The Arabidopsis *GolS1* promotor as a potential biosensor for heat stress and fungal infection?

by

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Abstract

Galactinol (Gol) has classically been considered to serve as a galactose donor during the biosynthesis of raffinose family oligosaccharides (RFOs). These sucrosyl oligosaccharides have been well characterised in their roles in carbon translocation and storage and, abiotic stress protection in plants. However, recent findings have demonstrated Gol to be an efficient free radical scavenger and it has also been suggested to act as signalling molecule during induced systemic resistance (ISR), upon pathogen infection. Collectively, these findings centres to the involvement of only a single galactinol synthase gene (*GolS*, synthesising Gol) in Arabidopsis (*AtGolS1*, At2g47180). The *AtGolS1* isoform has been shown to be transcriptionally up-regulated during heat stress and *Botrytis cinerea* infection. Further, it is also responsive to jasmonic acid, a key component of the ISR pathway. Here we targeted the *AtGolS1* promotor containing well defined heat shock transcription factor elements and a single putative jasmonate binding element, to develop a dual-functional biosensor with the ability to detect both heat stress and *Botrytis cinerea* infection. We created transgenic Arabidopsis lines where the reporter genes β-glucuronidase (GUS) and the green florescent protein (GFP) were under the control of the *AtGolS1* promotor. Using the native *AtGolS1* gene as a point of reference, we confirmed that the reporter genes were transcriptionally responsive to both heat stress and methyl jasmonate treatment in transgenic Arabidopsis. Under the same experimental conditions, both GUS assays and GFP imaging correlated with these transcriptional responses. Finally, we infected the transgenic lines with *Botrytis cinerea* infections to analyse reporter activity. Transcript analysis of transgenic lines clearly showed an increase in transcript abundance for both the native *AtGolS1* and the reporter genes in reponse to *B. cinerea* infection. Similarly, reporter assays revealed a distinct difference in activity between infected and uninfected plants from 24h to 96h after *Botrytis cinerea*

infection. These results provide sufficient proof-of-concept for the *AtGolS1* promotor to be used as a dual functional biosensor for both heat stress and fungal infection.

Opsomming

Galaktinol (Gol) is aanvanklik beskou as ’n galaktose skenker tydens die biosintese van raffinose familie van oligosakkariede (RFO). Hierdie sukrosiel oligosakkariede is goed gekenmerk vir hul funksies in koolstof translokasie en storing, sowel as die beskerming teen abiotiese stres in plante. Onlangse bevindinge het Gol geklassifiseer as 'n doeltreffende vry radikaal werwer, en is voorgestel om op te tree as 'n sein molekule tydens geïnduseerde sistemiese weerstand (ISR), tydens patogeen infeksie. Gesamentlik plaas hierdie bevindinge klem op die betrokkenheid van 'n enkele galaktinol sintase geen (*GolS*, sintetiseer Gol) in Arabidopsis (*AtGolS1*, At2g47180). Dit is voorheen bewys dat die *AtGolS1* isoform transkripsioneel op-gereguleer word tydens hitte-stres en *Botrytis cinerea* infeksie. Verder is dit ook sensitief vir jasmijnsuur, 'n belangrike komponent van die ISR pad. Gedurende hierdie studie het ons die *AtGolS1* promotor geteiken, wat die goed gedefinieërde hitte-skok transkripsie faktor bindings elemente en 'n enkele vermeende jasmijnsuur bindings element bevat, om 'n dubbele-funksionele biosensor te ontwikkel met die vermoë om beide hitte-stres en *Botrytis cinerea* infeksie op te spoor. Ons het transgeniese Arabidopsis lyne gegenereer waar die rapporteerder gene β--glukuronidase (GUS) en die groen fluoressent proteïen (GFP) onder die beheer van die *AtGolS1* promotor is. Deur gebruik te maak van die inheemse *AtGolS1* geen as 'n verwysingspunt, het ons bevestig dat die rapporteerder gene op ‘n transkriptionele vlak reageer op beide hitte-stres en metiel jasmijnsuur behandeling in transgeniese Arabidopsis. Onder dieselfde eksperimentele kondisies het beide GUS toetse en GFP fotografie gekorreleer met die transkripsie analise. Ten slotte, het ons die transgeniese lyne aan *Botrytis cinerea* infeksies blootgetel om die rapporteerder aktiwiteit te ontleed.

Transkripsie analise van transgeniese lyne het ’n duidelik toename in transkripsie vlakke getoon vir beide die plaaslike *AtGolS1* geen en die rapporteerder gene in reaksie op *B. cinerea* infeksie. Eenders, het rapporteerder toetse 'n duidelike toename in aktiwiteit tussen geïnfekteerde en ongeïnflekteerde plante getoon vanaf 24 h tot 96 h na *Botrytis cinerea* infeksie. Hierdie resultate bied voldoende bewys-van-konsep vir die *AtGolS1* promotor om gebruik te word as 'n dubbele funksionele biosensor vir beide hitte-stres en swam infeksie.

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Abbreviations

At	*Arabidopsis thaliana*
CBF3	C-repeat/DRE Binding Factor 1
cDNA	complementary DNA
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
FP	fluorescence Proteins
gDNA	genomic DNA
GFP	Green Fluorescence Protein
Gol	galactinol
GolS	galactinol synthase
GUS	β-glucuronidase
h	hours
HS	heat shock
HsfA	heat shock factor A
HSP	heat shock protein
HSF	heat shock transcription factor

ISR	induced systemic resistance
JA	jasmonic acid
kb	kilobase
MeJA	methyl jasmonate
MS	Murashige and Skoog
OA	osmotic adjustment
PCR	polymerase-chain-reaction
pGS	AtGolS1 promotor
PR	pathogenesis related
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
Raf	raffinose
RafS	raffinose synthase
RFOs	raffinose family oligosaccharides
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase-polymerase-chain-reaction
SAR	systemic induced resistance

SA	salicylic acid
SDS	sodiumdodecylsulphate
SS	stachyose synthase
SWEET	sugars will eventually be exported transporters
sqRT-PCR	Semi quantitative real time polymerease chain reaction
v	volume
w	weight
WT	Wild-type

Introduction

Plants experience a myriad of abiotic and biotic stresses throughout their life span. These vary in frequency and magnitude but nevertheless exposes the plant to unfavourable growth conditions, disrupting metabolic synergy (Bolton, 2009; Heil et al., 2002; Massad et al., 2012; Swarbrick et al., 2006). Ultimately the physiological manifestation of stress is the consequent reduction in fitness and output (Rejeb et al., 2014; Shao et al., 2008). In an agricultural context abiotic stress severely impacts plant growth and development and causes severe losses in crop production, often up to 50% reduction in yield (Wang et al., 2003; Rejeb et al., 2014). Commercial scale agriculture also leads to increased frequencies of biotic stress episodes such as pathogen infections and herbivory further compounding yield problems (Brown et al., 2002; Maron et al., 2006; Mordecai, 2011; Rejeb et al., 2014). While not as well reported as other abiotic stresses (e.g. drought and high salinity), heat stress is considered to be amongst the major abiotic stresses that lead to yield reductions in several crop species (Rienth et al., 2014; Pillet et al., 2012; Geatan, 2005; Kayum et al., 2016). Similarly, the necrotrophic fungal pathogen *Botrytis cinerea* is one of the most devastating biotic stresses experienced in commercial crops, causing severe damages and economic losses in over 200 plant species (Jarvis et al., 1977). A potential solution to improve crop management strategies in this regard is the development and use of molecular "biosensors" that act as an early warning system to stress episodes. This study considered the use of an Arabidopsis promotor to develop a dual functional plant based bio-sensor for both heat stress and fungal pathogen infection.

1.1 Temperature stress induces significant physiological changes in plants

Temperature is one of the major abiotic stress factors influencing plant growth and development and, due to climate change it is expected to increase significantly (Pillet et al., 2012). Acclimation of plants to both low- and high-temperature induces marked physiological responses in plants which, include signaling pathways, activated gene expression and ultimately leads to metabolic and/or biochemical changes (Stockinger et al., 1997; Gilmour et al., 1998; Haake et al., 2002; Panikulangara et al., 2004).

During temperature stresses, the photosynthetic processes are influenced the most in plants (Allakhverdiev et al., 2008). Several biochemical changes which are associated with low-temperature acclimation in Arabidopsis are related to the function of the *C*-repeat/DRE Binding Factor 1 (CBF3/DREB1) protein. Over-expression of this transcription factor in Arabidopsis leads to an increase in the levels of osmoprotective substances such as proline, sucrose, raffinose (Raf), glucose and fructose resulting in plants which are more resistant to both low temperature and drought stresses (Gilmour et al., 2000). Presumably these molecules function in osmotic adjustment (OA) to combat sub-cellular water deficit that is associated with these stresses.

During heat stress however, resistance/tolerance is associated with the expression of heat shock proteins (HSP). These proteins act as molecular chaperones, effectively protecting proteins from denaturation or, targeting stress-damaged proteins for degradation thereby conserving the metabolic integrity of cells (Panikulangara et al., 2004). The HSPs are regulated on a transcriptional level, through the heat-stress-dependant activation of transcription factors called heat shock transcription factors (HSFs, Panikulangara et al., 2004; Busch et al., 2005, Nishizawa et al., 2006, Schramm et al., 2006). These HSFs binds to a conserved heat shock binding elements (HSE) in the promotor regions upstream of heat stress

related genes. Nover et al., (2001) identified 21 different HSF genes in Arabidopsis. The genes *AtHSF1* and *AtHSF3* were shown to be rapid response regulators which are involved in the immediate early transcription of multiple heat stress genes (Lohmann et al., 2004). In contrast, over-expression of the *AtHSF3* in *Arabidopsis* showed low level HSP synthesis under normal temperatures, with an increased thermotolerance (Prändl et al., 1998). These findings have supported that HSPs play a critical role in protection against heat stress.

1.2 The biotic stress responses in plants are linked to classical phytohormones.

Plants have developed various mechanisms by which they defend themselves upon pathogen infections. They can induce resistance to pathogens and predators (herbivores) prior to significant infection/predation, that go beyond their physical barriers (the cell wall), upon the appropriate stimulus (Kim et al., 2008). This facilitates plants to effectively "prepare for" and defend themselves against breaches of the cell wall associated with pathogen infection and predation by herbivores. These general responses are associated with the production of several compounds which reduce and inhibit further attack and spread of infection.

The interaction between pathogens and the host plant can either lead to susceptibility (compatible response) or resistance (incompatible response) (Ryals et al., 1996). During resistance or incompatible interactions between plants and pathogens, a set of localized responses will be induced in and around the infected cells of the host. These responses usually lead to cell death (Kombrink et al., 2001) through the phenomenon known as the hyper-sensitive response (Lamb and Dixon, 1997). This allows plants to prevent the spread of infections through apoptosis of the cells surrounding the infected area.

1.2.1 Classical phytohormones elicit the expression of pathogen response genes

Subsequent to the initial stimulus (infection/predation), surrounding cells undergo responses which can include (i) the synthesis of novel antimicrobial compounds, (ii) activation of several pathogen related (PR) genes and (iii) alterations in cell wall composition which can inhibit further penetration of pathogens (Derckel et al., 1999). Subsequent to – or due to - these local responses, changes in gene expression occur, which are induced by signals that spread throughout the plant from the infected regions towards the uninfected parts of the plant. This systemic response is associated largely with the transcriptional upregulation of PR proteins and phytoalexins (Neuhaus, 1999). While phytoalexins are known to only be involved in local responses, the PR proteins occur at both local and systemic levels (Zhou, 1998; Nawrath and Metraux, 1999; Gupta et al., 2000). Initially PR proteins were thought to be absent in healthy plants and their levels increased during periods of infection (van Loon et al., 1970). However, they have been since described in over 40 species from at least 13 families and appear to generally be present (at low levels) during normal growth conditions (Nawrath and Metraux, 1999; Neuhaus, 1999; van Loon., 1999).

