Identification of novel transcriptional regulators controlling MtMYB1 and MtSWEET1b expression in the arbuscular mycorrhizal symbiosis.

Tadesse, Firehiwot

Author

Firehiwot Mekonnen Tadesse

930724821130

MSc. Minor thesis at the department plant science, molecular biology

Wageningen University & Research

Supervisor: Erik Limpens

Submission date final report
30/10/2020

Acknowledgment

I would like to thank dr.ir. Erik Limpens for allowing me to do my minor thesis at the department plant science, subdivision of laboratory of molecular biology. My deep and heartfelt gratitude for his daily supervision showed throughout the project and his patient showed in our communication and delayed report submission.

Table of Contents

Abstract

Arbuscular mycorrhizal symbiosis (AM) is arguably the most common symbiosis where most land plants establish intimate interaction with fungi of the subphylum Glomeromycotan. In AM symbiosis the two symbionts create a bidirectional nutrient exchange at the arbuscules. Arbuscule is a branched structure which is formed by the fungi in the root cortex cells of the host plant. To support and monitor the formation, development, and degeneration of the arbuscules, the host plant performs extensive transcriptional reprogramming. This is mediated via the activation of CSSP where a cascade of transcription factors regulates the expression of AM-specific genes. The transcriptional regulation of expression of some of these genes requires the activity of their Cis-regulatory element in their promoter region and has also been discussed in nutrient exchange. On the other hand, for some recently identified AM-associated genes their transcriptional regulators are yet to be discovered. Among these genes, the SWEET1b gene that encodes for glucose transporter, and MYB1 that regulates arbuscular degeneration associated genes, their transcriptional regulators are to be identified. While the presence of the cis-regulatory element called AW-box in their promoter region raises speculation that they may be regulated via RAM1-wri5a cascade.

Therefore this study attempts to identify the novel transcriptional factors that control the expression of MYB1 and SWEET1b genes and test if WRI5a binds to MYB1 and SWEET1b promoter. For this purpose i) MYB1/SWEET1B promoters fused with HIS reporter construct was generated by Gateway recombination reaction, ii) the linearised form MYB1-HIS reporter construct is integrated into the y187 yeast strain genome, iii) the expression level of MYB1-HIS reporter construct was tested over the rage of concentration of 3AT i.e (0,20,40,60,80Mm) and iv) 40mM3AT is selected as MYB1- HIS reporter expression limiting concentration and can be used in the Y1H assay. For the SWEET1B-His reporter construct, integration to a yeast genome and testing of its expression level can be done as an extension of this study.

1 Introduction

Arbuscular mycorrhizal (AM) is arguably the most common plant symbiosis where about 80 % of all land plants establish an intimate interaction with fungi of the subphylum Glomeromycotan and create a bidirectional nutrient exchange (Luginbuehl & Oldroyd, 2017; Schmitz & Harrison, 2014) The plants provide fixed carbon, sugars, and fatty acids to support fungal growth and survival, and in return, the plants receive nutrients from the fungus such as phosphorus from the soil and get protection against biotic and abiotic stress(Luginbuehl & Oldroyd, 2017). In the AM symbiosis, the growth of the fungi at the root surface of the plant marks the early stage of the symbiosis that subsequently leads to root cortex cell colonization by the fungi. The fungi colonize the roots of the plant either by growing in between cortex cells or via intracellular passages(Luginbuehl & Oldroyd, 2017). Ultimately, the fungi form highly branched hyphal structures, called arbuscules, inside the inner root cortex cells. At these arbuscules, the bidirectional nutrient exchange takes place (see

Figure 1).

The formation, development, and degeneration of the arbuscules can be recapitulated as follows; after fungal attachments to the plant's root surface, cellular reorganization in the plant's root cell facilitates fungal hyphal entrance and its differentiation (Luginbuehl & Oldroyd, 2017). This is accompanied by the formation of a specialized host membrane, called peri-arbuscular membrane, to envelop and separate the fungus from the host cytoplasm. The pre-arbuscular membrane defines the branched and trunk bases of the arbuscules where specific host transporters become localized to facilitate the nutrient exchange (Bonfante & Genre, 2010; Harrison, 2005; Vigneron et al., 2018; Wang et al., 2012).

The arbuscules are relatively short-lived (~ 2-7 days) after which they degenerate(Kobae & Hata, 2010). The host controls arbuscules life span depending on its nutritional status as well as arbuscules performance. When the arbuscules underperform in the symbiosis, the host imposes premature decay on the arbuscules while protecting itself and cleansing the degraded arbuscules out of the cortex cell. Consequently, preparing the root cortex cell to accommodate a new arbuscule (Breuillin-Sessoms et al., 2015; Kobae & Hata, 2010; Luginbuehl & Oldroyd, 2017).

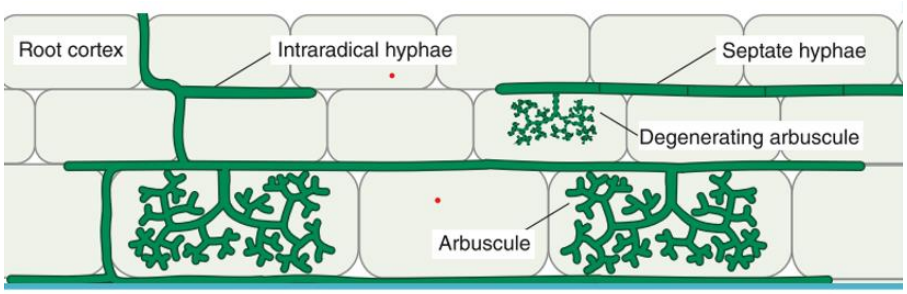


Figure 1 presents the appearance of arbuscules in the AM symbiosis(figure from Luginbuehl & Oldroyd, 2017).

Transcriptional regulation of arbuscule formation and development in AM symbiosis.

The formation and development of arbuscules involve the activation of the so-called common symbiotic signaling pathway (CSSP) (Wang et al., 2012). Briefly, at the early stage of AM symbiosis, the host plant roots exude strigolactones (SLs) which function as a plant hormone into the rhizosphere(Liu et al., 2011). This stimulates AM fungi and in response, the AM fungi secrete short-chain chitin oligosaccharides (Myc-COs) and lipochitooligosaccharides (Myc-LCOs) collectively called Myc-factors. Myc-factors triggers oscillations in calcium concentration in and around the nucleus (so-called calcium spiking) of the host plant(Camps et al., 2015; Maillet et al., 2011; Wang et al., 2012). This initiates the host plant's symbiotic response via the activation of CSSP (Maillet et al., 2011; Wang et al., 2012).

In the CSSP, the induced oscillations in calcium concentration are decoded by the nuclear-associated calcium- and calmodulin-dependent serine/threonine-protein kinase (CCaMK) (a protein which is regulated by Ca2+ and Ca2+/ CaM,). Subsequently, CCaMK interacts and phosphorylates the coiled-coil transcription factor CYCLOPS (Pimprikar et al., 2016). This prompts CYCLOPS activation and its binding to the AM *cis-regulatory* element in the promoter of *RAM1*(Required for Arbuscular Mycorrhization) to trigger its expression (Pimprikar et al., 2016). RAM1 is a GRAS-type transcription factor that interacts with other TFs to regulate genes required for AM development and peri-arbuscular membrane formation. The role of RAM1 in arbuscule development is shown in ram1 mutant where the plant unable to form fully developed arbuscules as development is being arrested during fungal hyphal branching(Luginbuehl & Oldroyd, 2017)

RAM1 is shown to act downstream of CYCLOPS because overexpression of *RAM1* in *cyclops* mutants can rescue arbuscule defects(Gobbato et al., 2012; Pimprikar et al., 2016; Rich et al., 2017). Another GRAS-protein essential for arbuscule development is DELLA. DELLA proteins repress gibberellin signaling which acts as the negative regulator of the symbiotic signaling pathway (Floss et al., 2013). DELLA proteins also interact with CCaMK and CYCLOPS to enhance CYCLOPS activity in transactivating *RAM1*. Active DELLA protein promotes the transcriptional induction of *RAM1* (Floss et al., 2017; Pimprikar et al., 2016).

Moreover, RAM1 and DELLAs interact with other GRAS-type TFs, such as RAD1(Required For Arbuscule Development1) and DIP1(Della Interacting Protein 1) to control arbuscule branching (Ho-Plágaro et al., 2019). RAD1 has been implicated with arbuscule development, as mutants display significant decreases in the number and formation of arbuscules. DELLA also interacts with the GRAS TF MIG1(Mycorrhiza-Induced Gras 1) which is proposed to play a role in regulating AM development and root development to accommodate fungal infection (Rich et al., 2017). Altogether, this shows interaction among these transcription factors is what partly constitutes the molecular mechanism underlying arbuscule development in AM symbiosis.

