A PROFILE OF THE EXPRESSION OF A METABOLIC GENE CLUSTER IN ARABIDOPSIS

by

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Abstract

Plant cells often display a microtubule reorganization event when encountered with stress. This has been found to be integral for the reaction to stresses such as aluminum toxicity and cold stress. A cDNA microarray was previously conducted that identified MARNERAL SYNTHASE (MRN1), an oxidosqualene cyclase that produces the triterpene marneral, as the most highly upregulated gene when microtubule dynamics are disrupted in Arabidopsis. This work identifies two cytochrome P450s, CYP71A16 and CYP705A12, that are highly coregulated with MRN1 and are located within close proximity to the MRN1 loci. Using GC-FID and GC-MS, MRN1 and CYP71A16 are shown to function together in a single pathway in what is known as a metabolic gene cluster, while further testing shows that they are not in fact regulated by microtubule dynamics.

The expression profile of these genes is explored since there is no known function for marneral or its related metabolites. Using a promoter-reporter and real time PCR analysis, it was found that the hormones ABA and methyl jasmonate induce expression of the three genes to different degrees depending on seedling age. Osmotic stressors, including mannitol and NaCl treatments, also induce the expression of these genes. MRN1, in particular, seems to show the highest level of induction suggesting that the pathway is transcriptionally regulated through MRN1. These conditions are shown to not affect the growth response in mutant plants unable to metabolize marneral or plants ectopically expressing different combinations of the three genes.

These conditions are intriguing because most triterpenes derived from secondary metabolism are generally thought to play roles in defense, yet these data suggest that the pathway is induced under abiotic stress conditions. The marneral cluster may have evolved to be expressed under osmotic stress conditions in a sense to protect the plants water from pathogens or herbivores. It is also reasonable to speculate that these compounds may play roles in signalling or membrane modification. Further experiments are proposed that could test these hypotheses.
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<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylate</td>
</tr>
<tr>
<td>ACT8</td>
<td>ACTIN 8</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>AUX1</td>
<td>AUXIN RESISTAT 1</td>
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<td>BSTFA</td>
<td>N, O-bis(trimethylsilyl)trifluoroacetamide</td>
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<td>BY-2</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>CHCl₃</td>
<td>chloroform</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>cytochrome P450</td>
</tr>
<tr>
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<td>DET3</td>
<td>DE-ETIOLATED 3</td>
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<tr>
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<td>dimethylallyl pyrophosphate</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EIC</td>
<td>extracted ion chromatogram</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FLIM-FRET</td>
<td>fluorescence lifetime imaging microscopy coupled with Förster resonance energy transfer</td>
</tr>
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<td>GA-3</td>
<td>gibberellic acid-3</td>
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<tr>
<td>GC-FID</td>
<td>gas chromatography flame ionized detector</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography coupled to a mass spectrometer</td>
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<td>GFP</td>
<td>GREEN FLUORESCENT PROTEIN</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>HMGR</td>
<td>3-HYDROXYL-2METHYLGLUTARYL-COA REDUCTASE</td>
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<td><em>Helicobacter pylori</em> protein Z1a</td>
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<tr>
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<td>LUPEOL SYNTHASE 4</td>
</tr>
<tr>
<td>m/z</td>
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</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>MCGs</td>
<td>marneral cluster genes</td>
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<tr>
<td>MeJA</td>
<td>methyl jasmonate</td>
</tr>
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<td>MEP</td>
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</tr>
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<td>magnesium chloride</td>
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<td>MOR1</td>
<td>MICROTUBULE ORGANIZATION 1</td>
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<tr>
<td>MPB2C</td>
<td>MOVEMENT PROTEIN BINDING PROTEIN 2C</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MRN1</td>
<td>MARNERAL SYNTHASE</td>
</tr>
<tr>
<td>MSBP1</td>
<td>MEMBRANE STEROL BINDING PROTEIN 1</td>
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<tr>
<td>MYB61</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>NADPH</td>
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</tr>
<tr>
<td>NaPO₄</td>
<td>sodium phosphate</td>
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<tr>
<td>OFP</td>
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<tr>
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<tr>
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<td>PIN-FORMED 2</td>
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<tr>
<td>PYL</td>
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<td>PYR</td>
<td>PYRABACTIN RESISTANCE</td>
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<td>SAD</td>
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<td>SQUALENE EPOXIDASE</td>
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<td>SQUALENE SYNTHASE</td>
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<td>THALIANOL SYNTHASE</td>
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<td>UBIQUITIN</td>
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<tr>
<td>XMAP215</td>
<td>MICROTUBULE ASSOCIATED PROTEIN 215 from <em>Xenopus laevis</em></td>
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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

There have been a number of observations describing changes in plant microtubule dynamics in relation to external stresses. While the function of this change is unknown, it has in some cases, been shown to be necessary in the stress response pathway within the plant (Sivaguru et al. 2003, Abdrakhmananova et al. 2003). A cDNA microarray was conducted prior to this work that compared the transcriptional response of plants with disrupted microtubules (Walia 2009). This experiment identified a metabolic gene cluster consisting of a triterpene synthase and two cytochrome P450s. This thesis explores the connections among these three genes and characterizes their expression.

1.2 MICROTUBULE STRUCTURE AND DYNAMICS

Cortical microtubules are essential to many cellular processes. Microtubules function through their ability to rapidly change. That ability is described as dynamic instability and is represented by the occurrence of five distinct events occurring at the + end: assembly, pause, catastrophe, disassembly, and rescue (reviewed in Hashimoto 2003; Valiron et al. 2001). Assembly is defined as the net polymerization of tubulin resulting in an increased length of the tubule, typically focused at one end. During assembly new tubulin dimers are added rapidly to the + end of a microtubule. These new tubulin dimers are bound to GTP, which generates a configuration that permits polymerization while resisting disassembly. Soon after the incorporation with the microtubule, the exposed GTP on the β-tubulin subunit is hydrolyzed, causing a conformational change that is required for microtubule disassembly. Normally, new GTP-bound subunit addition outpaces hydrolysis to generate a GTP cap but if all GTP is hydrolyzed at the plus end then rapid disassembly occurs, known as catastrophe. Rescue can sometimes occur if the microtubule regains the GTP cap and begins assembly again, but frequently the entire microtubule is lost. When subunit addition is matched by subunit loss, polymerization stalls and microtubules enter into a state of pause. Microtubule dynamics are modulated by microtubule associated proteins (MAPs). These proteins are closely associated with microtubules and facilitate specific aspects of microtubule dynamics. For instance, XMAP215 (the Xenopus homologue of Arabidopsis thaliana MOR1 protein) has been shown to facilitate both polymerization and depolymerization of microtubules (Brouhard et al. 2008).
sensitive mutant *mor1-1* has a microtubule cytoskeleton that spends more time in pause and has highly reduced growth and shrinkage rates at restrictive temperature (Kawamura and Wasteneys 2008). Chuong et al. (2004) used high affinity tubulin chromatography against crude Arabidopsis protein extracts in order to detect new proteins that can interact with tubulin. They identified 144 proteins, of which only 6% were identified as MAPs, while the major groups of proteins were involved with binding of RNA (21%), translation (19%), signalling (12%), and metabolism (21%). Doroshank et al. (2009) found similar results when probing the rice seed proteome. Furthermore, Winter et al. (2007b) characterized an association between microtubules and the transcription factor KNOTTED1 (KN1) in which the microtubule-associated protein MPB2C negatively regulates the movement of KN1 from cell to cell. These data indicate that microtubules may play a more direct role in gene regulation by acting as an intermediary in the signalling pathway between the surface of the cell and the nucleus.

1.3 MICROTUBULES AND STRESS INTERACTIONS

A number of studies have shown that a temporary microtubule disruption or reorganization event occurs after specific stresses and stimuli. Little is actually known about the activity and mechanisms the cytoskeleton plays in signalling, although the microtubule depolymerization event seems to be necessary for some of the stress response pathways (Wasteneys 2004).

1.3.1 Cold Stress

Freeze tolerance has been shown to occur after a period of cold induced microtubule disruption in wheat (*Triticum aestivum*). Initially, it was shown that a 4°C pretreatment, resulting in a temporary microtubule disassembly event, gave plants the ability to recover more quickly and tolerate cold shocks down to -7°C (Wang and Nick 2001). Later, three cultivars were examined that had varying levels of freeze tolerance (Abdrakhamanova et al. 2003). Their ability to recover from freeze shocks correlated with the amount of time it took for the cortical arrays to recover. Furthermore, the most sensitive cultivar acquired freeze-tolerance when temporarily pretreated with pronamide, a microtubule disrupting drug, and grown at 4°C. In another study, Sangwan et al. (2001) used a promoter-reporter construct for the cold-induced gene *BN115* from *Brassica napus* to show that drugs stabilizing the cytoskeleton (both microtubules and actin) prevented its expression, whereas cytoskeleton-destabilizing
drugs like oryzalin, colchicine, or latrunculin B promoted expression at 25°C. These studies suggest that microtubules and actin filaments are involved in a signalling pathway necessary for the activation of freeze tolerance-related processes.

1.3.2 Metal Stress

Aluminum exposure has been shown to cause microtubule disruption of root cells (Sivaguru et al. 2003). This microtubule disruption even was also found to be integral for the aluminum response pathway. When roots sense aluminum in the soil, glutamate channels open to facilitate efflux out of the cell. This binds to a glutamate receptor on the outer face the plasma membrane that triggers a Ca^{2+} influx event that depolarizes the membrane. This event leads to microtubule disassembly and subsequently production and secretion of organic acids such as citrate or malate that will ultimately chelate the toxic aluminum within the soil (Sivaguru et al. 2003). This pathway has also been shown to include PHOSPHOLIPASE D (PLD) in the production of phosphatidic acid (Pejchar et al. 2008). Lead poisoning in roots also produced aberrant microtubule arrays, but the mechanisms remain unknown. It is noted that the microtubules react to levels of lead (10 μM) below what is required to observe reduced root growth (20 μM) (Eun et al. 2000). Similar effects were found in Allium sativum cells treated with both lead and copper (Liu et al. 2009).

1.3.3 Osmotic Stress

Osmotic stresses have been shown to affect microtubule organization in maize roots (Blancaflor and Hasenstein 1995). Ionic and nonionic forms of osmotic stress induced reorganization in different ways, yet no depolymerization event was observed in this study. Nonionic stress rapidly caused transverse cortical microtubules to bundle and form “holes” in the array. The holes in the array partially recovered after nine hours of stress, but many bundles remained present. The microtubule response to KCl occurs much more slowly than what has been observed with nonionic stress. The cortical arrays began shifting from a transverse orientation (relative to the expansion axis) after 30 minutes. A strong response was seen after four hours by which time many of the cells had longitudinal microtubule arrays. It is likely that the specific microtubule responses are characteristic of the stressors. In a different study, salt stress tolerance was increased when the Arabidopsis microtubules were depolymerized and decreased when stabilized both using drugs (Wang et al. 2007). Both forms of osmotic stress have,
also been shown to trigger microtubule reorganization events in tobacco BY-2 cells (Dhonukshe et al. 2003).

The depolymerization of microtubules in BY-2 cells correlated with increased PLD activity (Dhonukshe et al. 2003). Interestingly, PLD has been found to bind microtubules in both tobacco (Gardiner et al. 2001) and Arabidopsis (Ho et al. 2009) cell cultures as well as in leek (*Allium porrum* L.) leaves (Andreeva et al. 2009). This suggests that PLD may act as a potential link between the plasma membrane, where environmental signals are sensed, and microtubules. To support this link, PLD has also been shown to be activated in response to stresses affecting microtubules (Li et al. 2004 and Bargmann et al. 2009).

### 1.3.4 Biotic Stress

Biotic stressors have been shown to affect microtubule organization. When examining the infections of the non-pathogenic *Phytophthora sojae* species and two isolates of *P. parasitica* that were virulent or avirulent (Noks1 and Cala2, respectively) in transgenic GFP-TUA Arabidopsis plants, Takemoto et al. (2003) observed localized dispersed fluorescence around the site of infection. This pattern of fluorescence is indicative of an accumulation of free tubulin dimers as it was not seen anywhere else in the cells. In another study it was shown that the nematodes *Meloidogyne* sp. and *Heterodera schachtii* induce a depolymerization event in the process of forming a gall. The cytoskeleton remained unstable during gall formation. When plants are treated with taxol, a microtubule-stabilizing drug that renders microtubules unable to depolymerize upon infection, the juvenile nematodes fail to mature (de Almeida Engler et al. 2004). Microtubule dynamics were also investigated in a well-characterized system involving the peptide cryptogein, which is secreted by *P. cryptogea*, treated tobacco cells (Binet et al. 2001). Similar to the effects of aluminum toxicity, cryptogein activates Ca$^{2+}$ influx, which in turn disrupts the microtubules. It was shown that microtubules failed to depolymerize when the Ca$^{2+}$ channels were blocked before application of cryptogein. The microtubule disruption event was necessary to activate programmed cell death. In a later study, cryptogein-induced Ca$^{2+}$ influx not only resulted in disrupted microtubules, but also increased activation of mitogen activated protein kinases as well as increased transcription of *PAL* and *hsr203J*. PAL is one of the initial enzymes involved in the phenylpropanoid pathway while hsr203J is involved in cell death, suggesting that
cryptogein activates defense mechanisms (Lecourieux et al. 2002). In a similar study, Qiao et al. (2010) showed that microtubule disruption occurs in response to Harpin, a plant pathogenic elicitor. This induces expression of defense-related metabolic pathways that can also be triggered by the microtubule-affecting drugs oryzalin or taxol. Lee et al. (2012) showed that the bacterial protein HopZ1a is injected into Arabidopsis cells by *Pseudomonas syringae* to specifically attack the microtubule network and promote virulence. This suggests that, in some cases, the disruption of microtubule arrays assists biotic infection. Although this study found that in some instances pathogens target microtubules, it could be speculated that the microtubule disruption event itself may have evolved to act as a signal of infection to the cell from similar or evolutionarily related pathways, as highlighted in the cryptogein system (Lecourieux et al. 2002) and Harpin-elicited grape cells (Qiao et al. 2010).

While Arabidopsis does not form any mycorrhizal associations (Stougaard 2001), other plants that develop these beneficial interactions can undergo microtubule reorganization events after the initiation of the association. For example, the obligate mycorrhizal fungus *Gigaspora margarita* will penetrate epidermal cells of susceptible host plants and grow deep into the cortex to form arbuscules. These structures form highly branched networks within the cell wall but they do not penetrate the plasma membrane. The development of the association between the legume *Lotus japonicus* and *G. margarita* shows cytoskeletal rearrangements upon infection of the epidermis (Genre and Bonfante 2005; Genre and Bonfante 2002). The *L. japonicus* mutant *Ljsym4-2* is unable to form mycorrhizal associations. It was shown that the mutant epidermal cells had a complete microtubule disorganization followed later by a complete absence of tubulin, likely resulting in cell death, indicating that SYM4 is important in recognizing and signalling the presence of beneficial fungi with detrimental ones, and that both processes result in specific responses from the cytoskeleton (Genre and Bonfante 2002).

Ca$^{2+}$ has commonly been shown to be an intermediate signal between the time of infection and the cytoskeletal response. In fact, it has been shown through the use of the calcium-sensitive luminescent protein aequorin that stresses such as drought, salt, and cold elicit tissue specific Ca$^{2+}$ influx events (Kiegle et al. 2000). It is likely that these calcium flux events and the microtubule disruption events are not exclusive. Calmodulin
has long been known to regulate microtubule dynamics through Ca\textsuperscript{2+} signalling and there are reports of other related proteins that may be involved and respond to specific calcium signatures (Webb et al. 1996). Calmodulin itself has been shown to localize to microtubules, but is thought to connect to microtubules through an intermediate protein (Fisher et al. 1996). A group of proteins known as centrins have been shown to have properties similar to calmodulin in that they have four Ca\textsuperscript{2+}-binding domains (Lecourieux et al. 2006) and some have been localized to interphase microtubules, suggesting that they play a role in the interaction between Ca\textsuperscript{2+} and cytoskeleton (Salisbury 1995; Del Vecchio et al. 1996). More recently MICROTUBULE-DESTABILIZING PROTEIN 25 was shown to be activated to destabilize microtubules in the hypocotyl upon increased cellular calcium levels (Li et al. 2011).

The common feature in each of the above described examples suggests that transient microtubule reorganization events play roles in signalling before new arrays are reoriented and/or polymerized. Figure 1.1 shows a possible pathway linking stress-related transcription to a transient microtubule transduction. This suggests that transcription factors or signalling proteins could lay dormant on a functional microtubule array. But once a signal is perceived at the plasma membrane through a membrane receptor, phosphatidic acid is produced from PLD (Gardiner et al. 2003, reviewed in Wasteneys 2003) or other phospholipid derived signals (DeWald et al. 2001). These signals induce the release of Ca\textsuperscript{2+} reserves (Munnik and Testernik 2009), which induce the disruption of microtubule dynamics and possible activation of transcription factors such as SCARECROW-like 14 found to bind tubulin (Chuong et al. 2004). SCARECROW-like 14 has been suggested to respond to stress by playing a role in xenobiotic defense through oxylipin metabolism (Fode et al. 2008) and/or signalling (Mueller et al. 2008).

To investigate this concept further, the conditional microtubule disruption in the temperature-sensitive mutant mor1-1 was utilized in a cDNA microarray experiment and compared to wild type (Columbia) tissue. The mor1-1 mutant maintains organized cortical microtubules below 21°C. At 29°C, the microtubules depolymerize and disorganize within 90 minutes and remain disorganized until the temperature is lowered back to 21°C (Whittington et al. 2001). RNA was isolated from mor1-1 and control wild type plants at multiple time points (2, 4, and 8 h) after the temperature was shifted
above the restrictive temperature (Walia 2009). The goal for this study was to identify transcripts involved in signalling and other processes that are dependent on the microtubule polymer status. These data were also thought to be important for reexamining the mor1-1 phenotype at 29°C and could potentially identify genes involved in producing the mor1-1 growth phenotypes at restrictive temperature, including cell elongation and expansion defects as well as organ twisting (Whittington et al. 2001). One of the transcripts that showed the strongest upregulation was MARNERAL SYNTHASE 1 (MRN1), a gene closely related to other Arabidopsis oxidosqualene cyclase (OSC) encoding genes (Husselstein-Muller et al. 2001), the product of which has been shown to produce a monocyclic triterpene known as marneral (Xiong et al. 2006).

While the function of marneral and existence of downstream metabolites is unknown, I will show that two cytochrome P450s (CYPs), CYP71A16 and CYP705A12, are highly co-regulated with MRN1 using bioinformatics data (See Chapter 2) (Toufighi et al. 2005). While these two enzymes would be likely candidates for metabolizing marneral, it is interesting to note that they are located next to MRN1 on the chromosome. If these genes function within a single pathway then the colocalization of the genes proves the existence of what is known as a metabolic gene cluster.

1.4 TERPENE METABOLISM

Terpenes are the largest class of natural products in plants with estimates of over 50,000 compounds known to date (McCaskill and Croteau 1997). Hormones such as cytokinins, gibberellins, brassinosteroids, and abscisic acid are all derived from the terpene pathway. Other primary metabolites represented in this pathway include membrane sterols and carotenoids (Chappell 1995). Most of the compounds produced are secondary metabolites, loosely defined as compounds synthesized in response to specific stimuli that are represented in taxonomically isolated groups. Terpenes are able to form such a diverse number of end products because of centralized enzymatic steps that allow basic carbon skeletons of the different classes of terpenes to be synthesized, followed by specific modifications in the later steps of individual pathways. Terpenes are made up of a series of C5 isoprene subunits derived from acetyl-CoA. There are two pathways in higher plants that commit carbon to the terpene pathway, and there is evidence suggesting that the two pathways can share intermediate
metabolites (Rodriguez-Concepción 2006). The mevalonate pathway utilizes cytosolic acetyl-CoA synthesized in the mitochondria, and generally leads to the production of sesquiterpenes ($C_{15}$) and triterpenes ($C_{30}$). An alternative pathway derived from the plastid, the 2-methyl-D-erythritol-4-phosphate (MEP) pathway, utilizes pyruvate to primarily produce monoterpenes ($C_{10}$), diterpenes ($C_{20}$), and tetraterpenes ($C_{40}$). There are occurrences of other classes of terpenes including hemiterpenes ($C_5$) sesterterpenes ($C_{25}$) and polyterpenes ($C_{>40}$), but their biosynthesis remains poorly understood. The synthesis of lower molecular weight terpenes ($C_{10}, C_{15}, C_{20}$) results from consecutive additions of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP) in a head to tail fashion, where the ‘head’ is represented by the prenyl pyrophosphate groups. The higher molecular weight terpenes result from head to head additions of the prenyl pyrophosphate groups. These reactions are catalyzed by a group of enzymes known as prenyl transferases. The first three steps into the triterpene pathway away from the sesquiterpene pathway are shown in Figure 1.2. Two IPPs are condensed with DMAPP by farnesyl pyrophosphate synthase to produce farnesyl pyrophosphate. Two farnesyl pyrophosphate molecules are then condensed in a head-to-head fashion by SQUALENE SYNTHASE (SQS), the first committed step to the triterpene pathway, to form squalene. This has been shown to occur in two steps: First by formation of presqualene pyrophosphate, then by the NADPH-dependent removal of the pyrophosphate group (Rilling 1966, 1969). Squalene is then oxidized by SQUALENE EPOXIDASE (SQE). The resulting 2,3-oxidosqualene is then passed on to an oxidosqualene cyclase (OSCs) that protonates and cyclizes it to form the initial triterpene. The overexpression of a SQS from Panax ginseng was found to promote the production of both native phytosterols and glycosylated triterpenes known as saponins (Lee et al. 2004). This study suggests that the production of squalene synthase may be a rate-limiting step in triterpene biosynthesis. However, the next step may also be under tight control because 2,3-oxidosqualene is at an important branch in the pathway leading to the production of sterols and brassinosteroids (primary metabolism) or a variety of other secondary metabolites (Chappell 1995). Once the basic triterpene skeleton is formed, more specialized modifications occur that modify the triterpene skeleton. These modifications are often catalyzed by cytochrome P450s, which promote reactions such as hydroxylations or epoxidations, but the enzyme family
is very diverse and can catalyze many different reactions (Werck-Reichhart and Feyereisen 2000).

1.4.1 2,3-Oxidosqualene Cyclases (OSCs)

In Arabidopsis, there are 13 biochemically characterized OSCs that have between 14 and 16 exons and whose size is highly conserved (Morlacchi et al. 2009). OSCs are considered to catalyze complicated transformations by initially protonating 2,3-oxidosqualene followed by a cyclization to form the triterpene backbone (Segura et al. 2003). The enzymes are generally considered to be membrane-bound and to contain a number of highly conserved amino acid sequences present even in the prokaryotic squalene hopene cyclases, from which OSCs are evolutionarily derived (Poralla 1994). A number of structural domains have been characterized by crystallization studies of the squalene hopene cyclases from Alicyclobacillus acidocaldarius (Wendt et al. 1997) and human lanosterol synthase (Thoma et al. 2004). It was shown that the active site is a cavity located in the centre of the enzyme that is lined with aromatic amino acids. The most prominent domain in the peptide is the QXXXXXW motif, which is repeated up to eight times and present in all eukaryotic OSCs where it is concentrated at both the N- and C- termini (Poralla 1994). These QW-motifs are connected to a series of outer helices. Together, these stabilize the whole protein and are thought necessary to absorb the reaction energy of the cyclization (Thoma et al. 2004; Wendt et al. 1997; Husselstein-Muller et al. 2001). The most likely motif for binding substrates is DCTAE. This was determined by mechanism-based irreversible inhibitors and mutational analysis of OSCs (Abe and Prestwich 1995). It seems that this motif specifies the binding of 2,3-oxidosqualene based on the fact that the hopene cyclases active site sequence is DDTAV and because it cyclizes squalene directly (Haralampidis et al. 2002). It is interesting to note that the MRN1 active site has been evolutionarily altered to DGTAE with an additional deletion of another highly conserved residue. These key residues could be responsible for the unique cyclization seen by MRN1 (Figure 1.3) (Xiong et al. 2006). This is in contrast to the cyclization of most triterpenes involved with primary metabolism (i.e. brassinosteroids and membrane sterols), which cyclize oxidosqualene through a protosteryl cation and many other common secondary triterpenes (i.e. β-amyrin- and lupeol-derived compounds), derived from the dammareneryl cation (reviewed in Phillips et al. 2006, Xue et al. 2011). The
ability to utilize a particular cation generally follows evolutionary patterns (Xiong et al. 2006, Phillips et al. 2006, and Xue et al. 2011).