Two types of induced resistance have been characterized to date *viz*. induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Fig. 1) (Ryals et al., 1996; Van Loon et al., 1998). Both these pathways rely mainly on the signalling molecules salicylic acid (SA), ethylene (ET) and jasmonic acid (JA), coupled with several response genes (Thomma et al., 1998; Guzman and Ecker, 1990; Staswick and Tiryaki, 2004) activated upon infection to provide resistance (Fig. 1). The SAR pathway is unique due to an early synthesis of endogenous SA and the activation of several SAR response genes (Ryals et al., 1996). This pathway is systemic, thus spreading from the site of infection throughout the plant (Ton et

al., 2002). SAR is usually activated when a plant is infected by a non-lethal pathogen (also known as non-necrotrophic). It has been demonstrated that the exogenous application of SA, leads to the activation of several pathogen related genes (PR) genes (Ryals, 1996). Interestingly, of the several PR genes involved during pathogen infection, only *PR3* is known to be activated by JA during ISR (Schweizer et al., 1998; Zheng et al., 2006). Salicylic acid is not considered to act as the signalling molecule during SAR, although it is necessary for the activation of this pathway (Vernooij et al., 1994). Signalling in this pathway is believed to be mediated by means of sugar-signalling pathways in the plant (Vlot et al., 2008; Wingler and Roitsch, 2008).

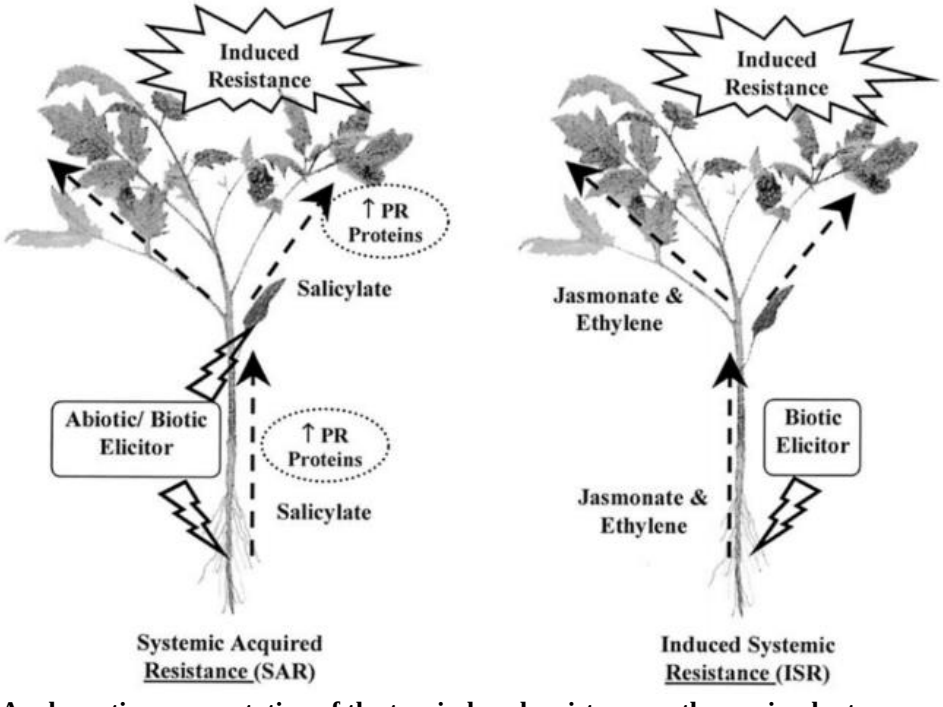

Figure 1: A schematic representation of the two induced resistance pathways in plants.

Systemic acquired resistance (SAR), relies on salicylic acid as a signalling molecule, and is activated by abiotic and biotic elicitors. SAR is associated with the accumulation of pathogenesis-related (PR) proteins. Induced systemic resistance (ISR) relies on jasmonic acid and ethylene as the signalling molecules. Although the two pathways use different signalling molecules, they overlap on a molecular level to control gene expression of the classical PR genes. From Vallad and Goodman (2004).

ISR mediated immunity is targeted to the site of infection (van Loon et al., 1998). This defence pathway is activated upon lethal (necrotrophic) pathogen infection, and largely uses JA as signalling molecule (Fig. 1) but, ethylene (ET) has also been demonstrated to accumulate with JA (Thomma et al., 1998; Ton et al., 2002). Regulation of the ISR pathway was established using Arabidopsis mutants (Knoester et al., 1999; Ton et al., 2002), where either the JA or ET biosynthetic pathways were disrupted. In these studies, it was shown that both JA and ET mutants were more susceptible to pathogen infection than wild type plants, firmly placing both these phytohormones as key facilitators of ISR.

2. Carbohydrates and their role in environmental stress

Apart from their function as prime energy and carbon sources in virtually all cells, carbohydrates serve critical regulatory roles in metabolism, growth and development and stress resistance (Gibson, 2005; Rolland et al., 2006). The importance of sugars and sugar signalling during environmentally challenging conditions have been studied to great depths (Gibson, 2005; Koch, 2004; Leon, 2003; Rolland et al., 2003; Vijn and Smeekens, 2000), highlighting the importance of carbohydrates under these conditions.

2.1 Carbohydrates play pivotal roles during both abiotic and biotic stress

Environmental stress factors affect plants negatively at both physiological and biochemical level leading to impaired growth and lowered yields. Unfavourable environmental conditions limit the plants access to the necessary growth requirements, therefore genetic and physiological compensations are made to allow basic survival of plants under these conditions (Mahajan and Tuteja, 2005). Plants experience several stress conditions including drought,

low temperature, heat, and oxidative stress on regular basis. All these factors influence the ability of plants to reach their full genetic and physiological potential, thus limiting the production of crops worldwide (Mahajan and Tuteja, 2005). Stresses occurring in nature are usually not in isolation, and several of these stresses can occur in a synchronised manner with each other. Upon perception of these stresses, several plant responses are induced which leads to the activation of signalling pathways, and changes in gene expression levels. These pathways combine in a cooperative manner to relieve and tolerate these stresses (Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki et al., 2006; Yamaguchi et al., 2005).

Plants synthesize phyto-hormones, reactive oxygen species, transcription factors and compatible solutes when faced with biotic and abiotic stresses to account for the damaging effects caused by these stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). Compatible solutes are thought to be one of the most important components during stress response mechanisms, as their accumulation (often to high intracellular concentrations) does not disrupt normal metabolic processes of the cell. Among these solutes are quaternary compounds, amino acids, and numerous sugars (Mahajan and Tuteja, 2005). The accumulation of soluble sugars during stress conditions, for instance the accumulation of raffinose family oligosaccharides (RFOs), are common under stress conditions, and is believed to serve multiple functions in carbon storage, membrane protection, free radical scavenging and osmotic adjustment. (Nishizawa et al., 2008; Van den Ende et al., 1996; Hoekstra et al., 2001).

2.2 Carbohydrates are involved in pathogen defence signalling

It is well known that sugars are involved in the defence mechanisms of plants during pathogen infection (Watson and Watson, 1951; Shalitin and Wolf, 2000). Studies have shown that in the absence of pathogen infection, sugars were able to regulate the expression of certain PR

genes (Herbers et al., 1996; Xiao et al., 2000). The expression of PR genes were inversely dependent on the level of hexose sugars in plants, suggesting that hexose sugars act as signalling sugars in the secretory pathway (Herbers et al., 1996). This was later confirmed by the ectopic expression of a cytosolic yeast derived invertase (catalysing the hydrolysis of sucrose) in tobacco plants that showed no activation of SAR in the presence of pathogen infection (Herbers et al., 1996). This led to the association between plant innate immunity and sugars (Rolland et al., 2006). The ability of sugars to act as signalling molecules during the physiological processes of plants is now well established (Rolland et al., 2006; Bolouri-Moghaddam et al., 2010). Hexose sugars such as glucose and fructose together with sucrose have been reported to be involved in the regulation of gene expression during carbon assimilation, hormone accumulation and the developmental and growth stages of plants (Moore et al., 2003; Cho et al., 2009; Koch 2004; Rolland et al., 2006; Tognetti et al., 2013).

It is known that a plants susceptibility to infection depends on the sugar content of its leaves (Horsfall and Dimond, 1957; Herbers et al., 1996). More recently it has been suggested that sugar-signalling pathways play a role in the defence responses in plants (Moghaddam and Van den Ende, 2012). Heil et al. (2012) showed that exogenous application of sucrose activated the ISR (JA mediated response) pathway. This led to the novel phenomenon called sweet immunity or sugar enhanced defence (Sonnewald et al., 2012; Bolouri Moghaddam and Van den Ende, 2012). During this defence approach, it is believed that sucrose gets transported actively towards the area of infection to account for the impaired photosynthetic ability. Opposing views about the exact function of SWEETs (Sugars Will Eventually be Exported Transporters) exist, as some results indicated resistance against pathogen infections in rice SWEETs loss-of-function mutants (Chen, 2014). This contradicts with the hypothesis that sucrose is required during the management of biotic stress (Lapin et al., 2013).

Nevertheless, sugars such as glucose, fructose, sucrose, trehalose, RFOs, and fructans have been shown to act as signalling molecules during pathogen infections in plants (Rolland et al., 2002; Rolland et al., 2006; Kim et al., 2008; Moghaddam and Van den Ende, 2012).

3. Raffinose family oligosaccharides (RFOs) are plant specific galacto-oligosaccharides with multiple physiological roles

The RFOs are a group of well-studied carbohydrates that represent galactose extensions of sucrose. Their accumulation in higher plants has been associated to a number of fundamental physiological functions which include (i) carbon transport in the phloem, (ii) carbon storage in sink tissues (roots, tubers and seeds) and (iii) potential roles in stress induced OA (Sprenger and Keller, 2000; Taji et al., 2002; Nishizawa-Yokoi et al., 2008; Elsayed et al., 2014; Blöchl et al., 2008; Angelovici et al., 2010). The precise mechanism/s by which RFOs exert their protective effects during stress are unclear.

3.1 RFO biosynthesis is a multi-enzymatic process

The biosynthesis of RFOs occurs by the stepwise transfer of galactosyl units from a suitable galactosyl donor to the acceptor molecule (Fig. 2) (Lehle and Tanner, 1973; Martinez-Villaluenga et al., 2008). The galactosyl donor is the unusual carbohydrate-cyclitol hybrid galactinol (Gol). Galactinol synthase (GolS, E.C 2.4.1.123) is responsible for Gol biosynthesis using UDP-galactose and *myo*-inositol as substrates (Lehle and Tanner, 1973; Martínez-Villaluenga et al. 2008). Subsequently, raffinose synthase (RS, E.C 2.4.1.82) is responsible for Raf (Suc-Gal_1, Fig. 2) biosynthesis using sucrose (Suc) and Gol as substrates. Stachyose synthase (SS, E.C. 2.4.1.67) synthesises Sta (Suc-Gal_2, Fig. 2) using Raf and Gol as substrates. Higher RFO oligomers (Suc-Gal_{3-15}) are synthesised by the unique

Gol-independent enzyme galactan:galactan galactosyl tranferase (GGT) which, uses RFOs as both Gol donors and acceptors (Bachmann and Keller, 1995; Haab and Keller, 2002; Tapernoux-Luthi and Keller, 2004).

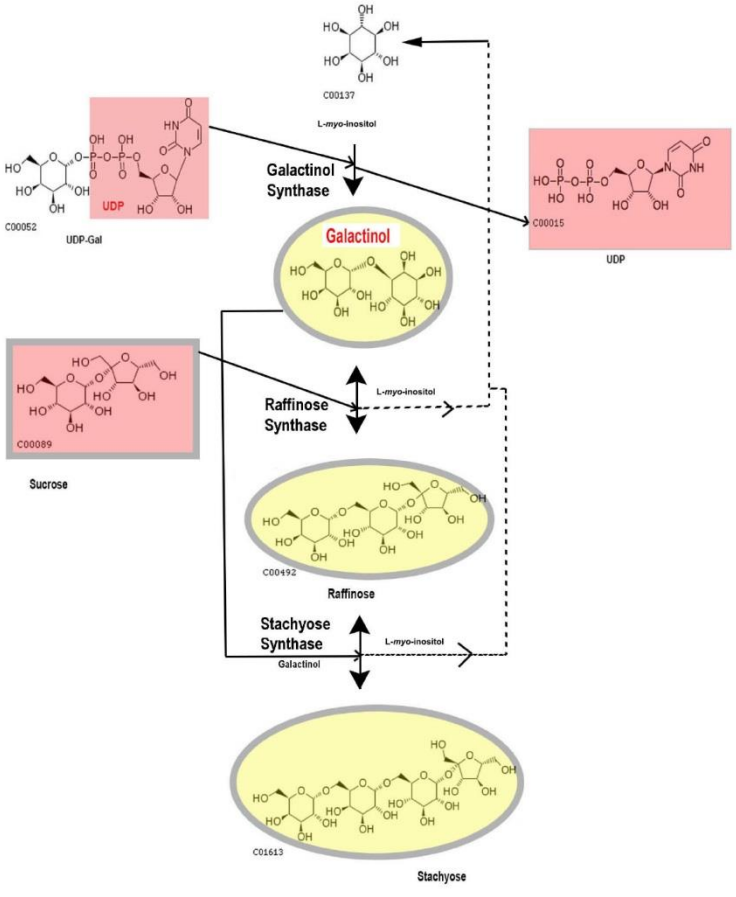

Figure 2: Schematic representation of the general RFO biosynthetic pathway in plants.