Transcriptional regulation of nutrient exchange in AM symbiosis

In previous studies, regarding nutrient exchange *in AM symbiosis,* carbon transfer from the fungus to the host plant was believed to be in the form of sugar(hexose)(Keymer et al., 2017). However, plant mutant for sugar degradation as well as sugar transporter genes studies showed no impact on root colonization or arbuscule development(Pimprikar & Gutjahr, 2018). This led to a suggestion that the AM fungi obtain its sugar source in the form of lipid, thus, the transcriptional regulation of lipid transporter genes are widely discussed(Luginbuehl & Oldroyd, 2017, 2017).

Meanwhile, a recent study showed that glucose transporters are being strongly upregulated at the arbuscules in response to AM fungi colonization. For instance, a study in M. truncatula showed that of the 26 SWEET genes, the MtSWEET1b gene that encodes for a glucose transporter protein is strongly induced in root cortex cells colonized by AM fungus(An et al., 2019). In addition to its upregulation in arbuscule containing roots cells, MtSWEET1b is also localized to the peri arbuscular membrane (An et al., 2019). Furthermore, the expression of Mt SWEET1b was shown to be consistent with carbon allocation from the plant to the fungi. As a result, it is anticipated that

MtSWEET1b may have a role in glucose transport to the fungus and stabilizing AM symbiosis. However, the transcriptional regulators of MtSWEET1b expression are yet to be revealed.

Efficient nutrients exchange at the arbuscules requires the transcriptional activation of specialized nutrient transporters (Bonfante & Genre, 2010). These include for example PT4, a phosphate transporter that mediates the transfer of inorganic phosphate from the fungus to the plant, and STR1/STR2 which have been associated with fatty acid biosynthesis and may play a role in lipid transfer to the fungus(Breuillin-Sessoms et al., 2015; Floss et al., 2017, 2017). PT4 and STR/STR2 are strongly dependent on RAM 1(Rich et al., 2017).

In M. truncatula, WRINKLED like AP2/EREBP transcription factor is shown to activate the expression of genes that are involved in fatty acid biosynthesis and control of lipid flux(Jiang et al., 2018). Among the WRINKLED like AP2/EREBP transcription factors, the overexpression of WRI5a is shown to enhance the expression of fatty acid biosynthesis associated genes whereas its disruption affects their expression and arbuscule formation(Maeo et al., 2009). In L. japonicus and M. truncatula, CBX and Wri5a target plant fatty acid biosynthesis genes which are required for AM fungal colonization via binding with AW box (CGnnn(n) 4CnAnG motifs in the STR and PT4 promoter region(Jiang et al., 2018; Keymer et al., 2017). AW box motifs are conserved among genes involved in fatty acid synthesis and function as cis-regulatory sequences and targeted by WRI5 transcription factors(Maeo et al., 2009).

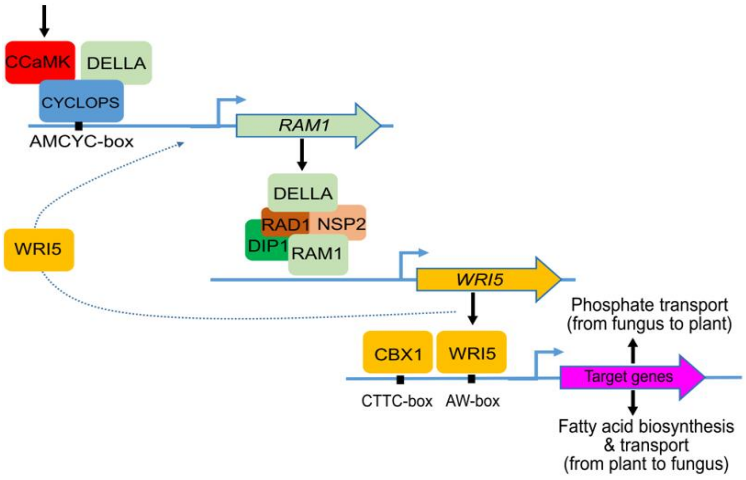


Figure 2 shows the overview of the transcriptional cascade that leads to the activation of genes that are implicated in nutrient exchange (figure from Limpens & Geurts, 2018b).

Transcriptional control of arbuscule degeneration

Furthermore, although most of the symbiotic transcriptional regulators are associated with arbuscule development, a recent study discovered the concerted activity of TFs that regulates arbuscule degeneration. Accordingly, MYB-like transcription factor 1 (MYB1) and its interaction partners have been identified as the main regulator of arbuscule degeneration (Vigneron et al., 2018). MYB1 is required for the induction of the expression of arbuscule degeneration-associated genes (Floss et al., 2017). MYB1 activity requires other GRAS transcription factors called NSP1 (Nodulation Signaling Pathway 1) and DELLA protein. A Yeast two-hybrid analysis showed that MYB1 interact with DELLA and NSP1 GRAS transcription factor, and forms MYB1/DELLA/NSP1 complexes (Gutjahr & Parniske, 2017; Schmitz & Harrison, 2014). On other hand, MYB1 interaction with DELLA enhances the expression of genes involved in arbuscule degeneration(Floss et al., 2013). This interaction provides an entry point to the molecular mechanism underlying arbuscule degeneration and transcriptional regulators of MYB1 is yet to be identified.

Aim and approach

In AM symbiosis, the host plant's TFs research reveals transcription factors that target AM-specific genes associated with the nutrient exchange as well as arbuscule formation. Studies showed Tfs act in large complexes to promote the function of other transcription factors or bind to the Cis-regulatory elements on the promoter regions of genes to regulate their expression(refe). In model plants, M. truncatula and Lotus. Japonicus, the transcriptional network which activates gene expression has been discussed related to arbuscule formation, degeneration, and nutrient exchange. Accordingly, transcription factors with distinct and/or redundant regulatory roles are starting to be identified. However, for other arbuscule specific genes associated with a nutrient exchange such as sugar transport (SWEET1B), and arbuscule degeneration (MYB1), their transcriptional regulator is yet to be identified.

This study aims to identify novel transcriptional regulators of MtMYB1 and MtSWEET1b genes. For this purpose, the Y1H approach allows to transform of TF-libraries or arrays into MtMYB1 and MtSWEET1b yeast strains will be used to screen TF libraries of Arabidopsis and Medicago. Furthermore, following the computational analysis of the promoter region of MtMYB1 and MtSWEET1b, the presence of the AW-boxes on Cis-regulatory elements raises a hypothesis that these genes are maybe regulated by the RAM1-WRI5a transcriptional cascade. Therefore, this study aims to test the binding of WRI5a to MYB1/SWEET1 promoters using yeast one-hybrid (Y1H) assays, in which interactions between TFs and DNA promoters which can be screened for reporter gene activation.

.

2 Material and Methods

All chemicals, instruments, and protocols that were used are provided in the appendices describing steps followed in the Gateway compatible-Y1H assay according to (Deplancke et al., 2004; Jiang et al., 2018; Part, 2012). In Gateway compatible-Y1H assay, it is attempted to generate MtMYB1/ MtSWEET1b promoter/reporter construct for TF screening, and check whether WRI5a transcription factor (TF) (1200bp) binds with MtMYB1 *(*702bp) *and* MtSWEET1b *(*1200bp) gene promoters. Modifications made by the supervisor Erik limpens were implemented and included in the protocol see Appendixes.

2.1 Generating MYB1/ SWEET1b gene promoters, and WRI5a with attB sites.

In this study, MYB1/SWEET1b gene promoters obtained from the roots of M.truncatula, and WRI5a (cDNA) was amplified using primers with attB sites. The primers designed with attB4 forward and attB1 reverse, and attB2 forward and attB1 reverse for amplifying MYB1/ SWEET1b promoters and WRI5a are provided 5.1. Following amplification by PCR, the molecular weight (bp sizes) of MYB1/ SWEET1b promoters and WRI5a PCR products were checked on gel electrophoresis. After the correct bp size was confirmed, gene fragments were purified from PCR products (MYB1) or the gel (SWEET1b and WRI5a) using Gene JET Gel Extraction Kit see 5.5. For the protocol followed during PCR amplification and gel electrophoresis see 5.4.

