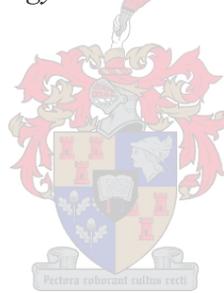


Functional roles of raffinose family oligosaccharides: Arabidopsis case studies
in seed physiology, biotic stress and novel carbohydrate engineering

by

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Summary

The raffinose family of oligosaccharides (RFOs) are α 1,6-galactosyl extensions of sucrose (Suc-Gal_n) unique to the plant kingdom. Their biosynthesis is mediated *via* α 1,6-galactosyltransferases which catalyse the formation of raffinose (Raf, Suc-Gal₁), stachyose (Sta, Suc-Gal₂) and higher oligomers (Suc-Gal_n, $n \geq 13$) in a stepwise manner. RFOs are well known for their historical roles as phloem translocates and general carbon storage reserves. In recent years their physiological roles have expanded to include potential functions in global plant stress-responses, where correlative mass increases are associated with abiotic stresses such as desiccation, salinity and low temperatures and, to a lesser extent biotic stress (pathogen infection).

This study focused on (i) the functional characterisation of a putatively annotated stachyose synthase from *Arabidopsis* seeds (RS4, *At4g01970*), (ii) dissection of the proposed functional role of the RFO precursor galactinol in biotic stress tolerance using the *Arabidopsis/Botrytis cinerea* pathosystem and, (iii) an attempt to engineer long-chain RFOs into *Arabidopsis* by constitutive over-expression of the unique RFO chain elongation enzyme galactan:galactan galactosyltransferase (ArGGT) from *Ajuga reptans*.

In *Arabidopsis* Raf is the only RFO known to accumulate in leaves, strictly during conditions of abiotic stress. However, seeds accumulate substantial amounts of both Raf and Sta. While RFO physiology in *Arabidopsis* leaves and roots is quite well characterised, little is known about the RFO physiology in the seeds. Apart from a single enzyme being described to partially contribute to seed Raf accumulation (RS5, *At5g40390*), no other RFO biosynthetic genes are known. In this work we functionally characterised an α 1,6-galactosyltransferase putatively annotated as a stachyose synthase (RS4, *At4g01970*) in the *Arabidopsis* database. Using two insertion mutants (*atrs4-1* and *4-2*) we demonstrated Sta deficiency in mature

seeds. A double mutant with the recently characterised *RS5*, shown to partially be responsible for Raf accumulation in mature seeds was completely deficient in seed RFOs. This provided the first hint that *RS4* could potentially also be involved in Raf biosynthesis. Seed specific expression of *RS4* was deregulated by constitutive over-expression in wild-type (Col-0) and the *atrs5* mutant background (RS and Raf deficient). Both Raf and Sta unusually accumulated in Col-0 leaves over-expressing *RS4*, under normal growth conditions. Further, leaf crude extracts from *atrs5* insertion mutants (RS and Raf deficient) over-expressing *RS4* showed enzyme activities for both RS and SS, *in vitro*. Collectively our findings have physiologically characterised *RS4* as a RFO synthase responsible for Sta and, partially Raf (along with *RS5*) accumulation during Arabidopsis seed development.

The galactosyl donor in RFO biosynthesis, galactinol (Gol) has recently been implicated in biotic stress signalling (pathogen response) in cucumber, tobacco and Arabidopsis. Those studies focused exclusively on Gol in their experimental approaches using both over-expression (tobacco, Arabidopsis) and loss-of-function (Arabidopsis) strategies. However, they did not address the invariable accumulation of Raf that is routinely obtained from such over-expression strategies. We therefore investigated if Raf could play a functional role in induced systemic resistance (ISR), a well-studied mechanism employed by plants to combat necrotrophic pathogens such as *Botrytis cinerea*. To this end we looked to the *RS5* mutant backgrounds (Raf deficient but Gol hyper-accumulating) reasoning that the Gol accumulating mutants should be resistant to *B. cinerea* (as previously described for transgenic over-expression of *GolSI* isoforms in tobacco and Arabidopsis). Such findings would then preclude a role for Raf, since the system would be Raf deficient. Surprisingly, two independent T-DNA insertion mutants for *RS5* (*atrs5-1* and *5-2*) were equally hyper-sensitive to *B. cinerea* infection as two independent T-DNA insertion mutants for *GolSI* (*atgols1-1* and *1-2*). The hyper-sensitivity of the *GolSI* mutant background has previously

been demonstrated. The *RS5* mutant backgrounds accumulate substantial amounts of Gol, comparable to those reported for transgenic plants (tobacco and Arabidopsis) where pathogen resistance was reported. Further, during the course of our investigations we discovered that both *AtGolsI* mutants also accumulated substantial amounts of both Gol and Raf under normal growing conditions. This was not reported in previous studies. Collectively our findings argue against a role for either Gol or Raf being responsible for the induction/signalling of ISR. However, we do not preclude that the RFO pathway is somehow involved, given the previous reports citing pathogen resistance when *GolsI* genes are over-expressed. We are further investigating a potential role for the *Gols* transcript and/or protein being the component of the suggested signalling function in ISR.

The unique enzyme from *A. reptans* (galactan:galactan galactosyltransferase, ArGGT) is able to catalyse the formation of higher oligomers in the RFO pathway without the use of Gol as a galactosyl donor but rather, using RFOs themselves as galactose donors and acceptors (Gol-independent biosynthesis). We constitutively over-expressed *ArGGT* in Arabidopsis as a way to engineer long-chain RFO accumulation to further dissect a role for them in improving freezing tolerance. To this end we have been unsuccessful in obtaining RFOs higher than Sta (which occurred in extremely low abundance) in the leaves. Since ArGGT would appear to show substrate preference for Sta, and Arabidopsis seeds accumulate substantial quantities of Sta, we further analysed the seed water soluble carbohydrate (WSC) profiles of three independent transgenic lines but detected no additional RFO oligomers beyond the normally accumulating Raf and Sta. We suggest further strategies to improve this approach (Chapter 4).

Collectively this work represents case studies of RFOs in seed physiology, their abilities/requirement in biotic stress and the use of unique enzymes to engineer long-chain RFO accumulation using the Arabidopsis model. At the time of submission of this

dissertation the following contributions have been made to the general scientific community:

- (i) Presentation of chapter 2 at the 26th International Conference for Arabidopsis Research (26th ICAR, 2015, Paris, France) and, (ii) Submission of chapter 2 as a manuscript presently under peer review for possible publication in *Plant and Cell Physiology*.

Opsomming

Die raffinose familie van oligosakkariede (RFO) is α 1,6-galactosyl uitbreidings van sukrose (Suc-Gal_n) uniek aan die plante koningryk. Hul biosintese word bemiddel deur α 1,6-galactosyltransferases wat in 'n stapsgewyse manier die vorming van raffinose (Raf, Suc-Gal₁), stachyose (Sta, Suc-Gal₂) en hoër oligomere (Suc-Gal_n, $n \geq 13$) kataliseer. RFOs is bekend vir hul historiese rol as floëem translokate en algemene koolstof reserwes. Meer onlangs was hul fisiologiese rolle uitgebrei om potensiële funksies te vervul in globale plant stres-reaksies, waar korrelatiewe massa toenames geassosieer word met abiotiese stresfaktore soos uitdroging, soutgehalte en lae temperature en tot 'n mindere mate biotiese stres (patogeen infeksie).

Hierdie studie fokus op (i) die funksionele karakterisering van 'n tentatief ge-annotateerde stachyose sintase van Arabidopsis sade (*RS4*, *At4g01970*), (ii) disseksie van die voorgestelde funksionele rol van die RFO voorloper galactinol in biotiese stres verdraagsaamheid, met behulp van die Arabidopsis/*Botrytis cinerea* patogeen sisteem en (iii) 'n poging om 'n lang-ketting RFOs in Arabidopsis te inisieer deur konstitutiewe oor-uitdrukking van die unieke RFO ketting-verlengings ensiem galactan:galactan galactosyltransferase (ArGGT) afkomstig van *Ajuga reptans*.

In Arabidopsis is Raf die enigste RFO bekend daarvoor om te versamel in die blare, eksklusief tydens toestande van abiotiese stres. Maar, sade versamel aansienlike konsentrasies van beide Raf en Sta. Terwyl RFO fisiologie in Arabidopsis (blare en wortels) baie goed gekenmerk is, is min bekend oor die RFO fisiologie in die saad. Afgesien van 'n enkele ensiem wat beskryf word om gedeeltelik by te dra tot Raf versameling (*RS5*, *At5g40390*), is geen ander RFO biosintetiese gene bekend in saad nie. In hierdie werk beskryf ons die funksionele karakterisering van 'n α 1,6-galactosyltransferase wat tentatief ge-annotateer word as 'n

stachyose sintase (RS4, *At4g01970*) in die Arabidopsis databasis. Met die gebruik van twee invoegings mutante (*atrs4-1* en *4-2*) het ons die verlies van Sta in volwasse sade gedemonstreer.

RFOs was heeltemal absent in sade van 'n dubbele mutant met die onlangs gekarakteriseerde RS5 (verantwoordelik vir gedeeltelike Raf versameling in volwasse sade). Dit het die eerste aanduiding daargestel dat RS4 potensieel ook betrokke kan wees in Raf biosintese. Saad-spesifieke uitdrukking van *RS4* was gedereguleer deur konstitutiewe oor-uitdrukking in wilde-tipe (Col-0) en die *atrs5* mutant agtergrond (RS en Raf gebrekkig). Oor-uitdrukking van *RS4* in Col-0 blare het gelei tot beide buitengewone Raf en Sta konsentrasies, onder normale groeitoestande. Verder, oor-uitdrukking van *RS4* in *atrs5* invoeg mutante (waar beide RS en Raf absent is) het *in vitro* ensiemaktiwiteite vir beide RS en SS getoon. Gesamentlik beskryf ons bevindinge die fisiologies karakterisering van RS4 as 'n RFO sintase, verantwoordelik vir Sta en gedeeltelik Raf (saam met RS5) sintese tydens Arabidopsis saad ontwikkeling.

Die galactosyl skenker in RFO biosintese, galactinol (Gol), was onlangs beskryf om 'n rol te speel in biotiese stres (patogeen reaksie) in komkommer, tabak en Arabidopsis. Daardie studies het uitsluitlik gefokus op Gol in hul eksperimentele benaderings deur die gebruik van beide oor-uitdrukking (tabak, Arabidopsis) en die verlies-van-funksie (Arabidopsis) strategieë. Maar hulle het nie die onveranderlike opeenhoping van Raf, wat gereeld verky word uit sulke oor-uitdrukking strategieë, aangespreek nie. Ons het dus ondersoek of daar 'n funksionele rol vir Raf in geïnduseerde sistemiese weerstand (ISR) kan wees. ISR is 'n goed-bestudeerde meganisme wat deur plante geïmplementeer word om nekrotrofiese patogene soos *Botrytis cinerea* te beveg. Vir hierdie doel het ons gekyk na die *RS5* mutant agtergronde (absent in Raf, maar hiper-akkumulاسie van Gol) met die redenasie dat die Gol akkumulerende mutante weerstandbiedig teen *B. cinerea* moet wees (soos voorheen beskryf

vir transgeniese oor-uitdrukking van *GolS1* in tabak en Arabidopsis). Sulke bevindings verhinder dan 'n rol vir Raf, aangesien die stelsel geen Raf akkumuleer nie. Verbasend, twee onafhanklike T-DNA invoeg mutante vir *RS5* (*atrs5-1* en *5-2*) was ewe hiper-sensitief vir *B. cinerea* infeksie as twee onafhanklike T-DNA invoeg mutante vir *GolS1* (*atgols1-1* en *1-2*). Die hiper-sensitiwiteit van die *GolS1* mutant agtergrond was reeds voorheen gedemonstreer.

Die *RS5* mutant agtergronde versamel aansienlike konsentrasies van Gol, vergelykbaar met dié berig vir transgeniese plante (tabak en Arabidopsis) waar patoogen-weerstandbiedigheid aangemeld is. Verder, in die loop van ons ondersoeke het ons ontdek dat beide *AtGolS1* mutante ook aansienlike konsentrasies van beide Gol en Raf onder normale groei-toestande akkumuleer. Dit was nie aangemeld in die vorige studies nie. Gesamentlik argumenteer ons bevindinge teen 'n rol vir óf Gol, of Raf, tydens die induksie van ISR. Alhoewel, ons elimineer nie 'n rol vir die RFO padweg nie, gegewe dat oor-uitdrukking van *GolS1* gene tydens patoogen-weerstandbiedigheid in vorige verslae verwysig was. Ons ondersoek verder 'n moontlike rol vir die aanwesigheid van die *GolS* transkrip en/of proteïen as 'n moontlike komponent van die voorgestelde funksie in ISR.

Die unieke ensiem van *A. reptans* (galactan:galactan galactosyltransferase, ArGGT) is in staat om die vorming van hoër oligomere in die RFO pad te kataliseer sonder die gebruik van Gol as 'n skenker galactosyl, maar eerder, met behulp van die RFO's hulself as galaktose skenkers en aanvaarders (Gol-onafhanklike biosintese). Ons het *ArGGT* konstitutief oor-uitgedruk in Arabidopsis as 'n manier om 'n lang-ketting RFO akkumulering daar te stel met die doel om 'n rol vir hulle in die verbetering van vriestoleransie verder te ontleed. Ons was tot dusver onsuksesvol in die verkryging van RFO's hoër as Sta in die blare (wat akkumuleer het in 'n baie lae konsentrasie). Sedert ArGGT 'n affiniteit vir Sta as substraat toon, en Arabidopsis sade versamel aansienlike hoeveelhede Sta, het ons verder die saad water oplosbare koolhidraat (WSC) profiele van drie onafhanklike transgeniese lyne ontleed, maar

bespeur geen bykomende RFO oligomere buite die normale Raf en Sta konsentrasie nie. Ons stel verdere strategieë voor om hierdie benadering (Hoofstuk 4) te verbeter.

Gesamentlik verteenwoordig hierdie werk gevallestudies van RFOs in saadfisiologie, hul vermoëns/vereiste in biotiese stres en die gebruik van unieke ensieme om lang-ketting RFO akkumulاسie daar te stel met behulp van die Arabidopsis model. Teen die tyd van die indiening van hierdie tesis was die volgende bydraes gemaak aan die algemene wetenskaplike gemeenskap: (i) Aanbieding van hoofstuk 2 op die 26^{ste} Internasionale Konferensie vir Arabidopsis Navorsing (26^{ste} ICAR, 2015, Parys, Frankryk), en (ii) indiening van hoofstuk 2 as 'n manuskrip tans onder nasiening vir moontlike publikasie in die joernaal 'Plant and Cell Physiology'.

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CHAPTER 1

General introduction

1.1 Plant sugars (saccharides) play major functional roles in the physiological processes required for development and survival

The simplest and most abundant form of carbohydrates is sugars (also termed saccharides) which can be classified into four biochemical groups based on their degree of polymerisation (DP). These groups are termed (i) monosaccharides [DP 1], (ii) disaccharides [DP 2], (iii) oligosaccharides [DP 3-9] and (iv) polysaccharides [DP \geq 10] (Kennedy and White, 1983). Plants produce many carbohydrates that are similar to those found in animals but, also synthesise a diverse set of carbohydrates unique to the plant kingdom. The major plant carbohydrates (starch and sucrose) usually account for nearly 75% of total dry mass. As the primary products of photosynthesis they provide the fundamental platform for further metabolism, structure and cellular communication (Bazzaz et al. 1987; Krogh 2008). Starch predominantly serves as a storage reserve. During periods of non-photosynthetic activity it is actively hydrolysed, providing the plant with 'renewable' carbohydrate resources that sustain essential physiological processes. Typically, carbohydrates are assembled from the monosaccharides and plants contain a plethora of sugars and sugar-derivatives along with the high-molecular weight polysaccharides such as cellulose and starch (Patrick et al. 2013). Despite the well documented diversity of carbohydrates, their metabolic roles in plant physiology are often complex and many of the mechanisms by which they exert their functional roles are unclear. This thesis will focus on sugars, more specifically the raffinose family of oligosaccharides (RFOs) and their roles in seed physiology and biotic stress in the *Arabidopsis* model.

1.2 Many exotic water-soluble sugars are sucrose based

Sucrose (Suc), a simple non-reducing carbohydrate is one of the main products of photosynthesis, alongside starch. As the most widespread disaccharide found in plants, synthesised in the cytosol and transported throughout the plant system via phloem, it serves as the energy molecule for all other cells (Koch et al. 2004; Patrick et al. 2013; Van den Ende 2013). Not only is Suc a major transport sugar in plants, it also serves as a structural backbone for numerous water soluble carbohydrates (WSC) which includes the Suc- and Fru-based oligosaccharides RFOs and fructans, respectively (Keller and Pharr, 1996; Vijn and Smeekens, 1999; Patrick et al. 2013). These WSCs are the most extensively studied of the various sucrosyl-oligosaccharides occurring in plants (**Fig. 1**).

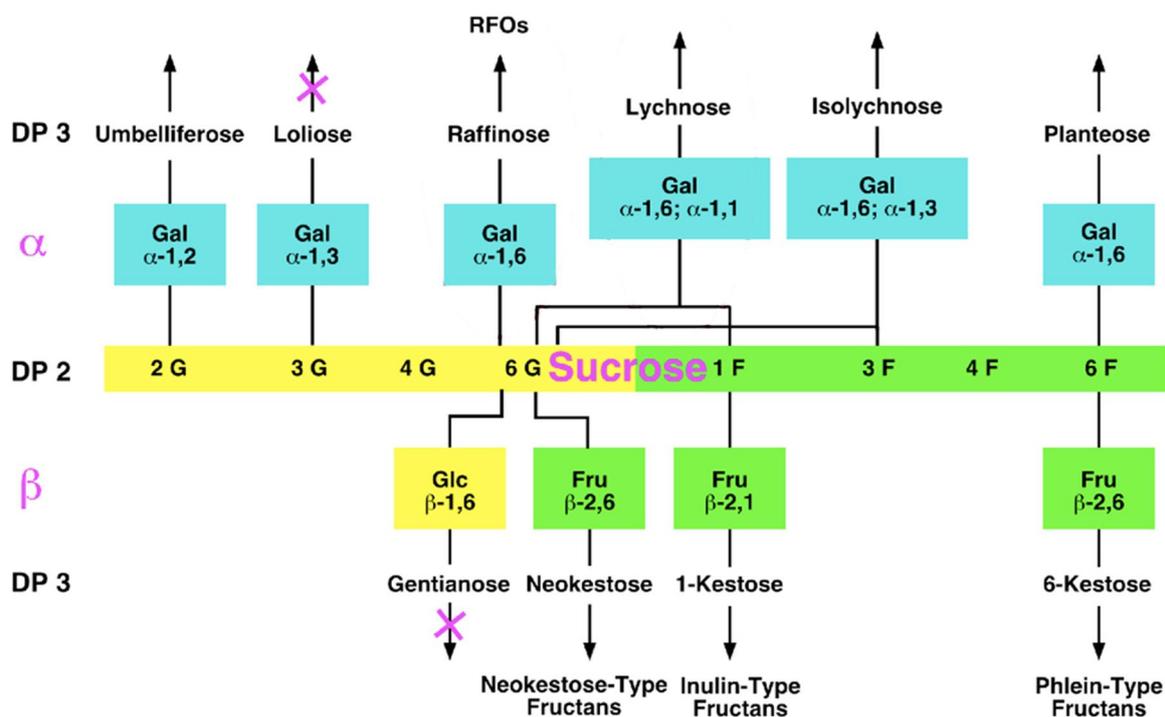


Fig. 1. Sucrose provides a structural backbone for numerous RFO- and fructan-based oligosaccharides (image adapted from Kandler and Hopf (1982) and provided courtesy of Prof. Dr. F Keller, University of Zurich).

The linkage configuration in these oligosaccharides determine the type of sugar and is generally defined with either alpha (α , RFO-based) or beta (β , fructan-based). Where applicable (Loliose and gentianose), the 'X' denotes no further elongation/higher oligomer synthesis. DP, degree of polymerisation; Gal, galactose; RFO, raffinose family oligosaccharides; G/Glc, Glucose; F/Fru, fructose.

1.2.1 Raffinose family oligosaccharides are galactosyl extensions of sucrose

“Classic” RFOs are α -galactosyl extensions of Suc. Their synthesis is mediated by α -galactosyltransferases in sequential steps to form the most common RFO oligosaccharides. The first oligosaccharide in this pathway is raffinose (Raf, DP 3), formed via the galactosyl transfer of galactinol (Gal) moieties to the glucose (Glc) moiety in Suc. Subsequent biosynthesis of RFOs are then built upon the Suc backbone leading to a series of oligosaccharides with varying degrees of polymerisation *viz.* stachyose (Sta, DP 4), verbascose (Ver, DP 5) and ajugose (DP 6). Higher oligomers (up to DP 13) have been reported (reviewed in, Van den Ende 2013) but their occurrence seems rare in the plant kingdom. Classic RFOs are well documented to in their roles in carbon translocation and storage (reviewed in Keller and Pharr, 1986) but also accumulate during periods of abiotic stress (Zuther et al. 2004; Peters and Keller 2009; Iftime et al. 2011; Egert et al. 2013, Tarkowski and Van den Ende 2015).

The so called “alternative” RFOs are derived from Suc, Raf and Sta (Van den Ende 2013). These “alternative” RFOs are specific to plant families. They include amongst others (refer to **Fig. 1**): planteose (DP 3, kiwi fruit, *Actinidia deliciosa*; Klages et al. 2004), lychnose (DP 4, chickweed, *Stellaria media*; Vanhaecke et al. 2006, 2008), stellariose (DP 5, chickweed, *Stellaria media*; Vanhaecke et al. 2010) and manninotriose (DP 5, red deadnettle, *Lamium purpureum*; dos Santos et al. 2013; Van den Ende 2013). Their synthesis and accumulation, like the classic RFOs, occurs either as carbon translocates and storage molecules or, as part of a stress response mechanism.

1.2.2 Fructans are fructosyl extensions of sucrose

Fructans are β -fructosyl extensions of Fru found in approximately 15% of flowering plants (Vijn and Smeekens 1999). They are represented by three main classes (i) Inulin-type

fructans (β -2,1-linkages), (ii) levan (phleins or phleans, β -2,6-linkages) and (iii) graminan-type fructans (both β -2,1-linkages and β -2,6-linkages) (**Fig. 1**). More complex fructans are formed from a 6G-kestotriose backbone where elongations occur on both sides of the molecule. Fructan biosynthesis occurs from Suc via trisaccharide intermediates, such as 1-kestose, 6-kestose or 6G-kestose, mediated by various fructosyltransferases (FTs) in the vacuole (Vijn and Smeekens 1999; reviewed in Valluru and Van den Ende 2008). An integral role for Suc has been demonstrated in (i) the biosynthesis of fructans, largely controlled by a Suc-specific pathway and (ii) the activation of FT gene expression in response to organ-specific Suc fluxes (Lu et al. 2002; Maleux and Van den Ende, 2007; Bolouri-Moghaddam and Van den Ende 2013; Van den Ende 2013).

Although fructans are generally regarded as storage carbohydrates, they have been shown to contribute significantly to abiotic stress tolerance (Livingston and Henson, 1998; De Roover et al. 2000; Yoshida et al. 2007). They have more recently also been suggested to be important in the defence response of plants to pathogen infection (Rolland et al. 2006; Bolouri-Moghaddam and Van den Ende 2013; Rudd et al. 2015).

1.3 The physiological roles of sugars encompass abiotic and biotic stress, apart from carbon translocation and storage

1.3.1 Sugars have been implicated in general abiotic stress responses

Abiotic stresses in plants lead to detrimental, and often fatal, physiological changes. Metabolic responses to environmental stress involve a multitude of regulatory processes during which (i) signalling, (ii) gene expression, (iii) hormonal fluxes, (iv) anti-oxidant accumulation and (v) carbohydrate dynamics in a cell changes dramatically.

Plants are able to manage environmental stresses (including water deficit, long periods of drought, increased salinity, high heat and light) through the activation of multiple molecular response pathways. Upon stress perception, plants are able to induce a signalling cascade which leads to modifications in gene expression and the subsequent accumulation of reactive oxygen species (ROS), phyto-hormones, transcription factors and compatible solutes (Yamaguchi and Blumwald 2005; Yamaguchi-Shinozaki and Shinozaki 2006). The latter has been proposed to function as one of the most important components in stress response mechanisms because compatible solutes (like some amino acids, sugars and sugar-alcohols) do not interfere with normal cellular metabolic processes even when they occur in substantial concentrations. The accumulation of sugars (e.g. RFOs and fructans) is thought to serve in osmotic adjustment and cellular protection (membrane stabilisation and ROS scavenging) (**Fig. 2**, Hoekstra 2001; Valluru and Van den Ende 2011).

RFOs are considered key WSCs during conditions of cold, drought and salinity tolerance where their accumulation possibly indicates roles as osmo-lytes/-protectants (Bachmann et al. 1995; Koster and Leopold 1988; Bartels and Sun-kar 2005) to stabilize cell proteins and membranes, and to support cell turgor (**Fig. 2**, Hoekstra 2001). During periods of cold acclimation, Raf concentrations accumulate to substantial amounts (Arabidopsis, Zuther et al. 2004; Iftime et al. 2010; Egert et al. 2013, Tarkowski and Van den Ende 2015) in the chloroplasts where it has been proposed to protect photosystem II (Knaupp et al. 2011). It has also been demonstrated in resurrection plants species that RFOs accumulate as part of a desiccation tolerance mechanism during water deficit to possibly (i) protect plants from membrane and sub-cellular damage during dehydration (Whittaker et al. 2001; Farrant 2007; Peters et al. 2007) and (ii) supply plant cells with the necessary energy levels to assist during rehydration and damage control (*Xerophyta viscosa*, Peters et al. 2007). The role of RFOs in abiotic stress is further discussed in section 1.5.2.