The first committed step in the synthesis of these galacto-oligosaccharides is the production of the galactose donor (galactinol) through the catalytic activity of galactinol synthase. Subsequently raffinose synthase and stachyose synthase catalyse the synthesis of the tri-saccharide raffinose and the tetra-saccharide stachyose. From Sengupta et al. (2015).

Both Gol and *myo*-inositol have been considered as key regulatory points in the RFO biosynthetic pathway (Elsayed et al., 2013). Evidence in this regard has reported Gol to be a key regulator in RFO synthesis in several plant species such as soybean (Handley et al., 1983; Saravitz et al., 1987) and cucumber (Handley et al., 1983). Similarly, *myo*-inositol 1-

phosphate synthase, the enzyme involved in the synthesis of *myo*-inositol have been shown to be a regulator of both Gol and Raf levels in plants (Keller and Pharr, 1996; Kellet et al., 1998, Lener et al., 2008). Collectively this shows the importance of the Gol synthesizing step during the RFO pathway.

3.1.1 The RFOs accumulate in response to abiotic stress

RFOs are known to serve a protective function during several abiotic stress conditions (Saravitz et al. 1987; Nakanishi et al. 1989; Hoekstra et al. 1997; Nelson, 1999; Sheveleva et al. 1997; Panikulangara et al. 2004; Sengupta et al. 2008). The enzymes involved in RFO biosynthesis have been studied to great extent under abiotic stress conditions such as heat (Panikulangara et al., 2004), desiccation (Taji et al., 2002; Zuther et al., 2004; Peters et al., 2007), cold (Bachman et al., 1995; Sprenger and Keller, 2000; Peters and Keller, 2009), and oxidative stress (Nishizawa et al., 2008).

RFOs have been identified to act as antioxidants which neutralise reactive oxygen species (ROS) build-up during stresses (Nishizawa et al. 2008; Van den Ende & Valluru 2009; Bolouri-Moghaddam et al. 2010; Van den Ende et al. 2011; Stoyanova et al. 2011; Peshev et al. 2013). Transgenic Arabidopsis plants that over-expressed the *AtGolS1*, *AtGolS2* and the heat-shock transcription factor (*HsfA*) showed tolerance against heat-induced oxidative stress when compared to wild-type plants. This observation was also associated with increased expression of *GolS* genes and the accumulation of both Gol and Raf. The accumulation of RFOs, specifically Raf, during chilling is the abiotic stress condition studied the most in Arabidopsis to date. A study conducted on rice showed that chilling treatment for an extensive period of time increased the levels of both Gol and Raf immensely (Saito and Yoshida, 2011). It has also been shown that cold-induced Raf accumulation in the chloroplast serves a protective function by stabilising photosystem II in Arabidopsis when freeze-thaw cycles

occur (Schneider & Keller 2009; Foyer & Shigeoka 2011; Knaupp et al. 2011). Although it is suggested that RFOs serves as osmoprotectants during cold stress (Bachman et al., 1995) and desiccation (Koster & Leopold 1988), they might only serve as a way in which plants store carbon within the vacuole (Gilbert et al., 1997).

In a study to identify novel target genes that are regulated by HSFs, *AtGolS1* mRNA was observed in the leaves of transgenic Arabidopsis plants over-expressing *AtHSF3*, in which HSP synthesis occur at normal temperatures (Panikulangara et al., 2004). This was compared to WT Arabidopsis plants grown at normal temperatures, where transcript levels of *AtGolS1* were nearly undetectable. Using promotor::reporter gene expression they were able to confirm that *AtGolS1* is a novel HSF-dependant heat stress gene in Arabidopsis. To further support this finding, they showed that the levels of Raf increased in the leaves of wild type plants, but not in mutant *GolS1* lines when exposed to heat stress. Interestingly, the *VvGolS1* gene in grapevine is routinely used as a marker for heat stress (Pillet et al., 2012). Analysis of the promotor region directly upstream of the *AtGolS1* gene revealed several HSEs (Panikulangara et al., 2004).

3.1.2 Galactinol synthases occur as small multigene families in plants

The *GolS* enzyme as mentioned previously, catalyses the production of Gol, the first committed step in the RFO pathway (Fig. 2). Total RFO contents have been shown to be directly dependant on *GolS* activity in the seeds and leaves of several plant species (Handley et al., 1983; Saravitz et al., 1987). Thus, *GolS* genes have frequently been used as an experimental tool (over-expression and knock-out strategies) to study the effect of RFO levels on abiotic and biotic stress tolerance (Taji et al., 2002; Panikulangara et al., 2004; Nishizawa et al., 2008; Kim et al., 2004; Cho et al., 2010). Further, *GolS* genes and their involvement (transcriptional up-regulation and enzyme activity increases) in abiotic and biotic stress have

been studied in several plant species such as *A. thaliana* (Taji et al., 2002; Panikulangara et al., 2004), *Xerophyta viscosa* (Peters et al., 2007), and *Ajuga reptans* (Sprenger and Keller, 2000).

A total of seven *GolS* isoforms have been identified in Arabidopsis on the basis of a unique C-terminal pentapeptide conserved sequence (APSAA) of GolSs (Taji et al., 2002 and Nishizawa et al., 2008). From extensive studies in Arabidopsis and other RFO accumulating higher plants, it is well established that specific differential up-regulation of these isoforms occurs in response to abtioic and biotc stress (Liu et al., 1998; Sprenger and Keller, 2000; Taji et al., 2002; Panikulangara et al., 2004; Blöchl et al., 2005; Kim et al., 2008). In Arabiodopsis, it is the *AtGolS1* and *2* genes that are specifically upregulated by osmotic stress (NaCl and drought, Taji et al., 2002). The *AtGolS1* isoform is further responsive to heat (Panikulangara et al., 2004) and oxidiative stress (Nishizawa et al., 2008). *AtGolS3* is uniquely upregulated only under conditions of low-temperature (Taji et al., 2002).

Thus it is clear that *GolS* genes not only play a key regulatory role in RFO biosynthesis but potentially also modulate the stress response through their differential expression patterns. This stress induced-modulation of *GolS* expression speaks to the occurrence of *cis*-elements in the promotors of these genes that lead to this differential expression. These *cis*-regulatory elements are controlled by several transcription factors that acts upstream in biotic and abiotic stress response pathways (Mahajan and Tuteja, 2005). These elements are used to predict possible gene functions according to the transcription factor binding elements in their promotors. Several of these binding elements have been identified in *GolS* genes such as the ABA responsive element (ABRE, Zhang et al., 2005), heat shock binding element (HSE, Panikulangara et al., 2004), low temperature responsive element (LTRE, Gao et al., 2002), and the dehydration and cold responsive element (DRE/CRT, Qin et al., 2004).

The historical role of Gol has been thought of strictly in the context of being the galactosyl donor in RFO biosynthesis. However, recent studies have challenged this view by demonstrating that it is also a very efficient free radical scavenger (Nishizawa et al., 2006; Nishizawa et al., 2008) and may have a very novel function as a signalling molecule during plant pathogen interaction (Kim et al., 2008; Cho et al., 2010). In these studies *GolS* overexpressing plants (cucumber, Kim et al., 2008 and Arabidopsis, Cho et al., 2010) were subsequently resistant to pathogen infection. Conversely an Arabidopsis *AtGolS1* mutant was sensitive (Cho et al., 2010), clearly suggesting a role for Gol and/or Raf in ISR-mediated pathogen interaction. However, the exact mechanism by which this may occur is unknown.

3.1.3 RFOs and galactinol as signalling molecules during biotic stress conditions

During pathogen induced-responses, the carbohydrate-cyclitol Gol has been suggested to act as a signalling molecule in ISR (Kim et al., 2004; Kim et al., 2008, Cho et al., 2010). Spencer et al., (2003) initially reported the elicitation of ISR by rhizobacterium *Pseudomonas chlororaphis O6* in both tobacco and cucumber plants by signifying protective effects against the foliar bacterial pathogens *Erwinia carotovora* subsp. *Carotovora* and *Pseudomonas syringae* pv. *tabaci.* In a study conducted by Kim et al. (2004), the *GolS* (*CsGolS1*) from cucumber (*Cucumis sativus L.*) were identified to be differentially expressed using a suppressive subtractive hybridization approach when plants were infected with the fungus *Corynespora cassiicola.*

The levels of *CsGolS1* expression and subsequent Gol content in plants increased when infected with *C. cassiicola* and several hours of O6 treatment (Kim et al., 2008). Transgenic tobacco plants over-expressing the *CsGolS1* gene, and a subsequent increase in Gol, showed resistance against the pathogen *B. cinerea* (Kim et al., 2008). These findings were

complimented when Arabidopsis mutants in the *AtGolS1* gene were more sensitive to *B. cinerea* infections, and transgenic tobacco plants over-expressing *AtGolS1* gene showed resistance (Cho et al., 2010). Exogenous application of Gol to wild-type tobacco plants showed enhanced resistance against infection as well as an increase in defence-related genes (Kim et al., 2008). These findings suggest that either Gol or RFOs may act as a molecular signal that activates the O6-mediated ISR in plants against fungal pathogens (Cho et al., 2010).

4. RFOs, heat stress, pathogen interaction and the development of a biosensor

4.1 What is a biosensor?

A biosensor is defined as the use of an entity to either detect or record a specific physiological change or process within a biological system, subsequently converting the event into a phenotypically visible response (Sadanandom and Napier, 2010). The development of biosensors that detect a specific signal, whether in biotechnological research, or for practical applications such as the detection of environmental toxins (Gil et al., 2000), and metabolite concentrations (Paige et al., 2012), has received considerable attention in recent years. Most biosensors rely on the specific interaction between a chemical or biological molecule with the biological "probe" utilised in the biosensor device.

In plants, genetically encoded biosensors (promotor::reporter) are mainly the preferred option due to the widespread success of this approach to date (Sadanandom and Napier, 2010). These genetic reporters have been successfully used for several years, specifically for the study of particular hormone interactions. The most commonly studied hormone via this approach is

auxins, by utilising the synthetic auxin sensitive promotor DR5 (Ulmasof et al., 1977). During the early stages of this approach, the β-glucuronidase (GUS) gene was the fusion gene of choice as it allowed researches to determine the position (specific tissue) and relative level of activation of a specific gene during set conditions. However, recently the focus has shifted towards optical based sensor systems that use fluorescence and bioluminescence proteins as the fusion partners (Sadanandom and Napier, 2010). For *in vivo* research based biosensors, the fluorescent proteins, mostly the green fluorescence protein (GFP) has become the reporter of choice in recent years (Ottenschlager et al., 2003; Meyer et al., 2007; Pagnusset et al., 2009).

Despite the use of these promotor-reporter fusion systems in research approaches, many industrial applications based attractions towards these systems has arisen. The main attraction towards these biosensors are their ability to be utilised without the need to puncture or damage the host cells, thus they can be analysed in real time, and that they can nowadays be driven by very specific and sensitive promotors. However, these promotors are not always perfect, as most of them are sensitive to several environmental stimuli. The second concern regarding this system, is the time-responsiveness of these promotors. From the induction to the actual functional reporter a time delay occurs, for instance the DR5::GFP could only be detected 1.5 hours after induction (Ottenschlager et al., 2003).