2.2 Cloning MYB1/SWEET1b gene promoters, and WRI5a PCR products into donor vectors to generate Entry Clones.

After purification, MYB1/SWEET1b promoter, and WRI5a with attB sites were used in Gateway BP reaction with pDONR242 (4-1) for MYB1/SWEET1b and pDONR221(2-1) for WRI5a that contain attP sites, see A 5.2. Gateway BP clonase reaction was performed to clone MYB1/SWEET1b gene promoters flanked by attB4 and attB1 sites into the donor vector that contains a toxic gene (ccdB), flanked by attP4 and attP1 sites. For WRI5a, flanked by attB2 and attB1 sites, cloning was performed in a donor vector where the ccdb gene is flanked by attP2 and attP1. The reagents and reaction setup are detailed in A 5.6. After the reaction was completed, E.coli strain (DH5α) was transformed from each reaction by electroporation and plated overnight in selection media. Given the donor vectors contain a kanamycin- resistance (kan^r) marker gene, the selection of transformed E.coli cells (positive colonies) harboring Entry Clones was done on kanamycin (Kan)-LB agar plates.

For this, electrocompetent E.coli cells (DH5α) was prepared beforehand following a procedure as mentioned in 5.8. Of the positive colonies, a single colony of E.coli was grown overnight in the LB liquid medium supplemented with Kan antibiotic. Then, the cell suspension was used for mini prepping and purifying clones according to the protocol in the plasmid DNA Mini Kit see A 5.10.

Then, restriction digestion of Entry Clones was performed to confirm the recombination of MYB1/SWEET1b and WRI5a into the donor vectors. This involves the incubation of MYB1/SWEET1b -Entry Clones with SacI and WRI5a -Entry Clones with EcoRV restriction enzymes and reaction buffer. Then digests were run on an agarose gel to identify fragments size A 5.11. Based on the restriction enzyme used fragments size of each entry clone digests were determined using the APE and Snap gene program. Therefore, the size of the fragment determined on the gel was compared to the expected fragments to verify the clones.

Then, Entry Clones which correspond to the expected fragment were further sequence-verified for the presence of MYB1/SWEET1b and WRI5a inserts. This includes, sample labeling and preparing the samples with the appropriate primer or M13 Forward and Reverse. Once inserts were verified, entry clones were selected for subsequent Gateway LR clonase reaction to transfer MYB1/SWEET1b and WRI5a to the Y1H reporter construct (Destination vectors) 5.3.

2.3 Transferring MYB1/SWEET1b gene promoters, and WRI5a from Entry Clones into a destination vector to generate the Y1H reporter construct.

An Entry Clone that contains MYB1/ SWEET1b gene promoters and WRI5a Tf insert flanked by attL sites were used to transfer inserts into their destination vectors. The destination vectors PMW#2 with a histidine (His) reporter gene and pDEST22 with tryptophan (Trp) reporter gene were used to generate MYB1/SWEET1b-His Y1H reporter construct, and WRI5a-Trp-Y1H construct respectively.

All three destination vectors were obtained from glycerol stock and were purified as mentioned in the protocol see A5.10. Consequently, purified vectors were used in the Gateway-LR reaction. When performing the LR clonase reaction, the same procedure was followed as BP reaction and the reaction setup is detailed in A 5.7. After the reaction was completed, electrocompetent E.coli strain (DH5α) was transformed from each reaction by electroporation and plated overnight. Then, transformed E.coli cells which contain MYB1/SWEET1b-His and WRI5a-Trp-Y1H construct were selected on Amp-LB agar plates as all the destination vectors carry ampicillin resistance

(Amp^r) marker gene. Of the positive colonies, a single colony of E.coli was grown overnight in the LB liquid medium supplemented with AMP antibiotic. Then, the cell suspension was used for mini prepping and purifying the MYB1/SWEET1b-His and WRI5a-Trp-Y1H constructs according to the protocol see A 5.10.

Then, the generated MYB1/SWEET1b-His and WRI5a-Trp-Y1H constructs were checked by restriction digestion and sequencing. For restriction digestion analysis the same procedure was followed as mentioned in section 2.2. Thus, MYB1/SWEET1b-His-Y1H reporter constructs that correspond to the expected fragment were selected for linearizing restriction digestion to be integrated into the yeast genome.

Integrating MYB1/SWEET1b-His-Y1H reporter constructs into Yeast strains (YM4271 and Y187) genome.

Yeast strains (YM4271 and Y187) that are obtained from -80^{o}c storage were streaked out on the YPAD agar plate and incubated at 30^{o}c for 2-3 days. Once colonies become visible, plates are checked for proper growth and stored in -4^{o}c until further use. The procedure followed for yeast cell growth and transformation is detailed in A5.12. After transformation, the yeast cells were grown on minimal media lacking histidine (Sc-His). This allows selecting those yeast colonies containing MYB1-His integrant in their genome. After confirming the appearance of these colonies, a single colony was picked, and regrow in the YPAD agar plate. Then, a single colony was picked and integrants were checked by performing PCR on yeast genomic DNA see A **Error! Reference source not found.**

2.4 Autoactivation testing of MYB1-His integrant.

After the confirmation of MYB1-His integrant, the expression level of His reporter gene. In doing so, a single colony from the same plate were picked and grown on a selective Sc-His media containing a range of concentrations of 3-amino triazole (3AT) (0,20,40,60 and 80 mM). 3AT is a competitive inhibitor of His enzyme and in the presence of increasing amounts of 3AT, more His needs to be expressed for the yeast cells to grow (Deplancke et al., 2004). Therefore, a given concentration of 3AT where the lowest auto activity of His reporter is achieved was selected of the plates that showed no growth**.**

3 Results and Discussion

This study aimed to identify transcriptional regulators of MtMYB1/MtSWEET1b genes expression and test whether WRI5a binds to the MYB1/SWEET1b promoters. For this purpose, the Gateway-compatible yeast one-hybrid(Y1H) approach is followed as follows; MYB1/SWEET1b promoters and WRI5a with attB sites are PCR-generated and cloned into donor vectors to create an Entry Clones. Consequently, MYB1/SWEET1b promoters and WRI5a are transferred from Entry Clones into the destination vectors. This generated MYB1/SWEET1b promoters-His and WRI5a-Trp-Y1H reporter construct. Then, MYB1-His reporter construct is linearized and integrated into a mutant *his* locus of Y187 yeast strains genome by site-specific homologous recombination. Transformed yeast colonies harboring MYB1-His integrant is selected on media lacking histidine (Sc-His) as a wild-type of His gene provides His expression. This was followed by checking the expression level of the reporter gene using a range of concentrations of 3AT (0, 20,40,60, and 80mM). Given the inhibitory activity of 3A on his reporter genes, the concentration where there is no growth of yeast cell is selected to ensure reduced levels of background expression of His gene in the subsequent Y1H assay. The results obtained during the Gateway recombination reaction and parts of the Y1H assay are detailed below.

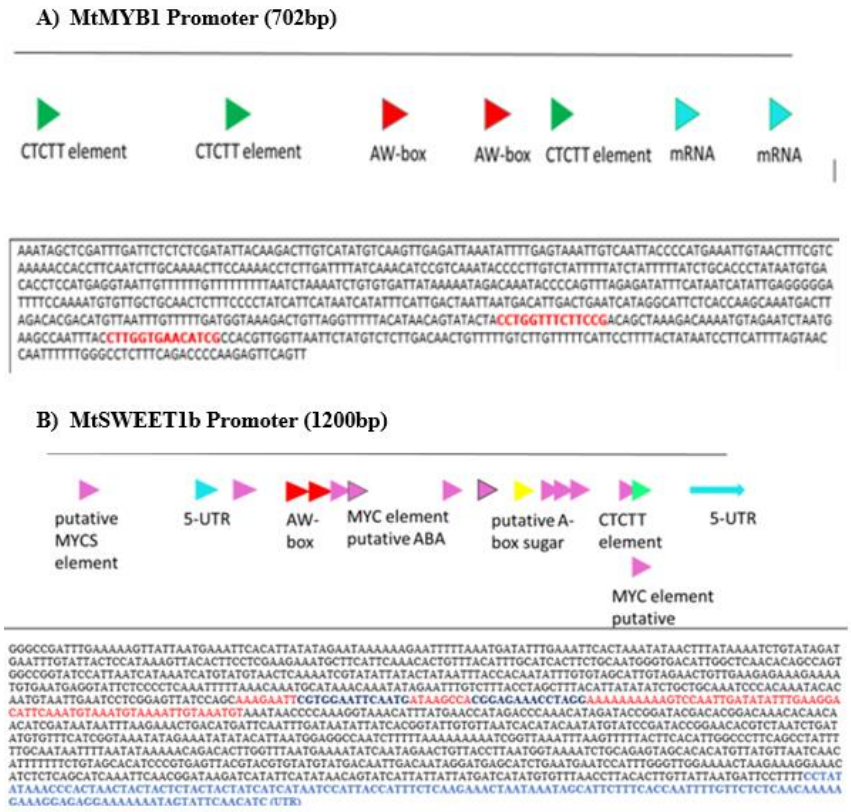


Figure 3 shows A Linear maps for the DNA sequence of MtMYB1 and MtSWEET1B promoters and their Cis-regulatory elements.

