu *et al*., 2015). The *LjNCED2* ABA biosynthetic gene is expressed in the roots in response to polyethylene glycol (PEG) treatments, which puts the plant under water stress by changing the osmotic potential of the soil or solution that the plants are in.

2.4.4 Leaf Senescence

Leaf and flower senescence are both processes promoted by ABA, jasmonic acid and ethylene, while being repressed by polyamines and cytokinins (Gan, 2003). In response to limiting phosphate-availability, leaf senescence is promoted in rice plants (Yamada *et al*., 2014). As previously stated, limiting phosphates is known to increase the biosynthesis and exudation of strigolactones, an effort by plants to acquire more nutrients *via* root development (Kapulnik and Koltai, 2014) or symbiotic relationship with AM fungi (Umehara *et al*., 2008). Several previous studies have shown that leaf senescence is delayed in strigolactone-biosynthetic and strigolactone-signalling mutants of *Arabidopsis*, rice and petunia plants (Woo *et al*., 2001; Snowden *et al*., 2005; Yan *et al*., 2007; Hamiaux *et al*., 2012; Yamada *et al*., 2014). A key component in leaf senescence is also MAX2, an F-box protein described in Section 2.2.5. Initially, MAX2 was identified as ORE9 (Woo *et al*., 2001), where the *ore9-1* mutant displayed a delayed senescence phenotype.

2.4.5 Secondary growth

Wild-type *Arabidopsis* (Col-0) plants are generally sturdier than strigolactone-biosynthetic or signalling mutants. This is mainly owing to increased secondary growth in the stem. Strigolactone-biosynthetic mutants had reduced secondary growth owing to reduced cambium activity and local treatments with GR24 induced secondary growth, suggesting that strigolactone signalling positively regulates cambium activity (Agusti *et al*., 2011). This makes sense as strigolactones probably inhibit branching to thicken the roots and shoots, ultimately

resulting in a non-bushy, sturdy phenotype. This stimulation of secondary growth is auxin-dependant (Agusti *et al*., 2011; Cheng *et al*., 2013).

2.4.6. Reproductive development

The *Slccd8* strigolactone biosynthesis mutant of tomato plants resulted in a phenotype that displayed smaller flowers that were quantified by measuring the length of petals, sepals and anthers during anthesis (Kohlen *et al*., 2012). After fruit development, the tomato fruits and seeds of the *Slccd8* mutant were also significantly smaller than the fruits borne by the wild-type plants. This phenomenon could be explained by the lack of apical dominance observed in strigolactone-deficient mutants. More nutrients are directed towards the formation of new branches than towards developing fruits.

2.5 Strigolactone interaction with other phytohormones

The major gaps within strigolactone-research are partly owing to its late discovery as a phytohormone and its biosynthesis and activity at pM concentrations, but mostly owing to the complex nature of the interactions with other phytohormones. These gaps occur at the biosynthesis, perception, and the signalling and response levels. Strigolactones are known to regulate branching together with auxins and cytokinins. Two models of this exact mechanism have been proposed to date.

2.5.1 Shoot branching – Regulation by auxins, cytokinins and strigolactones

Shoot branching can be defined as the outgrowth of axillary buds to form new branches and flowers (Ongaro and Leyser, 2008). Before the discovery of strigolactones as a novel group of phytohormones (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008), auxins and cytokinins were the only two groups of phytohormones known to influence branching. Auxins are transported basipetally from the apex to suppress the outgrowth of axillary buds in higher/vascular plants (Agusti *et al*., 2011), a process defined as apical dominance. The first hypothesis was that auxins move downward in the stem and are transported into the buds where they directly inhibit bud outgrowth. Cytokinins, produced in the roots, travel acropetally to promote the activation of lateral buds, which results in the formation of shoot branches (Ferguson and Beveridge, 2009).

However, a third unknown hormone of carotenoid-derived origin was also suggested to participate in the regulation of shoot branching and architecture (Booker *et al*., 2005; Simons *et al*., 2007; Ongaro and Leyser, 2008). It has now been confirmed that strigolactones are those carotenoid-derived chemicals (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Currently there are two models aiming to describe the regulation of bud outgrowth that leads to an increase in shoot branching. The first is known as the auxin canalization model and the second is known as the secondary messenger model. Auxins, cytokinins and strigolactones have an effect on one another and on bud outgrowth (Fig. 2.3), which eventually leads to increased or decreased branching in plants. This diagram (Fig. 2.3) does not specifically represent one of the models, but rather elements of both.

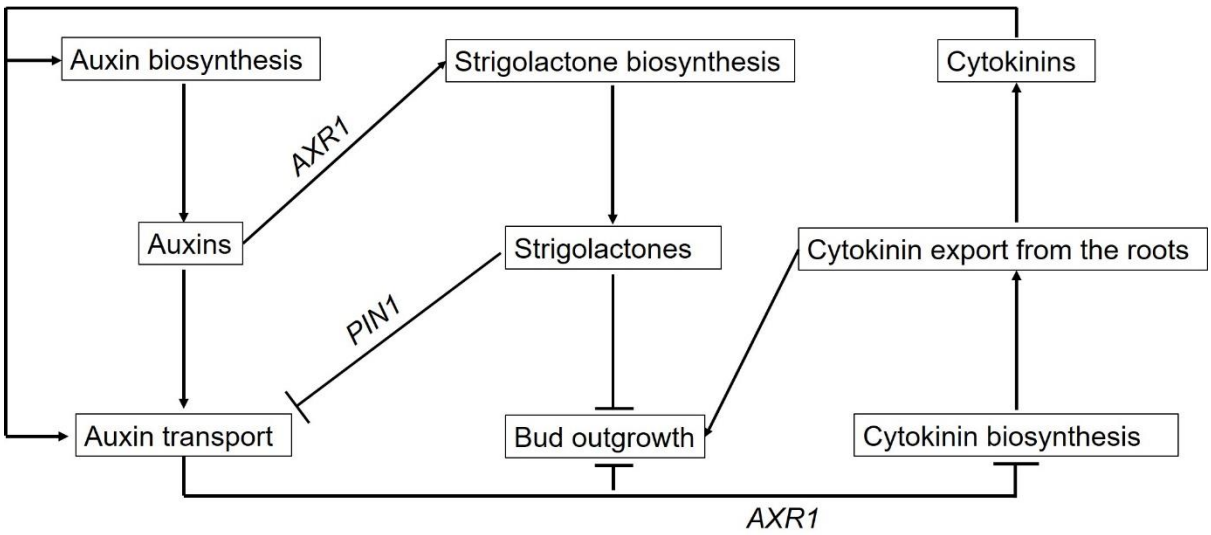

Figure 2.3: Schematic diagram of how auxins, cytokinins and strigolactones influence one another to eventually regulate bud outgrowth that leads to increased branching. Auxin inhibits bud outgrowth through its transport and by inhibiting cytokinins biosynthesis through *AXR1*, while cytokinin export stimulates bud outgrowth but stimulates auxin biosynthesis as well. Auxins, also through *AXR1*, stimulate strigolactone biosynthesis while strigolactones inhibits bud outgrowth and auxin transport through *PIN1*.

Bud outgrowth in higher plants are controlled by the TCP18 transcription factor, encoded by *AtBRC1* (At3g18550), a homologue of *TEOSINTE BRANCHED 1* (*TB1*) (Finlayson, 2007). In response to several stimuli, *BRC1* is either up-regulated, leading to axillary bud arrest, or down-regulated, leading to the outgrowth of these buds. The expression of *BRC1* is influenced by dense growth conditions affecting light, cytokinins, auxins (Aguilar-Martínez *et al*., 2007) and strigolactones (Rameau *et al*., 2015). Even before the discovery of strigolactones as a

novel phytohormone (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008), it was suggested that *BRC1* and *MAX* genes act within the same pathway (Aguilar-Martínez *et al*., 2007). As mentioned, auxins and cytokinins were used to formulate two models that aimed to describe the regulation of shoot branching in higher plants. They are known as the auxin-canalization and second messenger model. We now know that strigolactones also play a central role in shoot branching, acting directly in the bud to inhibit its outgrowth.

2.5.2 Auxin-canalization model

This auxin-canalization model was first proposed by Sachs (1991). Auxins are produced in the apex of higher plants. They were implicated in the regulation of branching because an increase in shoot branching was observed in plants where the apex was removed. In *max4* mutants, exogenous application of auxins did not inhibit the outgrowth of axillary buds (Sorefan *et al*., 2003; Bennett *et al*., 2006), suggesting that auxin-mediated inhibition of branching is *max4*-dependent. In return, auxin levels were elevated in *max4* rice mutants (Arite *et al*., 2007). During these studies, it was still unknown that *MAX4* is involved in strigolactone-biosynthesis and that strigolactones are involved in regulating shoot branching. Strigolactones inhibit the synthesis of PIN-FORMED proteins by inhibiting the expression of expression of *PIN1* (At1g73590) (Fig. 2.3), leading to a reduction of these proteins and the inhibition of auxin transport (Crawford *et al*., 2010). These PIN proteins are transmembrane protein known to transport auxin in a polar manner (Krecek *et al*., 2009). Owing to the decrease of auxin within the stem, auxins are exported out of the bud. This alleviates the suppression of the bud, allowing bud outgrowth. It is proposed that this process is regulated by PAT (polar auxin transport) in the stem (Li and Bangerth, 1999; Domagalska and Leyser, 2011), which is in turn regulated by strigolactones.

2.5.3 Secondary messenger model

This simplistic and classical model implies the direct action of strigolactones and/or cytokinins in the axillary buds of plants, with auxin controlling the biosynthesis of strigolactones and/or cytokinins Auxins regulate strigolactone and cytokinins synthesis in an *AXR1 (AUXIN RESISTANT 1*) (At1g05180) -dependent manner (Fig. 2.3) (Nordström *et al*., 2004). Through this mechanism, cytokinin biosynthesis is reduced, leading to less cytokinins being transported into the bud, preventing bud outgrowth. Auxins stimulate strigolactone biosynthesis. Strigolactones move directly into the bud to inhibit bud outgrowth (Gomez-Roldan *et al*., 2008;

Umehara *et al*., 2008). Evidence for strigolactones and cytokinins affecting bud outgrowth directly is that direct application of cytokinins to a bud is sufficient to stimulate its outgrowth, while strigolactone application in the same manner inhibits the outgrowth of the bud. In this model, it is difficult to conclude whether strigolactones, cytokinins or both are the secondary messenger. In one study, strigolactones were able to directly inhibit bud outgrowth independently of auxins (Brewer *et al*., 2009). Very little is known as to how cytokinins and strigolactones affect one another, which is one of the major reasons why neither one of the models have been rejected, although the canalization model has more support in the scientific community.

2.5.4 Interactions with ethylene, jasmonic acid, salicylic acid and gibberellic acid

Several interactions between auxin and ethylene have been described, with one example being that ethylene regulates root growth through auxin biosynthesis and transport (Růzicka *et al*., 2007). However, very little is known about possible interactions between ethylene and strigolactones. A common denominator between auxin, strigolactones and ethylene is their positive effect on root-hair elongation (Kapulnik *et al*., 2011b). The first observation was that root hair length increased when *max2-1* signalling mutants were exposed to the ethylene precursor ACC. This suggests that an ethylene response is dependent on strigolactone signalling. In the same study, it was observed that ethylene-signalling deficient mutants responded less effectively to GR24 in comparison to wild-type plants, suggesting that ethylene signalling is needed for a strigolactone-response.

Both jasmonic acid and salicylic acid are known to be involved in plant defence mechanisms. In tomato plants, the *Slccd8* strigolactone biosynthesis mutant, which has reduced levels of strigolactones, also had reduced levels of jasmonic acid, salicylic acid and abscisic acid, as determined by HPLC (High Performance Liquid Chromatography) (Torres-Vera *et al*., 2014). In the same study, wild-type plants were more resistant to *Botrytis cinerea* than the *Slccd8* mutant. This is suggestive of interactions between strigolactones and the three aforementioned phytohormones. However, with the strigolactone biosynthesis pathway not yet fully elucidated, the *CCD8* gene could prove to be more directly involved in the biosynthesis of these phytohormones and not just in strigolactone biosynthesis. Therefore, more research needs to be conducted in order to elucidate the role that strigolactones play in plant defence mechanisms. The bioactive gibberellin GA_1 is suggested to modulate decapitated pea plants response to applied GR24, possibly by affecting bud sensitivity (Luisi *et al*., 2011).

2.6 *BRC1 (BRANCHED1)*

Shoot branching as a common indicator of strigolactone activity can be directly linked to the expression of *BRC1 (BRANCHED1)* in axillary buds. *BRC1* expression is up-regulated in the presence of strigolactones, leading to a decrease in branching. The *brc1* mutant in *Arabidopsis* (Fig. 2.4) has a bushy phenotype similar to that of *max1, max3, max4* and *max2*. *AtBRC1* (*BRANCHED1*), locus ID *At3g18550*, is the most well characterized strigolactone-responsive gene to date. Although *brc2* has a similar phenotype to *brc1* (Fig. 2.4), it has been determined that *BRC1* plays a more relevant role in controlling branching than *BRC2* (At1g68800)(Aguilar-Martínez *et al*., 2007; Finlayson, 2007; Martín-Trillo *et al*., 2011).

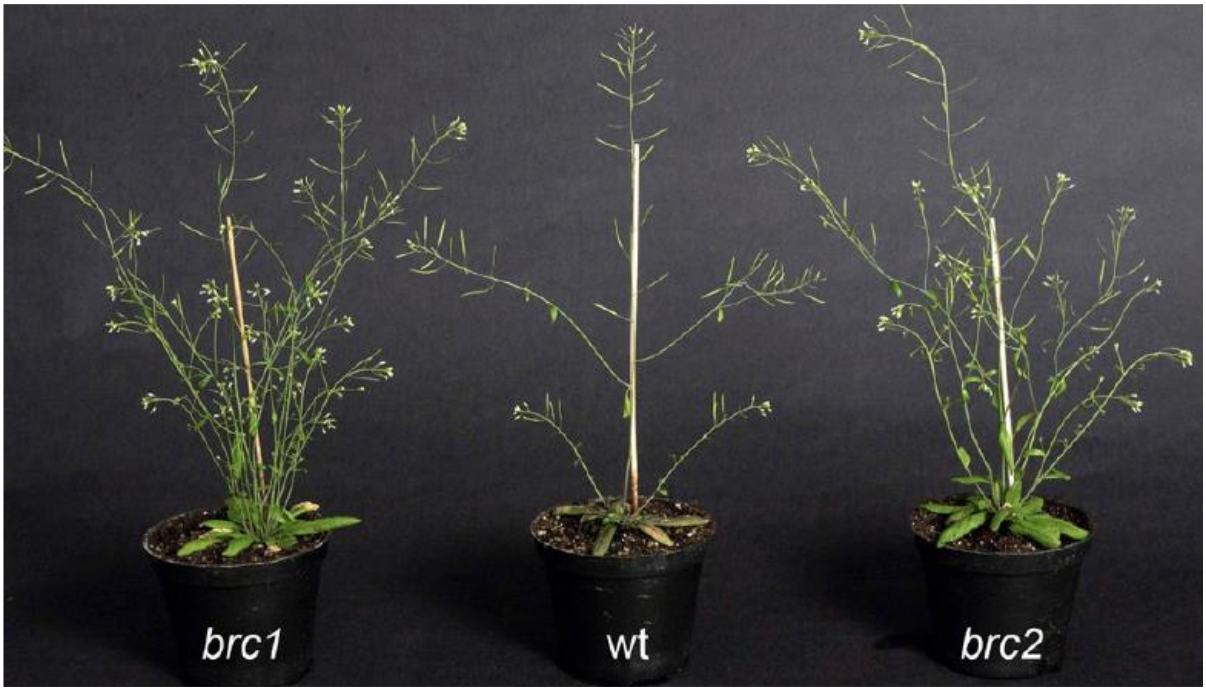

Figure 2.4: Phenotypes of *A. thaliana brc1* and *brc2* versus wild-type (Col-0) (Aguilar-Martínez *et al*., 2007). The wild-type plant has the normal apical dominance phenotype, while both *brc1* and *brc2* have numerous secondary branches, characteristic of strigolactone biosynthesis and perception mutants.

BRANCHED 1 (BRC1) is orthologous to *TEOSINTE BRANCHED 1 (TB1)*, a gene expressed in maize (*Zea mays*) axillary meristems. Expression of *TB1* prevents the outgrowth of buds at lower nodes and promotes female florescence at the upper nodes in maize plants (Cubas *et al*., 1999). Both *TB1* and *BRC1* encode putative transcription factors of the TCP family (Cubas *et al*., 1999; Martín-Trillo *et al*., 2011). TCP transcription factors, consisting of two subfamilies, are known to negatively regulate the expression of boundary-specific genes to control the morphogenesis of shoot organs (Koyama *et al*., 2007). The acronym TCP stands for TB1,

CYCLOIDEA and PCF, three types of proteins known to control cell cycling and cell division (reviewed by Choi *et al*., 2012).

TB1 is one of the few genes known to be involved in maize domestication, playing a role in maize apical dominance. The ancestor of modern domesticated maize, known as teosinte, is small and highly branched (Rameau, 2010). The *TB1* gene has unknowingly been selected for since the first domestication of maize plants. Less valuable nutrients are invested in the formation of branches and more nutrients are directed towards the formation and development of corn cobs.

In rice, *FINE CULM 1 (FC1)*, also known as *OsTB1* (Ab088343), is orthologous to *TB1*. An excess branching phenotype is observed in loss-of-function mutants of *FC1* (Minakuchi *et al*., 2010). In the same study, it was shown that the transcription of *FC1* was not up-regulated by exogenous application of GR24, but was down-regulated by cytokinin application. When maize *TB1* and rice *TB1* (*OsTB1*) were overexpressed in rice plants, the number of panicles and tillers were significantly reduced when compared to wild-type plants (Choi *et al*., 2012). In the same study, an increase in panicles and tillers was observed when *OsTB1* was silenced through an RNAi-mediated strategy.

In tomato, two paralogues, named *SlBRC1a* and *SlBRC1b*, resembling *BRC1* have been identified. In dormant vegetative axillary buds, *SlBRC1b* was highly expressed, as detected by quantitative Real-Time PCR analysis (Martín-Trillo *et al*., 2011). When subjected to stimuli known to promote branching (Table 2.4), expression of *SlBRC1b* was significantly down-regulated. In domesticated tomato, lateral shoot branching is an unwanted trait, because it diverts nutrients and water away from developing fruits, negatively affecting development (Martín-Trillo *et al*., 2011).

The expression of *PsBRC1*, the pea (*Pisum sativum*) homologue of *AtBRC1* and *TB1,* was up-regulated by GR24 (Braun *et al.,* 2012), which contrasts with what was observed in rice (Minakuchi *et al*., 2010) but correlates to the situation in *Arabidopsis thaliana* (Gomez-Roldan *et al*., 2008) when GR24 was directly applied to the axillary bud. When 6-benzylaminopurine (cytokinin) was applied in the same way, expression of *PsBRC1* was down-regulated. According to these authors, *PsBRC1* is almost exclusively expressed within the axillary bud. In 2008, Finlayson investigated the expression of *TCP18*, a synonym for *AtBRC1*, in *A. thaliana* and found that although the highest expression is in non-elongated rosette-buds, it is also expressed in rosette leaves and flowers. Low to no expression was detected in stems and roots. These expression levels were detected using northern blot analysis. Using qRT-PCR analysis, it was confirmed that *AtBRC1* is highly expressed in flowers, but not in seeds, roots, cauline leaves, stems and whole seedlings (Mashiguchi *et al*., 2009). However, treating

whole seedlings with GR24 resulted in a four-fold increase in expression of *AtBRC1* after just 6 hours.

Apart from being upregulated by strigolactones, it is known that the expression of *BRC1* is also up-regulated by auxin and low R:FR (red:far-red) light (González-Grandío *et al*., 2013) and down-regulated by the application of cytokinins and presence of sucrose (Mason *et al*., 2014; Barbier *et al*., 2015). A decrease in the R:FR light ratio is owing to an increase in shading by neighbouring plants (González-Grandío *et al*., 2013). This stimulus leads to the up-regulation of *BRC1*, leading to a shade avoidance response that results in a taller plant.

2.7 The aims and objectives of this study

2.7.1 Part 1

Due to TMB's antagonistic effect to karrikins and the similarity between the strigolactone receptor (D14) and the karrikin receptor (KAI2), it is hypothesized that TMB might have an antagonistic effect on strigolactone signalling either by binding to the D14 receptor affecting the KAI2 receptor that is also known to modulate some strigolactone-responses (Scaffidi *et al*., 2014). The aim of this part of the study was to determine if TMB is an inhibitor of strigolactone signalling.

The first objective was to find the optimal concentration at which TMB and furanone have any effects on the growth of *Arabidopsis in vitro*, by using lateral root formation as an indicator of strigolactone activity. The second objective was to determine the effect of TMB, furanone and GR24 on strigolactone-responsive genes, after short term and long term exposure.

2.7.2 Part 2

Identifying and quantifying strigolactones in *A. thaliana* has been challenging, and to date it has been almost impossible to accurately quantify strigolactone levels in this species due to the extremely low levels at which they are produced (Ruyter-Spira *et al*., 2011; Seto *et al*., 2014). Current methods used in strigolactone-research, such as forward- and reverse-genetics are limiting and slowing down the knowledge-gaining process. Because *Arabidopis* is a genetic and physiological model for higher plants, a tool that could ease the detection of strigolactones would aid in the further elucidation of the strigolactone pathway and provide further insight into strigolactone signalling and responses.

Having a reporter-construct in *A. thaliana* that is activated in the presence of strigolactones would be a valuable tool to further characterize the strigolactone pathway in model plant species. It could also be used in plant species where there is no available genetic data (sequence) and/or available mutants. Therefore, the aim of this part of the study was to develop a reporter-construct that is activated in the presence of strigolactones, using the promoter region of a known strigolactone-responsive gene, *AtBRC1*.

The first objective was to identify a well-known, well-characterized strigolactone-responsive gene and perform *in silico* analysis in order to identify a region of interest that might be a downstream target of the strigolactone pathway. The second objective was to isolate different sizes of the region directly upstream of *BRC1* (the strigolactone-responsive gene used in this project), clone them individually upstream of the *GUS* gene and transform the constructs into *A. thaliana* using *Agrobacterium tumefaciens*. The third objective was to analyse the transformants in order to investigate whether any of the constructs display strigolactone-responsiveness.

Chapter 3: Effect of TMB on the growth of *Arabidopsis thaliana*

''Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled'' – William Ramsay

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3.1 Introduction

Branching is an important trait in plants, as it can determine not only the survival and dominance, but ultimately the yield of certain crops. A plant with an increased branching phenotype might direct its acquired nutrients away from developing fruits, resulting in a lower yield. However, the bushy phenotype might be a survival tactic for more sustainable growth during its lifetime. The *BRC1* gene is known to play a central regulatory role in branching and ultimate architecture of plants (Aguilar-Martínez *et al*., 2007). Very recently, a group of chemicals known as strigolactones have been described as a novel group of phytohormones that inhibit shoot branching in higher plants (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Since this discovery, it has been determined that *BRC1* is highly expressed in the axillary buds where it arrests growth and subsequently prevents the outgrowth of the bud (Martín-Trillo *et al*., 2011) and many other functions of strigolactones have been uncovered.

Although many functions other than the regulation of shoot branching has been linked to strigolactones (Chapter 2), there are general consensus amongst the scientific community that not all of the components of the biosynthetic and signalling pathways have been elucidated, as is the case for many other phytohormone pathways. The reason for this is that phytohormones are active at relatively low concentrations in plants and they are involved in complex regulatory networks. The use of biosynthetic, perception and signalling mutants has been instrumental in uncovering important components of the strigolactone pathway. This strategy has its limitations, because some mutants may be unable to survive, or have phenotypes that could be a result of a deletion of a gene that is part of a completely different biochemical pathway (Matusova *et al*., 2005). With strigolactones seeming to play such a central and pivotal role in plant growth and development, it is important to not only characterize and describe the pathway in model plants, but also in plant species where no genetic data or mutant phenotypes are available, as it is possible that strigolactones have very different and unique roles in different plants.