1.4.2 Cytochrome P450s

Cytochrome P450s (CYPs) were originally identified as pigments that absorb light at 450 nm isolated from pig and rat liver microsomes (Garfinkel 1958, Klingenberg 1858, reviewed in Chapple 1998). CYPs typically contain a heme group that is necessary for monooxygenase functionality yet the family is highly divergent both functionally and evolutionarily. Even CYPs within the same family can show as low as 16% amino acid similarity (Werck-Reichhart and Feyereisen 2000). They generally catalyze hydroxylation reactions utilizing NADPH and O2 but have been shown to catalyze a diverse array of reactions. These enzymes typically are membrane bound and are targeted to the endoplasmic reticulum by 25-30 N-terminal amino acids, while the remainder of the enzyme remains in the cytoplasm (Schuler et al. 2006).

The two CYPs identified in this study belong to the CYP705 and CYP71 families (At5g42580 encodes CYP705A12 and At5g42590 encodes CYP71A16), which represent the largest two CYP families in Arabidopsis (Schuler et al. 2006; Nelson et al. 2004; and Nelson 2006). It is interesting to note that all the members of the CYP705 family have only been identified in the Brassicaceae, namely Arabidopsis thaliana and Brassica napus. This suggests that these proteins are involved in highly specialized and/or unique pathways (Schuler et al. 2006; Nelson et al. 2008). CYP705A22 was originally identified through a mutagenesis screen as gravity persistent signal-1 in which auxin is unable to redistribute in the stem when gravity stress is applied, although the substrate is still unknown (Nadella et al. 2006). CYP705A5 has been chemically characterized as a thalian-diol desaturase that is a part of another triterpene metabolic gene cluster (Field and Osbourn 2008).

In contrast to the CYP705 family, the CYP71 family is much more taxonomically widespread, with members found in all groups of land plants, thus raising the possibility of greater divergence of functionality between the different CYP71 proteins (Nelson 2006). Three members of the CYP71A subfamily have been previously characterized. CYP71A10, from Glycine max, was shown to be able to confer resistance to the phenylurea herbicides through a demethylation reaction when overexpressed in tobacco (Siminszky et al. 2000; Siminszky et al. 2003). CYP71A12 was similarly found to
1.5 Triterpene Function

All organisms utilize the triterpene pathway to build membrane sterols as well as many hormones. Beyond this, the roles for triterpenes are extremely diverse as are the individual mix of triterpenes found in specific plant species. Few ecophysiological functions have been identified for non-sterol triterpenes but some purified compounds have been shown to have defensive properties when used in assays against target organisms, including antimicrobial, antifungal, or antifeedant. One class of glycosylated triterpenes, known as saponins, has been shown through genetic analysis of both plants and pathogens to play a role in defense (Augustin et al. 2011). Avenacins, one of the best studied groups of saponins, are present in the epidermal root tissue and developing lateral roots in various *Avena* species (oats) (Wegel et al. 2009). Saponins are specifically noted for their defensive properties and are found within the vacuole (Kesselmeier and Urban 1982, Urban et al. 1982, Mylona et al. 2008, and reviewed in Augustin et al 2011). Chromosaponin I, a saponin isolated from *Pisum sativum*, can initiate physiological responses in other species. Arabidopsis seedling roots initially treated with chromatosaponin I showed up to a 10-fold increase in growth rate (Rahman et al. 2000). This response was later shown to result from an interaction between the saponin and the AUX1 protein, which is responsible for regulating the gravitropic response of the roots (Rahman et al. 2001).

Triterpene aglycones have even fewer characterized functions. Many of these have been identified as components of the plant cuticle, in which they are typically thought to function in defense (Wang et al. 2010, Wang et al. 2011). The cuticle of *Macaranga*, an ant-plant, which shows crystalline deposits comprised of up to 88% triterpenes, only allows symbiotic ants to walk on the surface of the stems (Markstädt et al. 2000). Certainly triterpene aglycones are known constituents of the plant cuticle and this localization could facilitate their utility in defense. Recently, a lupeol synthase
(an OSC) was found to be involved in nodulation of *Lotus japonicus*. RNAi lines with reduced lupeol synthase activity showed an early increase in nitrogen fixing nodule formation, suggesting the presence of lupeol (or related metabolites) negatively affects the nodulation process (Delis et al. 2011). Because of the diversity of triterpenes found in nature and the specificity of the mixture of triterpenes within individual species, it has been speculated that they have evolved towards ecophysiological functions to counteract both biotic and abiotic stresses specific to each species (Rasbery et al. 2006).

### 1.5.1 The Iridals

Iridals are triterpenes derived from the cyclization of 2,3-oxidosqualene into a bicyclic intermediate and subsequent cleavage of one ring (Marner 1997). MRN1 produces the only iridal outside of the Iridaceae (Xiong et al. 2006). Thus, marneral was named after Dr. Franz-Josef Marner for his pioneering work on these unique triterpenes (Xiong et al. 2006). Iridals have been shown to have a number of different biological characteristics, including being membrane constituents (Bonfils et al. 1996; Leconte et al. 1997), antiplasmodial activity (Benoit-Vical et al. 2003), and even tumor repressing activity (Bonfils et al. 2001). Leconte et al. (1997) showed that cycloiridals protected *Candida albicans* membranes from phosphate ion leakage, resulting from a pretreatment with saponins known to permeabilize cell membranes. The cycloiridals seemed to protect the membranes to a similar extent to ergosterol, a common fungal sterol. Ergosterol precipitates saponins and therefore removes the harmful substance but this also removes the membrane sterol. This trait would have deleterious results if there were a constant saponin supply. The cycloiridals, however, were unable to bind the saponins, but still prevented ion leakage, so it has been suggested that cycloiridals associate with and function to protect the plasma membrane. Interestingly, it has also been shown that a number of iridals are able to bind to and activate human protein kinase C (Takahashi et al. 2002), indicating that they have the ability, as a group, to act directly on signalling processes. In Arabidopsis, MRN1 is one of thirteen functionally divergent OSCs and its product, marneral, is the only instance outside of the Iridaceae of the production of a natural iridal product (Xiong et al. 2006). It remains to be seen whether marneral-derived compounds function similarly in Arabidopsis (or its herbivores and pathogens).
1.6 METABOLIC GENE CLUSTERS

It is interesting to note that the MRN1, CYP71A16, and CYP705A12 genes are located sequentially on the same chromosome, but in differing orientations and at considerable distances from each other (Fig 1.4). A phenomenon known as metabolic gene clustering, a situation in which neighboring genes encode enzymes that act within the same pathway, has just begun to be characterized in plants. A genome-wide investigation into gene clustering in Arabidopsis revealed that 10% of the genome belongs to co-expression domains consisting of up to nine genes. These domains were shown to contain homologous genes, in parallel orientation and with an average of only 1.5kb of intergenic sequence (Zhan et al. 2006). Interestingly, the MRN1, CYP71A16, and CYP705A12 cluster does not have any of these attributes, but does show high levels of co-regulation under the parameters set by the researchers.

1.6.1 Cluster Formation

The formation of metabolic gene clusters in plants has been speculated to be driven by the need to either inherit the separate genes as a single unit due, possibly, to the buildup of toxic intermediates or the need to co-regulate the genes at the point of transcription (Chu et al. 2011). In fact, from the characterization of metabolic gene clusters in plants, both of these possible theories seem to be supported. SAPONIN DEFICIENT 3 (SAD3), a member of a metabolic gene cluster in plants, plays a role in glycosylation of avenacin A-1. Mutations in SAD3 lead to shortened roots and reduced root hair production accompanied by an accumulation of the intermediate monodeglucosyl avenacin A-1 (Mylona et al. 2008). This suggests that the gene is linked to the cluster such that the group of genes is inherited as a whole. In the same cluster, it has been shown that the proximity of the genes to each other allows for chromatin level control over transcription. SAD1 and SAD2 were found to be located in condensed regions of the genome in cortical tissue, where the genes are not expressed, but decondense in the epidermal tissue where they are expressed (Wegel et al. 2009). This provides a clear scenario in which a cluster of genes can be co-regulated, at least partially, through close proximity of the component genes to each other.

Metabolic gene clusters have been more extensively studied in fungi where it seems mechanisms are involved in cluster maintenance. It was shown in the yeast
Saccharomyces cerevisiae that a gene cluster consisting of six genes involved in utilizing unusual nitrogen sources in a nitrogen-poor environment had an exceptionally low meiotic recombination rate when compared to other sequences of comparable size or chromosomal location (i.e. subtelomeric DNA) (Wong and Wolfe 2005). This suggests that evolutionary mechanisms are actively repressing genetic recombination within the cluster, even though the selective advantage provided by the cluster may not be utilized in any single generation. Alternative to the theories set forth about cluster formation in plants, the utility of the S. cerevisiae cluster suggests that evolutionary pressures that are only encountered in isolated generations pose a need for tight hereditary control over the maintenance of the clusters (Wong and Wolfe 2005).

1.6.2 Arabidopsis Gene Clusters in Triterpenoid Pathways

In addition to the marneral gene cluster there are at least two other clusters consisting of an OSC and neighbouring CYPs in Arabidopsis. The thalianol gene cluster has been shown to have an OSC (THALIANOL SYNTHASE) and two CYPs, CYP708A2 and CYP705A5 that were found to have hydroxylase and desaturase activities respectively and act sequentially in the thalianol pathway (Field and Osbourn 2008). This cluster also contains a putative acetyltransferase gene in a family specific to the Brassicales, but no metabolic activity was found. The third putative gene cluster contains the OSC BARUOL SYNTHASE and is surrounded by three other CYPs that are highly co-regulated, including CYP705A2, CYP705A3, and CYP702A2 (Mizutani and Ohta 2010), but have not been tested. Finally, Field et al. (2011) published a series of experiments characterizing the marneral gene cluster conducted in parallel to this work, which will be discussed further in Chapter 3.

The goals of this study were to:

1. Confirm a link between microtubule disruption and MRN1 transcription and to identify aspects of the signalling pathway between these two events.

2. Confirm that MRN, CYP71A16, and CYP705A12 are members of a metabolic gene cluster.

3. Identify conditions that induce the production of marneral-related metabolites and to test whether the marneral-related metabolites play a role in the physiological responses to those conditions.
Figure 1. Model describing the role microtubules may play in stress signalling pathways. Transcription factors may bind to microtubules and remain inactive until stresses known to induce a transient microtubule reorientation event occur. These events often are associated with a calcium influx event that may be responsible for the disruption of the microtubules. Once disassembled the transcription factors are free to induce transcription of gene appropriate for the response.
Figure 1. 2 Metabolic pathway leading to the production of triterpenes. Squalene synthase condenses two farnesyl pyrophosphate molecules in a head-to-head fashion to produce squalene. Squalene epoxidase catalyzes the production of 2,3-oxidosqualene from squalene. Each oxidosqualene cyclase commits 2,3-oxidosqualene to specific triterpene pathways.
Figure 1. 3 Amino acid alignment of the likely substrate binding site from multiple OSCs. These include MARNERAL SYNTHASE (AthMRN1), LUPEOL SYNTHASE (AtLUP1) that utilizes the dammarenyl cation in cyclization, CYCLOARTENOL SYNTHASE (AtCAS1) that utilizes the protosteryl cation, and the human LANOSTEROL SYNTHASE (HsERG7) that also utilizes the protosteryl cation. This data is based on an alignment in Xiong et al. (2006).
CHAPTER 2: MICROTUBULE-ASSISTED REGULATION OF TRANSCRIPTION

2.1 INTRODUCTION

Microtubules play roles in many different cellular functions. A new role has emerged as part of a response to various biotic and abiotic stresses. This idea is based on the observations that plant cells react to certain stimuli by rapidly and transiently reorganizing their cortical microtubules. Furthermore, it has been shown that this process can play a critical role in stress responses. For example, wheat seedlings subjected to temperatures low enough to disassemble microtubules are later resistant to freezing. This process can be mimicked using microtubule depolymerizing drugs as opposed to the low temperature treatment suggesting that microtubule depolymerization specifically activates steps to render the plant tolerant to freezing (Abdrakhamanova et al. 2003).

It is possible that microtubules are involved in the signal transduction pathway leading, through transcriptional activity, to the expression of adaptive proteins, as hypothesized by Wasteneys (2004). Microtubules could act as repositories for transcription factors such that their transient disruption could lead to the release and activation of the transcription factors. Indeed, there are some examples of transcription factors associating with microtubules. In animal systems, the Hedgehog signalling pathway is critical for proper development in animal embryos. This pathway is partially regulated by the transcriptional repressor, Costus Interruptus, whose activity is dependent on the association of the kinesin Costal2 with microtubules (Farzan et al. 2008, Sisson et al. 1997). Another example, in plants, involves the transcription factor KNOTTED1 that is targeted to microtubules by MPB2C where it becomes ubiquitinated and subsequently degraded (Winter et al. 2007b). While these are just two examples, other studies investigating protein-tubulin interactions through tubulin affinity chromatography in plants have identified a number of proteins involved in transcription and signal transduction (Chuong et al. 2004, Doroshenk et al. 2009).

In order to identify pathways utilizing microtubule depolymerization, a cDNA microarray experiment was conducted to identify changes in gene expression of pathways controlled by microtubule dynamics. To do this, the temperature-sensitive
mutant mor1-1 was used. In this mutant, shifting the temperature to 31°C dramatically reduces microtubule dynamics, causing loss of microtubule polymer mass, whereas in wild-type plants, microtubules remain well-organized and highly dynamic (Kawamura and Wasteneys 2008, Whittington et al. 2001). Shifting the temperature back to 21°C reverses the disruption. Thus, the mor1-1 mutant phenocopies the transient effects on microtubule arrays that occur when plants are exposed to certain stresses. For the microarray experiment, the transcript abundance of plants with (wild-type) and without (mor1-1) dynamic, well organized microtubules were compared at 2h, 4h, or 8h after the microtubule disruption at the restrictive temperature (Walia 2009). This microarray identified MARNERAL SYNTHASE (MRN1), a gene encoding a monocyclic triterpene synthase (Xiong et al. 2006), as the most highly upregulated gene when microtubule dynamics were disrupted. One of the goals of this work was to confirm the link between MRN1 expression and microtubule polymer status.

A co-expression analysis was conducted to help identify the function of MRN1 metabolites, and it identified two cytochrome P450s that were also identified on the mor1-1 microarray. The increased expression of MRN1, CYP71A16 and CYP705A12 (Marneral Cluster Genes or MCGs) was initially validated using real time PCR on both the original RNA and a separate biological replication. This caused me to ask if these genes play a role in the downstream growth defects observed in the mor1-1 mutant following microtubule disruption. A double mutant analysis of root growth at restrictive temperature using mrn1-1 and cyp71a16-1 each crossed to mor1-1 showed that neither MRN1 nor CYP71A16 play a role in the mor1-1 phenotype. I then began to probe the involvement of microtubules with MRN1 expression levels using mutations in other genes that may have been involved (rsw1 and myb61). However, as I continued to test new biological material, it became apparent in my control treatments comparing mor1-1 to wild type that MRN1 expression fluctuated acutely, but was not tied to microtubule polymer status in mor1-1. A promoter-reporter construct using the MRN1 promoter attached to a fluorescent reporter that predictably shifts from green to red fluorescence over time (Terskikh et al. 2000) was used to further test if there was a relationship between MRN1 expression and microtubule polymer status using both the mor1-1 mutations and the microtubule depolymerizing drug oryzalin. Neither condition was found to induce the expression of MRN1.
2.2 RESULTS

2.2.1 Validation of MRN1 Expression in mor1-1

The original mor1-1 microarray found few genes differentially regulated more than 2-fold. MRN1 expression was initially found to be dramatically upregulated in mor1-1 plants 2h and 8h after the transfer to 30°C in the microarray (Walia 2009) and I initially confirmed this increased expression across all three time points (2h, 4h and 8h) by 2.6 -fold, 11.6 -fold, and 16.7 -fold, respectively (Figure 2.1A). A co-expression analysis found that CYP71A16 and CYP705A12, the next two genes sequentially on the chromosome, were highly co-regulated with MRN1 (Table 2.1). As such, they were also identified as upregulated in mor1-1 from the microarray analysis. This was initially confirmed by real time PCR after 8h at 31°C such that CYP71A16 increased expression by 6.5 -fold and CYP705A12 increased by 10.9 -fold (Figure 2.1B).

2.2.2 Identifying T-DNA Mutants in Marneral Cluster Genes

T-DNA insertion mutants mm1-1 and cyp71a16-1 were acquired from TAIR (SALK_152492 and SALK_073803, respectively) (Alonso et al. 2003) and the presence and location of the T-DNA insertion were confirmed by PCR (Fig 2.2) and subsequent sequencing (data not shown). The T-DNA insertion in mm1-1 is located in the third intron which results in a reduction in the amount of total transcript. The PCR amplified product is directly downstream from the terpene cyclase domain. Its presence indicates that the transcript present in the plant likely results in functional protein (Figure 2.2A, B). The T-DNA insertion in cyp71a16-1 is located in the first exon near the start of the gene and results in a complete loss of transcript (Figure 2.2A, C).

2.2.3 Assessing the Contribution of MRN1 Gene Expression to the mor1-1 Phenotype

In order to determine if the increased expression of MRN1 and CYP71A6 contributed in any way to the strong growth defects presented by the mor1-1 mutant at its restrictive temperature, double mutants between mor1-1 and both mm1-1 and cyp71a16-1 were generated by crossing the parent lines. I hypothesized that if these genes were playing a role in expressing the mor1-1 phenotype at restrictive temperature, then the double mutants would appear more like wild-type plants. This was determined by measuring the root growth of plants shifted from 21°C to 31°C, or not, between 5-9 days of age. When grown at 21°C, both the single (mor1-1, mm1-1)
and double (mm1-1 mor1-1) mutants showed similar root growth. When the plants were shifted to 31°C five days after germination for an extra four days the growth of the roots was slowed, as expected. The presence of the mor1-1 mutation caused a significant reduction in root elongation also, as expected. Wild-type and mm1-1 root lengths were 4.2 and 4.6 cm, respectively, whereas mor1-1 and mm1-1mor1-1 root lengths were 2.9 and 2.7 cm, respectively. Similarly, cyp71a16-1 did not have an altered effect on the root growth of mor1-1 plants at 21°C or 31°C (Figure 2.3).

A series of experiments was conducted in order to identify other components of the regulatory pathway connecting microtubule disruption to MRN1 expression. The controls in these experiments did not show a significant change in MRN1 expression between mor1-1 and wild-type plants in contrast to the original observation. Table 2.2 summarizes the timeline of Real Time PCR-based expression studies and highlights the changes in expression levels as well as any alterations in conditions.

MRN1 expression levels in mor1-1 were first brought into question in an experiment asking if the mutant allele rsw1-1 (a temperature sensitive allele of cellulose synthase A1) would alter the expression of MRN1 in the mor1-1 background. This experiment was initially conducted to probe if the nature of the upregulation of MRN1 in the mor1-1 mutant was related to the downstream defects in cellulose. Surprisingly, in this experiment there was no difference in MRN1 expression in any of the samples in tissue 8h after the microtubules were disrupted and/or when cellulose synthesis was reduced (Figure 2.4). It was then considered that the lack of differential regulation could have been due to the fact that the plants were grown under continuous light, since in the initial microarray experiment conducted by Ankit Walia (2009), the plants had been grown in a 16h light - 8h dark cycle. Under the 16 h light -8 h dark cycle, two of three biological replicates showed a MRN1 induction of 3.5 and 4.2 -fold, but was not nearly to the extent that was initially observed. Furthermore, the third replicate showed a 12.2-fold downregulation in mor1-1. MRN1 expression was only minimally altered when the plants were raised under continuous light showing -1.9, -2.4, and +1.1 -fold differences (Figure 2.5). This seemed to suggest that there is a diurnal pattern to the expression of MRN1. This was supported by the strong increase in expression levels of MRN1 in a microarray comparing expression between wild type and MYB61 over-expressing plants (M. Campbell, University of Toronto, personal communication). MYB61 expression
naturally follows a strong diurnal pattern with the highest level of expression at the end of night (Liang et al. 2005). Semi quantitative PCR was used to test the diurnal expression pattern of MRN1 over five time points within a 24 h period under a 16 h photoperiod to show how the expression pattern changed. A day is considered completed at the end of the 16 h light phase and therefore the beginning of a day is at the beginning of the dark phase. Five time points were checked within a 24 h period: 8 days + 8 h light; 8 days + 15:35 h light; 9 days + 00:15 h dark; 9 days + 4:00 h dark; 9 days + 7:30 h dark; 9 days + 00:15 h light. Figure 2.6 confirms that MYB61 has the strongest expression at the end of the dark phase (Liang et al. 2005), whereas MRN1 expression was low, but did not fluctuate over the 24 h period, nor did it mimic expression of MYB61. Transcription factors are often post-translationally regulated thus it was important to test the effect of MYB61 mutations on the expression of MRN1. This was tested by treating myb61-1 with oryzalin and also generating mor1-1 myb61-1 double mutants. Isogenic lines selected from F2 plants included an azygous line, both single mutants, as well as the double mutant. The plants were exposed to 31°C for 4 h prior to harvest and grown under a 16 h light 8 h dark cycle. These plants did not show any differential regulation of MRN1, nor did the treatment with 1 mM oryzalin, a microtubule depolymerizing drug, change MRN1 expression (Figure 2.7). Due to the increasing number of experiments that did not show upregulation of MRN1 after microtubule disruption and because certain growth aspects of the microarray tissue were ambiguous, two different conditions were altered including planting density and whether or not growth of the mor1-1 and control plants on the same plates affected the expression of MRN1. Expression levels were initially qualitatively determined using semi-quantitative PCR, and if strong differences were apparent, then real time PCR was conducted. There was no difference in MRN1 expression found in any of the treatments even when comparing mor1-1 tissue to wild-type (Figure 2.8). In the samples with the strongest MRN1 upregulation (Fig 2.8 B rep2), MRN1 was quantified by real time PCR to only be upregulated 2.0 -fold (Figure 2.8 F).