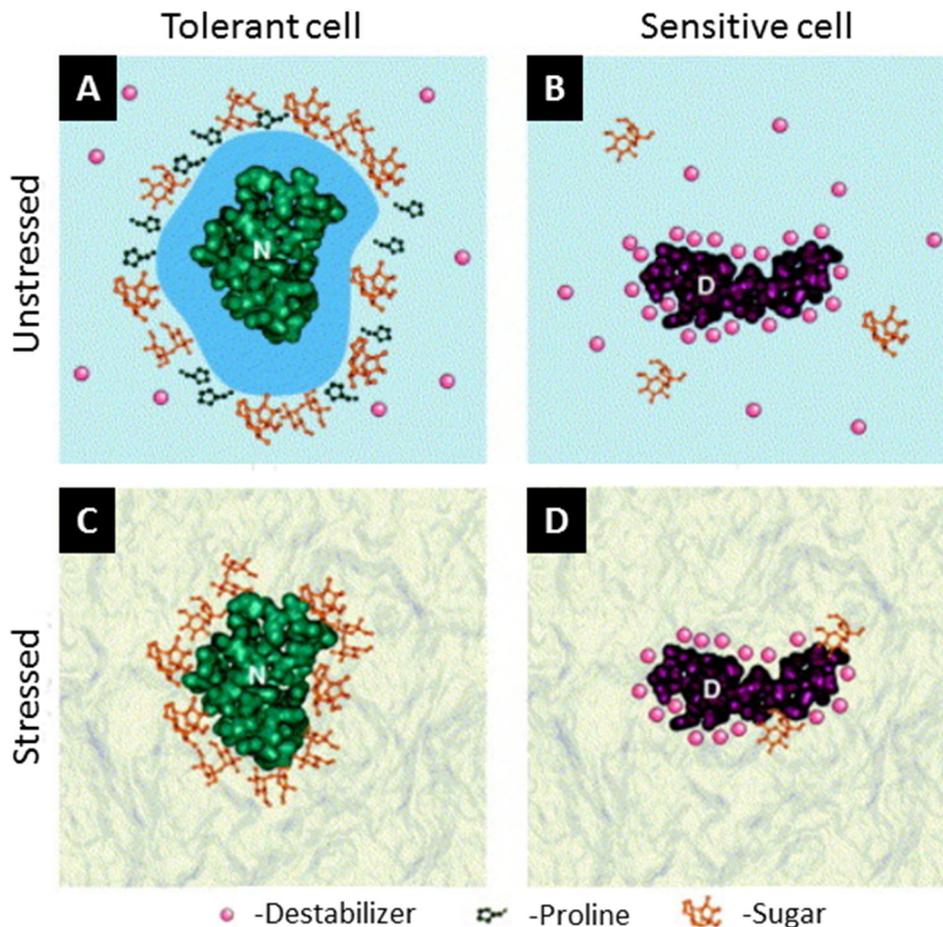


Fig. 2. Membrane stabilization mechanism via compatible solute (proline and sugars) accumulation in tolerant and sensitive systems, under non-stressed and abiotic stress conditions (image adapted from Hoekstra 2001).

During unstressed conditions tolerant cells (A) accumulate compatible solutes which protect cellular membranes during conditions of abiotic stress (C). Sensitive cells (B) do not accumulate compatible solutes resulting in degradation of proteins during conditions of abiotic stress (D) leading to irreversible cellular damage. N. normal cellular state, D. desiccated cellular state.

Furthermore, fructans are also considered to act as compatible solutes during conditions of abiotic stress (Valluru and Van den Ende 2008). The correlation of drought tolerance and fructan accumulation was primarily demonstrated in transgenic *Nicotiana tabacum* (tobacco, Pilon-Smits et al. 1995) and *Beta vulgaris* (sugar beet, Pilon-Smits et al. 1999). Recent exploration into environmentally-induced fructan accumulation provided insight that fructans may also play a role in ROS scavenging (Peshev et al. 2013; Peukert et al. 2014). Similar to RFOs, fructan-based oligomers are able to stabilize membranes during periods of

cold, drought and salinity by interacting with lipid-layers (Vereyken et al. 2001; Hinch et al. 2003; Tarkowski and Van den Ende 2015). However, due to its restricted localisation to the vacuole, it is proposed to only contribute to the stabilization of the tonoplast and possibly the plasma membrane (Valluru et al. 2008; Tarkowski and Van den Ende 2015). Fructans have been demonstrated to largely contribute to cold tolerance in fructan-accumulating plant species, such as wheat (Yokota et al. 2015). Further, it demonstrated that transgenic rice over-expressing the wheat FTs sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose:fructan 6-fructosyltransferase (6-SFT) showed improved tolerance to low, non-freezing temperatures (Kawakami et al. 2008). Although both 1-SST and 6-SFT are vacuolar enzymes (similar to ArGGT) their over-expression in plant systems is well documented and leads to accumulation of fructans to varying chain length and concentrations (reviewed in Cairns, 2003).

1.3.2 Sugars have been implicated in plant responses to fungal pathogen infection

Plants are sessile biological systems and therefore require rapid response mechanisms to activate their innate immunity against a broad range of microbes, insects, herbivores and fungi (reviewed in Bruce and Pickett 2007). Two major response pathways implicated in fungal attack have been described for induced plant immunity, termed (i) systemic acquired resistance (SAR) and (ii) induced systemic resistance (ISR) (Van Loon et al. 1998; Glazebrook et al. 2003; Durrant and Dong 2004; De Vos et al. 2005).

Immunity mediated by SAR is systemic, travelling throughout the plant from the site of infection (Ton et al. 2002; Durrant and Dong 2004). The activation of SAR is usually achieved after infection by a non-necrotrophic (non-lethal) pathogen which in turn promotes the production of salicylic acid (SA). A set of pathogenesis-related (PR) genes have been experimentally demonstrated to be transcriptionally responsive to both non-necrotrophic

pathogen infection and the exogenous application of SA (Pieterse et al. 1996; Ryals et al. 1996; Van Wees et al. 1997). Although SA is a pre-requisite for activation of SAR, it is not considered to be the systemic signalling molecule. This role has been suggested to be mediated via sugar-signalling (Vernooij et al. 1994; Kumar and Klessig 2003; Forouhar et al. 2005; Park et al. 2007; Vlot et al. 2008; Wingler and Roitsch 2008).

Immunity mediated by ISR is localised to the site of infection (reviewed in van Loon et al. 1998). The activation of ISR is triggered by necrotrophic (lethal) pathogen infection which predominantly promotes the accumulation of jasmonic acid (JA), but ethylene (ET) accumulation has also been demonstrated (Thomma et al. 1998; Ton et al. 2002; Glazebrook et al. 2003). Hormonal regulation of ISR was demonstrated in *Arabidopsis* mutants (disrupted in JA or ET, Knoester et al. 1999; Ton et al. 2002; Glazebrook et al. 2003). Those studies demonstrated that mutants were more susceptible to necrotrophic pathogen infection than the wild-type plants. Only a single PR-gene (PR3, *At3g12500*) is known to be transcriptionally responsive to ISR (and JA, Schweizer et al. 1998; Lorenzo et al. 2003; Zheng et al. 2006). However a number of well described R2R3-MYB and bHLH transcription factors have been shown to be induced by ISR (and JA, Chini et al. 2007; Kazan and Manners 2008; Fernandez-Calvo et al. 2011). Amongst these is MYB75, characterised to be the transcription factor regulating anthocyanin accumulation (Borevitz et al. 2000; Teng et al. 2005; Zuluaga et al. 2008; Ballare 2014; Tauzin and Giardina 2014).

Sugars have long been suggested to play active roles in plant defence mechanisms during pathogen infection (Watson and Watson, 1951; Shalitin and Wolf, 2000). A link between sugars and plant innate immunity was established after PR genes was demonstrated to be transcriptionally responsive to sugars, in the absence of pathogen infection (Herbers et al. 1996; Xiao et al. 2000; Rolland et al. 2006). Sugars are well characterised in

their ability to function as signalling molecules in plant physiological processes (reviewed in Rolland et al. 2002 and 2006). Both hexose sugars (glucose and fructose, Moore et al. 2003; Cho et al. 2009; Cho and Yoo 2011; Li et al. 2011) along with Suc (reviewed in Koch 2004; Rolland et al. 2006; Wind et al. 2010; Tognetti et al. 2013) have been experimentally demonstrated to regulate gene expression during (i) plant growth and developmental processes, (ii) carbon assimilation, (iii) hormone accumulation. Recently sugar-signalling pathways have been suggested to also function in plant defence responses (reviewed in Moghaddam and Van den Ende 2012). In most fungal pathogen-plant systems, a high level of sugars in plant tissues is suggested to enhance plant resistance (Herbers et al. 1996). Experimental findings include activation of ISR by exogenous Suc application (Heil et al. 2012). Further, anthocyanin biosynthesis is known to be triggered by Suc (Teng et al. 2005; Solfanelli et al. 2006). These secondary metabolites have been demonstrated to be effective ROS scavengers during pathogen infection (Bent et al. 1992; Zhang et al. 2013).

This has led to new terminology defining sugar-related plant defence responses (sweet immunity or sugar-enhanced defence, Bolouri-Moghaddam and Van Den Ende 2013; Trouvelot et al. 2014). During sugar-enhanced defence it is hypothesised that Suc is actively transported to sites of pathogen infection where photosynthetic capacity is impaired. The transcriptional up-regulation of SWEETs in tissues surrounding infected areas has led to the speculation that Suc is to some extent required in the management of biotic stress (Lapin et al. 2013). However, opposing views argue that these observations may represent the ability of pathogens to effectively hijack these Suc efflux systems to support their own growth. Experimental evidence for this comes from rice SWEET loss-of-function mutants which actually show resistance to pathogen infection (Chen 2014). The innate immunity of plants, which includes sugars, is evidently a complex process.

A number of sugars (Glc, Fru, Suc, trehalose, RFOs and fructans) have all been suggested to some extent to fulfil roles as signalling molecules triggering plant responses to pathogen infection (Sheen et al. 1999; Rolland et al. 2006, reviewed in Bolouri-Moghaddam and Van den Ende 2013). A signalling role for fructan oligosaccharides has been proposed in biotic stress but this remains unclear and requires further understanding as to the mechanism in which it operates (Van den Ende et al. 2004; Valluru and Van den Ende 2011; Eyles et al. 2013). It has recently been demonstrated in a next generation sequencing approach, that leaves of *Triticum aestivum* (wheat) infected with *Zymoseptoria tritici* (a necrotrophic pathogen) resulted in increased expression of the fructan biosynthetic pathway (Rudd et al. 2015). A similar role for RFOs has been implicated in biotic stress and is further discussed in section 1.5.3.

1.4 Biochemistry of the raffinose family of oligosaccharides (RFOs)

1.4.1 RFOs can be synthesised in both a galactinol-dependent and -independent manner by α 1,6-galactosyl transferases

The plant kingdom contains an exclusive range of sucrosyl oligosaccharides, termed raffinose family oligosaccharides (RFOs; Suc-(Gal)_n, 13 > n ≥ 1). RFOs are synthesised *via* α -1,6 galactosyltransferases that transfer galactosyl moieties in a galactinol-dependent fashion. This pathway is initiated by galactinol synthase (*Gols*, EC 2.4.1.123), forming galactinol (Gal; 1-O- α -D-galactopyranosyl-L-myo-inositol) as the primary galactosyl (Galactose; Gal) donor, allowing downstream RFO enzymes to transfer Gal units in a stepwise manner from one oligosaccharide to the other (Lehle and Tanner, 1973; Martínez-Villaluenga et al. 2008). Raffinose synthase (*RS*, EC 2.4.1.82) and stachyose synthase (*SS*, EC 2.4.1.67) are key enzymes in RFO biosynthesis catalysing the successive formation of raffinose (Raf, Suc-(Gal)₁) and stachyose (Sta, Suc-(Gal)₂), **Fig. 3A**.

Higher RFO oligomers in this pathway, including verbascose (Ver, Suc-(Gal)₃) and the longer-chain RFOs (Suc-(Gal)₄₋₁₃) are considered to be synthesised in a Gol-independent fashion via the unique RFO chain elongation-enzyme galactan:galactan galactosyltransferase (GGT, **Fig. 3B**). However, it is unclear whether Ver could be synthesised either *via* a multifunctional *SS* (with a broader acceptor specificity range) or an independent verbascose synthase (*VS*) (Tanner and Kandler 1968) in a Gol-dependent manner. The long-chain RFOs (up to Suc-(Gal)₁₃) in this pathway are typically chain-elongated in a Gol-independent fashion, utilising RFO molecules as both galactosyl donors and acceptors (see section 1.4.3, Bachmann et al. 1994; Bachmann and Keller 1995; Tapernoux-Lüthi et al. 2004).

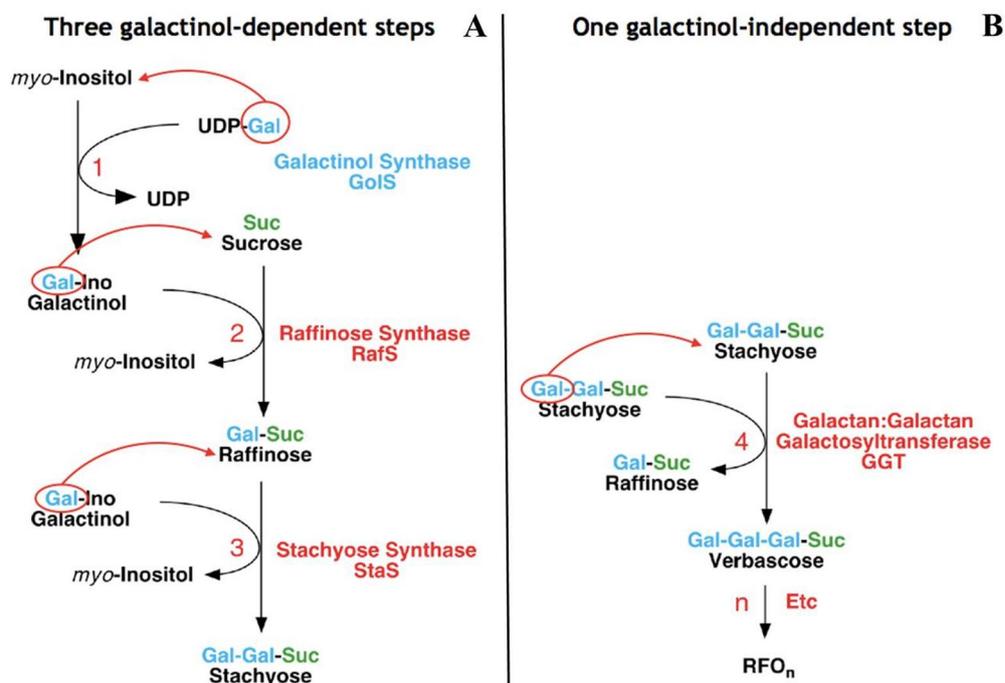


Fig. 3. Schematic representation of RFO oligomer synthesis in a (A) galactinol-dependent and (B) galactinol-independent manner. Image provided courtesy of Prof. Dr. F Keller, University of Zurich

(A) Schematic representation of Gol-dependent galactose transfer, up to the tetra-saccharide stachyose. (B) Schematic representation of Gol-independent galactose transfer illustrating the penta-saccharide verbascose and longer-chain RFO oligomers.

1.4.2 RFOs are hydrolysed by α 1,6-galactosyl hydrolases

The breakdown of RFOs is catalysed by specific α -galactosidases (α -Gals). Numerous forms of α -Gals have been isolated and their genes characterised, mainly from seeds in various species with few exceptions in leaves (Dey and Dixon 1985; Overbeeke et al. 1989; Zhu and Goldstein 1994; Keller and Pharr 1996; Davis et al. 1996, 1997; Peters et al. 2010). They have been described to be deposited into protein storage organs (vacuoles) in seeds where RFOs are believed to co-exist during seed maturation (Herman and Shannon, 1985; Sekhar and DeMason 1990). Thus, it seems that there is a continual synthesis and hydrolysis cycle of RFOs during seed maturation (Keller and Pharr 1996). The biochemical regulation of α -Gals are very complex and requires further insight. They can either be active in acidic or alkaline conditions, but this seems to be compartment and tissue specific (Keller and Pharr 1996). Seed α -Gals play an integral role during seed maturation and germination, possibly providing cells with carbon reserves by means of breaking down RFOs. In *Arabidopsis* the closest α -Gal homologs encode for seed imbibition proteins (*AtSIP1* and *AtSIP2*), similar to the characterised isoforms which are active in germinating barley seed and melon fruit (Heck et al. 1991; Carmi et al. 2003; Peterbauer and Richter 2001). The *AtSIP2* protein was functionally characterised as a true α -Gal, with substrate affinity for Raf and a sink-tissue specific pattern (Peters et al. 2010). Although *AtSIP1* has been linked indirectly to RFOs and abiotic stress, further investigations on *AtSIP* proteins will lend insight to alternative physiological contributions, including germination and seed maturation (Anderson and Kohorn 2001).

1.5 The precise physiological functions of raffinose family oligosaccharides (RFOs) have been well described but functional mechanisms remain unclear

Despite RFOs being renowned to accumulate almost ubiquitously in higher plants to fulfil key physiological functions during (i) source-to-sink phloem transport (Sprenger and Keller 2000), (ii) abiotic and biotic stresses and (Taji et al. 2002; Nishizawa-Yokoi et al. 2008; Knaupp et al. 2011; Egert et al. 2013; Keunen et al. 2013; Elsayed et al. 2014, Tarkowski and Van den Ende 2015) (iii) seed maturation (Blöchl et al. 2008; Angelovici et al. 2010), the precise mechanism/s by which this occur is unclear.

1.5.1 The RFOs are major agents of carbon translocation and storage

Phloem loading is an energised process that allows for the transport of sugar solutes, especially Suc, from source to sink tissues. This process is mediated via SWEETs (Sugars Will Eventually be Exported Transporters) and SUC/SUT (for Sucrose transporter/Sugar transporter) responsible for transfer of Suc from the phloem parenchyma into the sieve element companion cell complex for long-distance translocation (Riesmeier et al. 1992; Sauer 2007; Kühn and Grof 2010; Chen et al. 2012). Phloem loading occur *via* either apoplastic or symplastic phloem loading mechanisms (Turgeon and Ayre 2005; Chen et al., 2010; Ayre 2011, Lalonde and Frommer 2012). RFOs are implicated in symplastic phloem loading during which they are synthesised as a result of Suc polymerisation (**Fig. 4**). The latter process is termed a polymer trapping mechanism which allows for Suc to be imported into intermediary cells and polymerised to RFOs which are thought to be too large to diffuse back across the membrane (Turgeon and Gowan 1990; Turgeon 1996). As such, a highly controlled flux between Suc and RFO accumulation is maintained, allowing RFOs to potentially create an osmotic potential and consequently serve as the long distance translocates. This model has been extensively studied in Arabidopsis and recently in numerous herbaceous species (reviewed in Rennie and Turgeon 2009). This

transport ability of RFOs in leaves has also been well defined in *A. reptans*, a frost hardy labiate which transports mainly Sta in the phloem (Bachmann et al. 1994).

Apart from the role of RFOs during transport mechanisms, they also fulfil roles as storage compounds (Sheveleva et al. 1997; Sengupta et al. 2008; Miao et al. 2007). Besides their accumulation during seed maturation (Raf and Sta, reviewed in Peterbauer and Richter 2001), RFOs have also been shown to accumulate to significant amounts in other species such as *Stachys sieboldii* (tubers, Keller 1992) and *Ajuga reptans* (leaf and roots, Bachmann et al. 1994; Bachmann and Keller 1995) where they possibly serve as carbon reserves.

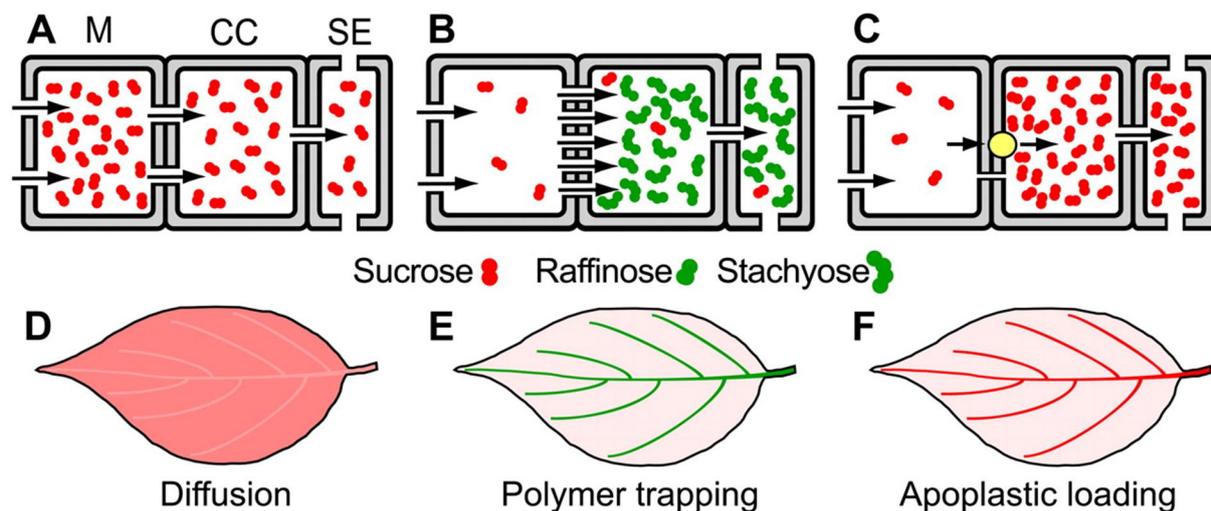


Fig. 4. Illustration taken from Turgeon 2010. Phloem loading in (i) minor veins (A-C) and (ii) whole leaf systems (D-F) by means of diffusion, polymer trapping or apoplastic loading

Suc passively diffuses through CC and SE (A and D) resulting in high *Suc* concentrations in M. *Suc* then diffuses to the intermediary cells where it is converted to Raf and Sta (B and E). These oligomers increases transport sugars in the phloem via the polymer trapping mechanism, causing M to maintain a low level of *Suc*. *Suc* is then further apoplastically loaded into the minor vein phloem via transporters (yellow circle), possibly SUT/SUC or SWEETs. M, mesophyll cells; CC, companion cells; SE, sieve elements; *Suc*, sucrose; Raf, raffinose; Sta, stachyose; SUT, sucrose uptake transporters; SUC, sucrose transporter; SWEET, sugars will eventually be exported transporters.

1.5.2 RFO mass increases are associated with abiotic stress events

The RFOs are well reported to accumulate in plants exposed to abiotic stress including water deficit, high salinity, heat shock, cold exposure (low temperature) and oxidative stress (Brenac et al. 1997; Gilbert et al. 1997; Pinheiro et al. 1998; Imanishi et al. 1998; Black et al. 1999; Pattanagul and Madore, 1999; Cunningham et al. 2003; Konrádová et al. 2003; Jouve et al. 2004; Panikulangara et al. 2004; Peters et al. 2007; Sanchez et al. 2008; Nishizawa et al. 2008; Egert et al. 2013). This has led to the suggestion that RFOs play functional roles in abiotic stress protection (reviewed in ElSayed et al. 2014). However, their exact mechanisms in this regard remain unclear.

The use of the Arabidopsis model has led to some of these mechanisms becoming clearer. The role of RFOs as ROS scavengers have recently been demonstrated (Nishizawa et al. 2008; Van den Ende and Valluru 2009; Van den Ende et al. 2011; Peshev et al. 2013) in transgenic Arabidopsis plants over-expressing *AtGolS1*, *AtGolS2* and heat-shock (*HsfA*) transcription factors (believed to orchestrate the expression of *GolS* isoforms during abiotic stress, Nishizawa et al. 2008; Busch et al. 2005; Nishizawa et al. 2006; Schramm et al. 2006). Arabidopsis wild-type and transgenic plants were exposed to methyl viologen (MV) treatment (inducing oxidative stress) and led to increased *GolS* gene expression and simultaneously accumulated high intracellular levels of Gol and Raf. The effects of oxidative damage were prominent in the wild-type plants, whilst transgenic plants had an improved tolerance to oxidative stress. These results collectively demonstrated the effectiveness of RFOs as anti-oxidants.

Low temperature conditions (cold-acclimation) have been extensively studied in Arabidopsis and possibly provides the most conclusive role for Raf. Raf accumulates in high amounts in Arabidopsis leaves during cold-acclimation and it has been proposed that Raf is transported to the chloroplast where it protects photosystem II (PSII). This was demonstrated in

knock-out *RS5* insertion lines (completely deficient of Raf, Egert et al. 2013) where the efficiency of PSII was perturbed during cold-acclimation, possibly due to a lack of Raf (Knaupp et al. 2011). The mechanism of this protection is not yet fully understood but it has been demonstrated that Raf is able to protect the chloroplast thylakoid membrane from photophosphorylation and electron transport during freezing (Knaupp et al. 2011), however it does not confer freezing- or cold-tolerance in *Arabidopsis* (Zuther et al. 2004).

Other studies which have proposed functions for RFOs in stress protection have traditionally associated correlative mass increases of RFOs to protective function (Peters and Keller 2009) or, they have drawn conclusions from tolerance obtained in transgenic systems over-expressing genes from the RFO biosynthetic pathway (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1999; Taji et al. 2002; Illing et al. 2005; Peters et al. 2007; ElSayed et al 2014). However, such reports do not address the mechanisms involved in such tolerance.

Recently, it has also been suggested that RFOs play an important role in biotic defence mechanisms, possibly as signalling molecules (Valluru and Van den Ende 2011; Kim et al. 2008, further discussed in section 1.5.3). Furthermore it seems that, as a collective, in abiotic and biotic stresses the individual roles for Gol and RFOs are not discriminated. It would be interesting to decipher and assign specific roles for these cyclitols and oligosaccharides, but this would require extensive analysis in both a Gol- and a Raf-free system, respectively.

