ENHANCED α-BISABOLOL PRODUCTION THROUGH METABOLIC ENGINEERING IN *NICOTIANA BENTHAMIANA*

MSc Report

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ABSTRACT

BACKGROUND Sesquiterpenes are secondary plant metabolites with extraordinary biological and chemical properties. In plants they serve as key molecules in defence, stress signalling etc. Besides their function in plants, sesquiterpenes have a great purpose as modulators in human health. For instance, the sesquiterpene α-bisabolol, originally isolated from chamomile, has remarkable wound healing properties. However, current production of natural α-bisabolol is not sustainable, thus an alternative production method is needed. Here I show that by modification of endogenous energy pathways in *Nicotiana benthamiana* α-bisabolol production can be enhanced. It was hypothesized that by metabolic engineering the energy pathways, the carbon flux can be redirected towards producing sesquiterpene precursors. To investigate this, I modified steps in the trehalose-6-phosphate (T6P) pathway, SNF1-related protein kinase (SnRK1) and the mevalonate (MVA) pathway by transient expression of several genes.

RESULTS To test how modification of these endogenous pathways enhances α-bisabolol production, the sesquiterpene emission, and the activity of the rate-limiting enzyme in the MVA pathway, HMG-CoA reductase (HMGR), was measured. Sesquiterpene emission was enhanced by higher T6P levels and upon inhibition of the catalytic subunit of SnRK1, KIN10. However, HMGR activity remained unchanged upon these modifications. Furthermore, the opposite was tested and likewise sesquiterpene emission was decreased upon lower T6P levels and overexpression of KIN10, and resulted in a decreased HMGR activity. Overexpression of HMGR by endogenous HMGR, instead of truncated HMGR has been shown to decrease sesquiterpene production and HMGR activity, indicating an inhibitory rather than a complementary effect. Lastly, combining higher T6P levels with inhibition of KIN10 also resulted in reduction in sesquiterpene production. This indicates that other mechanisms play a role when both T6P and SnRK1 are modified.

CONCLUSION By modifying the T6P pathway, SnRK1 activity and MVA pathway, sesquiterpene production can be enhanced. However, HMGR activity is not upregulated when sesquiterpene production is. Conversely, when HMGR is downregulated, the sesquiterpene production is decreased. Therefore, it can be concluded that sesquiterpene production is only partly dependent on HMGR activity. It is hypothesized that besides HMGR activity, carbon flux plays a crucial role in modifying the sesquiterpene production. Therefore, carbon partitioning needs to be regulated more tightly to further optimise sesquiterpene production.

KEYWORDS α-bisabolol, sesquiterpenes, T6P pathway, OtsA, OtsB, SnRK1, KIN10, MVA pathway, HMGR

ABBREVIATIONS

ABI1-1, *Arabidopsis thaliana* abscisic acid insensitive 1-1 (AT1G08810); **AGPase**, ADP-glucose pyrophosphorylase; **Bos**, *Artemisia annua* α-bisabolol synthase (GenBank: JQ717161.1); **KIN10**, *Arabidopsis thaliana* KIN10 (AT3G01090); **DMAPP**, Dimethylallyl diphosphate; **EP**, endoplasmic reticulum; **FPP**, Farnesyl diphosphate; **GC-MS**, Gas chromatography mass spectrometry; **HMG-CoA**, 3-hydroxy-3-methylglutaryl coenzyme A; **HMGR**, *Nicotiana benthamiana* 3-hydroxy-3-methylglutaryl-CoA reductase (LC015758.1); **IPP**, Isopentenyl diphosphate; **KIN10**, SNF1 kinase homolog 10; **MEP**, 2-C-methyl-d-erythritol-4-phosphate; **MVA**, Mevalonic acid; **RNAi KIN10_6** and **KIN10_8**, *Nicotiana benthamiana* silencing constructs KIN10 (AY919676.1); **OtsA**, *E.coli* trehalose-6-phosphate synthase (patent WO95/01446); **OtsB**, *E. coli* trehalose-phosphate phosphatase (patent WO97/42326); **SnRK1**, Sucrose non-fermenting-1-related protein kinase 1; **T6P**, Trehalose-6-phosphate; **tHMGR**, truncated *Arabidopsis thaliana* HMGR (AT1G76490); **TPP**, Trehalose phosphate phosphatase; **TPS**, Trehalose phosphate synthase.

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1 INTRODUCTION

In plants, secondary metabolites contain a plethora of bioactive agents, such as terpenoids, alkaloids, phenolics, and polyacetylenes (Heinrich et al., 1998). These metabolites are produced to improve the survival of the plants (Demain & Fang, 2000). They serve as defence against bacteria, fungi, amoebae, other plants, insects, and animals. Furthermore, they function as sexual hormones, differentiation effectors and as agents of symbiosis between other plants, nematodes, insects, and higher animals.