3.1 **PCR-Generated MYB/SWEET1b promoters with attB sites**

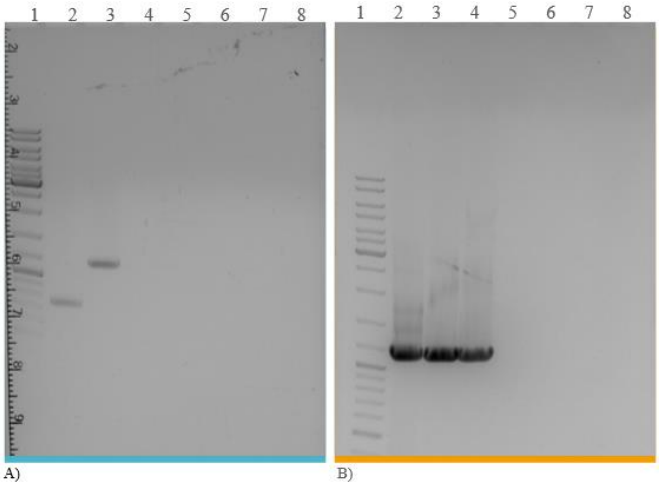


Figure 4 A shows the gel images of PCR-amplified MYB1(lane 2) and SWEET1b (lane 3) promoters using attB primers while figure B depicts PCR amplified SWEET1b promoters of three different plasmids templates i.e p-GFP, p-4-1, and p242 in lane 2, 3, and 4 respectively. The gel image includes a 10kb molecular marker ranging from 500bp to 10kb (lane 1).

In figure 3A, PCR amplified MYB1 promoter using attB primers is determined to be 700bp. Also, in fig 3 B, PCR amplified SWEET1b promoters are determined to be 1.2kb. This is in line with the expected sizes of MYB1/SWEET1B promoters; indicating that PCR products have had incorporated attB sites. Likewise, PCR-generated WRI5a with attB sites is also confirmed by gel electrophoresis (data not shown).

3.2 **Gateway generated MYB/SWEET1b and WRI5a Entry clones**

A **) MYB1-Entry clones**

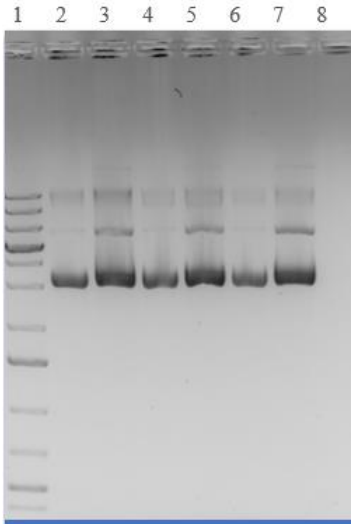


Figure 4 shows the gel images of restriction digestion of MYB1-Entry Clones using the SacI restriction enzyme. The image presents a 10kb molecular marker ranging from 500bp to 10kb in lane 1 while lane 3,5, and 7 depict the digested form of the respective undigested clones in lane 2, 4, and 6. For MYB1-Entry clone digests the expected fragments are 2377bp and 970bp.

According to the result found both digested and undigested clones result in fragments band at 3-3.5Kb is identified; indicating that digestion using SacI FD was unsuccessful. This could be due to the inactivity of SacI. On the other hand, results from sequencing verified that MYB1 insert is present in all the Entry clones that are used for restriction digestion; indicating that any of the clones can be used for the subsequent Gateway LR reaction, thus clone in lane 2 is selected.

B**) SWEET1b-Entry clones**

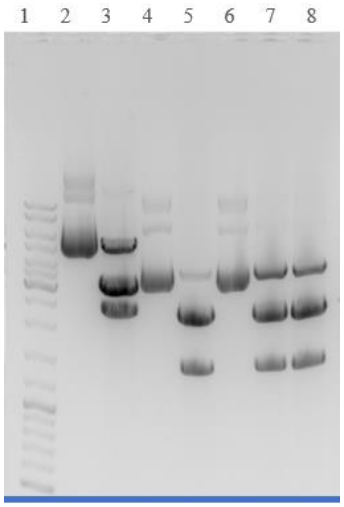


Figure 5 shows the gel image of the restriction digestion of SWEET1b-Entry Clones using the SacI restriction enzyme. The image presents a 10kb molecular marker ranging from 500bp to 10kb in lane 1 while lane 3,5, and 7 show the digested form of the respective undigested clones in lane 2, 4, and 6 respectively. For SWEET1B-Entry clone digests the expected fragments are 2377bp and 1435bp.

As shown in *Figure 5*, restriction digestion of SWEET1b-Entry Clones results in digests with a fragment size of 5kb, 3kb, and 2-2.5kb (lane3), and 3-3.5kb, 2-2.5 kb, and 1.2-1.5kb (lane 5 and 7). This indicates that the fragments identified in lane 5 and 7 seemed to correspond to the size of the expected fragments. However, for the fragments identified in lane 3, only a single band at ~ 2-2.5kb is matched with the expected fragment; implying the occurrence of an incomplete digest. Based on this observation, clones in lane number 5 and 7 are sequence-verified for containing SWEET1b promoter, therefore selected for the subsequent Gateway LR reaction.

C **) WRI5a- Entry clones**

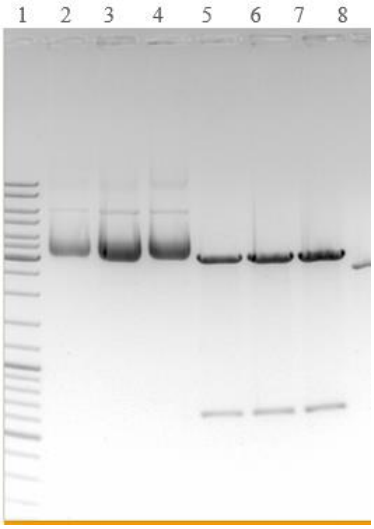
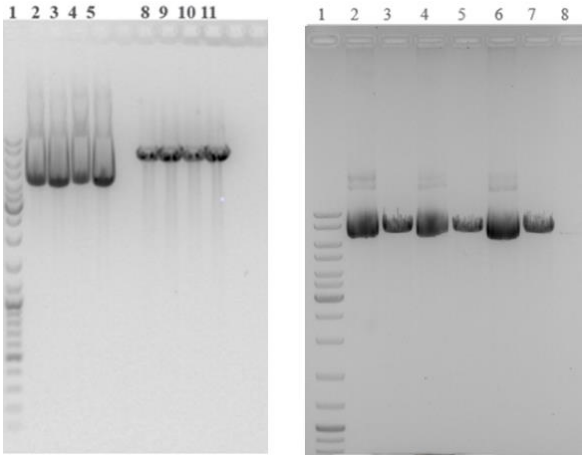


Figure 6 shows the gel images of the restriction digestion of WRI5a-Entry Clones using the EcoRV restriction enzyme. The image presents a 10kb molecular marker in lane 1 while lane 5,6, and 8 present the digested form of the respective undigested clones in lane 2, 3, and 4. For WRI5a -Entry clone digests the expected fragments are 3072bp and 678bp.

As shown in Figure 6 fragments bands at ~3kb and 600-700bp in lane 5,6 and 7 are determined from restriction digests of WRI5a Entry clones. This suggests that the fragments identified in lane 5,6, and 7 are matched with the expected fragments. Therefore, clones in lane number 5,6 and 7 are sequence-verified for containing WRI5a, and clone in lane 6 is selected for the subsequent Gateway LR reaction.