A possible way of further characterization of strigolactone-signalling and studying strigolactone-responses in non-model plant species is by using a strigolactone signalling

inhibitor. Currently, only strigolactone-biosynthesis inhibitors are available (Ito *et al*., 2010). The need for such an antagonist, and even more agonists, of strigolactone-activity has been described, with the proposed strategy being to modify current strigolactone agonists using the 3D structure of D14 and an *in silico* drug design method to screen for novel chemicals (Nakamura and Asami, 2014).

For this study, trimethylbutenolide (TMB) was investigated as a possible inhibitor of strigolactone signalling. TMB has mostly only been described as a germination inhibitor of certain plant species, specifically as a direct antagonist of karrikin activity (Soós *et al*., 2012; Ghebrehiwot *et al*., 2013a; Papenfus *et al*., 2015a). Two different studies on TMB have described it as a stimulator of pollen growth in three different plant species (Kumari *et al*., 2015) and an inhibitor of both germination and seedling development of *Ansellia africana* (Papenfus *et al*., 2015b). Other than the aforementioned studies, no research has been conducted on TMB, and its effect(s) on *A. thaliana* remains unknown.

The antagonistic effect of TMB on karrikin activity means that it could have a similar effect on strigolactone activity. Strigolactone and karrikins both act through the MAX2 pathway, but are perceived by two paralogues, D14 and KAI2 respectively. There have been reports that both D14 and KAI2 can mediate GR24 (strigolactone) responses, but only KAI2 can mediate karrikin responses (Waters *et al*., 2012b). The specific mechanism of how TMB inhibits karrikins is still unknown, but if it does block the KAI2 receptor, it could possibly block the D14 receptor as well. Even though TMB only affects KAI2, it could still act as an inhibitor of the strigolactone functions mediated via KAI2.

In this chapter, the effect of different furanone-derivatives were investigated, with a particular focus TMB. As a starting point to investigate the effect of these chemicals on the growth of *A. thaliana*, an *in vitro* approach was used to treat *A. thaliana* seedlings in controlled manner. The formation of lateral roots was used as an indicator of increased or reduced strigolactone activity. A small group of strigolactone-responsive genes were also used in a semi-quantitative reverse-transcription PCR (sqRT-PCR) gene expression analysis to determine if an increase of decrease in strigolactone-responsiveness was observed in the presence of TMB.

3.2 Materials and Methods

3.2.1 Chemicals

Phyto-agar as solidifying agent for media used for *in vitro* experiments was purchased from DUCHEFA Biochemie. GR24 (racemic) was obtained from Prof B Zwanenburg of the Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands. All other chemicals, including tissue culture media, were bought from Sigma-Aldrich unless explicitly otherwise stated. Primers used for semi-quantative RT-PCR analysis were designed by Marthinus Jacob Rossouw, using Oligo Explorer (version 1.1.2), and synthesized by Inqaba Biotechnical Industries (Pty) Ltd (South Africa).

3.2.2 Surface sterilization of *Arabidopsis thaliana* seeds

A. thaliana seeds were surface decontaminated by placing open microcentrifuge tubes containing the seed under a glass dome with a beaker containing 100 mL sodium hypochlorite and 2 mL hydrochloric acid (37%) for at least 4 h.

3.2.3 *In vitro* growth conditions and physiological growth quantification

Surface decontaminated seed was sown onto petri dishes containing ½ strength Murashige and Skoog (MS) media (2.2 $g.L^{-1}$) solidified with 9 $g.L^{-1}$ Phytoagar with the pH adjusted to 5.8 using potassium hydroxide (KOH). Growth media were sterilized by autoclaving for 20 min at a temperature of 121ºC and pressure of 103 kPa. Five days after germination (DAG), seedlings were transferred to a Greiner petri dish (120 mm x 120 mm) with media supplemented with the specific chemicals/treatments to be tested. These plates were placed almost vertically under cool white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 µmoles photons.$m^{-2}.s^{-1}$ in 16h light/8h dark growth conditions. The temperature of the growth room was 23ºC. Ten days later the plates were opened and photographed with a Nikon camera. The images were analysed using ImageJ (version 1.49) software. Physiological parameters investigated were lateral root number and primary root length.

3.2.4 *In vitro* application of treatments

The synthetic strigolactone analogue GR24 (racemic mixture of both enantiomers) was dissolved in acetone to a concentration of 500 µM and further diluted to 100 µM with ddH_20. The concentration of GR24 used in all the *in vitro* experiments was 0.1 µM. The second molecule, furanone (3-methyl-2(*5H*)-furanone), was obtained from Sigma-Aldrich in an 11.5 mM liquid solution and further diluted using ddH_20. The third molecule, TMB (3,4,5-trimethyl-2(*5H*)-furanone), was obtained as a 6.2 M solution (Key Organics Ltd., United Kingdom) and further diluted using ddH_20. All treatments were filtered sterilized using a 0.22 µm syringe filter unit directly into sterile ½ MS medium without sucrose. Optimal concentrations of TMB and furanone were determined by testing the effect of a range of different concentrations on the formation of lateral roots.

3.2.5 Semi-quantitative RT-PCR analysis

A. thaliana wild-type seedlings were surface decontaminated (Section 3.2.2) and grown vertically for 10 days on ½ MS media as described in Section 3.2.3, but with the addition of 2% (m/v) sucrose. Ten seedlings were submerged in a solution containing the appropriate treatment for 6 h, before being gently blotted dry, flash frozen in liquid nitrogen and ground to a fine powder in a microfuge tube using a plastic pestle. The RNA extractions were performed using the RNEasy Mini Kit (QIAGEN), according to the manufacturer's protocol. The concentration of total RNA was determined by a NanoDrop™ Lite Spectrophotometer (Thermo Scientific). Genomic DNA was removed by a DNase I (RNase-free) kit (Thermo Fisher Scientific), according to the manufacturer's protocol. The DNase I enzyme was inactivated by adding 1 µL 25 mM EDTA and incubating at 65ºC for 10 min. The mRNA was converted to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), according to manufacturer's protocol. Oligo(d)T primers were used for the reverse transcriptase reaction.

Each PCR reaction used 1 µL of cDNA, unless otherwise specified. The group of genes used were previously shown to be up- or down-regulated in response to GR24 treatment (Mashiguchi *et al*., 2009). The components of the optimization PCR reactions were 1X GoTaq® Reaction Buffer, 0.3 µM forward primer, 0.3 µM reverse primer, 0.3 mM dNTPs, 1 µL cDNA, 0.01 $U.\mu L^{-1}$ GoTaq® DNA Polymerase and ddH_20 to a final volume of 50 µL. Specific primer sequences (Table 3.1) and PCR cycle conditions (Table 3.2) were used for each PCR reaction.

Table 3.1: Primers used for semi-quantitative RT-PCR analysis of strigolactone-responsive genes in the absence and presence of GR24.

Gene name	Response to GR24	Forward (5'-3')	Reverse (5'-3')	T_m
(*At1g64380*) *AP2*	Up-regulated	GCCGCAAAACAG AATGAGGG	GAACGATGGCAT CCTCGCTA	62ºC
(*At2g42620*) *F-box*	Up-regulated	CGTGAATCAATGT CAACCAC	GATCGAAAATTAA CGGGTGA	56ºC
(*At4g14560*) *IAA1*	Down-regulated	ATGGAAGTCACC AATGGGCT	GGCAGTAGGAGC TTCGGATC	60ºC
(*At4g39070*) *STH7*	Up-regulated	ATGAAGATTTGGT GTGCTGT	GCTCGTAAATACC TCATTGG	56ºC
(*At3g18780*) *Actin2*	Unchanged	ATGGCTGAGGCT GATGATAT	CCATCACCAGAAT CCAGCAC	58 ºC

Table 3.2*:* PCR cycle conditions for semi-quantitative RT-PCR analysis of strigolactone-responsive genes.

Step	Temperature	Duration	Number of cycles
Initial Denaturation	95ºC	2 min	1
Denaturation	95ºC	1 min	
Annealing	*	1 min	25
Elongation	72ºC	2 min	
Final extension	72ºC	5 min	1

* Annealing temperatures were adjusted to the T_m described for each primer pair (Table 3.1)

3.2.6 Agarose gel electrophoresis

To visualize amplified DNA fragments, 25 µL of each of the PCR amplification products were loaded into the wells of an agarose gel (1% [m/v] agarose, 0.5x TBE buffer [5.4g.L^{-1} Tris base; 2.75 g.L^{-1} boric acid; 0.465 g.L^{-1} EDTA, pH 8.0]) stained with 2.5 µL per 50mL gel of Pronosafe (Laboratorios Conda). The amplified DNA fragments were separated in the gel at 110 V for approximately 35 minutes. The DNA was visualized and photographed under ultraviolet (UV)-light using the Alpha Imager 2000 (Alpha Innotech).

3.2.7 Statistical analysis

All experiments were replicated three times to ensure reproducibility. The one-way ANOVA function in Excel was utilized to determine any significant differences between the control/mock group and the treated groups.

3.3 Results and Discussion

3.3.1 Optimization of growth conditions

Standard *in vitro* growth conditions were used for all of the following experiments, as described in Section 3.2.2. Sucrose was excluded from the media as its presence can have confounding effects on strigolactone treatments (Jain *et al*., 2007). When investigating strigolactone activity in an *in vitro* setting with the addition of sucrose, some discrepancies occur between plants treated with low concentrations of GR24 versus plants treated with high concentrations of GR24 (Jain *et al*., 2007). With the addition of sucrose to the media, low concentrations of GR24 increase primary root length of *A. thaliana*, while higher concentrations of GR24 decrease the primary root length (Jain *et al*., 2007). All of the *in vitro* growth experiments in this chapter were repeated three times to ensure reproducibility.

Following experimentation to determine the best stage at which to transfer seedlings to new plates (data not shown), it was determined that 5 days after germination (DAG) was optimal. In order to determine the effect of different concentrations of furanone on lateral root formation, as well as to identify the optimal concentration to use in future experiments, *max4* seedlings were grown for 7 d on media containing 1 µM, 10 µM and 100 µM furanone (Fig. 3.1). The *max4* genotype, rather than the Col-0 wild type, was used since it would be easier to observe a change in phenotype as a result of strigolactone-like activity in this completely strigolactone-deficient mutant background.

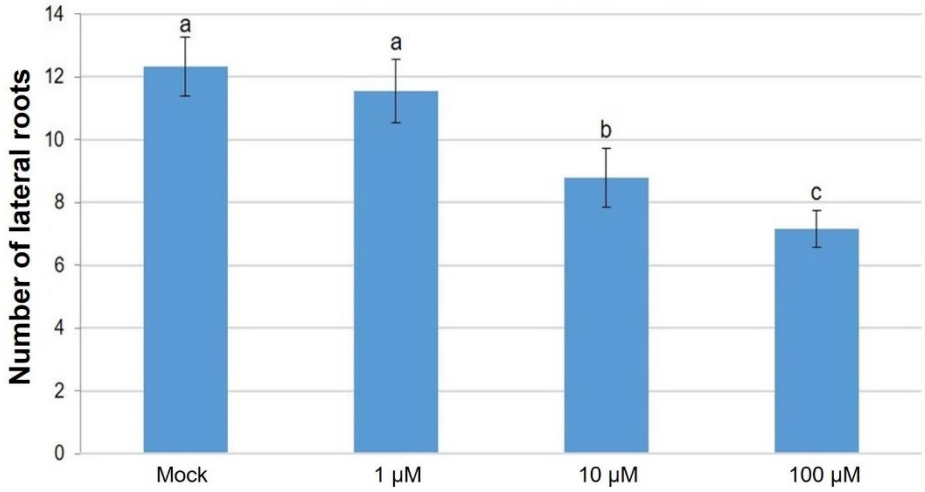


Figure 3.1: The effect of a mock treatment (control) and three different concentrations of furanone on lateral root formation in *A. thaliana max4* seedlings. Bars represent the mean of 50 replicates (n=50) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different ($P < 0.05$) from the control.

A reduction in lateral root number was observed when furanone was supplied at concentrations of 10 µM or 100 µM. Similar results were obtained when rice plants were treated this molecule (Nakamura *et al*., 2013). In that study, 50 µM of furanone slightly, but significantly, reduced the length of the second tiller of the rice plants, but not as much as 1 µM GR24. The hydrolysis of strigolactones by the enzymatic activity of D14, liberating the D-ring from the ABC-ring, seems to provide the energy needed to destabilize the D14 molecule, which starts the strigolactone-signalling process that eventually leads to the degradation of D53 proteins. The high concentration of furanone needed (100 µM) to simulate some strigolactone-activity might be explained by the lack of energy release because of the absence of the hydrolysis reaction. In *Petunia hybrida*, it was reported that neither the ABC-ring nor the D-ring (i.e. furanone) can stimulate D14-MAX2 interaction or modulate branching (Hamiaux *et al*., 2012). In that study, the concentration at which furanone was tested is not stipulated, which might explain the report of furanone's complete inactivity. Because there are numeral studies already published on the effect of furanone on plants growth, as described in Chapter 1 (General Discussion), this molecule was further utilized as a control in conjunction to GR24.

The use of furanone as a control in downstream experiments could prove to be valuable, because furanone has the same ring structure as TMB, without the additional two methyl groups. In this experiment, the effect of furanone on lateral root formation was only investigated on the *max4* phenotype, because *max4* is a strigolactone biosynthesis mutant and would show a greater effect in the presence of a treatment containing strigolactones or a strigolactone analogue. In the future, however, the wild-type should be included in order to make any definitive conclusions about the results.

To determine the optimal concentration of TMB for use in future experiments, *A. thaliana* Col-O wild type plants were treated with three different concentrations of TMB (Fig. 3.2). Since the overall aim of these experiments was to determine whether TMB is able to inhibit strigolactone signalling, wild type plants were used for this experiment instead of the *max4* phenotype, because the wild type Col-0 synthesizes strigolactones, whilst the *max4* biosynthetic mutant does not.

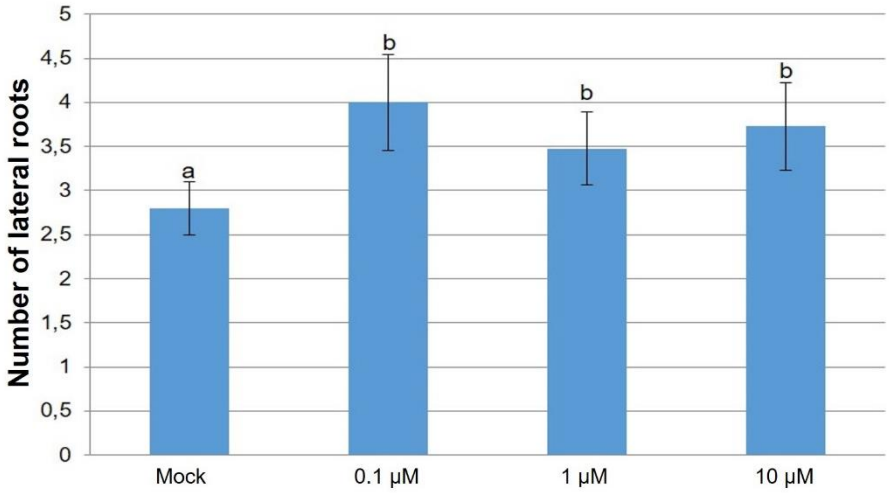


Figure 3.2: The effect of a mock treatments and three different concentrations of TMB on lateral root formation in *A. thaliana* Col-0 seedlings. Bars represent the mean of 50 replicates (n=50) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different ($P < 0.05$) from the control.

At all concentrations tested (0.1-10 µM), TMB slightly but significantly enhanced lateral rooting. The effect of TMB has never been investigated on *A. thaliana*, while most studies that have investigated this molecule mainly investigated its effect on germination (Light *et al*., 2010; Soós *et al*., 2012; Papenfus *et al*., 2015a; Papenfus *et al*., 2015b). It is often described as a direct antagonist to karrikins, because karrikins are known to stimulate germination (Ghebrehiwot *et al*., 2013a), but its possible interaction with strigolactone have never been described. In one of the germination studies, a range a TMB concentrations (1 µM-1 mM) inhibited the germination of *Lactuca sativa* (lettuce) seeds in a non-dose-dependent manner (Pošta *et al*., 2013), but clearly at higher concentrations than what was investigated in this study. This suggests that investigating the effect of TMB at concentrations higher than 10 µM would not have a greater effect on lateral root formation (Fig. 3.2). Also, TMB was obtained from the manufacturer in very low quantities (10 mg), which limited its uses for these experiments to concentrations of 10 µM and lower. The fact that TMB enhanced lateral rooting at the same concentration that strigolactones usually reduce lateral rooting does suggest that TMB may be acting here to inhibit strigolactone activity. In comparison, mock-treated *max4* seedlings (Fig. 3.1) also had more lateral roots than untreated (mock) Col-0 seedlings (Fig. 3.2). Without the synthesis of strigolactones, an increase in lateral root density is always observed (Kapulnik *et al*., 2011a).

3.3.2 Effect of different furanone-derivatives on *A. thaliana in vitro*

In order to provide further evidence that TMB inhibits strigolactone activity, *A. thaliana* Col-0 seedlings were treated not only with TMB (0.1 µM), but also GR24 (0.1 µM) and furanone (100 µM) (Fig. 3.3). Seedlings were grown for 10 d, as opposed to 7 d in the optimization experiments (Fig. 3.1, Fig. 3.2).

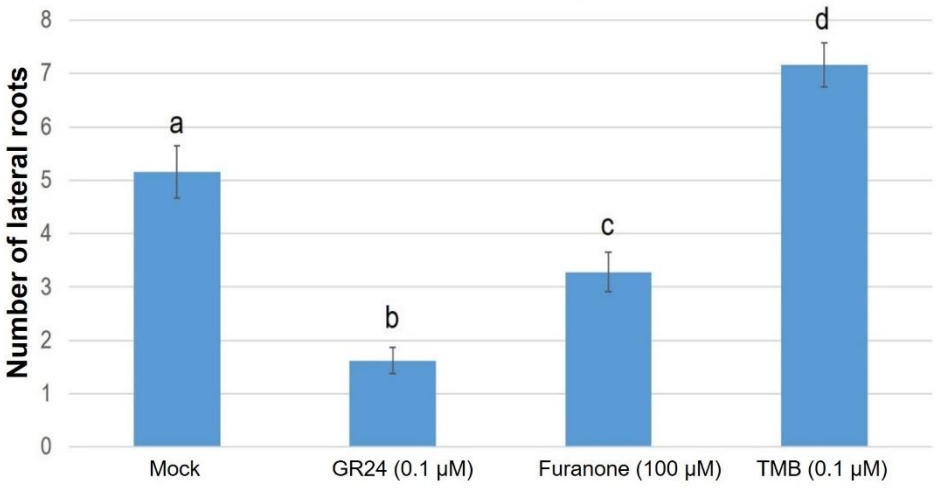


Figure 3.3: The effect of different furanone derivatives on lateral root formation in Col-0 *A. thaliana* seedlings. Bars represent the mean of 50 replicates (n=50) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different ($P < 0.05$) from the control

At a concentration of 0.1 µM, GR24 reduced lateral root formation, while furanone (100 µM)had the same effect but to a lesser extent and TMB (0.1 µM) slightly enhanced lateral root formation. In literature, GR24 is often used at the slightly higher concentration of 1 µM, while some research groups used it at 2.5 – 5 µM for a greater effect (Ruyter-Spira *et al*., 2011). In the *max4* phenotype, but not Col-0, a concentration of 0.01 µM GR24 significantly reduced lateral root formation (Kapulnik *et al*., 2011a). The fact that GR24 and TMB had the opposite effect on lateral root formation at the same concentration (0.1 µM) is interesting and suggests that TMB might be a competitive inhibitor of strigolactone signalling at the same concentration. In order to gain a deeper insight, a combined treatment of GR24 and TMB should also be tested on lateral root formation in the future. If TMB is in fact an inhibitor of strigolactone-activity, or more specifically an inhibitor of strigolactone signalling, it would be expected to either inhibit or ameliorate the reduction of lateral root formation by GR24. Unfortunately this experiment could not be performed due to time constraints.

3.3.3 Effect of furanone-derivatives on the expression of strigolactone-responsive genes

Using literature as reference, a set of genes that are known to be up- or down-regulated by GR24 (Mashiguchi *et al*., 2009) was selected. A wide variety of genes was tested, and only those that were clearly up-regulated after 25 cycles in a semi-quantitative manner were selected (Rossouw, 2015; unpublished data). Other genes were excluded due to inconsistent results and/or because their expression was unchanged in the presence of GR24 under the sqRT-PCR conditions tested.

The seedling tissue from the last growth experiment (Fig. 3.3) were flash frozen, RNA isolated, cDNA manufactured and the effect of GR24, furanone and TMB were tested on the expression of *At1g64380* (*AP2*), *At2g42620* (*F-box*), *At4g14560* (*STH7*) and *At4g14560* (*IAA1*) (Fig. 3.4). The expression of *At3g18780* (*Actin2*) was used as a reference gene.

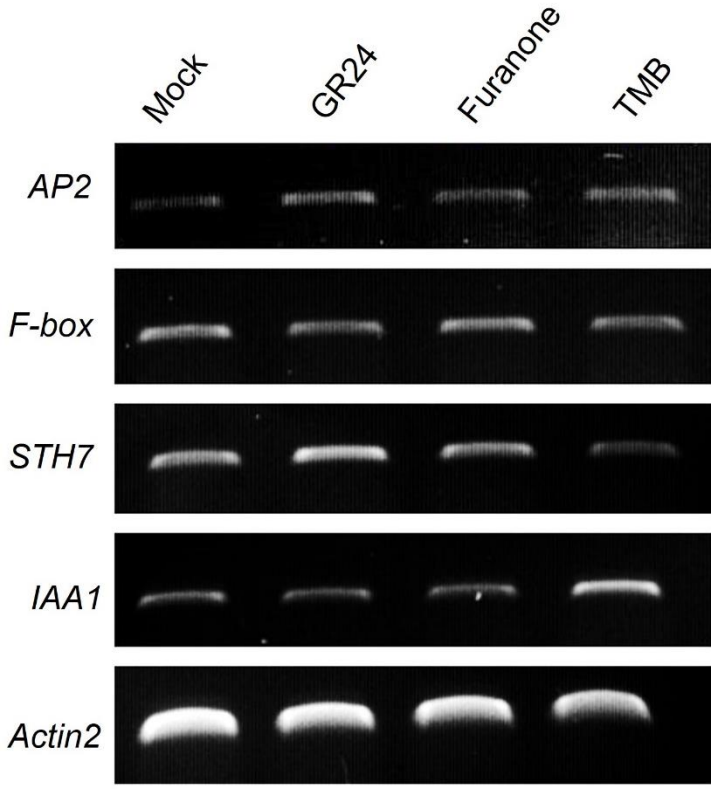


Figure 3.4: Gene expression of four different strigolactone-responsive genes in *A. thaliana* Col-0 seedlings after being treated with different furanone-derivatives and a control for 2 weeks. The expression of *Actin2* was used as a control.

During the development of the protocol, *AP2*, *F-box* and *STH7* were clearly up-regulated by GR24, while *IAA1* was down-regulated under the same conditions (Rossouw, 2015; unpublished data), which correlated with what was reported in literature (Mashiguchi *et al*.,

2009). In this experiment, of the genes supposed to be up-regulated by GR24, only *AP2* and *STH7* were very slightly up-regulated, while *F-box* seemed to remain unchanged under the same conditions. For *AP2* expression, all three treatments seemed to induce the expression of *AP2* in comparison to the mock treatments, which is unexpected if the assumption is that TMB has an antagonistic effect to strigolactones. For *F-box* expression, none of the treatments appeared to have any visible effect on expression levels. This, too, is the opposite to what was seen during the development of this protocol, because is believed to be antagonistic to any strigolactone-activity, based on its antagonistic effect on KAR_1 (Soós *et al*., 2012; Ghebrehiwot *et al*., 2013a) and the high degree of homology between the strigolactone receptor (D14) and the karrikin receptor (KAI2) (Kagiyama *et al*., 2013). For *STH7*, whilst GR24 induced expression as expected, TMB clearly down-regulated expression in comparison to the mock treatment, whilst furanone had no effect.