Sesquiterpenes are one of the most prevalent classes of secondary metabolites, and are naturally found in plants and invertebrates. In the plant kingdom, they are a large group with over 5,000 known compounds that are prevalent in numerous plant families (Chadwick et al., 2013). The most common plant family in which they are present is the *Asteraceae* family, an exceedingly large, diverse, and widespread family of flowering plants. One of the members of this family is *Helenium amarum*, and it has shown that sesquiterpenes can make up to 3% of the dry weight of plants, a relatively high concentration (Heinrich et al., 1998). Sesquiterpenes play a significant role in anti-herbivory defences (Koul, 2008; Kubo & Ganjian, 1981) and as antimicrobial agents (Cowan, 1999) in plants. However, besides their role in plants, many sesquiterpenoids are potent as modulators in human health (Chadwick et al., 2013).

1.1 Sesquiterpenes and human health

Sesquiterpenes have a wide variety of biological properties, making them interesting for their use in pharmaceuticals, a part of a balanced diet, or as cosmetics. Most studies into sesquiterpenes have focused on its antitumor properties and their use in treatment of cardiovascular disease. Additionally, sesquiterpenes are valuable in treatment of a wider variety of diseases. For instance, they can be used as antimalarials, antimicrobials, anti-inflammatory agents, analgesic, and sedative agents, for prevention of neurodegeneration and for treatment of burns, flu, and diarrhoea (Ahlemeyer et al., 1999; Canales et al., 2005; Heinrich et al., 1998; Prehn & Krieglstein, 1993; Wesolowska et al., 2006). In my thesis, I have focused on one of these fascinating sesquiterpenes, α-bisabolol.

1.2 Alpha-bisabolol

A-bisabolol is a naturally occurring sesquiterpene that was first isolated from chamomile (*Matricaria chamomilla*), a daisy-like plant in the *Asteraceae* family (Kamatou & Viljoen, 2010). Extraction of α-bisabolol from these flowers produces a blue essential oil (Singh et al., 2011). Therefore, this oil is not suitable for being used in cosmetics, a market that makes up a major demand for α-bisabolol. A-bisabolol has been commonly used as an ingredient in cosmetics and skin care products because of its anti-irritant, antibacterial, wound healing, and non-allergenic properties. Additionally, α-bisabolol is used as a pharmaceutical agent because of its anti-inflammatory, antispasmodic, anti-allergic, drug permeation, and vermifuge properties. Global production of α-bisabolol mainly takes place in Brazil, where α-bisabolol is harvested from the tree *Eremanthus erythropappus* (Clark, 2011). However, cultivation of *E. erythropappus* is not suitable because of its long production time and specific requirements for its growth. Therefore, the natural occurring trees are harvested, leading to environmental problems such as deforestation and sustainability of the species. Hence, an alternative method of production of α-bisabolol is of great importance.

An alternative way to produce α-bisabolol efficiently, is producing it in a plant production system. Plants can be used to produce homogenous and well-purified amounts of target molecules (Augustin et al., 2015). Additionally, plant production system can generate high-value molecules with minimal input. Advantages are that plants are easy to scale up, relatively cheap, and can produce the molecules relatively quickly (Sack et al., 2015). Furthermore, the benefit of a plant production system is the ability to perform posttranslational modifications, which is often essential in the production of biological active compounds. For example, adding N-glycans to recombinant proteins can increase the biological activity and half-life of many pharmaceuticals (Verpoorte et al., 2007). Furthermore, posttranslational modifications can be important in directing the mode of storage of volatiles. For example, α-bisabolol can be harvested more efficiently when it is stored in the plant cells instead of releasing it in the headspace (Ohgami et al., 2015). Together, the advantages of a plant production system make it a very suitable alternative to current production methods of α-bisabolol.

Furthermore, when a plant production system is used, it is crucial to understand the underlying mechanisms. Research in these underlying mechanisms, and thus in the endogenous pathways leads to better understanding the production mechanisms of α-bisabolol. It provides more insight into the related genes, and a major advantage of this is that those genes can be used as targets in classical breeding. The use of breeding targets can speed up the time-costly breeding process greatly. Thus, a secondary advantage of investigation into producing α-bisabolol in a plant production system is providing knowledge for a more classical alternative.