Several commercial biosensor approaches have been investigated in recent years, ranging from the detection of environmental toxins such as gas, using bioluminescence in bacteria (Gil et al., 2000) to the detection of explosives using the model plant *A. thaliana* (Nature, http://www.nature.com/news/2004/040129/full/news040126-10.html). Commercially used biosensors mainly rely on the activation of a promotor::reporter system (mainly a non-invasive reporter system) by a very specific stimuli, whether a chemical or a change in

environmental conditions (Sadanandom and Napier, 2010). One of the most promising examples recently is the utilisation of a GFP biosensor in Arabidopsis to detect reduced levels of oxygen on the International Space Station (Paul and Ferl, 2011). These promotors are being activated when the plant experience oxygen deprived conditions. This proves that biosensors are a very effective tool that can be utilised in the industry to detect a wide variety of stimuli.

4.2 Exploiting the *AtGolS1* promotor for a dual functional biosensor

As described above, the Arabidopsis *AtGolS1* gene is well described in the context of abiotic stress (Taji et al., 2002; Panikulangara et al., 2004) and most recently in biotic stress (Cho et al., 2010). Consequently, its transcriptional responses to both heat stress and pathogen infection in Arabidopsis, leads to the question as to whether the *AtGolS* promotor could serve as a dual reporter in the context of both heat stress and pathogen infection. To this end the *AtGolS* promotor has been shown to contain a number of heat shock binding elements (HSEs, Panikulangara et al., 2004). Only suggestive evidence exists that this promotor would be responsive to biotic stress (Cho et al., 2010). In that study both *B. cinerea* infection and exogenous JA application (mimicking ISR) led to *AtGolS1* expression.

The aim of this study was to develop a proof-of-concept dual functional biosensor by creating transgenic Arabidopsis (using the *AtGolS1* promotor fused to either GUS or GFP), which could then potentially detect (and respond to) both heat stress and fungal infection. This study provided a stepping stone for future applications in the grapevine industry, where both heat stress and *B. cinerea* causes extensive economical losses (See future applications p45).

Materials and methods

All chemicals utilised during this study were obtained from either SIGMA® (Steinheim, Germany) or MERCK® (Wadeville, Gauteng), unless specified otherwise. All primers used during this study were designed using the Oligo explorer® software, and synthesised by Inqaba Biotech®. All enzymes used were obtained from New England Biolabs® (NEB) (Inqaba, South Africa) unless stated otherwise.

1. Plant material

All *Arabidopsis thaliana* plants used in this study were from the Columbia-0 (Col-0) ecotype (Alonso et al., 2003). All plants were individually grown on Jiffy peat pellets (Jiffy™ nr. 7, South Africa), unless specified otherwise. Subsequent to stratification (24 h, 4°C), plants were maintained under controlled environment conditions (8 h light, 120 µmol photons m^{-2} s^{-1}, 22°C, 16 h dark, 18˚C, 60 % relative humidity). Plants were supplemented with 0.14 % (w/v) phostrogen (Bayer, Stark Ayres® Garden Center, Cape Town, South Africa) on days 7 and 14 after germination, as previously described (Peterson et al., 2010).

2. Genomic DNA isolation

Genomic DNA (gDNA) was isolated as previously described, with minor modifications (Edwards et al. 1990). Briefly, source leaves from four week old plants were macerated in 400 µl extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (*w/v*) SDS). Samples were centrifuged (13000 x *g*, 25°C, 10 min). Supernatant was transferred to a new tube, and equal amount of Isopropanol was added, mixed by inversion and incubated (-20^{0}C, 60 min). Samples were centrifuged (13000 x *g*, 25°C, 15 min), and the supernatant removed. The pellet was rinsed with 70 % (*v/v*) ethanol, air dried (25°C, 1 h) to allow evaporation of ethanol and subsequently resuspended in 100 µl TE (10 mM Tris-Cl and 1 mM

EDTA, pH 8.0). Samples were centrifuged (13000 x *g*, 25°C, 1 min) and the supernatant used in subsequent PCR reactions.

3. Isolation of AtGolS1 promotor (pGS) from *A. thalinana*

The *AtGolS1* promotor (https://www.arabidopsis.org/) was amplified from Col-0 gDNA.

PCR amplification was performed with Q5® High-Fidelity DNA Polymerase (New England Biolabs®) using the pGS forward and reverse primers respectively (Table 1). Primers were modified to include restriction overhangs (italicised) and furthermore designed to amplify a 3.5 kb fragment, including the 5'UTR region, upstream of the *AtGolS1* transcription initiation site. The purified amplicon was digested using *Hind*III and *Asc*I (New England Biolabs®), separated by means of gel electrophoresis on a 0.6 % (*w/v*) Agarose gel at 60 V and purified using Wizard® SV Gel and PCR Clean-up System (Promega, Anatech, South Africa).

4. Generation of respective reporter gene-promotor fusion constructs

4.1. Modification of Gateway compatible vector for reporter gene fusion

The Gateway® pMDC32 plant expression vector (Curtis and Grossniklaus, 2003) was used to generate a gateway compatible vector for reporter gene fusion constructs. The cauliflower mosaic virus (CaMV) 35S promotor was removed using the restriction enzymes *Hind*III and *Asc*I. The *AtGolS1* promotor (pGS) was directionally cloned into the pMDC32 backbone *via* restriction cloning, generating a plant expression vector containing the Gateway® cloning cassette driven by pGS (pMDCpGS). Ligations were transformed into DB3.1 competent cells *via* a heat shock transformation method (Sambrook and Russell, 2001). Positive clones were selected on Luria-Bertani (LB) plates containing 50 µg/mL kanamycin. Confirmation of transformation were conducted by PCR using the pGS forward (Table 1), and NosT reverse (Table 1) primer pair.

4.2. Isolation and cloning of reporter genes

The β-glucuronidase (GUS) and Green fluorescence protein (GFP) reporter genes were respectively amplified from the pMDC163 and pMDC85 vectors, using Q5® High-fedility DNA Polymerase (NEB) according to manufacturer's recommendations. Both genes were amplified using their respective forward and reverse primer pairs (Table 1). The resulting blunt-end reporter gene amplicons were subsequently A-tailed by incubating 1 µg of purified fragment with (0.025 U/µL) GoTaq® DNA polymerase (Promega), 200 µM dATP and 1x GoTaq DNA polymerase Buffer. The A-tailed fragments were cloned into the pCR8™/GW/ TOPO®-vector (Invitrogen, Life technologies, South Africa) according to manufacturer's protocol for TOPO® TA Cloning, generating entry clones. Entry clones were transformed by means of heat shock transformation into OneShot® Competent *Escherichia coli* cells (Invitrogen). Positive transformants were selected on LB medium containing 100 µg/mL spectinomycin. Directionality of transformants were determined via colony PCR, using the gene specific forward (GUS or GFP) and T7 reverse primers (Table 1). Transformants containing the gene of interest in the correct orientation were grown overnight in liquid LB containing 100 µg/mL spectinomycin, and plasmid minipreperations were performed using Wizard® *Plus* SV Minipreps DNA Purification System (Promega), according to the manufacturer's protocol. Plasmids were subsequently sequenced (Central Analytical Facility, Stellenbosch University, South Africa), using the M13 forward and reverse primer (Table 1).

4.3. Generation of destination vectors

A Gateway recombination cloning strategy was used to transfer the respective reporter genes from pCR8 entry vectors into the destination vector (pMDCpGS), according to manufacturer's protocol (Invitrogen). Recombination reactions were transformed into One Shot ® OmniMAX ™ 2 T1 PhageResistant Cells (Invitrogen), and transformants were

selected for on LB plates containing 50 µg/mL kanamycin. Clones containing the insert were confirmed with PCR, using the gene specific forward primer in combination with the NosT reverse primer. Destination vectors, containing reporter genes, were confirmed using the following primer combinations: reporter gene specific forward and reporter gene specific reverse, reporter gene specific forward and NosT Rev, pGS forward and reverse, pGS forward and reporter gene reverse, and pGS forward and NosT reverse (Table 1).

5. Plant transformation and selection procedures

5.1. Agrobacterium transformation

The two destination vectors (pMDCpGS::GUS, pMDCpGS::GFP) were introduced into *Agrobacterium tumefaciens* (GV3101) competent cells by means of electroporation (1.8 kV; 100 Ω; 25 µF in a 2 mm cuvette). Plasmid DNA (500 ng) were added to 100 µL *A. tumefaciens* (strain GV3101) cells. Cells were electroporated using a Gene Genepulser® (Bio-Rad, Bio Rad Laboratories, South Africa), recovered with 1 ml LB and incubated (28˚C, 2 h) with shaking (200 rpm). Transformants were selected on LB plates containing 50 µg/mL rifampicin, 25 µg/mL gentamycin and 50 µg/mL kanamycin. Positive clones were confirmed by means of colony PCR using the gene specific forward and NosT reverse primers (Table 1).

5.2. Agrobacterium mediated plant transformation

A. thaliana (Col-0) plants were transformed using a modified floral inoculation protocol (Narusaka M., 2010). A single colony of *A. tumefaciens* containing either pMDCpGS::GUS or pMDCpGS::GFP was selected and inoculated in 5 mL LB containing 10 µg/mL rifampicilin, 50 µg/mL gentamycin and 50 µg/mL kanamycin and incubated (28˚C , 16 h) with shaking (200 rpm). An aliquot of the culture (1.5 mL) was centrifuged (13 000 xg, 1

min, 25˚C), supernatant was removed, and cells were resuspended in 1 mL, 5 % (*w/v*) sucrose. Silwet L-77 was added to a concentration of 0.02 % (*v/v*) and vortexed prior to floral inoculation. Closed flower buds were inoculated with 5 µL of *Agrobacterium* inoculum. Inoculated plants were placed in the dark under high humidity conditions for 16 h. Seeds (T1) from the transformed plants (T0) were harvested and sterilized using the vapour sterilization method (Clough and Bent, 1998) and placed on half-strength MS (Duchefa, Labretoria, South Africa) media containing 17.5 µg/mL hygromycin for selection. Plates were stratified (4˚C, 24 h) and then placed under controlled growth conditions. Positive transformants were selected and transferred to Jiffy peat pellets (Jiffy™ nr. 7, South Africa) and maintained under greenhouse conditions described previously. Seeds from T2 plants were harvested and selection process repeated to obtain plants for subsequent experiments.

6. Heat stress

Heat stress experiments were performed as previously described by Keller et al. (2008). Transgenic Arabidopsis plants (pGS::GFP/Col-0 and pGS::GUS/Col-0) (21 day old) were transferred to a growth chamber for heat stress conditions (28˚C, 6 h). After 6 h heat stress, plants were assessed for reporter gene activity by *semi-quantitative* RT-PCR (sqRT-PCR) (see 8) and reporter gene assays (GUS stains and GFP imaging).

7. Jasmonic acid treatments

Chemical treatments were performed as previously described by Cho et al. (2010). Transgenic Arabidopsis plants (pGS::GFP/Col-0 and pGS::GUS/Col-0) (14 days old), grown on half strength MS media were transferred onto plates lined with filter paper containing 2 mL of either Methyl Jasmonate (½ MS liquid media, 50 μM MeJA (Sigma), 0.1 % (*v/v*) DMSO and 0.02 % (*v/v*) Silwet L-77) treatment or mock solution (½ MS liquid media, 0.1 % (*v/v*) DMSO

and 0.02 % (*v/v*) Silwet L-77). Samples were harvested at specific time intervals (0, 3, 6, 9, 12 and 15 h) after treatment and subject to sqRT-PCR to assess reporter gene activity.

8. RNA isolation and transcript analyses

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Whitehead Scientific, South Africa) according to manufacturer's protocol. Subsequently, complementary DNA (cDNA) was synthesised using M-MLV (H^-) Reverse Transcriptase (Promega), utilising the Oligo (dT)15 primer according to manufacturer's protocol. sqRT-PCRs were performed by designing primers from the coding sequence of the gene of interest that amplifies a fragment of 1 kb. A 50 µL PCR reaction was set up as follow: 3 μL cDNA, 5 U/μL GoTaq® DNA polymerase (Promega), 5x Green GoTaq® Reaction Buffer, forward and reverse primers (10 µM) and dNTP mix (10 mM). PCR amplification was limited to 25 cycles to avoid saturation of PCR reaction, and to exploit the linear phase of the reaction. Expression of the desired genes at 25 cycles were compared between the treated and untreated samples, using Actin (*AtACT2*, *At3g*18780) (Table 1) as reference gene.