1.5.3 Gol and RFOs have been suggested to participate as signalling molecules during biotic stress

Recently, the carbohydrate-cyclitol Gol has featured as a possible role player in signalling events linked to pathogen induced-responses (Kim et al. 2004; Kim et al. 2008,

Cho et al. 2010, Tarkowski and Van den Ende, 2015). The classical role of Gol has been thought to be the galactosyl donor during RFO biosynthesis. However, the role of Gol has since been expanded to include (i) free radical scavenging (Nishizawa et al. 2008) and (ii) protection against heat shock (Panikulangara et al. 2004) and (iii) protection against water-deficit (Albini et al. 1999; Taji et al. 2002). In the recent studies implicating Gol in pathogen infection, a differential screen of *Cucumis sativa* (cucumber) plants infected by *Corynespora cassiicola* (a leaf spot fungus) identified a galactinol synthase (*GolS*) isoform during infection (Kim et al. 2004). The *CsGolSI* gene was then constitutively over-expressed in tobacco, leading to resistance to infection by *B. cinerea* (Kim et al. 2008). This principle was further demonstrated in *Arabidopsis* where mutants in the *AtGolSI* isoform were susceptible to *B. cinerea* infection while *AtGolSI* over-expressing transgenic plants were resistant like tobacco (Cho et al. 2010). These experiments have implicated Gol accumulation in the ISR response and proposed a signalling role for Gol (in ISR) alike to phyto-hormones. Apart from demonstrating that *GolS* genes are JA-responsive, they have not addressed a potential mechanism by which this process occurs. A consideration not accommodated for by the studies is that Gol does not accumulate without the subsequent accumulation of Raf. This poses the question if Gol alone is responsible for the observation or if Raf is also involved? Nevertheless, it is clear that components of the RFO pathway have been implicated a novel physiological role (pathogen interaction) in plants.

However, these novel findings have provided proof-of-concept that a component of RFO biosynthesis exerts a signalling role in ISR. Chapter 3 of this work further explores this role, investigating if Gol alone is actually the signalling molecule or if Raf also contributes.

1.6 RFO accumulation in seeds is thought to facilitate the formation of a cytoplasmic glass

Mature seeds are quiescent organs and accumulate storage compounds such as carbohydrates and lipids. Proteins and lipids (contributing approximately 40% each to seed dry weight) are the most abundant components contained within seeds and accumulate during the mid-maturation phases. Conversely, Suc and RFOs are deposited during late-maturation phases (contributing approximately 2-20% of seed dry weight, Baud et al. 2002; Fait et al. 2006).

Seeds are divided into two categories based on their ability to survive moisture loss associated with maturation (Roberts 1973). These categories have been termed recalcitrant (desiccation-sensitive, does not survive water loss beyond 40%) and orthodox (desiccation tolerant, survives up to 5% of water content, Roberts 1973; Pritchard and Prendergast 1986; Farrant et al. 1989; Pritchard 1991; Finch-Savage 1992). Seed orthodoxy has long been associated with RFO (Raf and Sta) accumulation. Recalcitrant seeds accumulate no RFOs (or very low amounts). It is thus thought that RFO accumulation facilitates a protective role during seed maturation (when tissue desiccation occurs), initiating a glassy state in orthodox seeds (see **Fig. 2**, Crowe et al. 1987; Chen and Burris 1990; Leprince et al. 1990; Blackman et al. 1992; Blackman and Obendorf 1995; Leopold et al. 1994; Bailly et al. 2001; Hoekstra et al. 2001). This glassy state is proposed to prevent cytoplasmic crystallisation and the consequent drastic pH change which is detrimental to seed viability (Caffrey et al. 1988).

It has previously been thought that Suc fulfilled this function. However Suc alone (in the absence of RFOs) was unable to confer seed viability (Koster and Leopold, 1988). The mechanism of protection and metabolic regulation of RFO accumulation during seed development and germination is unclear (Sun et al. 1994; Steadman et al. 1996). However, it is tempting to speculate that RFOs may play a fundamental role in preventing the

crystallisation of Suc in mature seeds (5% of water content). This has also been suggested for desiccated resurrection plant leaves which accumulate RFOs along with large amounts of Suc (Peters et al. 2007). The desiccation tolerance of resurrection plant leaves have been compared to a state of seed orthodoxy, where vegetative tissues reach a quiescent state (Illing et al. 2006). To date, only one report has actually demonstrated the *in vitro* ability of small amounts of Raf in preventing the formation of the Suc crystal (Caffrey et al. 1988).

RFOs have also been implicated for their role as anti-oxidants and consequently impact longevity of orthodox seeds (Horbowicz and Obendorf 1994; Lin and Huang 1994). During oxidative stress (an active process during seed imbibition and early germination) a major burst of reactive oxygen species (ROS) destabilise macromolecules such as proteins, lipids and DNA. RFOs, especially Sta, are abundant in seeds (Arabidopsis seeds contain approximately 0.74 mM Raf and 3.4 mM Sta) and rapidly decline as germination proceeds, suggesting an important role as ROS scavenger protecting plant cells as anti-oxidant agents to maintain redox homeostasis (Mittler 2002; Nishizawa et al. 2008).

1.7 RFO-related enzymes in Arabidopsis are only partially characterised

1.7.1 Ten galactinol synthase (GolS) isoforms occur in Arabidopsis

GolS is thought to initiate the synthesis of RFOs by supplying the pathway with Gol, the primary galactosyl donor. The first step is to catalyse the transfer of a galactosyl moiety from UDP-D-Galactose to myo-inositol (Ino). This reaction yields Gol and UDP, of which Gol is the substrate required for downstream RFO enzymes to activate. In Arabidopsis seven putative GolS isoforms have been described, all containing the conserved amino acid site designated by 'APSAA'. Only three of these isoforms have been functionally characterised and their physiological roles determined in responses to drought, salt and cold stress, respectively (*AtGolS1*, *AtGolS2* and *AtGolS3*, Taji et al. 2002; Nishizawa et al. 2006).

However, *AtGolS1* has also been implicated to play an important role in pathogen interaction synthesising Gol as a possible signalling molecule (Kim et al. 2004; Kim et al. 2008, Cho et al. 2010, Tarkowski and Van den Ende, 2015). Currently, little is known about these genes and their function in seeds, but it is believed that they are involved in RFO synthesis (as intermediate cyclitol substrate) for the production of storage carbohydrates, given that RFOs are synthesised *de novo* and not imported into the seeds (Kandler and Hopf 1980; Peterbauer and Richter 2001). The specific roles of GolSs are often unclear and undefined and it is therefore important to consider the fact that the accumulation/synthesis of Gol is concomitant with that of Raf.

1.7.2 Only a single raffinose synthase (RS) has been reported from Arabidopsis leaves

The second reaction in the RFO pathway yields the trisaccharide Raf, a metabolite that has been widely reported to be involved in abiotic stress tolerance mechanisms (reviewed in ElSayed et al. 2014). RSs catalyse the transfer of galactosyl units from Gol as donor to Suc as acceptor. The biosynthetic actions of RSs are extremely specific in terms of substrates and acceptor molecules. Few RSs have been characterised, mainly from seeds (Lehle and Tanner 1973; Peterbauer et al. 1998; Watanabe and Oeda 1998; Hitz et al. 2002). In *Arabidopsis* only one true RS has been functionally characterised (Egert et al. 2013) and described to be solely active in leaves during abiotic stress, during which high amounts of Raf accumulate. In that study they also reported the possibility of a putative, as yet uncharacterised, seed-specific RS. No reports of a seed-specific RS in *Arabidopsis* seeds are available currently.

1.7.3 No stachyose synthase (SS) isoforms have been reported for Arabidopsis

To date, there are no reports concerning an *Arabidopsis* SS and its role in RFO biosynthesis. A putative sequence for AtSS (*At4g01970*) is annotated in the *Arabidopsis* database (TAIR) and shows amino acid identities of 58%, 58% and 59% to the known SSs from pea, adzuki

bean and mask flower, respectively. However it has also been reported previously as a RS (Lee et al. 2012, Nishizawa et al. 2008). This putative SS also shows amino acid identities of 53% and 47% to Arabidopsis AtSIP2 (*At3g57520*, Peters et al. 2010) and RS5 (*At5g40390*, Zuther et al. 2004), respectively. Recent partial characterisation of a bi-functional SS (RS4, *At4g01970*) in Arabidopsis seeds, synthesising both Raf and Sta in a Gol-dependent manner (Chapter 2, this study), could potentially resolve the sequence ambiguity and provide more insight as to the complexity of SSs in general.

1.7.4 No RFOs beyond Sta occur in Arabidopsis – an opportunity for engineering of long chain RFOs

In Arabidopsis, the only RFO found in vegetative tissues (leaves and roots) is Raf. Its accumulation is strictly associated with episode of abiotic stress (reviewed in ElSayed et al. 2014). In generative tissues (seeds) both Raf and Sta occur of which Sta is the most abundant RFO in the seeds.

While these short-chain RFOs (particularly Raf) have been proposed to function in the amelioration of abiotic stress, the long-chain RFOs (> Sta) have only been studied in the common bugle (*Ajuga reptans*, Peters and Keller 2009). All reported RFO biosynthetic enzymes from Arabidopsis (RS5, Egert et al. 2013; RS4, this study) are galactinol-dependent in the RFO synthesis abilities. The unique RFO chain elongating enzyme galactan:galactan galactosyltransferase (GGT) identified and characterised from *A. reptans* displays the Gol-independent ability to use short-chain RFOs (like Raf and Sta) as both the galactosyl donors and acceptors. This allows for the accumulation of substantial amounts of long-chain RFOs (up to Suc-(Gal)₁₃).

Arabidopsis RFO physiology may thus present a unique opportunity to engineer long-chain RFO accumulation (outside *A. reptans*) by simply over-expressing *ArGGT* constitutively.

Such a transgenic system may prove useful in dissecting the functional role/s of long-chain RFOs which have been previously suggested to encompass improved freezing tolerance (Peters and Keller 2009). While Raf has been demonstrated to fulfil a membrane protection function in the *Arabidopsis* chloroplasts (Knaupp et al. 2011) a single *in vitro* study has reported on the improved efficacy of higher DP RFOs in protecting artificial liposomes from desiccation (Hincha et al. 2003). Chapter 4 of this work provides the preliminary data of an approach to engineer long-chain RFO accumulation in *Arabidopsis* using this strategy.

1.8 Aims of this work

In this study we aimed to further elucidate the RFO pathway by (i) characterisation of the putatively annotated SS in *Arabidopsis* (*At4g01970*, *RS4*), (ii) investigating the role of Raf in biotic stress and (iii) attempting to engineer long-chain RFOs into *Arabidopsis* in order to further investigate its suggested role in abiotic stress tolerance.

We have successfully characterised and reported on RS4 as being the sole SS responsible for Sta accumulation in *Arabidopsis* seeds using a reverse genetic-approach. We furthermore elucidated the ability of this enzyme to be of a multi-functional nature, catalysing the formation of both Raf and Sta, in a forward genetic-approach.

To further explore the nature of Raf in biotic stress, we exploited the knowledge of the linear fashion of the RFO pathway. We used the Raf-free system, provided by RS5 T-DNA insertion lines (previously characterised as Raf-deficient, Egert et al. 2013), hyper-accumulating Gol. We were able to demonstrate that Raf does not necessarily impact the ISR pathway. However, we were also able to conclusively demonstrate that the hyper-accumulation of Gol did not improve ISR, contradictory to previous belief.

We furthermore attempted to engineer exotic carbohydrates into *Arabidopsis* in a forward genetic-approach, constitutively over-expressing the *Ajuga reptans* GGT. We aimed to obtain transgenic plants accumulating long-chain RFOs ($DP \geq 5$) and investigate their role in physiological stress responses. To this end we have established three independent transgenic lines, but have not yet been successful at accumulating long-chain RFOs in *Arabidopsis* leaves or seeds.

CHAPTER 2

A Single Gene (*RS4*, *At4g01970*) is Responsible for Seed Specific-biosynthesis of the Raffinose Family Oligosaccharides (RFOs) Raffinose and Stachyose in Arabidopsis

Running head: *At4g01970* Synthesises Both Raffinose and Stachyose

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Chapter 2 has been submitted to Plant and Cell Physiology (www.pcp.oxfordjournals.org) and was under peer review at the time of submission of this thesis. The following content represents the actual PDF of the manuscript generated during the online submission process and, includes a supplementary dataset.

Author contributions:

BL conducted all experimental work presented in the manuscript and participated in the writing thereof. FH assisted with LC-MS sample loading via the Central Analytical Facility (CAF, Stellenbosch University). SP conceived of this study, its design and coordination, and participated in the manuscript writing.

A Single Gene (RS4, At4g01970) is Responsible for Seed Specific-biosynthesis of the Raffinose Family Oligosaccharides (RFOs) Raffinose and Stachyose in Arabidopsis

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Abbreviations: DAF, days after flowering; Gal, galactose; GGT, galactan:galactan galactosyl transferase; Gol, galactinol; Gent, gentamicin; GUS, β -glucuronidase; Hyg, hygromycin; Kan, kanamycin; Raf, raffinose; RFOs, raffinose family oligosaccharides; RS, raffinose synthase; Rif, rifampicin; SS, stachyose synthase; Sta, stachyose; sqPCR, semi-quantitative reverse transcription PCR; Suc, sucrose; UDP-Gal, uridine diphosphate galactose; UTR, untranslated region; qPCR, quantitative real-time PCR (qPCR)

Abstract

Raffinose family oligosaccharides (RFOs) are galactose extensions of sucrose (Suc-Gal_n). Mature Arabidopsis generative tissues (seeds) accumulate the RFOs raffinose (Raf, Suc-Gal₁) and stachyose (Sta, Suc-Gal₂) presumably *via* the catalytic activities of raffinose synthase (RS) and stachyose synthase (SS), respectively. The RFO biosynthesis pathway has been extensively characterised in Arabidopsis vegetative tissues (leaves and roots) but little is known from the seeds. A single gene (*RS4*, *At4g01970*) is annotated as either an RS or SS. Using two insertion mutants we demonstrated Sta deficiency in mature seeds. A double mutant with the recently characterised *RS5* (*At5g40390*), shown to partially be responsible for Raf accumulation in mature seeds was completely deficient in seed RFOs. This provided the first hint that *RS4* could potentially also be involved in Raf biosynthesis. The only RFO accumulating in Arabidopsis leaves is Raf, occurring strictly in response to various abiotic stresses. Seed specific expression of *RS4* was deregulated by constitutive over-expression in Col-0 and the *atrs5* mutant background (RS and Raf deficient). Both Raf and Sta unusually accumulated in Col-0 leaves over-expressing *RS4*, under normal growth conditions. Further, leaf crude extracts from *atrs5* insertion mutants (RS and Raf deficient) over-expressing *RS4* showed enzyme activities for both RS and SS, *in vitro*. Collectively our findings have physiologically characterised *RS4* as a RFO synthase responsible for Sta and, partially Raf (along with *RS5*) accumulation during Arabidopsis seed development.

Keyword index

Arabidopsis; seeds; raffinose family oligosaccharides; raffinose synthase; stachyose synthase

Introduction

Raffinose family oligosaccharides (RFOs; Suc-Gal_n, 13 < n ≤ 1) are unique to plants. These sucrosyl oligosaccharides are synthesised *via* α1,6-galactosyltransferases that generally catalyse the transfer of galactosyl moieties using the unusual cyclitol-carbohydrate hybrid galactinol (Gol) as the galactosyl donor. Galactinol synthase (GolS, EC 2.4.1.123) is responsible for the biosynthesis of Gol (using myo-inositol and UDP-Gal as substrates). Raffinose synthase (RS, EC 2.4.1.82) and stachyose synthase (SS, EC 2.4.1.67) successively catalyse the formation of raffinose (Raf, Suc-Gal₁) and stachyose (Sta, Suc-Gal₂) in a Gol-dependent manner. Verbascose (Ver, Suc-Gal₃) has been reported to be synthesised either *via* a multifunctional SS in *Pisum sativum* (pea seeds, Peterbauer and Richter 2002) or potentially *via* a Gol-dependent verbascose synthase (VS, unreported). Higher RFO oligomers (up to Suc-Gal₁₃) have been reported to accumulate in the freeze tolerant labiate *Ajuga reptans* (common bugle, Bachman et al. 1994; Haab and Keller 2002; Peters and Keller 2009). These higher RFO oligomers are synthesised by the novel Gol-independent enzyme galactan:galactan galactosyl transferase (GGT) which, uses RFOs as both galactosyl donors and acceptors.

The RFOs have been extensively physiologically characterised in their roles as phloem translocates and in carbon storage in sink tissues (roots and tubers, reviewed in Keller & Pharr 1986). However, they frequently also occur in generative tissues (seeds, reviewed in Peterbauer and Richter 2001) where their accumulation has been proposed to function in both carbon storage and protection from water deficit-processes linked to seed maturation. In vegetative tissues (leaves and roots) Raf is frequently reported to increase in response to various abiotic stresses, suggestive of a role in stress protection (Taji et al. 2002; Zuther et al. 2004; Knaupp et al. 2008; Nishizawa et al. 2008; Egert et al. 2013). Accumulation of Sta in leaves is rare but, has been reported for *Alonsoa meridionalis* (mask flower, Voitsekhovskaja et al. 2009), *Cucumis melo* (melon, Holthaus and Schmitz 1991) and *Cucurbita pepo* (pumpkin, Gaudreault and Webb 1981) where it appears to serve as a long distance translocate.

The biosynthesis of RFOs in *Arabidopsis* vegetative tissues is well described with 10 *GolS*s reported (Taji et al. 2002; Nishizawa et al. 2008). The only RFO accumulating in vegetative tissues is Raf. This accumulation is strictly associated with abiotic stresses and a single RS (*RS5*, At5g40390) has recently been described to be responsible for this stress induced-Raf accumulation (Egert et al. 2013). Both Raf and Sta accumulate in *Arabidopsis* generative tissues, with Sta being the most abundant RFO in mature seeds (Taji et al. 2002; Egert et al. 2013). The recently characterised *RS5* has been shown to partially contribute to seed Raf

accumulation (Egert et al. 2013). However, beyond this little else is known of the identities of the genes responsible for RFO accumulation in seeds. Despite Sta being the predominant RFO in the seeds, there are no reports concerning the molecular identity of an Arabidopsis SS or its role in RFO biosynthesis.

A single coding sequence (*RS4*, *At4g01970*) is annotated in the Arabidopsis database (www.arabidopsis.org) as either a RS or SS. It shares amino acid identities of only 58%, 58% and 59% to the known SSs from pea, adzuki bean and mask flower, respectively. It has been reported previously as a RS (*RS4*, Nishizawa et al. 2008; Lee et al. 2012) on the basis of expression (transcript abundance). This putative RS (or SS) also shares amino acid identities of 53% and 47% to the Raf specific alkaline α -galactosidase SIP2 (*At3g57520*, Peters et al. 2010) and the recently characterised RS5 (*At5g40390*, Zuther et al. 2004; Egert et al. 2013), respectively. Its expression pattern however, appears strictly associated to Arabidopsis reproductive structures (developing seeds, [Arabidopsis eFP browser](#); [the plant proteome database](#)).

Only three SSs have been functionally characterised (biochemically identified) from; *Vigna angularis* (adzuki bean, Peterbauer et al. 1999), pea seeds (Peterbauer and Richter 2002) and to a lesser extent mask flower (Voitsekhovskaja et al. 2009). The unclear functional annotation of *At4g01970*, its seed specific expression pattern and the lack of molecular identities for seed RFO biosynthetic genes led us to functionally characterise *RS4* using both reverse- (T-DNA insertion mutants) and forward-genetic (constitutive over-expression) approaches.

Results

Promoter activity, transcript abundance of RS4 and Sta accumulation is restricted to generative tissues

Promoter activity was evaluated by GUS staining using a 1.5 kB fragment of the *RS4* promoter fused to β -glucuronidase and, determined to be prominent throughout seed development (**Fig. 1A**). The highest promoter activity was observed in closed flowers and immature siliques (0.75 cm, 2 DAF) and was almost absent in mature siliques (1 cm, 5 DAF, **Fig. 1A**). The expression of *RS4* was monitored over these developmental stages using a real-time PCR methodology. Unlike the promoter activity, transcripts were most abundant in mature siliques (where promoter activity was almost absent), 2-fold higher than in young siliques (where promoter activity was highest, **Fig. 1B**). The WSC profiles of these developmental stages were determined using quantitative LC-MS. The RFOs Raf and Sta began accumulating in young siliques (0.50cm, 1 DAF) but, the highest concentrations occurred in mature seeds (2.15 ± 0.12 and 7.68 ± 1.12 mg g⁻¹ DW, respectively; **Supplementary Fig. S2**).

Seed Sta accumulations shows perturbations in RS4 loss-of-function mutants

Using a quantitative real-time PCR (qPCR) methodology, the *atrs4-1* T-DNA insertion line was determined to be a knock-down mutant (reduced transcript) while *atrs4-2* was a knock-out (ablated transcript, **Supplementary Fig. S1C**). Quantitative LC-MS was conducted on WSC extracts from mature seeds of the two mutants (**Fig. 2**). The only RFOs accumulating in wild-type (Col-0) seeds were Raf (2.15 ± 0.22 mg g⁻¹ DW) and Sta (7.68 ± 0.97 mg g⁻¹ DW). In mature seeds from the *atrs4-1* line, Sta was present in low concentrations (1.25 ± 0.07 mg g⁻¹ DW) and was completely absent in the *atrs4-2* line (**Fig. 2D**). Hyper-accumulation of Raf (approximately 4-fold, **Fig. 2C**) was evident in mature seeds of both *atrs4-1* and *1-2*, accounting for concentrations of 5.3 ± 0.08 and 4 ± 0.2 mg g⁻¹ DW, respectively. Similarly, Gol hyper-accumulation (nearly 2-fold) occurred in mature seeds from both lines to concentrations of 4.2 ± 0.2 and 4.25 ± 0.1 mg g⁻¹ DW, respectively (**Fig. 2B**). Accumulation of Suc was similar in the mature seeds of all the backgrounds (23.36 ± 0.03 , 25 ± 0.5 and 28.68 ± 1.82 mg g⁻¹ DW for Col-0, *atrs4-1* and *1-2*, respectively).

Mature seeds of the atrs4/atrs5 double mutant are completely deficient in RFOs

The T-DNA insertion line *atrs5-1* (Egert et al. 2013) and *atrs4-2* were manually cross-pollinated to create the double mutant, *atrs4/atrs5*. Subsequent to propagating F1 and F2 generations, plants were genotyped to confirm zygosity (**Supplementary Fig. S3**). Concentrations of WSCs were analysed in the mature seeds of homozygous

F3 plants. The only WSCs to accumulate in the seeds of the double mutant (*atrs4/atrs5*) were Suc (6.45 ± 0.86 mg g⁻¹ DW) and Gol (3.2 ± 0.98 mg g⁻¹ DW). Relative to mature seeds of the wild-type (Col-0) Suc was 4-fold lower (24 ± 0.90 mg g⁻¹ DW) and Gol 1.5-fold higher (2.44 ± 0.12 mg g⁻¹ DW) in mature seeds of the double mutant. Both Raf and Sta accumulation were completely ablated in mature seeds of the double mutant (**Fig. 3C, D**).

Raf and Sta unusually accumulates in the leaves of transgenic Arabidopsis constitutively over-expressing RS4, under normal growing conditions

Using a constitutive over-expression strategy, *RS4* expression was de-regulated into the leaves of transgenic Arabidopsis (35S:*RS4*/Col-0). Constitutive transcript abundance of *RS4* and cold acclimation-induced (4°C, 14 d) accumulation of the *RS5* transcript was confirmed using sqRT-PCR (**Supplementary Fig. S4B**). Total WSCs were analysed from the leaves of the two independent 35S:*RS4*/Col-0 lines using quantitative LC-MS under conditions of cold acclimation (4°C, 14 d). The only WSC accumulating in non-acclimated wild-type (Col-0) leaf samples was Suc (6.1 ± 1 mg g⁻¹ DW), with complete absence of Gol, Raf and Sta (**Fig. 4**). Cold acclimation resulted in a nearly 20-fold increase (to 103.86 ± 2 mg g⁻¹ DW) of Suc in leaves from wild-type plants, accompanied by accumulation of Gol and Raf (4.46 ± 0.4 and 45.51 ± 3 mg g⁻¹ DW, respectively). No Sta occurred in cold acclimated wild-type leaves.

Both 35S:*RS4*/Col-0 lines hyper-accumulated Suc up to 3-fold higher (vs. wild type) in non-acclimated leaves (**Fig. 4A**). Accumulation of Gol (9.72 ± 0.25 and 10.15 ± 0.02 mg g⁻¹ DW), Raf (32.6 ± 1.2 and 34.9 ± 0.6 mg g⁻¹ DW) and Sta (1.57 ± 0.08 and 1.62 ± 0.1 mg g⁻¹ DW) also occurred in non-acclimated leaves of both over-expressing lines, respectively (**Fig. 4B, C and D**, respectively).

Cold-acclimated leaves of the two 35S:*RS4*/Col-0 lines accumulated approximately 4-fold higher Suc (77.05 ± 4 and 76.6 ± 1.8 mg g⁻¹ DW, respectively) and 1.7-fold higher Raf (56.4 ± 1.2 and 54.8 ± 3 mg g⁻¹ DW, respectively). When compared to non-acclimated leaves, Gol was slightly lower (7.9 ± 0.2 and 8.3 ± 0.3 mg g⁻¹ DW) in acclimated leaves from the two transgenic lines, respectively. The occurrence of Sta in cold-acclimated leaves from the two transgenic lines was comparable to the concentrations observed in the non-acclimated leaves of both lines (**Fig. 4D**).

Constitutive over-expression of RS4 in the atrs5-1 mutant background reveals that RS4 synthesises both Raf and Sta in vitro

Using the T-DNA insertion line *atrs5-1* (Egert et al. 2013), transgenic plants constitutively over-expressing *RS4* (*35S:RS4/atrs5-1*) were generated. Marker resistant (hygromycin) plants of the T2 generation were genotyped to establish zygosity of the T-DNA insertion into *RS5* (**Supplementary Fig. S4A**). Further, sqRT-PCR was used to ascertain the transcript abundance of *RS4* in the leaves of transgenic lines and confirm the absence of the *RS5* transcript under cold acclimation conditions (4°C, 14 d). Both transgenic lines showed constitutive transcript abundance of *RS4* in the leaves and were deficient of the *RS5* transcript (**Supplementary Fig. S4B**). Leaf crude extracts from *35S:RS4/atrs5-1* lines were incubated with either 100 mM Suc and 10 mM Gol (RS activity) or 100 mM Raf and 10 mM Gol (SS activity). No RS or SS activities were detected in leaf crude extracts from the *atrs5-1* background. However, both RS and SS activities were observed in leaf crude extracts from the two transgenic lines (**Fig. 5**).