3.3 Gateway-generated MYB/SWEET1b-His reporter construct



A) B)

Figure 7 A, represents the restriction digestion of MYB1-His Y1H reporter construct using the SacI restriction enzyme. The fragment bands appear in lane 8,9,10 and 11 are the digested form of MYB1-His reporter construct of the respective undigested constructs in lane 2,3,4 and 5. Similarly, Figure 7 B shows the restriction digestion of the SWEET1B-His reporter constructs using the SacI restriction enzyme. The fragment bands appear in lane 3,5 and 7 are the digested form of SWEET1B-His reporter construct of the respective undigested clones in lane 2,4 and 6. The image presents a 10kb molecular marker in lane 1. The expected fragment of the MYB1/SWEET1B-His Y1H reporter construct is 7506bp.

As shown in Figure 7 A restriction digestion of MYB1-His Y1H reporter construct results in digest with a fragment size of 7.5-8kb in lane 8,9,10, and 11. This indicates that the fragments identified seemed to correspond to the size of the expected fragment. Based on this result, MYB1-His Y1H reporter constructs used in lane 8 and 9 are sequence-verified for containing MYB1, and construct in lane 8 is selected for the subsequent linearizing restriction digestion. Also, restriction digestion of SWEET1b-His Y1H results in digests i.e 7.5-8Kb in lane 3,5, and 7 which could correspond to the expected fragment. According to this observation, all the constructs used in lane 3,5, and 7 are sequence-verified for containing SWEET1b in the construct. Thus, the SWEET1b-His Y1H reporter construct in lane 3 is selected for the subsequent linearizing restriction digestion

3.4 **MYB1-His reporter integration into the y187 yeast strain and Auto activity testing**

After MYB1-His reporter construct is linearized, its integration into y187 yeast strains results in 5 colonies in Sc-His media; indicating that yeast transformation is low. Thus, for improved transformation efficiency the use of a higher concentration of plasmid DNA from 500ng-1ug and longer incubation up to (2h) of transformation mix at 30 ^{o}C should be considered. While performing the yeast transformation, the PEG solution should be freshly prepared to yield a final concentration of 50%. Also, the quality of single-stranded – carrier DNA needs to be checked on the agarose gel(Editor, n.d.)

Furthermore, the colonies found in media lacking histidine suggests that the integrated MYB1-His reporter gene is auto active. Auto activity implies the expression of the reporter gene, which could occur due to an endogenous yeast activator that binds to MYB1-His reporter. Since such autoactivation is observed in the absence of WRI5A-TF it is necessary to select MYB1-His reporter integrant with the lowest auto activity before the subsequent Y1H assay. so that WRI5a-MYB1 DNA interactions can be retested without background reporter expression For this, 3AT which is a competitive inhibitor of the His enzyme is used in Y1H assays to suppress His auto activity. The result showed at starting from 40 mM to 80mM no growth of yeast strain is observed in Sc-His media.

Furthermore, other findings of this study showed that MYB1-His reporter integration into the YM4271 yeast strain wasn't successful; which could due to i) the correct strain may not be used ii) The size and conformation of MYB1-His reporter construct, iii) yeast growth used for transformation, and iv) the quality of PEG and single-stranded DNA, and a short incubation time of yeast in the transformation mix.

4 Conclusion and recommendation

- Following the Gateway recombination system MYB1/SWEET1bpromoters-His and WRI5a(Tf) -Trp Y1H reporter constructs are generated.
- MYB1-His reporter construct is integrated into the Y187 yeast genome successfully.
- For Y187 yeast strain with MYB1-His integrant shows no background expression of His reporter gene on Sc-His media supplemented with 40mM 3AT.
- Transformation with WRI5a in Sc-HIS-Trp media and TF Screening of Arabidopsis TF library needs to be done for Y187 yeast strain with MYB1-His integrant. For this, a recent improved Y1H assay called enhanced Y1H (eY1H) that uses a high-density array (HDA) robotic platform to mate yeast DNA-bait strains with a collection of yeast strains each expressing a different TF should be considered(Chatterjee, Nimrat Walker, 2017).
- Furthermore, check if the Tfs to be used in the Y1H assays require post-translational modifications, or whether the transcription factors are heteromeric as the Y1H assay may not detect interaction involving heteromeric TFs
- The integration of SWEET1b-His reporter constructs into the yeast genome needs to be done and the working concentration of 3AT should be determined

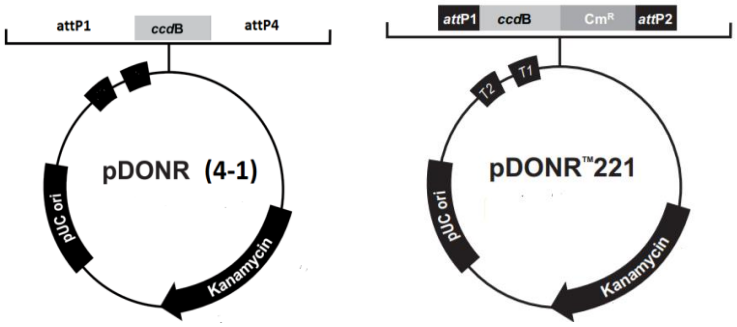
5 Appendixes

5.1 attB primer design

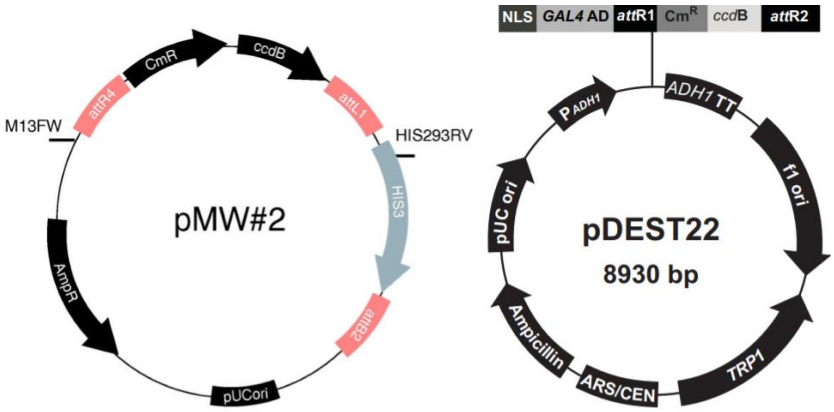
For MYB1, SWEET1B, and WRI5a used in a Gateway BP recombination reaction with donor vectors, attB sites were incorporated into the PCR products. Forward, and reverse primers incorporated attB4 and attB1 sites to *MtMYB1 and MtSWEET1b* gene promoters and *attB*2 and attB1 sites to WRI5a. When designing forward and backward PCR primers, the following point was considered. For each primer, **four** guanine(G) nucleotides followed by 25bp and (18-25 gene-specific bp) were added. This facilitates the binding of clonase enzymes to the terminal attB sites.

MtSWEET1bP-attB4	**GGGGCAACTTTGTATAGAAAAGTTGATGTTCCTCGAAGAAATGCTTCAT**
MtSWEET1bP-attB1	GGGGCTGCTTTTTTGTACAAACTTGTGAGAACAAAATTGGTGAAAGAATGC
MtMYB1p-attB4	GGGGCAACTTTGTATAGAAAAGTTGGATGAAATAGCTCGATTTGATTCTCTCTCG
MtMYB1p-attB1	GGGGCTGCTTTTTTGTACAAACTTGTAACTGAACTCTTGGGGTCTGA
MtWRI5a-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGGAGGTTTCCAATGT
MtWRI5a-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGTTAGAAATGTTGGAAGGG

5.2 DONOR Vectors (pDonor)



5.3 Destination vectors (pDEST)



5.4 Polymerase Chain Reactions (PCR) amplification and Gel electrophoresis

- The optimal conditions for the concentrations of each reagent used to amplify target genes were provided by the supervisor. Depending on the stock solutions and the system being used, the PCR cycling parameters were set by the supervisor and adjusted according to the primers being used.

- A master mix which contains all the required reagents except template DNA was prepared in a 1.5ml Eppendorf tube
- Template DNA was added to PCR tubes containing the master mix, while the master mix without template DNA was prepared as a negative control.
- The molecular weight of the amplified PCR products then was checked by agarose gel electrophoresis following ethidium bromide staining

PCR reaction setup

Reagents	Amount used.
5X HF buffer	10µL
10mM dNTPS	1 µL
10µM F	2.5µL
10µM R	2.5µL
Milli Q water	31.5 µL
Phusion taq Polymerase	0.5 µL
Template DNA	2 µL

	Temperature	Duration
Denaturation	98^{o}c	10 sec
Primers annealing	60/57^{o}c	30 sec
Extension	72^{o}c	1min
-----	72^{o}c	5min

Gel-electrophoresis

- 0.5 gm of agarose powder was weighed and transferred into an Erlenmeyer flask, and 50 ml of buffer tris-acetate-EDTA (TAE) was added to make 0.1% agarose gel.