2.2.4 Promoter Analysis

A promoter-reporter construct was built as an alternate approach to determining changes in expression of MRN1 after altering microtubule dynamics due to the discrepancy between the original and subsequent expression experiments, as well as screening for other MRN1 activating stimuli (see Chapter 4). The dsRED variant E5,
also known as TIMER because the protein’s fluorescence predictably shifts from green to red over time (Terskikh et al. 2000), was attached 4205bp upstream of MRN1. Four independent transgenic $pMRN1_{4205}$::TIMER lines were isolated from the progeny of Agrobacterium-transformed plants and confirmed to be homozygous through antibiotic resistance in the T3 lines. These lines were then examined to identify the general expression profile for MRN1 and compare it to available bioinformatics data using Genevestigator (Zimmerman 2004) and Arabidopsis eFP browser (Brady et al. 2007). Publicly available microarray analyses suggest that expression peaks in 6 to 13 day-old tissue and is isolated in the older regions of the root (Figure 2.9). The eFP data (Brady et al. 2007) also suggests the strongest expressing tissues are the cortex and procambium tissues. Analysis of fluorescence patterns in root tissues suggested that expression of $pMRN1_{4201}$::TIMER was strongest within the vascular cylinder and there was little or no signal present in the cortical tissue (Figure 2.10). While this pattern did not match up with the eFP data, the region of expression in the root was in agreement, i.e. the longitudinal expression pattern. The oldest part of the root showed moderately high expression (the region approximately 5 mm from the root/hypocotyl junction) compared to younger tissue (at the beginning of the region where root hairs are fully elongated). Since the TIMER protein shifts from green (emission 500 nm; TIMER$_{\text{green}}$) to red (emission 583 nm; TIMER$_{\text{red}}$) fluorescence over time, red fluorescence was examined in the older root tissue. When non-transgenic wild-type Col-0 roots were examined there was often strong red autofluorescence in the same tissues in which TIMER was expressed in the reporter line. This autofluorescence, however, appeared as small foci with diffuse fluorescence throughout the interior of the root. TIMER$_{\text{red}}$ fluorescence was only discernible from the autofluorescence when looking at TIMER$_{\text{green}}$ as well (Figure 2.11). While this prevented the determination of localization of TIMER$_{\text{red}}$, the nature of the fluorescent shift guaranteed that imaging TIMER$_{\text{green}}$ provided only the youngest protein, and finding differences in subsequent treatments would most likely indicate induction or suppression from the various treatments.

$pMRN1_{4205}$::TIMER line 5 was crossed into mor1-1 plants and lines homozygous for $pMRN1_{4205}$::TIMER line 5 and both homozygous and azygous for mor1-1 were segregated from the F2 seeds. Since MRN1 expression is strongest 10-14 days after germination, it was important to check if MRN1 induction could also be induced outside
of this timeframe. I examined the fluorescence profile of plants at 5, 10, and 15 days after germination and placed the plants into 30°C conditions for 4h prior to microscopic analysis. Figure 2.12 shows TIMER\textsubscript{green} fluorescence (under false colour to highlight any differences in intensity) between the \textit{MRN1\textsubscript{4205}::TIMER-5} with or without the \textit{mor1-1} allele. The lines were grown under similar conditions as in the \textit{mor1-1} microarray analysis, under 16/8 light/dark diurnal cycles at 21°C until the temperature shift (Walia 2009). The range of fluorescence was similar with or without the \textit{mor1-1} mutation.

In addition to the induction of \textit{MRN1} expression in the \textit{mor1-1} mutant, Walia (2009) also showed an induction of \textit{MRN1} in five day-old seedlings treated with oryzalin. In order to test this, the \textit{MRN1\textsubscript{4205}::TIMER-5} fluorescence was observed after treatment of 5, 10, and 15 day-old seedlings with 1 mM oryzalin for 4h (Figure 2.13). It appears as if there could be a slight increase of TIMER\textsubscript{green} in the 1 mM oryzalin-treated roots across all three ages therefore I quantified any changes in expression with real time PCR over a larger population of roots. I also tested if \textit{CYP71A16} or \textit{CYP705A12} were induced by the oryzalin treatment (Figure 2.14). None of the plants showed greater than a 1.5-fold change in expression level at any age, similar to the results of the controls for the \textit{myb61-1} experiment mentioned previously.

2.3 DISCUSSION

2.3.1 Growth of \textit{mor1-1} is Not Influenced by \textit{MRN1} or \textit{CYP71A16}

A \textit{mor1-1} microarray analysis originally found \textit{MRN1} as the most highly upregulated gene after the disruption of microtubule dynamics (Walia 2009). After initially confirming this using real time PCR, it seemed reasonable that secondary metabolic pathways would be initiated in response to many of the stresses microtubule reorganization events are known to be a part of, such as salt and drought stress, cold temperatures, or metal ion toxicity.

The \textit{mor1-1} phenotype is most pronounced in elongating cells, of all types, where microtubules are not able to maintain growth anisotropy. \textit{MRN1} was not expected to be involved in expression of the \textit{mor1-1} phenotype because \textit{MRN1} expression is limited to root cells that have slowed or ceased elongation. Furthermore, the marneral pathway is unique to Arabidopsis whereas the loss of microtubule dynamics has similar effects on cell expansion in most species. Even with these facts in mind, it was important to
determine if the increased expression of MRN1 or CYP71A16 was responsible for any part of the mor1-1 phenotype. I found no change in growth between mor1-1 plants with or without mutations in MRN1 or CYP71A16. While it is possible that the MRN1 transcripts still present in mm1-1 could be sufficient to help manifest the mor1-1 phenotype, subsequent expression analysis decoupling the link between microtubule disruption and MRN1 expression strongly suggests that MRN1 is not involved.

2.3.2 MRN1 is Not Differentially Regulated by Microtubule Polymer Status

While real time PCR analysis on the original sample of RNA confirmed that MRN1 was upregulated, I began to probe this phenomenon using mutations in genes that might have been involved (rsw1-1 and myb61-1). rsw1-1 was tested to determine if MRN1 expression was related to alterations in cellulose function as a result of microtubule disruption (Williamson et al. 2001). If this were the case we would expect increased MRN1 expression in either the rsw1-1 mutant or a lack of MRN1 expression in the rsw1-1 mor1-1 plants. This could not be tested because the upregulation of MRN1 in mor1-1 plants at restrictive temperature did not occur. It was found on Genevestigator that plants overexpressing MYB61 have a much higher level of MRN1 expression (Zimmerman et al. 2004). Since MYB61 is thought to be responsible for inducing the closure of stomata in response to drought (Liang et al. 2005), a stimulus that affects microtubule polymer status, it seemed possible that MYB61 could be a transcription factor involved in the induction of MRN1 expression in mor1-1. However, as I began to conduct these experiments, it became apparent through control treatments that MRN1 expression was not tied to microtubule polymer status in mor1-1 although it fluctuated acutely. During the testing of different growth conditions, as well as the double mutant analysis with myb61-1 mor1-1 and rsw1-1 mor1-1, only a weak or no induction of expression in mor1-1 occurred. This was also confirmed in the reporter construct pMRN14205::TIMER. This leaves two possibilities for the original observation: MRN1 displays such large swings in expression, especially in relation to age, that it is possible that the original plants used for the microarray analysis were not from comparable seeds, i.e. the parent plants were grown under different conditions and/or the seeds were stored separately resulting in differences in germination/growth rates between seeds. The other possibility is that MRN1 expression is linked to microtubule polymer status but requires a second or more specific stress that was not mimicked in
my experiments, similar to a Ca$^{2+}$ signature which is able to play a role in diverse signal transduction pathways using a single signalling molecule (McAinsh and Pittman 2008). In fact, some of the stresses that induce microtubule reorganization events also involve Ca$^{2+}$ influx, for example in the response to aluminum in Arabidopsis roots (Sivaguru et al. 2003) or in the response of grape cells to the bacterial elicitor Harpin (Qiao et al. 2010). It would not be surprising to find out that the status of microtubules/tubulin in response to a given stress is specific to each stress.

2.3.3 MRN1 Expression Pattern

According to an analysis of a fluorescent reporter, MRN1 expression is strongest in the vascular cylinder in the root differentiation zone, where cells have ceased elongating. These expression data agree temporally and longitudinally in the root with the available bioinformatic data, but MRN1 expression is also predicted to be in the cortex according to both protoplasting/fluorescence activated cell sorting expression data (Brady et al. 2007). This data is brought into question by the possible effects of the protoplasting process itself on transcription. The authors controlled against this by comparing transcripts between protoplasted root cells and an identical treatment not subjected to protoplasting, and in so doing identified 356 genes induced by protoplasting (Brady et al. 2007). This control, however, might not pick up subtle inaccuracies such as an incorrect radial expression pattern (but the correct longitudinal pattern) as I have shown for MRN1. Alternatively, there could be regulatory elements outside of the promoter region used in the reporter lines that could control the tissue level expression.

2.3.4 Conclusion

These data indicate that microtubule disruption is not sufficient to induce MRN1 transcription. This was shown using both real time PCR analysis and a promoter-reporter construct in the temperature sensitive mor1-1 mutant, in which microtubules rapidly disassemble upon exposure to 31°C conditions, as well as the treatment with the microtubule depolymerizing drug oryzalin. This discrepancy in data cannot rule out the possibility that the induction of MRN1 by changes in microtubule dynamics occurs with an unknown secondary factor. If this were the case I show that planting density, neighbouring plants, or diurnal cycle conditions are not involved. It will be interesting to identify conditions in which MRN1 expression is upregulated, since many stress
response pathways involve a microtubule reorganization event. Furthermore, the functions of manerl-related metabolites are unknown, yet the observation that two neighboring CYPs are highly co-expressed is suggestive of a metabolic gene cluster, in which all three genes function enzymatically in the same biochemical pathway. Their activity must still be identified in order to confirm the existence of the manerl gene cluster.

2.4 MATERIALS AND METHODS

2.4.1 Plant Growth Conditions

Plants were grown for ten days on flat plates containing 3% sucrose in Hoagland’s medium in 0.7% agar. The plants were grown under a 16h day/8h night light regime at 21°C and on the 10th day were moved to 29°C for the appropriate amount of time before harvest. The plants were carefully removed from the agar and frozen in liquid nitrogen before being placed in a frozen 50 ml falcon tube and stored at -80°C until the RNA was extracted. Replicates of paired genotypes (i.e. mutant vs. wild-type) were grown in opposite halves of the same Petri dishes in order to control for any variances in media quantity between plates unless otherwise noted. Furthermore, one to three plants of each genotype were left growing at 29°C for three extra days to be certain that plants were homozygous for the mor1-1 allele. Similarly, one to three plants were left on plates treated with microtubule-destabilizing drugs for an extra three days to qualitatively confirm the drug’s efficacy.

2.4.2 RNA Extraction Protocols

RNAeasy plant mini Kit (Qiagen) extraction (i.e. Silica Column): This protocol was used to extract RNA for the initial microarray validation experiments. The kits were used according to the manufacturer’s instructions. There was a tendency to have significant DNA contamination in the samples. Therefore, I would conduct an On-Column DNase Digestion (Qiagen) according to the manufacturer’s protocol.

Trizol RNA extraction (Invitrogen): RNA was extracted according to the manufacturer’s instructions.

Combined Trizol and Silica Column extraction: This protocol is adapted from a protocol used for total RNA extraction of *Helianthus* sp. as outlined in Lai et al. (2006). Approximately 100 mg of frozen homogenized tissue was added to 1.5 ml Eppendorf
tubes (to approximately 400 µl) and extracted with 1 ml of Trizol reagent according to the manufacturer’s instructions. After phase separation with CHCl₃, the aqueous phase was mixed with 0.53x volumes of 100% ethanol, mixed, and applied to a silica column (Epoch Biolabs). The RNA was bound to the column after a 30 second centrifugation. To be certain that the samples were free of DNA contamination, the samples were always treated with the On-Column DNase digestion set (Qiagen) according to the manufacturer’s protocol and the columns were subsequently treated according to the RNAeasy Plant RNA extraction Kit (Qiagen) protocol. It is important to note that two extra centrifugation steps were needed to completely remove the residual ethanol from the final wash of the column in Wash buffer (70% ethanol in RNase free water). After the RNA was eluted from the columns, the RNA quantity and solution purity were determined using a standard spectrophotometer or a nanodrop spectrophotometer to determine the 260/280 and 260/230. The RNA quality and spectrophotometer accuracy were determined by running 1 µg of total RNA on a TAE gel containing ethidium bromide and assessing the clarity and evenness of intensities of the rRNA bands.

2.4.3 Real Time PCR

All materials used for real time PCR were prepared in accordance with the MIQE guidelines for qPCR (Bustin et al 2009). Total RNA was extracted as described above. First strand cDNA synthesis was conducted using Superscript II or Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Between 1 µg and 4 µg of total RNA was used during each reaction. The reactions were primed with oligo dT₁₈ primers. When the total RNA extraction from any sample in a particular experiment yielded less than 90 ng/µl (the minimum concentration to use 1 µg in a standard 20 µl reaction) the total RNA was lowered across all samples or that sample was concentrated with a silica column or precipitated and resuspended in a smaller volume. The PCR was conducted on either an MJ Mini Thermalcycler with a continuous fluorescence detector (BIORAD) and was analyzed using the Opticon MONITOR 3 analysis software (BIORAD) or a MyiQ 5 Real Time PCR Detection System (BIORAD) coupled with the iQ5 software. Reactions were mixed using the iQ SYBR Green Supermix (BIORAD) and conducted in quadruplicate. Analysis conducted using the MJ mini Thermalcycler were normalized by taking the average Cₚ value from four replicate PCR reaction from primers targeting a reference gene (UBQ10 or ACT8) according the ΔΔCₚ method of quantification. The Cₚ value (quantification cycle) refers
to what is also known as threshold cycle or crossing point (Ct or Cp respectively) and is determined based on when the fluorescent intensity of a reaction passes a certain threshold. My threshold on all experiments conducted on the MJ mini were manually adjusted to the lowest possible setting while allowing the standard curve to produce the optimal R² value as well as the optimal efficiency value. For experiments using the ΔΔC_q method for quantification, the R_q values representing the theoretical maximum and minimum relative expression values were calculated and expressed as error bars in the corresponding figures. This is determined by taking the standard deviation of the pool of technical replicates of the target and reference gene C_q values with the following formula.

\[ s = \sqrt{S_{\text{target}}^2 - S_{\text{reference}}^2} \]

These values are calculated for both the test and control samples and the standard deviation of the ΔΔC_q is calculated by:

\[ \Delta \Delta C_q = \Delta C_q (\text{test}) - \Delta C_q (\text{control}) \]

These values were then incorporated into the ΔΔC_q values to show the maximum or minimum fold changes of each sample by \(2^{\Delta \Delta C_q \pm s}\), where s is the standard deviation of the ΔΔC_q. The experiments conducted on the MyiQ5 machine used a consistent value of 42 which always crossed the logarithmic portion of each reaction.

For real time experiments following MRN1_2401::TIMER analysis, the Pfaffl method of quantification was used as it removes the assumption that all primers amplify to the same efficiency by including the efficiency of each primer set as opposed to the ΔΔC_q method (Pfaffl 2001). This is calculated by the following equation:

\[ \text{Fold Change} = \frac{(Eff_{\text{target}})^{\Delta C_q_{\text{target}}(\text{control} - \text{sample})}}{(Eff_{\text{ref}})^{\Delta C_q_{\text{ref}}(\text{control} - \text{sample})}} \]

The standard deviation of the fold change values of three separate biological experiments were calculated for experiments analyzed under this method. The efficiencies of each primer set were calculated by mixing 5 µl of each sample of cDNA samples from 5, 10, and 15 day-old; ethanol control, 10 mM MeJA, and 10 mM ABA treated tissues (discussed in Chapter 4). Serial dilutions (5-fold) were made and used as a template in Real Time PCR reactions to create a standard curve. The slope of this
curve was then used to calculate the efficiency of the primer pairs in the following equation:

\[ E_{ff} = 5^{1/m} \]

These efficiencies are reported in appendix 1. PCR programs are reported in appendix 2.

2.4.4 Double Mutant Analysis

The \textit{mm}1-1 and \textit{cyp}71a12-1 mutants were crossed to \textit{mor}1-1 plants and double and single mutants as well as azygous control lines were segregated from each cross. Alleles were identified by PCR, for primers and reaction conditions see appendix 1 and 2, respectively. Root lengths were measured and a Student t test was used to compare the effects of each mutation. Plants were grown for five days at 21°C under 16h light/8h dark cycle then shifted to 31°C under the same light cycle. The plates were scanned on a flatbed scanner daily and measurements were made on the resulting images using Image J (http://rsb.info.nih.gov/ij/).

2.4.5 Cloning the \textit{pMRN4201::TIMER} Construct

The \textit{MRN1} promoter was cloned using a PCR fragment as well as KPN1 fragments of the MIFO20 bacterial artificial chromosome (TAIR). PCR could only amplify 689bp upstream of the start site, presumably due to the high AT percentage of the promoter. Attempts to amplify longer fragments failed even with the addition of 60 mM TMAC (tetramethyl ammonium chloride), which has been shown to ameliorate amplification of AT-rich templates (Chevet et al. 1995). The genomic region between At5g42600 (\textit{MRN1}) and At5g42590 (\textit{CYP71A16}) spans two available BACs, with MIFO20 covering 12.5 kb upstream from \textit{MRN1}. KPN1 was used to digest MIFO20 because there are only six \textit{Kpn I} sites on the BAC, four of which cover up to 9.5kb of the promoter. The BAC was digested, purified, and ligated into \textit{Kpn I} digested and dephosphorylated pBSI-KS. After transformation into DH10B cells useful colonies were identified based on a restriction analysis. pBS-Fragment I spanned from -2401 to +3710 into the \textit{MRN1} gene. pBS-Fragment II spanned from -6664 to -2401 in the \textit{MRN1} promoter. pBS-Fragment III spanned from -9534 to -6664. The small PCR fragment from -689 was subcloned into the BamHI-BseGI fragment of pRL-null to make \textit{proMRN1689::LUC}. A \textit{Kpn I} to \textit{HindIII} fragment was isolated from pBS-Fragment I and ligated into the same sites of a partially digested \textit{proMRN1689::Luc} plasmid to produce
proMRN12401::LUC. The following steps were conducted by Paulus Parkboswachter den Hollander (Wageningen University) under my supervision. The Luciferase gene was removed with NotI and PstI and PCR was used to produce the pTIMER insert with appropriate ends for ligation using Alw21I and Eco 31I (Fermentas). The cassette was removed from the parent plasmid with Xhol and EcoRI and was subcloned into the SalI and EcoRI sites of pCAMBIA1390 containing the pTIMER fluorophore, which is the E5 variant of dsRED (Clonetech). This construct was introduced into Col-0 plants using the floral dip method (Clough and Bent 1998). Independent transformants were isolated by screening on agar nutrient plates containing Hygromycin (25 µg/mL) and single insert homozygous plants were isolated by screening 60 T2 seedlings on hygromycin looking for a 3:1 segregation ratio. I then screened 30 T3 seedlings from up to eight separate T2 progeny on Hygromycin-supplemented media and identified homozygous lines from those displaying 100% survival.
Figure 2. 1 Real Time PCR validation of the mor1-1 microarray. 

**A.** Expression of *MRN1* in mor1-1 compared to Col-0 at 2, 4, and 8 h after the shift to restrictive temperature (30°C) relative to *UBQ10*. 

**B.** Expression of *CYP71A16* and *CYP705A12* in mor1-1 8 h after the shift to restrictive temperature relative to *UBQ10*. Data is representative of two biological replicates. Error bars represent ΔΔCq± s.
Table 2. Expression analysis of genes co-expressed with *MRN1* (Toufighi et al. 2005)

<table>
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<tr>
<th>Locus ID</th>
<th>Gene name</th>
<th>r-value</th>
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<tbody>
<tr>
<td>At5g42600</td>
<td>Manneral Synthase</td>
<td>1</td>
</tr>
<tr>
<td>At5g42590</td>
<td>CYP71A16</td>
<td>0.921</td>
</tr>
<tr>
<td>At5g42580</td>
<td>CYP705A12</td>
<td>0.865</td>
</tr>
<tr>
<td>At5g35940</td>
<td>Mannose-binding lectin superfamily</td>
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<td>At4g15340</td>
<td>PEN1</td>
<td>0.831</td>
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<td>At3g59710</td>
<td>NAD(P)-Binding Rossmann-fold superfamily protein</td>
<td>0.831</td>
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<td>At5g48010</td>
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<tr>
<td>At5g23840</td>
<td>MD-2-related lipid recognition domain-containing protein</td>
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Figure 2. 2 RT-PCR analysis of T-DNA insertion mutants in MRN1 and CYP71A16. A. Diagram showing the location of the T-DNA insertion. Grey arrowhead indicates presumed catalytic site of MRN1. Not to scale. Red region indicates the amplified portion of the cDNA used in the RT-PCR analysis B. *mrn1-1* shows a reduction of *MRN1* transcript level. Semi-quantitative PCR gel showing low expression of *MRN1* after 35 PCR cycles from *mrn1-1* tissue as compared to wild type. Evidence of PCR product likely indicates functional transcript. C. *cyp71a16-1* lacks expression. Semi-quantitative PCR gel showing an absence of expression of *CYP71A16* after 35 PCR cycles from *cyp71a16-1* tissue as compared to wild type. B-C *Actin 8* expression is shown as an internal control after 23 PCR cycles.
Figure 2. Double mutant analysis of root length at 21°C and 31°C. Single mutants do not mimic the mor1-1 root growth phenotype, nor do double mutants enhance the mor1-1 phenotype at permissive (21°C) or restrictive (31°C) temperature. Plants were grown for 5 days at 21°C and transferred (or not) to 31°C. A. mnr1-1. B. cyp71-1. Bars are ± standard error of the mean.
**Table 2. Summary of real time PCR experiments.** This table shows the variation in results from Real Time PCR of different replicates/treatments. The RNA source describes the experiment/replicate for each data set. When data is extrapolated from a double mutant experiment, it only represents MRN1 expression levels compared between mor1-1 and wild type tissue. The changes in MRN1 expression column describes the differences in expression found, if any, and is colour coded according its agreement with microarray data. Green indicates an agreement with microarray data, yellow indicates partial agreement with the microarray data, red indicates no agreement with the microarray data. The quality score is based on the efficiency of the standard curve reactions, accuracy of the $r^2$ value (i.e. Pearson Coefficient of Determination) from the standard curve, and presence or absence of contamination in one or more primer pairs. Quality scores of A have efficiencies between 95-105%, $r^2$ values better than 0.99, and no contamination in the negative controls. B have efficiencies between 85-115%, $r^2$ values better than 0.99, contamination in blanks that appear after the 30th cycle. C have efficiencies between 85-115%, $r^2$ values better than 0.985, and contamination may be present after the 27th cycle. The RNA extraction column shows the method used to extract the RNA. Figure showing column refers to the figure where this data is shown. RNA extractions marked with an * were extracted by Ankit Walia.
<table>
<thead>
<tr>
<th>RNA source</th>
<th>Changes in MRN expression</th>
<th>Experimental Conditions</th>
<th>Quality</th>
<th>RNA extraction</th>
<th>Figure showing</th>
</tr>
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<tbody>
<tr>
<td>RNA from microarray</td>
<td>+8-3 fold in mor1-1</td>
<td>8 h at 30°C under 16/8 light/dark cycles on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>C</td>
<td>RNAeasy kit*</td>
<td>not shown</td>
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<tr>
<td>1st Biological Replicate after Microarray</td>
<td>+2.6-fold in mor1-1</td>
<td>2 h at 30°C under 16/8 light/dark cycles on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>C</td>
<td>RNAeasy kit*</td>
<td>2.1</td>
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<tr>
<td>1st Biological Replicate after Microarray</td>
<td>+11.6-fold in mor1-1</td>
<td>4 h at 30°C under 16/8 light/dark cycles on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>B</td>
<td>RNAeasy kit*</td>
<td>2.1</td>
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<tr>
<td>1st Biological Replicate after Microarray</td>
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<td>8 h at 30°C under 16/8 light/dark cycles on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>B</td>
<td>RNAeasy kit*</td>
<td>2.1</td>
</tr>
<tr>
<td>rsw1/mor1-1 experiment first replicate</td>
<td>No difference</td>
<td>4 h at 29°C under 24 hour light horizontally on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>C</td>
<td>RNAeasy kit</td>
<td>not shown</td>
</tr>
<tr>
<td>1st replicate comparing 24hr light regime to 16/8 light/dark in mor1-1</td>
<td>No difference under 24hr light; +3.5-fold under 16hr light</td>
<td>4 h at 29°C under variable light conditions on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>A</td>
<td>RNAeasy kit</td>
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<td>2nd replicate comparing 24hr light regime to 16/8 light/dark in mor1-1</td>
<td>-2.9-fold under 24hr light; +4.2-fold under 16hr light</td>
<td>4 h at 29°C under variable light conditions on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>B</td>
<td>RNAeasy kit</td>
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<td>3rd replicate comparing 24hr light regime to 16/8 light/dark in mor1-1</td>
<td>No difference under 24hr light; -12.2-fold under 16hr light</td>
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<td>RNAeasy kit</td>
<td>2.4</td>
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<td>1st and 2nd replicates comparing expression in WT, mor1-1, myb61-1, and mor1-1/myb61-1</td>
<td>No difference with or without the myb61-1 mutation</td>
<td>4 h at 31°C under 16hr light conditions on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>A</td>
<td>Trizol</td>
<td>2.6 B</td>
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<tr>
<td>Experiment checking the growth on the same plates as well as plant density</td>
<td>+2.1-fold in mor1-1</td>
<td>4 h at 31°C under 16hr light conditions on Hoagland’s media with 3% sucrose and 0.8% agarose</td>
<td>B</td>
<td>Trizol</td>
<td>2.7 F</td>
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Figure 2. 4 MRN1 expression analysis upon microtubule disruption and reduced cellulose deposition when grown in continuous light conditions. The real time PCR analysis shows that MRN1 is differentially expressed by less than 2-fold in mor1-1, rsw1-1, and mor1-1 rsw1-1 after transfer to 31°C for 8 h. The only difference in growth conditions to the original microarray tissue was that the plants were grown for 10 days under continuous light, as opposed to 16/8 day/night cycle. Error bars represent ΔΔCq± s.
Figure 2. 5 Expression of *MRN1* at restrictive temperature in plants grown in different light regimes. Bars indicate fold change in expression between *mor1-1* and wild type at 29°C for 4 h in either 16h days/8h nights (blue) or in continuous light (red). A, B, C indicate three separate biological replicates. Error bars represent ΔΔCq± s.
Figure 2.6  Semiquantitative PCR analysis showing the expression of *MRN1* over day and night cycle. Image is of a semi-quantitative PCR gel showing how expression changes over a 24 h period. *MYB61* expression is shown as a positive control as its expression is known to be induced in the dark and quickly repressed at the beginning of a new light cycle (i.e. 15 minutes light). *ACT8* was included as a reference gene and expression should remain constant.
Figure 2.7 Real Time PCR analysis of *MRN1* expression after microtubules are disrupted in *myb61-1*.  