9. GUS activity assays

GUS staining was performed using an adapted protocol from Parcy et al. (1998). Tissue were harvested and placed in ice cold 90 % Acetone until all samples were harvested. Samples were then placed at RT for 20 min, acetone was removed and replaced with staining buffer (0.2 % Triton X-100, 50 mM $NaHPO_4$ buffer (pH 7.2), 2 mM Potassium Ferrocyanide, 2mM Potassium Ferricyanide). X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) (Thermo Fisher Scientific, Inqaba biotech, South Africa) were then added to a final concentration of 2 mM. Samples were vacuum infiltrated on ice for 15 to 20 minutes. Samples were then incubated overnight at 37˚C. Staining buffer was removed and samples were incubated in successive ethanol concentrations (20 %, 35 %, and 50 %) for

30 min each. Tissue was fixed by incubating in FAA (50 % (*v/v*) ethanol, 5 % (*v/v*) formaldehyde, 10 % (*v/v*) acetic acid) for 30 min. FAA was removed, and samples were examined and stored in 70 % ethanol.

10. Quantitative GFP expression analyses

GFP-expression analyses were conducted using the IVIS® Lumina II imaging and the Living Image software version 3.0 (Caliper Life Science). Imaging was conducted by using an optimised set of parameters for the system previously used by Stephan et al. (2011). The locked GFP filter was used for both excitation and emission for the fluorescent image: exposure time 0.5 sec, binning medium, subject height 0.5 cm, f/stop 2, field of view 12.5 cm and a high lamp level. A black and white image of each sample was taken by utilising the standard settings for exposure time, binning medium, and f/stop 16. An overlay image of the black and white and GFP image were conducted using the Living Image software version 3.0 (Caliper Life Sciences).

11. Fungal preparation and infection procedures

11.1. Culturing of Botrytis cinerea spores

Botrytis cinerea (GrapeVine strain, obtained from the Institute for Wine Biotechnology, Stellenbosch University) was cultivated on sterile apricot halves (Weigh less®, South Africa) on a petri dish (14 d, 25˚C, in the dark).

11.2. Harvesting B. *cinerea* spores

Spores were harvested with 2 ml wash solution (ddH2O containing 1 % (*v/v*) Tween-20) by repetitively washing the mycelium, allowing spores to be captured within the wash solution. Spores were subsequently filtered through glass wool to remove excess mycelium and allowed to hydrate (16 h, 4˚C, in the dark). Germination efficiency (>80 %) was determined by spreading an aliquot of the spore suspension (5 µL) onto a 20 % agar plate and incubated (16 h, 25˚C). Spore germination efficiency were determined according to germination plate results. Spore concentration were determined using a hemocytometer and the following equation:

TOT=LT+LB+C+RT+RB

TOT – total

LT – left top corner

LB – left bottom corner

C – center

RT – right top corner

RB – right bottom corner

$$[\text{Spores/mL}] = \frac{\frac{TOT}{5}}{16} \times 4 \times 10^6$$

11.3. *B. cinerea* plant infections

B. cinerea spores were diluted to the desired concentration (1 x 1 spores/mL) using infection buffer (50 % water, 50 % grape juice). Four week old Arabidopsis source leaves, three leaves per plant, were infected with 5 µL of either infection buffer or mock solution (infection buffer

containing no spores). Infected plants were maintained at high humidity (90% relative humidity) to allow infection to occur. Plants were assessed at 24 h intervals by means of expression analysis (sqRT-PCR) and reporter gene activity (GUS assays and GFP imaging).

Table 1 Primer names and sequences used in this study

Primer name	**Sequence**
pGS forward restriction	GATATAAGCTTGACCGGTCTTTTGTGTC
pGS reverse restriction	GATATGGCGCGCCGTGATTAGCACGTGATCTG
pGS forward	GACCGGTCTTTTGTGTC
pGS reverse	GTGATTAGCACGTGATCTG
NosT reverse	AAGACCGGCAACAGGATTC
GUS forward	ATGTTACGTCCTGTAGAAACCC
GUS reverse	TCATTGTTTGCCTCCCTGCT
GFP forward	ATGAGTAAAGGAGAAGAACTTTTCACT
GFP reverse	TTATTTGTATAGTTCATCCATGCCATG
T7 reverse	CCCTATAGTGAGTCGTATTA
M13 forward	GTAAAACGACGGCC
M13 reverse	GTCATAGCTGTTTCCTG
AtActin2 forward	ATGGCTGAGGCTGATGATAT
AtActin2 reverse	TTAGAAACATTTTCTGTGAACGAT

Results

1. Confirmation of reporter gene-promotor constructs (pGS::*gus* and pGS::*gfp*)

The final reporter constructs (pGS::*gus* and pGS::*gfp*) were confirmed using a step-wise PCR-based approach (Fig. 3 A-B). This allows conformation of the newly modified pMDC32 vector backbone (Curtis and Grossniklaus, 2004) to contain the *AtGolS1* promotor (lane 3 and 4), as well as the insertion of the reporter genes (lane 1 and 2) within the Gateway cassette by yielding incrementally larger PCR amplicons associated with the assemblage of the reporter construct within the vector. These final reporter constructs were used in subsequent Agrobacterium mediated plant transformation.

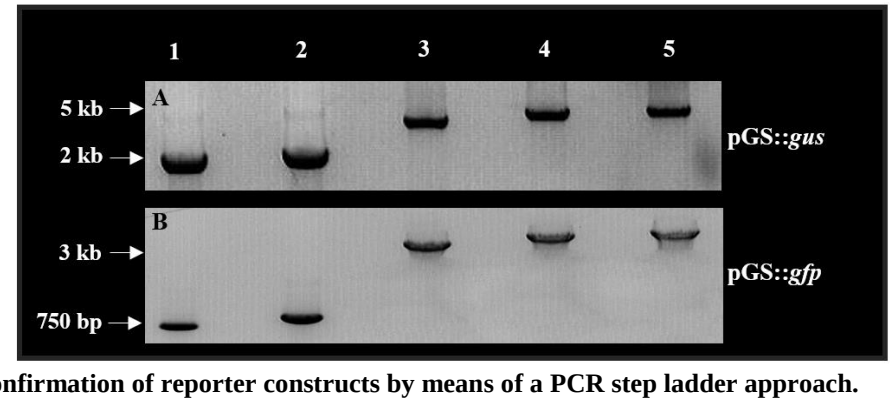

Figure 3: Confirmation of reporter constructs by means of a PCR step ladder approach.

Confirmation of the assembly of the two reporter constructs by means of a PCR based step ladder approach. The following combinations of primers were used for lanes 1 to 5 for the two respective vectors (**A**- pGS::*gus*; **B**- pGS::*gfp*): **1)** reporter gene forward and reporter gene reverse; **2)** reporter gene forward and NosT reverse; **3)** pGS forward and pGS reverse; **4)** pGS forward and reporter gene reverse; **5)** pGS forward and NosT reverse.

2. Confirmation of reporter gene expression in transgenic Arabidopsis (T2) lines (pGS::*gus* and pGS::*gfp*)

Following selection of T2 transgenic plants for hygromycin resistance, plants were genotyped for the presence of the respective reporter genes (GUS and GFP, data not shown). Positive transgenic plants were then analysed for expression of the reporter genes using RT-PCR (Fig. 4). Transcripts of the reporter genes GUS and GFP, were absent in the Col-0 control plants, but detected for all transformed lines (1-3) for both pGS::*gus*/Col-0 and pGS::*gfp*/Col-0 (Fig. 4).

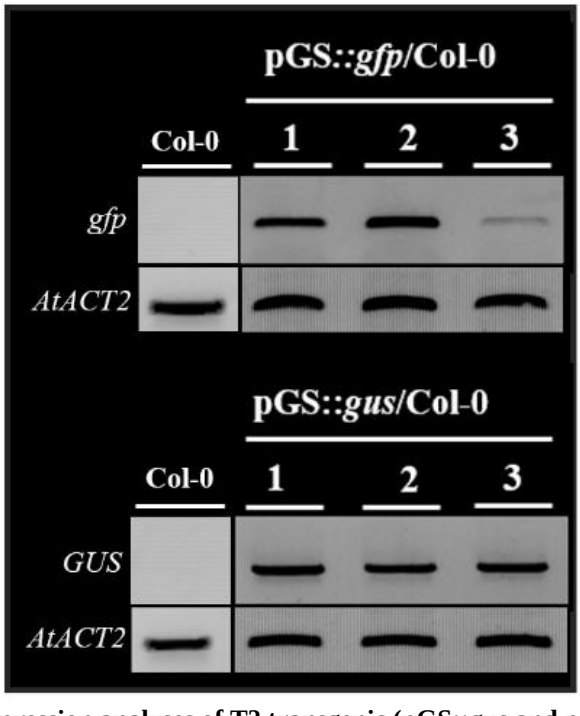

Figure 4: Reporter gene expression analyses of T2 transgenic (pGS::gus and pGS::gfp) plants.

Expression of reporter transgenes, *gfp* and *gus,* were confirmed by means of RT-PCR using gene specific primers (Table 1). Actin was used as reference gene for each line. PCR reactions were performed at 30 cycles. **1-3** refers to transgenic lines 1-3 for each of the reporter genes.

3. Selection of highest expressing reporter line for pGS:*gus*/Col-0 and pGS:*gfp*/Col-0.

3.1 Heat shock element exploited to determine transcript levels and validation of promotor - reporter gene activation during heat stress

The highest expressing line for each of the reporter genes were selected for experiments in response to *B. cinerea* infection. To identify the highest expressing lines, the well characterised heat shock binding element (Panikulangara et al., 2004) occurring in pGS was exploited. Three confirmed transgenic lines for each reporter construct (shown in Fig. 3, pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) were subjected to heat stress as previously described in Panikulangara et al. (2004). Reporter gene expression were assessed by means of sqRT-PCR, and the relative expression between the stressed and unstressed plants for each of the lines was considered (Fig. 5 and 7). From these analyses a single line was selected for each of the reporter gene constructs.

3.1.1 Heat stress induces activity of the AtGolS1 promotor in pGS:gus/Col-0

The level of expression of GUS, in the pGS::*gus*/Col-0 lines, was consistent between the three transgenic lines, showing a distinct increase in transcript levels for the heat stressed plants compared to non-stressed plants (Fig. 5). However, line 1 and 3 showed higher levels of expression than line 2. The three transgenic lines selected for transcript analysis (Fig. 5), were also analysed by means of GUS reporter assays. For each of the three lines, a plant subjected to heat stress (28˚, 72h) and a plant grown under normal conditions were assessed (Fig. 6).

For all three lines, GUS activity were observed under normal growth conditions, mainly in young leaves and petioles (Fig. 6, line 1 - 3 C). During heat stress conditions however, GUS

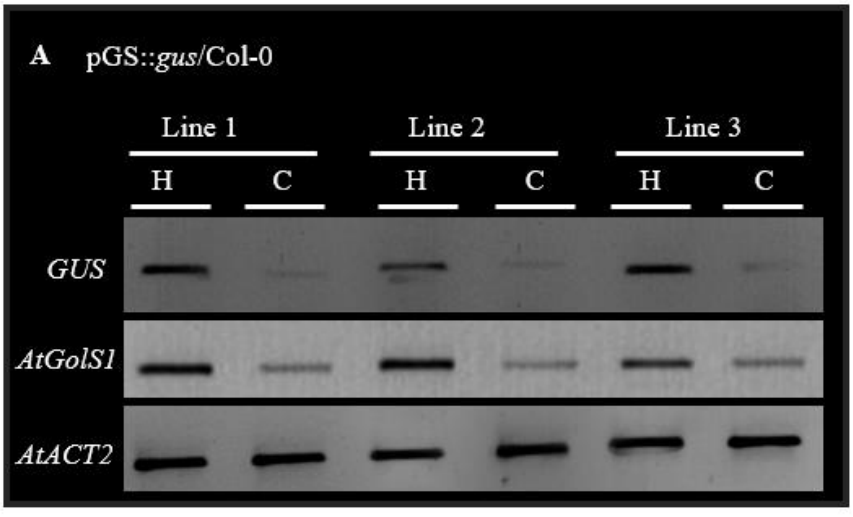

Figure 5: Analysis of transcript levels during heat stress for the pGS::gus/Col-0 transgenic lines.