The expression of *STH7* is not only strongly induced by strigolactones, but also by KAR_1 (Smith and Li, 2014a; Waters *et al*., 2015). For *IAA1*, TMB induced its expression in comparison to the mock treatment, whilst a slight reduction in expression following treatment with GR24 was observed. The expression of *IAA1* is known to be repressed by GR24 (Waters *et al*., 2012b), but also by karrikins (Nelson *et al*., 2011). It is therefore clear that the expression of both *STH7* and *IAA1* is affected by both GR24 and karrikins in a similar way. For greater clarity on whether the effect of TMB is specific to strigolactones and not karrikins, *d14* and *kai2* mutants can be included in a future experiment to exclude either strigolactone or karrikin signalling. From what has been seen in literature and in this study, TMB might affect the expression of *STH7* and *IAA1* in a similar way. Karrikins can also be used in the same experiment as a further control.

The expression analysis thus offered some support for the possibility that TMB can inhibit strigolactone signalling, since TMB induced the opposite effect on expression compared with GR24 in two of the four genes tested (*STH7* and *IAA1*). However, unexpected results were observed for both the *AP2* and the F-box genes. No changes in expression of the F-box gene were observed for any of the treatments, whilst the expression of the *AP2* gene was slightly increased by all three treatments. If TMB is in fact an inhibitor of strigolactone activity, it would not have the same effect on a strigolactone-responsive gene as GR24. The F-box gene has previously been shown to be up-regulated by GR24 treatment (Mashiguchi *et al*., 2009). Before this experiment was conducted, the assumption was made that TMB would have an opposite effect on the expression of strigolactone(GR24)-responsive genes. It is, however, possible that TMB could still be an inhibitor of strigolactone activity without affecting the expression of every single strigolactone-responsive gene in an opposite way. Another

explanation could be that this may be due to the short half-life (approximately 7 d) of GR24 (Zwanenburg and Pospísil, 2013). A recent study even suggested that the half-life of GR24 is even shorter than previously described (Rasmussen *et al*., 2013). The sqRT-PCR analysis was optimised using 2 week old seedlings that were treated by immersion in a fresh GR24 solution for 6 h and then immediately tested for the expression of the genes (Rossouw, 2015; unpublished data). However, since this experiment lasted two weeks, it is possible that the physiological effect (decreased lateral root formation) remained, but that changes in gene expression could no longer be easily detected due to the breakdown of GR24 during the course of the experiment. Although the half-life of TMB has not been investigated, the assumption is that it is stable for long periods of time, because in nature, its antagonistic effect on germination is only alleviated after rainfall, which washes away the high concentration of TMB to increase the concentration of karrikins which leads to germination (Ghebrehiwot *et al*., 2013b). The implication is that TMB did not become inactive in experiments that run for as long as two weeks. In the future, this experiment can be repeated where the effect of TMB after medium term exposure can be investigated, for example after periods of 24h, 48h, 72h and one week, whilst repeating the experiment at 6 h and two weeks. Another synthetic analogue of strigolactones, Nijmegen-1, could also be included for verification of the obtained results.

From the first semi-quantitative RT-PCR analysis results (Fig. 3.4), not many conclusions could be made, with the exception that TMB repressed the expression of *STH7* and induced the expression of *IAA5*. Since it was initially determined that the up- or –down-regulating of these genes is optimal after 6 h (Rossouw, 2015; unpublished data), a similar experiment was conducted using ten 14-day old *A. thaliana* Col-0 seedlings per treatment to investigate the effects of GR24 (0.1 µM), furanone (100 µM), TMB (0.1 µM), GR24 (0.1 µM) in conjunction with furanone (100 µM) and GR24 (0.1 µM) in conjunction with TMB (0.1 µM) on the expression of the aforementioned genes after 6 h (Fig. 3.5).

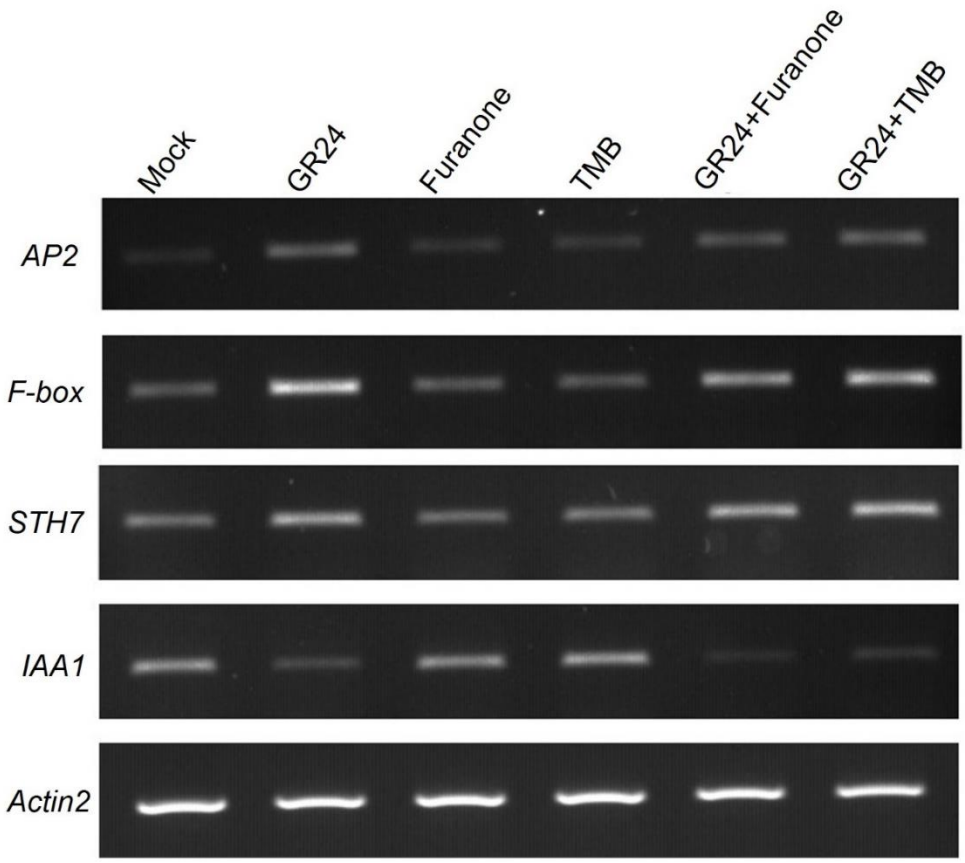


Figure 3.5: Gene expression of four different strigolactone-responsive genes in two-week old *A. thaliana* seedlings after being treated with different furanone-derivatives and a control for 6 h. The expression of *Actin2* is used as a control.

The expression of *AP2*, *F-box* and *STH7* was up-regulated in the presence of GR24 in comparison to the mock treatment, while the expression of *IAA1* was down-regulated. This correlated exactly with what has been seen before (Mashiguchi *et al*., 2009) and during the development of the protocol (Rossouw, 2015; unpublished data). Furanone, on its own, appeared to have no effect on the expression of any of the genes in comparison to the mock treatments. Treatment with TMB alone also had no effect on the expression of any of the gene in comparison to the mock treatment, except for a slight induction of *AP2*. It is possible that TMB acts over a longer time frame, and that the 6h treatment used here was too short to observe any effects of TMB on gene expression. For the combined treatments, GR24 in conjunction with furanone followed the same general pattern as for GR24 alone, except that the double treatment showed a greater reduction in the expression of *IAA1,* which suggests that furanone slightly enhances the effect of GR24. However, this enhancing effect was not observed at the expression of the other strigolactone-responsive genes. Seedlings treated with a combination of GR24 and TMB showed similar changes in gene expression for all genes as did seedlings treated with GR24 alone, suggesting that TMB was not able to inhibit the

effects of GR24 when the two compounds were applied in tandem. As stated earlier, it is also possible that TMB may only exert an effect over a longer term and so would not have affected gene expression, as GR24 did, at this point.

3.4. Concluding remarks and future prospects

This part of the study was aimed at determining whether TMB could act as a specific inhibitor of strigolactone signalling, using furanone and GR24 as indicators of strigolactone-activity. Parts of the results do suggest that TMB might have an antagonistic effect on strigolactone activity, at least in terms of lateral root formation and the expression of some strigolactone-responsive genes. The presence of TMB enhanced lateral root formation in comparison to a mock treatment, while GR24 reduced lateral root formation under the same conditions. Although the lateral root experiments suggest that TMB might have an inhibitory effect on strigolactones, it is also possible that this effect could be modulated through a completely different pathway. One such an example is that of auxins, whose role in lateral root formation have previously been investigated and partly characterized (Laskowski *et al*., 2006; Mayzlish-Gati *et al*., 2012).

From these results, it can be concluded that TMB could have some strigolactone inhibitory activity, but it does not seem to outcompete a similar amount of exogenous strigolactones. In a future experiment, different concentrations of GR24, as well as TMB, could be tested against one another. The racemic mixture, GR24, used in this study is a synthetic analogue of natural occurring strigolactone, but only one enantiomer is similar to naturally occurring strigolactones, which means that racemic GR24 may elicit responses that might not actually be elicited naturally by strigolactones (Mangnus *et al*., 1992; Rasmussen *et a*l., 2013; Scaffidi *et al*., 2014).

In other future experiments, it would be valuable to investigate the same treatments and combinations of treatments (Fig. 3.5) in on the growth of *A. thaliana* in more *in vitro* growth experiments. The use of furanone as a control in these experiments was valuable, since furanone has an identical ring structure to TMB, without the addition of two methyl groups. In the initial experiments, its effect on lateral root formation was only investigated on the *max4* phenotype, because *max4* is a strigolactone biosynthesis mutant. In the future, however, the wild-type should be included in order to make any definitive conclusions about the results. Also, because the aim of this study was to determine if TMB inhibits strigolactone signalling, a few mutant phenotypes should be incorporated in both the growth and sqRT-PCR analysis experiments. One or more biosynthetic mutants (*max4*, *max3* or *max1*) and the perception and signalling mutants, *max2* and *d14*, can be used to provide evidence that TMB inhibits

strigolactone signalling. A karrikin-perception mutant, *kai2*, could also be used as a control, as it would remove karrikin signalling from the equation. If TMB elicits an effect in the *kai2* mutant and wild-type, but not in *d14*, it would mean that it only involves strigolactone signalling. If a similar effect is seen in both *d14* and *kai2*, it would mean that it is modulated through KAI2, because KAI2 is known to modulate some strigolactone signalling, while D14 only modules strigolactone signalling (Scaffidi *et al*., 2014). If TMB enhances lateral root formation in *A. thaliana* Col-0 plants, but not in the *max2* and *d14* mutants, it would be further evidence that TMB is in fact an inhibitor of strigolactone signalling.

The effect of TMB on both of the enantiomers in GR24 should also be tested, separately. The use of natural strigolactones, strigol, would be best, but the half-life of these molecules are only 12-24 h (Akiyama *et al*., 2010; Chen *et al*., 2010; Rasmussen *et al*., 2013) and the results does suggest that the effect of TMB can only be seen after longer exposure. In the future, these experiments can be repeated where the effect of TMB after medium term exposure can be investigated. This could include measurements after 24h, 48h, 72h and one week, whilst also repeating the experiment at 6 h and two weeks. Both the growth experiments, along with gene expression analysis, possibly including more genes, should be performed at these time points. In conjunction with these experiments, *A. thaliana* plants grown *ex vitro* can also be treated with optimized concentrations of TMB to investigate whether a change in growth and shoot architecture may be observed.

Another proposed method is to perform similar experiments in plant species in which higher concentrations of strigolactones are produced. In conjunction with this, proteomic experiments could also be used. One aspect that can be focussed on is to investigate the presence of D14 proteins. If strigolactones are prevented from binding to the D14 receptor by TMB, more D14 proteins would remain stable, therefore increasing the concentration of D14 proteins in plants treated with TMB.

In conclusion, the results could be interpreted that TMB elicited a response suggestive of a strigolactone inhibitor. However, the results are very preliminary and further investigation, using these introductory findings, is needed to make a definitive conclusion of the effect of TMB on the growth of *A. thaliana*.

Chapter 4: Developing a strigolactone-responsive reporter construct

"There is no way around the hard work. Embrace it." – Roger Federer

4.1 Introduction

In the last ten years, strigolactones as a novel phytohormone has been further described and functions ranging from the root to the shoot linked to it. The biosynthetic and signalling pathways have also been partially elucidated (de Saint Germain *et al*., 2013), with general consensus among the scientific community that more components of this pathway are yet to be identified. Strigolactones, although a phytohormone like auxins and cytokinins, are bioactive at much lower concentrations than most other phytohormones (Sato *et al*., 2005). Strigolactones have been isolated and quantified in some plants, usually from root exudations in mycorrhizal species. This is because they are biosynthesized in the roots (Seto and Yamaguchi, 2014) and exuded from the organ where they play a vital role in the establishment of the symbiotic relationship with arbuscular mycorhizal fungi (Akiyama *et al*., 2005). However, in *A. thaliana*, the most widely-used plant model, the detection and quantification of strigolactones is challenging (Kohlen *et al*., 2011), with some research groups unable to isolate them (Goldwasser *et al*., 2008; Seto and Yamaguchi, 2014). Because *Arabidopsis* is a non-mycorrhizal plant, strigolactones are produced only as a phytohormone in this species and consequently at levels which are too low for consistent isolation or analysis. This poses a problem and major limitation to further the characterization of the strigolactone pathway in *A. thaliana*. The expression of *BRC1*, a TCP transcription factor known to inhibit the outgrowth of branches, is currently used by research groups as an indicator of increased strigolactone activity, as its expression is consistently up-regulated by strigolactones, mostly in the axillary buds in plants (Aguilar-Martínez *et al*., 2007; Mashiguchi *et al*., 2009; Finlayson *et al*., 2010; Domagalska and Leyser, 2011; Braun *et al*., 2012; González-Grandío *et al*., 2013; Bennett and Leyser, 2014). The up-regulation of *BRC1* is indicative of enhanced strigolactone-activity, while its down-regulation is indicative of the opposite (Aguilar-Martínez *et al*., 2007). Other genes have also been identified that are known to be up- or down-regulated in the presence of strigolactones (Mashiguchi *et al*., 2009; Mayzlish-Gati *et al*., 2010; Ito *et al*., 2015).

The use of reporter constructs in plant biology have been instrumental in determining the localization of specific transcripts at particular stages of development and under specific conditions. Similarly, a reporter construct has also been developed and used for the detection

of auxins in plants (Ulmasov *et al*., 1997). This reporter construct consists of a tandem repeat of the auxin-responsive TGTCTC DNA binding element, fused to the *GUS* reporter gene. A similar reporter construct that is activated only in the presence of strigolactones would be a valuable research tool for strigolactone-research groups, because of the challenges faced in isolating and quantifying this phytohormone. Unfortunately, there are no known strigolactone-responsive motifs. A starting point to create a strigolactone-responsive promoter construct would be to use the promoter region/s of strigolactone-responsive genes. Two obvious candidate genes for this purpose would be *BRC1*, involved in the regulation of shoot branching (Aguilar-Martínez *et al*., 2007; Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008; Martín-Trillo *et al*., 2011) and already widely used as a genetic marker for strigolactone signalling in Arabidopsis, and *PDR1*, the *Petunia hybrida* ABC transporter which has been shown to transport strigolactones *in planta* (Kretzschmar *et al*., 2012). For this part of the project, an attempt was thus made to create a strigolactone-responsive reporter construct that is only activated in the presence of strigolactones, using the 1500 bp regulatory region immediately upstream of *AtBRC1* (At3g18550).

4.2 Materials and Methods

4.2.1 *At3g18550* (*AtBRC1*) *in silico* promoter analysis

The promoter and intergenic sequence upstream directly upstream of the start codon from the coding sequence of *AtBRC1* (*At3g18550*) was retrieved from the Athena Pantheon online database (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl). The *Arabidopsis* Gene Regulatory Information Server (AGRIS) (http://arabidopsis.med.ohio-state.edu/), the Anthena Pantheon motif analysis tool and The Database of Arabidopsis Transcription Factors (DATF) (http://datf.cbi.pku.edu.cn/) were used to predict the occurrence of putative transcription factor binding sites in this 1500 bp upstream region.

4.2.2 Chemicals

All primers were designed using the Primer3 online tool (http://simgene.com/Primer3) and synthesized by Inqaba Biotechnical Industries (Pty) Ltd. Enzymes and vectors used for Gateway® cloning were purchased from Invitrogen™. All other chemicals were purchased from Sigma-Aldrich, unless explicitly otherwise stated.

4.2.3 Extraction of genomic DNA (gDNA) from *A. thaliana*

Wild-type *A. thaliana* Col-0 seedlings were grown on ½ strength MS media supplemented with 2% (m/v) sucrose and solidified with 9 $g.L^{-1}$ Phytoagar. Ten two week-old seedlings that had been grown under white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 µmoles $photons.m^{-2}.s^{-1}$, temperature of 25ºC and a 16h light/8h dark photoperiod were collected, flash frozen using liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. Approximately 500 mg of the powdered tissue was incubated with 5 mL CTAB buffer (2% [m/v] CTAB; 2% [m/v] PVP; 100 mM Tris-HCl, pH 8.0; 25 mM EDTA; 2M NaCl; 0.5 $g.L^{-1}$ spermidine) at 65ºC for 30 min. A chloroform:iso-amyl alcohol (24:1) extraction was performed and centrifuged for 15 min at 3000 x*g*. The DNA was precipitated by incubating 2.5 mL isopropanol with the aqueous phase at -20ºC for 60 min. After centrifugation for 5 min at 10 000 x*g*, the supernatant was removed and the pellet resuspended in 500 µL ddH_20. The gDNA was precipitated by adding 0.1 volume 5M NaCl and 2 volumes ice-cold absolute ethanol and incubating the tube overnight at -20ºC. The gDNA was spun down at 10 000 x*g* at 4ºC for 60 min and the pellet briefly washed with 70% ethanol and resuspended in 20 µL ddH_20. The integrity and concentration of the gDNA was determined by a NanoDrop Lite Spectrophotometer and gel electrophoresis on a 1% (m/v) agarose gel.

4.2.4 Preparation of chemically competent *E. coli*

Chemically competent *E. coli* were manufactured and transformed according to the method described by Sambrook *et al*. (1989). Aliquots of chemically component cells were flash frozen and stored at -80 ºC.

4.2.5 Preparation of electro competent *Agrobacterium tumefaciens*

A single colony of *Agrobacterium tumefaciens* GV3101 was inoculated into 100 mL LB medium (10 $g.L^{-1}$ bacterial peptone; 5 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ NaCl; 10 $g.L^{-1}$) containing 25 $\mu g.mL^{-1}$ rifampicin and 25 $\mu g.mL^{-1}$ gentamycin and grown for 18 h at 28 ºC with shaking at 200 rpm. Cells were harvested by centrifugation at 4000 x*g* for 10 min at 4 ºC and washed three times in 40 mL ice-cold ddH_20. Cells were again harvested by centrifugation at 4000 x*g* for 5 min at 4 ºC, resuspended in 40 mL ice-cold 10% glycerol, re-centrifuged and finally resuspended in 1 mL ice-cold 10% (v/v) glycerol. Aliquots of 40 µL were flash frozen by liquid nitrogen and stored at -80 ºC.

4.2.6 Promoter isolation and cloning into pMDC163.gb vector

Three different promoter sizes (1480, 990 and 500 bp fragments immediately upstream of *AtBRC1*) were amplified via PCR using *A. thaliana* gDNA as template. The components of the optimization PCR reactions were 1X GoTaq® Reaction Buffer, 0.3 µM forward primer, 0.3 µM reverse primer, 0.3 mM dNTPs, 4 $ng.\mu L^{-1}$ gDNA, 0.01 $U.\mu L^{-1}$ GoTaq® DNA Polymerase (Promega) and ddH_20 to a final volume of 50 µL. The components of the PCR reactions used for cloning were 1X Phusion HF Buffer, 0.5 µM forward primer, 0.5 µM reverse primer, 0.3 mM dNTPs, 4 $ng.\mu L^{-1}$ gDNA, 0.02 $U.\mu L^{-1}$ Phusion® DNA Polymerase (Thermo Fisher Scientific) and ddH20 to a final volume of 50 µL. The PCR cycle conditions in Table 4.1 and primer sequences in Table 4.2 were used for all PCR reactions.

The DNA fragments (1480bp, 990bp and 500bp fragments immediately upstream of *AtBRC1*) were initially PCR amplified using GoTaq® DNA Polymerase, in order to optimise the PCR conditions. Following successful amplification, the PCR reaction was repeated using Phusion® High-Fidelity DNA Polymerase.

Table 4.1: PCR cycle conditions for the amplification of the 1480, 990 and 500 bp fragments immediately upstream of *AtBRC1* for both GoTaq® and Phusion® reactions.

Step	Temperature		Duration		Cycles
	GoTaq	Phusion	GoTaq	Phusion	
Initial Denaturation	95ºC	98ºC	2 min	30 s	1
Denaturation	95ºC	98ºC	1 min	10 s	
Annealing	*		1 min		35
Elongation	72ºC		2 min		
Final extension	72ºC		5 min		1

*Annealing temperatures were determined according to the specific annealing temperature (T_m) for each primer set (Table 4.2) for the GoTaq® PCR reactions. The annealing temperatures for the Phusion® PCR reactions were 3ºC higher than the T_m indicated in Table 4.2.

Table 4.2: Primer sequences used to isolate the 1480, 990 and 500 bp fragments immediately upstream of *AtBRC1*, as well as the T7 and GUS forward and reverse primer sequences.

	Forward (5'-3')	Reverse (5'-3')	T_m
1480 bp	TGAAGTCATAAGAATTACACCAAAA	GCCTTTTTAGGGGTTTTTGAA	55 ºC
990 bp	AAGATCTGTTTATTTAAAGTGAAATGT	GCCTTTTTAGGGGTTTTTGAA	56 ºC
500 bp	CCCCCATAATGATCTCTTTGC	GCCTTTTTAGGGGTTTTTGAA	55 ºC
T7	TAATACGACTCACTATAGGG	-	45 ºC
GUS	AGTGGATCCATGTTACGTCCTG	TGCGAGCTCTCATTGTTTGCCTC	56 ºC

Amplified fragments were isolated after the PCR reaction using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's recommendations. To prepare the fragments for cloning into the pCR®8/GW/TOPO® vector (Invitrogen), adenine-overhangs were attached by mixing adding 5 µL of the purified PCR product, 2 µL deoxyadenosine triphosphate (dATP), 2 µL 5X Colorless GoTaq® Reaction Buffer and 1 µL GoTaq® DNA Polymerase to a PCR tube and incubating at 72ºC for 20 min. The fragments were again purified using the Wizard® SV Gel and PCR Clean-Up System before cloning the PCR product into the pCR®8/GW/TOPO® vector. The purified A-tailed PCR product was then ligated into the pCR®8/GW/TOPO® vector, according to manufacturer's protocol.

Two microliters of the ligation reaction was added to 40 µL One Shot® TOP10 *Escherichia coli* competent cells (Section 4.2.4) and incubated at a temperature of 4ºC for 20 min before the

cells were heat shocked at 42ºC for 45 s. The vial was incubated again at 4ºC for another 2 min. One millilitre Super Optimal Broth with Catabolite repression (SOC) medium (20 $g.L^{-1}$ bacterial peptone; 5 $g.L^{-1}$ yeast extract; 0.6 $g.L^{-1}$ NaCl; 0.5 $g.L^{-1}$ KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) was added to the vial and the culture incubated at 37ºC with shaking at 200 rpm. The culture was centrifuged for 15 s at maximum speed in a desktop centrifuge, resuspended in 150 µL fresh SOC and spread on Luria Broth (LB) plates (10 $g.L^{-1}$ bacterial peptone; 5 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ NaCl; 10 $g.L^{-1}$ bacteriological agar) supplemented with 50 $\mu g.mL^{-1}$ spectinomycin. The plates were incubated at 37ºC for 18 h until colonies appeared.