**A.** Real Time PCR analysis of 10 day-old plants treated with 1 mM oryzalin for 4 hours. Expression data is relative to the expression levels of DMSO treated plants.  

**B.** Real Time PCR analysis of 10 day-old double mutant plants *myb61-1 mor1-1* after the transfer to 31°C for 4 h. Expression data is relative to the expression levels of azygous plants.  

A and B normalized against *ACT8*. Error bars represent ΔΔCq±s.
Figure 2. 8 Expression analysis of MRN1 relative to planting density. A-E Semiquantitative PCR analysis comparing planting density (B-D) or co-planting (D-E) different genotypes on the same plate along with treatments at 31°C for 4 h. Expression differences of MRN1 are compared between wild type and mor1-1. ACT8 was used as a reference gene. The MRN1 PCR reaction completed 30 cycles and ACT8 reaction completed 24 cycles. A. Control PCR reactions. B-D Wild type and mor1-1 plants grown on separate plates. B. Low density planting (60 plants/plate). C. Medium density plant (150 plants/plate). D. High density planting (250 plants/plate). E. Wild type and mor1-1 plants planted on the same plate under high density (125 plants/genotype/plate). For B-E “1” and “2” represent separate biological replicates for each treatment. F. Real time PCR analysis on replicate 2 from B, low density planting. Error bars represent ΔΔCq± s.
Figure 2.9 Bioinformatics data showing the expression profile of MRN1. A. Genevestigator graphical representation of the developmental expression pattern of MRN1 showing the strongest expression between 6 and 13 days after germination (Zimmerman 2004). B. Genevestigator anatomical expression profile showing that MRN1 expression is isolated to roots, and is more prominent in older roots. Blue heat map key is true for A and B. C. eFP browser graphical representation of root cell type expression profile of MRN1 (Brady 2007). Note this graphic is based on the same data represented in “root cell” section of “B”. This shows the strongest expression in cortical cells and procambial cells.
Figure 2. 10 MRN1 promoter expression analysis in roots of young seedlings. A. Expression of pMRN425::TIMER between 7 and 14 days after germination. Older tissue is approximately 5 mm from the root-hypocotyl junction and younger tissue is in the region of the root hair zone where root hairs have ceased elongating. B. Autofluorescence of non-transgenic wild type plants at 7 or 14 days after germination. Bars = 100 µm.
Figure 2. 11 Red fluorescence of \textit{pMRN4205::TIMER}. Fluorescence in \textit{pMRN_{4205}::TIMER} plants (top) is slightly more diffuse, which likely results from the cytoplasmic localization of TIMER within the vascular cylinder. This is highlighted by the additional green fluorescence on the right. Nontransgenic wild type shows red fluorescent puncta in similar tissues that TIMER is present. Scale bars = 200 µm.
Figure 2. 12 Expression profile of \textit{pMRN}_{4205}::\textit{TIMER} in wild type and \textit{mor1-1} roots after incubation at 30°C. A. Key showing the regions photographed along with the eFP browser expression data for \textit{MRN1}. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Wild type plants (left) and \textit{mor1-1} plants (right in B, middle in C and D) expressing \textit{pMRN}_{4205}::\textit{TIMER} or wild type (absent in B right in C and D) as a control for autofluorescence. Images were pseudocoloured after acquisition to more easily view differences in fluorescence. Only the fluorescence at 488nm was imaged in order to only account for new fluorescent protein (\textit{TIMER}_{\text{green}}). Three replicates are shown to provide a range of expression B. 15 day-old plants. C. 10 day-old plants D. 5 day-old plants. Scale bars = 150 µm.
Figure 2. 13 Expression profile of pMRN4205::TIMER treated with oryzalin for 4 h. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Plants treated for 4 h with DMSO (left) or 1 mM oryzalin in DMSO (right). After image acquisition images were pseudocoloured to more easily view differences in fluorescence. Only the fluorescence at 488nm was imaged in order to only account for new fluorescent protein (TIMERgreen). Non-transgenic wild type plants were always imaged under similar conditions to account for autofluorescence. Three replicates are shown to provide a range of expression B. 15 day-old plants. C. 10 day-old plants D. 5 day-old plants. Scale bars = 150 µm.
Figure 2. Real time PCR analysis of the effect of oryzalin on the expression of *MRN1*, *CYP71A16*, and *CYP705A12*. Plants were treated with 1 mM oryzalin or DMSO for 4 h prior to harvest. Data shown is an average of three biological replicates. Bars represent ± standard error of mean fold change values.
CHAPTER 3: CHARACTERIZATION OF THE MARNERAL GENE CLUSTER

3.1 INTRODUCTION

Metabolic gene clusters are groupings of phylogenetically unrelated genes on a chromosome that metabolize products in a common pathway. They likely result from the evolutionary benefits associated with co-inheriting a suite of genes or co-regulating the suite of genes. The linkage between genes in a metabolic gene cluster would facilitate the inheritance of a complete pathway. It has been found that genes whose products function epistatically build and maintain strong linkages (Nei 1967). Furthermore, it has been proposed that toxic intermediates help to maintain metabolic gene clusters by lowering the fitness of offspring that do not inherit a complete cluster and thus maintaining the linkage of the functional cluster in those that do (Chu et al. 2011). This is exemplified in a gene cluster in *Avena strigosa* comprised of eight genes that lead to the production of the saponin avenacin A-1, which confers an evolutionary advantage through defence (Mylona et al. 2008). The *sad3* mutant, with a defect in an allele of one of the avenacin cluster genes, accumulates monodeglucosyl avenacin A-1 that results in stunted roots and a reduced number of root hairs. This cluster may have been formed and maintained by the positive effect from the presence of the saponin, but also by the negative effect from the accumulation of the toxic intermediate found in *sad3*.

Co-regulation has also been implicated in maintenance of metabolic gene clusters. It has been shown that the chromosomal region in which the avenacin cluster mentioned above occurs is only decondensed in the nuclei of root epidermal cells in which avenacin production occurs (Wegel et al. 2009). The chromatin remains condensed in tissues not expressing the biosynthetic genes. These observations suggest that clusters have evolved and are maintained through genetic selection because of the advantages conferred by co-inheriting a set of genes in addition to the regional sub-chromosomal regulation of gene expression.

After the co-expression analysis was conducted on *MRN1* to help elucidate a function for the triterpene, two cytochrome P450-encoding genes, *CYP71A16* and *CYP705A12*, were identified that were highly co-regulated and located sequentially on
the chromosome (Table 2.1). In order to characterize MRN1, CYP71A16, and CYP705A12 as members of a metabolic gene cluster, the sequential biochemical activities of these respective enzymes must be shown. Yeast expression systems are commonly used to explore the activity of new triterpene synthase genes found in nature. The yeast strains used typically have a mutation in the only oxidosqualene cyclase (OSC) in their genome, LANOSTEROL SYNTHASE (Kushiro 1998). Without this gene, the yeast will accumulate 2,3-oxidosqualene, the precursor to all triterpenes, for cyclization by the introduced triterpene synthase. It is unclear if the plant OSCs are as efficient in yeast expression systems, or whether they will produce similar mixtures of compounds from multifunctional enzymes. The utility of these heterologous systems also becomes questionable when multiple enzymes are expressed constituting different classes of enzymes. For example, Kribii et al. (1997) identified SQUALENE SYNTHASE 1 as the predominantly expressed squalene synthase in Arabidopsis, but found that the native C-terminus blocked activity in yeast. It was only after this region was replaced with one from the Schizosaccharomyces pombe SQS that the gene could rescue the mutant yeast (strain 5302). Furthermore, downstream reactions with enzymes such as CYPs require cytochrome P450 reductases to provide electrons for their activity (Urban et al. 1997). It would therefore be useful to develop an in planta expression system to characterize multistep pathways such as metabolic gene clusters to avoid these inconsistencies between organisms.

In order to identify the activity of each Marneral Cluster Gene (MCG) (i.e. MRN1, CYP71A16 and CYP705A12), the cDNAs of these genes were cloned into a multicistronic vector that includes the Foot and Mouth Disease Virus -2A sequence, which results in a single RNA molecule that produces separate peptides during translation to produce multiple proteins (El Amrani et al. 2004, Donnelly et al. 2001). These constructs were then stably transformed into Arabidopsis to determine their functions.

In this chapter, I also explore the possibility of using mesophyll protoplasts to show activity of triterpene synthases as a quick, transient system. Previous reports show that enhancing the expression of genes upstream to the OSCs in the triterpene pathway, including squalene synthase and squalene epoxidase, enhances the output of the pathway in different long-term expression systems (Lee et al. 2004, Seo et al. 2005, Mirjalili et al. 2011). I expressed SQS1 (SQUALENE SYNTHASE 1), SQE1
(SQUALENE EPOXIDASE 1) and SQE4 from Arabidopsis in protoplasts isolated from transgenic plants expressing 35S::LUP4 (LUPEOL SYNTHASE 4) to explore the efficacy of using protoplasts for metabolite production. These plants have been shown to produce β-amyrin in leaf tissue (Buschhaus 2011). I was able to increase the production of β-amyrin in protoplasts and will highlight future steps needed to develop this into a transient expression system.

3.2 RESULTS

MRN1 was expressed in GIL77, a yeast strain used to demonstrate activity of triterpene synthases because it accumulates oxidosqualene. FRIEDELIN SYNTHASE from Kalanchoe diagremontiana (Wang et al. 2010) showed large amounts of new triterpene product, friedelin, when expressed in the GIL77 strain, as expected (Figure 3.1A). In these experiments, MRN1 expression did not typically identify any new products when compared to yeast transformed with the empty vector (Figure 3.1B). These data are representative of two separate cultures. Marnerol was, however, sporadically found in yeast samples expressing MRN1 after induction (Figure 3.2A). Marnerol TMS ether was only identified by GC-MS using Extracted Ion Chromatograms (EICs) for the M+ at m/z 500 as well as m/z 191 identified as an abundant ion from the mass spectrum shown by Xiong et al. (2006). The mass spectrum for the MRN1-expressing GIL77 extracts at 20.5 minutes corresponds to the published mass spectrum for marnerol TMS ether (Figure 3.2B).

3.2.1 Triterpene Pathway Manipulation in Protoplasts

Since expression in the GIL77 yeast expression system was inconsistent and possibly lacked plant-specific interactions or signals necessary for enzymes downstream from the OSC-catalyzed step, it became necessary to develop a transient in planta system for expression of multiple enzymes within a triterpene pathway such as the marneral cluster. An attractive system used in a number of plant molecular assays is transfected mesophyll protoplasts. Previous reports showed that enhancing the expression of genes early in the triterpene pathway, including squalene synthase and squalene epoxidase, enhances the output of the pathway in different systems (Lee et al. 2004, Seo et al. 2005, and Mirjalili et al. 2011). These studies ectopically expressed different squalene synthases using the 35S promoter and showed an increase in the production of downstream products after one month of culture. I hypothesized that
enhancing expression of squalene synthase or squalene epoxidase genes by introducing plasmids containing the different genes into protoplasts would rapidly increase the flux through the triterpene pathway. To determine if this is the case, SQS1, SQE1, and SQE4 were all transfected into mesophyll protoplasts isolated from plants stably expressing 35S::LUP4, an OSC known to produce β-amyrin (Buschhaus 2011). The plasmids used for transfection contained a separate 35S::GFP reporter gene that would allow for the quantification of transfection efficiency. While transfection efficiency was highly variable (from 25% to 75% in experiments not shown), expression of SQS1 was found to significantly increase the production of β-amyrin more than 5-fold from 0.51 pg/protoplast to 2.74 pg/protoplast (Fig 3.3). The transient expression of both SQE1 and SQE4 showed an insignificant increase in production of β-amyrin to 2.31 pg/protoplast and 1.43 pg/protoplast, respectively.

Marnerol was initially identified in planta from mesophyll protoplasts isolated from plants expressing pUBQ1::MRN1 (Fig 3.4). In the original characterization of MRN1, it had been suggested that the cyclization of oxidosqualene leads to the production of marnerol but the authors typically found marnerol in higher quantities (Xiong et al. 2006). They speculated that the alcohol was either formed enzymatically in the yeast or during the saponification step of the extraction process. To test whether the aldehyde was being degraded during the saponification step, lipid extracts were derivatized in a second step involving hydroxylamine HCl followed by BSTFA. Lupenone, a triterpene ketone, was first derivatized in a separate reaction to identify the product. The initial reaction produced lupenone oxime TMS ether with the expected molecular weight of 511 g/mol (Figure 3.5). This was used as the internal standard in subsequent lipid extracts of protoplasts.

Protoplast lipids were extracted with or without saponification from pUBQ1::MRN1 or Col-0 protoplasts followed by the two derivatizations previously mentioned. These extracts yielded small amounts of marnerol, labeled as peak 1, in the pUBQ1::MRN1 protoplasts but not in the Col-0 plants, with no new detectable products resembling marneral oxime TMS ether (Fig 3.6a). To confirm that the oximation reaction proceeded to completion, the EIC m/z 205, was examined as this is the dominant ion from the mass spectrum of lupenone (Fig. 3.6b). The absence of a peak at the expected retention time in the EIC, labeled with a red line on each chromatogram,
confirmed that none remained in the samples. The absence of any marneral derivatives was confirmed by looking at the EIC for \( m/z \) 513 (Fig. 3.6c), which is the expected molecular mass of marneral oxime TMS ether as well as \( m/z \) 69 (Fig. 3.6d), which is the molecular weight of single isoprene units and is characteristic of all terpenes. As such, this should help to identify retention times for any new triterpene products formed, including lupenone oxime TMS ether.

### 3.2.2 Characterization of Stable Lines

After identifying marnerol in mesophyll protoplasts, I examined whether marnerol could be identified from whole leaves. After transformation with \( pUBQ1::MRN1 \), I identified 16 transformants, of which nine were identified as having a single insert based on the progeny having survival rates of 3:1 on antibiotics. Both the expression level of \( MRN1 \) and the level of marnerol were measured in the leaves of these transformants. \( MRN1 \) is not normally expressed in leaves and therefore any transcript present should be the product of the transgene. Three high expressing lines were identified (lines 1, 3, and 13) (Fig 3.7 A). The marnerol content in the leaves of these transgenic lines mimics the relative amount of transcript (Fig 3.7). The experiment was originally designed to identify the presence of marnerol, not quantify it; therefore marnerol was quantified against the native triterpene alcohol \( \beta \)-sitosterol. One of the three replicates received the addition of the internal standard lupenone against which marnerol TMS ether was quantified. The quantification of marnerol TMS ether against the native \( \beta \)-sitosterol TMS, as well as lupenone oxime TMS ether, from this single replicate shows similar results based on the two quantifications (Figure 3.8 A). Similarly, the ratio of \( \beta \)-sitosterol TMS to lupenone oxime TMS was considered and should give a similar value across all 9 samples, most show the ratio to be close to 6.0, with two samples varying by +/- 45% (\( MRN1OE4 \) and \( MRN1OE7 \)) (Figure 3.8 B).

\( MRN1OE3 \) leaves were also used to purify marnerol using TLC. Extracts from \( MRN1OE3 \) and Col-0 were applied to TLC plates using CHCl\(_3\) as the mobile phase. Compounds were extracted from the plate and identified by GC-MS. Marnerol was identified to have an \( R_f \) of 0.29.

When I stably expressed different combinations of the MCGs, I was unable to detect any new products, but plants expressing either \( MRN1 \) alone or both \( CYP705A12 \) and \( MRN1 \) accumulated trace levels of marnerol, while none was found from plants
expressing both CYP71A16 and MRN1 (Figure 3.9). This implies that CYP71A16 catalyzed the next metabolic step in the marneral pathway. This is supported by the fact that the knockout mutant cyp71a16-1 accumulates marnerol to detectable levels when MRN1 is highly expressed, whereas it is below detection in wild type control plants (Figure 3.10). I was not able to identify any new compounds in the leaves of plants expressing pUBQ::CYP71A16-2A-MRN1 or pUBQ::CYP705A12-2A-CYP71A16-2A-MRN1 or in the leaves of F1 plants from crosses between pUBQ::CYP71A16 and pUBQ::MRN1 (data not shown).

3.3 DISCUSSION

3.3.1 Production of Marnerol

The initial characterization of MRN1 by Xiong et al. (2006) found that marneral was the native product and was thought to be reduced by native yeast enzymes or possibly during saponification since they were only able to detect the aldehyde in non-saponified extracts. In contrast, I found that marnerol was produced in stably transformed plants but I was not able to detect the aldehyde (Fig 3.2). Therefore, I tried to derivatize any marneral present in extracts from these plants using hydroxylammonium chloride followed by BSTFA. These reactions efficiently converted lupenone, a triterpene ketone, to lupenone oxime TMS with no trace of marneral oxime TMS ether (Fig 3.1). I only detected marnerol in both saponified and non-saponified samples. These data suggest that the conversion of marneral to marnerol either occurs in vivo or during the extraction process prior to saponification. This is supported by the findings of Field et al. (2011) that also did not identify marneral in either saponified or non-saponified extracts, leading them to the same conclusion.

3.3.2 Transient System

Using protoplasts as a transient system was found to increase production of β-amyrin of transgenic cells after expressing SQUALENE SYNTTHASE 1. Expression of a Panax ginseng squalene synthase (PgSS1) was also found to increase production of phytosterols in transgenic adventitious roots of both P. ginseng and Eleutherococcus senticosus, an unrelated plant that produces a number of medicinal saponins (Lee et al. 2004, Seo et al. 2005). SQS1, the only functional SQS in Arabidopsis (Busquets et al. 2008), was found to boost triterpene production in long term hairy root cultures of Withania coagulans (Mirjalili et al. 2011). These data show that even a transient
increase in expression can result in an increased production of triterpenes due to the fact that SQS1 may act as a rate limiting step in triterpene pathways and can be manipulated to optimize a rapid in planta transient system for studying triterpene pathways.

In an attempt to characterize six of the SQE genes in Arabidopsis, Rasbery et al. (2007) expressed each gene in a yeast strain deficient in ERG1, the native squalene epoxidase, and only found activity with SQE1, SQE2, and SQE3. It was further shown that SQE4 and SQE5 were not able to rescue sqe1-3 defects. Considering this, along with the fact that phylogenetic analysis place SQE4, SQE5, and SQE6 into their own clade (Squalene Epoxidase Like genes) that is distinct from all other characterized plant SQEs, these genes may have adopted a new role or they could maintain functionality, but have acquired regulatory features that allow their activity only in specific compartments or under specific conditions. However, when expression data for all the genes in Arabidopsis thought to be involved in the triterpene pathway from squalene synthesis to cyclized triterpenes, including both SQSs, all six SQEs, and all triterpene synthases in Arabidopsis are put into a hierarchical clustering analysis, SQE4 was found to be the most highly coregulated with MRN1, followed by SQE1 (see chapter 5). It would be interesting to determine what role if any SQE4 is playing alongside the MCGs.

There are a number of simple transient systems available for testing the subcellular localization or RNA/protein production of constructs, but these are rarely used to test biosynthetic pathways. While this research has shown that SQS1 can be used to rapidly increase the production of triterpenes in protoplasts, it is only the first step in developing a useful system. A number of studies have found that manipulating other steps in the triterpene pathway may also be useful for induction. For instance, it has long been known that 3-hydoxy-3-methylglutaryl-coA reductase (HMGR) is an important regulatory enzyme not only in plants, but is likely to be the key enzyme regulating sterol biosynthesis in other eukaryotes (Stermer et al. 1994, Goldstein and Brown 1990, Suzuki et al. 2004, Schaller et al. 1995, and Manzano et al. 2004). This has resulted in the class of drugs known as statins that have also been shown to be inhibitors of plant HMGRs including lovastatin (Bach and Lichtenthaler 1982, Chappell and Nable 1987, and Hemmerlin and Bach 1998). While this is an attractive step in
developing a transient system, it is the first committed step in the production of terpenes within the mevalonate pathway, and altering its activity could influence many terpene products. It makes sense that plants require more specialized regulation of terpene pathways because of the diversity found among the different compounds. Control of SQS or SQE would alter the production of triterpenes specifically, as these catalyze the first two steps of the pathway, respectively. In fact, Vögeli and Chappell (1988) showed that an induction of the sesquiterpene pathway by an elicitor treatment coincided with a decrease in sterol production. This makes sense because both pathways utilize farnesyl pyrophosphate, but the regulation of the common precursor was later shown to be a direct result of the regulation of TSS (Tobacco Squalene Synthase) RNA and protein activity, although protein levels remained the same (Devarenne et al. 2002). This suggests that, at least for suppression of the pathway, SQS is transcriptionally and post-translationally regulated. It has also been shown that HMGR activity is induced by inhibiting SQS or SQE with drugs in tobacco BY-2 cells (Wentzinger et al. 2002). Their data further show that neither SQS expression nor the resulting enzyme’s activity changed based on the presence or absence of native levels of squalene, suggesting that a feedback mechanism is lacking in plants for monitoring SQS levels in relation to squalene levels. My data support this concept by showing that the triterpene pathway in protoplasts is more sensitive to ectopic expression of SQS1, which can therefore promote the production of downstream products. As such, SQS1 rather than SQE1, appears to be a more suitable target for developing a transient triterpene synthase expression system. The next step would be to create stable lines with an inducible promoter driving SQS1 expression that could be transfected with triterpene synthase genes to identify their activity.