- The solution was mixed thoroughly and boiled for 1 min in the microwave, and cooled down for 5 to 10 min. Then, 5 µL ethidium bromide was added, and the solution was poured over the gel casting plate.
- After the gel is solidified, the comb was removed, and the gel was placed into the gel box.
- Once the gel box was fully covered by TAE buffer, the molecular weight gene ladder (Gene Mix ruler 10kbp) was loaded into the first well of the gel and samples with gel loading dye into the other wells.
- Finally, 5 µL of ethidium bromide was added to the buffer and gel electrophoresis was run.

5.5 **Gene JET gel extraction kit for the purification of DNA fragments**

- All purification steps were carried out at **room temperature.**
- Centrifugation was carried out in a table-top microcentrifuge at > 12000 x g (10 000 – 14 000 rpm, depending on the rotor type).

Protocol A. DNA extraction from the gel using a centrifuge

Step	**Procedure**
1	Gel containing the DNA fragment was excised using a clean razor blade and was as close to the DNA as possible to minimise the gel volume. The gel slice was then placed into a pre-weighed 1.5 mL tube and the weight of the gel slice was recorded. **Note.** UV exposure was allowed for a few seconds to avoid DNA damage by UV illumination.
2	**1:1 volume of Binding Buffer** was added to the gel slice (volume: weight) (e.g., add 100 µL of Binding Buffer for every 100 mg of agarose gel)
3	The gel mixture was Incubated at **50-60°C** for **10 minutes** or until the gel slice was completely dissolved and the tube was mixed by inversion, every few minutes to facilitate the melting process. Once the gel has completely dissolved, it was vortexed briefly, and the colour was Checked. After a yellow colour that

	shows an optimal pH for DNA binding was checked the sample was loaded on the column
5	Up to 800 µL of the solubilized gel solution (from step 3) was transferred to the Gene JET purification column and centrifuged for 1 min. The flow-through was discarded, and the column was placed back into the same collection tube. Note • If the total volume exceeded 800 µL, the solution could be added to the column in stages. After each application, centrifuge the column for 30-60s and discard the flow-through after the spin. Repeated until the entire volume has been applied to the column membrane. Did not exceed 1 g of total agarose gel per column.
6	*Optional:* This binding step was performed given the purified DNA is to be used for sequencing 100 µL of Binding Buffer was added to the Gene JET purification column and Centrifuged for 1 min. The flow-through was discarded, and the column was placed back into the same collection tube.
7	**700 µL** of **Wash Buffer** (diluted with ethanol) was added to the Gene JET purification column and Centrifuged for 1 min. The flow-through was discarded, and the column was placed back into the same collection tube.
8	The empty Gene JET purification column was centrifuged for an additional 1 minutes to remove residual buffer. Note: this step is essential to avoid residual ethanol in the purified DNA solution, as its presence may inhibit downstream enzymatic reactions.
9	Then, the Gene JET purification column was transferred into a clean 1.5 mL microcentrifuge tube and 50 µL of Elution Buffer was added to the centre of the purification column membrane and Centrifuged for 1 min. **Note.** For low DNA amounts, the elution volumes could be reduced to increase the DNA concentration. An elution volume between 20-50 µL did not significantly reduce the DNA yield. However, elution volumes less than 10 µL were not recommended.

10	Finally, the Gene JET purification column was discarded, and the purified DNA was stored at -20°C

5.6 **Performing the Gateway BP reaction to generate entry clones**

Materials

- attB-PCR product
- pDONR vector (150 ng/µL)
- Gateway®BP Clonase® II enzyme mix kept at −20°C.
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Proteinase K solution

Reactions	Components		
1	attb-myb (15-150ng), 1-7 µl	150ng/µl,1µl pDONR4-1 (P4-P1)	TE buffer, PH 8.0 to 8 µl
2	attb-SWEET1b (15-150ng), 1-7µl		
3	attb-WRI5a (15-150ng), 1-7µl	150ng/µl,1µl pDONR 221 (P1-P2)	
-ve control	----------	--------	
+ve control	----------	PSWEET1b	

- These components were added into a 1.5ml micro-centrifuge tube at a room
- the Gateway® BP Clonase® II enzyme mix was taken from −20°C and thaw on ice (~ 2 minutes).

- 2 µL of Gateway® BP Clonase® II enzyme mix was added to the reaction samples and reactions were incubated at room temperature for 1 hour. Note: For most applications, a 1-hour incubation will yield enough entry clones.
- Depending on the needs, the time for recombination reaction was extended up to 18 hours.
- 1 µL of the Proteinase K was added to each reaction and incubated for 10 minutes at room temperature and transformed electrocompetent E. coli cells.

5.7 Performing the Gateway LR reaction to generate expression clones

Materials

- pEntr with MYB1, SWEET1b and wri5a inserts
- pDEST vector (100 ng/µL, glycerol stock) i.e. PMW#3(pUra), PMW#2(pHIS) and pDEST22
- Gateway®LR Clonase® II enzyme mix kept at −20°C.
- TE Buffer, pH 8.0
- Proteinase K solution

Reactions	**Components**		
1	attL-myb-pEntr (15-150ng), 1-7 µl	100ng/µl,1µl pHIS and pUra	TE buffer, PH 8.0 to 8 µl
2	attLSWEET1b-pEntr (15-150ng), 1-7µl		
3	attbL-pEntr-WRI5a (15-150ng), 1-7µl	100ng/µl,1µl pDEST 22 R1-R2	
4	-ve control (mock transformation)		

- These components were added into a 1.5ml microcentrifuge tube at a room

- the Gateway® LR Clonase® II enzyme mix was taken from −20°C and thaw on ice (~ 2 minutes).
- 2 µL of Gateway® LR Clonase® II enzyme mix to the sample and reactions were incubated at room temperature for 2 hours.
- Depending on the needs, the length of the recombination reaction was extended to 18h
- 1 µL of the Proteinase K was added to each reaction, incubated for 10 minutes at room temperature, and used for E. coli transformation.

5.8 Preparation of electro-competent E.coli and transformation by electroporation

Material

- Autoclave all:
- 500 ml LB medium (without NaCl!!!) in a 2 Liter Erlenmeyer.
- 3 large PVC buckets
- 2 small PVC tubes optional
- 1-liter mQ
- 8,5% glycerol (200 ml)
- Eppendorf tubes.
- 5 ml pipet tips
- All materials should be on ice before starting the procedure!

Procedure

- During the whole procedure, the cells were kept on ice as much as possible
- For the preparation of electro-competent cells, E. coli stain DH5α was inoculated from glycerol frozen stock, in 10 ml LB medium and incubated overnight at 37°C.
- The next day, cultured cells were diluted to 1:100 by adding the preculture to 500 ml Lb medium without NaCl and incubated with vigorous shaking at 37°C until the OD600 is 0.5–0. 8 (3-4 hours).
- Once the right OD achieved the cell suspension Spun for 10 min at 4000 rpm at 4°C
- Then the bacterial pellet was re-suspended in 500 ml ice-cold mQ. (Make sure to re-suspend well, no clumps should be visible anymore) and spun for 10 min at 4000 rpm and 4°C.

- The supernatant was discarded, and the pellet was re-suspended in 500 ml ice-cold mQ. (Make sure to re-suspend well, no clumps should be visible anymore) and spun for 10 min at 4000 rpm and 4°C.
- The supernatant was discarded, and the pellet was re-suspended in 40 ml 8,5% ice-cold glycerol and spun for 10 min at 4000 rpm and 4°C,
- The supernatant was discarded, and the pellet was re-suspended in 2.5 ml of 8,5% ice-cold glycerol.
- 80 µl of resuspension in precooled Eppendorf tubes was stored at -80°C.

5.9 Transforming E.coli by electroporation

Materials

Transforming DH5α™ E. coli strain				
Gateway-BPrxn	Donor Vectors			Selection media
	pDONR 221	pDONR (4-1)		LB agar plate + 50 µg/ml kanamycin
Gateway-LRrxn	Destination vectors			
	pDEST (pHIS)	pDEST (pUra),	pDEST 22	LB agar plate + 50 µg/ml Ampicillin

Procedure

- 2 µL of the BP and LR recombination reaction was added into 80 µL of electrocompetent E. coli
- Samples were mixed gently and transferred into a 0.1-cuvette, and electroporated at 2500v using an electroporator
- Electroporation read measuring the salt concentration of the solution ranging from 4.0ms-5.0ms was taken as an acceptable range.
- 300 µL of liquid LB medium was prepared in the 15-ml snap-cap falcon tube and the electroporated solution was transferred to this tube and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.
- Then, 150 µL from each transformation was spread on a pre-warmed selective plate and incubated overnight at 37°C.