Three colonies were selected and inoculated into 2mL liquid LB medium. The cultures were incubated at 37ºC with shaking at 200 rpm for 18 hours. Pure plasmid DNA was isolated from the 2 mL cultures using the Thermo Scientific GeneJET Plasmid Miniprep Kit, according to manufacturer's protocol. Plasmid DNA concentration was determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific). To determine the orientation of the fragment inserted into the pCR®8/GW/TOPO® vector, the T7 vector-specific forward primer and promoter-specific reverse primer (Table 4.2) were used in the same PCR conditions for GoTaq®DNA Polymerase as previously described (Table 4.1). The PCR products were visualised via electrophoresis on a 1% (m/v) agarose gel.

Seven microliters (150 ng) of the confirmed pCR®8/GW/TOPO® vector with the promoter inserted in the correct orientation was then added to 1 µL (150 ng) of the pMDC163.gb binary vector (Addendum C) and 2 µL of the LR Clonase™ II enzyme and incubated at room temperature for 1 h. One microliter of proteinase K solution was added and the reaction incubated at 37ºC for 10 min.

A 1 µL aliquot of the LR Clonase reaction was used to transform chemically competent One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Thermo Fisher Scientific) as described previously. Transformed cells were plated out onto selective LB plates containing 50 $\mu g \cdot mL^{-1}$ kanamycin. After incubating at 37ºC for 18 h, bacterial colonies were selected and incubated at 37ºC in liquid LB containing 50 $\mu g \cdot mL^{-1}$ kanamycin. The purified final construct was isolated using the Thermo Scientific GeneJET Plasmid Miniprep Kit. To determine the success of the LR Clonase reaction and confirm the construct, two PCR reactions were set up using GUS-specific and promoter-specific primers (Table 4.2).

4.2.7 Transforming *A. tumeficiens* GV3101 by electroporation

A total of 1 µg of the confirmed construct was added to 40 µL of electrocompetent *A. tumeficiens* GV3101 cells (Section 4.2.5) and gently mixed in by pipetting up and down. The

cells were incubated on ice (4ºC) for 20 min. The mixture was then transferred to a Gene Pulser®/MicroPulser™ Electroporation Cuvettes (0.1 cm gap) (Bio-Rad) and electroshocked using the *Agrobacterium* pre-set protocol on the Gene Pulser Xcell™ Electroporation System (Bio-Rad). Immediately after the shock pulse had been applied, 1 mL liquid SOC medium was added directly to the mixture, which was then transferred to a 1.5 mL microfuge tube.

After 1.5 h of shaking at 200 rpm and a temperature of 28ºC, different volumes of the small cell culture were plated out on YEP medium (10 $g.L^{-1}$ bacterial peptone; 10 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ NaCl) solidified with 12 $g.L^{-1}$ bacteriological agar and supplemented with 10 $\mu g.mL^{-1}$ rifampicin, 30 $\mu g.mL^{-1}$ gentamicin and 50 $\mu g.mL^{-1}$ kanamycin. Plates were incubated at 28ºC for 48 h. To confirm the presence of the pMDC163 expression vector containing the promoter sequences, individual bacterial colonies visible on the plates were selected and inoculated into fresh liquid YEP medium. After 12 h of growth, a total of 500 µL of the cell culture was centrifuged at 10 000 x*g* for 20 s and resuspended in ddH_2O before being boiled at 100ºC for 5 min. A 5 µL aliquot of the boiled suspension was used in a PCR reaction using promoter gene-specific primers and/or GUS-specific primers. Where the PCR procedure described above failed, an alkaline plasmid isolation was performed and the resulting plasmid(s) transformed into chemically competent *E. coli* DH5α. Standard colony PCR reactions, where the PCR reaction was prepared without the addition of a DNA template but rather adding the *E. coli* cells from the colony directly to the reaction using a pipette tip, were then performed on a few the resulting colonies.

4.2.8 *Agrobacterium*-mediated transformation of *A. thaliana* Col-0, *max4* and *max2*

Arabidopsis thaliana Col-0, *max4* and *max2* plants were grown in a 10h light/ 14h dark growth environment with a light intensity of 90 $\mu E.ms^{-2}.s^{-1}$, temperature of 25 ºC and relative humidity of 90% in peat disks (Jiffy-7® bags, Jiffy Products Int.). Primary inflorescences were clipped of at the base 7 days before transformation, to allow for the production of fresh, young inflorescences. Confirmed *A. tumefaciens* colonies containing the desired constructs were inoculated into 50 mL YEP medium containing 10 $\mu g.mL^{-1}$ rifampicin, 30 $\mu g.mL^{-1}$ gentamicin and 50 $\mu g.mL^{-1}$ kanamycin and incubated at 28ºC for 48 h. This starter culture was used to inoculate 500 mL YEP medium containing the aforementioned antibiotics. This culture was incubated at 28ºC with shaking at 200 rpm for 24 h. The cell culture was centrifuged at 4000 x*g* for 10 min at room temperature and resuspended in 500 mL 5% (m/v) sucrose. Silwet- L-77 was added to the solution to a concentration of 0.02% (v/v). Plants were inverted and dipped into the *Agrobacterium*-sucrose cell suspension for 10 s, before being wrapped in plastic film and placed on their side in the dark at ambient temperatures for 18 h. The plastic

film was then removed and the plants were transferred back to the growth room. This entire procedure was repeated one week later to improve the transformation efficiency.

As an alternative to floral dipping, individual buds were treated directly with the *Agrobacterium* culture. The transformed *A. tumefaciens* were grown as described in Section 4.2.8, but 1.5 mL of the starter culture was centrifuged for 20 s at 10 000 x*g* and resuspended in 2 mL 5% (m/v) sucrose. Silwet-L77 was added to a concentration of 0.02 % (v/v). Each closed floral bud was treated with 10 µl of this suspension. Plants were placed in a closed box for 18 h before being transferred back to the growth room. This procedure was repeated one week later to improve the transformation efficiency.

4.2.9 Transformant selection and confirmation

Seeds were collected after seed maturation, surface decontaminated (Section 3.2.2) and sown on ½ strength MS media supplemented with 17.5 µg.mL^{-1} hygromycin B. After two weeks, primary transformants were transferred to peat disks. Owing to the fact that all seeds germinated in the presence of hygromycin B, only seedlings that developed a complete root system and true leaves were selected. Transformants were grown until the emergence of primary bolts, which were clipped to stimulate inflorescence growth to produce more seed. The T_2 seed was collected and used for further analysis. T_2 seeds were also selected on ½ MS media supplemented with 2% (m/v) sucrose and 17.5 µg.mL^{-1} hygromycin B. Seedlings were grown for two weeks under white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 µmoles photons.m^{-2}.s^{-1}, temperature of 25ºC and a 16h light/8h dark day period before either being transplanted to a peat disk or analysed by GUS staining.

4.2.10 Treatments of T_2 transformants

The two-week old seedlings were placed on sterile filter-paper soaked by a solution containing GR24 (0.1 µM), Nijmegen-1 (0.1 µM, also a synthetic strigolactone analogue), 6-benzylaminopurine (BAP, 0.5 µM) or 1-naphthaleneacetic (NAA, 0.5 µM) or only dd$H_2$0 (mock) for 6 h. Seedling tissue was either analysed by GUS staining or blotted dry and flash frozen for semi-quantitative RT-PCR analysis.

4.2.11 Histochemical GUS staining of T_2 transformants

In vitro grown seedlings were placed in ice-cold 90% acetone and fixed at room temperature for 25 min. The acetone was removed and the seedlings submerged in GUS staining buffer (50 mM $NaPO_4$ buffer, pH 7.2; 0.2% [v/v] Triton X-100; 2 mM Ferricyanide; 2 mM ferrocyanide) without 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) and incubated at room temperature for 25 min. The $NAPO_4$ buffer (200 mL) was prepared by mixing 28 mL 0.2 M NaH_2PO_4, 72 mL 0.2 M Na_2HPO_4 and 100 mL ddH_20. The GUS staining solution was removed and replaced with the same staining solution containing 2 mM X-Gluc and incubated at 37ºC for 18 h. The staining buffer was removed and the samples incubated successively in 20%, 35% and 50% (v/v) ethanol at room temperature for 30 min. The samples were incubated in FAA solution (50% [v/v] ethanol; 3.7% [v/v] formaldehyde; 5% [v/v] acetic acid) for 30 min at room temperature to fix the tissue. The FAA solution was removed and 70% ethanol added to the samples. Plants grown on peat disks were stained in a similar manner to that described above, except that the plants were subjected to a constant 160 Torr vacuum for 20 min at room temperature.

4.2.12 Semi-quantitative RT-PCR analysis

Seedling tissue was flash frozen using liquid nitrogen. Total RNA isolation, DNAse treatment and cDNA synthesis were performed as described in Section 3.2.7. The primer sequences in Table 4.3 were used to perform semi-quantitative RT-PCR analysis (Section 3.2.6). The GUS primer sequences were as described by Ditt *et al*. (2001).

Table 4.3: Primer sequences used to perform semi-quantitative RT-PCR analysis on T_2 transformants treated with GR24, Nijmegen-1, NAA and BAP.

	Forward (5'-3')	Reverse (5'-3')	T_m
GUS	TATCAGCGCGAAGTCTTTATACC	CAGTTGCAACCACCTGTTGAT	56ºC
BRC1	ATTCCTCACAACCATTGCTC	TCAAATCCAAGCATGTCTTG	56ºC
Actin2	ATGGCTGAGGCTGATGATAT	CCATCACCAGAATCCAGCAC	58ºC

4.3 Results and Discussion

4.3.1 *At3g18550* promoter analysis

To start analysing the intergenic region of *AtBRC1* (At3g18550) in order to identify a possible promoter region or regions of interest, The Arabidopsis Information Resource (TAIR) database was consulted. According to this database, the *AtBRC1* gene is situated on chromosome 3, upstream of At3g18535 and the intergenic region between these coding sequences is 6.3 kb (Fig. 4.1)

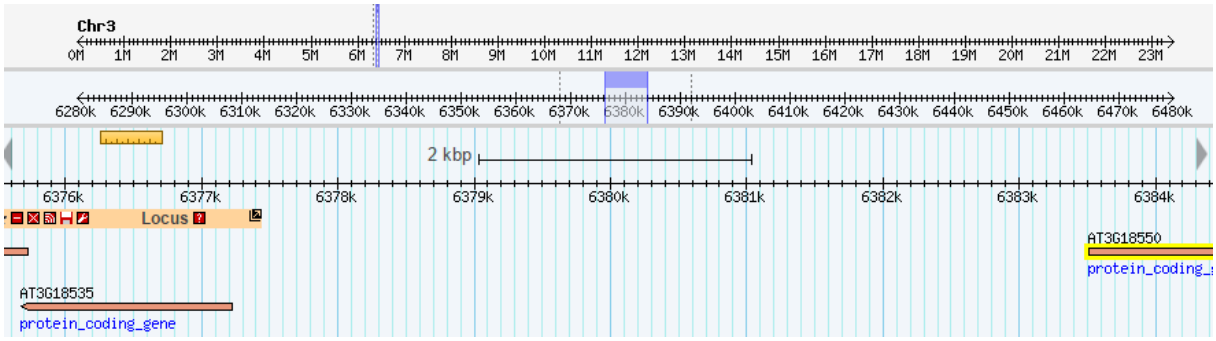


Figure 4.1: Location of At3g18550 on chromosome 3, obtained from The Arabidopsis Information Resource (TAIR), tairvm17.tacc.utexas.edu/cgi-bin/gb2/gbrowse/arabidopsis/?name=AT3G18550, on www.arabidopsis.org, Aug 22, 2015.

A promoter can be defined as a DNA region that directs the transcription of a downstream unit (Sandelin *et al*., 2007; Juven-Gershon and Hsu, 2008). In one study, it was determined that the average effective promoter length in *Arabidopsis* is 500 bp (Korkuc *et al*., 2014). It was therefore decided to start by isolating the 1500 bp upstream of the *AtBCR1* starting codon to ensure that all promoter elements that might influenced its expression are included. Since the first genome sequencing project was completed and researchers started to characterize genes, the main focus has been on protein coding sequences, which means that intergenic regions remain largely uncharacterized. There is also no literature available describing any strigolactone-responsive promoter binding element. This gap between the signalling components and the physiological effects made it almost impossible to select the size of the upstream region of *AtBRC1* that might contain a binding site for a transcriptional factor downstream of the strigolactone signalling pathway. Any conclusions based on the *in silico* analysis of the 1500 bp intergenic region would have been purely speculative. It was therefore estimated that the 1500 bp region directly upstream of *AtBRC1* should be an adequate starting point. Using databases of promoter elements (Section 4.2.1), putative motifs of the 1500 bp region directly upstream op *AtBRC1* were mapped (Fig. 4.2; Addendum A), and each motif defined, described and described according to literature (Addendum B).

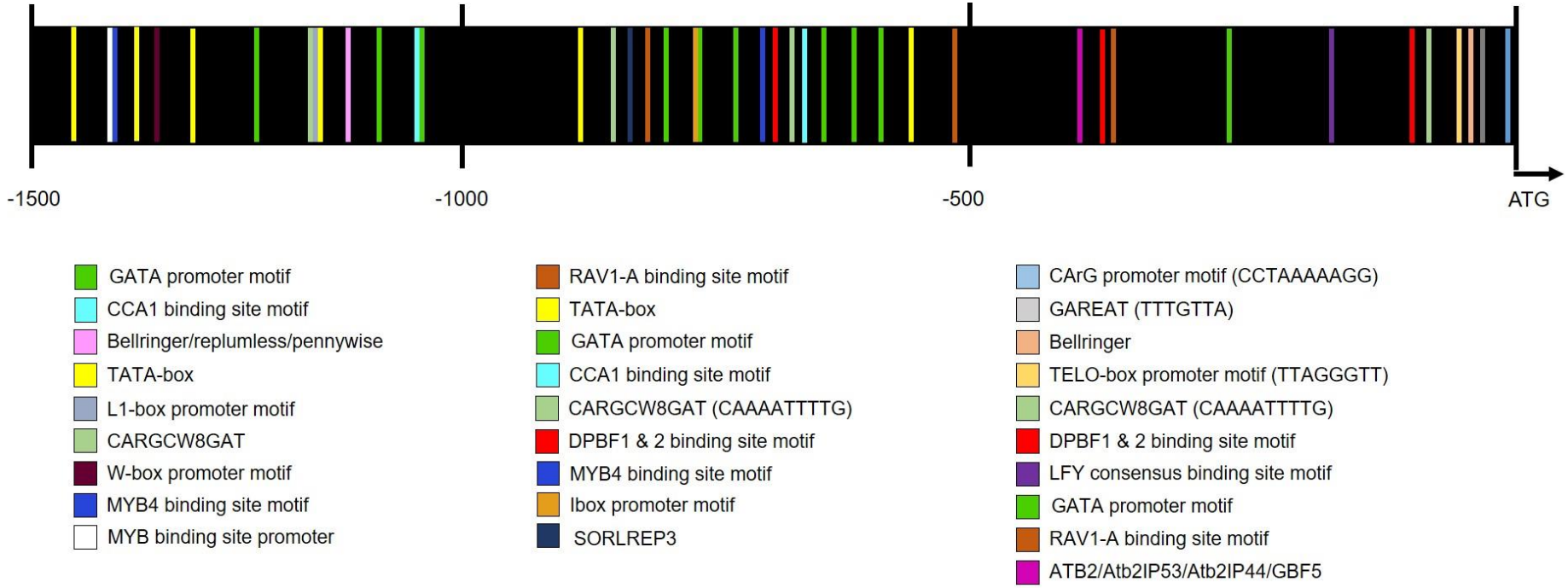


Figure 4.2: The 1500 bp region upstream of At3g18550 with putative promoter binding elements obtained from three different transcription factor databases.

A wide variety of putative promoter binding sites were discovered and mapped, none of which have been experimentally verified within this promoter region. Most of the promoter binding sites only occur once, although others occur in multiple copies (Table 4.4).

The most recent component of strigolactone-signalling that has been characterized is SMAX- and SMAX-like proteins (SMXLs), with the degradation of SMAX1 (D53) acting downstream of strigolactone perception as a prerequisite for strigolactone signalling (Zhou *et al*., 2013). Currently it has only shown to be protein-binding (Stanga *et al*., 2013b; Rameau *et al*., 2015; Wang *et al*., 2015), which means that it is most likely not directly involved in binding to the promoters of strigolactone-responsive genes. However, this does not preclude the possibility that it exerts some degree of control by binding to other transcription factors, activators or repressors. From this *in silico* analysis, no motif/s or specific region was identified that could be important for the development of a strigolactone-responsive reporter construct.

Table 4.4: Most frequently occurring putative promoter binding elements in the 1500 bp immediately upstream of *At3g18550*.

Promoter element	Copies	Function
GATA promoter motif	10	Light-responsive element (LRE)
TATA-gbox	6	Initiates transcription through RNA Pol recognition
DPBF1 & 2 binding site motif	3	Binding site for the putative transcription factor family bZIP
CARGCW8GAT	3	AGAMOUS-like 15 binding site
RAV1-A binding site motif	3	Binding site for RAV1
MYB4 binding site motif	2	The MYB4 protein is responsive to jasmonic- and salicylic-acid.

Other regions of interest might be those containing binding sites for proteins involved in the signalling pathways of other phytohormones. Strigolactones have only been defined as phytohormones since 2008 (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). The complexity of the interactions between strigolactones and other phytohormones (Chapter 2, Literature Review) means that motifs that have previously been identified are downstream targets for the signalling pathway of other phytohormones might in fact be strigolactone-responsive motifs as well. During the discovery and characterization of phytohormones, functions that were connected to one phytohormone, were often reassigned to another. For the same reason, it is also possible that some of these previously identified motifs might also be strigolactone-responsive.

The GATA promoter motif, which is a light responsive element (LRE), occurs 10 times in the 1500 bp region directly upstream of *AtBRC1*, which was to be expected owing to the fact that *AtBRC1* is also controlled by light intensity and wavelength, specifically up-regulated by shade (decrease in red:far-red light) (Martín-Trillo *et a*l., 2011; González-Grandío *et al*., 2013). Shade, which could be due to neighbouring plants, induces the expression of *BRC1*, which leads to the repression of bud outgrowth and branching and the plant growing taller in order to avoid the shade. However, it is unclear whether the LREs in the 1500 bp promoter region is responsive to low R:FR or high R:FR light. With the aim of the project in mind, to develop a reporter construct that is only activated in the presence of strigolactones, it might be essential to remove these LREs to ensure that the construct only responds in the presence of strigolactones. The decision to determine the length of the region upstream of *AtBRC1* to use was difficult, due to no prior literature or knowledge on strigolactone-responsive promoter elements. The *in silico* analysis of the region also did not provide any clear insight. In fact, the presence of motifs that are related to other phytohormones (gibberellic acid, jasmonic acid, salicylic acid, and ethylene) and light-responses already highlighted the complexity of the promoter region.

To enable us to identify a strigolactone-responsive promoter region, it was decided to start at approximately 1500 bp and shorten the promoter in fragments of 500 bp in order to remove any promoter binding elements that might respond to any biochemical process other than strigolactone signalling. This incremental deletion would also help to narrow down the search areas for possible strigolactone-responsive motifs, based on potential differences in expression driven by these different constructs.

.4.3.2 Selection of transgenic lines

Following *Agrobacterium* transformation with the various reporter constructs (Section 4.2.6), T_1 plants were selected (Section 4.2.9) and grown until seed set. Seeds were collected by Cedrick Matthys at the Flanders Institute for Biotechnology (VIB), Belgium. The seeds of the several individual transgenic lines (Table 4.5) obtained were planted out in soil and grown until the production of T_2 seeds.

Table 4. 5*:* The number of individual transgenic lines obtained after every individual transformation event.

	Col-0	*max4*	*max2*
	Number of transformants		
p*BRC1*(-1480):*GUS*	15	15	11
p*BRC1*(-990):*GUS*	15	3	15
p*BRC1*(-500):*GUS*	-	-	-

No transformants were obtained for the p*BRC1*(-500):*GUS* construct in any of the genotypes. More transformations were performed after retransforming *A. tumefaciens* and confirming the presence of the expression vector via colony PCR. At the time, the Silwet L-77 used as a surfactant for the floral dip transformation became comprised (turned a cloudy brown colour). Other surfactants such as tween20 and glycerol were used as alternatives, but no transformants were obtained. Once a fresh stock of Silwet-L77 was obtained, floral bud inoculation as an alternative method was used for transformation. To date, no transformants have been obtained for this construct.

One individual line each of p*BRC1*(-1480):*GUS:*Col-0*,* p*BRC1*(-1480):*GUS:max4*, p*BRC1*(-1480):*GUS:max2*, p*BRC1*(-990):*GUS*:Col-0*,* p*BRC1*(-990):*GUS*:*max4,* p*BRC1*(-990):*GUS*:*max2* were used for further analysis. A second line for each was also used for certain downstream experiments.

4.3.3 Transgenics response to GR24 *in vitro*

As a pilot experiment, six seedlings of six different transgenic lines were grown on ½ strength MS media supplemented with 2 % (m/v) sucrose and 17.5 µg.mL^{-1} hygromycin B. Three were left untreated, while the other three were treated with 0.1 µM GR24 for 6 hours, before GUS staining was performed (Fig. 4.3)

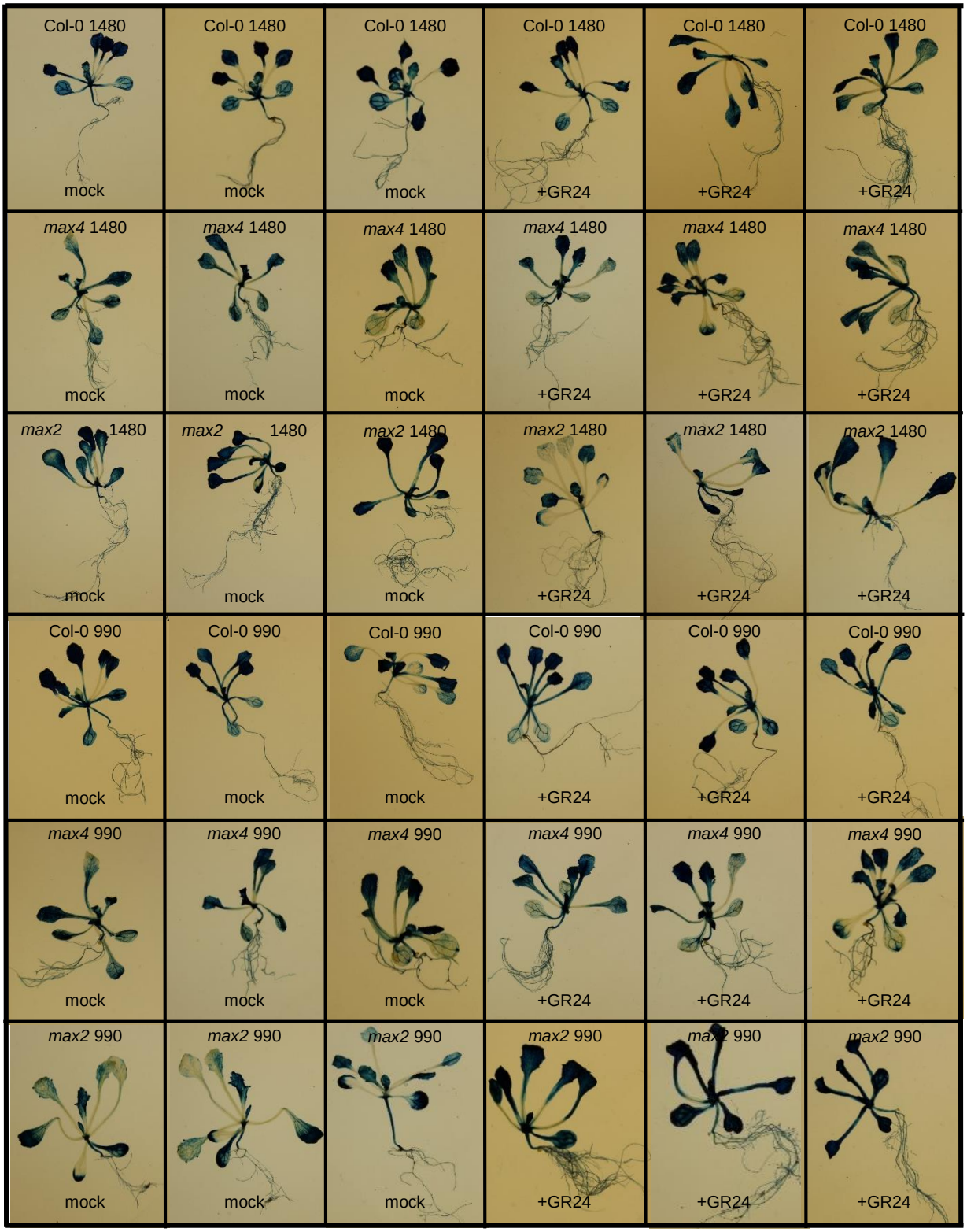


Figure 4. 3: The presence and location of GUS in p*BRC1*(-1480):GUS:Col-0, p*BRC1*(-1480):GUS:*max4*, p*BRC1*(-1480):GUS:*max2*, p*BRC1*(-990):GUS:Col-0, p*BRC1*(-990):GUS:*max4*, p*BRC1*(-990):GUS:*max2* seedlings untreated (mock) versus treated with 0.1 µM GR24 for 6 h.