3.3.3 Cytochrome P450 Reactions

These data clearly show that MRN1 and CYP71A16 function together in a single pathway. This is in agreement with the recent study showing the evolution of the marneral and thalianol clusters in Arabidopsis (Field et al. 2011). While the Field et al. (2011) study confirms my data showing that cyp71a16-1 accumulates marnerol, they also stop short of determining a role for CYP705A12 and assume it is a part of the marneral gene cluster based not only on genomic location and transcriptional regulation but by the fact that it shows homology to THAD, a desaturase member of the thalianol cluster. They also determined that CYP71A16 is actually a marnerol oxidase that
produces, at least in transgenic leaves expressing both 35S::MRN1 and 35S::CYP71A16, multiple products. The four most abundant products resemble isomers of hydroxylated desaturated marnerol and display a molecular ion of 586 (C$_{36}$H$_{66}$O$_2$Si$_2$) for the TMS derivative. These data add another function to the diverse CYP71A family of proteins, which include three characterized members, two of which (CYP71A10 and CYP71A12) have been shown to be involved in detoxifying herbicides through demethylation + hydroxylation or demethylation alone (Siminszky et al. 2000; Siminszky et al. 2003 Hayashi et al. 2007). The third, CYP71A13, represents an atypical reaction for a CYP in camalexin biosynthesis in the dehydration of indole-3-acetaldoxime to indole-3-acetonitrile (Nafisi et al. 2007).

### 3.3.4 Conclusion

These data confirm that CYP71A16 and MRN1 are members of a metabolic gene cluster. Similar to Field et al. (2011), we can still only speculate on the function of CYP705A12. While there is promise for the development of a transient triterpene expression system in protoplasts, a more useful tool identified was the manipulation of SQS. This experimental system shows that a fairly rapid increase in production of β-amyrin results from increased production of SQS. While other studies have shown that overexpression of SQS results in increased triterpenes, the process requires the culturing of plant tissue for weeks at a time (Lee et al. 2004, Seo et al. 2005, Mirjalili et al. 2011), not 24 h.

### 3.4 MATERIALS AND METHODS

All primers used are reported in appendix 1

All PCR programs are reported in appendix 2

#### 3.4.1 Cloning cDNAs

Primers were initially phosphorylated using T4 Polynucleotide Kinase (Fermentas) in a reaction containing 10 mM Primer, 1x buffer A (50 mM Tris-HCL (pH 7.6) 10 mM MgCl$_2$, 5 mM DTT, 0.1 mM Spermidine), 1 mM dATP, and 1 U enzyme. This reaction was incubated at 37°C for 90 minutes then the reaction was stopped by incubating at 75°C for 10 minutes and stored on ice or placed at -20°C until use.

_Smal_ (Invitrogen), a blunt cutting enzyme, was used extensively in cloning the various plasmids and was always accompanied by a dephosphorylation reaction using
either Calf Intestinal Alkaline Phosphatase (Invitrogen) or Antarctic Phosphatase (NEB) according to the manufacturer’s instructions.

The cDNAs for the individual inserts of \textit{MRN1}, \textit{CYP71A16}, and \textit{CYP705A12} were amplified from 10 day-old seedling cDNA using phosphorylated primers (EJ-169/EJ-170 for \textit{MRN1}, EJ-92/EJ-93 for \textit{CYP71A16}, and EJ-94/EJ-95 for \textit{CYP705A12}) with Phusion DNA polymerase (Finnzyme, NEB) which leaves blunt ends on amplified products. Each of the CYP PCR products were ligated into the \textit{SmaI} site of pBluescript I KS using T4 ligase (Invitrogen) according to the manufacturer’s protocol and transformed into competent DH5α cells. The \textit{MRN1} PCR product was digested with \textit{EcoRI} and \textit{XbaI} and ligated into the same sites of pBluescript I KS. Colonies were selected on LB ampicillin (100 µg/ml) plates and were identified by restriction analysis. Colonies with successful inserts were then sequenced through the entire insert and glycerol stocks were made for each plasmid. This created pBS-MRN1, pBS-CYP71A16, and pBS-CYP705A12.

The plasmids for the various clusters were created by linking each cDNA with the short 19 amino acid Foot and Mouth Disease 2A peptide that produces multiple peptides from a single transcript (Ryan and Drew 1994). Primers were designed to include three cysteines at the 3’ end of the PCR product so they could be ligated into the \textit{SmaI} site of pBluescript I KS and the site would be maintained and used for the second and/or third cDNA to be ligated in. The plasmids were otherwise created similarly to the single cDNA constructs mentioned above using primers EJ-104/EJ-105 and EJ-108/EJ-109 to create pBS-CYP71A16-2A and pBS-CYP705A12-2A, respectively. The pBS-CYP705A12-2A-CYP71A16-2A construct was created using primers containing the appropriate sequences on either side of the CYP71A16 cDNA to maintain the 2A sequences (EJ-116/EJ-105). \textit{MRN1} cDNA was added to the end of each of those plasmids using primers containing the remaining 2A sequence attached to the 5’ end of the PCR product (EJ-156/EJ-157).

\textbf{3.4.2 Yeast Expression Plasmids}

The \textit{MRN1} cDNA was subcloned into the \textit{KpnI} – \textit{XbaI} sites of the yeast expression vector pYES2 (Invitrogen). Site directed mutagenesis was conducted on this plasmid in order to introduce an A at the -3 position to optimize expression in yeast, as recommended by the manufacturer. This was conducted by designing
prephosphorylated primers, one with the appropriate mutations to introduce the Kozak sequence and an extra restriction site for colony identification (EEJ-172), and another to bind to the opposite strand that would amplify the entire plasmid (EEJ-173). A PCR reaction was conducted using the original pYES2-MRN1 plasmid as the template and the Phusion DNA polymerase. This reaction was digested with DpnI to destroy the original DNA and then transformed into TOP10 cells (Invitrogen). Minipreps were conducted on cultures from colonies selected on LB plates containing Ampicillin (100 µg/ml). These were identified by restriction analysis and confirmed by sequencing from the promoter through the terminator of the gene.

3.4.3 Plant Expression Plasmids

The UBQ1 promoter was used to drive expression of each construct in plants in order to avoid silencing that occurs from using viral promoters like 35S. UBQ1 promoter was amplified using the primers EJ-151 and EJ-152 and ligated into the Smal similarly to previous descriptions. This produced the pBS-UBQ1pro construct. The BamHI - HindIII fragment was cloned into the binary pCambia 1380 plasmid to create pC-UBQ1pro. To confirm the expression profile of the UBQ1 promoter, GFP was PCR amplified using EJ-158 and EJ-159 primers, and both pC1380-UBQ1pro and the PCR product were digested with SpeI and SstI and ligated together to produce pC-pUBQ::GFP. UBQ1pro was also ligated into pCambia 1390 to create a second vector with a different series of restriction sites using the BamHI – EcoRI fragment from pBS-UBQ1pro to produce pC1390- UBQ1pro. pBS-CYP71A16 and pBS-CYP705A12 were digested and ligated into pC1380-UBQ1pro using the XhoI – Smal and EcoRI I – XhoI fragments, respectively, to create pC-pUBQ1::CYP71A16 and pC-pUBQ1::CYP705A12. pC-pUBQ1::MRN1 was created by ligating the SstI – EcoRI fragment from pBS-MRN1 into pC1390::UBQ1pro. Since pBS-CYP705-MRN1 and pBS-CYP705A12-2A-CYP71A16-2A-MRN1 both have SstI sites on both sides of the construct, these fragments were digested with SstI I and ligated non-directionally into pC1380-UBQ1pro. Restriction analysis was used to identify plasmids with inserts ligated in the correct direction. This formed pC-pUBQ1::CYP705A12-2A-MRN1 and pC-pUBQ1::Cluster. To make the pC-pUBQ1::CYP71A16-2A-MRN1, pC-pUBQ1::CYP71A16 was digested with KpnI – EcoRI and ligated into pBS-CYP71A16-2A-MRN1 to make pBS-pUBQ1::CYP71A16-2A-MRN1. This plasmid was the digested with KpnI and SpeI and ligated into the same sites of pC1390-UBQ1pro.
3.4.5 Protoplast Expression Plasmids

The plasmids used for protoplast transfection are based on a pUC19 vector containing 35S-OFP1-HA construct that was a gift from Shucai Wang (University of British Columbia). The cDNA for SQUALENE EPOXIDASE 1 (SQE1, At1g58440), SQUALENE EPOXIDASE 4 (SQE4, At5g24140), and SQUALENE SYNTHASE 1 (At4g34640) were amplified from 10 day-old seedling cDNA using the primers EJ-180 and EJ-181; EJ-182 and EJ-183; EJ-189 and EJ-194, respectively. The SQE1 product was digested with Sac I-HF (NEB) to completion and then partially digested with Ncol at room temperature and the band at ~1500bp was gel-purified and ligated into the digested pUC19-35S-OFP1-HA plasmid to create pUC19-SQE1. The SQE4 cDNA was digested with PstI – Ncol fragment from the SQE4 PCR product and was ligated into the pUC19-35S-OFP1-HA plasmid to create pUC19-SQE4. The SQS1 PCR product was digested with Ncol and BstXI. The SQS1 primer EJ-189 included a BstXI site that would leave a SacI overhang. BstXI also cuts SQS1 near the middle of the gene but produces a degenerate recognition site that would only allow ligation at the same point. Therefore, both fragments were included in a ligation reaction with pUC19-SQS1 cut with Ncol and SacI. These three products were ligated to create pUC19-SQS1.

To quantify the transformation efficiency of the protoplasts, each of these 3 constructs needed to also express GFP. To accomplish this, a PCR reaction amplifying the entire UBQ1::GFP::NOS region of pC-UBQ1::GFP was run with the primers EJ-190 and EJ-191. These include restriction sites for Eco31I and PstI. Eco31I is a degenerate enzyme and the primer was designed to leave a BamHI overhang after digestion. The PCR product was digested and ligated into BamHI – PstI digested pUC19-SQE1, pUC19-SQP2, and pUC19-SQS1.
3.4.6 Plant Growth Conditions

Plants used for protoplast analysis and leaf hexane extractions were grown from seed on soil (Sunshine mix #4) for 3-6 weeks under a 12/12 light regime. Plants grown for the identification of marneral in cyp71-1 were grown from seeds in liquid cultures. 30-40mg of seeds were sterilized and left at 4°C for 2 days. These were then placed in 250 ml Erlenmeyer flasks containing 125 ml liquid Hoagland’s media supplemented with 1% sucrose. The cultures were grown for 10 days under continuous light conditions on a circular shaker running at 200 rpm.

The yeast strain GIL77 (gal2 hem3-6 erg7 ura3-167) was created as an in vivo system for studying the function of plant triterpene synthase gene (Kushiro et al. 1998). This is typically an excellent system because yeast cells contain only a single triterpene pathway, leading to the production of ergosterol and have a much less complex chemical profile than a typical plant. The erg7 mutation halts this pathway at the step between 2,3-oxidosqualene and ergosterol (Karst and Lacroute 1977). While this requires exogenous ergosterol to be added to the media, it also allows for an accumulation of the unstable 2,3-oxidosqualene within the yeast.

3.4.7 Transformation of pYES2-MRN1 into GIL77

GIL77 was transformed using the LiAce/SS method described by Gietz and Woods (2002). A 4 ml culture was made by adding a small amount of a glycerol stock of GIL77 to 2xYEPD +HE and growing it overnight to density at 30°C on a rotary shaker at 200 rpm. The OD_{600} was determined the next day and approximately 10ml/transformation of 2xYEPD +HE was inoculated to a final concentration of approximately 5 x 10^6 cells/ ml. This was left to grow to a density of 2 x 10^7 cells/ ml (approximately 5 hours). The cells were then centrifuged at 3000g in a 50 ml conical tube for 5 minutes. The pellet was rinsed with 25 ml water and centrifuged again. The final pellet was resuspended in 250 µl water/transformation. The yeast cells were then divided among each reaction and 360 µl of transformation mixture (33% PEG 3500, 100 mM LiAc, 300 ng/µl boiled Salmon Sperm DNA, 2.5 ng/ µl plasmid DNA) was added. The cells were incubated at 42°C for 45 – 60 minutes. The yeast cells were then pelleted at 14,100g and washed once with water. The pellet was resuspended after the wash in 1 ml water and 200 µl of the culture was gently spread on SC-U +HE plates and incubated at 29°C for 3 days. This typically resulted in 50-1000 transformed colonies.
3.4.8 Expression of pYES2-MRN1 in GIL77

A 4 ml culture of a single transformed colony were grown in SC-U+glucose+HE media overnight and a miniprep was performed on half the culture to confirm the insertion of the plasmid while the second half was used to seed a new culture (from 15-200 ml) in SC-U+glucose+HE and grown for 2 days shaking at 200 rpm at 29° C. The yeast were then transferred to the same volume of SC-U+Galactose+HE for another 12-16 hours. In some experiments, this culture was then transferred to Resting Buffer for another 24 hours.

Occasionally the portion of yeast taken for the miniprep was taken after induction. Some experiments also excluded the initial growth step in SC-U+glucose+HE and instead used SC-U+Galactose+HE. The negative control for marneral/marnerol typically was transformed with pYES2-GFP, which allowed me to determine if the Gal induction promoter was functioning properly as determined by looking a sample of the yeast under a fluorescent microscope.

3.4.9 Yeast Hexane Extraction and Analysis

The cultures were centrifuged at 1500 g for 5 minutes. The pellet was washed in 20 ml dH2O and centrifuged again. The pellet was then saponified by resuspending in 15 ml 50% EtOH and 1g KOH and placed in 70°C for 60 minutes. This mixture is then extracted 3 times with 3-5 ml hexane. The extract was then dried under a gentle N2 flow, resuspended in 100 µl CHCl3 and derivatized by mixing the extract with 10 µl pyridine (Sigma) and 10 µl N,O-bis(trimethylsilyl)-trifluoracetamide (Sigma) and the mixture was refluxed for 1 hour at 70°C. The samples were then dried under N2 and resuspended in 100 µl CHCl3, then injected into FID-GC or GC/MS.

Occasionally TLC was used. In this case, prior to derivatization, the hexane extracts were applied to a silica plate (250 mM x 20 cm x20 cm). The mobile phase used was CHCl3 and was run for approximately 90 minutes in an enclosed glass chamber. Once the mobile front travelled to approximately 85% of the distance to the end of the plate, it was removed from the chamber, and allowed to dry. The plate was then sprayed with a solution of primuline and acetone and viewed under λ256 light. Different fractions were scraped off of the plate and soaked in 2-5 ml CHCl3 for 2 -16 hours, filtered into a new glass vial, dried under N2, and resuspended in 100 µl CHCl3. At this point, the samples were derivatized as described before.
Samples were analyzed using a Hewlett-Packard 6890 Network GC equipped with a mass spectrometric detector (Agilent 5973N) and/or on an Agilent 7890A GC-FID. Both machines were equipped with an HP-1 column (Agilent, 30m, 320 mM bore, 0.10 mM film), although oven programs were different. The program used on the 7890A GC-FID was as follows: A splitless injection was used at 300°C. The oven program started at 50°C for 2 min and increased at a rate of 40°C/min up to 200°C, held for 1 minute, then increased at a rate of 3°C/min to 320°C, held at 320°C for 15 minutes. The FID detector was heated to 300°C, with a hydrogen flow of 30 ml/min, compressed air of 350 ml/min, and helium at 23 ml/min. The beginning column pressure was 4.9 psi with H₂ carrier gas flow of 2 ml/min for the first 30 minutes. After that, the flow increased to 100 ml/minute for 31.75 minutes before it dropped to 4 ml/minute. The total run time was 61.75 minutes. The GC-MS program was slightly different in that each sample was injected directly on column into a flow of helium gas with a constant flow rate of 1.4 ml/min. The oven temperature was programmed for 2 min at 50°C, followed by a 40°C/min ramp to 200°C, held at 200°C for 2 min, increased by 3°C/min up to 320°C, and held at 320°C for 30 min. The total run time was 77.75 min.

3.4.10 Protoplast Analysis

The following technique was modified and worked up from Tiwari et al. (2006). To harvest mesophyll protoplasts approximately 1 g of leaf tissue was cut into thin strips (< 1mm). These strips were incubated in 30 ml fresh cellulose-macerozyme solution (1% w/v Cellulase R10, 0.25% Macerozyme R10, 400 mM Mannitol, 10 mM CaCl₂, 5 mM MES, pH 5.7) in a glass Petri dish under vacuum for 20 minutes. The strips were left in solution for an additional 90-120 minutes on a rotary shaker (40 rpm). The leaf sections were then shaken more vigorously at 80 rpm for an additional minute before the protoplasts were filtered through 200 mM nylon mesh. 1/3 the volume of the protoplast mix of 200 mM CaCl₂ was added. The cells were pelleted at 200 g for 3 minutes using a swinging bucket rotor (SX4250 Beckman Coulter). They were washed and then resuspended in W5 solution (154 mM NaCl, 5 mM KCl, 125 mM CaCl₂, 5 mM glucose). The protoplasts were quantified using a haemocytometer and resuspended in the appropriate volume of Mg-mannitol (400 mM mannitol, 15 mM MgCl₂, 4 mM MES, pH 5.7) solution to attain a concentration of 4 x 10⁵ cells/ ml.
In order to transfect the protoplasts, 3 ml protoplast solution was mixed with 15 µg plasmid DNA (purified from an endotoxin free maxiprep (Qiagen) or an E.Z.N.A. Endo-Free Plasmid Mega kit (Omega Bio-Tek) according to the manufacturers’ protocols) and added to 3 ml PEG solution (40% PEG-3350 in Ca-mannitol solution pH 10.0). This was gently mixed and placed at room temperature for 20 minutes. 11 ml W5 solution was added dropwise and the mixture incubated for another 10 minutes at room temperature. The protoplasts were pelleted and resuspended in 10 ml WI solution (500 mM Mannitol, 20 mM KCl, 4 mM MES, pH 5.7). The cells were placed at a shallow angle to minimize the pressure of the solution on the cells in vented 50 ml conical tubes at 27°C overnight.

For protoplasts, the transfection efficiency was calculated by screening for GFP fluorescence compared to autofluorescence of chlorophyll using a Texas red filter on a Zeiss Axiovert microscope. The cells were then pelleted and washed with dH2O prior to the hexane extraction as described for yeast above.

3.4.11 Plant Chemical Analysis

For stable expression lines, tissue was weighed and harvested. Three leaves were harvested from ~21 day-old plants that typically had a combined fresh weight of 150 – 250mg. They were frozen in liquid nitrogen and stored at -80°C until further processing. The frozen leaves were placed in 400 µl of 0.1 M NaPO4 at pH7.0 and the tissue was sonicated three times using a probe sonicator (Branson Sonifier 150) for no more than 30 seconds each time to homogenize the tissue. When appropriate, an internal standard was included. The slurry was saponified by transferring to conical tubes with a final concentration of 80% EtOH, 4 mg KOH, and 100 mg/ml butylated hydroxytoluene and placed at 70°C for 2 h. After saponification the mixture was diluted with ddH2O up to 9 ml and extracted 3 times with 3 ml hexane. The hexane extract was passed through a filter made of glass wool and a glass pipette to remove any residual plant material. When appropriate, the mixture was first derivatized with 50 µl of 20 mg/ml hydroxylamine HCl in pyridine and the reaction was refluxed for 2 h at 70°C. Once cooled, the reaction was dried under N2 and the residual hydroxylamine HCl was removed by washing with 500 µl ddH2O and partitioned with 200 µl hexane three times. This extract was dried and derivatized with 10 µl BSTFA in 10 µl pyridine at 70°C for 1h. The reaction was then dried under N2 and resuspended in CHCl3. When the oximation
reaction was unnecessary, the extracts were only derivatized with BSTFA and pyridine. These samples were analyzed similarly to yeast hexane extracts (described previously).