Expression levels of GUS and *AtACT2* during heat stress conditions for the different transgenic lines were determined using a semi-quantitative PCR based approach. Line 1 to represent three independent T2 transgenic lines. For each line: **H-** represents heat stress plants and **C-** represents unstressed, control plants.

activity was more prominent within the older leaves, and less so within young leaves (Fig. 6, Line 1 - 3 H). An increase in the activity of GUS activity between the heat stressed (Fig. 6 H) and unstressed (Fig. 6 C) plants were observed for all three transgenic lines. For the three lines, it is clear that line 1 showed higher levels of GUS activity in the heat stressed plants compared to line 2 and 3. Line 1 was selected for further study (described below).

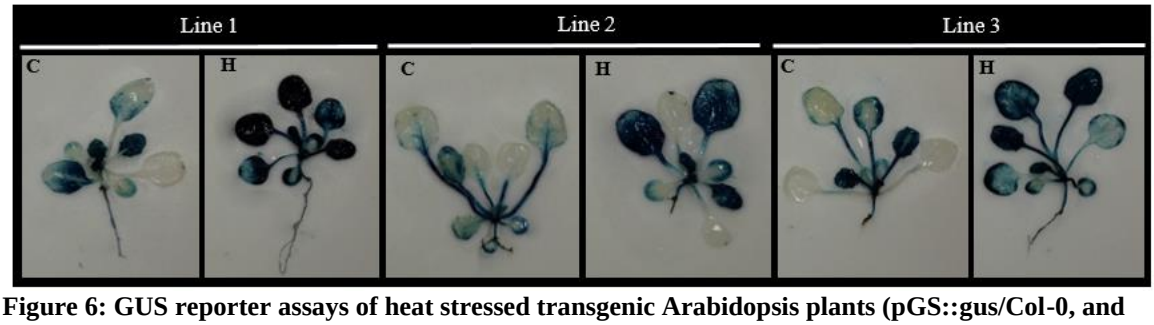

Figure 6: GUS reporter assays of heat stressed transgenic Arabidopsis plants (pGS::gus/Col-0, and pGS::gfp/Col-0)

GUS reporter assay of transgenic Arabidopsis plants (pGS::*gus*/Col-0), during heat stress. Three independent lines were subjected to heat stress at 28˚C for 72h and GUS activity was analysed. For each line, a plant subjected to heat stress (**H**) and a plant unexposed to heat stress (**C**) were assessed.

3.1.2 Heat stress induces activity of the AtGolS1 promotor in pGS:gfp/Col-0

Transcript levels of the reporter gene, GFP, in pGS::*gfp*/Col-0 (Fig. 7) was up-regulated in line 2 for the stressed plants compared to unstressed plants. Line 1 showed relative low levels of expression for both stressed and unstressed conditions, with little or no levels of expression for line 3.

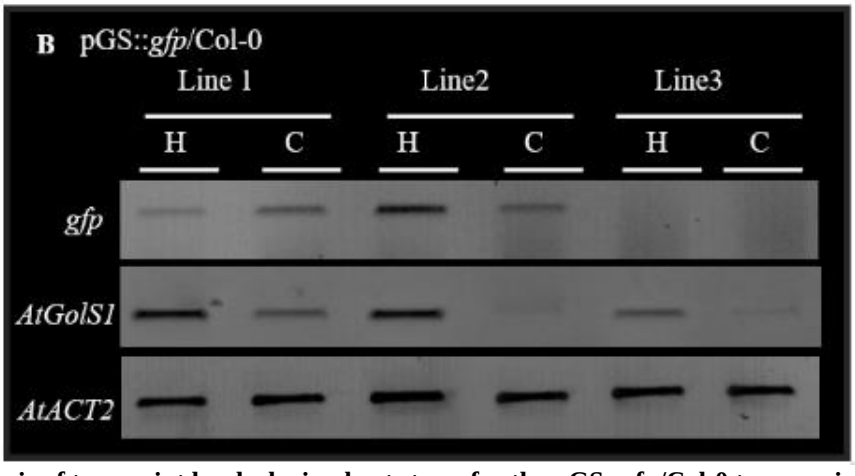

Figure 7: Analysis of transcript levels during heat stress for the pGS::gfp/Col-0 transgenic lines.

Expression levels GFP and *AtACT2* during heat stress conditions for the different transgenic lines were determined using a semi-quantitative PCR based approach. Line 1 to 3 for each reporter gene represent three independent T2 transgenic lines. For each line: **H-** represents heat stress plants and **C-** represents unstressed, control plants.

Of the three transgenic lines selected for transcript analyses (Fig. 7), only line 2 (highest transcript abundance under heat stress) was analysed with a GFP imaging assay. GFP imaging of heat stressed (28˚C, 72h) reporter plants from line 2 revealed strong GFP expression in the stressed plants compared to unstressed ones (Fig. 8) While the unstressed plant (Fig. 8 C) showed background fluorescence only within the younger leaves, the stressed plant (Fig. 8 H) showed high fluorescence throughout all leaves.

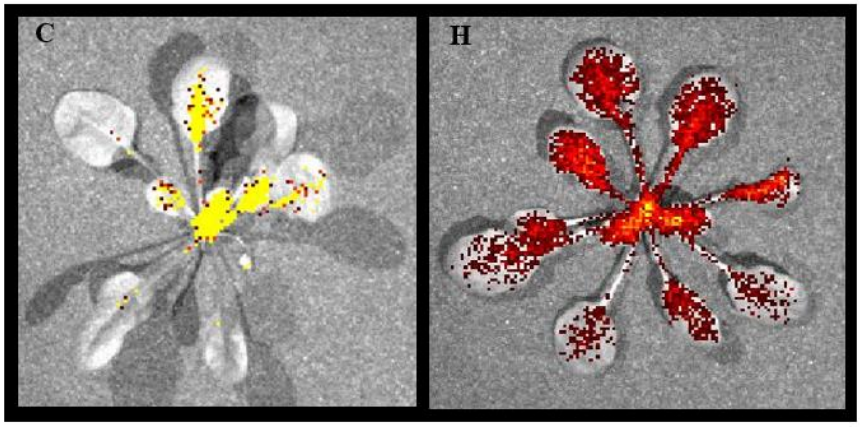

Figure 8: GFP imaging of heat stressed transgenic Arabidopsis plants (pGS::gfp/Col-0)

GFP imaging of heat stressed transgenic Arabidopsis plants (pGS::*gfp*/Col-0) using the IVIS® Lumina II imaging and the Living Image software version 3.0 (Caliper Life Science). Imaging was conducted using line 2 (Fig. 7 B), the highest expressing transgenic line. Plants were subjected to heat stress at 28˚C for 72h and GFP fluorescence were assessed for both unstressed (**C**), and stressed (**H**) plants.

4. Methyl jasmonate induces activity of the *AtGolS1* promotor

To determine whether the cloned *AtGolS1* promotor is activated by jasmonic acid, 14 day old Arabidopsis transgenic plants (pGS::*gus*/Col-0) grown on ½ MS media were treated with (50µM) MeJA.). Transcript levels of *AtActin2*, GUS and *AtGolS1* were also assessed by

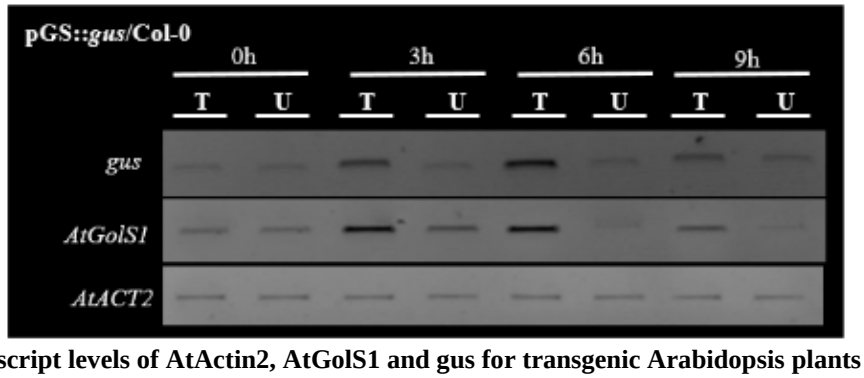

Figure 9: Transcript levels of AtActin2, AtGolS1 and gus for transgenic Arabidopsis plants (pGS::gus/Col-0) treated with 50µM MeJA.

The analysis of transcript levels of *AtActin2*, *AtGolS1* and GUS by means of semi-quantitative PCR in transgenic Arabidopsis plants (pGS::*gus*/Col-0) treated with 50µM MeJA at time points 0, 3, 6, and 9 hours after treatment. **T-** Represents plants treated with 50µM MeJA at specific time point, and **U-** represents untreated plants.

means of *sq*RT-PCR at time points 0h, 3h, 6h and 9h for the treated and untreated plants (Fig. 9). The activation of the pGS::*gus* reporter construct were assessed by performing GUS assays on treated and untreated plants at 0h, 6h, and 12 h after application of MeJA (Fig. 10).

An increase in the level of expression for both GUS and *AtGolS1* were observed at 3h and 6h respectively when compared to untreated plants (Fig. 9). After 9h the transcript levels of both GUS and *AtGolS1* decreased significantly for the treated plants, but were still higher than untreated plants (Fig. 9). GUS assays revealed similar results, where untreated plants show little to no GUS activity over the 12h period (Fig. 10 A, C and E). Treated plants however, showed an increase in the level of activity at 6h (Fig. 10 D) after treatment when compared to immediately after treatment, 0h (Fig. 10 B), and untreated plants, and a decrease in activity towards 12h (Fig. 10 E). From the GUS assays (Fig. 10 D) it is clear that expression occurs mainly in the younger leaves and roots of the treated plants and to a lower extend in older leaves.

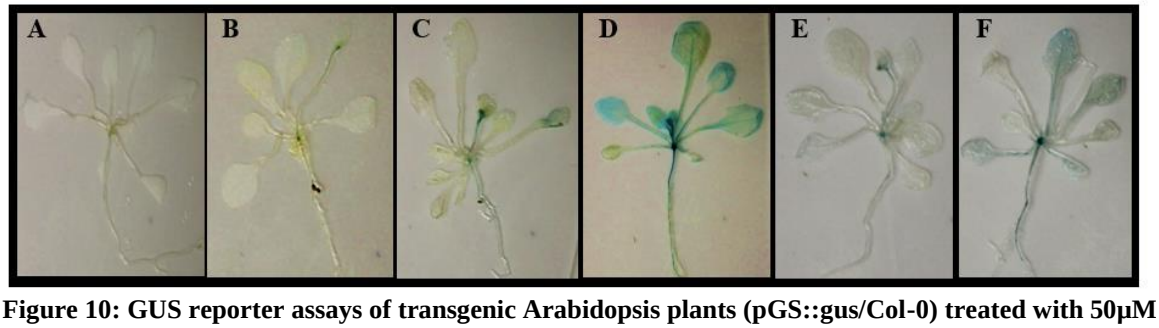

Figure 10: GUS reporter assays of transgenic Arabidopsis plants (pGS::gus/Col-0) treated with 50µM MeJA.

GUS assays of transgenic Arabidopsis plants (pGS::*gus/Col-0*), treated with 50µM MeJA at time points 0h, 6h, and 12h after treatment. **A** and **B -** 0h after treatment, **C** and **D-** 6h after treatment, and **E** and **F-** 12h after treatment. **A**, **C**, and **E** represent untreated plants, and **B**, **D**, and **F** represents plants treated with 50µM MeJA.

5. Expression analysis and reporter assays of transgenic Arabidopis plants (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) during *B. cinerea* infection.

5.1 Expression analysis of transgenic Arabidopis (pGS::gus/Col-0 and pGS::gfp/Col-0) plants during B. cinerea infection.

The highest expressing transgenic Arabidopis (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) lines (Fig. 5 and 7) were subjected to *B. cinerea* infections, and transcript levels of *AtACT2*, the reporter genes (GFP and GUS), *MYC2*, *PR3* and *AtGolS1* were assessed by means of sqRT-PCR every 24h (Fig. 11). Three source leaves per plant were infected with *B. cinerea* spores (1 × 1 spores/mL), and gene expression in the infected leaves, uninfected leaves of an infected plant and the mock infected plant (no spores) were compared over a 72h period (Fig. 11).