Across all of the lines tested in this experiment, GUS expression was high and not localized to a specific part of the seedling. This high expression of p*BRC1*(-1480):*GUS* and p*BRC1*(-990):*GUS* was unexpected, because *AtBRC1* is not known for its high expression in *A. thaliana*, especially in seedlings (Mashiguchi *et al*., 2009). According to The Arabidopsis Information Resource (TAIR), [https://www.arabidopsis.org/servlets/TairObject?id=37813&type=locus], on www.arabidopsis.org, Nov 24, 2015, the expression of *BRC1* is almost exclusively restricted to the axillary bud. It should be noted that GUS expression was not quantitatively measured and the term 'high expression' only refers to the clear presence and ubiquitous presence of the GUS protein in the T2 transformants grown *in vitro* (Fig. 4.3).

The genotype background (Col-0, *max4* and *max2*) of the transformants played no role in expression of the construct, with only a slight deviation seen in the lines p*BRC1*(-1480):*GUS:max2* and p*BRC1*(-990):*GUS*:*max2*. A slight induction of *GUS* expression was observed in p*BRC1*(-990):*GUS*:*max2* seedlings treated with GR24. With the *max2* mutant being a strigolactone-perception mutant, it was expected that GUS expression would be less than in transformants with a Col-0 background in the absence of GR24, but this was not the case. With the *max2* mutant being a strigolactone-signalling mutant, it is known that strigolactones are still synthesized, but not perceived. There is also no known feedback perception, which means that the strigolactone concentration will only increase in the *max2* mutant (Foo *et al*., 2005; Hayward *et al*., 2009). The high expression of GUS could also have been induced by the growth conditions in an *in vitro* environment, but because the 1500 bp region upstream of *AtBRC1* is uncharacterized, any of the promoter elements could be responsible for the high expression of GUS.

According to literature, *AtBRC1* is a downstream component of the strigolactone signalling pathway (Zhou *et al*., 2013), which means that its expression in a signalling mutant such as *max2* would be expected to be unchanged in the presence of GR24. This contradicts to what is seen in this result and highlights the fact that even the 1480 bp immediately upstream of *AtBRC1* is already a shortened version of the full promoter region of *AtBRC1*. The high expression of GUS in the *max2* background could mean that the main strigolactone-dependant motifs lie further upstream of -1480 bp or that the *in vitro* growth condition stimulated higher expression, or a combination of both. The fact that such high expression was observed without the addition of GR24, in all three backgrounds, means that both reporter-constructs are highly responsive to other expression cues in the plant, but also that possible silencer motifs lie further upstream of -1480, resulting in major deregulation of the promoter. If such a major regulatory motif lies so far upstream of the starting codon of *AtBRC1*, it is possible be

that motifs responsible for the stimulation of *AtBRC1* by strigolactones also lies further upstream. At this stage, the deregulation of the *AtBRC1* has been achieved, but the presence of many other promoter binding elements resulted in a highly responsive reporter-construct that seems unchanged in the presence of GR24.

From the high expression of GUS by these transformants, it is clear that the expression of *BRC1* is already de-regulated by shortening the promoter region to 1480 bp and 990 bp. This shortening of the promoter seems to have removed binding sites for transcriptions factors that would repress expression. Further investigation of the intergenic region upstream of the -1500 bp may be required to reveal the presence of binding sites for SMX-like proteins, which are known to repress *AtBRC1* expression (Wang *et al*., 2015). Very recently, SMXLs in *Arabidopsis* have shown to play an opposite role to D53-like proteins, with SMXLs acting with the TPR2 protein to repress transcription and allowing bud outgrowth, while D53-like proteins plays a role in activating transcription to inhibit bud outgrowth (Wang *et al*., 2015). With this information, the DNA region of interest would have been a DNA binding site that is recognized by a D53-like protein/transcription factor, but, as mentioned, this information is still unknown. It is also unknown whether D53-like proteins are DNA-binding, with the only evidence being that they are protein binding (Zhou *et al*., 2013). Therefore, the proteins/transcription factors/mechanisms downstream of proteosome-mediated degradation of D53 is yet to be identified. When this project was started, the existence of D53 / SMXLs was known, but detail of its downstream activity remained largely unknown (Zhou *et al*., 2013). The results thus far suggest that potential binding site(s) or regions of interest downstream of SMXL degradation might have been removed already and therefore lie upstream of -1480 bp of *AtBRC1*.

4.3.4 GUS expression of plants grown in peat disks

With the high expression of GUS and redundancy among all the genotype backgrounds in seedlings grown *in vitro*, it was decided to include only transformants with a Col-0 background in all the downstream experiments. In order to analyse the expression of *GUS* in older p*BRC1*(-1480):*GUS*:Col-0 and p*BRC1*(-990):*GUS*:Col-0 plants grown under different conditions, seedlings were sown and selected *in vitro* on hygromycin B and transferred to peat disks after two weeks. After growing for another two weeks on the peat disks in a short daylength environment (10 h light: 14 h dark), GUS staining analysis was performed on the untreated plants (Fig. 4.4).

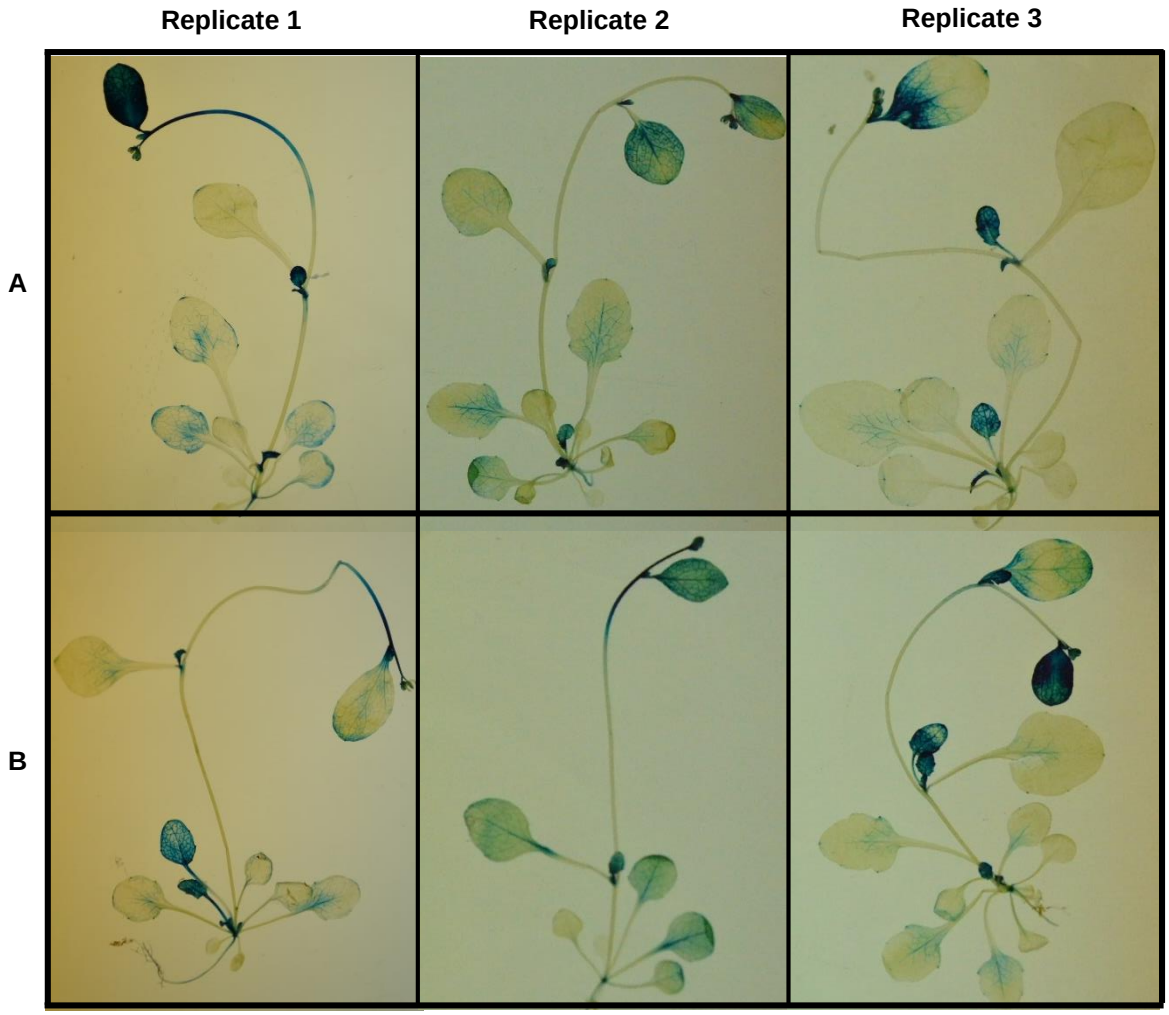


Figure 4.4: Native expression of *GUS* in of three four-week old **(A)** p*BRC1*(-1480):*GUS*:Col-0 and **(B)** p*BRC1*(-990):*GUS*:Col-0 transformants grown in peat disks.

For the two different constructs, GUS expression was almost indistinguishable between the two different promoter sizes (1480 bp and 990 bp). However, the intensity of the GUS expression was considerably less than was observed in the *in vitro* grown plants, suggesting that the *in vitro* environment was at least partially responsible for the intense GUS expression that was observed in that experiment. The highest expression of GUS in this experiment was in the axillary buds and the shoot meristem region. Young rosette leaves were also darkly stained and therefore sites of high GUS expression. Some GUS staining could also be seen in rosette leaves, flowers and cauline leaves. The fact that literature has reported only low levels of *BRC1* expression in cauline leaves (Mashiguchi *et al*., 2009) also points towards at least partial de-regulation of the control of spatial expression by the *BRC1* promoter in these shortened promoter constructs.

Because it was difficult to separate the root system from the wet, expanded peat disk and these had to be left out of the analysis. Based on the GUS staining results of *in vitro* grown two-week old seedlings (Fig. 4.3), it is at least possible that the construct might have been expressed in the roots in this experiment as well. With the major differences between *in vitro* growth conditions and the *ex vitro* growth conditions, a difference in the staining pattern was anticipated. Plants grown *in vitro* (Fig. 4.3) and GUS stained directly thereafter, were subjected to a longer day-length and lower light intensity (50 $\mu mol.m^{-2}.s^{-1}$) in comparison to plants grown in peat disks (90 $\mu mol.m^{-2}.s^{-1}$). This might be explained by the presence of the LREs previously described, but it is unknown whether these motifs are responsive to a certain light intensity and/or wave length. The higher light intensity might be responsible for the significantly lower GUS expression in this experiment, as *BRC1* expression is usually stimulated by low R:FR light and low intensity light and repressed in opposite light conditions (González-Grandío *et al*., 2013). The 1480 bp reporter construct contains 10 LREs, while the 990 bp reporter construct contains 7. In these 990 bp transformants, there was no clear indication of reduced GUS expression compared with those containing the 1480 bp construct. The 500 bp reporter-construct, of which no transformants were obtained, contains only one LRE and it would suggest that an increase in GUS expression would have been seen.

Table 4.6: Relative levels of expression of *AtBRC1* in mature *A. thaliana* plants (Mashiguchi *et al*., 2009).

High	Low	None
Axillary bud meristem	Cauline leaves	Seeds
Flowers	Whole seedlings	
Rosette leaves	Stem	
	Roots	

In order to observe GUS expression in full grown plants, T_2 transformants were selected on hygromycin B as previously described and transferred to expanded peat disks after two weeks. Four weeks after the transfer, GUS staining were performed on three untreated plants from the p*BRC1*(-1480):*GUS*:Col-0 and p*BRC1*(-990):*GUS*:Col-0 lines (Fig. 4.5).

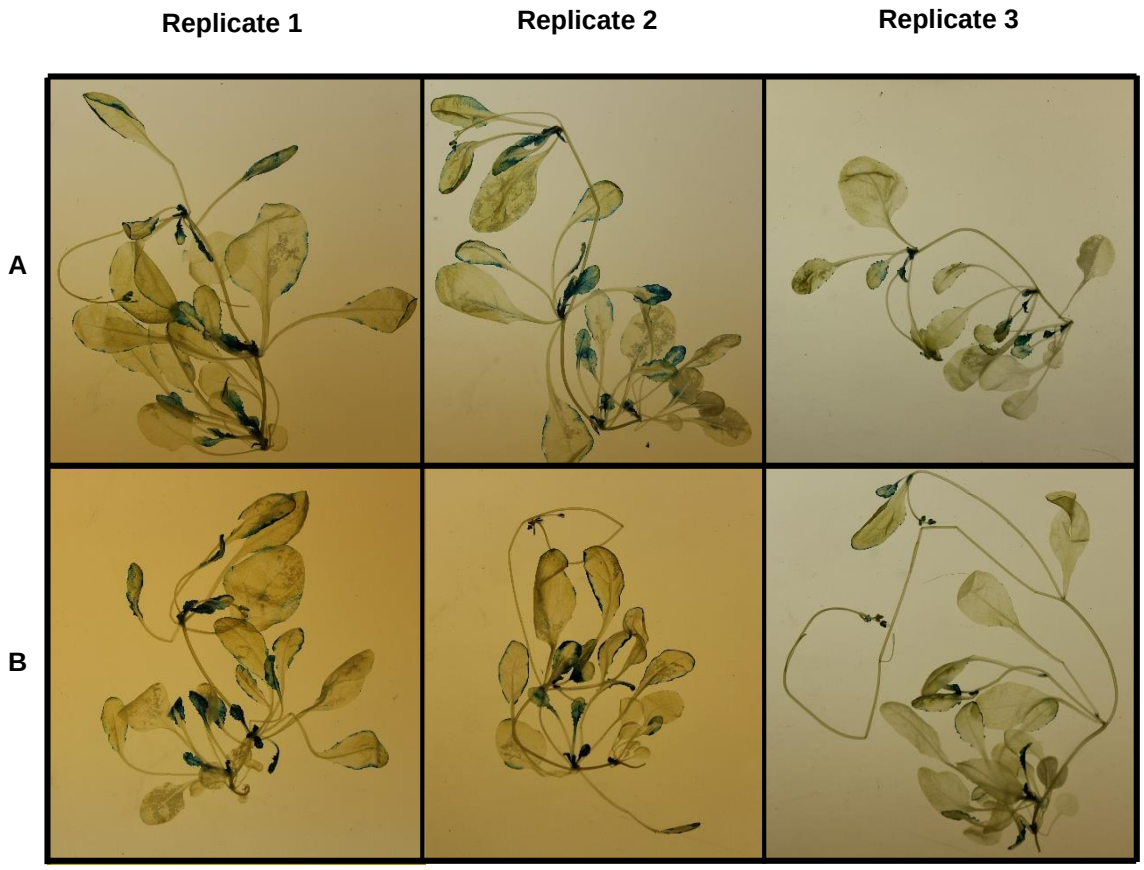


Figure 4.5: Native expression of GUS in three six-week old **(A)** p*BRC1*(-1480):*GUS*:Col-0 and **(B)** p*BRC1*(-990):*GUS*:Col-0 transformants grown in peat disks.

Again, the highest expression of *GUS* was in the axillary buds and shoot meristem regions, and also in young, developing leaves. Overall, GUS expression appeared to be much lower than in either the *in vitro* grown or four-week old plants (Fig. 4.4), but quantitative analysis needs to performed to accurately determine GUS activity. Full grown leaves also had no to very little GUS expression, with traces of the GUS protein found on the edges of these mature, but still expanding leaves. In some of the bigger, actively growing leaves, small symmetrical sites of GUS expression was observed (Fig. 4.6). These sites are leaf margins protrusions, also known as serrations, and have shown to a play a vital role in the determining the final shape of the leaf (Bilsborough *et al*., 2011)

Figure 4.6: Small symmetrical sites of GUS expression in the serrations of actively growing leaves of both p*BRC1*(-1480):*GUS*:Col-0 and p*BRC1*(-990):*GUS*:Col-0 transformants.

High expression of GUS could be seen at these serrations of almost mature leaves. In full grown leaves, the staining pattern was not observed, suggesting that the leaf has completed its expansion and formation. Leaf expansion is controlled by both auxins (Keller *et al*., 2011) and cytokinins (Werner *et al*., 2003; Vercruyssen *et al*., 2015), two groups of phytohormones known to have interactions with strigolactones, specifically in controlling bud outgrowth in plants (Aguilar-Martínez *et al*., 2007; Ferguson and Beveridge, 2009; Prusinkiewicz *et al*., 2009; Hu *et al*., 2014; de Jong *et al*., 2014) . Cytokinins are known to promote mitotic cell division in leaves, but also inhibit leaf senescence and delay leaf differentiation (Fregni *et al*., 2011). As discussed in Chapter 2, strigolactones and cytokinins generally have opposite functions, for example strigolactones would inhibit bud outgrowth in plants while cytokinins would stimulate it. The transcription factors known to control these actively expanding sites are also TCP factors (Koyama *et al*., 2007). An increase in serrations was observed when TCP activity was observed (Bilsborough *et al*., 2011; Ballester *et al*., 2015). Increased TCP

activity would therefore lead to a decrease in serrations, subsequently inhibiting the expansion of the leaves. In the context of the aim of this project, to develop a strigolactone-responsive reporter construct, this staining pattern is probably irrelevant, but further investigation into the promoter regions of these TCP factors compared with the promoter region of *AtBRC1* (also a TCP factor) might reveal an overlapping promoter binding element that could be used in future studies.

Literature suggests that this GUS staining pattern is an auxin-response, because auxins are known to control leaf expansion during vegetative growth (Ljung *et al*., 2001; Koyama *et al*., 2007; Keller *et al*., 2011). To date, no evidence linking strigolactones to leaf expansion has been reported. In a recent study using pea plants, the possible link between strigolactones and photomorphogenesis (leaf expansion and elongation) was investigated, but no supporting evidence was found (Urquhart *et al*., 2015). As discussed in Chapter 2, auxins and cytokinins were the only phytohormones implicated in the regulation of shoot branching before the discovery that strigolactones are also involved. This could mean that strigolactones play a similar role in leaf growth in development that has yet to be discovered.

After the initial GUS staining results of transformants grown *in vitro* (Fig. 4.3), it was decided to create a transgenic plant that contains an empty pMDC163.gb vector to demonstrate that the vector alone induces no GUS expression. Only one transgenic line, with a *max4* background was obtained (Fig. 4.7)

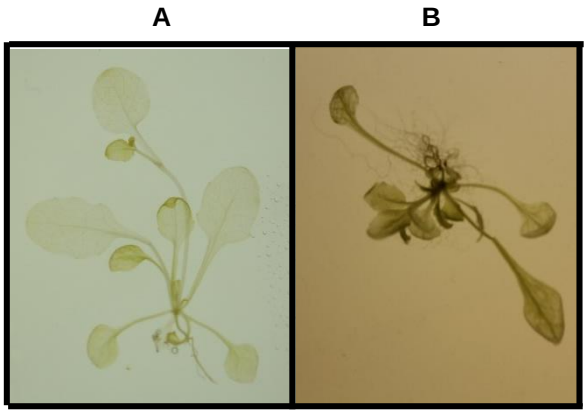


Figure 4.7: Two-week old **(A)** Untransformed Col-0 and **(B)** pMDC163 (empty):*max4* plants after being subjected to GUS staining.

After GUS staining, no GUS protein was observed in either of the untransformed Col-control or the pMDC163(empty):*max4* transformant. It was concluded that the pMDC163.gb binary vector with an inserted fragment is unable to induce GUS expression, which was to be expected, as no promoter elements are present in the empty pMDC163.gb ((The Arabidopsis

Information Resource (TAIR), [https://www.arabidopsis.org/servlet/TairObject?id=501100123&type=vector], on www.arabidopsis.org, Nov 24, 2015). Any GUS staining patterns and GUS expression (Section 4.3.6) are therefore entirely due to the promoter fragments fused to GUS.

4.3.5 Semi-quantitative RT-PCR

With the GUS staining analysis not giving any indication of GR24-responsiveness by the 1480 bp or 990 bp promoter fragments, a different approach was sought to accurately determine if these constructs are indeed strigolactone-responsive. As previously described, plants were subjected to different treatments before being flash frozen, ground, RNA isolated, cDNA manufactured and semi-quantitative RT-PCR analysed focussing on the expression of *BRC1*, *GUS* and *Actin2* (Fig. 4.8).

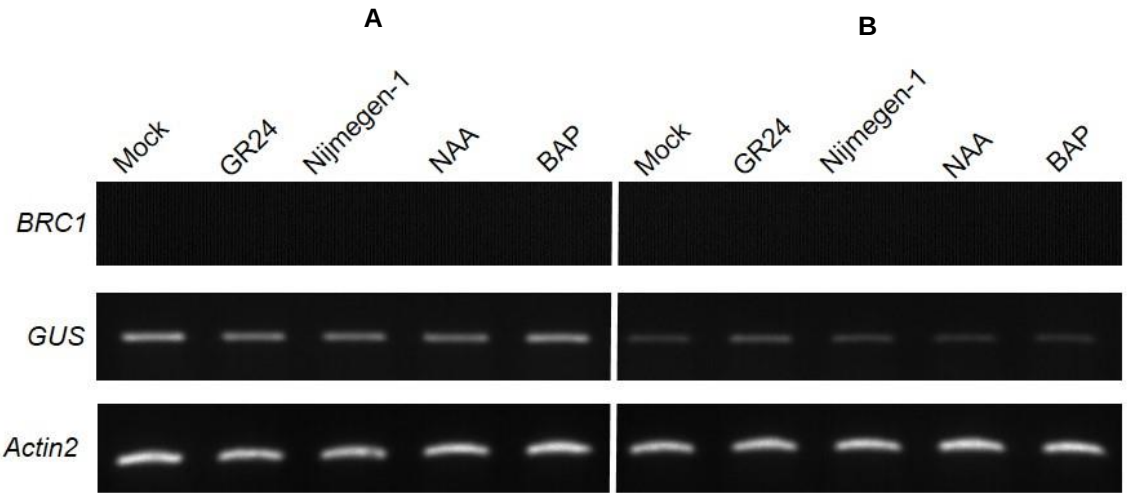


Figure 4.8: The expression of *GUS*, *Actin2* and *BRC1* in pooled samples of **(A)** p*BRC1*(-1480):*GUS* and **(B)** p*BRC1*(-990):*GUS* in a Col-0 background.