1.3 The MVA pathway

Sesquiterpenes are colourless, lipophilic molecules that are biosynthesised from three branched, unsaturated C_5 isoprene units (Chizzola, 2013). In plants, there are two pathways that synthesize precursors for isoprenoids: the mevalonate (MVA) pathway, and the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway (Vranova et al., 2013). The MVA pathway is located in the cytosol, whereas the MEP pathway is located in the plastids. Both pathways synthesize isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), however only the MVA pathway synthesizes the sesquiterpene precursor farnesyl diphosphate (FPP) from these precursors. FPP is subsequently used for the formation of isoprenoids, which are the precursors for a wide variety of compounds such as sterols, carotenoids, and sesquiterpenes (Zhang et al., 2015). Sesquiterpenes are subsequently further modified by oxidation and glycosylation, which is essential for their functioning.

Because the production of sesquiterpenes is dependent on the levels of sesquiterpene precursors, the MVA pathway and its regulators are an important topic of investigation. The rate limiting step in the MVA pathway is the reduction of hydroxymethylglutaryl coenzyme A (HMG-CoA) to mevalonate (MVA) by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Chappell et al., 1995; Rodríguez-Concepción, 2006). However, HMGR enzyme activity is tightly regulated at transcriptional and posttranscriptional levels (Choi et al., 1992; Lumbreras et al., 1995). Therefore, understanding which genes and pathways influence HMGR activity is an important strategy to optimize sesquiterpene production in plants.

1.4 SnRK1 (KIN10)

SnRK1 is the major cellular energy sensor in plants, and has an essential role in energy signalling. It is a key component of plant cell homeostasis and is activated under energy-depleting stress conditions (Broeckx

et al., 2016). Activated SnRK1 stimulates catabolism and represses growth-related processes and in turn, inhibited SnRK1 leads to stimulation of metabolic processes such as the MVA pathway. It acts by extensive transcriptional regulation, down-regulation of TOR kinase signalling and by phosphorylation of a wide variety of metabolic enzymes and regulatory proteins.

SnRK1 consists of three subunits, a catalytic kinase α subunit and regulatory β and γ subunits. The active, catalytic α subunit consists of the kinases KIN10 and KIN11. SnRK1 activity is regulated by various regulatory molecules in the cell. One of the negative regulators of SnRK1 is ABI1, a protein that is activated in response to abscisic acid (ABA) (Rodrigues et al., 2013). ABI1 dephosphorylates and thereby inactivates the SnRK1 α-catalytic subunit. Another regulator of HMGR is sucrose non-fermenting-1-related protein kinase 1 (SnRK1), a heterotrimeric kinase complex (Dale et al., 1995; Halford et al., 2003). Furthermore, SnRK1 is regulated by a plethora of other factors, such as stress, sucrose availability and trehalose-6-phosphate (T6P) (Halford & Hey, 2009; Smeekens, 2015). Besides being a target of various regulatory molecules, SnRK1 also has an extensive influence on regulatory molecules. One of the target of SnRK1 is the metabolic enzyme HMGR (Halford et al., 2003). HMGR is phosphorylated by KIN10 and thereby becomes inactivated. Inactivation of HMGR subsequently leads to substantial downregulation of the MVA pathway. In conclusion, SnRK1 is of key influence on the production of various metabolic compounds, such as sesquiterpenes.

1.5 The trehalose-6-phosphate pathway

The Trehalose-6-phosphate (T6P) pathway is an important stress responsive signalling pathway in plants. T6P has a role in embryonic regulation, vegetative developments, flowering time, determination of the meristem and cell fate specification (Tsai & Gazzarrini, 2014). The T6P pathway is responsive to sugar availability and affects the global plant metabolism. Regulation by the T6P pathway is mediated through T6P levels, which is adjusted by trehalose phosphate synthase (TPS) and T6P phosphatase (TPP). TPS catalyses the conversion of UDP-glucose and glucose-6P to T6P and TPP catalyses the conversion of T6P to trehalose, whose activities are dependent on the carbon availability in the plant cell (Wingler et al., 2012).

Several studies have investigated the effect of the T6P pathway by overexpression and knocking down of TPS and TPP. It was observed that altered T6P levels lead to an altered phenotype, such as increased kernel set and harvest index in maize when TPP is overexpressed (Nuccio et al., 2015; Schluepmann et al., 2011). Furthermore, when TPS is overexpressed by OtsA encoding *Escherichia coli* TPS, carbon utilization in seedlings is influenced (Schluepmann et al., 2003).