For *cyp71a16-1* analysis, plants were harvested from the liquid Hoagland's media and ground into a fine powder using a mortar and pestle in liquid nitrogen. The tissue was then freeze dried and stored at -80°C until further processing. These extracts were processed as described by Field and Osbourn (2008). The total extract was weighed and 12 mg of each sample was applied to a 2 mM silica TLC plate (20cm x 20cm) (Merck). The lipids were separated using chloroform and a fraction representing *R*<sub>f</sub> values of 0.25 – 0.35 was scraped into a test tube and extracted overnight with CHCl<sub>3</sub>. 20% of the final extract was derivatized and applied to the GC-MS as described previously.
Figure 3. 1 Heterologous expression of OSCs in *Saccharomyces cerevisiae* GIL77. **A.** GC-FID traces of extracts from cultures expressing empty *pYES2* (black) or *pYES2-KdFRS* (green) (Wang et al. 2010). **B.** GC-FID of extracts from cultures expressing empty *pYES2* (black) or *pYES2-MRN1* (red) demonstrating *MRN1* expression in GIL77 without the production of new products.
Figure 3. 2 Expression of *MRN1* in GIL77 to produce marnerol. A. Overlay of TIC (Left axis) and EIC traces from GC-MS (Right axis) m/z 191 (blue) and m/z 500 (green) showing the elution of marnerol at 20.5 min. * indicates marnerol  B. Mass spectrum of marnerol from A.
Figure 3. Induction of the triterpene pathway in 35S::LUP4 protoplasts. 480,000 protoplasts were transfected with plasmids containing SQS1, SQE1, or SQE4 along with a separate gene expressing GFP on the same plasmid in order to determine the transfection efficiency for each line. The efficiencies for SQS1 were 28.5%, 28.2%, and 44.4% for three replicates. They were 25.9%, 21.4%, and 40.3% for SQE1. For SQE4 they were 38.5%, 38.6%, and 38.0%. Total lipids were extracted 16h after transfection, purified using TLC, and quantified against an internal standard using GC-FID. Bars indicate standard deviation. * indicates p-value < 0.01.
Figure 3.4 Identification of marnerol in protoplasts stably expressing pUBQ1::MRN1 line 3.
Extracted Ion Chromatogram of m/z 500 of extracts from protoplasts of wild type plants (black) or of plants expressing pUBQ1::MRN1 (red). Asterisk shows peak with a mass spectrum indicative of marnerol.
Figure 3. Derivatization of lupenone with hydroxylamine HCl followed by BSTFA to form lupenone oxime TMS ether. A. GC/MS Total Ion Chromatograms of lupenone (blue) compared to the products of the derivatizations (black). B. Mass spectrum of lupenone. C. Mass spectrum of lupenone oxime TMS ether.
Figure 3. *Marneral is not present in extracts from protoplast.* A. Total Ion Chromatograms of samples from protoplasts of plants expressing pUBQ1::MRN1 that was saponified (green) or not (black) or Col-0 plants extracted under the same conditions (blue or orange, respectively). After saponification, the extracts were transformed into oxime TMS ether derivatives. Extracted Ion Chromatograms from the same samples at $m/z$ 205 (B) $m/z$ 513 (C) or $m/z$ 69 (D). Red lines in C indicate expected elution time of lupenone. “1” indicates marnerol TMS ether; “2” indicates β-sitosterol; “3” indicates lupenone oxime TMS ether. Saponified traces are offset by 0.8 minutes in all chromatograms and by $4.0 \times 10^5$ in A, by 5000 in B, by 2000 in C, and by $2.0 \times 10^5$ in D.
Figure 3. 7 Expression of *MRN1* correlates with the production of marnerol. **A.** Semi-quantitative PCR analysis illustrating varying expression levels of different *pUBQ1::MRN1* lines in leaves of transgenic plants. *ACT8* was used as the internal control. Both *MRN1* and *ACT8* PCR reactions were conducted for 25 cycles. **B.** Quantification of marnerol relative to native β-sitosterol extracted from leaves from GC-FID.
Figure 3. 8 Validation of the quantification of marnerol in pUBQ1::MRN1 lines. A. Amounts of marnerol TMS ether relative to either the derivatized internal standard lupenone oxime TMS ether or to the derivatized endogenous β-sitosterol, the most abundant triterpene alcohol. B. Ratios of lupenone oxime TMS ether to native β-sitosterol TMS ether. This ratio should be constant in every sample.
Figure 3. 9 GC-FID of hexane extracts of various transgenic lines. Each line represents independent transformants. A. pUBQ::MRN1 (red) compared with wild type (black) showing marnerol TMS ether eluting at 20.5 minutes. B.-D. Red trace represents pUBQ::MRN1 line 3, Blue trace represents the indicated multicistronic “cluster” line. B. Marnerol is absent in all pUBQ::CYP71A16-2A-MRN1 lines. C. Marnerol is present in two of three pUBQ::CYP705A12-2A-MRN1 lines. D. Marnerol is absent in all pUBQ::CYP705A12-2A-CYP71A16-2A-MRN1 lines. Samples showing a peak at 20.5 minutes were confirmed to have marnerol TMS ether by GC-MS.
Figure 3. 10 Identification of marnerol in cyp71a16-1. A. Total Ion Chromatograms from extracts of cyp71a16-1 (red) compared to Col-0 (black). Note other triterpene derivatives were identified from the mass spectra. B. Overlay of extracted ion chromatograms m/z 191 (grey) and m/z 500 (green) from cyp71a16-1 showing marnerol eluting at 22 minutes.
CHAPTER 4: EXPRESSION ANALYSIS OF THE MARNEERAL GENE CLUSTER

4.1 INTRODUCTION

Few functions have been positively identified for triterpenes derived from secondary metabolism and non-sterol triterpenes, although many are generally expected to act in defense (Phillips et al. 2006). Triterpene saponins and cuticle-associated aglycones are the most extensively studied non-hormone and non-steroid triterpenes. Triterpene aglycones have been shown to be found in high proportions within the cuticle (20%-80%) (Buschhaus and Jetter 2011). While their general function is not well characterized, some seem to play specific ecophysiological roles. For instance, *Macaranga*, which has an epicuticular crystalline layer comprised predominantly of triterpenes, allows only certain symbiotic ant species to walk over it (Markstädter et al. 2000). Cuticular triterpenes on fruits may also be involved in ripening, such that the chemical makeup of the fruit cuticle in which the ratio of aliphatic molecules (such as alkanes) to triterpenes decreases as the fruit loses water (Vogg et al. 2004, Parsons et al. 2012). It has been speculated that the relative increase in triterpenes creates “nanomolecular pathways” to allow for the diffusion of water out of the fruit over the course of ripening (Parsons et al. 2012). The functions of many saponins are generally better characterized than aglycones and seem to play roles in defense (Augustin et al. 2011). Many of these roles, such as antifungal or antimicrobial, have only been ascertained from a pharmacological perspective by probing purified saponins for these activities (Zhang et al. 2005, Deng et al. 2008, Avato et al. 2006). It is clear, however, that these compounds are created by the plants for defensive purposes because some phytopathogens produce saponin-detoxifying enzymes to allow infection. Examples of this are found in the relationship between *Avena* sp. and pathogenic fungi that are only harmful when expressing saponin hydrolyzing enzymes that detoxify the avenacoside saponins (Bowyer et al. 1995, Quidde et al. 1999, Morrissey et al. 2000).

Bioinformatic analysis of the MCGs suggests that the pathway is upregulated in response to osmotic stresses related to high concentrations of NaCl and mannitol (See section 4.2) (Winter et al. 2007a). Some studies have identified secondary metabolites
produced under abiotic stress conditions through metabolic profiling. For example, freezing stress conditions, which effectively limit the available water in cells, elicit the production of compounds such as proline, sugars and sugar alcohols to act as osmoprotectants and antifreeze compounds (Maruyama et al. 2009). In addition, an increase in flavonoid production has also been noted (Korn et al. 2008, Zhang et al. 2011). It is not unreasonable to speculate that triterpenes may be produced under conditions in which membranes need to be modified such as high and low temperatures or drought conditions. In fact, it was recently shown that a transcription factor involved in drought and gravity responses binds to the THAS promoter to repress expression (de Silva et al. 2011), suggesting that thalianol-related metabolites may function in these conditions. THAS is, like MRN1, another OSC encoding gene located within a metabolic gene cluster in Arabidopsis (Field and Osborne 2008).

The complete biochemical characterization of every OSC from Arabidopsis has illustrated a large diversity of triterpenes capable of being produced in a species previously assumed to have relatively few triterpenes (Morlacchi et al. 2009). It also exemplifies the fact that there may be a number of uncharacterized biological functions of non-sterol triterpenes, such as the marneral-related metabolites. In order to elucidate the function of these metabolites, expression analysis on the genes involved should hint at the function of the end metabolites. For example, enzymes responsible for producing defensive compounds are generally induced by wounding or signals such as MeJA. Three global expression analyses using cDNA microarrays have found a rapid increase in MRN1 transcript levels, as follows: When ectopically expressed, Membrane Sterol Binding Protein 1 (MSBP1) stimulates the expression of the marneral cluster of genes between 3- and 14-fold compared to wild type seedlings while also generating shorter roots and hypocotyls (Yang et al. 2005). MSBP1 is a protein shown to bind to 24-epi-brassinolide in vitro (Yang et al. 2005), and to interact directly with and negatively regulate the brassinosteroid signalling pathway (Song et al. 2009). Another study investigating vacuolar H+-ATPase activity used a microarray analysis to compare the transcripts of the det3 mutant to wild type under two conditional treatments known to restrict cell expansion within the det3 mutant, including treatment with KNO₃ and 16°C growth conditions. MRN1 was upregulated in both scenarios (Brüx et al. 2008). In a third microarray study, MRN1 expression was down-regulated in the tudorSN1
tudorSN2 (tsn1tsn2) double mutant only after the addition of salinity stress. These RNA-binding proteins seem to enhance the stability of bound transcripts under salinity stress conditions. There is increased mortality and reduced cell elongation in the double mutant under long term exposure to treatments of NaCl (150mM and 100mM, respectively) (dit Frey et al. 2010). Each of these studies investigates conditions related to root elongation and it is intriguing to note that the region of the root in which elongation slows is the same region where MRN1 is primarily expressed (see Chapter 2).

The goal of this work was to determine if a functional marneral pathway is necessary for the cessation of root growth in response to conditions found to induce the expression of the MCGs. To achieve this goal, I identified the expression profile of the MCGs using bioinformatics data and confirmed those data using pMRN4205::TIMER fluorescence and real time PCR. These conditions were then tested by specifically looking at root elongation rates of plants treated under these conditions. Possible functions of marneral-related metabolites will be discussed.

4.2 Results

4.2.1 Root Growth Analysis of mrn1-1

Previous microarray analyses suggest a possible involvement of MRN1 in regulating root elongation. The involvement in root elongation and cell expansion can be tested by simply altering the amount of sucrose in a growth medium (Benfey 1993). The hypothesis that MRN1 expression ceases elongation was tested based on the localization of MRN1 expression. Wild type and mrn1-1 plants were imaged after 8 days of growth on media supplemented with 0% or 3% sucrose, and it was found that the absence of sucrose resulted in longer roots in mrn1-1 compared to wild type, whereas the addition of 3% sucrose resulted in nearly identical growth (Figure 4.1).

Sucrose can dramatically alter the metabolism of germinating seeds by affecting ABA and ethylene pathways and delaying the biosynthesis of photosynthetic components within the cotyledons (Rognoni et al. 2007). Since the root measurements were conducted on young seedlings, it was important to consider whether the metabolism of these seeds was responsible for the altered root growth and not simply the decreased expression of MRN1. To test this, mrn1-1 and wild-type seeds were
germinated on varying concentrations of sucrose (0%-9%) for 48 h and scored based on their stage of germination that included: not germinated, emerging radicle, emerging apical hook, or cotyledons fully open (Figure 4.2 A). These stages correspond to the Arabidopsis growth stages 0.1, 0.5, 0.7, and 1.0, respectively, from Boyes et al. (2001). It was initially found using the same seeds as in Figure 4.1 that \textit{mrn1-1} proceeded through the stages much slower than wild type across all concentrations indicating that \textit{MRN1} may play a role in seedling germination.

The conditions experienced during embryo development can change how young seedlings react to the hormones ABA and ethylene (Finch-Savage and Leubner-Metzger 2006); both hormone signalling pathways are linked closely to sucrose sensing in germinating seeds (Rognoni et al. 2007). The seeds used in Figure 4.1 and 4.2 A were stored together and were of comparable age, but the parent plants were not grown under identical conditions. I therefore produced three sets of wild-type and \textit{mrn1-1} seeds, with each set grown under similar but separate conditions. Once mature, the seeds were put through a similar germination test on media lacking sucrose. After 48 h \textit{mrn1-1} seedlings were found to germinate at a similar rate as wild-type plants (Figure 4.2 B). Interestingly, there seems to be a variation in the speed of germination when comparing the same genotypes across different replicates. When the seeds from replicate A were again grown on media containing 0% or 3% sucrose for 8 days, there was no significant difference in root length between \textit{mrn1-1} and wild type plants (Figure 4.3). This indicates that the initial difference in root growth found on media lacking sucrose likely resulted from differences in the growth conditions of the parent plant and not the reduction in expression in \textit{mrn1-1}. More importantly, this highlights the necessity to maintain control plants paired with mutant plants for at least one generation prior to testing growth and physiology between plants.

4.2.2 Hormone Induction of MCGs

In order to help identify a function for marneral-related metabolites, the expression profiles of MCGs were examined using the Arabidopsis eFP browser (Winter et al. 2007a). The hormone data set suggests that \textit{MRN1} is upregulated by both ABA and MeJA, but not other hormones including ethylene, cytokinin, auxin, or brassinosteroids (Figure 4.4). This analysis also shows an up-regulation of \textit{CYP71A16} and \textit{CYP705A12} by MeJA but not by ABA. While neither gene is very highly expressed
in these data sets, *CYP71A16* appears to be more highly expressed than *CYP71A16* (Figure 4.5). It should be noted that the original cDNA microarrays used to produce these data were conducted on 7 day-old seedlings.

Treatments with 10 µM ABA and 10 µM MeJA were conducted on plants expressing the fluorescent reporter *pMRN*<sub>4205::TIMER</sub> (for construct details see Chapter 2) and quantified using real time PCR, which also allowed the measurement of changes in *CYP71A16* and *CYP705A12* expression levels. Plants of various ages (5, 10, 15 days-old) were assessed to determine whether the sensitivity of *MRN1* changes outside of the optimal point of expression, between 10-13 days-old. Set points along the root were also imaged to determine if the induction changes the expression profile along the longitudinal axis of the root, as *MRN1* was shown to be primarily expressed in the oldest region of the root. Three individuals for each treatment are shown in order to display the range of expression after each treatment. Treatment with 10 µM ABA for 4 h appeared to have slightly increased the fluorescence of *MRN*<sub>4205::TIMER</sub>, but only in 10 day-old roots. There seemed to be no difference in 15 day-old roots and a slight decrease in expression of 5 day-old roots (Figure 4.6). MeJA treatment caused a slight increase in fluorescence in 5 day-old seedlings, a stronger increase in fluorescence in 10 day-old seedlings, and no change in fluorescence of 15 day-old seedlings (Figure 4.7). Furthermore, the increase in fluorescence occurs as expected in the oldest region of the root.

Real Time PCR analysis of these same treatments on wild-type Col-0 seedling tissue revealed much more dramatic increases in *MRN1* expression after treatment with either 10 µM ABA or 10 µM MeJA across all ages, compared with experiments using *pMRN*<sub>4205::TIMER</sub> expressing plants (Figure 4.8). In general, MeJA treatment had a stronger effect on younger seedlings, as was also true for *CYP71A16* and *CYP705A12*. ABA, in contrast, had a much stronger effect on the older roots, displaying up to 243-fold increase in expression. It should be pointed out that the expression values for *MRN1* were highly variable because the mock treatments tended to have a very low basal expression level. *CYP71A16* had a surprisingly minimal response displaying no more than 4-fold upregulation in any treatment. This is in agreement with the eFP browser data that showed a consistently present expression level of *CYP71A16*.
between mock and hormone treatments (Figure 4.5). *CYP705A12* followed the same trends as *MRN1*, as expected with the strong level of co-regulation.

### 4.2.3 Stress Induction of MCGs

ABA and MeJA are generally involved in regulating stress responsive pathways. Therefore, the Arabidopsis eFP browser (Winter et al. 2007a) was used again to determine if the MCGs were induced under stress conditions. *MRN1* was found to be induced by osmotic stresses, both ionic and non-ionic, but not under conditions of drought (Figure 4.9 A). It is important to note that *MRN1* was very weakly expressed under these conditions with a maximal absolute expression value of 25.13 (24 h NaCl treatment), whereas the maximum value displayed in other conditions was 2621. This value represents the normalized abundance of any particular transcript on the microarray slide. The plants used to collect these data were 18 days old, outside of peak expression period of *MRN1* (Figure 2.9). The Arabidopsis eFP browser provides data on a series of microarrays conducted to identify expression levels in specific cell types of the root using fluorescently activated cell sorting to compare expression profiles of roots treated with 140mM NaCl and those transferred to iron-free media (Dinneny et al. 2008). These experiments used five day-old seedlings. Both conditions enhanced the expression of *MRN1* and showed absolute expression values of 1701 and 1628 for NaCl and iron-free treatments, respectively, in cortical tissue compared to a control treatment with an absolute expression value of 787 (Figure 4.9 B). The upregulation by NaCl and mannitol agrees with both the real time PCR data showing an induction by ABA as well as a previous study investigating the plant’s response to NaCl treatments (dit Frey et al. 2010). Therefore *pMRN1*<sub>4502::TIMER</sub> plants were treated with a number of osmotic stress conditions, including 7% sucrose, 300mM mannitol, 150mM NaCl, and a treatment with ferrozine (300 µM), the iron-chelating compound used in the eFP data set. These treatments were given to 5, 10, and 15 day-old plants 24 hours before imaging. The roots were imaged along the root from the root-hypocotyl junction to the youngest region of the maturation zone. As the TIMER protein matures its fluorescent emission changes from green to red (500 nm to 580 nm), only TIMER<sub>green</sub> fluorescence was imaged in order to specifically show the youngest protein. The resulting images were then pseudo-coloured and the contrast was adjusted to highlight any differences in fluorescence between each treatment and a mock treatment. The sucrose treatment did not induce further expression of TIMER<sub>green</sub> at any time point (figure 4.10); whereas
300mM mannitol- (Figure 4.11) and 150mM NaCl- (Figure 4.12) treated plants both induced a stronger fluorescent signal than the mock treatments. This increase was apparent in both 5 and 10 day-old seedlings along the length of the imaged regions of the root. Roots of 15 day-old plants did not show an increase in expression. Plants transferred to iron-deficient media showed a slight induction of expression in 10 day-old seedlings, but 5 and 15 day-old plants showed no differences in fluorescence intensity (Figure 4.13).

A bioinformatics analysis shows that CYP71A16 expression was not differentially regulated by the osmotic stress treatments in the 18 day-old plants used in the abiotic stress data set from the eFP browser (Figure 4.14) (Winter et al. 2007a). CYP705A12 is only slightly differentially regulated by salinity stress. Similar to MRN, both CYPs are very weakly expressed in this data set with maximum absolute expression values of 64.1 and 24.5 for CYP71A16 and CYP705A12, respectively, with maximum expression values of 2891 and 3766, respectively, within all comparable datasets. CYP71A16 seems to be highly expressed in the root data series but does not seem to be affected by a NaCl treatment. The absolute expression level increased from 675 in the control to 1327 in iron-free media in the mature zone (zone 4), i.e. just more than 2-fold (Figure 4.15 A). CYP705A12 likewise was not upregulated in any zone by the NaCl treatment, but was upregulated 3-fold in the mature zone upon ferrozine treatment (Figure 4.15 B). Interestingly, CYP705A12 showed a high level of expression in columella cells of the root tip. Both treatments increased the expression of CYP705A12 less than 2-fold. These data do not seem to agree with co-expression analysis; however the tissue used for both data sets were collected outside of the peak expression period identified for MRN1 (6-13 days after germination, see Figure 2.9).

It was therefore necessary to directly test the expression of these genes under the conditions known to induce MRN1 using real time PCR (Figure 4.16). Wild-type plants were grown and treated under identical conditions to the pMRN14502::TIMER plants. Only 10 day-old plants were analyzed since this was found to be the developmental period at which ABA had the strongest effect on MRN1 expression. This analysis largely supported the expression profiles identified by the Arabidopsis eFP browser data set for both CYPs, with the exception of the ferrozine treatment, which showed CYP71A16 decrease expression by 2.2-fold and CYP705A12 decrease
expression by 1.4-fold. No difference in expression was found in any other treatment. 

*MRN1* expression was most highly upregulated by the treatment with 300 mM mannitol (40-fold). Treatment with 150 mM NaCl increased expression by 6-fold (Figure 4.16 A). It is surprising that neither CYP was differentially regulated by these stressors but it should be noted that the basal level of both CYPs was much greater than that of *MRN1* (Figure 4.16 B) There was 87-fold and 43-fold more CYP71A16 and CYP705A12 transcript, respectively, when comparing the transcript levels between control samples. This low basal expression level and high increase in expression suggests that the marneral pathway may be regulated through transcriptional control of *MRN1* as opposed to either CYP.

### 4.2.4 ABA activation of the marneral pathway

Since 10 µM ABA was identified as the treatment leading to the strongest induction of the MCGs, I wanted to determine if an increase in the production of marnerol could be observed. This treatment was conducted on *cyp71a16-1* tissue since this was previously shown to accumulate marnerol whereas it could not be identified in wild-type tissue (see Figure 3.10). Seedlings were grown on plates for 10 days and then transferred to liquid media containing 10 µM ABA or in a mock treatment and left at 21°C for another 48 h. The original characterization of *MRN1* upon hormone treatment was after a 4 h treatment and I speculated that more time would be needed to identify an increased production of marnerol. This was based on other studies measuring the accumulation of secondary triterpenoid pathways after hormonal induction that found the peak production of these metabolites to occur between 12 h and 8 days after induction (Suzuki et al. 2005, Achnine et al 2005, Scholz et al 2009). To confirm that ABA induction of *MRN1* is maintained over the course of this assay, real time PCR analysis shows that *MRN1* is induced upon treatment and remains up-regulated between 24 h and 48 h after induction by 9.5-fold and 5.9-fold, respectively. It is interesting to note that the mock treatment dramatically decreases *MRN1* expression level by 40.5-fold and 37.0 fold at 24 h and 48 h after the mock treatment, respectively (Figure 4.17a). Production of marnerol was enhanced by 12.4-fold from 6.3 ng/mg of non-saponifiable lipids (NSL) to 77.6 ng/mg NSLs (Figure 4.17b). These data show that ABA not only induces expression of *MRN1*, but that the gene product is also active.
4.2.5 Stress Tolerance

While the expression studies help to identify conditions with which the MCGs are expressed, the function of marneral related metabolites in the plant is still unknown. Other studies involving conditions effecting root elongation have also identified an upregulation of MRN1 through cDNA microarray analyses (Yang et al. 2005, dit Frey et al. 2010, Brüx et al. 2008). MRN1 expression was also found to be highest in the region where root elongation is slowing (see chapter 2). Together, these data indicate that MRN1 expression may be linked to the cessation of root elongation. To determine if the marneral pathway is involved in the cessation of root elongation as a response to hormone or osmotic stress treatments, 5 day-old seedlings of cyp71a16-1, and transgenic plants expressing pUBQ1::MRN1, pUBQ1::CYP71A16-2A-MRN1 and pUBQ1::CYP705A12-2A-CYP71A16-2A-MRN1 were treated with varying concentrations of ABA, MeJA, mannitol, or NaCl. The average root elongation rate was then calculated over the next four days of growth (Figures 4.18 – 4.21, respectively). mrn1-1 was not used because it was found to produce MRN1 transcript (See Chapter 2). In the root growth assay, there was less than a 10% difference in relative root elongation rates in every plant compared to control plants. Salt and osmotic stresses can also affect other aspects of plant morphology such as leaf/hypocotyl expansion or lateral root formation (Burssens et al. 2000). There were, however, no noticeable changes in these traits between the different lines and their respective controls. These data suggest that the induction of the MCGs within these treatments is not responsible for changes in root growth.

4.3 DISCUSSION

4.3.1 Sucrose Effects on Seed Germination

I initially found that mrn1-1 roots grew longer compared to wild type when grown on media lacking sucrose. While testing this observation over a range of sugar concentrations, I noticed that the difference in root lengths seemed to be correlated with the speed of germination. Rognoni et al. (2007) showed previously that the effect of sucrose on germination rates is directly linked to the growth conditions of the parent plants. Once three independent sets of seeds were grown it was found that mrn1-1 had no effect on the relationship between sucrose concentration and germination speed or root elongation. These data exemplify the importance of obtaining mutant or transgenic
seed paired with control seeds grown side by side to pair physiological traits of the offspring.

4.3.2 Marneral Cluster Gene Expression

Bioinformatic data showed that the MCGs are induced developmentally between 8-13 days after germination and that their expression can be stimulated by ABA, MeJA, and both ionic and non-ionic osmotic stress treatments (eFP browser; Winter et al. 2007a; Dinneny et al. 2008). These data were generally confirmed by visual comparison of pMRN4201::TIMER plants using confocal microscopy followed by real time PCR. The only strange occurrence was that the pMRN4201::TIMER plants did not change expression levels upon treatment with ABA, despite the fact that, according to real time PCR analysis, ABA was the strongest inducer of MRN1 expression. This may be due to the short treatment (4 h) not providing enough time to allow the TIMERgreen protein to accumulate to levels sufficient for fluorescence detection. This would also help to explain why greater differences in fluorescence occurred in the osmotic treatments (24 h), whereas real time PCR data show that hormones have the strongest effect on MRN1 expression. Alternatively, there could be an ABA regulatory element not included in the promoter-reporter construct.