For the WT (Col-0), and both transgenic lines, *MYC2* were expressed at higher levels in the infected plant (both infected and uninfected leaves) compared to the mock uninfected plant over the period of infection (Fig. 11). Transcript levels of *MYC2* were constantly being expressed at low levels within the uninfected plants during all three time points (24h, 48h, and 96h). Transcripts for *PR3* could only be detected at extremely low levels within the infected leaves for the WT and both transgenic lines (Fig. 11). Expression levels of *PR3* remained consistent over the infection period only within the infected leaves. Transcript levels of *AtGolS1* and both reporter genes (GFP and GUS) in the transgenic lines followed similar patterns over the period of infection. Expression of these genes were predominantly within the infected leaves at 24h, 48h and 72h after infection, and at lower levels in the uninfected leaves and uninfected plants. However, transcripts could be detected within the uninfected leaves and uninfected plants over the period of infection (Fig. 11). For the WT,

expression of the *AtGolS1* were similar in both the infected leaves, uninfected leaves and uninfected plants 24h after infection, but were up-regulated in the uninfected leaves 48h and 72h after infection (Fig. 11).

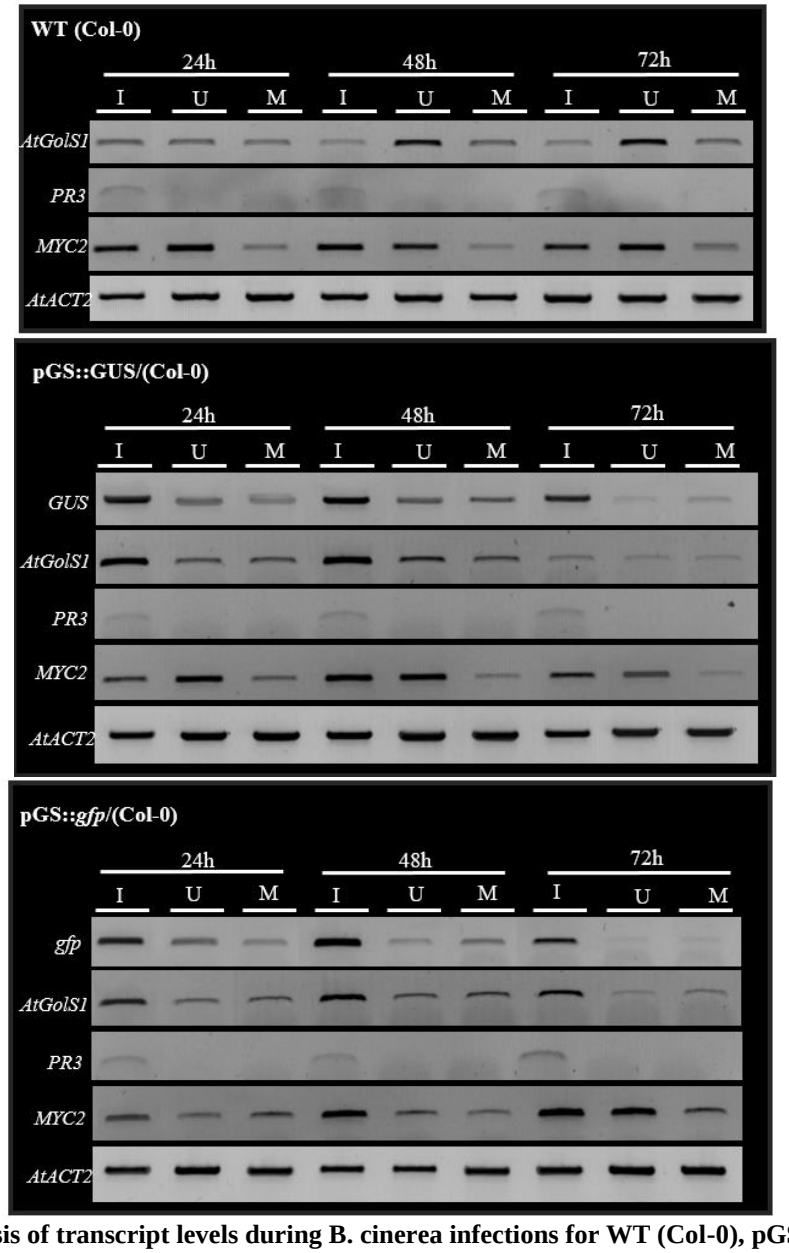

Figure 11: Analysis of transcript levels during B. cinerea infections for WT (Col-0), pGS::gfp/Col-0 and pGS::gus/Col-0.

Transcript levels of *AtACT2, MYC2, PR3, AtGolS1* and either GUS or GFP at 24h, 48h and 72h after *B. cinerea* infection of WT (Col-0) and transgenic (pGS::*gfp*/Col-0 and pGS::*gus*/Col-0) Arabidopsis plants. Three leaves per plant were infected with *B. cinerea* spores. For each time point (24h, 48h and 72h), transcript levels were assessed for the infected leaf (**I**), uninfected leaf of the infected plant (**U**), and the mock infected plant (**M**) (no spores).

5.2 Reporter assays of transgenic Arabidopis (pGS::gus/Col-0 and pGS::gfp/Col-0) lines during B. cinerea infection.

The highest expressing transgenic Arabidopis (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) lines (Fig. 5 and 7) were subjected to *B. cinerea* infections and were assessed for reporter gene activity. Infected plants were assessed by either GUS assays (pGS::*gus*/Col-0) (Fig. 12) or

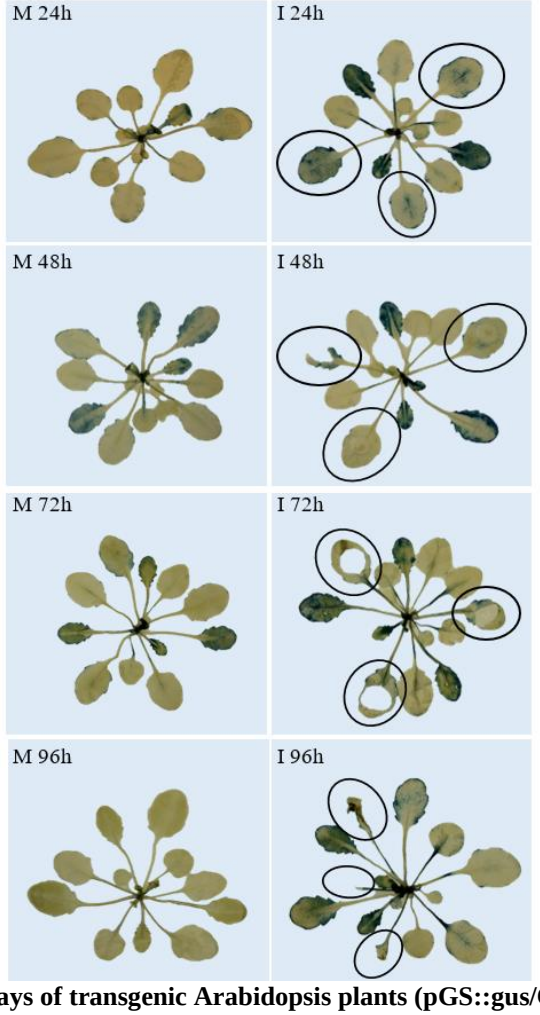

Figure 12: GUS reporter assays of transgenic Arabidopsis plants (pGS::gus/Col-0) infected with B. cinerea.

GUS reporter assays performed on transgenic Arabidopsis plants (pGS::*gus*/Col-0) 24h, 48h, 72h and 96h after *B. cinerea* infection. Infected (**I**) and mock infected (no spores) (**M**) plants were assessed every 24h. The three infected leaves are circled for each plant.

GFP imaging (pGS::*gfp*/Col-0) (Fig. 13) at time points 24h, 48h, 72h, 96h after infection.

GUS reporter assays showed the highest activity 24h after infection (Fig. 12), spreading throughout the entire plant. However, activity within the infected leaves and young leaves (sink leaves) were higher compared to the uninfected older leaves (source leaves) (Fig 12). After 48h, the levels of GUS activity decreases and localised towards the areas surrounding the infection and remains high within younger leaves. This patterns remains similar over the next 24h, and activity increases throughout the entire plant 96h after infection. GUS activity was prominent within the younger leaves of both infected and uninfected leaves over the period of infection.

GFP imaging of infected plants revealed high levels of fluorescence in the infected leaves, and lower levels in the uninfected leaves at 24h after infection (Fig. 13). The relative fluorescence increased in the infected leaves from 24h to 48h after infection, but decreased in the uninfected leaves (Fig. 13). Fluorescence was detected throughout the infection period within the areas surrounding infection and low levels in the uninfected leaves. Fluorescence was concentrated towards the centre of the plants (younger plant tissue) for all four time points. Uninfected plants showed low levels of fluorescence only within the younger tissue (Fig. 13 M, 24h and 72h), and no activity in the rest of the plant.

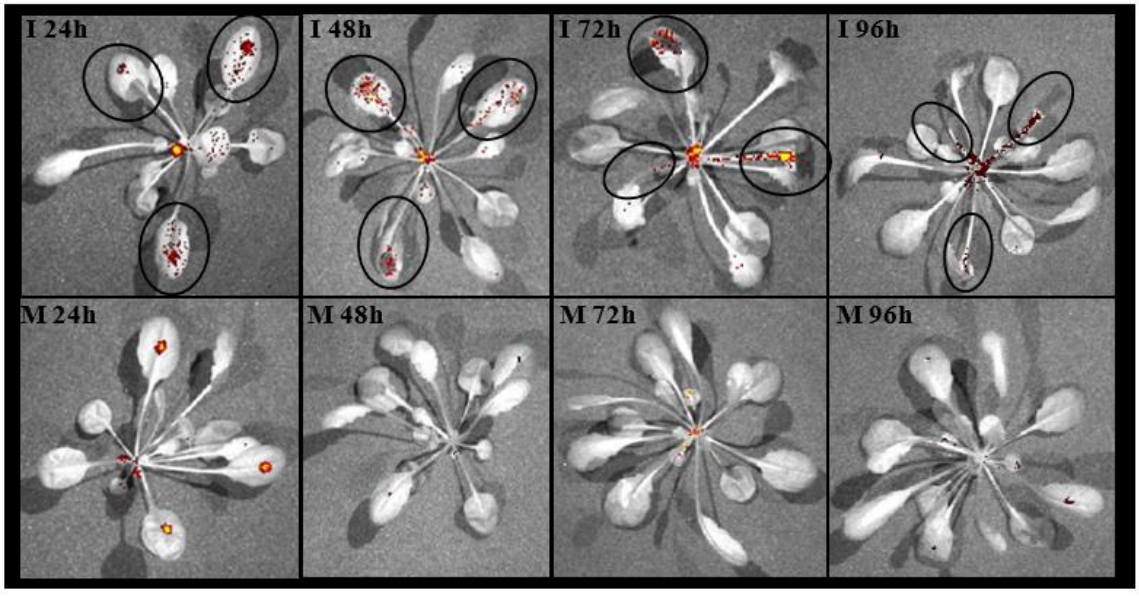

Figure 13: GFP fluorescence imaging of transformed Arabidopsis plants (pGS::gfp/Col-0) infected with B. cinerea.

Transformed Arabidopsis plants (pGS::*gfp*/Col-0) were analysed using the IVIS® Lumina II imaging and the Living Image software version 3.0 (Caliper Life Science) 24h, 48h, 72h and 96h after *B. cinerea* infection. Infected plants, and mock infected (no spores) plants are represented as **I** and **M** respectively. The level of fluorescence at each time point represents relative fluorescence to a WT negative control. The three infected leaves are circled for each plant.

Discussion

Gol has historically been thought to exclusively serve as the galactose donor during the biosynthesis of RFOs. However, reports have now demonstrated additional functions in free radical scavenging (Nishizawa et al., 2008) and as a signalling molecule in ISR (Kim et al., 2008; Cho et al., 2010). Together with heat stress, these reported functions are all related to the transcriptional up-regulation of a single *GolS* isoform in Arabidopsis, *AtGolS1* (AT2G47180).