Discussion

A major component of the storage carbohydrates in seeds are RFOs (Peterbauer and Richter 2001). The RFO tetra-saccharide Sta (Suc-Gal₂) features prominently as the major RFO in the seeds of many plants. Despite its occurrence in Arabidopsis seeds (Taji et al. 2002; Egert et al. 2013), there are no reports confirming the molecular identity of a SS in Arabidopsis. A single gene (*RS4*, At4g01970) is putatively annotated as both a RS and SS. This study addressed the functional characterisation of *RS4* and the contextualisation of its physiological role in the RFO metabolism of Arabidopsis.

Promoter activity, RS4 transcript abundance and Sta accumulation are strictly associated with Arabidopsis reproductive structures

Using the β -glucuronidase (GUS) reporter system we ascertained that the *RS4* promoter was active only in the reproductive structures of Arabidopsis. This activity was strongly associated to developing seeds (**Fig. 1A**) but did not correlate to the transcript profile of *RS4* in these tissues. While promoter activity was highest in immature siliques, *RS4* transcripts were most abundant in mature siliques (containing maturing seeds). Due to the large quantities of fresh material required to conduct enzyme assays in the reproductive structures we opted to evaluate the WSC profiles of these tissues using a quantitative LC-MS platform. Consistent with previous reports (Taji et al. 2002; Zuther et al. 2004; Knaupp et al. 2008; Nishizawa et al. 2008; Iftime et al. 2011; Egert et al. 2013) we did not observe any Sta accumulation in vegetative tissues (leaves) of wild-type (Col-0) plants. Its accumulation (along with Raf) was observed to occur only at the onset of silique development and accumulation was highest in mature seeds (**Supplementary Fig. S2D**)

Seed Sta accumulation shows perturbations in the two T-DNA insertion mutants for atrs4-1 and 4-2

Using two T-DNA insertion mutants from the SALK collection we identified a knock-down (*atrs4-1*, transcript repressed) and a knock-out (*atss4-2*, transcript deficient) mutant (**Supplementary Fig. S1C**). We could demonstrate that (i) mature seeds of the knock-down mutant showed reduction in seed Sta accumulation and (ii) mature seeds of the knock-out mutants were deficient of Sta (**Fig. 2**). This finding clearly demonstrated that *RS4* functions as a seed specific SS, responsible for Sta deposition into mature seeds. As a consequence of impaired *RS4* function, mature seeds of both mutants hyper-accumulated Gol and Raf (substrates for SS, **Fig. 2B, C**). Similar observations have been made in insertion mutants for *RS5* which, led to hyper-accumulation of Gol but not Suc (substrates for RS, Zuther et al. 2004; Egert et al. 2013). These observations clearly demonstrate the

“linear” nature of the RFO biosynthesis pathway and how the use of a reverse genetic approach can conclusively demonstrate gene function in this context (by substrate hyper-accumulation).

The atrs4/atrs5 double mutant provides a hint that RS4 is also responsible for Raf biosynthesis

In the recent study which characterised *RS5* (*At5g40390*, Egert et al. 2013), two independent T-DNA insertion lines (*atrs5-1* and *5-2*) contained 50% less Raf in mature seeds when compared to mature seeds from wild-type controls (Col-0). It was postulated that an additional (unidentified) seed-specific RS was responsible for the synthesis of the remaining 50% of seed Raf. Using the *atrs5-1* line we created the *atrs4/atrs5* double mutant, hypothesising that if *RS4* coded for a *bona fide* SS then the seeds of the double mutant would hyper-accumulate Raf (and Gol) in higher concentrations than the seeds of *atrs5-1*. Surprisingly, we obtained seeds that were completely deficient in RFOs (Raf and Sta, **Fig. 3**). Similar to mature seeds from the parental *atrs5-1* and *atrs4-2* mutants, mature seeds of *atrs4/atrs5* hyper-accumulated Gol 1.5-fold higher than, mature seeds from wild-type controls (Col-0, **Fig. 3**). The seed RFO deficiency of the double mutant provided an indirect line of evidence to *RS4* also fulfilling the function of an RS, suggesting that the 50% seed Raf of the *atrs5-1* mutant was not due to an unidentified RS but rather to *RS4* being a bi-functional α 1,6-galactosyltransferase.

De-regulating the seed specific expression of RS4 demonstrates that it synthesises both Raf and Sta in the leaves of transgenic Arabidopsis

Multi-functional α 1,6-galactosyltransferases are not unprecedented. The pea seed SS (Peterbauer et al. 2002) was shown to have “multi-functional” activity *in vitro*. In context, this multi-functional activity was both Gol-dependent (using Raf and Gol to synthesise Sta) and Gol-independent (using Raf and Sta to synthesise Ver). Similarly the RFO chain elongation enzyme GGT has been demonstrated to function in a Gol-independent manner using RFOs as both galactosyl donors and acceptors (Bachmann and Keller, 1995; Haab and Keller, 2002; Tapernoux-Luthi et. al. 2004). To further validate our findings we looked to the heterologous expression of *RS4*. The few reports which demonstrate *in vitro* biochemical characterisation of SS enzymes all employed the *Sf9/21* insect cell system (Peterbauer et al. 1999; 2002). We were unsuccessful in obtaining recombinant *RS4* via an *E. coli* heterologous platform and thus considered using *Arabidopsis* itself as the “heterologous” expression system generating both 35S:*RS4*/Col-0 and 35S:*RS4*/*atrs5-1* transgenic lines. Our justification for adopting this approach is the strict abiotic stress-inducible accumulation of only Raf in Col-0 leaves and, the complete absence of both RS activity and Raf accumulation in *atrs5* mutant backgrounds.

A recent report characterised the Raf and Sta accumulating ability of transgenic Arabidopsis (Col-0) constitutively over-expressing the adzuki bean SS (Iftime et al. 2011). In that study Sta was only able to accumulate under conditions of cold-acclimation (4°C) and not under normal growing conditions. This is presumably due to RS5 activity and Raf accumulation (substrate for SS activity) only occurring in Arabidopsis leaves under abiotic stress conditions (Taji et al. 2002; Zuther et al. 2004, Knaupp et al. 2008; Iftime et al. 2011; Egert et al. 2013), like cold-acclimation. When we analysed leaf WSC profiles of 35S:RS4/Col-0 transgenic lines we found significant amounts of both Raf and Sta in leaves from plants growing under normal conditions. Cold-acclimation only slightly increased these Raf concentrations but not the Sta. Similar to previous reports, leaves from our wild-type control (Col-0) plants were Raf and Sta deficient under these normal growing conditions, accumulating Raf only as a response to cold-acclimation (**Fig. 4**). Taking into account the previous study where neither Raf nor Sta was observed in the leaves of normally grown transgenic plants when using the adzuki bean SS (Iftime et al., 2011), our observations provided further *in vivo* evidence of a dual Raf and Sta biosynthetic capacity for RS4.

Finally, to conclusively demonstrate that RS4 was able to synthesise both Raf and Sta we looked to the 35S:RS4/*atrs5-1* transgenic lines which would be both RS and Raf deficient due to the insertion into *RS5* (previously reported in Zuther et al.2004; Egert et al. 2013). We analysed the *in vitro* Raf and Sta biosynthetic activities of leaf crude extracts from normally grown plants. We could show these leaf crude extracts from two independent 35S:RS4/*atrs5-1* transgenic lines were indeed able to synthesise both Raf and Sta , *in vitro*. Further, this dual activity was Gol-dependent unlike that reported for the pea seed SS. This proved unambiguously that RS4 was indeed able to synthesise Raf and Sta (in a Gol-dependent manner).

In summary, we have identified Arabidopsis *RS4* (*At4g01970*) as a seed specific-SS that is responsible for the deposition of Sta into maturing seeds. During the course of our investigations we uncovered an additional function, showing unequivocally that it is also partially responsible for Raf deposition during seed maturation (together with *RS5*). Most importantly our findings have uncovered the first reported bi-functional α 1,6-galactosyltransferase (RFO synthase) from Arabidopsis. We are currently investigating the biochemical parameters of the RS and SS activities of RS4 and are further employing a site directed-mutagenesis approach to elucidate if there are specific catalytic domains imparting these activities.

Materials and Methods

Plant material and cold acclimation treatment

All Arabidopsis T-DNA insertion mutants used were obtained from the Salk Institute's T-DNA insertion mutant collection, in the Col-0 ecotype (Alonso et al. 2003). Two insertion lines for *At4g01970* (Salk_088817, *atrs4-1* and Salk_026853, *atrs4-2*) contained a T-DNA insertion in the 5'-UTR and the third exon, respectively. Subsequent to seed stratification (24 h, 4°C), plants were propagated and maintained on peat disks (Jiffy™ no.7, South Africa) under controlled environment conditions (8 h light, 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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), at 7 and 21 d post germination as previously described (Petersen et al. 2010). Homozygous insertion lines were verified by PCR on genomic DNA (gDNA) isolations as previously described (Egert et al. 2012; 2013). Briefly, aliquots of genomic DNA (5 μl) were used in PCR reactions containing primer combinations for the wild-type allele (LP + RP, ~1 kB, **Supplementary Table S1**) and for the mutant allele (RP + LBb1.3, ~0.7 kB). The T-DNA specific LBb1.3 primer (5'GCGTG GACCGCTTGCTGCAACT) was used in combination with the respective RP primer for each of the two independent lines.

The *RS4* (*At4g01970*), *RS5* (*At5g40390*) double mutant was created by manually crossing the *atrs4-2* (pollen acceptor, this study) to the homozygous insertion line *atrs5-1* (pollen donor, Egert et al. 2013). Progeny (F_1) were analysed for the heterozygous state of both the *RS4* and *RS5* alleles using the same PCR strategy described above. Subsequent progenies (F_2) were then similarly screened for the homozygous state of both alleles (mutant) to establish progeny (F_3) carrying T-DNA insertions in both *RS4* and *RS5* genes. This mutant was designated *atrs4/atrs5*.

Where applicable, four week-old plants (rosette stage 1.14) were used for cold-acclimation treatments as previously described (Zuther et al. 2004; Egert et al. 2013). Briefly, plants were transferred to controlled environment chambers with the same settings as listed above but, subject to a constant temperature of 4°C over 14 d. Experiments were conducted thrice using pools of 12 plants each.

Promoter β -Glucuronidase fusions and constitutive over-expression of RS4

The full length Arabidopsis *RS4* (*At4g01970*) cDNA was obtained from the Riken Arabidopsis full-length cDNA database (pda19731, www.brc.riken.jp, Seki et al. 1998; 2002). The coding sequence (CDS) was amplified using the Expand High Fidelity PCR System (Roche Applied Science, Roche products, South Africa), according to manufacturer's instructions using primers *RS4*_{for} 5' ATGGCTCCACTTCACGAATC and *RS4*_{rev} 5' TTTAAAAGGTGAAAGACAGATGAGAG. For promoter β -Glucuronidase analyses a 1.5 kb fragment of Arabidopsis genomic DNA, immediately upstream of the start codon of the *At4g01970* and including a putative TATA box consensus sequence, was amplified in the same manner, using primers *RS4*_{prom_{for}} 5' AAAGATGAATAAAAAGATGATTGTTAGG and *RS4*_{prom_{rev}} 5' TTGGATTCGATTACATCGTTG.

Both the *RS4* CDS and the 1.5 kb promoter fragment were cloned using the pCR[®]8/GW/TOPO[®]TA Cloning Kit (Invitrogen, Life technologies, South Africa) according to manufacturer's instructions. Following PCR verification of colonies containing clones in the sense (5' to 3') orientation, the pMDC binary vector range (Curtis and Grossniklaus 2003) was used to create constructs via a conventional LR clonase[™] reaction (Life technologies) to obtain pMDC32::*RS4* (constitutive *RS4* expression driven by dual 35S promoter) and pMDC163::*RS4*_{prom} (β -glucuronidase expression driven by native *RS4* promoter).

Plant transformations and marker selection procedures

Electro-competent *A. tumefaciens* (GV3101) was transformed with pMDC32::*RS4* and pMDC163::*RS4*_{prom} plasmid mini-preparations using a Genepulser[®] (1.8 kV; 100 Ω ; 25 μ F, Bio-Rad, Bio Rad Laboratories, South Africa). Arabidopsis wild-type (Col-0) and the *atrs5-1* mutant were transformed using the floral inoculation method (Narusaka et al. 2010), with minor modifications. Briefly, *A. tumefaciens* colonies were propagated to mid-log phase ($OD_{600} = \sim 1.2$) in 5 ml Luria Broth (LB), supplemented with antibiotics (⁵⁰Rif., ²⁵Gent., ⁵⁰Kan.). Aliquots (1 ml) were centrifuged (10 000 g, 2 min) and the pellet re-suspended in 5% (w/v) sucrose solution supplemented with 0.02% (v/v) Silwet L-77. Floral buds were inoculated with 5 μ l of the suspension and plants were incubated in the dark (16 h, 90% relative humidity), prior to continued growth under our controlled environment conditions.

Seeds (T1) were surface sterilised, using the vapour-phase seed sterilisation method (Clough and Bent 1998), stratified (4°C, 24 h), and placed on half-strength MS (Duchefa, Labretoria, South Africa) media containing the

selection antibiotic ($^{17.5}\text{Hyg}$). Plantlets were propagated in controlled growth conditions (8 h light, 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 22°C, 16 h dark, 18°C, 60% relative humidity) for two weeks. Marker resistant-plantlets were transferred to Jiffy™ disks and grown under our controlled environment conditions (described above). Seeds (T2) were harvested, re-selected for (marker resistance) and, propagated in Jiffy™ disks for further characterisation.

GUS activity assays

Following propagation under our controlled environment conditions six to eight week-old plants were used to harvest developmental organs (obtained from T2 plants). In the case of the reporter construct (pMDC163:: $RS4_{\text{prom}}$ /Col-0) these organs were harvested and analysed at different developmental stages [leaf, closed flower, open flower, immature silique (0.5 cm, 1 DAF), young silique (0.75 cm, 2 DAF) and mature silique (1 cm, 5 DAF)] and used to stain for GUS activity as previously described (Parcy et al. 1998).

RNA isolation and transcript analysis

Total RNA was isolated using the RNeasy® Plant Mini kit (Qiagen, Whitehead Scientific, South Africa), following the manufacturer's instruction. The complementary DNA (cDNA) template was obtained *via* reverse transcription of 1 μg of total RNA, using an oligo (dT₁₅) primer and M-MLV (H) reverse transcriptase (Promega, Anatech, South Africa) following the manufacturer's instruction. Seed developmental organs representing closed and open flowers and; immature, young and mature siliques were harvested from Arabidopsis plants (Col-0). Transcript analysis ($RS4$) was performed either using semi quantitative RT-PCR (sqPCR) or real-time quantitative PCR (qPCR) methodologies (described below).

The sqPCRs were conducted using GoTaq® DNA polymerase (Promega, Anatech, South Africa) in a 50 μl reaction (3 μl cDNA, 1.25 U DNA polymerase, 5 \times green PCR buffer, 0.5 mM of each dNTP, and 0.5 μmol of each primer) for 24 cycles, with a primer annealing temperature optimum of 58°C for all respective sqPCR primer pairs. The constitutively expressed gene $ACT2$ ($At3g18780$), was used to determine the number of cycles used for the sqPCR, where expression occurred in the linear range. The $ACT2$ and $RS4$ primer pairs (ACT_{for} 5' ATGGCTGAGGCTGATGATAT, ACT_{rev} 5' TTAGAAACATTTTCTGTGAACGAT; $RS4_{\text{sqfor}}$ 5' ATG GCTCCACTTCACGAATC, $RS4_{\text{sqrev}}$ 5' GCATCTTCGGCTTGAGAGGA) were designed to amplify fragments of 1 kb from the cDNA.

The templates used in the qPCR experiments represented 1:9 volumetric dilutions of first strand cDNA. All samples evaluated were prepared in parallel and three independent experiments were executed. The qPCR reactions were conducted using the GoTaq[®] qPCR master mix (Promega, Anatech, South Africa) that exhibits a BRYT[®] green dye fluorescent signal (excitation at 493nm and emission at 530nm). Using the Rotor-Gene[®] Q 6000 thermocycler (Qiagen, Whitehead Scientific, South Africa), qPCR reactions were conducted in 50 μ l reactions (1 μ l cDNA, 25 μ l qPCR master mix and 0.5 μ mol of each primer) in a 36-well rotor. Cycling conditions consisted of one initial denaturation step at 95°C for 10 min, followed by 40 cycles of a two-step denaturation/annealing process (95°C, 15 s/60°C, 1 min). Primer pairs were designed, with annealing temperature optimums of 60°C, to amplify products of between 60 and 130 bp. Three seed specific reference genes (**Supplementary Table S2**; Dekkers et al. 2012) and *RS4* were used in the analyses. The threshold cycle number (Δ Ct) was used to calculate relative fold change with the $\Delta\Delta$ Ct method, using the closed flower state as the calibrator sample (Livak and Schmittgen 2001). All qPCR experimentation was conducted in compliance with the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE, Bustin et al. 2009).

Water soluble carbohydrate (WSC) extractions

WSCs extractions from dry seeds (50 mg) or macerated, freeze-dried leaf (50 mg), flower (50 mg) and silique (50 mg) material was conducted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), with minor modifications. Extractions were executed in a three-step sequential series [1 ml 80% (v/v) EtOH, 1 ml 50% (v/v) EtOH, 1 ml de-ionised H₂O (dH₂O)]. Each consecutive extraction was conducted twice at 80°C for 10 min, centrifuged (13 000 g, 10 min, RT) and supernatants transferred to a new Eppendorf tube prior to the next step in the series. The supernatants for each individual extraction were pooled, vacuum centrifuged and re-suspended in dH₂O to a final volume of 200 μ l. Samples were desalted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), prior to LC-MS analysis.

Leaf crude extracts and enzyme assays

Crude extracts and enzyme assays were executed as previously described (Peters et al. 2007; Peters and Keller 2009; Peters et al. 2010; Egert et al. 2013), with minor modifications. Freshly collected leaf material (200 mg) was macerated in 400 μ l pre-cooled extraction buffer [50 mM HEPES/KOH pH 7.0, 1 mM EDTA, 20 mM DTT, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na ascorbate, 2% (w/v) PVP]. Extracts

were centrifuged (13 000 g, 10 min, 4°C) and 200 µl of the supernatant desalted *via* gel filtration (1400 g, 2 min, 4°C) through a Sephadex G-25 (fine, Sigma, South Africa) column (final bed volume of 3 ml). Sephadex columns were pre-equilibrated twice by centrifugation (1400 g, 2 min, 4°C) with 2 ml of assay buffer (50 mM Hepes/NaOH pH7.0, 10 mM DTT). Enzyme activities were assayed, using 25 µl aliquots of the desalted extracts, in a final volume of 50 µl assay buffer containing either 100 mM Suc and 10 mM Gol for RS activity, or 100 mM Raf and 10 mM Gol for SS activity. Assays were performed for 1 h at 30°C and reactions were subsequently stopped by flash-freezing in liquid N₂ and boiling (5 min, 80°C). Samples were desalted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), prior to LC-MS analysis.

LC-MS analysis and WSC quantification

LC-MS analysis was performed with a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UPLC. Samples were separated on a Waters UPLC BEH Amide column (2.1 x 100 mm; 1.7 µm) at a flow rate of 0.17 ml/min at 35°C. Solvent A consisted of acetonitrile/water (30:70) containing 0.1% ammonium hydroxide and solvent B was acetonitrile/water (80:20) containing 0.1% ammonium hydroxide. The mobile phase gradient was from 0% to 60% solvent A over 5 min, maintained for 2 min at 60% solvent A before the column was re-equilibrated to the initial conditions. Electrospray ionization was applied in the negative mode and the scan range was from m/z 150 to 1500. The capillary voltage was set a 2.5 kV, the cone voltage was 15 V, the source temperature 120 °C and the desolvation temperature was 275 °C. The desolvation gas and cone gas flows were 650 L/h and 50 L/h, respectively. Quantification was conducted against a series of standard sugars after standard curves were created within the linear response range of the apparatus. All WSCs were monitored using their deprotonated quasi-molecular ions and quantified with the TargetLynx application manager (Waters MassLynx V4.1V software).

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Fig. 1 Analyses of *RS4* promoter activity and expression during various stages of silique development:

(A) Representative β -glucuronidase (GUS) activity stains from various silique developmental stages in transgenic plants where β -glucuronidase expression is driven by a 1.5 kB fragment of the native *RS4* promoter. GUS activity was stained for during seed developmental stages representing closed flowers (CF), open flowers (OF), immature siliques (1 DAF, 0.5 cm), young siliques (2 DAF, 0.75 cm) and mature siliques (5 DAF, 1 cm). DAF, days after flowering. (B) Expression analyses of *RS4* during Col-0 seed developmental phases using quantitative real-time PCR (qPCR). Data represent the fold change of *RS4* relative to the Arabidopsis ubiquitin 11 (*UBQ11*, *At4g05050*) control gene, using the CF state as the calibrator sample (indicated as a solid black bar). Values represent the mean \pm standard error of three independent experiments calculated from the Ct values using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). A value of 1 represents no expression/transcript deficiency. Experimentation was conducted in compliance with the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE)

Fig. 2 Water soluble carbohydrate (WSC) profiles in the seeds of wild-type (Col-0) and, the *atrs4-1* and *4-2* T-DNA insertion lines:

Accumulation of the WSCs sucrose (Suc), galactinol (Gol), raffinose (Raf) and stachyose (Sta) in mature seeds are represented in A, B, C and D, respectively. Error bars represent the mean \pm standard error of three independent experiments where samples were pooled from 24 plants. n.d., not detected. Statistical significance is indicated by stars as determined by a two-tailed t-test, using Col-0 as the comparison control for *atrs4-1* and *4-2*, respectively (Gol * $p \leq 0.024$ and 0.011 , Raf ** $p \leq 0.003$ and * $p \leq 0.014$, Sta * $p \leq 0.012$ and ** $p \leq 0.007$ for *atrs4-1* and *4-2*, respectively).

Fig. 3 Water soluble carbohydrate (WSC) profile in the seeds of wild-type (Col-0) and the *atrs4/atrs5* double mutant:

Accumulation of the WSCs sucrose (Suc), galactinol (Gol), raffinose (Raf) and stachyose (Sta) in mature seeds are represented in A, B, C and D, respectively. Error bars indicate the mean \pm standard error of three independent experiments where samples were pooled from 24 plants (F3), genotyped to be homozygous for insertions in both *RS4* (*atrs4-2* mutant, this study) and *RS5* (*atrs5-1* mutant, Egert *et al.*, 2013). n.d., not detected. Statistical significance is indicated by stars as determined by a two tailed t-test, using Col-0 as the comparison control for the double mutant *atrs4/atrs5* (Suc ** $p \leq 0.001$, Raf ** $p \leq 0.001$, Sta ** $p \leq 0.007$).

Fig. 4 Water soluble carbohydrate (WSC) profiles in the leaves of wild-type (Col-0) and transgenic lines constitutively over-expressing *RS4* in the Col-0 background:

The WSCs sucrose (Suc), galactinol (Gol), raffinose (Raf) and stachyose (Sta) are represented in A, B, C and D, respectively. Leaves were harvested from normally grown plants (non-acclimated) or following cold-acclimation (4°C, 14d). Error bars indicate the mean \pm standard error of three independent experiments where samples were pooled from 18 plants per line. L1, line 1; L2, line 2; n.d., not detected. Statistical significance is indicated by stars as determined by a two tailed t-test, using Col-0 as the comparison control for each transgenic line (L1 and 2), respectively. Non-acclimated state (Suc **p \leq 0.001 and 0.001, Gol **p \leq 0.002 and ***p \leq 0.003, Raf **p \leq 0.002 and ***p \leq 0.002, Sta **p \leq 0.002 and **p \leq 0.004 for L1 and L2, respectively). Acclimated state (Suc **p \leq 0.006 and ***p \leq 0.0008, Gol ***p \leq 0.0006 and **p \leq 0.007, Raf **p \leq 0.002 and p \leq 0.09, Sta **p \leq 0.001 and ***p \leq 0.0006 for L1 and L2, respectively).

Fig. 5 Enzyme activities in crude extracts obtained from the leaves of transgenic lines constitutively over-expressing *RS4* in the *atrs5-1* background:

Raffinose synthase (RS) and stachyose synthase (SS) activities were measured from the leaves of *atrs5-1* insertion mutants (Egert *et al.*, 2013) constitutively over-expressing *RS4*. Crude extracts were incubated in the presence of either 100 mM Suc and 10 mM Gol (RS activity) or, 100 mM Raf and 10 mM Gol (SS activity) for 1 h at 30°C (50 mM HEPES, pH 7.0). The *atrs5-1* mutant background was used as the negative control for leaf RS and SS activity.