The expression of *Actin2* in p*BRC1*(-1480):*GUS*:Col-0 and p*BRC1*(-990):*GUS*:Col-0 was consistent at 25 cycles. In p*BRC1*(-1480)*GUS*:Col-0, a slight reduction of *GUS* expression was observed in plants treated with GR24, Nijmegen-1 and NAA, whilst *GUS* expression levels in the mock- and BAP-treated plants were similar. The expression of *BRC1* is not known to be inhibited by these treatments (de Jong *et al*., 2014), but is known to be inhibited by cytokinins (BAP). These sqRT-PCR results are thus in complete contrast to what would have been expected, but the fact that a clearly reduced promoter of *AtBRC1* was investigated could explain the slight reduction in *GUS* expression to some extent, especially because this

promoter region and strigolactone signalling as a whole remains largely uncharacterized. In an attempt to compare the expression of *BRC1* with *GUS,* cDNA was diluted to obtain the optimal dilution where *Actin2* is not saturated at 35 cycles (Fig. 4.9). This was done because *BRC1* expression occurs naturally at such low levels that it can only detected at 35 cycles, while it appears that *Actin2* reaches saturation after 25 cycles.

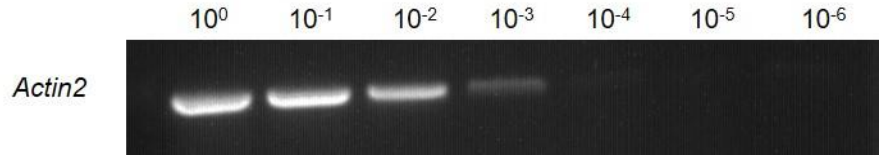


Figure 4.9: The expression of *Actin2* in ten two-week old *A. thaliana* Col-0 seedlings. A dilution series of the same cDNA sample was prepared and the PCR reaction repeated for 35 PCR cycles using the different dilutions of the one cDNA sample.

Using the diluted cDNA as a template, PCR reactions was set up to compare *BRC1*, *GUS* and *Actin2* expression in two-week old *A. thaliana* Col-0 seedlings. Ten microliter of 10^{-3} dilutions for each sample was used a template for PCR reactions for 35 cycles. The cDNA used as template for *BRC1* expression was left undiluted, but the PCR reactions were still repeated for 35 cycles (Fig. 4.10).

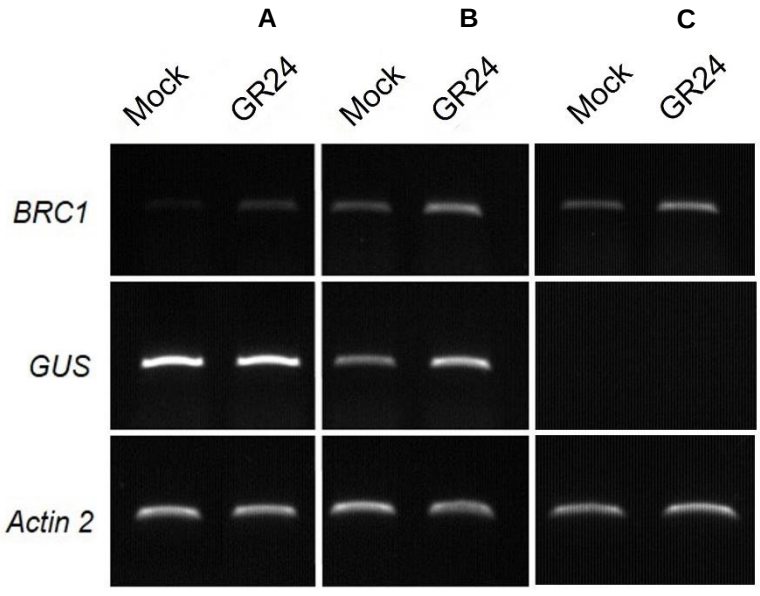


Figure 4.10: Expression of *BRC1*, *GUS* and *Actin2* in **(A)** p*BRC1*(-1480):*GUS*:Col-0 **(B)** pBRC1(-990) and **(C)** Col-0 wild type plants. All the PCR reactions were performed for 35 cycles. The template for the *GUS* and *Actin2* PCR reactions were 10 µL of a 1000-time diluted cDNA sample. Half (25 µL) of the total PCR reaction volume was loaded onto the agarose gel.

It is unclear whether the GUS expression was induced by GR24 in p*BRC1*(-1480):*GUS*:Col-0 transformants, but a slight induction of GUS expression by GR24 in p*BRC1*(-990):*GUS*:Col-0 was observed. The expression of *Actin2* was consistent in p*BRC1*(-1480):*GUS*:Col-0 and p*BRC1*(-990):*GUS*:Col-0 transformants and Col-0 plants for both untreated and GR24-treated samples. This apparent strigolactone-responsiveness was not observed by GUS staining analysis in this study. In a repeat experiment, using the same cDNA as previously synthesized (Fig. 4.8), the expression of *BRC1* (using undiluted cDNA as template), *GUS* (using 10 µL of 1000x diluted cDNA) and *Actin2* (using 10 µL of 1000x diluted cDNA) for analysed at 35 PCR cycles (Fig. 4.11).

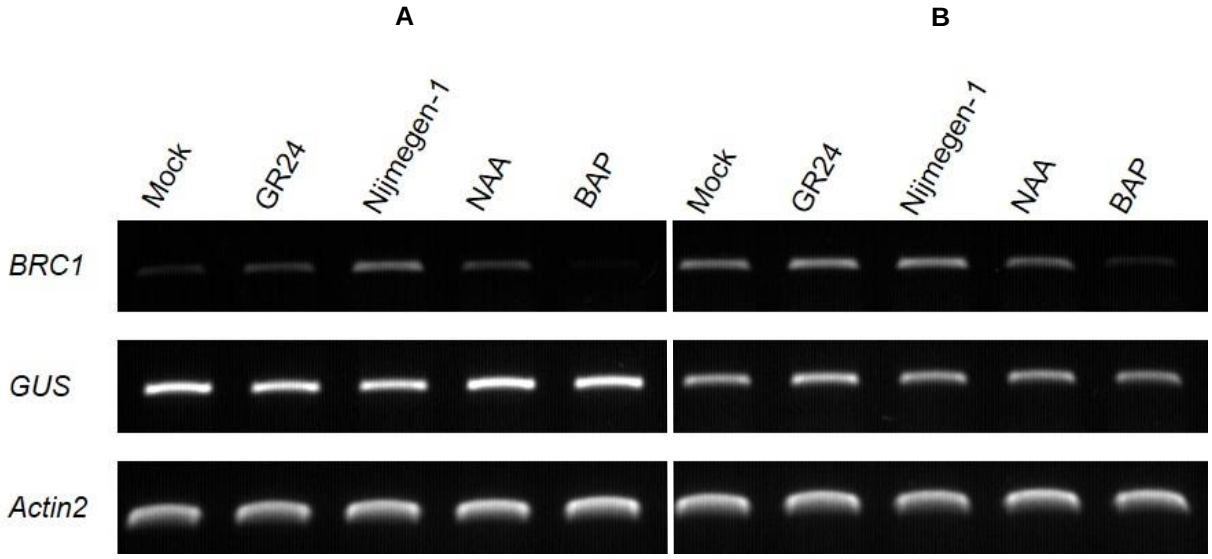


Figure 4.11: Expression of *BRC1*, *GUS* and *Actin2* in **(A)** p*BRC1*(-1480):*GUS*:Col-0 and **(B)** p*BRC1*(-990):*GUS*:Col-0 plants. The PCR reactions were repeated for 35 cycles. The template for the *GUS* and *Actin2* PCR reactions was 10 µL of a 1000-time diluted cDNA, while the template for the *BRC1* PCR reactions was 2 µL of undiluted cDNA.

The expression of *GUS* was consistent for all the treatments and the mock treatment in p*BRC1*(-1480):*GUS*:Col-0 plants, but a slight increase in *GUS* expression is observed in GR24-treated p*BRC1*(-990):*GUS*:Col-0 plants, which correlates with the previous analysis (Fig. 4.14; Fig. 4.16). Even by using a cDNA dilution of 10^{-2}, the expression of *GUS* was still much higher when compared to *BRC1* expression in PCR reactions using 2 µL undiluted cDNA. This, again, highlights the degree of deregulation that the *AtBRC1* promoter has undergone during the construction of these reporter-constructs. The dilution of cDNA to achieve the gene expression results observed (Fig. 4.16; Fig. 4.17) is not an ideal method for

sqRT-PCR and quantitative RT-PCR (qRT-PCR) would have been a superior method to accurately determine the levels *BRC1* expression. However, from these sqRT-PCR analyses it can be concluded that the expression of *GUS* is slightly induced in p*BRC1*(-990):*GUS*:Col-0 plants. This suggests that at least one strigolactone-responsive motif may be present in the 990 bp region immediately upstream of *AtBRC1*.

In these experiments, the expression of *GUS* was compared to the expression of *BRC1*. The expression of *BRC1*, *in planta*, is controlled by numerous biochemical pathways (Chapter 2), as it regulates the outgrowth of axillary buds that result in a major change in plant architecture. The expression of *TCP* genes, a synonym for *BRC1* is *TCP18*, are generally controlled in plants at both transcriptional and post-transcriptional levels (Koyama *et al*., 2007) This control of bud outgrowth does not only occur during the development and initiation, but also throughout the development of the branch, with *BRC1* being the central role player (González-Grandío *et al*., 2013). With such tight regulation, *BRC1* transcriptional levels can be controlled and regulated at the post-transcriptional level, as is the case during biosynthesis of strigolactones as well (Marzec and Muszynska, 2015). In a recent review paper, the authors also described *BRC1* as target for multiple stimuli at both the transcriptional and post-transcriptional level (Rameau *et al*., 2015). These regulation mechanisms can explain why such low levels of *BRC1* were observed, in comparison to *GUS*. The expression of *GUS* would not be subjected to the same post-transcriptional regulation mechanisms, and therefore would appear relatively high in comparison to *BRC1* expression.

4.4 Concluding remarks and future prospects

The aim of this part of the project was to develop a reporter construct that is activated only in the presence of strigolactones. The strategy to shorten the promoter of *At3g18550* was to remove promoter binding elements that are binding sites for proteins (transcription factors) downstream of any other pathway except the strigolactone signalling pathway. This strategy was challenging because there is no information in literature about strigolactone-specific promoter binding elements. The fact that the strigolactone signalling pathway is so poorly understood also made it impossible to predict regions of interest immediately upstream of *AtBRC1*, even with recent studies filling in some of the gaps in our understanding of strigolactone signalling and physiological effects (Zhou *et al*., 2013; Wang *et al*., 2015).

The first conclusion that can be made by the results obtained is that promoters generated from both the 1480 bp and 990 bp regions immediately upstream of *AtBRC1* are severely

deregulated in comparison to the natural *BRC1* promoter. This can be seen from both the GUS staining and sqRT-PCR analysis. This means that the promoter binding element/s downstream of the action of SMX-like protein, known to repress the expression of *AtBRC1* (Wang *et al*., 2015), probably lies further upstream from -1480. The second conclusion is that the 990 bp region immediately upstream of *AtBRC1* might contain a strigolactone-responsive motif/s which has yet to be identified.

For future experiments, the p*BRC1*(-500):*GUS* needs to be transformed into wild-type, *max4* and *max2 A. thaliana plants*, in order to determine if this construct is still strigolactone-responsive and if less background *GUS* expression can be seen. In the broader context, these results can then be used as a starting to point to identify possible strigolactone-specific motif/s that are responsible for the up-regulation of *AtBRC1*. The same can be done with the region -3000 to -1480 in order to identify any strigolactone-specific motif/s in that region. This/these motif/motifs could even be used to create a synthetic reporter-construct, in a similar way to which the auxin-responsive reporter-construct was made (Ulmasov *et al*., 1997). The motif/s responsible for repression can be used in a relief of restraint promoter whereby strigolactone signalling would destroy the SMXL repressor proteins to allow for the expression of GUS. Other reporter genes should also be experimented with, as it seems like the use of the GUS reporter system would not allow for the detection of changes in a sensitive system like strigolactone signalling.

Other future experiments could include the investigation of other strigolactone-responsive genes. The use of *AtBRC1* was motivated by the fact that it is mostly used as an indicator of strigolactone-activity, but because of wide variety of external stimuli and biochemical reactions in the plants that also affects its expression (Aguilar-Martínez *et al*., 2007; Finlayson *et al*., 2010; González-Grandío *et al*., 2013), it is clear that its regulation is a complex process as evidence from studying parts of its promoter region and an extensive literature search (Addendum B).

Chapter 5: Final conclusions and future prospects

"If your experiment needs statistics, you ought to have done a better experiment."

– Ernest Rutherford

5.1. Conclusions

Since the discovery that strigolactones are a novel group of phytohormones and not just the detrimental signal that stimulates the germination of the seeds of parasitic plants (Cook *et al*., 1966; Cook *et al*., 1972), numerous research groups have embarked on the journey to elucidate the strigolactone biochemical pathway. Biosynthesis inhibitors, forward and reverse genetics and yeast-two-hybrid methods have been instrumental in the determination and characterization of key components in the strigolactone biosynthesis and signalling pathway. Although more functions have been connected to strigolactones, the discovery of more role-players in especially the signalling pathway has been halted to a certain extent. There is a need for novel tools that could aid in the elucidation of the full strigolactone pathway. One such a tool that currently does not exist is an inhibitor of strigolactone signalling. Another valuable tool would be a reporter-construct that is activated only in the presence of strigolactones.

The aim of part one of this project was to determine if TMB inhibits strigolactone signalling. The hypothesis was generated by the observation that TMB is known to have an antagonistic effect to karrikins, which are known to stimulate seed germination. It is also known that the receptor for karrikins, KAI2, can modulate some strigolactone responses (Scaffidi *et al*., 2014). Strigolactones are also known to stimulate germination, albeit mainly the seeds of parasitic plants. Both karrikins and strigolactones affect plants in other ways as well. This resulted in the question of whether the fact that TMB has an antagonistic role to karrikins could mean that there is a possibility that it also has an inhibitory effect on strigolactones, particularly at the signalling level. The receptor for strigolactones is D14, while KAI2 is the receptor for karrikins (Kagiyama *et al*., 2013), effectively inhibiting the binding of karrikins. TMB could therefore either bind D14 to inhibit strigolactone signalling, or block strigolactone effects modulated by KAI2. From the preliminary results of this study, there are some indications that TMB might be an inhibitor of strigolactone signalling, although perhaps not as potent an inhibitor as would be desirable.

For the second part of this study, the aim was to develop a strigolactone-responsive reporter construct that is only activated in the presence strigolactones. The results observed during

this study suggests that at least one possible strigolactone-responsive motif may lie in the 990 bp region upstream of *At3g18550*. It is possible that more copies of this or motifs, which might represent the binding site/s for targets downstream of the SMX-like proteins (Wang *et al*., 2015), lie upstream of -1480 bp, as evident from the deregulation of the promoter seen in this study. There is, of course, also the possibility that these elements lie downstream of the start codon of *At3g18550*.

Before and during the course of this study, some limitations of this study was identified. One such limitation if the fact that strigolactones are synthesized at low concentrations (pg). Another limitation is the lack of any experimentally verified promoter binding elements related to a strigolactone response, making it nearly impossible to identify regions of importance without experimental verification. Later in this study it became clear that the GUS reporter system also has some limitations, especially in terms of responding to such a tightly regulating system like strigolactone signalling. The use of the *AtBRC1* promoter region was also limiting, owing to the fact that its expression can be influenced by a wide variety of biological processes.

5.2. Future work

In order to fully understand the effect of TMB on the growth on *A. thaliana*, more experiments need to be conducted, including the incorporation of mutants such as *d14* and *kai2* into the experimental trials. The use of karrikins in these experiments would also possibly indicate if TMB is indeed an inhibitor of strigolactone signalling. For the second part of this project, it would be valuable to investigate the GUS expression pattern of the 1480 bp and 990 bp construct in the *max4* and *max2* background as well, as it could provide further insight into the promoter region of *AtBRC1*. These transformants have already been created. In future efforts to create a strigolactone-responsive reporter construct, further investigation into the genomic region upstream of the region already cloned, -3000 to -1480, must be performed until a strigolactone-response is observed in the form of increased GUS expression. The 500 bp construct already created must also be transformed into *A. thaliana*.

A method known as Chromatin immunoprecipitation (ChIP) could be implemented to determine the binding sites of a particular protein, such as SMX1 or D53. This could prove to be vital in the quest to identify strigolactone-responsive motifs. When strigolactone-responsive motifs are identified, a synthetic reporter construct, similar to that of the auxin-responsive reporter-construct, can be created. The use of reporter systems other than GUS should also be investigated.

References

Aguilar-Martínez JA, Poza-Carrión C, Cubas P (2007) Arabidopsis *BRANCHED1* acts as an integrator of branching signals within axillary buds. Plant Cell **19**: 458–72

Agusti J, Herold S, Schwarz M, Sanchez P, Ljung K, Dun EA, Brewer PB, Beveridge CA, Sieberer T, Sehr EM, *et al* (2011) Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. Proc Natl Acad Sci **108**: 20242–20247

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature **435**: 824–827

Akiyama K, Ogasawara S, Ito S, Hayashi H (2010) Structural requirements of strigolactones for hyphal branching in AM fungi. Plant Cell Physiology **51**: 1104–1117

Alder A, Jamil M, Marzorati M, Bruno M, Vermathen M, Bigler P, Ghisla S, Bouwmeester H, Beyer P, Al-Babili S (2012) The path from β-carotene to carlactone, a strigolactone-like plant hormone. Science **335**: 1348–51

Arite T, Iwata H, Ohshima K, Maekawa M, Nakajima M, Kojima M, Sakakibara H, Kyozuka J (2007) *DWARF10*, an *RMS1/MAX4/DAD1* ortholog, controls lateral bud outgrowth in rice. The Plant Journal **51**: 1019–29

Arite T, Umehara M, Ishikawa S, Hanada A, Maekawa M, Yamaguchi S, Kyozuka J (2009) *d14*, a Strigolactone-Insensitive Mutant of Rice, Shows an Accelerated Outgrowth of Tillers. Plant Cell Physiologoly **50**: 1416–24

Ballester P, Navarrete-Gómez M, Carbonero P, Oñate-Sánchez L, Ferrándiz C (2015) Leaf expansion in Arabidopsis is controlled by a TCP-NGA regulatory module likely conserved in distantly related species. Physiol Plant **155**: 21 - 32

Bao X, Franks RG, Levin JZ, Liu Z (2004) Repression of AGAMOUS by BELLRINGER in floral and inflorescence meristems. Plant Cell **16**: 1478–1489

Barbier F, Péron T, Lecerf M, Perez-Garcia MD, Barrière Q, Rolčík J, Boutet-Mercey S, Citerne S, Lemoine R, Porcheron B, *et al* (2015) Sucrose is an early modulator of the key hormonal mechanisms controlling bud outgrowth in Rosa hybrida. J Exp Bot **66**: 2569–2582

Bennett T, Leyser O (2014) Strigolactone signalling: standing on the shoulders of DWARFs. Curr Opin Plant Biol **22**: 7–13

Bennett T, Sieberer T, Willett B, Booker J, Luschnig C, Leyser O (2006) The *Arabidopsis MAX* pathway controls shoot branching by regulating auxin transport. Curr Biol **16**: 553–63

Beveridge CA, Symons GM, Murfet IC, Ross JJ, Rameau C (1997) The *rms1* Mutant of Pea Has Elevated Indole-3-Acetic Acid Levels and Reduced Root-Sap Zeatin Riboside Content but Increased Branching Controlled by Graft-Transmissible Signal(s). Plant Physiol **115**: 1251–1258

Beveridge CA, Kyozuka J (2010) New genes in the strigolactone-related shoot branching pathway. Curr Opin Plant Biol **13**: 34–9

Bierfreund NM, Reski R, Decker EL (2003) Use of an inducible reporter gene system for the analysis of auxin distribution in the moss Physcomitrella patens. Plant Cell Rep **21**: 1143–1152

Bilsborough GD, Runions A, Barkoulas M, Jenkins HW, Hasson A, Galinha C, Laufs P, Hay A, Prusinkiewicz P, Tsiantis M (2011) Model for the regulation of *Arabidopsis thaliana* leaf margin development. Proc Natl Acad Sci U S A **108**: 3424–3429

Booker J, Sieberer T, Wright W, Williamson L, Willett B, Stirnberg P, Turnbull C, Srinivasan M, Goddard P, Leyser O (2005) *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a carotenoid-derived branch-inhibiting hormone. Dev Cell **8**: 443–9

Boyer FD, de Saint Germain A, Pillot JP, Pouvreau J-B, Chen VX, Ramos S, Stévenin A, Simier P, Delavault P, Beau J-M, *et al* (2012) Structure-activity relationship studies of strigolactone-related molecules for branching inhibition in garden pea: molecule design for shoot branching. Plant Physiol **159**: 1524–44

Braun N, de Saint Germain A, Pillot J-P, Boutet-Mercey S, Dalmais M, Antoniadi I, Li X, Maia-Grondard A, Le Signor C, Bouteiller N, *et al* (2012) The Pea TCP Transcription Factor PsBRC1 Acts Downstream of Strigolactones to Control Shoot Branching. Plant Physiol **158**: 225–238

Brewer PB, Dun EA, Ferguson BJ, Rameau C, Beveridge CA (2009) Strigolactone acts downstream of auxin to regulate bud outgrowth in pea and Arabidopsis. Plant Physiol **150**: 482–93

Brewer PB, Koltai H, Beveridge CA. (2013) Diverse roles of strigolactones in plant development. Mol Plant **6**: 18–28

Brown NAC (1993) Promotion of Germination of Fynbos Seeds by Plant-Derived Smoke. New Phytol **123**: 575–583

Bythell-Douglas R, Waters MT, Scaffidi A, Flematti GR, Smith SM, Bond CS (2013) The Structure of the Karrikin-Insensitive Protein (KAI2) in *Arabidopsis thaliana*. PLoS One **8** (1): e54758. Doi:10.1371/journal.pone.0054758

Calder WJ, Lifferth G, Moritz MA, St. Clair SB (2010) Physiological Effects of Smoke Exposure on Deciduous and Conifer Tree Species. Int J For Res **2010**: 1-7

Chen VX, Boyer F-D, Rameau C, Retailleau P, Vors J-P, Beau J-M (2010) Stereochemistry, total synthesis, and biological evaluation of the new plant hormone solanacol. Chemistry **16**: 13941–5

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang H-S, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, *et al* (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14**: 559–574

Chen Y, Yordanov YS, Ma C, Strauss S, Busov VB (2013) DR5 as a reporter system to study auxin response in Populus. Plant Cell Rep **32**: 453–463

Cheng X, Ruyter-Spira C, Bouwmeester H (2013) The interaction between strigolactones and other plant hormones in the regulation of plant development. Front Plant Sci **4**: 199

Choi MS, Woo MO, Koh EB, Lee J, Ham TH, Seo HS, Koh HJ (2012) Teosinte Branched 1 modulates tillering in rice plants. Plant Cell Rep **31**: 57–65

Cook CE, Whichard LP, Turner B, Wall ME, Egley GH (1966) Germination of Witchweed (Striga lutea Lour.): Isolation and Properties of a Potent Stimulant. Science **154**: 1189–90

Cook CE, Whichard LP, Wall M, Egley GH, Coggon P, Luhan PA, McPhail AT (1972) Germination stimulants. II. Structure of strigol, a potent seed germination stimulant for witchweed (Striga lutea). J Am Chem Soc **94**: 6198–6199