- An efficient BP and LR recombination reaction was expected to produce hundreds of colonies.

5.10 Plasmid extraction and purification from 1-5ml E. coli culture.

- 1-5ml Culture grew overnight in a 10-20ml culture tube.
- The cell suspension was centrifuged at 10,000x g for 1 minute at room temperature, and the culture media was discarded
- Pellet was resuspended with 250ul solution I mixed with RNase and vortexed thoroughly and the suspension was transferred into a new 1.5ml.
- 250ul solution II was added, and the tube was gently rotated several times to get a clear lysate
- 350ul solutions III was added, and the solution was immediately inverted several times until a flocculent white precipitate forms. Then the solution was centrifuged at maximum speed: > 13,000 xg for 10 minutes.
- Once a compact white pellet was formed, the cleared supernatant transferred into HiBind DNA Mini column inserted into a 2ml collection tube.
- The transferred supernatant was centrifuged at maximum speed for 1 minute and the filtrate was discarded and the tube was reused
- 500ul HBC buffer diluted with isopropanol was added in the HiBind DNA Mini column and centrifuged at maximum speed for 1minute and the filtrated was discarded and the collection tube was reused.
- 700ul DNA wash buffer diluted with ethanol was added, the column was centrifuged at maximum speed for 30 sec, and the filtrate was discarded and the collection tube was reused. This step was repeated.
- Then, the empty HiBind DNA Mini column was centrifuged at maximum speed for 2 minutes to dry the column. This step is critical for the removal of trace ethanol that may interfere with downstream applications
- The HiBind DNA Mini column was transferred into a nuclease-free 1.5 ml microcentrifuge tube
- 30-100ul Elution buffer or sterile deionized water was added and left to sit for 1 minute and centrifuged at maximum speed for 1 minute.

- Eluted DNA was stored at -20^{O}C.

5.11 **Restriction digestion reaction setup**

Restriction digestion of Entry clone				
100ng/ µL MYB1-pEntr	0.5µL SacI FD	2 µL 10x R buffer	MQ water to 20 µL	Inc ,37^{o}c ,30 minutes
100ng /µL SWEET1b-pEntr	0.5µL SacI FD			
100ng /µL Wri5a -pEntr	1µL EcoRV			Inc ,37^{o}c,45 minutes
Restriction digestion of Expression clone				
100ng /µL MYB1: pHIS	0.5µL SacI FD	2 µL 10x R buffer	MQ water to 20 µL	Inc ,37^{o}c,30 minutes
100ng /µL MYB1: pUra				
100ng /µL SWEET1b: pHIS				
SWEET1b: pUra				
Linearizing restriction digestion of expression clones				
100ng /µL MYB1: pHIS	1 µL xho	2 µL 10x R buffer	MQ water to 20 µL	Inc ,37^{o}c,45 minutes
100ng /µL MYB1: pUra				
100ng /µL SWEET1b: pHIS				
SWEET1b: pUra				

5.12 **Preparation of yeast strains YM4271 and Y187 and transformation**.

A Dual membrane Kit 3 User Manner for Yeast growth and transformation.

- From a fresh YPAD agar plate, a single colony of (YM4271 and Y187) strains were inoculated into 50 ml liquid YPAD medium and grew overnight at 30°C with shaking. colonies from a re-streaked plate that is no older than 2 weeks were also used.
- After overnight shaking at 30°C, the OD_{546} of the cultures was measured which should be between 0.6 – 0.8. If the OD_{546} reading was above 1.0, the cultures were diluted to OD_{546} of 0.2 and regrew to OD_{546} 0.6
- 50 ml Cultures with appropriate OD were pelleted for 5 minutes at 2500 g and suspended in 2.5 ml water (MQ).

The following PEG/LiOAc master sufficient for 5 transformations was prepared from the kit.

PEG/LiOAc master mix	
Component	Amount
50% PEG	1.2 ml
1 M LiOAc	180µl
Single-stranded carrier DNA	125 µl

The amounts of constructs used.

Reaction	Amount	Plasmid
1	300ng	MYB1: pHIS
2	300ng	MYB1: pUra
3	300ng	SWEET1b: pHIS
4	300ng	SWEET1b: pUra
5	300ng	Wr5a: PDEST22

- 300 µl PEG/LiOAc master mix was added to each tube and vortexed briefly

- 100 µl of resuspended yeast cells from step 3 was then added to each tube and vortexed for 1 minute to thoroughly mix all components
- The mix was Incubated in 42°C water bath for 45 minutes
- The reactions were pelleted for 5 minutes at 700g
- each pellet was dissolved in 100 µl of 0.9% NaCl and each transformation was plated onto the following plates

Reaction	Plasmid	SD-HIS agar plate	SD-Ura agar plate
1	MYB1::pHIS	100 µl	-
2	MYB1::pUra	-	100 µl
3	SWEET1b::pUra	-	100 µl
4	SWEET1b::pHIS	100 µl	-
-ve		100 µl	100 µl

- All plates were then sealed with parafilm and incubated for 4 days at 30°C

Note: After 4 days at 30°C, approximately 100-1000 colonies on every plate expected depending on the transformation efficiency. If you observe significantly fewer colonies, it is recommended that you repeat the entire transformation procedure.

Preparation before:

Glycerol stocks

A single bacterial colony was incubated at 37°C overnight with vigorous shaking in LB liquid medium containing the appropriate antibiotic

The other day, 0.25 ml glycerol (8.5% sterile! = autoclaved) was added to 0.75 ml of incubated medium and froze in liquid nitrogen and stored at -80°C.

Testing Autoactivation of DNA Bait Yeast Strains

- Using sterile toothpicks, transfer 12 integrant colonies (see Note 4) from the plate in 3.1.1.20 to a 150 mm Sc-His plate If positive control yeast strains that express known levels of reporters are available, they should also be transferred to these plates (Fig. 3).
- Incubate the plates at 30°C for 1 to 2 days. 2. Replica-plate (see Note 5) the yeast from 3.1.2.1 onto several 150 mm Sc-His plates, each containing a different concentration of 3AT (0, 10, 20, 40, 60, 80 mM) (see Note 6),
- and a 150 mm YEPD plate onto which a nitrocellulose (NC) filter has been placed (so that the yeast will grow on the filter).
- Replica-clean (see Note 5) the plates containing 3AT and incubate them for 5 days at 30°C. Incubate the other two plates overnight at 30°C. The Sc-His plate without 3AT is used to maintain the integrants and can be stored at room temperature for 14 days.
- The NC filter/YEPD plate is used for the βGal assay (steps 3 to 7 below). 3. For each NC filter/YEPD plate to be analyzed, place two Whatman filters in an empty 150 mm Petri dish. Move to a fumehood for the next three steps.
- 4. For each Petri dish in 3.1.2.3, set up a reaction mix containing 6 mL Z-buffer, 11 µL β-mercapto-ethanol, and 100 µL X-gal solution (see Note 7). Use this entire mix to soak the Whatman filters, and remove any air bubbles using forceps to lift the filters and squeeze the bubbles to the sides, then remove excess liquid into a waste bottle by tipping the plate.
- 5. Lift the NC filter from the YEPD plate using forceps and place the filter yeast side up in a liquid nitrogen bath for 10 seconds. Discard the YEPD plate.
- 6. Use the forceps to place the frozen NC filter with the yeast facing up onto the wet Whatman filters from 3.1.2.4, and use forceps (or a needle) to remove air bubbles under the NC filter quickly as the NC filter (and yeast lysate) thaws.
- 7. Incubate each βGal assay plate at 37°C overnight. Record the amount of blue compound generated by each yeast lysate.
- 8. Observe the amount of growth each integrant displays after 5 days on the Sc-Ura,- His plates containing 3AT (3.1.2.2), recording how much 3AT was required to inhibit the growth of each integrant strain.
- 9. Choose 1 to 4 integrant strains showing the lowest autoactivity for both reporters (see Note 8). 3.1.3: Yeast PCR to.