Fig. 1A Analyses of RS4 promoter activity and expression during various stages of silique development:
Representative β -glucuronidase (GUS) activity stains from various silique developmental stages in transgenic plants where β -glucuronidase expression is driven by a 1.5 kB fragment of the native RS4 promoter
209x297mm (300 x 300 DPI)

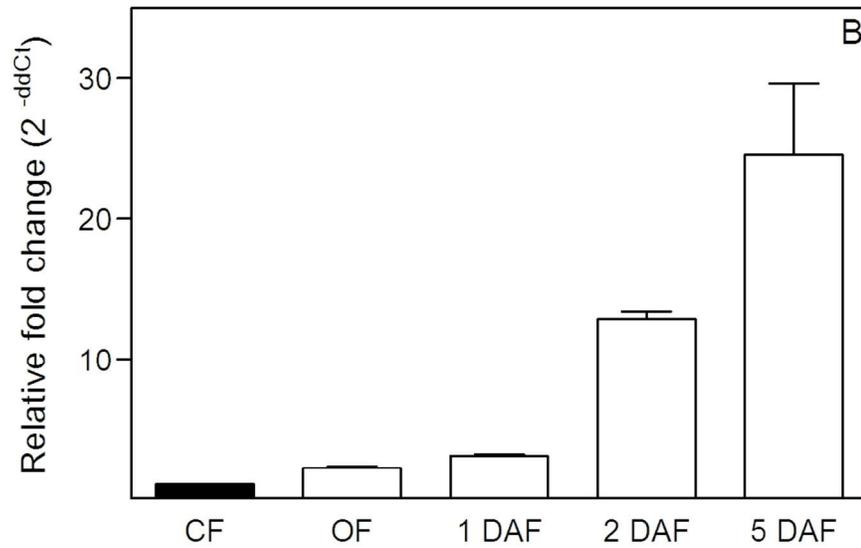


Fig. 1B Analyses of RS4 promoter activity and expression during various stages of silique development: Expression analyses of RS4 during Col-0 seed developmental phases using quantitative real-time PCR (qPCR)
103x65mm (300 x 300 DPI)

Review

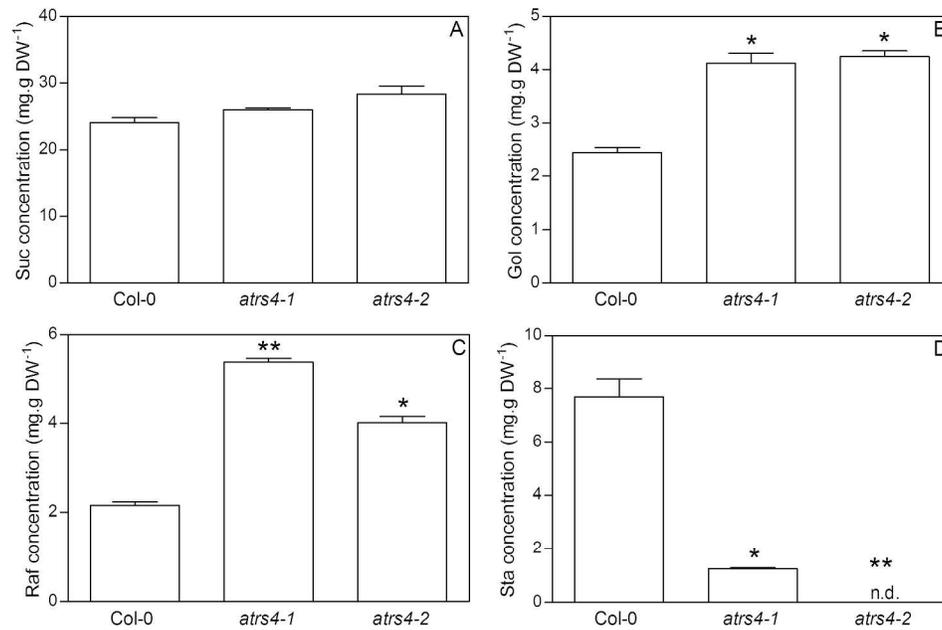


Fig. 2 Water soluble carbohydrate (WSC) profiles in the seeds of wild-type (Col-0) and, the *atrs4-1* and 4-2 T DNA insertion lines
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Review

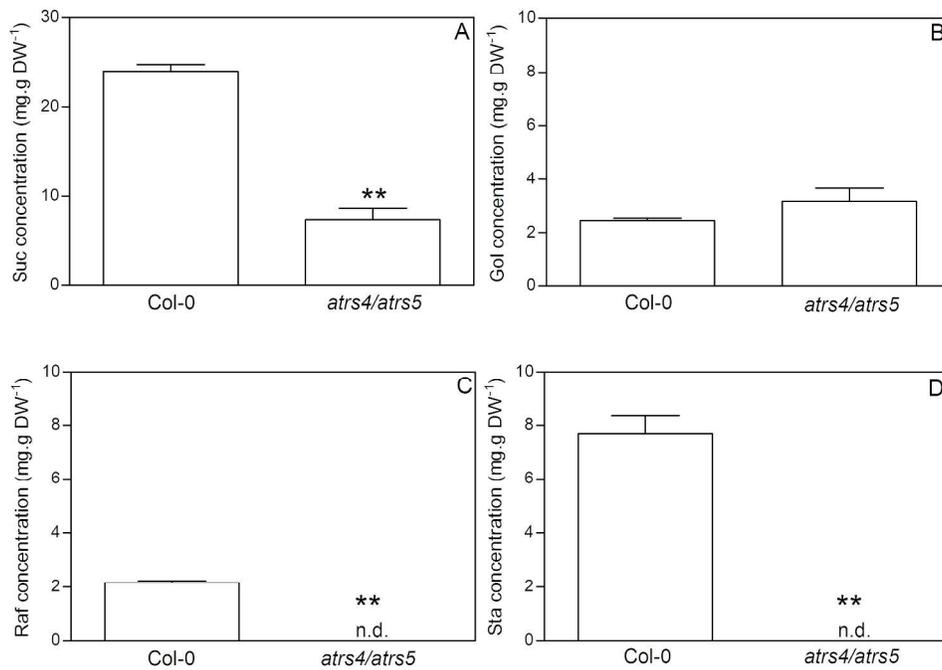


Fig. 3 Water soluble carbohydrate (WSC) profile in the seeds of wild-type (Col-0) and the *atrs4/atrs5* double mutant
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review

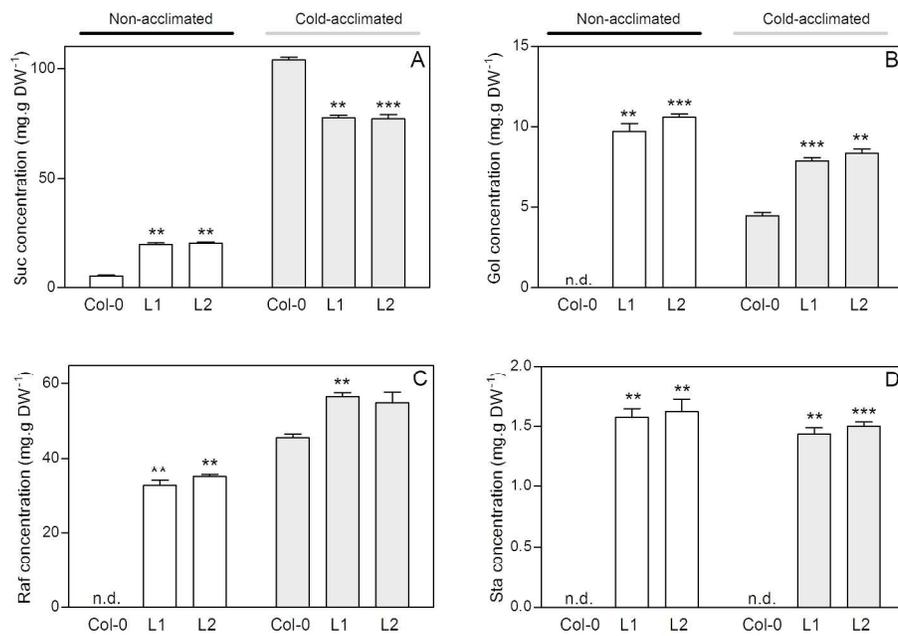


Fig. 4 Water soluble carbohydrate (WSC) profiles in the leaves of wild-type (Col-0) and transgenic lines constitutively over-expressing RS4 in the Col-0 background
258x172mm (300 x 300 DPI)

Review

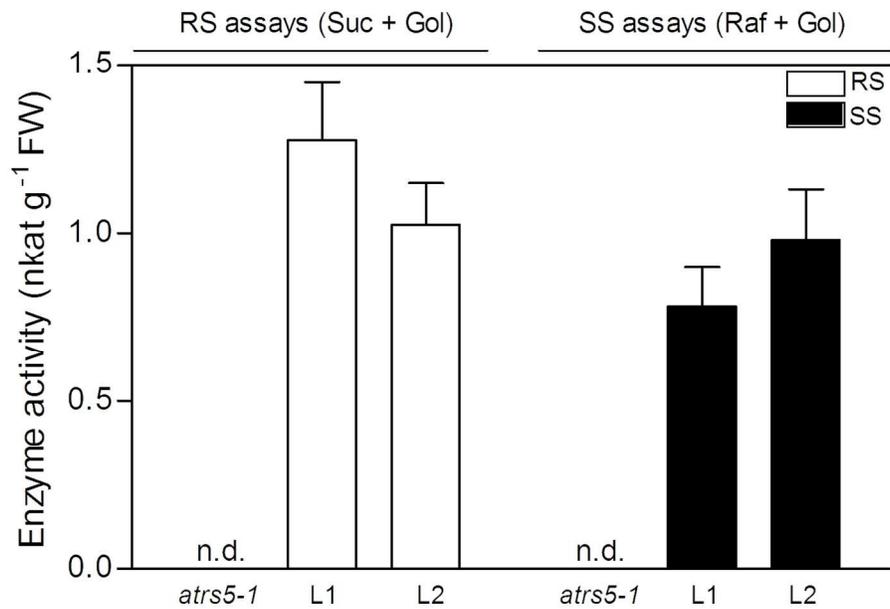


Fig. 5 Enzyme activities in crude extracts obtained from the leaves of transgenic lines constitutively over-expressing RS4 in the *atrs5-1* background
139x92mm (300 x 300 DPI)

PCP Supplementary information (Chapter 2 of thesis)

Table S1. Primers used for zygosity determination of the *RS4* (*atrs4-1* and *4-2*) and *RS5* (*atrs5-1*) T-DNA insertion lines

T-DNA insertion mutant-line	Forward primer (LP)	Reverse primer (RP)
Salk_088817 (<i>atrs4-1</i>)	5' GAGCCACTCTCTGCACAAATC	5' GCATCATAGTTTGCCAAGTAGC
Salk_026853 (<i>atrs4-2</i>)	5' TCGAATACGCCATGAATCTTC	5' CAGAAGAACATGGAGGACGAG
Salk_049583 (<i>atrs5-1</i>)	5' CTCTTCTTGAAGGCTCCTTCC	5' ATGACATCAACTTTAACGCCG

Table S2. Primer pairs used for quantitative real-time PCR (qPCR) analyses. Three independent seed specific reference genes, *UBC*, *ARP6* and *ASAR1* were selected for normalisation of the data.

Gene name	TAIR Gene code	Forward primer	Reverse primer	Amplicon size (bp)
<i>AtUBC2</i>	<i>At5g25760</i>	5' CTGCGACTCAGGGAATCTTCTAA	5' TTGTGCCATTGAATTGAACCC	61
<i>AtARP6</i>	<i>At3g33520</i>	5' TAACAACCTCAGGAGGACCCCA	5' CTACGACACCGAGCTGAT	130
<i>AtASAR1</i>	<i>At4g02080</i>	5' GCTGTGTTATTATTAAGCCGTAAG	5' AAAGCTAGGTACGGTTTAAGAC	121
<i>AtRS4</i>	<i>At4g01970</i>	5' GCTTGCTACTTGACCGTTGA	5' CCCACCATCTTCAAACCTCT	66

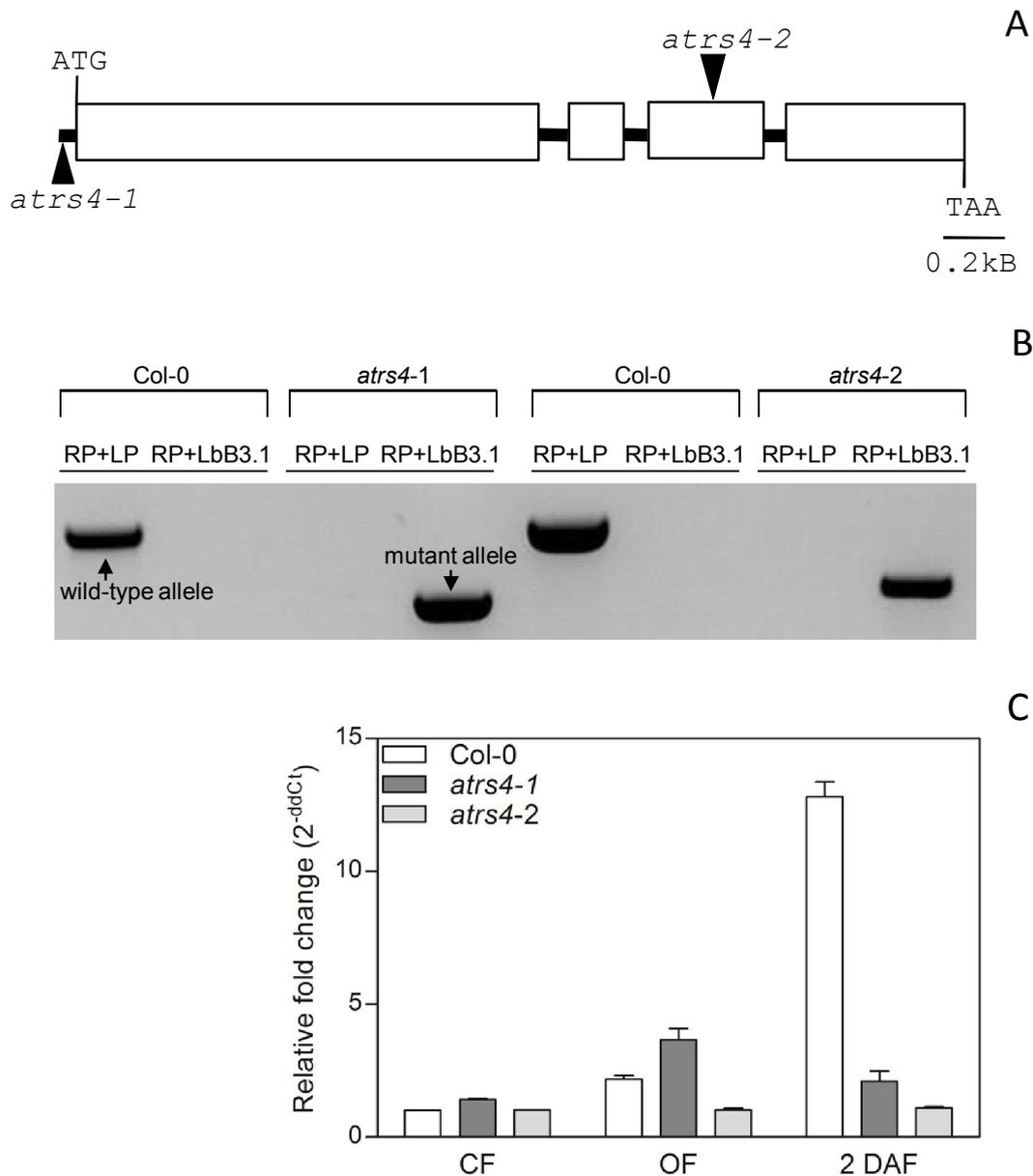


Figure S1. Analyses of Arabidopsis *RS4* (*atrs4-1* and *4-2*) T-DNA insertion lines (A) Schematic representation of *RS4* (*At4g01970*) indicating T-DNA insertions in the 5'-UTR (*atrs4-1*) and third exon (*atrs4-2*). (B) Zygosity determination of *atrs4-1* and *4-2* using genomic DNA PCRs. Primer pairs amplified the wild-type (LP+RP) and mutant (RP+LbB1.3) alleles. (C) Expression levels of *RS4* were determined by quantitative real-time PCR (qPCR) in Col-0 and *atrs4* insertion mutants. The threshold cycle number (ΔC_t) was used to calculate relative fold change with the $\Delta\Delta C_t$ method, using the closed flower state (Col-0) as the calibrator sample (Livak and Schmittgen 2001). All qPCR experimentation was conducted in compliance with the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE, Bustin et al. 2009). CF – closed flower; OF – open flower; 2 DAF – 2 days after flowering. Error bars indicate the mean \pm standard error of three independent experiments. A value of 1.0 represents no expression//transcript deficiency.

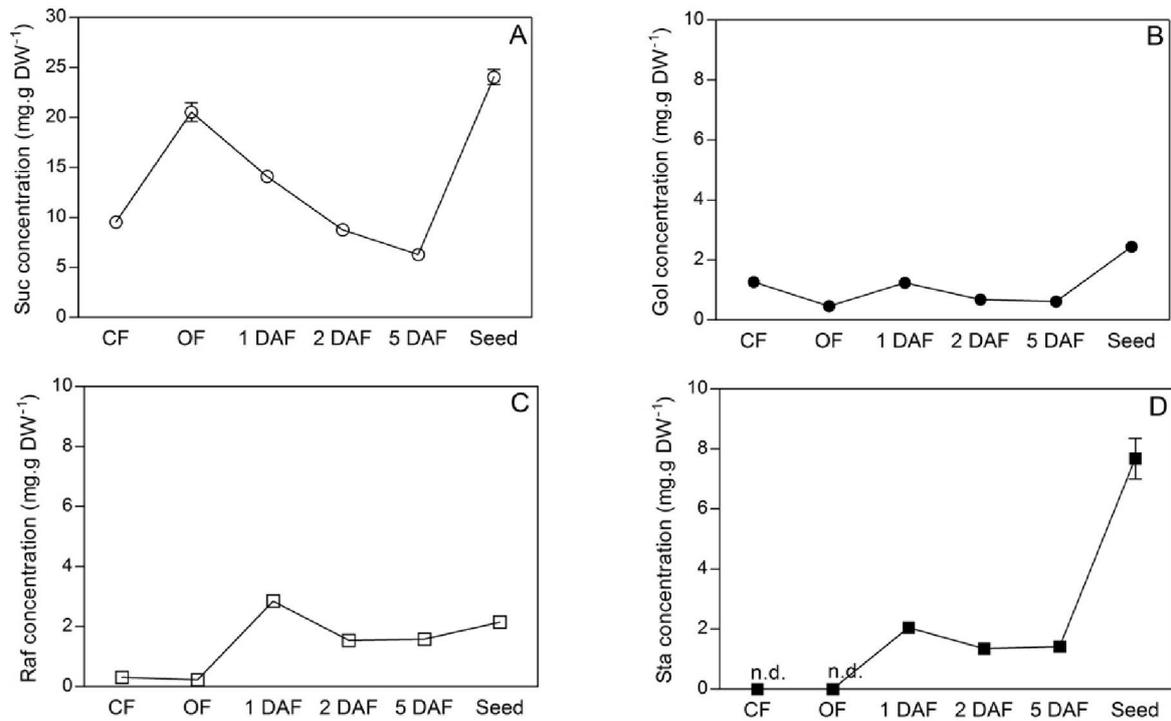
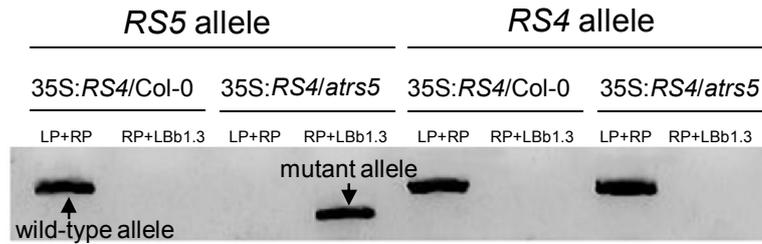


Figure S2. Changes in water soluble carbohydrates (WSCs) in wild-type (Col-0) during various stages of silique development. (A), (B), (C) and (D) indicate changes in the WSCs sucrose (Suc), galactinol (Gol), raffinose (Raf) and stachyose (Sta) during the various developmental stages in seed production/maturation, respectively. Error bars indicate the mean \pm standard error of three independent experiments where samples were pooled from 24 plants. CF – closed flower; OF – open flower; DAF – days after flowering.



Figure S3. Representative image identifying the homozygous double mutant *atrs4/atrs5*. Genomic DNA PCRs were conducted on DNA isolated from the leaves of plants (F3) that had previously been genotyped to be heterozygous for the T-DNA insertion into both *RS4* and *RS5*, following a manual cross between the homozygous single mutants *atrs4-2* (this study) and *atrs5-1* (Egert et al. 2013). Primer pairs amplified the wild-type (LP+RP) and mutant (RP+LBb1.3) alleles.

A



B

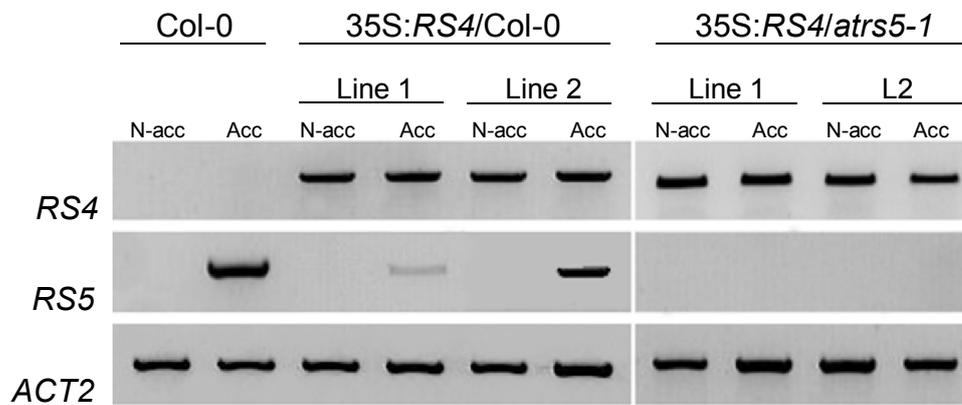


Figure S4. Analyses of transcript abundance in the leaves of Col-0 and *atrs5-1* transgenic lines constitutively over-expressing *RS4*. (A) Genomic DNA PCR analyses was conducted on the respective transgenic lines (T2) to confirm their background genotypes as Col-0 or *atrs5-1* (Egert et al. 2013). Primer pairs amplified the wild-type (LP+RP) and mutant (RP+LBb1.3) alleles. (B) Transcript abundance was assessed, using semi-quantitative RT-PCR, in each transgenic line to confirm constitutive over-expression of *RS4* and deficiency of *RS5* transcripts (*atrs5-1* background). Leaves were harvested from normally grown plants (non-acclimated) or following cold-acclimation (4°C, 14d). The *ACTIN2* gene (*ACT2*, *At3g18780*) was used as a constitutively expressed control. N-acc – non-acclimated; Acc – cold-acclimated.

CHAPTER 3

Hyper-accumulation of galactinol, in the absence of priming, does not improve induced systemic resistance in the *Arabidopsis/Botrytis cinerea* pathosystem

3.1 Introduction

Galactinol (Gol; 1-O- α -D-galactopyranosyl-L-*myo*-inositol) is an unusual sugar conjugate found in plants and is formed *via* glycosylation of *myo*-inositol (Ino), a reaction catalysed by galactinol synthase (GolS, EC 2.4.1.123). Its classical (and only) role has been thought to serve as the galactose (Gal) donor in the biosynthesis of the raffinose family oligosaccharides (RFOs), α 1,6-galactosyl oligomers of sucrose (Suc-Gal_n). The RFOs have been widely reported to be (i) agents of phloem transport and carbon storage (reviewed in Keller and Pharr, 1986) and (ii) associated with abiotic stress tolerance responses (reviewed in ElSayed, 2013).

The first step in RFO biosynthesis is initiated by GolS which catalyses the formation of Gol, using UDP-galactose (UDP-Gal) and Ino as substrates. The biosynthesis of RFOs then occurs by the sequential transfer of galactosyl moieties from (i) Gol to the C₆ position of the glucose (Glc) moiety in Suc, forming an α 1,6-galactosidic linkage to yield the trisaccharide raffinose (Raf, Suc-Gal₁) and (ii) from Gol to the C₆ position of the Gal moiety in Raf to yield the tetra-saccharide stachyose (Sta, Suc-Gal₂). These reactions are catalysed by raffinose synthase (RS, EC 2.4.1.82) and stachyose synthase (SS, EC 2.4.1.67), respectively. Both RS and SS predominantly function in a Gol-dependent manner. Biosynthesis of higher RFO oligomers occurs *via* a Gol-independent biosynthetic pathway. In *Ajuga reptans*, galactan:galactan galactosyltransferase (GGT) utilizes RFOs as both galactosyl donors and

acceptors during chain elongation, facilitating the synthesis of higher RFO oligomers (up to Suc-Gal₁₃; Bachmann and Keller 1995; Haab and Keller 2002; Tapernoux-Lüthi et al. 2004; Peters and Keller, 2009).

Historically, Gol was only thought to play a role as the galactosyl donor in RFO biosynthesis. However, it has since been demonstrated to be an effective free radical scavenger *in vitro* (Nishizawa et al. 2006; Nishizawa et al. 2008) and its intracellular accumulation during abiotic stresses suggests its involvement in stress-protection (Taji et al. 2002; Kaplan et al. 2004, 2007; Panikulangara et al. 2004; Peters et al. 2007). Increases in GolS transcript abundance and enzyme activity during cold exposure has been reported for *Phaseolus vulgaris* (kidney beans, Liu et al. 1998), *Arabidopsis* (Taji et al. 2002; Zuther et al. 2004; Egert et al. 2013), *Ajuga reptans* (Sprenger and Keller, 2000) and *Solanum lycopersicum* (tomato, Downie et al. 2003). Further, expression of *Arabidopsis GolSs* (*AtGolS1* and *AtGolS2*) has been reported for heat shock (Panikulangara et al. 2004; Busch et al. 2005; Schramm et al. 2006; Nishizawa et al. 2006).

There are ten *GolS* isoforms in *Arabidopsis* (Taji et al., 2002; Nishizawa et al., 2008) of which the most well reported are *AtGolS1* (*At2g47180*) and *AtGolS2* (*At1g56600*). Their occurrence is associated with seed maturation and a myriad of abiotic stresses (Taji et al. 2002; Panikulangara et al. 2004; Egert et al. 2013). The *AtGolS3* (*At1g09350*) isoform is responsive only to cold-acclimation conditions (Taji et al. 2002; Panikulangara et al. 2004). Little is known of the other *Arabidopsis GolS* isoforms (Taji et al. 2002; Nishizawa et al. 2006; 2008).

Recent reports have proposed a novel function for Gol being involved as a signalling molecule during biotic stress (Kim et al. 2004; Kim et al. 2008; Cho et al. 2010, Tarkowski and Van den Ende 2015). The mechanisms underpinning pathogen

induced-responses (biotic stress) in plants are of a complex nature and mainly consist of two major arms termed systemic acquired resistance (SAR) and induced systemic resistance (ISR). These pathways are intricately linked to salicylic acid (SA) and jasmonic acid (JA) hormonal regulation, respectively. While carbohydrates have been long suggested to be involved/associated with both the ISR and SAR plant response (Sequeira 1983; Herbers et al. 1996), the recent reports outlining a functional role for Gol in eliciting ISR responses in both tobacco and Arabidopsis are quite novel (Kim et al. 2008; Cho et al. 2010). A *Cucumis sativus* GolS (cucumber, *CsGolSI*) homolog was identified in a differential screen of cucumber plants (primed with *Pseudomonas chlororaphis* O6) infected with *Corynespora cassiicola* (a leaf spot fungus, Kim et al. 2004). In that study, transcript increases of *CsGolSI* positively correlated with increases in Gol concentrations, implicating Gol in the ISR pathway. In a subsequent study, over-expression of the *CsGolSI* in tobacco constitutively increased resistance to *Botrytis cinerea* infections, concomitant with an increase in Gol accumulation (Kim et al. 2008). Further, exogenous feeding of Gol to wild-type tobacco plants led to an increased resistance to *B. cinerea* infection, suggesting a signalling role for Gol alike to phyto-hormones. The principle was further demonstrated using Arabidopsis (Cho et al. 2010). In that study, over-expression of *AtGolSI* in Arabidopsis led to transgenic plants that were resistant to *B. cinerea* infection. Conversely, *AtGolSI* T-DNA insertion mutants were sensitive to infection. These findings evidently link both GS and Gol to the plant ISR response/s. However, the mechanism by which this occurs is unknown.