Crawford S, Shinohara N, Sieberer T, Williamson L, George G, Hepworth J, Müller D, Domagalska MA, Leyser O (2010) Strigolactones enhance competition between shoot branches by dampening auxin transport. Development **137**: 2905–2913

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol **48**: 355–381

Cubas P, Lauter N, Doebley J, Coen E (1999) The TCP domain: a motif found in proteins

regulating plant growth and development. Plant J **18**: 215–22

De Jong M, George G, Ongaro V, Williamson L, Willetts B, Ljung K, McCulloch H, Leyser O (2014) Auxin and strigolactone signaling are required for modulation of *Arabidopsis* shoot branching by N supply. Plant Physiol **166**: 384–395

DeMason DA, Polowick PL (2009) Patterns of DR5::GUS Expression in Organs of Pea (*Pisum sativum*). Int J Plant Sci **170**: 1–11

De Saint Germain A, Bonhomme S, Boyer FD, Rameau C (2013) Novel insights into strigolactone distribution and signalling. Curr Opin Plant Biol **16**: 583–589

Ditt RF, Nester EW, Comai L (2001) Plant gene expression response to *Agrobacterium tumefaciens*. PNAS **98** (19): 10954–10959

Domagalska MA, Leyser O (2011) Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol **12**: 211–221

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in *Arabidopsis*. Trends Plant Sci **15**: 573–581

Estabrook EM, Yoder JI (1998) Plant-Plant Communications: Rhizosphere Signaling between Parasitic Angiosperms and Their Hosts. Plant Physiol **116**: 1–7

Farooq M, Wahid A, Kobayashi, N, Fujita, D, Basra, SMA (2009) Plant drought stress : effects, mechanisms and management. Agron Sustain Dev Springer Verlag **29**: 185–212

Ferguson BJ, Beveridge CA (2009) Roles for auxin, cytokinin, and strigolactone in regulating shoot branching. Plant Physiol **149**: 1929–44

Fett-Neto AG, Fett JP, Veira Goulart LW, Pasquali G, Termignoni RR, Ferreira AG (2001) Distinct effects of auxin and light on adventitious root development in Eucalyptus saligna and Eucalyptus globulus. Tree Physiol **21**: 457–464

Finlayson SA, Krishnareddy SR, Kebrom TH, Casal JJ (2010) Phytochrome regulation of branching in Arabidopsis. Plant Physiol **152**: 1914–27

Finlayson SA. (2007) Arabidopsis *TEOSINTE BRANCHED1-LIKE 1* regulates axillary bud outgrowth and is homologous to monocot *TEOSINTE BRANCHED1*. Plant Cell Physiol **48**: 667–677

Flematti GR, Chisalberti EL, Dixon KW, Trengove RD (2004) A compound from smoke that promotes seed germination. Science **305**: 977

Foo E, Bullier E, Goussot M, Foucher F, Rameau C, Beveridge CA (2005) The Branching Gene *RAMOSUS1* Mediates Interactions among Two Novel Signals and Auxin in Pea. The Plant Cell **17**: 464–474

Foo E, Turnbull CG, Beveridge CA (2001) Long-distance signaling and the control of branching in the *rms1* mutant of pea. Plant Physiol **126**: 203–9

Fukui K, Ito S, Asami T (2013) Selective mimics of strigolactone actions and their potential use for controlling damage caused by root parasitic weeds. Mol Plant **6**: 88–99

Gan S (2003) Mitotic and postmitotic senescence in plants. Sci Aging Knowledge Environ **2003** (38): re7

Ghebrehiwot HM, Kulkarni MG, Szalai G, Soós V, Balázs E, Van Staden J (2013) Karrikinolide residues in grassland soils following fire: Implications on germination activity. South African J Bot **88**: 419–424

Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Van Aken O, Millar AH, Murcha M, Whelan J (2010) TCP Transcription Factors Link the Regulation of Genes Encoding Mitochondrial Proteins with the Circadian Clock in *Arabidopsis thaliana*. Plant Cell **22**: 3921–3934

Goldwasser Y, Yoneyama K, Xie X, Yoneyama K (2008) Production of strigolactones by *Arabidopsis thaliana* responsible for *Orobanche aegyptiaca* seed germination. Plant Growth Reg **55**: 21–28

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pillot J-P, Letisse F, Matusova R, Danoun S, Portais JC, *et al* (2008) Strigolactone inhibition of shoot branching. Nature **455**: 189–94

González-Grandío E, Poza-Carrión C, Sorzano COS, Cubas P (2013) *BRANCHED1* promotes axillary bud dormancy in response to shade in *Arabidopsis*. Plant Cell **25**: 834–50

Goulet C, Klee HJ (2010) Climbing the branches of the strigolactones pathway one discovery at a time. Plant Physiol **154**: 493–6

Grove MD, Spencer GF, Rohwedder WK, Mandava N, Worley JF, Warthen JD, Steffens GL, Flippen-Anderson JL, Cook JC (1979) Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. Nature **281**: 216–217

Guo Y, Zheng Z, La Clair JJ, Chory J, Noel JP (2013) Smoke-derived karrikin perception by the α/β-hydrolase KAI2 from *Arabidopsis*. PNAS **110**: 8284–9

Ha CV, Leyva-González MA, Osakabe Y, Tran UT, Nishiyama R, Watanabe Y, Tanaka M, Seki M, Yamaguchi S, Dong N Van, *et al* (2014) Positive regulatory role of strigolactone in plant responses to drought and salt stress. PNAS **111**: 851–6

Hamiaux C, Drummond RSM, Janssen BJ, Ledger SE, Cooney JM, Newcomb RD, Snowden KC (2012) DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. Curr Biol **22**: 2032–6

Hayward A, Stirnberg P, Beveridge C, Leyser O (2009) Interactions between auxin and strigolactone in shoot branching control. Plant Physiol **151**: 400–12

Herold S (2009) The role of strigolactones in the regulation of secondary growth in *Arabidopsis thaliana*.

Hu Z, Yamauchi T, Yang J, Jikumaru Y, Tsuchida-Mayama T, Ichikawa H, Takamure I, Nagamura Y, Tsutsumi N, Yamaguchi S, *et al* (2014) Strigolactone and cytokinin act antagonistically in regulating rice mesocotyl elongation in darkness. Plant Cell Physiol **55**: 30–41

Hu Z, Yan H, Yang J, Yamaguchi S, Maekawa M, Takamure I, Tsutsumi N, Kyozuka J, Nakazono M (2010) Strigolactones negatively regulate mesocotyl elongation in rice during germination and growth in darkness. Plant Cell Physiol **51**: 1136–42

Humphrey AJ, Beale MH (2006) Strigol: biogenesis and physiological activity. Phytochem **67**: 636–40

Humphrey AJ, Galster AM, Beale MH (2006) Strigolactones in chemical ecology: waste products or vital allelochemicals? Nat Prod Rep **23**: 592–614

Ito S, Kitahata N, Umehara M, Hanada A, Kato A, Ueno K, Mashiguchi K, Kyozuka J, Yoneyama K, Yamaguchi S, *et al* (2010) A new lead chemical for strigolactone biosynthesis inhibitors. Plant Cell Physiol **51**: 1143–50

Ito S, Nozoye T, Sasaki E, Imai M, Shiwa Y, Shibata-Hatta M, Ishige T, Fukui K, Ito K, Nakanishi H, et al (2015) Strigolactone Regulates Anthocyanin Accumulation, Acid Phosphatases Production and Plant Growth under Low Phosphate Condition in *Arabidopsis*. PLoS One **10**: e0119724

Ito S, Umehara M, Hanada A, Kitahata N, Hayase H, Yamaguchi S, Asami T (2011) Effects of triazole derivatives on strigolactone levels and growth retardation in rice. PLoS One **6**: e21723

Jain A, Poling MD, Karthikeyan AS, Blakeslee JJ, Peer WA, Titapiwatanakun B,

Murphy AS, Raghothama KG (2007) Differential effects of sucrose and auxin on localized phosphate deficiency-induced modulation of different traits of root system architecture in *Arabidopsis*. Plant Physiol **144**: 232–247

Jamil M, Charnikhova T, Verstappen F, Bouwmeester H (2010) Carotenoid inhibitors reduce strigolactone production and *Striga hermonthica* infection in rice. Arch Biochem Biophys **504**: 123–31

Joel DM (2000) The long-term approach to parasitic weeds control: Manipulation of specific developmental mechanisms of the parasite. Crop Protection **19**: 753–758

Johnson X, Brcich T, Dun EA, Goussot M (2006) Branching Genes Are Conserved across Species. Genes Controlling a Novel Signal in Pea Are Coregulated by Other Long-Distance Signals. Plant Physiol **142**: 1014-1026

Juven-Gershon T, Hsu J (2008) The RNA polymerase II core promoter—the gateway to transcription. Curr Opin cell Biol. **20**: 253–259

Kagiyama M, Hirano Y, Mori T, Kim S-Y, Kyozuka J, Seto Y, Yamaguchi S, Hakoshima T (2013) Structures of D14 and D14L in the strigolactone and karrikin signaling pathways. Genes Cells **18**: 147–60

Kapulnik Y, Delaux PM, Resnick N, Mayzlish-Gati E, Wininger S, Bhattacharya C, Séjalon-Delmas N, Combier J-P, Bécard G, Belausov E, *et al* (2011a) Strigolactones affect lateral root formation and root-hair elongation in *Arabidopsis*. Planta **233**: 209–16

Kapulnik Y, Koltai H (2014) Strigolactone involvement in root development, response to abiotic stress and interactions with the biotic soil environment. Plant Physiol **166**: 1–29

Kapulnik Y, Resnick N, Mayzlish-Gati E, Kaplan Y, Wininger S, Hershenhorn J, Koltai H (2011b) Strigolactones interact with ethylene and auxin in regulating root-hair elongation in *Arabidopsis*. J Exp Bot **62**: 2915–24

Keller CP, Grundstad ML, Evanoff MA, Keith JD, Lentz DS, Wagner SL, Culler AH, Cohen JD (2011) Auxin-induced leaf blade expansion in *Arabidopsis* requires both wounding and detachment. Plant Signalling & Behavior **6**: 1997–2007

Kgosi RL, Zwanenburg B, Mwakaboko AS, Murdoch AJ (2012) Strigolactone analogues induce suicidal seed germination of *Striga* spp. in soil. Weed Res **52**: 197–203

Kohlen W, Charnikhova T, Lammers M, Pollina T, Tóth P, Haider I, Pozo MJ, de Maagd RA, Ruyter-Spira C, Bouwmeester HJ, *et al* (2012) The tomato carotenoid cleavage dioxygenase8 (SlCCD8) regulates rhizosphere signaling, plant architecture and affects

reproductive development through strigolactone biosynthesis. New Phytol **196**: 535–547

Kohlen W, Charnikhova T, Liu Q, Bours R, Domagalska MA, Beguerie S, Verstappen F, Leyser O, Bouwmeester H, Ruyter-Spira C (2011) Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. Plant Physiol **155**: 974–987

Koltai H (2011) Strigolactones are regulators of root development. New Phytol **190**: 545–9

Korkuc P, Schippers JHM, Walther D (2014) Characterization and Identification of cis-Regulatory Elements in *Arabidopsis* Based on Single-Nucleotide Polymorphism Information. Plant Physiol **164**: 181–200

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. Plant Cell **19**: 473–484

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot **63**: 1593–1608

Krecek P, Skupa P, Libus J, Naramoto S, Tejos R, Friml J, Zazímalová E (2009) The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biol **10**: 249

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature **483**: 341–4

Kumari A, Papenfus HB, Kulkarni MG, Pošta M, Van Staden J (2015) Effect of smoke derivatives on *in vitro* pollen germination and pollen tube elongation of species from different plant families. Plant Biol **17**: 825–830

Laskowski M, Biller S, Stanley K, Kajstura T, Prusty R (2006) Expression profiling of auxin-treated Arabidopsis roots: Toward a molecular analysis of lateral root emergence. Plant Cell Physiol **47**: 788–792

Li C, Bangerth F (1999) Autoinhibition of indoleacetic acid transport in the shoots of two-branched pea (*Pisum sativum*) plants and its relationship to correlative dominance. Physiol Plantarum **106**: 415–420

Light ME, Burger BV, Staerk D, Kohout L, Van Staden J (2010) Butenolides from plant-derived smoke: natural plant-growth regulators with antagonistic actions on seed germination. J Nat Prod **73**: 267–269

Light ME, Gardner MJ, Jäger AK, Van Staden J (2002) Dual regulation of seed germination by smoke solutions. Plant Growth Regulation **37**: 135–141

Liu J, He H, Vitali M, Visentin I, Charnikhova T, Haider I, Schubert A, Ruyter-Spira C, Bouwmeester HJ, Lovisolo C, *et al* (2015) Osmotic stress represses strigolactone biosynthesis in Lotus japonicus roots: exploring the interaction between strigolactones and ABA under abiotic stress. Planta 1435–1451

Ljung K, Bhalerao RP, Sandberg G (2001) Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. Plant J **28**: 465–474

López-Ráez JA, Charnikhova T, Fernández I, Bouwmeester H, Pozo MJ (2011) Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato. J Plant Physiol **168**: 294–297

López-Ráez JA, Bouwmeester H (2008) Fine-tuning regulation of strigolactone biosynthesis under phosphate starvation. Plant Signalin Behavior **3**: 963–965

López-Ráez JA, Charnikhova T, Gómez-Roldán V, Matusova R, Kohlen W, De Vos R, Verstappen F, Puech-Pages V, Bécard G, Mulder P, *et al* (2008) Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytologist **178**: 863–74

Luisi A, Lorenzi R, Sorce C (2011) Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*). Plant Growth Regulation **65**: 415–419

Mangnus EM, Dommerholt FJ, De Jong RLP, Zwanenburg B (1992) Improved synthesis of strigol analog GR24 and evaluation of the biological activity of its diastereomers. J Agric Food Chem **40**: 1230–1235

Martín-Trillo M, Grandío EG, Serra F, Marcel F, Rodríguez-Buey ML, Schmitz G, Theres K, Bendahmane A, Dopazo H, Cubas P (2011) Role of tomato *BRANCHED1*-like genes in the control of shoot branching. Plant J **67**: 701–14

Mashiguchi K, Sasaki E, Shimada Y, Nagae M, Ueno K, Nakano T, Yoneyama K, Suzuki Y, Asami T (2009) Feedback-Regulation of Strigolactone Biosynthetic Genes and Strigolactone-Regulated Genes in *Arabidopsis*. Biosci Biotechnol Biochem **73**: 2460–2465

Mason MG, Ross JJ, Babst BA, Wienclaw BN, Beveridge CA. (2014) Sugar demand, not auxin, is the initial regulator of apical dominance. Proc Natl Acad Sci 201322045

Matusova R, Rani K, Verstappen FW a, Franssen MCR, Beale MH, Bouwmeester HJ

(2005) The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. Plant Physiol **139**: 920–934

Mayzlish-Gati E, De-Cuyper C, Goormachtig S, Beeckman T, Vuylsteke M, Brewer PB, Beveridge C a, Yermiyahu U, Kaplan Y, Enzer Y, *et al* (2012) Strigolactones are involved in root response to low phosphate conditions in *Arabidopsis*. Plant Physiol **160**: 1329–41

Mayzlish-Gati E, LekKala SP, Resnick N, Wininger S, Bhattacharya C, Lemcoff JH, Kapulnik Y, Koltai H (2010) Strigolactones are positive regulators of light-harvesting genes in tomato. J Exp Bot **61**: 3129–36

Sorin C, Bussell JD, Camus I, Ljung K, Kowalczyk M, Geiss G, McKhann H, Garcion C, Vaucheret H, Sandberg G, Bellini C (2005) Aucin and Light Control of Adventitious Rooting in *Arabidopsis* Require *ARGONAUTE1*. The Plant Cell **17**: 1343-1359

Minakuchi K, Kameoka H, Yasuno N, Umehara M, Luo L, Kobayashi K, Hanada A, Ueno K, Asami T, Yamaguchi S, *et al* (2010) FINE CULM1 (FC1) works downstream of strigolactones to inhibit the outgrowth of axillary buds in rice. Plant Cell Physiol **51**: 1127–35

Nakamura H, Asami T (2014) Target sites for chemical regulation of strigolactone signaling. Front Plant Sci **5**: 1–9

Nakamura H, Xue YL, Miyakawa T, Hou F, Qin H-M, Fukui K, Shi X, Ito E, Ito S, Park S-H, *et al* (2013) Molecular mechanism of strigolactone perception by DWARF14. Nat Commun **4**: 2613-2623

Nelson DC, Flematti GR, Ghisalberti EL, Dixon KW, Smith SM (2012) Regulation of Seed Germination and Seedling Growth by Chemical Signals from Burning Vegetation. Annu Rev Plant Biol **63**: 107–130

Nelson DC, Riseborough JA, Flematti GR, Stevens J, Ghisalberti EL, Dixon KW, Smith SM (2009) Karrikins discovered in smoke trigger Arabidopsis seed germination by a mechanism requiring gibberellic acid synthesis and light. Plant Physiol **149**: 863–73

Nelson DC, Scaffidi A, Dun E a, Waters MT, Flematti GR, Dixon KW, Beveridge CA, Ghisalberti EL, Smith SM (2011) F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. PNAS **108**: 8897–8902

Niwa M, Daimon Y, Kurotani K, Higo A, Pruneda-Paz JL, Breton G, Mitsuda N, Kay SA, Ohme-Takagi M, Endo M, *et al* (2013) BRANCHED1 interacts with FLOWERING

LOCUS T to repress the floral transition of the axillary meristems in *Arabidopsis*. Plant Cell **25**: 1228–42

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. PNAS **101**: 8039–8044

Ongaro V, Leyser O (2008) Hormonal control of shoot branching. J Exp Bot **59**: 67–74

Overvoorde P, Fukaki H, Beeckman T (2010) Auxin control of root development. Cold Spring Harbor Perspectives in Biology **2**: a001537

Papenfus HB, Kulkarni MG, Pošta M, Finnie JF, Van Staden J (2015a) Smoke-Isolated Trimethylbutenolide Inhibits Seed Germination of Different Weed Species by Reducing Amylase Activity. Weed Sci **63**: 312–320

Papenfus HB, Naidoo D, Pošta M, Finnie JF, Van Staden J (2015b) The effects of smoke derivatives on *in vitro* seed germination and development of the leopard orchid *Ansellia africana*. Plant Biology **18**: 289-294

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol **6**: 763–775

Pošta M, Light ME, Papenfus HB, Van Staden J, Kohout L (2013) Structure-activity relationships of analogs of 3,4,5-trimethylfuran-2(5H)-one with germination inhibitory activities. J Plant Physiol **170**: 1235–1242

Pozo MJ, Azcón-Aguilar C (2007) Unraveling mycorrhiza-induced resistance. Curr Opin Plant Biol **10**: 393–398

Proust H, Hoffmann B, Xie X, Yoneyama K, Schaefer DG, Yoneyama K, Nogué F, Rameau C (2011) Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. Development **138**: 1531–9

Prusinkiewicz P, Crawford S, Smith RS, Ljung K, Bennett T, Ongaro V, Leyser O (2009) Control of bud activation by an auxin transport switch. PNAS **106**: 17431–17436

Qin X, Zeevaart JA (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. PNAS **96**: 15354–15361

Rameau C (2010) Strigolactones, a novel class of plant hormone controlling shoot branching. Comptes Rendus Biologies **333**: 344–9

Rameau C, Bertheloot J, Leduc N, Andrieu B, Foucher F, Sakr S (2015) Multiple pathways regulate shoot branching. Frontiers in Plant Science **5**: 741-755

Rasmussen A, Beveridge CA, Geelen D (2012a) Inhibition of strigolactones promotes adventitious root formation. Plant Signal Behav **7**: 694–697

Rasmussen A, Heugebaert T, Matthys C, Deun R Van, Boyer FD, Goormachtig S, Stevens C, Geelen D (2013) A fluorescent alternative to the synthetic strigolactone GR24. Mol Plant **6**: 100–112

Rasmussen A, Mason MG, De Cuyper C, Brewer PB, Herold S, Agusti J, Geelen D, Greb T, Goormachtig S, Beeckman T, *et al* (2012b) Strigolactones suppress adventitious rooting in *Arabidopsis* and pea. Plant Physiology **158**: 1976–87

Reyes JC, Muro-Pastor MI, Florencio FJ (2004) The GATA family of transcription factors in *Arabidopsis* and rice. Plant Physiology **134**: 1718–1732

Rocher A, Dumas C, Cock JM (2005) A W-box is required for full expression of the SA-responsive gene *SFR2*. Gene **344**: 181–192

Rohilla R, Garg M, Kumar G (2011) A newly discovered Phytohormone : Strigolactones. Der Pharmacia Sinica **2**: 164–171

Ruyter-Spira C, Kohlen W, Charnikhova T, van Zeijl A, van Bezouwen L, de Ruijter N, Cardoso C, Lopez-Raez JA, Matusova R, Bours R, *et al* (2011) Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: another belowground role for strigolactones? Plant Physiol **155**: 721–34

Růzicka K, Ljung K, Vanneste S, Podhorská R, Beeckman T, Friml J, Benková E (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell **19**: 2197–2212

Sachs T (1991) Cell polarity and tissue patterning in plants. Development **113**: 83–93

Sandelin A, Carninci P, Lenhard B, Ponjavic J, Hayashizaki Y, Hume DA (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies. Nat Rev Genet **8**: 424–436

Sato D, Awad AA, Takeuchi Y, Yoneyama K (2005) Confirmation and quantification of strigolactones, germination stimulants for root parasitic plants *Striga* and *Orobanche*, produced by cotton. Biosci Biotechnol Biochem **69**: 98–102

Scaffidi A, Waters MT, Sun YK, Skelton BW, Dixon KW, Ghisalberti EL, Flematti GR, Smith SM (2014) Strigolactone Hormones and Their Stereoisomers Signal through

Two Related Receptor Proteins to Induce Different Physiological Responses in Arabidopsis. Plant Physiol **165**: 1221–1232

Schwartz SH, Qin X, Loewen MC (2004) The biochemical characterization of two carotenoid cleavage enzymes from *Arabidopsis* indicates that a carotenoid-derived compound inhibits lateral branching. J Biol Chem **279**: 46940–5

Seto Y, Sado A, Asami K, Hanada A, Umehara M, Akiyama K, Yamaguchi S (2014) Carlactone is an endogenous biosynthetic precursor for strigolactones. Proc Natl Acad Sci U S A **111**: 1640–5

Seto Y, Yamaguchi S (2014) Strigolactone biosynthesis and perception. Curr Opin Plant Biol **21**: 1–6

Shahbaz M, Ashraf M (2013) Improving Salinity Tolerance in Cereals. CRC Crit Rev Plant Sci **32**: 237–249

Shrivastava P, Kumar R (2015) Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci **22**: 123–131

Simons JL, Napoli CA, Janssen BJ, Plummer KM, Snowden KC (2007) Analysis of the *DECREASED APICAL DOMINANCE* genes of petunia in the control of axillary branching. Plant Physiol **143**: 697–706

Smith SM, Li J (2014) Signalling and responses to strigolactones and karrikins. Curr Opin Plant Biol **21**: 23–29

Snowden KC, Simkin AJ, Janssen BJ, Templeton KR (2005) The Decreased apical dominance1 / Petunia hybrida *CAROTENOID CLEAVAGE DIOXYGENASE8* Gene Affects Branch Production and Plays a Role in Leaf Senescence, Root Growth, and Flower Development. The Plant Cell **17**: 746-759

Soós V, Sebestyén E, Posta M, Kohout L, Light ME, Van Staden J, Balázs E (2012) Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce (*Lactuca sativa*) achenes. New Phytol **196**: 1060–1073

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, Foo E, Chatfield S, Ward S, Beveridge C, Rameau C, *et al* (2003) *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. 1469–1474