6 Reference

An, J., Zeng, T., Ji, C., de Graaf, S., Zheng, Z., Xiao, T. T., Deng, X., Xiao, S., Bisseling, T., Limpens, E., & Pan, Z. (2019). A Medicago truncatula SWEET transporter implicated in arbuscule maintenance during arbuscular mycorrhizal symbiosis. *New Phytologist*, *224*(1), 396–408. https://doi.org/10.1111/nph.15975

Bonfante, P., & Genre, A. (2010). Mechanisms underlying beneficial plant - Fungus interactions in mycorrhizal symbiosis. *Nature Communications*, *1*(4), 1–11. https://doi.org/10.1038/ncomms1046

Breuillin-Sessoms, F., Floss, D. S., Karen Gomez, S., Pumplin, N., Ding, Y., Levesque-Tremblay, V., Noar, R. D., Daniels, D. A., Bravo, A., Eaglesham, J. B., Benedito, V. A., Udvardi, M. K., & Harrison, M. J. (2015). Suppression of arbuscule degeneration in Medicago truncatula phosphate transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3. *Plant Cell*, *27*(4), 1352–1366. https://doi.org/10.1105/tpc.114.131144

Camps, C., Jardinaud, M. F., Rengel, D., Carrère, S., Hervé, C., Debellé, F., Gamas, P., Bensmihen, S., & Gough, C. (2015). Combined genetic and transcriptomic analysis reveals three major signalling pathways activated by Myc-LCOs in Medicago truncatula. *New Phytologist*, *208*(1), 224–240. https://doi.org/10.1111/nph.13427

Chatterjee, Nimrat Walker, G. (2017). 乳鼠心肌提取 HHS Public Access. *Physiology & Behavior*, *176*(10), 139–148. file:///C:/Users/Carla Carolina/Desktop/Artigos para acrescentar na qualificação/The impact of birth weight on cardiovascular disease risk in the.pdf

Deplancke, B., Dupuy, D., Vidal, M., & Walhout, A. J. M. (2004). A Gateway-compatible yeast one-hybrid system. *Genome Research*, *14*(10 B), 2093–2101. https://doi.org/10.1101/gr.2445504

Field Code Changed

Editor, W. B. (n.d.). *Genomics*.

Floss, D. S., Gomez, S. K., Park, H. J., MacLean, A. M., Müller, L. M., Bhattarai, K. K., Lévesque-Tremblay, V., Maldonado-Mendoza, I. E., & Harrison, M. J. (2017). A Transcriptional Program for Arbuscule Degeneration during AM Symbiosis Is Regulated by MYB1. *Current Biology*, *27*(8), 1206–1212. https://doi.org/10.1016/j.cub.2017.03.003

Floss, D. S., Levy, J. G., Lévesque-Tremblay, V., Pumplin, N., & Harrison, M. J. (2013). DELLA proteins regulate arbuscule formation in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(51), 5025–5034. https://doi.org/10.1073/pnas.1308973110

Gobbato, E., Marsh, J. F., Vernié, T., Wang, E., Maillet, F., Kim, J., Miller, J. B., Sun, J., Bano, S. A., Ratet, P., Mysore, K. S., Dénarié, J., Schultze, M., & Oldroyd, G. E. D. (2012). A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Current Biology*, *22*(23), 2236–2241. https://doi.org/10.1016/j.cub.2012.09.044

Gutjahr, C., & Parniske, M. (2017). Cell Biology: Control of Partner Lifetime in a Plant–Fungus Relationship. *Current Biology*, *27*(11), R420–R423. https://doi.org/10.1016/j.cub.2017.04.020

Harrison, M. J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology*, *59*, 19–42. https://doi.org/10.1146/annurev.micro.58.030603.123749

Ho-Plágaro, T., Molinero-Rosales, N., Fariña Flores, D., Villena Díaz, M., & García-Garrido, J. M. (2019). Identification and expression analysis of GRAS transcription factor genes involved in the control of arbuscular mycorrhizal development in tomato. *Frontiers in Plant Science*, *10*(March). https://doi.org/10.3389/fpls.2019.00268

Jiang, Y., Xie, Q., Wang, W., Yang, J., Zhang, X., Yu, N., Zhou, Y., & Wang, E. (2018). Medicago AP2-Domain Transcription Factor WRI5a Is a Master Regulator of Lipid Biosynthesis and Transfer during Mycorrhizal Symbiosis. *Molecular Plant*, *11*(11), 1344–1359. https://doi.org/10.1016/j.molp.2018.09.006

Keymer, A., Pimprikar, P., Wewer, V., Huber, C., Brands, M., Bucerius, S. L., Delaux, P. M., Klingl, V., von Röpenack-Lahaye, E., Wang, T. L., Eisenreich, W., Dörmann, P., Parniske,

M., & Gutjahr, C. (2017). Lipid transfer from plants to arbuscular mycorrhiza fungi. *ELife*, *6*, 1–33. https://doi.org/10.7554/eLife.29107

Kobae, Y., & Hata, S. (2010). Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. *Plant and Cell Physiology*, *51*(3), 341–353. https://doi.org/10.1093/pcp/pcq013

Limpens, E., & Geurts, R. (2018). Transcriptional Regulation of Nutrient Exchange in Arbuscular Mycorrhizal Symbiosis. *Molecular Plant*, *11*(12), 1421–1423. https://doi.org/10.1016/j.molp.2018.11.003

Liu, W., Kohlen, W., Lillo, A., den Camp, R. O., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W. C., Hooiveld, G. J. E. J., Charnikhova, T., Bouwmeester, H. J., Bisseling, T., & Geurts, R. (2011). Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. *Plant Cell*, *23*(10), 3853–3865. https://doi.org/10.1105/tpc.111.089771

Luginbuehl, L. H., & Oldroyd, G. E. D. (2017). Understanding the Arbuscule at the Heart of Endomycorrhizal Symbioses in Plants. *Current Biology*, *27*(17), R952–R963. https://doi.org/10.1016/j.cub.2017.06.042

Maeo, K., Tokuda, T., Ayame, A., Mitsui, N., Kawai, T., Tsukagoshi, H., Ishiguro, S., & Nakamura, K. (2009). An AP2-type transcription factor, WRINKLED1, of Arabidopsis thaliana binds to the AW-box sequence conserved among proximal upstream regions of genes involved in fatty acid synthesis. *Plant Journal*, *60*(3), 476–487. https://doi.org/10.1111/j.1365-313X.2009.03967.x

Maillet, F., Poinsot, V., André, O., Puech-Pagés, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez, H., Bécard, G., & Dénarié, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature*, *469*(7328), 58–64. https://doi.org/10.1038/nature09622

Part, P. (2012). *Gateway ® Technology with Clonase ® II A universal technology to clone DNA*. *25*.

Pimprikar, P., Carbonnel, S., Paries, M., Katzer, K., Klingl, V., Bohmer, M. J., Karl, L., Floss, D.

S., Harrison, M. J., Parniske, M., & Gutjahr, C. (2016). A CCaMK-CYCLOPS-DELLA complex activates transcription of RAM1 to regulate arbuscule branching. *Current Biology*, *26*(8), 987–998. https://doi.org/10.1016/j.cub.2016.01.069

Pimprikar, P., & Gutjahr, C. (2018). Transcriptional Regulation of Arbuscular Mycorrhiza Development. *Plant and Cell Physiology*, *59*(4), 673–690. https://doi.org/10.1093/pcp/pcy024

Rich, M. K., Courty, P. E., Roux, C., & Reinhardt, D. (2017). Role of the GRAS transcription factor ATA/RAM1 in the transcriptional reprogramming of arbuscular mycorrhiza in Petunia hybrida. *BMC Genomics*, *18*(1), 1–14. https://doi.org/10.1186/s12864-017-3988-8

Schmitz, A. M., & Harrison, M. J. (2014). Signaling events during initiation of arbuscular mycorrhizal symbiosis. *Journal of Integrative Plant Biology*, *56*(3), 250–261. https://doi.org/10.1111/jipb.12155

Vigneron, N., Radhakrishnan, G. V., & Delaux, P. M. (2018). What have we learnt from studying the evolution of the arbuscular mycorrhizal symbiosis? *Current Opinion in Plant Biology*, *44*, 49–56. https://doi.org/10.1016/j.pbi.2018.02.004

Wang, E., Schornack, S., Marsh, J. F., Gobbato, E., Schwessinger, B., Eastmond, P., Schultze, M., Kamoun, S., & Oldroyd, G. E. D. (2012). A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Current Biology*, *22*(23), 2242–2246. https://doi.org/10.1016/j.cub.2012.09.043