There are general interactions between ABA and MeJA that help to regulate responses between biotic and abiotic stresses (Fujita et al. 2006). It has recently been noted that a plant’s response to stress is likely tuned to the environment as a whole, and that the responses do not accumulate additively for multiple individual stresses (Atkinson and Erwin 2012). With this in mind, it is not surprising that different gene networks can be induced by combined stimuli, whereas some of these pathways would fail to respond to single stressors. This has been speculated to occur in order for the plant to optimize growth under any particular condition while protecting itself from any biotic or abiotic stressors. This is exemplified by the fact that constitutive activation of defense-related pathways often result in plants with suboptimal growth (Herms and Mattson 1992). This could also help to explain the weak growth and development found in 35S::MRN1 and 35S::MRN1x 35S::CYP71A16 plants (Field et al. 2011) or in 35S::THAL plants (Field and Osborne 2008). As researchers are beginning to probe plant defense against simultaneous stresses, ABA, jasmonates, ethylene, and salicylic acid all seem to be involved in crosstalk to coordinate expression of appropriate
responses. Of these hormones, ABA seems to be central to most stress responses, and regulates the activation of the classically defined biotic stress hormone pathways of salicylic acid and jasmonates. It has been implicated in signalling pathways contributing to both the resistance and increased susceptibility to plant diseases and pathogens.

A model was recently proposed to explain how ABA can be involved in biotic stress response pathways (Ton et al. 2009). The model describes the response to fungal or bacterial pathogens, both proceeding in three phases. Phase I is universal to both responses by closing stomata and blocking salicylic acid, jasmonate, and ethylene signalling pathways to prevent resource waste. Phase II sees the induction of post-invasion defenses occurring, which involves the production of callose. At this point ABA continues to play a role in defense signalling for fungal defense, but not for bacterial defense. As the fungal infection proceeds into Phase III, ABA gradually plays less of a signalling role while jasmonate activity increases. At the same time ABA inhibits any ethylene and salicylic acid pathways. It is at this point that marneral production might be induced to function in defence, assuming the presence of an osmotic stress. This model could help to explain the involvement of both ABA and MeJA in \textit{MRN1} activation and supports my root stress physiology data, which found no alterations in root growth when MCG expression was altered.

\textbf{4.3.3 Sensitivity Over Development}

The real time PCR data found the sensitivities of the MCGs to ABA and MeJA changed inversely with the age of the seedling. This introduces an additional factor when considering how plants react biochemically to their environment. The developmental regulation of defense genes has only been reported once before in a relationship between VSP1 and VSP2 (vegetative sorting protein 1 and 2), in relation to induction by MeJA (Matthes et al. 2008). \textit{VSP1/2} (combined) levels were induced by MeJA to various degrees in different Arabidopsis accessions, but the expression in Col-0 and Ge-3 was less sensitive in leaves over time. \textit{VSP2} has been shown to affect insect development and mortality through digestion and is thought to be produced as an anti-insect protein in the plant (Liu et al. 2005). This indicates that the utility of \textit{VSP2} defense decreases as the plant develops. This supports the idea that marneral related metabolites, if they function in defence, are important for protecting the roots of pre-bolting plants, especially while the growing environment is experiencing osmotic stress.
On the other hand, PYRABACTIN RESISTANCE (PYR) and PYR LIKE (PYL) proteins have recently been found to be integral components of ABA signalling pathways (Joshi-Saha et al. 2011). Three members of this gene family in Arabidopsis have been identified as the first signalling components specifically involved in the interaction between ABA and jasmonic acid (JA), including PYL4, PYL5, and PYL6 (Lackman et al. 2011). In that study, the authors found that ply4 and ply5 loss of function mutants had diminished responses to JA treatment and that the genes are regulated by both JAs and JA-related stressors such as wounding, flagellin, syringolin, or infections by Blumeria graminis and Psuedomonas syringae. Interestingly, these proteins are characterized by the presence of a START domain (Steroidogenic acute regulatory-related lipid transfer domain) that has been shown to use lipids, including sterols, as ligands (Joshi-Saha et al. 2011). It would be interesting to determine if triterpene aglycones, such as metabolites from the marnerol pathway, function as signalling molecules in pathways involving proteins like PYLs, although no changes in physiology to MeJA or ABA were identified in this study. It is also possible that the PYL proteins regulate transcription of MRN1 (indirectly) in both ABA and MeJA treatments. Further analysis of the MRN1 promoter is needed to determine if the induction by ABA and MeJA are independent or not.

4.3.4 Other Aspects of MRN1 Transcript Levels

It was found previously that histone modifications occur in the region containing the MCGs (Zhang et al. 2007, discussed in Field et al. 2011). Histone modifications affect how nucleosomes interact with and position themselves on chromatin. This ultimately controls access of the DNA to transcription factors that will regulate transcription of DNA (reviewed in Fransz and de Jong 2011). The chromatin surrounding the MCGs is known to be targeted by histone H3 lysine 27 trimethylation (H3K27me3), which represses expression of target genes (Field et al. 2011, Zhang et al. 2007). Beyond H3K27me3 modifications, there are many histone modifications that occur in ABA and salt stressed plants (Kim et al 2010) that are likely to contribute to the overall expression profile of MRN1.

It is also important to look at the lifespan of mRNAs under stress conditions. TudorSN proteins have been shown to bind to target transcripts in order to both stabilize and transport them to the ER (dit Frey et al. 2010). They are also involved in
germination and growth responses to ABA and NaCl. *MRN1* was underrepresented in salt-stressed plants lacking TudorSN proteins, suggesting that *MRN1* transcripts are bound by these proteins, which increases their longevity. While I have shown that there is an induction of *MRN1* in response to salinity stress, this could be showing that in some cases *MRN1* transcription is unaffected but the stability of the RNA molecule is enhanced.

### 4.3.5 Marneral-Derived Metabolite Function

It is interesting to find that mutant and transgenic plants show no alteration in growth when treated with ABA, MeJA, NaCl, or Mannitol treatments, suggesting that although the MCGs are expressed under these conditions and the pathway was shown to be active upon ABA treatment, marneral-derived metabolites do not seem to be involved in the cessation of root growth in response to these treatments. The only other reports of marneral-like compounds are in the Iridaceae and are known as iridals. Originally identified by Franz-Joseph Marner, for whom marneral was named, these compounds display defensive properties, including cytotoxic (Bonfils et al. 2001), antiplasmodial and antifungal characteristics (Benoit-Vical et al. 2003). It is possible that the marneral-related metabolites share analogous functions. Since *MRN1* expression seems to be highly developmentally regulated, with peak expression around 10-13 days post germination and highest in the inner tissue layers of the root, it is possible that the pathway is expressed in order to protect the oldest regions of the root from herbivory, especially when considering the lack of physiological changes from the root growth assays, despite the data supporting differential regulation of the *MRN1* cluster in response to environmental stress.

Other cDNA microarrays have identified *MRN1* as being one of the most differentially regulated genes under the conditions tested (Yang et al. 2005, Brüx et al. 2008). While it is likely that the magnitude of the induction of *MRN1* found in any transcriptional data set results from the fact that it normally has such low expression levels, the idea that *MRN1* transcription is an important control point for the whole cluster suggests that any inducing conditions are useful for determining a function for the metabolites, and less so for either CYP. Yang et al. (2005) identified MEMBRANE STEROL BINDING PROTEIN 1 (MSBP1) based on its homology to a similar protein that can bind progesterone in pigs. These types of proteins (steroid binding proteins)
can act as either steroid receptor molecules that relay cellular signals or as steroid carrier proteins that control the transport and metabolism of steroids. Yang et al. (2005) overexpressed MSBP1 and found MRN1 as one of the highest upregulated genes in their cDNA microarray analysis. This was interpreted to be evidence for the involvement of a novel sterol (or in this case, non-sterol triterpene). In subsequent studies they found that MSBP1 is involved in regulating the turnover of the polar auxin transport protein PIN2 through endocytosis (Yang et al. 2008) as well as negatively regulating BAK1 (BRI1-associated receptor kinase) (Song et al. 2009) which localizes to both the plasma membrane and endosomes (Russinova et al. 2004). Through these actions, MSBP1 inhibits cell elongation (Yang et al. 2005). While BAK1 is best characterized for its role in brassinosteroid signalling, it has also been shown to be involved early in biotic stress elicitor signalling pathways in a brassinosteroid independent manner (Chinchilla et al. 2007). While there is no evidence that marner related metabolites are involved in brassinosteroid or auxin signalling, the induction of MRN1 by the overproduction of MSBP1 could indicate that the metabolites play a role in this signalling process within the plant and not necessarily directly in defence, as most secondary metabolites are considered to do.

Brüx et al. (2008) found that the conditional vacuolar ATPase mutant det3, which codes for a V-ATPase located in the Golgi apparatus and early endosomes (VHA-c), has defects in cell elongation. It is susceptible to treatments in 16°C or upon treatments with KNO₃. It is under these treatments that MRN1 is upregulated (12.6-fold and 190-fold, respectively, when compared to wild type). While the 16°C treatment caused defects in splicing VHA-c transcripts, and thus lower levels of RNA, the nature of the KNO₃ phenotype is unclear. It led to a response indicative of oxylipin signalling. My data agree with these in that they show how the global reaction of det3 plants is indicative of oxylipin signalling and I show that JA, an oxylipin, induces MRN1 expression. Other studies found that insufficient V-ATPase activity in the Golgi-network/early endosomes causes hypersensitivity to NaCl treatments (Batelli et al. 2007, Krebs et al. 2010) and point to the possibility that Golgi/early endosomal V-ATPases are necessary to power Na⁺/H⁺ antiporters that pump Na⁺ out of the cytosol and into endosomes for storage and/or disposal (Schumacher and Krebs 2010). How or if marneral-related metabolites are associated with these processes remains to be
determined. There is an interesting commonality between det3 and MSBP1 in that the two studies provide a link between the perturbation of the Golgi-network/early endosome pathways and MRN1 expression.

4.3.6 Conclusion

Although stress hormones and osmotic stresses induce transcription of the marneral pathway, there is no change in the root elongation rate of plants lacking a functional pathway. There is evidence that plants respond to abiotic stresses by protecting against biotic stresses. This may be the primary function of marneral-related metabolites. There is evidence, however, that MRN1 is expressed in lines with defects in Golgi/endosome related trafficking, which leaves a question as to whether these metabolites play a role in signalling pathways. Further experiments are required to identify these functions.

4.4 MATERIALS AND METHODS

4.4.1 Plant Growth Conditions

Media for RNA analysis of stress treatments and microscopic analysis of pMRN14205::TIMER were prepared as following. Seeds were germinated on 0.8% agar, 1% sucrose, Hoagland’s media and grown for the indicated amount of time. For each treatment, seedlings were added to 150ml liquid 1x Hoagland’s solution supplemented with the indicated treatments for 4h (for MeJA and ABA treatments) or 24h (for mannitol, NaCl, and Ferrozine treatments).

4.4.2 Root Growth Analysis and Conditions

Seeds were sterilized and placed in water at 4°C in the dark for at least 48h. They were then germinated on 0.8% agar, 1% sucrose in Hoagland’s media and grown at 21°C under continuous light for 5 days. Seeds were planted on six rows with 18 seeds/row on a square 10 x 10 x 1.5 cm Petri dish. Five plates per treatment per genotype were prepared by transferring seedlings of similar sizes (between transgenic/mutant plants and controls) to new plates at a density of seven seedlings per genotype per plate. These plates were then scanned on a flatbed scanner, and returned to a 21°C growth chamber under continuous light. They were then scanned daily until the seedlings were 9 days old. The images from the scanner were analyzed using image J (http://rsb.info.nih.gov/ij/) by taking the first and last image in the series, converting them to a stack, stabilizing the two images within the stack, converting to
grayscale, and then using the “split to RGB” function to colour the image of the young seedlings green and the older seedling red and merging the images. The new growth was then measured and recorded for analysis. The measurements from the five pooled plates of each treatment were used to conduct student T-tests to calculate significant differences between each genotype and the control plants grown on the same plate.

Supplements to media for root growth analysis were prepared as follows. Plates containing MeJA and ABA were prepared by adding 1 ml/l of 1000 times the target concentration of each hormone in EtOH after the media was autoclaved. NaCl and ferrozine (Sigma Aldrich) were added to the media in the appropriate concentrations by adding the salts prior to autoclaving. Mannitol-containing plates were prepared at two times the concentration and the mannitol was prepared separately at two times the concentration and was added to the autoclaved media through a vacuum filter (0.22 µm pore size, Millipore Steritop Bottle- Top Filter). There was 50 ml of media added to each plate.

4.4.3 Growth Conditions for Sucrose Analysis
For germination analysis, seeds were placed on Petri dishes containing Hoagland’s media supplemented with the appropriate amount of sucrose and 1.2% agar. They were then grown under continuous light and the seedlings were scored for their stage of germination daily. For root growth analysis, the seeds were prepared in the same way, but left to grow for 8 days before the plates were scanned on a flatbed scanner daily and measurements were made on the resulting images using Image J (http://rsb.info.nih.gov/ij/).

4.4.4 Expression Analysis Growth Conditions
Plants were grown on vertical Petri plates containing Hoagland’s media supplemented with 1% sucrose and 0.8% agar and were grown under continuous light for the indicated number of days. For ABA and MeJA, 4-6 h before imaging/RNA extraction, the plants were placed into liquid Hoagland’s media containing hormones or ethanol at a concentration of 0.1%, for a final molarity of 10 µM. For 150 mM NaCl, 300 mM mannitol, 0.3mM ferrozine, and 7% sucrose treatments, the plants were transferred onto fresh Hoagland’s Petri plates supplemented with 1% sucrose (with the exception of the 7% sucrose treatments), 0.7% agar, and the indicated stressors 24 h before imaging/RNA extraction. At least nine plants were imaged for each treatment and age.
and non-transgenic plants were always grown on the same plates and imaged under identical conditions. Three 1 mg samples of tissue were collect for RNA extraction for real time PCR analysis.

4.4.5 Microscopic Analysis of pMRN14205::TIMER

Images were acquired on a Zeiss Pascal confocal microscope using the 488-nm line from an argon laser using a 20x objective. TIMERgreen fluorescence was detected through a 505-530nm band pass filter.

Images were then analyzed using ImageJ (http://rsb.info.nih.gov/ij/) by first putting all images of the same treatments into a stack, then making a montage. The contrast was then optimized for each treatment data set and the lookup table “Rainbow RGB” was applied.

4.4.6 RNA Extraction

All RNA extractions were conducted using the combined Trizol and Silica Column method as described in Chapter 2.

4.4.7 Real Time PCR analysis

All materials used for real time PCR were prepared in accordance with the MIQE guidelines for qPCR (Bustin et al 2009). First strand cDNA synthesis was conducted using Superscript II or Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. A total of 3.5 µg of RNA was used in each reaction. The reactions were primed with oligo dT18 primers. When the total RNA extraction from any sample in a particular experiment yielded less than 320 ng/µl (the minimum concentration to use 3.5 µg in a standard 20 µl reaction) the sample was concentrated with a silica column and eluted in a smaller volume. The PCR was conducted on a MyiQ 5 Real Time PCR Detection System (BIORAD) coupled with the iQ5 software. The data were analyzed according to the Pfaffle method as described in Chapter 2 (Pfaffl 2001). For the relative comparison between MRN1 and each CYP (Figure 4.16b), the Pfaffle equation was modified to include the efficiencies of all three sets of primers using the following:

\[
\text{Fold difference} = \frac{(\text{Eff}_{\text{target A}})^{C_{\text{qtarget A}}}}{(\text{Eff}_{\text{reference}})^{C_{\text{qreference A}}}} \times \frac{(\text{Eff}_{\text{reference}})^{C_{\text{qreference B}}}}{(\text{Eff}_{\text{target B}})^{C_{\text{qtarget B}}}}
\]
Target A represents MRN1 whereas target B represents either P450. Reference A or B refer to the C_q value for the reference gene (ACT8) from each experiment.

4.4.8 Quantification of marnerol

Plants were grown as described in section 4.4.4 with the exception that plates were prepared at a density of 40 seeds/plate and 9 plates/ treatment were prepared and grown for 10 days. Plants used for the 0 h timepoint for both RNA and metabolite analysis were harvested directly from plates. Both treated and mock samples were placed in 100 mL liquid Hoagland’s medium with either ABA in ethanol or ethanol alone and cultured at 21°C. After 24 h 200 mg of tissue was harvested out of each replicate and prepared for RNA extraction. After 48 h, the tissue was harvested and approximately 100mg of tissue was set aside for RNA extraction. The remaining tissue was prepared and analyzed as described in section 3.4.11 with the exception that 4 mg of non-saponifiable lipids were applied to a 0.5 mm TLC plate. The GC-FID peak corresponding to marnerol TMS ether was identified based on the retention time of the peak corresponding to the confirmed marnerol TMS ether from the cyp71a16-1 analysis in figure 3.10 because quantities were below detection using GC/MS.
Figure 4. 1 Alterations of root length by sucrose. Graph showing longer average root length of \textit{mm1-1} compared to wild type grown on 0\% sucrose media after 8 days at 21\degree C, but no difference is found in plants grown on 3\% sucrose media. \( n = 27 \). * indicates \( p < 0.01 \).Bars represent \( \pm \) standard error.
Figure 4. 2 Analysis of sucrose effects on germination and root growth. A. Graph indicates percentage of seedlings in any particular germination stage for \textit{mmr1-1} and wild type-seeds on media containing 0%, 1%, 3%, 5%, and 9% sucrose after 48 h at 21°C. B. Graph indicates percentage of seedlings in any particular germination stage between three sets of \textit{mmr1-1} and wild-type seeds germinated on 0% sucrose after 48 h at 21°C. \( n = 45 \).
Figure 4. 3 Root length of paired seeds in relation to sucrose Graph indicates root length of 8 day-old seedlings on media containing 0% or 3% sucrose. Seeds were from "set A" in Fig 4.2 B. n = 27. Bars indicate ± standard error.
Figure 4. 4 Bioinformatic analysis of the induction of MRN1 transcription from hormone treatments using the Arabidopsis eFP browser (Winter et al. 2007a). Most hormones do not induce the transcription of MRN, including ethylene (ACC), cytokinin (zeatin), auxin (IAA), gibberelin (GA-3), or brassinosteroids (brassinolide). MRN1 transcription is induced 3h after treatment with ABA or MeJA. Global transcript distribution graph represents a distribution of average expression levels for all genes within the data set used by the eFP browser. Red bar indicates MRN1 expression level. The grey bar indicates the highest level of MRN1 expression in any data set. Heat map shows the absolute expression values and their distribution within this data set.
Figure 4. 5 Bioinformatic analysis of the induction of CYP71A16 and CYP705A12 transcription by ABA and methyl jasmonate using the Arabidopsis eFP browser (Winter et al. 2007a). ABA did not induce either cytochrome P450, MeJA however, induced transcription within an hour of treatment, which reached a high level of expression after 3 h. Global transcript distribution graph represents a distribution of average expression levels for all genes within the eFP browser data set indicating that both genes are relatively weakly expressed within the hormone data set. Red bar indicates cytochrome P450 expression level. The grey bar indicates the highest level of expression in any data set used by the eFP browser. Heat map shows the absolute expression values and is accurate for data from both cytochrome P450s.
Figure 4. 6 Expression profile of pMRN::TIMER treated with 10 µm ABA for 4 hours. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. After acquisition, images were pseudo-coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Heat map shows colour profile used. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence B-D. Plants treated for 4 h with EtOH (left) or 10 µM ABA in EtOH (right). Three replicates are shown to provide a range of expression B. 15 day-old plants. C. 10 day-old plants D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. 7 Expression profile of pMRN::TIMER treated with 10 µm Methyl Jasmonate for 4 hours. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. After acquisition, images were pseudo-coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Heat map shows colour profile used. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence. B-D. Plants treated for 4 h with EtOH (left) or 10 µM ABA in EtOH (right). Three replicates are shown to provide a range of expression. B. 15 day-old plants. C. 10 day-old plants. D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. 8 Real time PCR analysis of MCGs treated with 10 µM ABA and 10 µM MeJA. Plants were treated for four hours in liquid media containing 0.1% ethanol alone or with each hormone, respectively. Data is the average expression from whole plant tissue of three biological replicates relative to ACT8. Bars indicate standard error. Grey line indicates 2-fold change.
A

Control

0 hour 0.25 hour 0.5 hour 1 hour 3 hours 6 hours 12 hours 24 hours

Osmotic 300 mM Mannitol

0 hour 0.5 hour 1 hour 3 hours 6 hours 12 hours 24 hours

Salt 150 mM NaCl

0 hour 0.5 hour 1 hour 3 hours 6 hours 12 hours 24 hours

Drought plants were exposed to an air stream for 15 min with loss of app. 10% fresh weight

0 hour 0.25 hour 0.5 hour 1 hour 3 hours 6 hours 12 hours 24 hours

Global transcript distribution

Absolute Expression Value

B

Salt Response

Control

140 mM NaCl

Iron Deficiency

Control

Fe minus

Global transcript distribution

Absolute Expression Value
Figure 4. 9 Bioinformatic analysis of the induction of *MRN1* transcription from water related stressors using the Arabidopsis eFP browser (Winter et al. 2007a).  A. Ionic and non-ionic osmotic stresses induce expression of *MRN1* after 24 hours, but not drought stress through transpiration. Note that the absolute expression values were very low, indicated by the lack of a red bar on the global transcript distribution graph. This represents a distribution of average expression levels for all genes within the eFP browser data set. The grey bar indicates the highest level of expression in any data set used by the eFP browser. Heat map shows the absolute expression values. Note materials used to generate these data were from 18 day-old plants (Kilian et al. 2007).  B. eFP browser data from root data source for salt and iron deficiency treatments (Dineny et al. 2008). Longitudinal (right) and cross section (centre) diagrams for each treatment representative of data derived from fluorescence activated cell sorting experiments. Diagram of root (right) represents a separate dataset derived from roots dissected into the four different zones along the longitudinal axis of the root. Global transcript distribution represents a histogram of all expression values across data sets. The red bar indicates the expression level of *MRN1* within that set and the grey bar indicates the maximum level of expression of *MRN1* across any eFP browser dataset. Note plants used to generate these data were 5 days old.
Figure 4. Expression profile of pMRN::TIMER treated with 7% sucrose for 24 h. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Plants transferred to fresh plates (left) or to fresh plates with 7% sucrose (right) 24 h before imaging. After acquisition, images were pseudo-coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence. Three replicates are shown to provide a range of expression. B. 15 day-old plants. C. 10 day-old plants. D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. 11 Expression profile of pMRN::TIMER treated with 300 mM mannitol for 24 h. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Plants transferred to fresh plates (left) or to plates containing 300 mM mannitol (right) 24 h before imaging. After acquisition, images were pseudo-coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence. Three replicates are shown to provide a range of expression B. 15 day-old plants. C. 10 day-old plants D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. 12 Expression profile of *pMRN::TIMER* treated with 150 mM NaCl for 24 h.  A. Key showing the regions photographed along with the eFP browser expression data for *MRN1*. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Plants transferred to fresh plates (left) or to plates containing 150 mM NaCl (right) 24 h before imaging. After acquisition, images were pseudo coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence. Three replicates are shown to provide a range of expression. B. 15 day-old plants. C. 10 day-old plants. D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. Expression profile of pMRN::TIMER treated with 300 mM ferrozine for 24 h. A. Key showing the regions photographed along with the eFP browser expression data for MRN1. The roman numerals indicate the region imaged for the subsequent treatments. B-D. Plants transferred to fresh plates (left) or to plates containing 300 mM ferrozine (right) 24 h before imaging. After acquisition, images were pseudo-coloured and the contrast was enhanced to more easily view differences in fluorescence. Only the fluorescence at 488 nm was imaged in order to only account for new fluorescent protein. Non-transgenic wild type Col-0 control plants were always imaged under similar conditions to account for autofluorescence. Three replicates are shown to provide a range of expression B. 15 day-old plants. C. 10 day-old plants. D. 5 day-old plants. Scale bars = 150 µm.
Figure 4. 14 Bioinformatic analysis of the induction of CYP71A16 and CYP705A12. Analysis conducted from the abiotic stress series from the Arabidopsis eFP browser (Winter et al. 2007a). CYP71A16 (left) is not induced by mannitol or NaCl. CYP705A12 (right) is induced only by NaCl. Note that the absolute expression values were very low. This represents a distribution of average expression levels for all genes within the eFP browser data set. The grey bar indicates the highest level of expression in any data set used by the eFP browser. Heat map shows the absolute expression values. Plants used to generate these data were from 18 day-old plants (Kilian et al. 2007).
Figure 4. 15 Bioinformatic analysis of the induction of *CYP71A16* and *CYP705A12* in root dataset from Arabidopsis eFP browser (Winter et al. 2007a). *CYP71A16* (left) is not induced by mannitol or NaCl. *CYP705A12* (right) is induced only by NaCl. Note that the absolute expression values were very low. This represents a distribution of average expression levels for all genes within the eFP browser data set. The grey bar indicates the highest level of expression in any data set used by the eFP browser. Heat map shows the absolute expression values. Note plants used to generate these data were from 18 day-old plants (Kilian et al. 2007).
Figure 4.16 Real time PCR analysis of MCG after stress treatments.  