It is important to note that neither of the studies associating Gol with ISR consistently analysed (or reported) Gol and/or RFO content of the systems used in those experiments (cucumber, tobacco and Arabidopsis). The accumulation of Gol is obligately linked to the appearance of RFOs. In the context of Arabidopsis, whenever *GolSs* are used in a constitutive

over-expression strategy transgenic plants hyper-accumulate significant amounts of both Gol and Raf (Taji et al. 2002, Nishizawa et al. 2006, 2008; Kim et al. 2008, Cho et al. 2010). Conversely, Arabidopsis T-DNA insertion mutants in either *AtRS5* (*At5g40390*, Zuther et al. 2004; Knaupp et al. 2011; Egert et al. 2013) or *AtRS4* (*At4g01970*, this work, Chapter 2) hyper-accumulate Gol, demonstrating the “linear” nature of the Gol-dependent RFO biosynthesis pathway. The recent studies which propose a novel function for Gol as a signalling molecule in ISR (Kim et al. 2008, Cho et al. 2010) did not experimentally address the invariable accumulation of Raf in their systems (tobacco and Arabidopsis, respectively) over-expressing *GolS* isoforms. It is Raf that is the most widely reported RFO implicated in protective functions during episodes of abiotic stress (Taji et al. 2002; Zuther et al. 2004; Knaupp et al. 2011; Nishizawa et al. 2008; Egert et al. 2013). It is thus feasible that Raf may also be involved in the proposed ISR signalling function.

Since the Arabidopsis RFO biosynthetic pathway obligately requires Gol for Raf biosynthesis, a Gol free (but Raf accumulating) system was not physiologically feasible. However, we reasoned that if Raf was a component of the ISR response then a Raf free (but Gol accumulating) system should reveal this function during pathogen infection. To this end we looked to the recently characterised *RS5* (*At5g40390*) T-DNA insertion mutants *atrs5-1* and *5-2* (Egert et al. 2013). The *atrs5* mutant backgrounds accumulate no Raf in the leaves but hyper-accumulate Gol, as a consequence of RS loss-of-function (Zuther et al. 2004; Egert et al. 2013). We anticipated that a Raf deficient (but Gol hyper-accumulating) system should show resistance equal to the previously described *AtGolS1* over-expressing lines (Cho et al. 2010), if Gol alone was the elicitor of the ISR response. We further included two additional *AtGolS1* (*At2g47180*) T-DNA insertion mutants (*atgols1-1* and *1-2*) to reproduce the recently reported findings (sensitive to *B. cinerea*, Cho et al. 2010).

In this work we report, and argue, against either Gol and/or Raf being signalling molecules eliciting the ISR pathway in the Arabidopsis/ *B. cinerea* pathosystem. We reproduced the susceptibility reported for T-DNA insertion lines for *AtGolS1* (Cho et al. 2010) but could also demonstrate that the two *atgols1* insertion lines accumulated substantial amounts of both Gol and Raf under normal growing conditions (prior to *B. cinerea* infections). Further, we demonstrated that two independent *atrs5* insertion lines were equally susceptible as the *atgols1* insertion lines (despite hyper-accumulating Gol to concentrations similar to *GolS* over-expressing plants).

3.2 Materials and Methods

3.2.1 Plant material

All Arabidopsis T-DNA insertion mutants used were obtained from the Salk Institute's T-DNA insertion mutant collection, in the Col-0 ecotype (Alonso et al. 2003). Two independent insertion lines for *At2g47180* (*AtGols1*, Salk_128044, *atgols1-1*; Salk_121059, *atgols1-2*) and *At5g40390* (*AtRS5*, Salk_049583, *atrs5-1*; Salk_085989, *atrs5-2*), respectively were used in this study. Subsequent to seed stratification (24 h, 4°C), plants were propagated and maintained on peat disks (Jiffy™ no.7, South Africa) under controlled environment conditions (8 h light, 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 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), at 7 and 21 d post germination as previously described (Petersen et al. 2010). Homozygous insertion lines were verified by PCR on genomic DNA (gDNA) isolations as previously described (Egert et al. 2012; 2013). Briefly, aliquots of genomic DNA (5 μl) were used in PCR reactions containing primer combinations for wild-type alleles of *AtGols1* and *AtRS5* (LP+RP, ~1 kB, **Supplementary Table S1**) and for mutant alleles (RP+LBb1.3, ~0.7 kB). The T-DNA specific LBb1.3 primer (5'GCGTGGACCGCTTGCTGCAACT) was used in combination with the respective RP primer for each of the two independent lines.

Where applicable, three week-old plants (rosette stage 1.08) were used for cold-acclimation treatments as previously described (Zuther et al. 2004; Egert et al. 2013). Briefly, plants were transferred to controlled environment chambers with the same settings as listed above but, subject to a constant temperature of 4°C over 14 d, followed by a de-acclimation period under controlled conditions as described above (14 d) prior to further assessment.

Where applicable, all plants (subsequent to the acclimation/de-acclimation periods) were acclimated to fungal infection environment conditions [24 h prior to infection (8 h light, 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 22°C, 16 h dark, 22°C, 90% relative humidity)]. Experiments were conducted thrice using pools of 18 plants each.

3.2.2 RNA isolation and transcript analysis

Total RNA was isolated using the RNeasy[®] Plant Mini kit (Qiagen, Whitehead Scientific, South Africa), following the manufacturer's instruction. The complementary DNA (cDNA) template was obtained *via* reverse transcription of 1 μg of total RNA, using an oligo (dT₁₅) primer and M-MLV (H⁻) reverse transcriptase (Promega, Anatech, South Africa) following the manufacturer's instruction. Transcript analysis (for *At2g14610*, *At3g12500*, *At5g40390*, *At1g09350*, and *At3g18780*) was performed using semi-quantitative RT-PCR (sqPCR).

The sqPCRs were conducted using GoTaq[®] DNA polymerase (Promega, Anatech, South Africa) in a 50 μl reaction (3 μl cDNA, 1.25 U DNA polymerase, 5 \times green PCR buffer, 0.5 mM of each dNTP, and 0.5 μmol of each primer) for 24 cycles, with a primer annealing temperature optimum of 58°C for all respective sqPCR primer pairs. The constitutively expressed gene, *AtACT2* (*At3g18780*), was used to determine the number of cycles used for the sqPCR, where expression occurred in the linear range. The primer pairs were designed to amplify fragments of 1 kb from the cDNA (**Supplementary Table S2**).

3.2.3 Water soluble carbohydrate (WSC) extractions and desalting procedures

WSCs extractions from freeze-dried leaf (50 mg) material was conducted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), with minor modifications. Extractions were executed in a three-step sequential series [1 ml 80% (v/v) EtOH, 1 ml 50% (v/v) EtOH, 1 ml de-ionised H₂O (dH₂O)]. Each consecutive extraction was conducted twice at 80°C for 10 min, centrifuged (13 000 g, 10 min, RT) and supernatants

transferred to a new Eppendorf tube prior to the next step in the series. The supernatants for each individual extraction were pooled, vacuum centrifuged and re-suspended in dH₂O to a final volume of 200 µl. Samples were desalted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), prior to LC-MS analysis. Briefly, samples were desalted through pre-rinsed 1 ml Mobicol spin columns (MoBiTec) mediated by centrifugation (2000 g, 2 min, 4°C). The columns were packed with a 10 µm frit, 150 µl anion exchange resin [HCO₂⁻ form, 200–400 mesh (Bio-Rad AG 1-X8)], 100 µl PVPP and 50 µl cation exchange resin [H⁺ form, 200–400 mesh (Bio-Rad AG 50W-X8)]. Carbohydrate samples were desalted through the columns and centrifuged-rinsed four times with 250 µl dH₂O. Aliquots of 100 µl were analysed with LC-MS as described below.

3.2.4 LC-MS analyses and WSC quantification

LC-MS analysis was performed with a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UPLC. Samples were separated on a Waters UPLC BEH Amide column (2.1 x 100 mm; 1.7 µm) at a flow rate of 0.17 ml/min at 35°C. Solvent A consisted of acetonitrile/water (30:70) containing 0.1% ammonium hydroxide and solvent B was acetonitrile/water (80:20) containing 0.1% ammonium hydroxide. The mobile phase gradient was from 0% to 60% solvent A over 5 min, maintained for 2 min at 60% solvent A before the column was re-equilibrated to the initial conditions. Electrospray ionization was applied in the negative mode and the scan range was from m/z 150 to 1500. The capillary voltage was set a 2.5 kV, the cone voltage was 15 V, the source temperature 120 °C and the desolvation temperature was 275 °C. The desolvation gas and cone gas flows were 650 L/h and 50 L/h, respectively. Quantification was conducted against a series of standard sugars after standard curves were created within the linear response range of the apparatus. All WSCs were monitored using

their deprotonated quasi-molecular ions and quantified with the TargetLynx application manager (Waters MassLynx V4.1V software).

3.2.5 Fungal cultivation and plant infection

Botrytis cinerea (GrapeVine strain, obtained from the Institute for Wine Biotechnology, Stellenbosch University) was cultivated on sterile canned apricot halves in Petri dishes (14 d, 25°C, without light). Spores were harvested with a 2 ml wash solution (dH₂O, 1% tween 20) in ten repetitive steps and subsequently filtered through glass wool to remove fungal hyphae. Cultures were (i) assessed for germination efficiency ($\geq 80\%$) by placing an aliquot (5 μ l) onto an empty Petri dish [10 h prior to infection (8 h light, 120 μ mol photons $m^{-2} s^{-1}$, 22°C, 16 h dark, 22°C, 90% relative humidity)] and (ii) allowed to hydrate (10 h, 4°C, without light) in 1 ml wash solution prior to inoculum preparation. Spore inoculums were prepared in sterile half-strength grape juice to a final spore concentration of 10^5 spores/ml.

Susceptibility of acclimated/de-acclimated (described above) plants was assessed by placing a single droplet (10 μ l per source leaf) of *B. cinerea* spore inoculum or sterile half-strength grape juice (mock control) in the centre of each leaf. Each experimental setup contained 18 plants per line (Col-0, *atgols1-1* and *1-2*, *atrs5-1* and *5-1*) where three leaves per plant were infected. Experiments were conducted thrice. Disease assessment (lesion formation) was observed by image capturing (24 h cycle, 7 d) and virtual measurement of lesions.

3.3 Results

3.3.1 *Botrytis cinerea* infects source leaves more efficiently than sink leaves

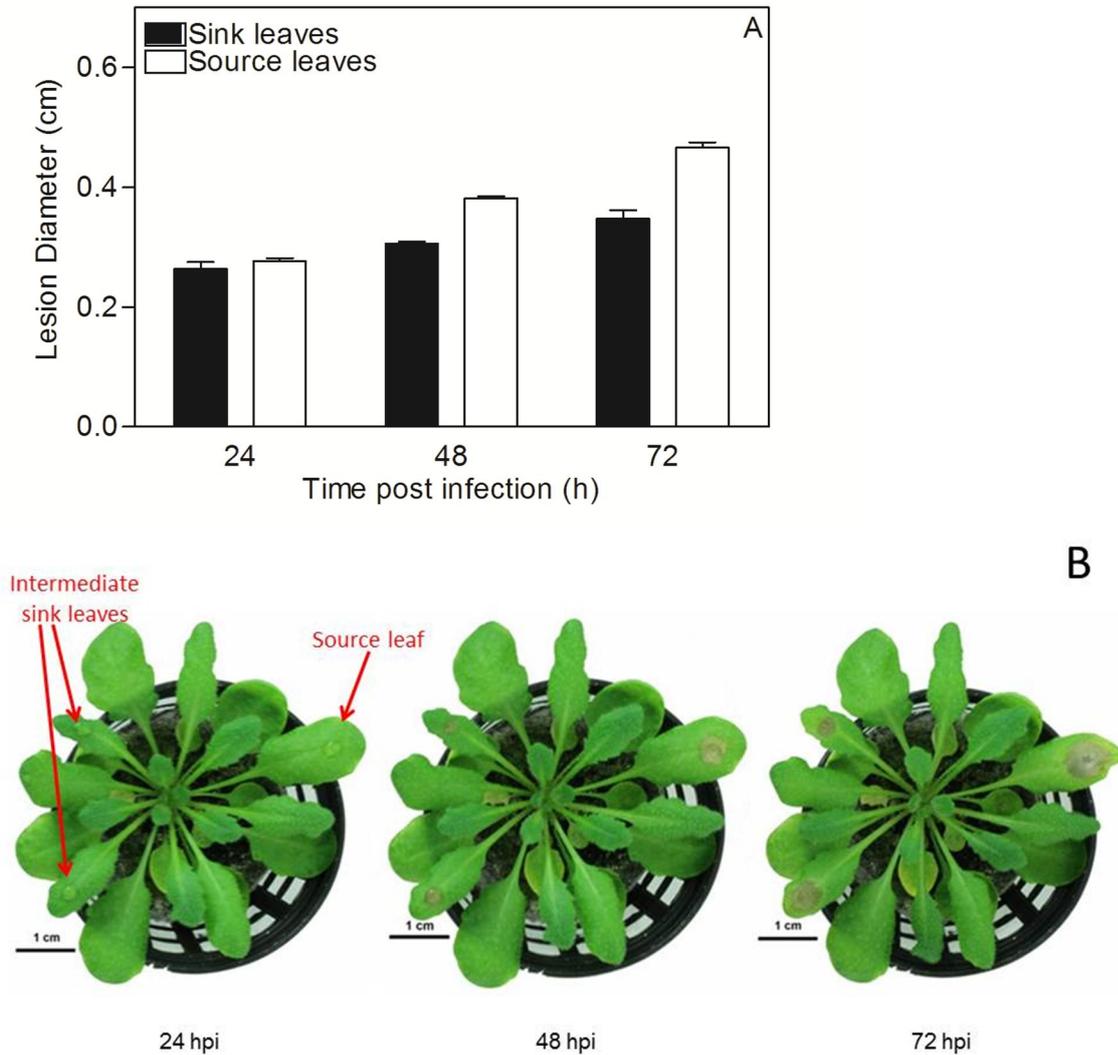


Fig.1 Progression of lesion development and assessment of cell death in *Arabidopsis* (Col-0) leaves infected with *B. cinerea*

(A) Progression of lesion development in *Arabidopsis* (Col-0) source and sink leaves infected with *B. cinerea* (10^5 spores/mL, 80% germination efficiency), over a period of 72 h. (B) Representative image of lesion development, over a period of 72 h, on a whole plant system where intermediate sink and source leaves were infected.

Using *Arabidopsis* wild-type plants (Col-0, rosette stage 1.16, non-acclimated), we infected sink and source leaves with *B. cinerea* (10^5 spores/ml) to establish reproducible infection parameters. Lesion diameters were captured over a period of 72 h (Fig. 1A and B). Lesion development was consistently progressive over the 72 h period in both sink and source leaves. Source leaves were more susceptible to infection showing increased lesion diameters over 72 h as compared to intermediate sink-leaves (Fig. 1A).

3.3.2 Cold-acclimation ‘priming’ does not improve Gol hyper-accumulation in the *atrs5* mutant background

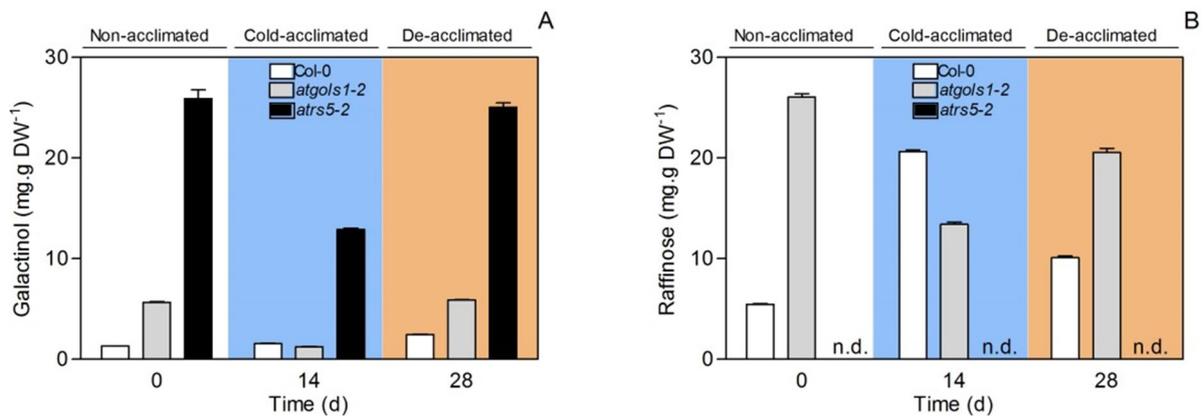


Fig.2 Accumulation of galactinol (Gol) and raffinose (Raf) in leaves of Col-0, *atgols1-2* and *atrs5-2*, respectively during non-acclimated, cold-acclimated and de-acclimated conditions.

(A) and (B) Representative figures of Gol and RFO accumulation for Col-0 and insertion lines, *atgols1-2* and *atrs5-2*, at the end of each acclimation cycle, respectively. d. days, n.d. not detected.

Prior to pathogen (*B. cinerea*) infection, all plant backgrounds (Col-0, *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*) were subject to periods of cold-acclimation (4°C, 14 d) and de-acclimation (22°C, 14 d), respectively. Total WSCs were analysed from the leaves of Col-0 and respective insertion lines using quantitative LC-MS under specified conditions of acclimation. Both Gol and Raf was detected in low levels of the non-acclimated state of

Col-0 leaves ($1.3 \pm 0.02 \text{ mg g}^{-1} \text{ DW}$ and $5.5 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$, respectively). Cold-acclimation of Col-0 resulted in an insignificant increase of Gol ($1.5 \pm 0.8 \text{ mg g}^{-1} \text{ DW}$) and a 4-fold increase in Raf ($20.6 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$). No Sta was detected in the leaves of these plants. During the de-acclimation period, Gol levels increased ($2.5 \pm 0.6 \text{ mg g}^{-1} \text{ DW}$) and Raf levels stabilised at $10 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$, 2-fold lower than the cold-acclimated state (**Fig.2A**).

Two independent insertion lines carrying insertions in either the *AtGols1* (*atgols1-1* and *atgols1-2*) or *AtRS5* (*atrs5-1* and *atrs5-2*) gene, respectively, were analysed for their WSC content before and after the same cold-acclimation conditions as described above. The *atgols* insertion lines contained significant amounts of Gol and Raf before cold-acclimation, 5-fold higher than Col-0 (up to 5.6 ± 0.8 and $26.05 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$, respectively; representative figure indicating only the *atgols1-2* line, **Fig.2A**). After a period of cold-acclimation (14 d), these lines had a nearly 5-fold decrease in Gol ($1.25 \pm 0.04 \text{ mg g}^{-1} \text{ DW}$) and a 2-fold decrease in Raf ($13.3 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$). Subsequent to the de-acclimation phase, the insertion lines had Gol and Raf levels similar to those before the cold-acclimation period (5.85 ± 0.05 and $20 \pm 1.3 \text{ mg g}^{-1} \text{ DW}$, respectively).

The *atrs5* insertion lines hyper-accumulated Gol under conditions of non-acclimation, 25-fold higher than Col-0 and 5-fold higher than *atgols* lines ($25 \pm 1.6 \text{ mg g}^{-1} \text{ DW}$). Gol decreased 2-fold during cold-acclimation ($12.9 \pm 0.7 \text{ mg g}^{-1} \text{ DW}$) and subsequent to the de-acclimation period concentrations were similar to that of the non-acclimated state ($24.8 \pm 0.6 \text{ mg g}^{-1} \text{ DW}$)(representative figure showing only *atrs5-2*, **Fig.2B**). Raf was completely absent in both *atrs5* insertion lines throughout (representative figure showing *atrs5-2*, **Fig.2B**).

3.3.3 Pathogenesis-related gene transcripts were not affected by the cold-acclimation/de-acclimation priming strategy

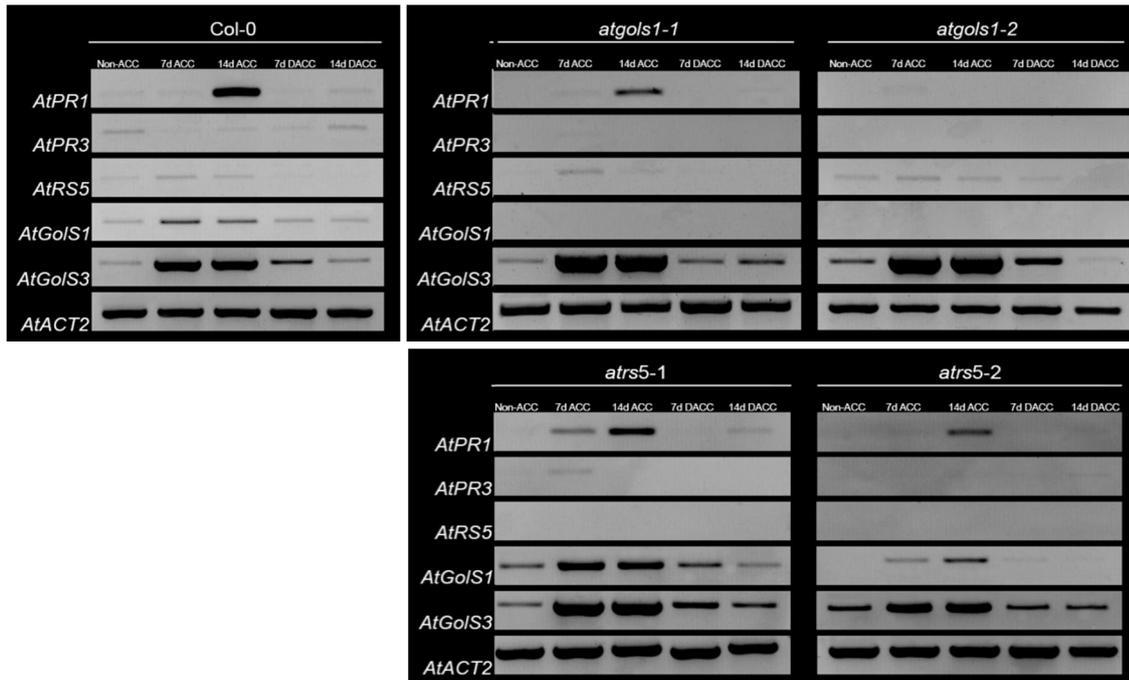


Fig.3 Transcript analyses of RFO-related and pathogen-related gene expression during cold-acclimation “priming” in Col-0, *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*, respectively.

A semi-quantitative PCR approach was followed to determine gene expression changes for *AtACT2*, *AtGoS3*, *AtGoS1*, *AtRS5*, *AtPR3* and *AtPR1*, respectively during the acclimated and de-acclimated periods

Total RNA was extracted from the leaves of Col-0, *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2* insertion lines, respectively. Transcript abundance/expression was determined via a semi-quantitative PCR approach, using *AtACT2* (*At3g18780*) as the constitutive reference gene (**Fig. 3**). During cold-acclimation (7 d and 14 d, respectively) the *AtGoS3* (*At1g09350*) transcript increased as anticipated. This is the only known *GS* isoform in Arabidopsis known to be responsive to cold stress (Taji et al. 2002; Panikulangara et al. 2004). Transcript absence of *AtGoS1* (*At2g47180*) in both *atgols1-1* and *atgols1-2* insertion lines confirmed true loss-of-function lines. However, these transcripts were slightly increased (akin to *AtGoS3*) during periods of cold-acclimation in the Col-0, and *atrs5-1* and *5-2* insertion lines,

respectively. Using the same approach, true loss-of-function *atrs5* insertion lines were confirmed by the absence of *RS5* transcripts. Transcripts for *AtRS5* was present in all other lines (Col-0, *atgols1-1* and *1-2*, respectively), indicating slight increased expression levels during the cold-acclimated period.

The responsiveness of pathogen-related (PR) genes to cold-acclimation was further analysed. The induced systemic resistance (ISR)/jasmonic acid (JA) related *AtPR3* (*At3g12500*) gene showed no significant response to cold-acclimation in any of the lines. The systemic acquired resistance (SAR)/salicylic acid (SA) related *AtPRI* (*At2g14610*) gene had consistent transcript increases in all lines after 14 d of cold-acclimation. All transcript levels reverted back to the non-acclimated state subsequent to the de-acclimation period.

3.3.4 Both *atgols1* and *atrs5* mutant backgrounds are comparably sensitive to *B. cinerea* infection

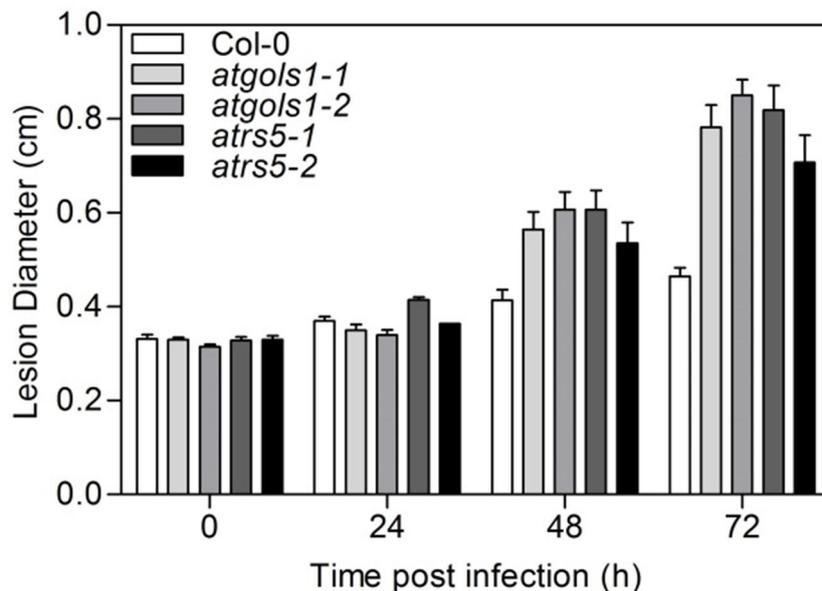


Fig.4 Lesion development in metabolically primed Col-0, *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*, respectively.