To date, two potential mechanisms of action are proposed for T6P (Lunn et al., 2014). In the first scenario, there is a direct interaction between T6P and SNRK1, in which it is hypothesized that T6P acts as a direct inhibitor of this key metabolic regulator. In the second scenario, it is hypothesized that T6P has overlapping targets with SnRK1 rather than acting through SnRK1. It was argued that, between those two scenarios, intermediate scenarios are very likely. Thus, T6P has an important influence on the metabolic processes, either by direct modification of SnRK1, direct modification of the metabolic pathways or by a combination of this. Therefore, the T6P pathway is another target for investigating its role in sesquiterpene production.

1.6 Hypotheses and research strategy

In this study, I described gene silencing, inactivation, and overexpression of several genes by transient transformation in *N. benthamiana* leaves. The aim was to optimize production of sesquiterpenes, and more specifically α-bisabolol in a plant production platform. Since HMGR is the rate limiting step in the MVA pathway, the HMGR activity was investigated in addition to sesquiterpene production. It was hypothesized that higher HMGR activity would lead to higher production of sesquiterpene precursors, IPP and DMAPP, and subsequently to higher total sesquiterpene production.

The genes were tested separately and combined to find the most optimal gene combination to enhance sesquiterpene production. First, a constitutive expression system was used to investigate how the genes affect the sesquiterpene production and HMGR activity. Subsequently, a selection of these genes was tested separately and combined in an inducible expression system. I used an inducible system because metabolic engineering can lead to negative effects on plant growth and development and lead to toxicity of newly introduced compounds (Dudareva et al., 2013). Inducible expression of the genes circumvents downregulation and activation of other regulatory responses by the plant. The inducible vector contains a GVG system that allows induction of the target genes reducing pleiotropic effects on the plant (Aoyama & Chua, 1997). The GVG gene is a chimeric transcription factor that is induced by dexamethasone (DEX) and subsequently targets the upstream activation sequence (UAS) that controls the target gene. Two inducible constructs were designed using different promoters, a Cauliflower mosaic virus 35S (35S) promoter and an ubiquitin 10 (UB10) promoter. The 35S promoter is a highly active promoter, however its main disadvantage is being leakier than UB10. The UB10 promoter is less active, but also less leaky, making it potentially a more suitable promoter in this system. In conclusion, in this investigation several expression strategies were tested to discover the most optimal way to enhance sesquiterpene production.