A. Analysis of how 150 mM NaCl (blue), 300 mM mannitol (red), 7% sucrose (green), or iron-deficient media (purple) treatments for 24 h affect the expression of MRN1, CYP71A16, and CYP705A12.  

B. Amount of CYP71A16 and CYP705A12 transcript relative to MRN1 in control treatments from A. Data is the average expression of three biological replicates relative to ACT8. Bars are mean ± standard error.
Figure 4. **Induction of the marnerol metabolic pathway.** A. Real time PCR analysis of *MRN1* in 10-day old plants treated with 10 µM ABA (black) or not (grey) 24 h and 48 h after treatment relative to levels prior to treatment. B. Amount of marnerol TMS ether in plants 48 h after treatment relative to pretreated levels. Tissue used for A and B were from the same source. Values are an average of 2 separate biological replicates.
Figure 4. Analysis of relative root elongation rates in response to various concentrations of ABA. Graphs show Col-0 (black) compared to mutant or transgenic plants (grey). A. cyp71a16-1 B. pUBQ1::MRN1 C. pUBQ1::CYP71A16-2A-MRN1 D. pUBQ1::CYP705A12-2A-CYP71A16-2A-MRN1. Averaged root elongation rates calculated from 5 day-old seedlings transferred to new plates containing varying concentrations of ABA for 4 days. These rates were relative to rates calculated from mock treatments. Bars indicate ± standard error. n = 21-56.
Figure 4. Analysis of relative root elongation rates in response to various concentrations of MeJA. Graphs show Col-0 (black) compared to mutant or transgenic plants (grey). A. cyp71a16-1 B. pUBQ1::MRN1 C. pUBQ1::CYP71A16-2A-MRN1 D. pUBQ1::CYP705A12-2A-CYP71A16-2A-MRN1. Averaged root elongation rates calculated from 5 day-old seedlings transferred to new plates containing varying concentrations of MeJA for 4 days. These rates were relative to rates calculated from mock treatments. Bars indicate ± standard error. n = 21-56.
Figure 4. Analysis of relative root elongation rates in response to various concentrations of mannitol. Graphs show Col-0 (black) compared to mutant or transgenic plants (grey). A. cyp71a16-1 B. pUBQ1::MRN1 C. pUBQ1::CYP71A16-2A-MRN1 D. pUBQ1::CYP705A12-2A-CYP71A16-2A-MRN1. Averaged root elongation rates calculated from 5 day-old seedlings transferred to new plates containing varying concentrations of mannitol for 4 days. These rates were relative to rates calculated from mock treatments. Bars indicate ± standard error. n = 21-56.
Figure 4. Analysis of relative root elongation rates in response to various concentrations of NaCl. Graphs show Col-0 (black) compared to mutant or transgenic plants (grey). A. cyp71a16-1 B. pUBQ1::MRN1 C. pUBQ1::CYP71A16-2A-MRN1 D. pUBQ1::CYP705A12-2A-CYP71A16-2A-MRN1. Averaged root elongation rates calculated from 5 day-old seedlings transferred to new plates containing varying concentrations of NaCl for 4 days. These rates were relative to rates calculated from mock treatments. Bars indicate ± standard error. n = 21-56.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF MAJOR FINDINGS

In this thesis, I identified a metabolic gene cluster consisting of an OSC and two unrelated CYPs, including \( \textit{MRN1} \), \( \textit{CYP71A16} \), and \( \textit{CYP705A12} \). These phylogenetically unrelated genes are neighboring each other, and two were found to function in a single pathway. While these genes were previously identified as being upregulated when microtubules are disrupted (Walia 2009); I later found that this was not the case. In order to confirm the activity of these genes in a single pathway, I expressed them in \textit{Arabidopsis} and identified the presence of marnerol in leaves of plants ectopically expressing \( \textit{MRN1} \) and in seedlings of \( \textit{cyp71a16-1} \). \( \textit{CYP705A12} \) was not characterized but is assumed to be part of the metabolic gene cluster due to its high level of co-expression with \( \textit{MRN1} \) and neighbouring location in the genome. I identified conditions that induce the expression of these genes using bioinformatics and confirmed that data by developing a \( \textit{MRN1} \)-specific fluorescent promoter-reporter construct, as well as using real time PCR to measure expression of all three genes. Using the treatment that induced the MCGs the greatest (ABA), I showed a greater accumulation of marnerol \textit{in planta} 48 hours after induction. I then tested the response of plants ectopically expressing different combinations of the three genes, as well as the mutant \( \textit{cyp71a16-1} \), to the treatments shown to induce transcription of the cluster in order to identify a function for the pathway. Surprisingly, no changes in root growth were found in the response to these conditions. Listed below is a summary of all the major findings of this thesis.

Summary of major findings

- \( \textit{MRN1} \) is not induced by microtubule depolymerization alone. (Ch. 2)
- \( \textit{MRN1} \) expression is concentrated in the vascular cylinder of the root. (Ch. 3)
- \( \textit{CYP71A16} \) is the second enzyme in the marnerol pathway. (Ch. 3)
- \( \textit{SQS1} \), but not \( \textit{SQE1} \) or \( \textit{SQE4} \), expression can be induced to rapidly enhance triterpene production (Ch. 3)
- Between the three genes in the cluster, \( \textit{MRN1} \) shows the most dynamic transcriptional profile, suggesting it as a regulation point in the pathway. (Ch.4)
• Transcription of the marneral gene cluster is induced most strongly by the hormones ABA and MeJA, with inversely varying sensitivities over the course of seedling development. (Ch. 4)

• Osmotic stresses induce expression of the marneral gene cluster. (Ch. 4)

• ABA promotes the production of marnerol (Ch. 4)

• The marneral pathway is not necessary for cessation of root elongation in response to ABA, MeJA, ionic and non-ionic osmotic stresses. (Ch. 4)

5.2 MICROTBULES AND STRESS SIGNALLING (CHAPTER 2)

5.2.1 Is There a Relationship Between MRN1 and Microtubules?

I found that MRN1 expression was not induced by a disruption in microtubule dynamics, as opposed to what was previously shown (Walia 2009). It is possible that the microtubule array’s response to any particular stress carries information specific for that stress, which my experiments did not capture, similar to what has become known as a calcium signature that describes the very specific patterns of Ca^{2+} concentration fluxes in the cytosol under different stresses (McAinsh and Pittman 2009). It is evident from Table 2.2 that there was a secondary factor involved in tissue preparation that initially found MRN1 induction in mor1-1 (see Table 2.2 for expression levels to compare experiments in which tissue was prepared by me or Ankit Walia*). What is particularly interesting is that the stress and ABA treatments found to upregulate MRN1 are also known to involve changes in microtubule dynamics, specifically including ABA treatment (Sakiyama and Shibaoka 1990), and both ionic and non-ionic osmotic stresses (Blancaflor and Hasenstein 1995, Komis et al. 2002, 2008). A rigorous analysis of the MRN1 promoter is needed to clarify the relationships between stresses related to microtubule disruption and MRN1 transcription. This could identify transcription factors responsible for inducing the expression of MRN1 under these treatments and would allow testing of the activity of these transcription factors under these treatments (ABA, NaCl, and mannitol) with or without functional microtubules. Of these treatments, salt stress has the most described connections between microtubules and stress response.

Specific connections between microtubules and salt treatments include the SALT OVERLY SENSITIVE (SOS) pathway genes (Shoji et al. 2006, Wang et al. 2007) and mutants in the PREFOLDIN complex that are responsible for folding tubulin proteins
SOS1 is a Na\(^+\)/H\(^+\) antiporter and SOS2 is a kinase that regulates SOS1 (reviewed in Zhu 2003). Mutants of these genes are both hypersensitive to NaCl treatments and have abnormal responses to microtubule-affecting drugs (Shoji et al. 2006, Wang et al. 2007). The PREFOLDIN complex mutants pfd3 and pfd5 both have reduced levels of tubulin that are also hypersensitive to NaCl, but not LiCl or mannitol (Rodriguez-Milla and Salinas 2009). While it is not clear if these two phenotypes are linked, it suggests that microtubules play a role in salt stress response. While my data show an increase in \(MRN1\) in salt stressed plants, dit Frey et al. (2010) found that the tudorSN proteins may protect the \(MRN1\) transcripts from degradation under this stress. Interestingly, tudorSN proteins were shown in multiple studies to associate with microtubules (Sami-subbu et al. 2001, Abe et al. 2003, and Chuong et al. 2004). The interactions between microtubules, tudorSN, and \(MRN1\) transcript level under salt stress needs to be characterized further.

5.2.2 Elucidating a Relationship Between Microtubule Depolymerization and Transcription.

The original \(mor1-1\) cDNA microarray falsely identified a number of genes as being differentially regulated as determined by a real time PCR analysis on the same RNA (data not shown). The different responses of the MCGs in subsequent analyses also question the validity of this analysis. However, using a cDNA microarray is still a reasonable approach to identifying microtubule-dependent signalling pathways. By identifying the differentially regulated genes, common promoter elements might then be elucidated that respond to changes in microtubule dynamics, and subsequently the transcription factors that bind to them.

An alternative approach to the characterization of the role of microtubules in controlling gene transcription would be to further characterize the relationships between known microtubule-interacting transcription factors and microtubules. Some examples include OFPs (Hackbusch et al. 2005) or other bHLH proteins such as KNAT1 (Winter et al. 2007b), or SCARECROW LIKE 14, a tubulin binding transcription factor (Chuong et al. 2004) shown to be involved in salicylic acid signalling and activation of chemical detoxifying pathways (Fode et al. 2008). This approach would test the model proposed in Figure 1.1 describing the interaction between microtubules and stress signalling pathways more directly than using a microarray to analyze changes in global transcript
levels because many of the differentially regulated genes identified using this technique may be poorly characterized. An important goal would be to identify and characterize the domains on these transcription factors necessary for microtubule binding that would allow the identification of more transcription factors with related domains. The genes and pathways activated or repressed by these transcription factors can be identified through chromatin immunoprecipitation (ChIP) assays, followed by electromobility shift assays, yeast one-hybrid systems, or cDNA microarrays utilizing modified constitutively active transcription factors or their mutants to identify a suite of pathways downstream of the microtubule reorganization event. These targeted analyses would provide a clearer understanding of the interactions between stress responses and microtubules.

5.3 MARNERAL-RELATED METABOLITES AND DEFENSE (CHAPTER 4)

Secondary metabolic enzymes arise from gene duplication events involving enzymes from primary metabolism. Primary metabolic pathways have been honed over billions of years of evolution, whereas newly duplicated genes/pathways provide an opportunity for plants to adapt to their ever changing environment by developing secondary metabolic pathways (Ober 2010). An example of this was recently shown by Shoji and Hashimoto (2011) who describe the recruitment of a duplicated nicotinamide adenine dinucleotide (NAD) biosynthetic gene from primary metabolism to function in nicotine biosynthesis. This initial functionalization of the duplicated gene likely occurred from newly formed cis-elements in the promoter of the new gene, allowing for specialization of the new pathway. This seems like a logical progression for the recruitment of duplicated genes into secondary metabolic pathways. It is also reasonable to assume that linking the expression of these pathways to multiple stresses could arise if the stresses frequently occur together, even if the pathway guards against a single stress.

It is apparent that the environment is sensed as a whole and that signalling pathways for both biotic and abiotic stress are often coordinated through similar sets of hormones and proteins, including similar mitogen activated protein kinases and transcription factor networks (Fujita et al. 2006). Some secondary metabolic pathways are induced under specific combinations of stresses or stimuli and have likely evolved because the activation of defense responses allows the organism to adapt to the environment as a whole (Atkinson and Erwin 2012). For example, plants might be the
only source of water available to certain pests and herbivores under conditions where water is scarce, even if they are otherwise unpalatable. It would then be beneficial for the plant to automatically produce extra defensive compounds when under osmotic stress, whereas under normal conditions the plant could utilize its energy for growth and development. This has been described in tomato plants that were found to accumulate defensive metabolites upon drought stress, which was further shown to deter herbivorous insect larvae (English-Loeb et al. 1997). In lacking any noticeable altered responses to the stresses inducing expression of the MCGs, it is possible that the function of the marneral pathway could be analogous to this by functioning passively in defense during osmotic stressed conditions. Further analyses are needed to test the defensive properties of marneral-related metabolites as well as the susceptibility of plants, in which MCG expression is altered, to combined biotic and abiotic stresses.

An analysis of potential cis-regulatory elements in the MRN1 promoter is warranted to help identify specific functions of the marneral pathway. A yeast-one hybrid assay would aid in the identification of these proteins followed by mobility shift assays and genetic studies using any available mutants. These follow up analyses could clarify if the sensitivity of induction over development to MeJA and ABA are linked to a common transcription factor, or result from the activity of different ones.

5.4 THE MARNERAL GENE CLUSTER (CHAPTER 3)

The use of a co-expression analysis identified three neighboring genes that were highly coregulated. My research found that at least two of these genes function in a single pathway making them a part of a metabolic gene cluster. Through heterologous and ectopic expression of MRN1, I confirmed that the gene product produced is marneral, though this probably follows the initial production of marneral. In the initial characterization of MRN1, Xiong et al. (2006) showed that the aldehyde is the primary product by comparing the production of marneral and marneral both in vivo and in vitro without including the saponification step. After primarily finding the production of marneral occurred when skipping the saponification step, they concluded that the reduction to marneral was most likely to occur during this step, but could not rule out the possibility that a native yeast alcohol dehydrogenase catalyzed the reaction. My data clearly show that no marneral was extracted from plants overexpressing MRN1 with or without the saponification step. Both these data and the experimentation from Field et
al. (2011) lead to the conclusion that MRN1 is reduced by an unknown mechanism in planta.

CYP71A16 was identified as the enzymatic able to metabolize marnerol through the identification of high levels of marnerol in plants lacking a functional CYP71A16 gene and the absence of marnerol in leaves of plants expressing both MRN1 and CYP71A16. These data are corroborated by Field et al. (2011) who identified the biochemical nature of MRN1 and CYP71A16 in parallel to these studies. They also found through co-overexpression of these genes that CYP71A16 likely hydroxylates marnerol to produce at least seven new compounds. The function of CYP705A12 remains to be determined, but may play a similar role to CYP705A5 (also known as THALIANOL DESATURASE), a member of another triterpene metabolic gene cluster in Arabidopsis (Field and Osbourn 2008). Further analyses of CYP705A12 are needed to confirm the activity of this gene. Experiments similar to those used here and by Field et al. (2011) using heterologous and ectopic expression can be used to elucidate a function for CYP705A12. An RNAi knockdown strategy is also needed since no mutations are available.

5.5 Gene Clusters and Metabolons in Secondary Metabolism (Chapters 3 and 4)

Metabolic gene clusters likely result from the need to both co-regulate a set of enzymes because the intermediate metabolic products might be toxic as well as to promote the likelihood of inheriting the whole pathway, which is increased when the genes are so closely linked (Qi et al. 2004, Chu et al. 2011). It has been shown in the avenicin pathway in oats (Avena strigosa) that the chromosomal region in which the avenicin cluster is located is regulated through chromatin condensation, whereby the region is heterochromatic in cell types in which the secondary metabolites are produced and euchromatic in the adjacent tissues (Wegel et al. 2009). Both of these scenarios (toxic intermediates or pathway regulation) are also speculated to be the reason for the formation of “metabolons” or “metabolic channels” (Jørgensen et al. 2005, Bassard et al. 2011). Metabolons are defined as an interaction between multiple enzymes in a metabolic pathway to create a channel for a compound to be passed from one enzyme to the next. This concept has been used to engineer a gene fusion between a FARNESYL PYROPHOSPHATE SYNTHASE and PATCHOULOL SYNTHASE, a
sesquiterpene synthase (Albertsen et al. 2011). The fused enzymes doubled the production of the terpene compared to co-expression of free enzymes in yeast. This shows that the close association between functioning enzymes can make a pathway more efficient. In another example, a glycosyltransferase from *Sorghum bicolor* was found to change its subcellular localization from cytoplasm to the ER only when coexpressed with two CYPs known to be involved in the same pathway (Nielsen et al. 2008). This was the expected localization since most CYPs are predicted to be anchored to the cytoplasmic surface of the ER membrane (Werck-Reichhart et al. 2002). It seems possible that the metabolic gene clusters aids in the co-expression of key members of metabolons. A recent study developed an assay using FLIM-FRET (Fluorescence lifetime imaging microscopy coupled with Förster resonance energy transfer) to show a relationship between one enzyme and two separate partners, thus forming two small competing metabolons (Crosby et al. 2011). This system provides a tool to help identify both the presence and longevity of metabolons. The short lifetime of metabolons has long been thought to be the reason why their presence is so difficult to identify (Jørgensen et al. 2005). It would be interesting to determine if the MCGs form a metabolic gene cluster using this FLIM-FRET system. Identification of a metabolon could also provide a link between the formation of metabolic gene clusters and metabolons as well as a tool to identify more enzymes within a metabolon.

In Arabidopsis, the gene families for squalene synthase (2 members) and squalene epoxidase (6 members) have specific genes in these families that are coexpressed with the *MRN1* and *THAS* clusters (*SQS1* and both *SQE1* and *SQE4*) suggesting these genes could also be members of a maner real metabolon (Fig 5.1). It would be very interesting to determine if *SQE4* is involved in any way with the maner real pathway since it was previously found that it does not function as a true squalene epoxidase in both a heterologous expression system and in the *sque1-3* mutant (Rasbery et al. 2007). These enzymes could be incorporated into a FLIM-FRET study as described above. They could also be used in a protoplast expression analysis, similar to what I described in Chapter 3 using 35S::LUP4 plants, to determine if the MRN1 activity is altered to different degrees when these enzymes are present.
5.6 TRANSIENT IN PLANTA EXPRESSION SYSTEM FOR TRITERPENE PATHWAYS
(CHAPTE 3)

I showed in chapter 3 that triterpene production can be rapidly enhanced by increasing the expression levels of SQS1 in mesophyll protoplasts. There are now more than 40 OSCs characterized from plants that represent a tiny fraction of those found in nature (Phillips et al. 2006, Vranová et al. 2012). While ERG7-deficient yeast strains that accumulate the precursor to cyclized triterpenes have proven to be very useful in the characterization of OSCs, an in planta system that can be used to express plant-specific pathways is needed to rapidly elucidate triterpene pathways. Using an accurate understanding of how to manipulate the regulation of key enzymes within the triterpene pathway is the first step in developing an in planta system. Coupled with transient expression, this system could provide a tool for triterpene-related gene and pathway discovery or expression and could be useful in both drug discovery and the bioengineering of defense pathways in crops. To further develop this system, inducible SQS1 overexpression plants should be developed to determine if products from a transfected OSC are identifiable from this system. One pitfall from this protoplast system is the highly variable rates of transfection efficiency that could be overcome by using fluorescence activated cell sorting that would be able to purify only the OSC expressing cells out of the whole population of protoplasts.

The value of this system is the expression within a single population of plant cells. A system with a similar goal has also been developed for expressing biosynthetic enzymes in only plant trichomes (Wang et al. 2002) and has been used to characterize CYP725A4 (Rontein et al. 2008). This is also an attractive system since trichomes can be isolated from the rest of the plant (Yerger et al. 1992, Marks et al. 2008) and in many species trichomes specialize in the production of secondary metabolites (Schilmiller et al. 2008). However, this process requires time to produce stable transformants for each enzyme needing to be characterized and this can be avoided by using mesophyll protoplasts.

5.7 IMPLICATIONS

This thesis confirms the existence of a metabolic gene cluster in a secondary metabolic pathway and shows that the pathway is induced under osmotic stress conditions. The initial goals of this work were threefold: 1) To characterize a
relationship between microtubule depolymerization and the signalling pathway leading to stress responses within plants. 2) To characterize the activity of MRN1, CYP71A16, and CYP705A12 within a single pathway, thus defining them as a metabolic gene cluster. 3) To determine the function of the marneral gene cluster in Arabidopsis. While I determined that MRN1 was not a suitable gene to study the relationship between microtubules and plant stress responses, this initial research led to the observed co-expression between MRN1, CYP71A16, and CYP705A12. MRN1 and CYP71A16 were identified to be involved in the same pathway. CYP705A12, while lacking biochemical data, is still considered part of the metabolic gene cluster based on the gene’s location and coexpression with MRN1 and CYP71A16. This was followed by an analysis of the expression profile of the three genes.

Conditions were identified that induce transcription of the marneral cluster, one of which was shown to activate the marnerol pathway, yet plants with altered enzyme abundance within this pathway did not show changes in growth under the same conditions. This highlights the fact that pathways may be activated under stresses in which they function passively in defense of other stresses. If this concept is true, then the two stress conditions (in the likely case of marneral-related metabolites, these include osmotic and biotic stresses) would have occurred frequently during the evolution of the pathway and this concept could have important implications for breeding or engineering plants, such that defensive pathways may only need to be induced under abiotic stress conditions as opposed to constantly producing defensive compounds.

An important feature of metabolic gene clusters may be the existence of metabolons made up of the encoded enzymes. Since both metabolic gene cluster and metabolon formation are thought to increase the efficiency of metabolic pathways in plants, this could be another important tool for the production of plant metabolites. Although this research does not address the existence of a marneral metabolon, its possible identification is clearly one of the next objectives to be attempted. Identifying links between gene localization and enzyme cooperation could give important clues on increasing the viability of engineering plants as sources of valuable natural products.
Figure 5.1 Hierarchical clustering analysis from Expression Browser (Toufighi et al. 2005). Genes are clustered based on their expression profiles including all SQS, SQE, and OSC genes from Arabidopsis. The analysis was conducted on the AtGenExpress - Tissue Series using the “Average of replicate treatments relative to average of appropriate control” option. *MRN1* is clustered with a *SQS1*, two squalene epoxidases (*SQE1* and *SQE4*) and *THAS* highlighted by the red box.
REFERENCES


APPENDICES

APPENDIX 1: LIST OF PRIMERS

<table>
<thead>
<tr>
<th>Cloning Primers</th>
<th>Real Time</th>
<th>PCR Primers</th>
<th>Sequence</th>
<th>Target Gene</th>
<th>Efficiency</th>
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* indicated the primer matched to Lba1. ** indicates primer specific to T-DNA region.
## APPENDIX 2: PCR PROGRAMS

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**PCR rxn**

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<td>94°C -3 min</td>
<td>51.2°C -20 s</td>
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<td>cyp71A16-1 (SALK_073803)</td>
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**Real Time PCR program**

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**Extend**

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