Leaves of respective lines were infected with *B. cinerea* (10^5 spores/mL, 80% germination efficiency) subsequent to metabolic priming. Lesion development was captured over a period of 72 h, at a 24 h interval. h. hours.

Subsequent to cold-acclimation and de-acclimation of Col-0, *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*, plants were inoculated with *B. cinerea* (10 μ L droplet/source leaf of a 10^5 spores/mL culture concentration) and lesion development was photographically monitored over a period of 72 h. Lesion diameter was determined manually for each respective site of infection. The progression of lesion development in the insertion lines were measured for susceptibility (or resistance) using Col-0 as the control. Col-0 infection resulted in lesion sizes of approximately 0.45 ± 0.58 cm after 72 h (**Fig.4**). Lesion diameter increased nearly 0.5-fold after 48 h in the respective insertion lines (0.56 ± 0.19 , 0.6 ± 0.2 , 0.6 ± 0.18 and 0.53 ± 0.14 cm for *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*, respectively). Insertion lines showed nearly 2-fold increase in lesion diameter after 72 h of infection when compared to the wild-type (0.78 ± 0.21 , 0.85 ± 0.22 , 0.81 ± 0.24 and 0.70 ± 0.21 cm for *atgols1-1*, *atgols1-2*, *atrs5-1* and *atrs5-2*, respectively, **Fig.4**).

3.4 Discussion

The recent reports which propose a function for Gol as a signalling molecule in ISR (Kim et al. 2004; Kim et al. 2008; Cho et al. 2010) used a biotic priming methodology where plants were primed with *P. chlororaphis* (inducer of ISR) and subsequently infected with a necrotrophic fungus. They also demonstrated that GS over-expression in tobacco and Arabidopsis resulted in transgenic plants that were constitutively resistant to necrotrophic pathogen infection in the absence of biotic priming, thus suggesting that priming induces Gol biosynthesis which in turn elicits the ISR signalling responses. Further, the well described pathogenesis related marker genes PR1 (*At2g14610*, salicylic acid, SA, responsive) and PDF1.2 (*At5g44420*, jasmonic acid, JA, responsive) were both Gol-responsive (exogenous feeding of excised leaves). However, *AtGolS1* was specifically shown to be only JA-responsive thus suggesting a role for this GS isoform in JA-mediated ISR signalling.

3.4.1 Cold-acclimation/de-acclimation as a method to hyper-accumulate Gol in RS5 mutant plants does not affect PR gene-expression nor improve leaf Gol concentrations

Upon establishing a reproducible pathosystem using source leaves (**Fig. 1**), we further investigated a non-invasive priming strategy *via* cold-acclimation/de-acclimation. Plants are often subject to harsh chemical or biotic priming prior to pathogen infection (e.g. BABA and *P. chlororaphis* priming, respectively, Hulten et al. 2005; Ton et al. 2005; Schreiber and Desveaux 2008) in order to enhance their response time to the pathogen. These priming strategies are often harmful to the plant and could trigger undesirable pleiotropic responses (Hulten et al. 2005; Goellner and Conrath 2008). Priming further also induces ISR and SAR responses (Conrath 2009).

Our reasoning around the use of cold-acclimation/de-acclimation to “prime” Arabidopsis leaves was primarily due to the RS5 loss-of-function mutants. We believed that the well described cold-acclimation induced Raf accumulation in Arabidopsis wild-type (Col-0)

leaves (Zuther et al. 2004, Iftime et al. 2011, Egert et al. 2013) could be manipulated in *atrs5-1* and *5-2* (RS and Raf deficient, Gol hyper-accumulating) to hyper-accumulate substantial amounts of Gol (during cold-acclimation), since Gol is biochemically hydrolysed by an α -1,6 galactosyltransferase like RS. We further presumed that de-acclimation would then result in Gol remaining in the leaves of the *atrs5* mutant lines but returning to “normal” levels in our wild-type controls.

Since we wished to induce Gol hyper-accumulation in the absence of an active ISR response (elicited by chemical or biotic priming) we confirmed that our cold-acclimation/de-acclimation “priming” strategy did not elicit the classical PR gene-response. We could demonstrate that PR1 (SA inducible, SAR response, Lee et al. 2006; Spoel and Dong 2012; Mettraux 2015) and PR3 (JA inducible, ISR response, Zhang et al. 2010; Spoel and Dong 2012) were either unaffected or returned to levels comparable to controls in all of the backgrounds, following the cold-acclimation/de-acclimation priming (**Fig.3**).

Our Gol hyper-accumulating RS5 mutants did not show increased transcript abundance for PR1 and PR3 subsequent to the de-acclimation period (plants were subsequently challenged with *B. cinerea* infection). Previously, feeding experiments (Gol) on excised leaves (Col-0) have been reported to increase both PR1 and PDF1.2 (Cho et al. 2010). We suggest that the act of excision could have elicited the PR gene response in this regard (and not Gol). Wounding is well reported to induce both ISR and SAR (Conrath 2009). This could explain why the feeding experiments reported both an ISR and SAR-associated gene response, whilst *AtGolSI* was responsive to JA feeding only (and not SA feeding).

Our wild-type controls (Col-0) followed typical reported trends in cold-acclimation induced RFO accumulation (Zuther et al. 2004; Knaupp et al. 2011; Egert et al. 2013;

Nagele and Heyer 2013). Trace amounts of Gol and Raf were detected in the leaves (**Fig. 2**) under our normal growing conditions but during cold-acclimation leaves showed transcriptional up-regulation of *AtGolS3* and *AtRS5* (**Fig.3**) and, increased concentrations of Gol and Raf (**Fig.2**). Following de-acclimation, Gol and Raf concentrations returned to trace amounts in the leaves of wild-type controls. Both *atrs5* mutant lines hyper-accumulated Gol to levels comparable to those reported for GS over-expressing Arabidopsis lines (resistant to *B. cinerea* infection, Cho et al. 2010; **Supplementary Table S3**) under normal growing conditions. Cold-acclimation/de-acclimation did not result in further increases of Gol concentrations (**Fig. 2**).

A surprising finding was the substantial concentrations of both Gol and Raf in the leaves of both *atgols1* mutant lines under normal growing conditions. This finding has neither been reported, nor addressed in previous studies using similar T-DNA insertion lines (Cho et al. 2010). Surprisingly, during conditions of cold-acclimation, Gol and Raf concentrations decreased in these mutant lines, and it is tempting to speculate that there is a de-regulation of the RFO pathway when *AtGolS1* transcription is interrupted. However, transcript levels of both *AtGolS3* and *RS5* in these mutant lines did not display unusual expression profiles (**Fig. 3**).

3.4.2 The Gol hyper-accumulating RS5 insertion mutant line is susceptible to B. cinerea infection

Our Raf-deficient system (*atrs5-1* and *5-2*) accumulated Gol to concentrations comparable to previous reports describing GS over-expression strategies (**Supplementary Table S3**), including the *AtGolS1* over-expressing line described to be resistant to *B. cinerea* infection (Cho et al. 2010). That study did not report on Gol or Raf concentrations in the transgenic lines over-expressing *AtGolS1*.

Importantly, we reproduced the findings reported in Cho et al. (2010), demonstrating that two additional *AtGolS1* insertion lines were sensitive to *B. cinerea* infection (in the absence of traditional biotic priming). However, we also observed that these lines accumulated substantial amounts of Gol and Raf that were not previously reported (**Fig. 2**), under normal growing conditions. Surprisingly, our Raf-deficient system (*atrs5-1* and *5-2*) was sensitive to *B. cinerea* infection (comparable to the *atgols1* mutant lines, **Fig. 4**). Given that we measured Gol concentrations (in the *AtRS5* mutants) comparable to those previously reported for GS over-expressing Arabidopsis, it was clear that Gol itself could thus not be the elicitor of the ISR response, as previously reported.

However, the occurrence of high concentrations of both Gol and Raf in the *atgols1* lines (also sensitive to infection) seem to argue against Raf being involved in the ISR response. It is unclear why the *atgols1* mutant background shows de-regulated Gol and Raf accumulation. It is obvious that Gol accumulation in this mutant background must be due to the activity of another isoform (not *AtGolS1*). A tempting speculation is the possibility that the link to the Gol/RFO pathway and ISR may involve the actual GolS1 transcript or protein. The promoter of *AtGolS1* contains the JA binding cis-element (AACGTG, Boter et al. 2004) and it has been reported to be JA-responsive (Cho et al., 2010). While it is clear that *GolS* over-expression in both tobacco and Arabidopsis elicits pathogen resistance via ISR, the mechanism is unknown. Our findings argue against the metabolite (Gol and/or Raf) in this process. However, this signalling mechanism might involve the binding of small coding and non-coding RNA which has previously been demonstrated to play similar roles in abiotic stress responses (reviewed in Sunkar et al. 2007).

In conclusion we do not discount an underlying mechanism involving the RFO pathway in plant innate immunity. However, our findings for the Gol hyper-accumulating *RS5* mutants have clearly demonstrated that Gol accumulation alone is insufficient to elicit ISR based

resistance, as previously suggested (Kim et al. 2008; Cho et al. 2010) and suggests that neither is Raf.

Our findings warrant further investigation and we are currently ascertaining which *Gols* isoform may be up-regulated in the *AtGols1* mutant backgrounds, that is responsible for the unusual accumulation of Gol and Raf under normal growing conditions. Future investigations could potentially employ a strategy where the uniquely cold-responsive *AtGols3* is used in a constitutive over-expression approach to establish if transgenic plants are resistant to infection (comparable to *AtGols1* over-expressers), since only the *AtGols1* gene contains the JA responsive cis-element in its promoter region. Furthermore, we suggest that future experimentation would benefit from a combination of (i) a genome-wide transcriptome approach (for example *via* next generation transcriptome analyses using an Illumina HiSeq2500 sequencing platform) to determine any differences in transcript profiles and, (ii) a targeted global LC-MS/GC-MS metabolite analyses to profile the carbohydrate changes in the *AtGols1* over-expressing and *AtRS5* mutant backgrounds prior to and, following *B. cinerea* infections.

Supplementary Figures (Chapter 3)

Table S1. Primers used for zygosity determination of *AtGolS1* and *AtRS5* T-DNA insertion lines

T-DNA insertion mutant line	Forward primer (LP)	Reverse primer (RP)
Salk_128044 (<i>atgols1-1</i>)	5' TCTACATTTTCTGGGTGACGC	5' CACTTCCAGAACTTTCACGG
Salk_121059 (<i>atgols1-2</i>)	5' TTGTTTGACCTACCAGATGGC	5' TTCGAAACAAAAATTGAACCG
Salk_049583 (<i>atrs5-1</i>)	5' CTCTTCTGAAGGCTCCTTCC	5' ATGACATCAACTTTAACGCCG
Salk_085989 (<i>atrs5-2</i>)	5' ATGGAECTCAGCACAAGGATG	5' TTATTGAAA TCCTCACACC

Table S2. Primers used for sqRT-PCR transcript analysis. Primer pairs were designed to amplify 1 kb fragments

Gene name and AGI code	Forward primer (LP)	Reverse primer (RP)
PR1 (<i>At2g14610</i>)	5' ATGAATTTACTGGCTATTCTCG	5' TTAGTATGGCTTCTCGTTCAC
PR3 (<i>At3g12500</i>)	5' ATGCCTCCACAAAAGAAAAC	5' CTAATAGCAGCTTCGAGGAG
RS5 (<i>At5g40390</i>)	5' ATGGCTTCGCCGTGTTGACC	5' CGGAGCTTCAGGACGGAGAC
GolS3 (<i>At1g09350</i>)	5' ATGGCACCTGAGATGAACAACAAGT	5' CTAAGCCGCGGATGGAGCT
GolS1 (<i>At2g47180</i>)	5' ATGGCTCCGGGGCTTACTCA	5' TCAAGCACGGACGGTGCGGT
ACT2 (<i>At3g18780</i>)	5' ATGGCTGAGGCTGATGATAT	5' TTAGAAACATTTTCTGTGAACGAT

Table S3. A summary of Gol and Raf accumulation in (A) forward genetic-approaches and (B) reverse genetic-approaches

(A) Species	GolS isoform	AGI code	Accession number	[Gol] accumulation (mg g ⁻¹ DW)	[Raf] accumulation (mg g ⁻¹ DW)	Reference
Arabidopsis	GolS1	AT2G47180	AY091426	2.7	13.4	Nishizawa et al. 2008
Arabidopsis	GolS2	At1g56600	KJ138960	23	83	Nishizawa et al. 2008
Arabidopsis	GolS2	At1g56600	KJ138960	42	80	Taji et al. 2002
Arabidopsis	GolS1	AT2G47180	AY091426	Not reported	Not reported	Cho et al. 2010
<i>Cucumis sativus</i>	GolS1	-	AY237112	63	Not reported	Kim et al. 2008
(B) T-DNA insertion line	Affected gene	AGI code	Accession number	[Gol] accumulation (mg g ⁻¹ DW)	[Raf] accumulation (mg g ⁻¹ DW)	Reference
<i>atgols1</i>	GolS1	AT2G47180	AY091426	5.7	26	This study
<i>atrs5</i>	RS5	AT5G40390	NM_123403	25	0	This study

CHAPTER 4

Constitutive over-expression of *Ajuga reptans* galactan:galactan galactosyltransferase (*ArGGT*) in *Arabidopsis* to engineer the accumulation of long-chain raffinose family oligosaccharides (RFOs)

4.1 Introduction

A myriad of exotic carbohydrates, such as verbascose, ajugose, planteose and stellariose are acquainted with the raffinose family of oligosaccharides (RFOs, Suc-(Gal)_n, 13 < n ≤ 1) (Kandler and Hopf 1982). RFOs are α-galactosyl extensions of sucrose (Suc) initiated by the transfer of galactosyl moieties from galactinol (Gol, 1-O-α-D-galactopyranosyl-L-*myo*-inositol). The first enzyme committed to RFO biosynthesis is galactinol synthase (GS, EC 2.4.1.123) which catalyse the formation of Gol using UDP-galactose and *myo*-inositol as substrates. Subsequent reactions are catalysed by raffinose synthase (RS, EC 2.4.1.82) and stachyose synthase (SS, EC 2.4.1.67) in a stepwise manner, using either Suc or raffinose (Raf, Suc-Gal₁) in a Gol-dependent fashion to synthesise Raf and stachyose (Sta, Suc-Gal₂), respectively. Higher oligomers in this pathway are often synthesised in a Gol-independent manner. Verbascose (Ver, Suc-Gal₃) has been reported to be synthesised in such a fashion in *Pisum sativum* (pea seeds, Peterbaur and Richter 2002) *via* a multi-functional SS. A uni-functional verbascose synthase (VS) has not yet been reported. These short-chain RFOs, particularly Raf, have been frequently associated with episodes of abiotic stress and their stress induced-accumulation has thus been linked to sub-cellular protection.

In the leaves of *Ajuga reptans* (common bugle, Bachman et al. 1994; Haab and Keller 2002; Peters and Keller 2009) and *Coleus blumei* (variegated coleus, Gilbert et al. 1997), an

enzyme activity responsible for the Gol-independent synthesis of higher RFO oligomers has been reported. This enzyme, galactan:galactan galactosyltransferase (GGT) has been cloned and characterised from *A. reptans*. It utilizes RFOs as both galactosyl donors and acceptors to synthesise long-chain oligomers (up to Suc-(Gal)₁₃, Tapernoux-Lüthi et al. 2004). Both GGT and long-chain RFOs are localised to the vacuoles. In *A. reptans* RFOs are the most abundant carbohydrates with Sta being the main carbon translocate (Peters and Keller, 2009). Higher RFO oligomers are major carbon store molecules (Bachmann et al. 1994) and they are synthesised by GGT in the vacuolar compartments (Bachmann et al. 1994; Haab and Keller 2002; Tapernoux-Lüthi et al. 2004). To date, these long-chain RFOs have been mainly implicated for their role in carbon storage during winter months.

However, the long-chain RFOs have also been suggested to facilitate abiotic stress tolerance. This suggestive role has been demonstrated in an *A. reptans* excised leaf system where an increase in GGT activity positively correlated to the accumulation of long-chain RFOs (up to Suc-(Gal)₇) during conditions of freezing (Bachmann et al. 1994; Bachmann and Keller 1995; Peters and Keller 2009). A similar study has been done in *C. blumei* where the increase of GGT activity positively correlated to increased levels of long-chain RFOs in response to salinity stress (Gilbert et al. 1997). A single study has addressed the efficacy of different RFO oligomers (up to Ver) in protecting artificial liposomes from desiccation induced-damage (Hincha et al. 2003). It reported that the higher RFO oligomers afforded improved protection in this *in vitro* system. In *Arabidopsis*, cold acclimation induced-Raf has been demonstrated to fulfil a similar membrane stabilisation function improving photosynthetic efficiency under these conditions (Knaupp et al, 2008). Given the large quantities in which these higher RFO oligomers occur in their natural systems (*A. reptans* and *C. blumei*) they may fulfil a far more critical physiological role stress responses, other than merely behaving as osmo-lytes.

With the exception of GGT being transiently expressed on tobacco protoplasts, all other studies associated with GGT have been conducted in *A. reptans*. There are no reports outlining a constitutive over-expression approach in a stable transgenic system in order to further to investigate the physiological role/s of long-chain RFOs synthesised via GGT. In this study we explored a genetic engineering strategy for the accumulation of long-chain RFOs by constitutively over-expressing *ArGGT* in the model plant Arabidopsis. Our preliminary results are outlined using three independent transgenic lines exposed to long term cold-acclimation (30 d, 4°C) to ascertain if constitutive GGT overexpression results in these lines accumulating RFOs beyond Raf, in the leaves.

4.2 Materials and methods

4.2.1 Plant material and cold acclimation treatment

The *Arabidopsis* Col-0 ecotype was used in this study (Alonso et al. 2003). Subsequent to seed stratification (24 h, 4°C), plants were propagated and maintained on peat disks (Jiffy™ no.7, South Africa) under controlled environment conditions (8 h light, 120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, 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), at 7 and 21 d post germination as previously described (Petersen et al. 2010).

Where applicable, four week-old plants (rosette stage 1.14) were used for cold-acclimation treatments as previously described (Zuther et al. 2004; Egert et al. 2013). Briefly, plants were transferred to controlled environment chambers with the same settings as listed above but, subject to a constant temperature of 4°C over 30 d. Experiments were conducted thrice using pools of 12 plants each.

4.2.2 The constitutive over-expression of *ArGGT*

The full length *Ajuga reptans ArGGT* (Acc. no. AY386246) cDNA was obtained from leaf material. The coding sequence (CDS) was amplified using the Expand High Fidelity PCR System (Roche Applied Science, Roche products, South Africa), according to manufacturer's instructions using primers *ArGGT*_{for} 5' ATGGAGGCATCAGTGTTCTT and *ArGGT*_{rev} 5'TC ACGACATTTGGAGTCACTT. The *ArGGT* CDS was cloned using the pCR®8/GW/TOPO®TA Cloning Kit (Invitrogen, Life technologies, South Africa) according to manufacturer's instructions. Following PCR verification of colonies containing clones in the sense (5' to 3') orientation, the pMDC binary vector range (Curtis and Grossniklaus 2003) was used to create the construct via a conventional LR clonase™ reaction (Life

technologies) to obtain pMDC32::*ArGGT* (constitutive *ArGGT* expression driven by dual 35S promoter).

4.2.3 Plant transformations and marker selection procedures

Electro-competent *A. tumefaciens* (GV3101) was transformed with pMDC32::*ArGGT* plasmid mini-preparations using a Genepulser[®] (1.8 kV; 100 Ω ; 25 μ F, Bio-Rad, Bio Rad Laboratories, South Africa). Arabidopsis wild-type (Col-0) was transformed using the floral inoculation method (Narusaka et al. 2010), with minor modifications. Briefly, *A. tumefaciens* colonies were propagated to mid-log phase ($OD_{600} = \sim 1.2$) in 5 ml Luria Broth (LB), supplemented with antibiotics (⁵⁰Rif., ²⁵Gent., ⁵⁰Kan.). Aliquots (1 ml) were centrifuged (10 000 g, 2 min) and the pellet resuspended in 5% (w/v) sucrose solution supplemented with 0.02% (v/v) Silwet L-77. Floral buds were inoculated with 5 μ l of the suspension and plants were incubated in the dark (16 h, 90% relative humidity), prior to continued growth under our controlled environment conditions.

Seeds (T1) were surface sterilised, using the vapour-phase seed sterilisation method (Clough & Bent 1998), stratified (4°C, 24 h), and placed on half-strength MS (Duchefa, Labretoria, South Africa) media containing the selection antibiotic (^{17.5}Hyg.). Plantlets were propagated in controlled growth conditions (8 h light, 120 μ mol photons $m^{-2} s^{-1}$, 22°C, 16 h dark, 18°C, 60% relative humidity) for two weeks. Marker resistant-plantlets were transferred to Jiffy[™] disks and grown under our controlled environment conditions (described above). Seeds (T2) were harvested, re-selected for (marker resistance) and, propagated in Jiffy[™] disks for further characterisation.

4.2.4 RNA isolation and transcript analysis

Total RNA was isolated using the RNeasy[®] Plant Mini kit (Qiagen, Whitehead Scientific, South Africa), following the manufacturer's instruction. The complementary DNA (cDNA)

template was obtained *via* reverse transcription of 1 µg of total RNA, using an oligo (dT₁₅) primer and M-MLV (H⁻) reverse transcriptase (Promega, Anatech, South Africa) following the manufacturer's instruction.

The sqPCRs were conducted using GoTaq[®] DNA polymerase (Promega, Anatech, South Africa) in a 50 µl reaction (3 µl cDNA, 1.25 U DNA polymerase, 5× green PCR buffer, 0.5 mM of each dNTP, and 0.5 µmol of each primer) for 24 cycles, with a primer annealing temperature optimum of 58°C for all respective sqPCR primer pairs. The constitutively expressed gene, *AtACT2* (*At3g18780*), was used to determine the number of cycles used for the sqPCR, where expression occurred in the linear range. The *AtACT2* and *ArGGT* primer pairs (*AtACT*_{for} 5' ATGGCTGAGGCTGATGATAT, *AtACT*_{rev} 5' TTAGAAACATTTTCTGTGAACGAT; *ArGGT*_{for} 5' ATGGAGGCATCAGTGTTCTT, *ArGGT*_{sqrev} 5' TCC TCCCCGGTTTAGCAGCA) were designed to amplify fragments of 1 kb from the cDNA.

4.2.5 Water soluble carbohydrate (WSC) extractions

WSCs extractions from seeds (50 mg) and freeze-dried leaf (50 mg) material was conducted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), with minor modifications. Extractions were executed in a three-step sequential series [1 ml 80% (v/v) EtOH, 1 ml 50% (v/v) EtOH, 1 ml de-ionised H₂O (dH₂O)]. Each consecutive extraction was conducted twice at 80°C for 10 min, centrifuged (13 000 g, 10 min, RT) and supernatants transferred to a new Eppendorf tube prior to the next step in the series. The supernatants for each individual extraction were pooled, vacuum centrifuged and re-suspended in dH₂O to a final volume of 200 µl. Samples were desalted as previously described (Peters et al. 2007; Peters and Keller 2009; Egert et al. 2013), prior to LC-MS analysis.

4.2.6 LC-MS analysis and WSC quantification

LC-MS analysis was performed with a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UPLC. Samples were separated on a Waters UPLC BEH Amide column (2.1 x 100 mm; 1.7 μ m) at a flow rate of 0.17 ml/min at 35°C. Solvent A consisted of acetonitrile/water (30:70) containing 0.1% ammonium hydroxide and solvent B was acetonitrile/water (80:20) containing 0.1% ammonium hydroxide. The mobile phase gradient was from 0% to 60% solvent A over 5 min, maintained for 2 min at 60% solvent A before the column was re-equilibrated to the initial conditions. Electrospray ionization was applied in the negative mode and the scan range was from m/z 150 to 1500. The capillary voltage was set a 2.5 kV, the cone voltage was 15 V, the source temperature 120 °C and the desolvation temperature was 275 °C. The desolvation gas and cone gas flows were 650 L/h and 50 L/h, respectively. Quantification was conducted against a series of standard sugars after standard curves were created within the linear response range of the apparatus. All WSCs were monitored using their deprotonated quasi-molecular ions and quantified with the TargetLynx application manager (Waters MassLynx V4.1V software).

4.3 Results

4.3.1 Transcript abundance of *ArGGT* is constitutive over a period of cold-acclimation

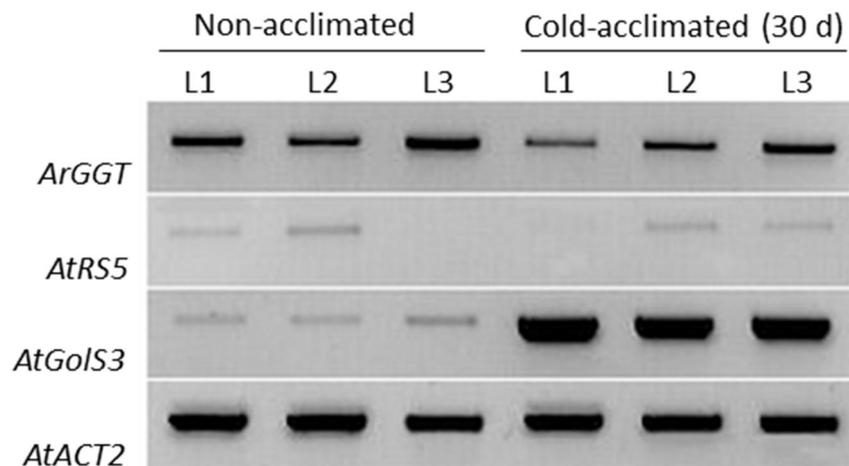


Fig. 1 Transcript analysis of RFO-related genes and *ArGGT* in Arabidopsis (Col-0) transgenic leaves exposed to cold-acclimation (4°C) over a period of 30 d:

A semi-quantitative PCR approach was followed to determine gene expression changes, during the long-term cold-acclimation, for *AtACT2*, *AtGolS3*, *AtRS5* and *ArGGT* in three independent lines constitutively over-expressing *ArGGT*, respectively. d. days, L1. Line 1, L2. Line 2, L3. Line 3.