Spartz AK, Gray WM (2008) Plant hormone receptors: new perceptions. Genes Dev **22**: 2139–2148

Spensley M, Kim JY, Picot E, Reid J, Ott S, Helliwell C, Carré IA (2009) Evolutionarily conserved regulatory motifs in the promoter of the Arabidopsis clock gene *LATE ELONGATED HYPOCOTYL*. Plant Cell **21**: 2606–2623

Stanga JP, Smith SM, Briggs WR, Nelson DC (2013) SUPPRESSOR OF MORE AXILLARY GROWTH2 1 controls seed germination and seedling development in *Arabidopsis*. Plant Physiol **163**: 318–30

Stevens JC, Merritt DJ, Flematti GR, Ghisalberti EL, Dixon KW (2007) Seed germination of agricultural weeds is promoted by the butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one under laboratory and field conditions. Plant Soil **298**: 113–124

Stirnberg P, Furner IJ, Ottoline Leyser HM (2007) MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. Plant J **50**: 80–94

Stirnberg P, van De Sande K, Leyser HMO (2002) MAX1 and MAX2 control shoot lateral branching in *Arabidopsis*. Development **129**: 1131–41

Sun H, Tao J, Hou M, Huang S, Chen S, Liang Z, Xie T, Wei Y, Xie X, Yoneyama K, *et al* (2015) A strigolactone signal is required for adventitious root formation in rice. Ann Bot **4**: 1155–1162

Tang W, Perry SE (2003) Binding site selection for the plant MADS domain protein AGL15. An *in vitro* and *in vivo* study. J Biol Chem **278**: 28154–28159

Torres-Vera R, García JM, Pozo MJ, López-Ráez JA. (2014) Do strigolactones contribute to plant defence? Mol Plant Pathol **15**: 211–216

Tsuchiya Y, Vidaurre D, Toh S, Hanada A, Nambara E, Kamiya Y, Yamaguchi S, McCourt P (2010) A small-molecule screen identifies new functions for the plant hormone strigolactone. Nature Chemical Biology **6**: 741-749

Ueda H, Kusaba M (2015) Strigolactone regulates leaf senescence in concert with ethylene in *Arabidopsis*. Plant Physiol **169**: 138-147

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA Proteins Repress Expression of Reporter Genes Containing Natural and Highly Active Synthetic Auxin Response Elements. The Plant Cell **9**: 1963-1971

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, *et al* (2008) Inhibition of shoot branching by new

terpenoid plant hormones. Nature **455**: 195–200

Urquhart S, Foo E, Reid JB (2015) The role of strigolactones in photomorphogenesis of pea is limited to adventitious rooting. Physiol Plant **153**: 392–402

Vanneste S, Friml J (2009) Auxin: a trigger for change in plant development. Cell **136**: 1005–16

Van Staden J, Jager A, Light ME, Burger B (2004) Isolation of the major germination cue from plant-derived smoke. South African J Bot **70**: 654–659

Vercruyssen L, Tognetti VB, Gonzalez N, Van Dingenen J, De Milde L, Bielach A, De Rycke R, Van Breusegem F, Inzé D (2015) GROWTH REGULATING FACTOR5 Stimulates *Arabidopsis* Chloroplast Division, Photosynthesis, and Leaf Longevity. Plant Physiol **167**: 817–832

Wang L, Wang B, Jiang L, Liu X, Li X, Lu Z, Meng X, Wang Y, Smith SM, Li J (2015) Strigolactone Signaling in *Arabidopsis* Regulates Shoot Development by Targeting D53-Like SMXL Repressor Proteins for Ubiquitination and Degradation. The Plant Cell **27**: 3128–3142

Waters MT, Brewer PB, Bussell JD, Smith SM, Beveridge CA (2012a) The *Arabidopsis* ortholog of rice *DWARF27* acts upstream of *MAX1* in the control of plant development by strigolactones. Plant Physiol **159**: 1073–85

Waters MT, Nelson DC, Scaffidi A, Flematti GR, Sun YK, Dixon KW, Smith SM (2012b) Specialisation within the DWARF14 protein family confers distinct responses to karrikins and strigolactones in *Arabidopsis*. Development **139**: 1285–95

Waters MT, Scaffidi A, Moulin SLY, Sun YK, Flematti GR, Smith SM (2015) A *Selaginella moellendorffii* Ortholog of *KARRIKIN INSENSITIVE2* Functions in Arabidopsis Development but Cannot Mediate Responses to Karrikins or Strigolactones. Plant Cell **27**: 1–21

Werner T, Motyka V, Laucou V, Smets R, Onckelen H Van, Schmuelling T (2003) Cytokinin-Deficient Transgenic Arabidopsis Plants Show Functions of Cytokinins in the Regulation of Shoot and Root Meristem Activity. Plant Cell **15**: 2532–2550

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. Plant Cell **13**: 1779–90

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot **95**: 707–35

Xie X, Yoneyama K, Harada Y, Fusegi N, Yamada Y, Ito S, Yokota T, Takeuchi Y, Yoneyama K (2009) Fabacyl acetate, a germination stimulant for root parasitic plants from *Pisum sativum*. Phytochemistry **70**: 211–5

Xie X, Yoneyama K, Yoneyama K (2010) The strigolactone story. Annu Rev Phytopathol **48**: 93-117

Yamada Y, Furusawa S, Nagasaka S, Shimomura K, Yamaguchi S, Umehara M (2014) Strigolactone signaling regulates rice leaf senescence in response to a phosphate deficiency. Planta **240**: 399–408

Yamaguchi S, Kyozuka J (2010) Branching hormone is busy both underground and overground. Plant Cell Physiol **51**: 1091–4

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci **10**: 615–620

Yan H, Saika H, Maekawa M, Takamure I, Tsutsumi N, Kyozuka J, Nakazono M (2007) Rice tillering dwarf mutant *dwarf3* has increased leaf longevity during darkness-induced senescence or hydrogen peroxide-induced cell death. Genes Genet Syst **82**: 361–366

Yoneyama K, Awad A a, Xie X, Yoneyama K, Takeuchi Y (2010) Strigolactones as germination stimulants for root parasitic plants. Plant Cell Physiol **51**: 1095–103

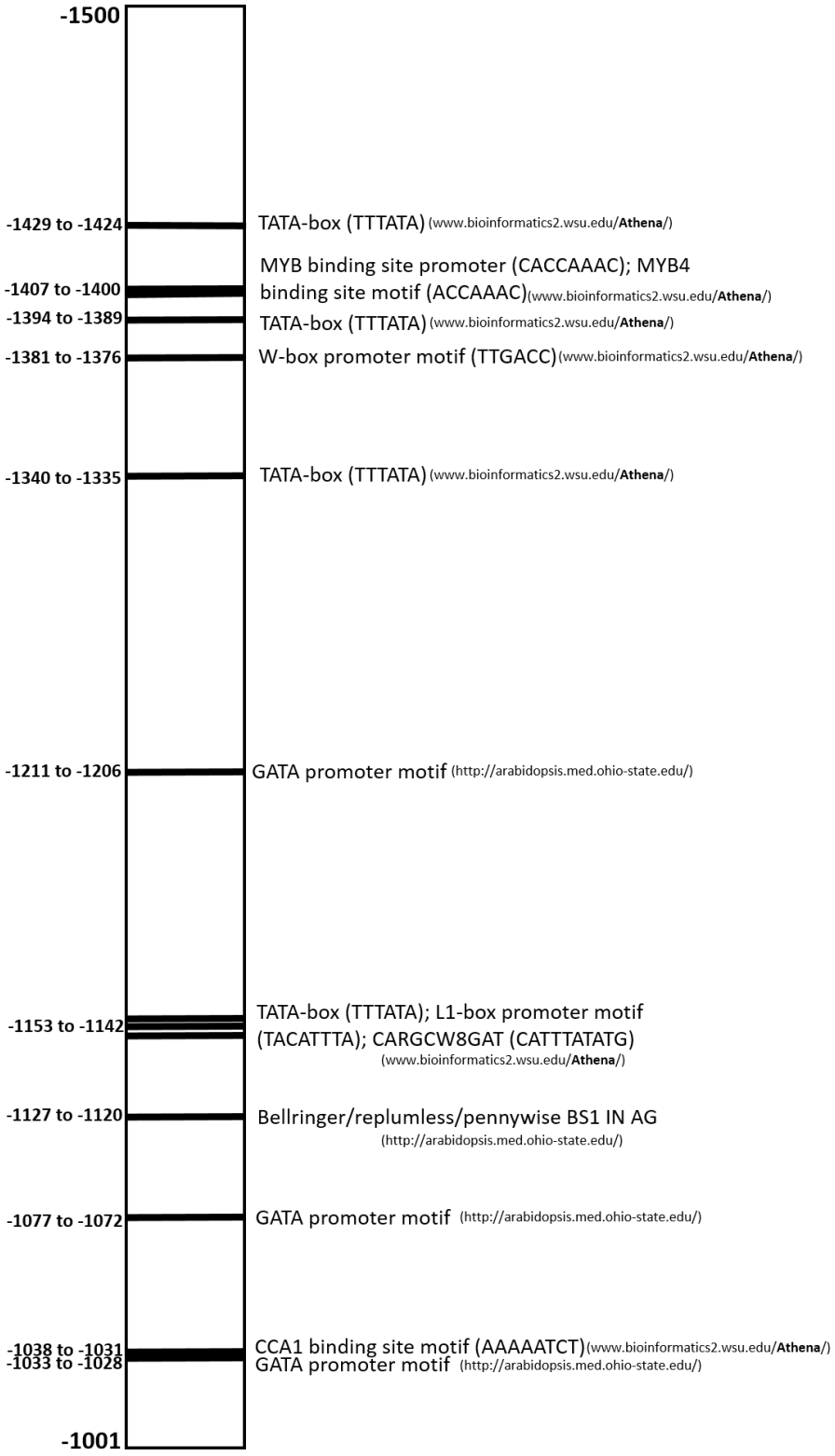
Yoneyama K, Xie X, Yoneyama K, Takeuchi Y (2009) Strigolactones: structures and biological activities. Pest Management Science **65**: 467–470

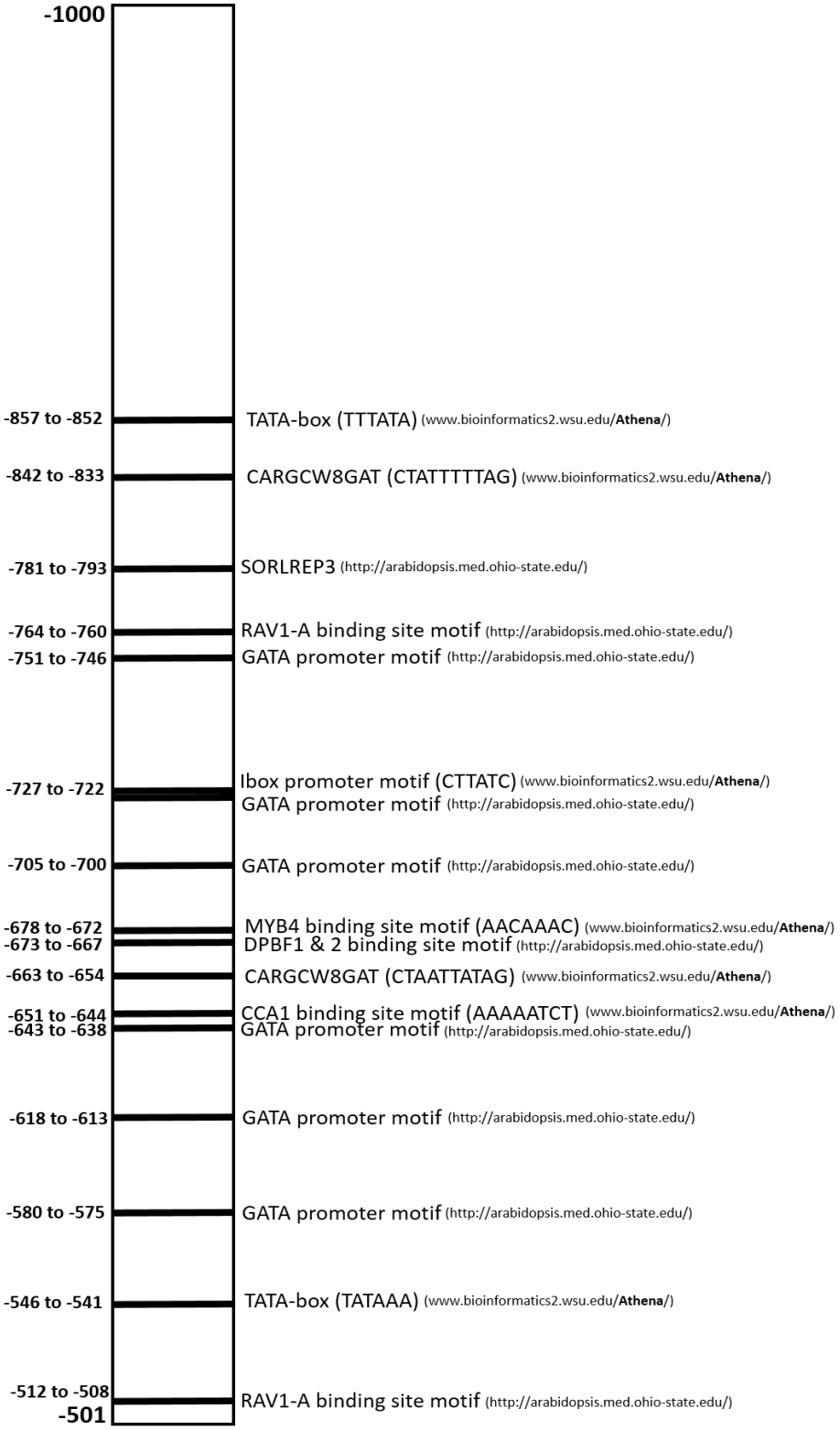
Zhou F, Lin Q, Zhu L, Ren Y, Zhou K, Shabek N, Wu F, Mao H, Dong W, Gan L, *et al* (2013) D14-SCF(D3)-dependent degradation of D53 regulates strigolactone signalling. Nature **504**: 406–10

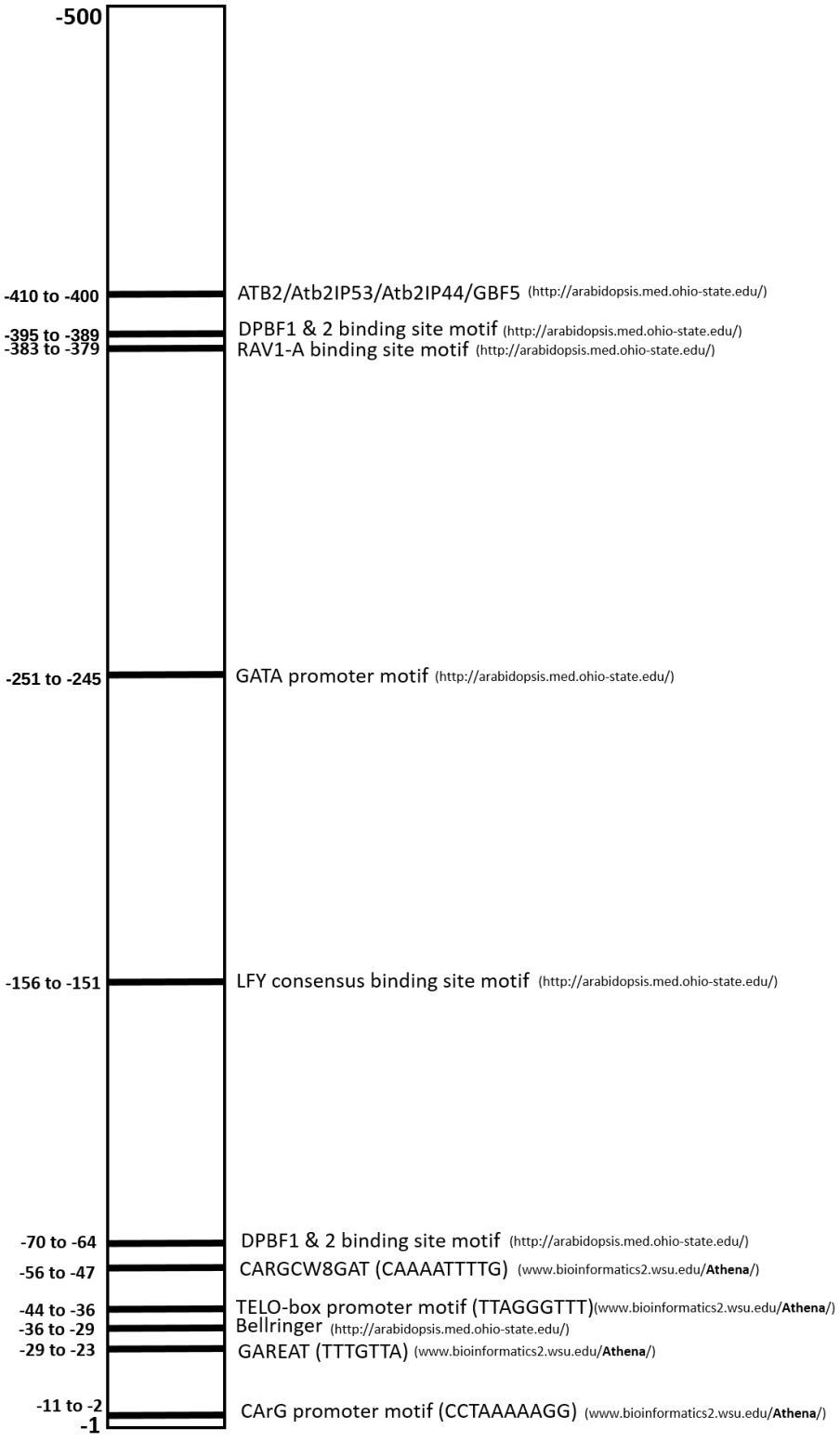
Zwanenburg B, Mwakaboko AS, Reizelman A, Anilkumar G, Sethumadhavan D (2009) Structure and function of natural and synthetic signalling molecules in parasitic weed germination. Pest Management Science **65**: 478–491

Zwanenburg B, Pospísil T (2013) Structure and activity of strigolactones: new plant hormones with a rich future. Mol Plant **6**: 38–62

Addendum A: Promoter diagram of the 1500 bp upstream of *AtBRC1*







Addendum B: Explanations of some of the putative motifs in the upstream region of *At3g18550*

-1500 bp to -991 bp immediately upstream of the starting codon (ATG) of *At3g18550*

TATA-box (4x)

Usually situated 25-35 base-pairs downstream of the start site of a gene (http://www.nature.com/scitable/). The TATA-box DNA motif occur very abundantly in promoter sequences of all types of organisms.

MYB binding-site

MYB-proteins are a large family of transcription factors that are known to control development, metabolism and stress (both biotic and abiotic) responses in plants (Dubos *et al*., 2010).

MYB4 binding-site

The MYB4 protein is a repressor of transcription and is involved in the regulation of UV-protection. This protein is responsive to jasmonic- and salicylic-acid (Chen *et al*., 2002).

W-box

Two W-boxes is found in the promoter sequence of a salicylic acid responsive gene (Rocher *et al*., 2005) and specifically recognized by salicylic acid-induced WRKY DNA binding proteins (http://bioinfo.cau.edu.cn/ProFITS/).

GATA promoter motif (3X)

This promoter element is known to be responsive to light and found in many light responsive genes (Reyes *et al*., 2004). This was to be expected, due to the fact that *BRC1* is a light responsive gene.

L1-box

This promoter element is found within the promoter of *AtPDF1*, involved in L1 layer-specific expression (http://bioinfo.cau.edu.cn/ProFITS/).

CARGCW8GAT

This promoter element provides a binding site for AGL15 (AGAMOUS-like 15), a MADS domain protein that is highly expressed directly after germination during embryo development (Tang and Perry, 2003).

Bellringer/replumless/pennywise BS1 IN AG

In a study by Bao *et al*., (2004), it was proposed that expression of the *BELLRINGER* gene represses *AGAMOUS,* a gene responsible for the formation of stamens and carpels in flowers.

CCA1 binding site

The CCA1 (Circadian associated 1) protein is closely related to the MYB-transcription factor. (http://bioinfo.cau.edu.cn/ProFITS/). This protein plays a central role alongside MYB-transcription factors in the very complex circadian clock.

-990 bp to -501 bp immediately upstream of the starting codon (ATG) of *At3g18550*

TATA-box (2X)

Usually situated 25-35 base-pairs downstream of the start site of a gene (http://www.nature.com/scitable/). Very abundant sequence found in promoter sequences.

CARGCW8GAT (2X)

This promoter element provides a binding site for AGL15 (AGAMOUS-like 15), a MADS domain protein that is highly expressed directly after germination during embryo development (Tang and Perry, 2003).

SORLREP3

This motif is over-represented in promoters that is repressed by light (http://bioinfo.cau.edu.cn/ProFITS/).

RAV1-A binding site motif (2X)

Binding site for the RAV1-A protein that is highly expressed in the rosette leaves and roots (http://bioinfo.cau.edu.cn/ProFITS/). RAV1-A is implicated in lateral root and leaf development, playing a role in ethylene-signalling, negative regulation of flower development and is down-regulated in response to brassinosteroids and zeatin (http://www.uniprot.org/uniprot/).

GATA promoter motif (6X)

This promoter element is known to be responsive to light and found in many light responsive genes (Reyes *et al*., 2004).

Ibox promoter motif

Found in the promoter sequence of light-regulated genes and is a binding-site for LeMYB1, a MYB-like protein (http://bioinfo.cau.edu.cn/ProFITS/). This conserved domain also plays a role in circadian control.

MYB4 binding-site

The MYB4 protein is a repressor of transcription and is involved in the regulation of UV-protection. This protein is responsive to jasmonic- and salicylic-acid (Chen *et al*., 2002).

DPBF1 & 2 binding site motif

Binding site for the putative transcription factor family bZIP (http://bioinfo.cau.edu.cn/ProFITS/). The Basic Leucine Zipper Domain (bZIP) family, contains transcription factors that are known to regulate pathogen defence, light and stress responses, flower development and seed maturation. The specific bZIP transcription factor that binds to DPBF2 is coded by *At3g44460*, also known as *ATBZIP67. ATBZIP67* is expressed in the cotyledon of *Arabidopsis thaliana* and responds to xenobiotic stimuli (https://www.arabidopsis.org).

CCA1 binding site

The CCA1 (Circadian associated 1) protein is closely related to the MYB-transcription factor. (http://bioinfo.cau.edu.cn/ProFITS/). This protein plays a central role alongside MYB-transcription factors in the very complex circadian clock.

-500 bp to the starting codon (ATG) of *At3g18550*

ATB2/Atb2IP53/Atb2IP44/GBF5

Found in the promoter region of proline dehydrogenase (ProDH) gene (http://bioinfo.cau.edu.cn/ProFITS/).

DPBF1 & 2 binding site motif (2X)

Binding site for the putative transcription factor family bZIP (http://bioinfo.cau.edu.cn/ProFITS/).

RAV1-A binding site motif

Binding site for the RAV1-A protein that is highly expressed in the rosette leaves and roots (http://bioinfo.cau.edu.cn/ProFITS/).

GATA promoter motif

This promoter element is known to be responsive to light and found in many light responsive genes (Reyes *et al*., 2004).

CARGCW8GAT

This promoter element provides a binding site for AGL15 (AGAMOUS-like 15), a MADS domain protein that is highly expressed directly after germination during embryo development (Tang and Perry, 2003).

TELO-box promoter motif

Found in the eEF1AA1 gene promoter. The presence of this motif is required to activate expression in the root primordial (http://bioinfo.cau.edu.cn/ProFITS/).

Bellringer

In a study by Bao *et al*. (2004), it was proposed that expression of the *BELLRINGER* gene represses *AGAMOUS,* a gene responsible for the formation of stamens and carpels in flowers.

GAREAT

A gibberellin-responsive element.

CArG promoter motif

Also recognized by MADS box transcription factors (Spensley *et al*., 2009).