The *AtGolS1* gene has been demonstrated to be transcriptionally responsive to external application of JA (Cho et al., 2010) as well as heat stress (Panikulangara et al., 2008). In this regard the promotor region of *AtGolS1* contains key determinants such as heat shock transcription factor binding elements (perfect sequence at −192 bp from ATG followed by several imperfect sequences further upstream) and several putative JA binding elements (-1046, -2687, -2701, and -2321 bp from the ATG start codon).

This study investigated the potential of the *AtGolS1* promotor (pGS) as a tool to develop a dual functional molecular biosensor that can detect both heat stress and fungal pathogen infection. The aim of this study was to develop this biosensor in the Arabidopsis model by using two reporter gene (GUS and GFP) fusions that can detect when plants are exposed to either heat stress or fungal pathogen infection by utilising pGS as the "detector" and a reporter gene as the "signal". For the purpose of this study, two reporter genes, in parallel, were used in a fusion with pGS namely GUS and GFP.

Confirmation of pGS::reporter construct

During this study, two reporter genes were assessed namely GFP and GUS. Firstly, we developed a binary destination plant expression vector by replacing the 2 × 35S promotors of the Gateway® pMDC32 plant expression vector (Curtis and Grossniklaus, 2003) with pGS.

The reporter genes were sub cloned into the destination vector to obtain the final vector, pGS::reporter (GUS/ GFP). Vector construction was confirmed by performing a step-wise PCR reaction (Fig. 3) as described previously (materials and methods) for each of the destination vectors. The incrementally larger PCR amplicons associated with the assemblage of the reporter construct within the vector confirmed the insertion of the pGS (Fig. 3, lane 3 and 4) and reporter genes (Fig. 3, lane 1 and 2). These two constructs were used in Agrobacterium mediated transformation of Arabidopsis (Col-0).

Both heat stress and exogenous MeJA application elevated transcript abundance of *AtGolS1*, GFP and GUS in transgenic reporter lines.

Following transformation of Arabidopsis (Col-0) with the respective reporter constructs, expression analyses was conducted on putative transgenic lines (T2) which were hygromycin resistant. We examined native *AtGolS1*, GUS and GFP expression under conditions of heat stress (28 ˚C, Fig 5 and 7) and exogenous MeJA treatment (Fig 9). The highest expressing line for both GUS (line 3) and GFP (line 2) was selected (Fig. 5 and 7) based on the response of the reporter genes to heat stress for further experiments. For the purpose of this study, only one line per reporter gene was selected based on comparative results from previous work on the AtGolS1 promotor during heat stress (Panikulangara et al., 2004).

Since our reporter lines contained a native *AtGolS1* (and promotor), we looked to using the *AtGolS1* gene as a point of reference for induced promotor activity. During heat stress experiments the *AtgolS1* transcript expression was similar to what was previously reported (Panikulangara et al., 2008). Similarly, native *AtGolS1* expression under exogenous MeJA closely resembled previous reports (Cho et al., 2010). Both the reporter genes (GUS, GFP) (pGS:*gus*/Col-0 and pGS:*gfp*/Col-0) under the control of the *AtGolS1* promotor, showed

increases in transcript abundance that were responsive to heat stress. Similarly, the reporter gene GUS (transgenic lines pGS:*gus*/Col-0) showed an increase in transcript abundance during exogenous MeJA application (Fig. 9). This demonstrated, that our 3.5 kB promotor sequence contained intact HSF binding elements and MeJA responsive elements. These experiments provided the foundation to further analyse if the promotor response would result in phenotypic manifestation of the reporter genes.

Both heat stress and exogenous MeJA application elevated GFP and GUS activities in transgenic reporter lines

Expression analysis conducted on transgenic plants (pGS:*gus*/Col-0 and pGS:*gfp*/Col-0) showed an increase in transcript levels for both the native *AtGolS1* and the two reporter genes (GUS and GFP) under control of pGS during heat sress (Fig. 5 and 7) and MeJA treatments (Fig. 9).

Following the initial molecular screens to determine the transcriptional response of our reporter genes under heat stress and MeJA treatment, we looked to actual reporter assays (GUS assays and GFP imaging) to determine if these correlated with our observed transcript abundance patterns under heat stress and exogenous MeJA treatment. We could clearly observe very strong reporter activities for both GUS (Fig. 6) and GFP (Fig. 8) under heat stress, and GUS (Fig. 10) during MeJA treatment. Interestingly, for the non-stressed plants, low levels of reporter gene (GUS and GFP) (Fig. 6 and 8) expression as well as reporter gene activity for both GUS assays (Fig. 6) and GFP imaging (Fig. 8) was observed. This may be due to the several Drought Response Elements (DREs) identified in the *AtGolS1* promotor when *in-silico* promotor analysis was conducted (data not shown). These DREs are extremely sensitive to changes in soil moisture content, high salinity, and cold (Yamaguchi-Shinozaki

and Shinozaki, 1994) and could have been activated under our growth conditions and/or watering regime.

However, our results still correlate with the expression patterns, showing a distinct difference between heat stressed and unstressed plants (Fig. 6 and 8) as well as the MeJA treated and untreated plants (Fig. 10). These results correlate with previous findings (Panikulangara et al., 2008; Cho et al., 2010) showing the involvement of *AtGolS1* during biotic and abiotic stresses. Using these results we introduced our reporter lines into the *B. cinerera* pathogen system.

Expression analysis and reporter assays of transgenic Arabidopis (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) during *B. cinerea* infection

Previous studies showed that root colonization by rhizobacterium, *Pseudomonas chlororaphis O6*, induced the expression of *GolS* and subsequently conferring systemic resistance against fungal pathogen infection in cucumber (Spencer et al., 2003). Further studies revealed that of all the *GolS* genes in Arabidopsis, *AtGolS1* was the only one to be up - regulated upon *B. cinerea* infection (Cho et al., 2010). O6-mediated ISR against *B. cinerea* were disrupted in *AtGolS1* mutant lines, suggesting the involvement of *AtGolS1* as a possible signalling molecule in ISR (Cho et al., 2010). Jasmonate treatment in wild-type *Arabidopsis* plants induced the expression of *AtGolS1*, whereas salicylic acid did not (Cho et al., 2010). Interestingly, most other JA-dependant defence genes are repressed through transcription factors such as WRKY33 and MYC2 during pathogen infection (Rainer et al., 2012; Kazan and Manners, 2013). Together these findings suggest that *AtGolS1* expression due to O6 colonization is facilitated via the jasmonate-dependant pathway, therefore acting as a stimulant of ISR against *B. cinerea* in Arabidopsis.

To determine whether our highest expressing transgenic lines (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0) were able to detect fungal pathogen infection, we analysed them by means of expression studies and reporter assays over a 4 day period of *B. cinerea* infection. During expression studies we looked at the pathogenesis related gene, *PR3*, the transcription factor *MYC2*, *AtGolS1* and finally the reporter transgenes (GUS, GFP) (Fig. 11). Here we showed that transcript levels of *PR3*, were only up-regulated in the infected leaves for both transgenic lines and wild-type plants, thus very specific towards the area of infection and not in areas distant to infection (Fig. 11). It is well established that *PR3* is induced in plants when experiencing wounding or fungal infections (Senthilraja et al., 2009; Senthilraja et al., 2013). Induction of *PR3* and *PR4* occurs in a SA-independent manner, and occurs via the JA dependant pathway (Thomma et al. 1998). During necrotrophic pathogen infections, such as *B. cinerea*, the JA-dependant resistance, including the activation of *PR3* (Thomma *et al*., 1998), is believed to be more effective than SA-dependant resistance in Arabidopsis (Staswick *et al*., 1998; Vijayan *et al*., 1998).

The MYC2 transcription factor is a key regulator of the two branches of the JA signalling pathway. MYC2 is required for ISR induced by beneficial soil microbes (Kazan and Manners, 2013). In contrast, MYC2 functions as a suppressor of JA-dependant defence gene expression (Lorenzo et al., 2004). Upon pathogen infection or wounding, JA is rapidly synthesised which leads to the removal of suppressors from the MYC2 protein (Kazan and Manners, 2013). The MYC2 protein in response activates the expression of several early JA-responsive genes. However, MYC2 also function as a transcriptional suppressor of JA-responsive genes during pathogen infection. We showed that *MYC2* transcript levels were up-regulated in both the infected leaves and uninfected leaves of infected plants (areas distant to infection) 24h after

infection, showing the repression of JA-mediated defence genes through the activity of the MYC2 protein.

Finally, transcript levels of *AtGolS1* and both reporter genes (GUS and GFP) were up-regulated in the infected plants, mostly within the infected leaves (Fig. 11). Transcript levels of *AtGolS1* in the WT plants only increased 48h after infection whereas the transgenic lines a response is seen 24h after infection (Fig. 11). This might be due to a delay in infection or pathogen response in the particular plant sampled, as no visible lesions were observed for any of the lines 24h after infection. *B. cinerea* is a necrotrophic pathogen which can occur in a latent state in the plant until conditions are favourable, thus causing this possible delay in response observed in the WT plants. Interestingly, although several JA-response genes are down-regulated during *B. cinerea* infection, *AtGolS1* transcript abundance increased. This provides further evidence that either Gol or Raf might serve as a signalling molecule during ISR mediated resistance as proposed by Cho et al. (2010).

Reporter assays conducted (GUS assays and GFP imaging) revealed the activation of both reporter genes 24h after infection, and remained activated as the infections progressed (Fig. 12 and 13). We could clearly observe elevation in reporter activity between the infected and uninfected plants (Fig. 12 and 13). Reporter activity correlates with expression studies on both the native *AtGolS1* and the reporter genes (Fig. 11). For the GFP imaging (Fig. 13), the reporter gene was mainly activated in the area surrounding the infection, the petioles of infected leaves and the younger leaves, whereas with the GUS assays (Fig. 12), activation were throughout the entire plant. As mentioned previously, this might only be due to fluorescence not being detected at lower levels, whereas GUS assays are more sensitive to low levels of reporter gene expression (Sagi et al., 2003). A clear difference between the infected and uninfected plants were observed for both sets of reporter genes. Activation of

pGS occurs due to the synthesis of JA immediately after pathogen infection and it seems that *AtGolS1* is regulated independently from other JA-responsive genes.

In conclusion, we developed transgenic Arabidopsis lines (pGS::*gus*/Col-0 and pGS::*gfp*/Col-0), and confirmed the activation of the transgenes by both heat stress and MeJA treatment as described previously (Panikulangara et al., 2004; Cho et al., 2010). Finally we showed that during *B. cinerea* infection, both reporter genes were activated (expression analysis) from 24h to 96h after infection. This correlated with reporter activities captured during pathogen infection. These results indicate that pGS can be utilised as a molecular biosensor in future studies to detect both heat stress and fungal pathogen infection.

Future applications of the study

From an economical point of view, grapevine is considered one of the most important fruit crops in the world (Vivier et al., 2012). It is used in the wine industry, dried fruits and table grapes. High temperatures and *B. cinerea* infection collectively contributes to massive losses in the grapevine industry both pre-and-post-harvest. High temperatures are associated with the decrease of favourable compounds such as organic acids (Champagnol 1984) and several secondary metabolites (Mori et al. 2007) within the berries. Further, high temperatures drive metabolism towards the accumulation of sugars, instead of qualitative compounds (Greer and Watson, 2010). *B. cinerea* also accounts for major losses in the grapevine industry of both vines (McLellan et al., 1973) and berries (Nair et al., 1995). A heat responsive galactinol synthase gene (*VvGolS1)* is published as a marker gene for heat stress in *V. vinivera* berries (Pillet et al., 2012). This provides suggestive evidence that the *VvGolS1* gene in grapevine is transcriptionally regulated similarly to *AtGolS1*.

The proof-of-concept results from our study could be applied in the context of the wine industry. In this regard it is plausible that a YFP reporting transgenic grapevine can be used in conjunction with a low cost imaging system such as the handheld ROFIN Polilight forensic flashlight (ROFIN forensics, Australia) that could then be employed as an early warning system for heat stress and pathogen infection to improve the management strategies of these problems in the vineyard. This does not suggest the use of transgenic grapevine for the production of wine and table grapes, rather the use of transgenic grapevine planted at strategic positions to act as an early signal for heat stress and fungal infection. Thus, the transgenic vines will not be used directly for the production of consumables.

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