Total RNA was extracted from the leaves of three independent transgenic lines, constitutively over-expressing *ArGGT* in the Col-0 background. Transcript abundance was determined *via* a semi-quantitative PCR approach, using *AtACT2* (*At3g18780*) as the constitutive reference gene (**Fig. 1**). Subsequent to a long-term cold-acclimation period (30 d) the *AtGolS3* (*At1g09350*) transcript increased as anticipated. This is the only known *GS* isoform in Arabidopsis known to be responsive to cold stress (Taji et al. 2002; Panikulangara et al. 2004). Transcripts for *AtRS5* did not indicate increased levels of expression subsequent to cold-acclimation, despite its implication in cold stress responses (Zuther et al. 2004, Knaupp et al. 2011; Egert et al. 2013). However, constitutive over-expression of *ArGGT* seemed to have deregulated *RS5* transcripts where two transgenic lines (L1 and L2) showed impairment of *RS5* expression (down-regulated after

cold-acclimation constitutive expression for L1 and L2, respectively). Transcript levels of *ArGGT* were constitutively expressed in all transgenic lines regardless of cold-acclimation. Given the well described responses of wild-type (Col-0) *Arabidopsis* leaves (Egert et al. 2013; Chapter 2, this study; Chapter 3, this study), in non-acclimated and cold-acclimated conditions, we did not report results for the wild-type leaves (with regards to transcripts and WSC analysis) in this chapter and merely focused on preliminary evidence of the ability of *ArGGT* to induce long-chain RFO synthesis.

4.3.2 Constitutive over-expression of *ArGGT* results in the accumulation of *Sta* in transgenic *Arabidopsis* leaves

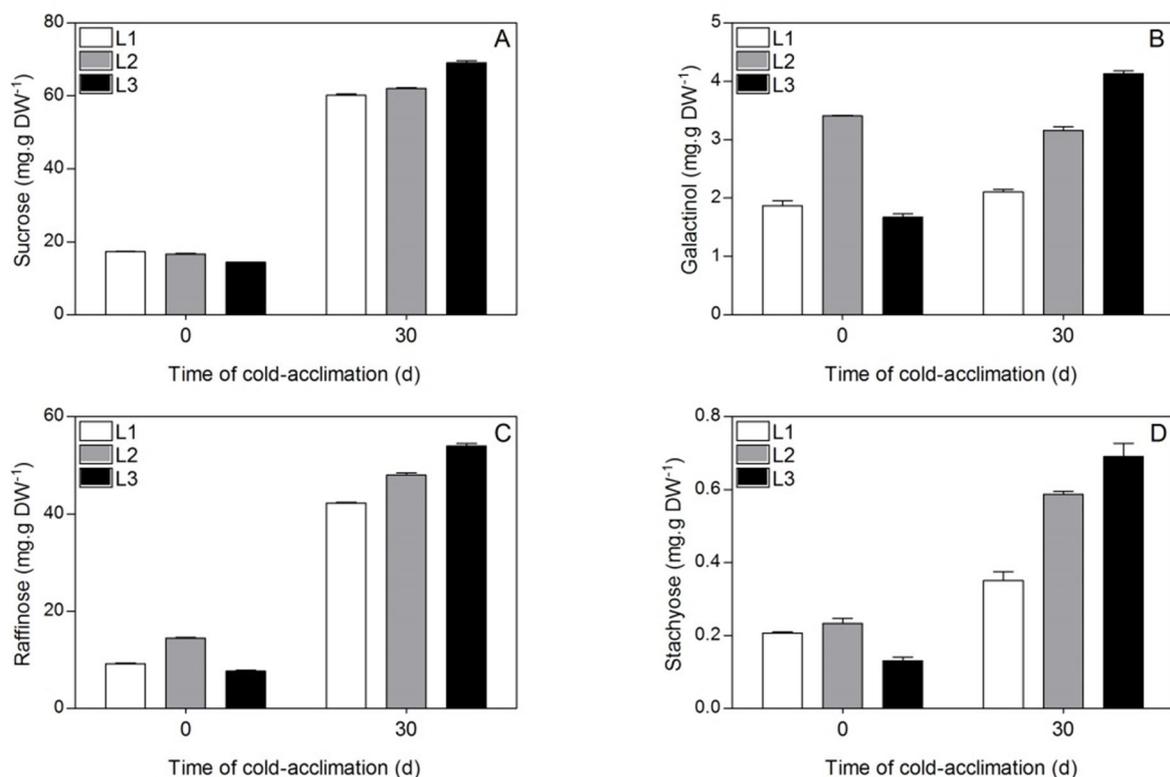


Fig. 2 Water soluble carbohydrate (WSC) profiles in the leaves of three independent transgenic lines constitutively over-expressing *ArGGT* in the Col-0 background:

The WSCs sucrose (*Suc*), galactinol (*Gol*), raffinose (*Raf*) and stachyose (*Sta*) are represented in A, B, C and D, respectively. Leaves were harvested from normally grown plants (non-acclimated) or following cold-acclimation (4 °C, 30 d). d, days, L1. Line 1, L2. Line 2, L3. Line 3.

Using a constitutive over-expression strategy, *ArGGT* expression was de-regulated into the leaves of transgenic *Arabidopsis* (35S:*ArGGT*/Col-0). Total WSCs were analysed from the leaves of the three independent 35S:*ArGGT*/Col-0 lines using quantitative LC-MS under conditions of long-term cold-acclimation (4°C, 30 d). The three 35S:*ArGGT*/Col-0 lines unusually accumulated Suc (**Fig. 2 A**, 17.34 ± 0.3 , 16.71 ± 0.4 and 14.44 ± 0.02 mg g⁻¹ DW for L1, L2 and L3, respectively), Gol (**Fig. 2 B**, 1.86 ± 0.2 , 3.41 ± 0.01 and 1.67 ± 0.1 mg g⁻¹ DW for L1, L2 and L3, respectively), Raf (**Fig. 2 C**, 9.20 ± 0.3 , 14.47 ± 0.2 and 7.72 ± 0.3 mg g⁻¹ DW for L1, L2 and L3, respectively). Interestingly, Sta accumulation (**Fig. 2 D**, 0.20 ± 0.01 , 0.23 ± 0.3 and 0.13 ± 0.02 mg g⁻¹ DW for L1, L2 and L3, respectively) occurred in non-acclimated transgenic leaves.

Cold-acclimated leaves of the three 35S:*ArGGT*/Col-0 lines accumulated approximately 3-fold higher Suc (**Fig. 2 A**, 60.15 ± 0.4 , 62.02 ± 0.8 and 69.12 ± 0.9 mg g⁻¹ DW for L1, L2 and L3, respectively) and 4-fold higher Raf (**Fig. 2 C**, 42.27 ± 0.3 , 47.95 ± 0.8 and 53.9 ± 0.6 mg g⁻¹ DW for L1, L2 and L3, respectively) when compared to non-acclimated leaves. Gol accumulation was marginally unchanged in the transgenic lines with the exception of one (L3) which showed an approximate 2-fold increase (**Fig. 2 B**, 2.1 ± 0.1 , 3.1 ± 0.08 and 4.1 ± 0.1 mg g⁻¹ DW for L1, L2 and L3, respectively) in cold-acclimated leaves. The occurrence of Sta in cold-acclimated leaves from the three transgenic lines increased up to 6-fold (**Fig. 2 D**, 0.35 ± 0.3 , 0.58 ± 0.02 and 0.69 ± 0.09 mg g⁻¹ DW for L1, L2 and L3, respectively) when compared to the non-acclimated leaves.

4.3.3 Mature seeds from transgenic lines constitutively over-expressing *ArGGT* neither improves *RFO* accumulation nor induce long-chain *RFO* accumulation

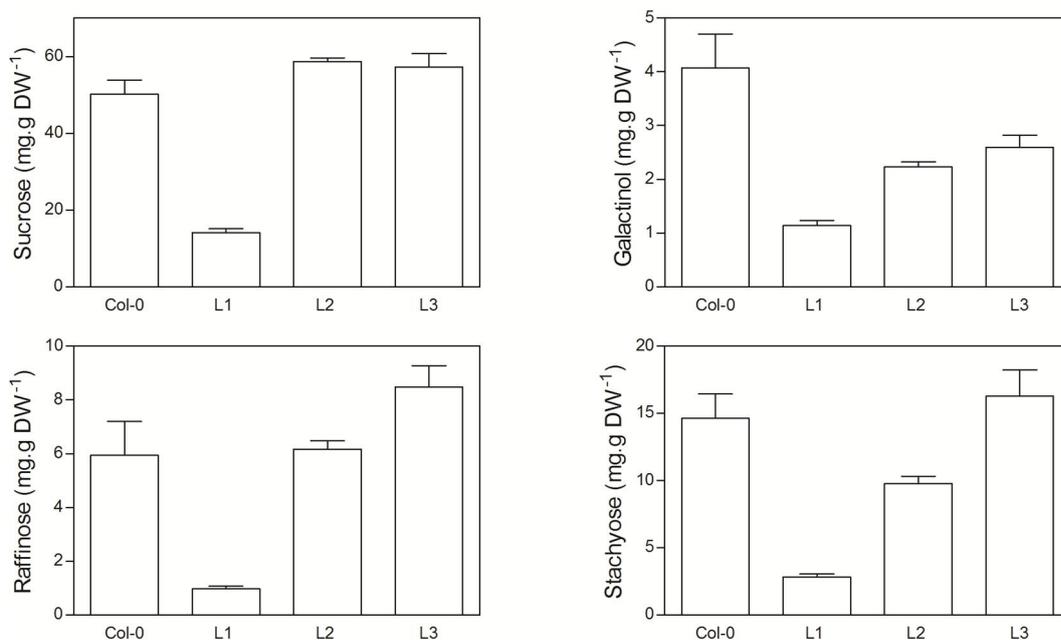


Fig. 3 Water soluble carbohydrate (WSC) profile in the seeds of Col-0 and transgenic lines over-expressing *ArGGT*:

Accumulation of the WSCs sucrose (*Suc*), galactinol (*Gol*), raffinose (*Raf*) and stachyose (*Sta*) in mature seeds of wild-type (*Col-0*) and transgenic (constitutively over-expressed *ArGGT*) lines are represented in A, B, C and D, respectively. L1. Line 1, L2. Line 2, L3. Line 3.

Total WSCs were analysed from mature seeds of wild-type (*Col-0*) *Arabidopsis* and the three independent *35S:ArGGT/Col-0* lines using quantitative LC-MS. The only RFOs accumulating in both wild-type (*Col-0*) and transgenic (*35S:ArGGT/Col-0*) seeds were *Raf* and *Sta*. In mature wild-type seeds WSC accumulated to normal concentrations of 50.13 ± 3.8 , 4.07 ± 1.9 , 5.94 ± 2.4 and 14.63 ± 3.6 mg g⁻¹ DW for *Suc*, *Gol*, *Raf* and *Sta*, respectively (**Fig. 3 A, B, C and D**, respectively).

The accumulation of WSCs in two transgenic lines (L2 and L3) was comparable to that of the wild-type. *Suc* accumulated to concentrations of 58.70 ± 2.1 and 57.22 ± 3.2 mg g⁻¹ DW, for L2 and L3, respectively (**Fig. 3 A**). Accumulation of *Gol* occurred in mature seeds from both

lines to concentrations of 2.23 ± 0.3 and 2.59 ± 0.3 mg g⁻¹ DW, respectively (**Fig. 3 B**). Raf accumulation was similar in the mature seeds of these transgenic lines (**Fig. 3 C**, 6.17 ± 0.6 and 8.48 ± 0.8 mg g⁻¹ DW for L2 and L3, respectively). Sta accumulated in varying concentrations (**Fig. 3 D**, 9.79 ± 0.3 and 16.28 ± 2.1 mg g⁻¹ DW for L2 and L3, respectively).

Mature seeds from one of the *ArGGT* transgenic lines (L1) showed decreased levels of all seed carbohydrates (**Fig. 3**, 14.17 ± 1.4 , 1.13 ± 0.2 , 0.96 ± 0.3 and 2.82 ± 2.1 mg g⁻¹ DW for Suc, Gol, Raf and Sta, respectively) and could simply be ascribed to a pleiotropic insertion.

4.4 Discussion

In *Ajuga reptans* RFOs are major carbon translocates and storage reserves, with Sta being the predominant phloem mobile WSC (Bachmann et al. 1994, Peters and Keller, 2009). It has been demonstrated that the accumulation of higher RFO oligomers potentially contributes to improved frost tolerance in an *A. reptans* excised leaf system and their mass increases in the context of high salinity (*C. blumei*), suggests that they might have far greater roles as compatible solutes during abiotic stress conditions (Gilbert et al. 1997; Haab and Keller, 2002; Peters and Keller 2009). In this study we investigated the ability of *A. reptans* galactan:galactan galactosyltransferase (ArGGT) to initiate the formation of higher RFO oligomers in Arabidopsis *via* a constitutive over-expression strategy.

The stress induced-RFO response of Arabidopsis is well characterised, with Raf being the only RFO oligomer to accumulate in the leaves when Arabidopsis is exposed to multiple abiotic stresses (Taji et al. 2002, Egert et al. 2013, ElSayed et al. 2013). However, it is cold acclimation (4°C) that results in the highest mass increases of Raf in the leaves (Zuther et al. 2004; Iftime et al. 2011; Egert et al. 2013). In this context it has previously been reported for *A. reptans* that (i) *ArGGT* transcript abundance (and enzyme activity) increase (Tapernoux-Lüthi et al. 2004; Egert et al. 2013) and, (ii) long chain RFOs accumulate in leaves and roots (Bachmann and Keller 1995; Tapernoux-Lüthi et al. 2004; Peters and Keller 2009). Given that long-chain RFOs have been suggested to impart frost tolerance in *A. reptans* (Peters and Keller, 2009) our objective was to evaluate whether ArGGT could initiate the synthesis of higher oligomers in Arabidopsis under conditions of cold exposure and subsequently improve freezing tolerance.

Our preliminary data representing three independent transgenic lines constitutively over-expressing *ArGGT* demonstrate the accumulation of small amounts of Sta in the leaves

(**Fig. 2 D**) of plants exposed to long term cold-acclimation (4°C, 30d). Since Sta is not known to accumulate in Arabidopsis leaves, we attribute this to the Gol-independent transferase activity of ArGGT. We considered that our over-expression strategy did not account for the well reported vacuolar localisation of both GGT and long-chain RFOs. However, similar strategies exploiting fructan FTs have been described to result in fructan-oligosaccharide accumulation in transgenic plants (reviewed in Cairns, 2003). FTs like sucrose:sucrose 1-fructosyltransferase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT) and fructan:fructan 1-fructosyltransferase (1-FFT) function very similarly to ArGGT in the chain elongation of fructan oligosaccharides that occur exclusively in the vacuole (Vijn and Smeekens 1999; Kawakami et al. 2008; Valluru and Van den Ende 2008; Peshev et al. 2013).

Our transgenic lines did not accumulate any RFO oligomer beyond Sta. We further analysed WSCs in the seeds of the transgenic plants but determined that only Raf and Sta accumulated (**Fig. 3 C and D**). A number of *in vitro* studies point to ArGGT showing a substrate preference for Sta (to synthesise long-chain RFOs, Sta $K_m = 58$ mM, Haab and Keller 2002). It is, however, also able to use Raf (Raf $K_m = 42$ mM, Haab and Keller, 2002). We presumed that Sta concentrations in Arabidopsis seeds (approximately 4 mM, Nishizawa et al. 2008) would have been sufficient for ArGGT to initiate the syntheses of oligomers higher than Sta. In the absence of sub-cellular compartmentation studies in our work, we can only assume that the amount of Sta accumulating in the leaves and seeds of our transgenic lines is insufficient for ArGGT to initiate further synthesis. However, the cold-acclimation induced accumulation of Raf appears sufficient for ArGGT to synthesise the small amounts of Sta we observed in the leaves of independent transgenic lines. Sub-cellular compartmentation studies have reported cold-acclimation induced Raf percentage distribution of 50% in the cytosol, 30% in the chloroplast and 20% in the vacuole of wild-type Arabidopsis (Iftime et al. 2011). It is thus

likely that some ArGGT activity is occurring in the cytosol but under physiologically neutral pH (7.0). Although the *in vitro* biochemical studies on ArGGT report an acidic pH (4.5 – 6.0) optimum, some activity was also demonstrated at a neutral pH (Haab and Keller 2002).

Another possibility for the failure of our transgenic lines accumulating long-chain RFOs is the possible inhibitory effects of the high mass increases of both Gol and Suc during cold acclimation of Arabidopsis. They are both significant inhibitors of ArGGT activity *in vitro* (Haab and Keller, 2002; Tapernoux-Lüthi et al. 2004) reducing GGT activity (up to 80%) when occurring at concentration of between 10 -15 mM. Sucrose is well known to occur in both the cytosol and vacuoles (transport mediated by specific vacuolar sucrose transporters, reviewed in Neuhauser et al. 2007).

To overcome this, we propose two strategies. Firstly, using our *RS4* transgenic lines (Chapter 2, this study) we could create transgenic plants that are super-transformed with ArGGT (35S:*ArGGT*//35S:*AtRS4*/Col-0). Given that our *RS4* transgenic lines accumulate Sta under normal growing condition, we may create a system where two Sta synthesising enzymes (*RS4* and ArGGT) may produce enough Sta to at least initiate Ver synthesis. Secondly, we suggest a multipronged strategy to employ an approach of classical gene-stacking which would include over-expression of *AtRS4* (synthesises both Raf and Sta), *AtRS5* (synthesises Raf) and *ArGGT* (utilise Raf and predominantly Sta) in the Col-0 background to create a single transgenic line able to synthesise the required substrates for ArGGT activity under normal growing conditions (without the accumulation of inhibitory substrates, Gol and Suc). It is not inconceivable that plants would naturally transport excessive RFOs to the vacuole for storage. A single study demonstrated sub-cellular compartment percentage distribution of RFOs in transgenic lines (over-expressing the adzuki bean SS, Iftime et al. 2011). In that study they demonstrated that at least 20% of each Raf and Sta accumulates in the vacuole of transgenic plants (Iftime et al. 2011). However,

Arabidopsis has not been unequivocally reported to utilise the vacuole as a RFO storage compartment and it would be interesting to evaluate the ability of ArGGT to synthesise extra-vacuolar long-chain RFOs under such stringent conditions of gene-stacking and substrate production.

In summary, we have partially demonstrated that *ArGGT* over-expression in an Arabidopsis system is sufficient to accumulate only small amounts of Sta in the leaves. We consider that this may be due to accumulation of Gol and Suc under our experimental system, that inhibits ArGGT activity and have proposed alternate strategies to improve the methodological approach to engineering long-chain RFO accumulation in Arabidopsis.

CHAPTER 5

General conclusion

5.1 General summary

The raffinose family oligosaccharides (RFOs) are well known sucrosyl-galactosyl oligosaccharides (Suc-Gal_n, 13 < n ≤ 1). Their biosynthesis occurs sequentially and involves (i) galactinol synthase (GOLS) which synthesise the galactosyl donor galactinol (Gol), (ii) raffinose synthase (RS) which produces raffinose (Raf) and (iii) stachyose synthase (SS) which produces stachyose (Sta). Higher RFO oligomers (up to Suc-(Gal)₁₃) are known to occur but their biosynthesis occurs via the unique Gol-independent enzyme galactan:galactan galactosyl tranferease (GGT, Bachman et al. 1994; Gilbert et al. 1997; Haab and Keller 2002; Tapernoux-Lüthi et al. 2004; Peters and Keller 2009). Apart from their demonstrated roles in in phloem translocation and carbon storage, RFOs have also been implicated in abiotic stress protection (reviewed in ElSayed et al. 2014). More recently, a novel function in biotic stress protection (fungal pathogen infection) has been ascribed to the galactose donor, Gol (Kim et al. 2008; Cho et al. 2011; Bolouri-Moghaddam and Van den Ende 2013).

Despite the Arabidopsis genome being fully sequenced (and annotated), its RFO physiology is not completely known, particularly in the context of the known biosynthetic genes, their tissue specific localisation/s and their physiological roles. Ten *Gols* genes have been proposed but only three isoforms (*AtGols-1*, -2 and -3, *At2g47180*, *At1g56600* and *At1g09350*, respectively) have been characterised (Taji et al. 2002; Nishizawa et al. 2006). Further only one RS has been functionally characterised (RS5, *At5g40390*, Egert et al. 2013) and there are no reports concerning an SS. This work has described experimental approaches to (i) identify new RFO biosynthetic genes in Arabidopsis (Chapter 2), (ii) further explore the

proposed functional role of Gol in biotic stress tolerance using the Arabidopsis/*Botrytis cinerea* pathosystem (Chapter 3) and, (iii) constitutively over-express the *ArGGT* RFO chain-elongation enzyme in Arabidopsis in an attempt to engineer long-chain RFOs into Arabidopsis (Chapter 4).

The key findings of this work are highlighted below:

5.1.1 Functional characterisation of RS4, a bi-functional α 1,6-galactosyltransferase in Arabidopsis seeds

We report on the successful functional identification of Arabidopsis *RS4* (*At4g01970*) and demonstrated that it was a SS responsible for the accumulation of Sta during Arabidopsis seed development. During the course of our investigations it became apparent that *RS4* could also synthesise Raf. Collectively our findings uncovered a novel RFO biosynthetic enzyme active in Arabidopsis seeds that is responsible for the biosynthesis (and deposition) of the RFOs Raf and Sta into Arabidopsis seeds. To our knowledge, this represents the first report of such a “bi-functional” RFO biosynthetic enzyme from Arabidopsis.

5.1.2 Neither Gol nor Raf induces resistance to *Botrytis cinerea* infection

We further investigated the role of Gol and RFOs (Raf) in Arabidopsis during induced systemic resistance (ISR, pathogen infection) using *GolS1* and *RS5* T-DNA mutant backgrounds. We reproduced the *B. cinerea* susceptibility of *AtGolS1* (*atgols1-1* and *1-2*) mutants, as previously reported. However, during our analyses of water soluble carbohydrate (WSC) profiles, we discovered that these lines hyper-accumulate both Gol and Raf (not previously reported or addressed). This finding discounted both Gol and Raf as possible

signalling molecules during ISR. Nonetheless, we tested the Raf-deficient RS5 (*atrs5-1* and *5-2*, Raf deficient, Gol hyper-accumulation) mutants and could demonstrate that they were equally susceptible as the *AtGolSI* mutants, regardless of the hyper-accumulation of Gol. Speculatively, the presence of the *AtGolSI* transcript or protein (and not Gol) might be a key regulator in ISR.

5.1.3 The RFO chain-elongation enzyme (ArGGT) did not induce the accumulation of long-chain RFO oligomers in Arabidopsis

Higher RFO oligomers (>DP 4, Sta) have not been reported to accumulate in Arabidopsis. In this study (Chapter 4) we describe an attempt to engineer the synthesis of long-chain RFO oligomers (>DP 4) in Arabidopsis by constitutive over-expression of the *Ajuga reptans* galactan:galactan galactosyltransferase (*ArGGT*). To this end we could demonstrate the marginal accumulation of Sta in transgenic Arabidopsis leaves and, despite the high abundance of RFOs in seeds, we were unsuccessful in obtaining higher RFO oligomer synthesis in the generative tissues of these transgenic lines.

Appendix A: List of abbreviations

At Arabidopsis thaliana

α-Gal galactosidase enzyme

cDNA complementary DNA

Col-0 *Arabidopsis thaliana* ecotype Columbia-0

d days

dNTP deoxynucleotide triphosphate

DP degree of polymerization

DTT dithiothreitol

DW dry weight

EDTA ethylenediaminetetraacetic acid

Fru fructose

FT frucosyltransferase enzyme

Gal galactose

gDNA genomic DNA

GGT galactan:galactan galactosyltransferase

Glc glucose

Gol galactinol

GolS galactinol synthase

h hours

Ino *myo*-inositol

ISR induced systemic resistance

JA jasmonic acid

kb kilobase

LC-MS Liquid Chromatography Mass Spectrometry

MS Murashige and Skoog

n.d. not detected

NASC Nottingham Arabidopsis Stock Centre

nkat nano katal

PCR polymerase-chain-reaction

PMSF phenylmethylsulfonylfluoride

PR pathogenesis related

PSII photosystem II

PVP polyvinylpyrrolidone

PVPP polyvinylpolypyrrolidone

Raf raffinose

RS raffinose synthase

RFOs raffinose family oligosaccharides

RNA ribonucleic acid

rRNA ribosomal RNA

ROS reactive oxygen species

RT-PCR reverse transcriptase-polymerase-chain-reaction

SAR systemic induced resistance
SA salicylic acid
SDS sodiumdodecylsulphate
SE standard error
SFT sucrose:fructose transferase enzyme
Sf9 insect cell line from *Spodoptera frugiperda*
SIP seed imbibition proteins
Sta stachyose
SS stachyose synthase
SST sucrose:sucrose transferase enzyme
Suc sucrose
SUC sucrose carriers
SUT sucrose transporters
SWEET sugars will eventually be exported transporters
T-DNA transferred DNA
v volume
Ver verbascose
VS verbascose synthase
w weight
WSC water soluble carbohydrates

Appendix B: List of figures

CHAPTER 1

Figures

Fig. 2. Sucrose provides a structural backbone for numerous RFO- and fructan-based oligosaccharides (image adapted from Kandler and Hopf (1982) and provided courtesy of Prof. Dr. F Keller, University of Zurich)

Fig. 2. Membrane stabilization mechanism *via* compatible solute (proline and sugars) accumulation in tolerant and sensitive systems, under non-stressed and abiotic stress conditions (image adapted from Hoekstra 2001)

Fig. 3. Schematic representation of RFO oligomer synthesis in a (A) galactinol-dependent and (B) galactinol-independent manner. Image provided courtesy of Prof. Dr. F Keller, University of Zurich

Fig. 4. Illustration taken from Turgeon 2010. Phloem loading in (i) minor veins (A-C) and (ii) whole leaf systems (D-F) by means of diffusion, polymer trapping or apoplastic loading

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Fig. 3 Water soluble carbohydrate (WSC) profile in the seeds of Col-0 and transgenic lines over-expressing *ArGGT*

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