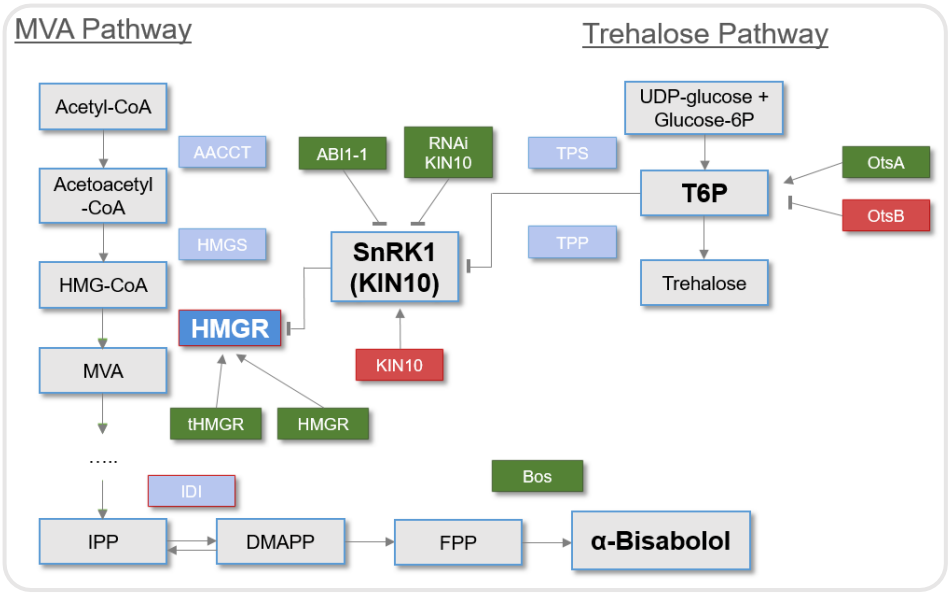

Figure 1 The hypothesized link between the trehalose pathway, SnRK1 and MVA pathway. Gene overexpression, inactivation and silencing are schemetically shown for all genes used in this study. Grey boxes, actors in the MVA and T6P pathways; Dark blue and light blue boxes, enzymes affecting the actors; Green boxes, infiltrated genes with a hypothesized positive effect on the α-bisabolol production and HMGR activity; Red boxes, infiltrated genes with a hypothesized negative effect on the α-bisabolol production and HMGR activity. Genes used: OtsA, trehalose-6-phosphate synthase; OtsB, trehalose-phosphate phosphatase; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; RNAi KIN10, RNAi hairpin silencing construct *N. benthamiana* KIN10; KIN10, *Arabidopsis thaliana* KIN10; tHMGR, truncated *A. thaliana* HMGR; HMGR, *N. benthamiana* HMGR; Bos, *A. annua* α-bisabolol synthase. Steps in the patway: Acetyl-CoA, Acetyl coenzyme A; KIN10, SNF1 kinase homolog 10; DMAPP, dimethylallyl; FPP, farnesyl diphosphate; HMG-CoA, hydroxymethyl-glutaryl coenzyme A; IPP, isopentenyl diphosphate; MVA, mevalonic acid; SnRK1, sucrose non-fermenting-1-related protein kinase 1; T6P, trehalose-6-phosphate. Enzymes are indicated in blue: AACT, acetoacetyl CoA thiolase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IDI, IPP isomerase; TPS, trehalose phosphate synthase; TPP, T6P phosphatase. (MVA pathway derived from Rodríguez-Concepción (2006), trehalose pathway derived from Schluepmann et al. (2003)).

Genes that were hypothesized to have an impact on the sesquiterpene production were expressed transiently in *Nicotiana benthamiana* leaves, and their hypothesized effect is shown in figure 1. Genes in the T6P and MVA pathway were overexpressed, inactivated or silenced, to direct the metabolic status of the plant toward sesquiterpene production. The following genes were expressed:

- *A. annua* α-bisabolol synthase (**Bos**) converts the sesquiterpene precursor farnesyl diphosphate (FPP) to α-bisabolol (Li et al., 2013). This gene is co-expressed in all treatments in order to investigate the effect of the other co-expressed genes not just on the general sesquiterpene production, but more specifically on the α-bisabolol production.
- *A. thaliana* N-terminal truncated 3-hydroxy-3-methylglutaryl-CoA reductase (**tHMGR**) overexpresses HMGR and is co-expressed in all treatments, except for the treatments in which overexpression of HMGR by endogenous full length HMGR is tested. tHMGR lacks the membrane domain, which is therefore expressed in the cytosol instead of the membranes of the endoplasmic reticulum (ER) (Leivar et al., 2005; Tomé et al., 2014). It was shown in previous research by Cankar et al. (2015) that co-expression of tHMGR leads to enhancement of sesquiterpene production.
- *A. thaliana* abscisic acid insensitive 1-1 (**ABI1-1**) is a protein with a dominant mutation. ABI1 causes dephosphorylation, and thereby inactivation of KIN10, and the dominant mutation in ABI1-1 causes it to be activated independent of ABA (Rodrigues et al., 2013). It was hypothesized that inactivation of the SnRK1 complex by ABI1-1 would lead to a greater stability of endogenous HMGR and thereby higher production of sesquiterpenes.
- *A. thaliana* KIN10 (**KIN10**) overexpresses KIN10. Overexpression of KIN10 is hypothesized to lead to stronger SnRK1 activity, and this is argued to result in a lower stability of HMGR. Consequently the sesquiterpene production is hypothesized to be reduced.
- *N. benthamiana* endogenous HMGR (**HMGR**) codes for the full length endogenous gene, and thus contains the membrane domain. It is hypothesized to overexpress and enhance endogenous HMGR. Therefore it was hypothesized that overexpression of HMGR by HMGR results in a higher production of sesquiterpenes.
- *N. benthamiana* KIN10 (**RNAi KIN10**) gene fragments of 221 (RNAi KIN10_6) and 250 base pairs (RNAi KIN10_8) are used for creation of hairpin RNAi constructs, and are hypothesized to silence endogenous KIN10. Silencing of KIN10 would have a similar effect on HMGR activity and sesquiterpene production as the ABI1-1 protein, however by a different mechanism. Thus, it is hypothesized that silencing KIN10 leads to enhancement of HMGR stability and thereby sesquiterpene production.
- *E. coli* trehalose-6-phosphate synthase (TPS), **otsA**, catalyses the conversion of UDP-glucose and glucose-6P into T6P. T6P inhibits SnRK1, and is thereby an indirect strategy of inhibiting KIN10 (Y. Zhang et al., 2009). As stated before, inhibition of KIN10 is hypothesized to increase HMGR activity and thereby enhance sesquiterpene production.
- *E. coli* trehalose-6-phosphate phosphatase (TPP), **otsB**, catalyses the conversion of T6P to trehalose, and thereby decreases T6P levels in the plant (Y. Zhang et al., 2009). Lower T6P levels lead to less inhibition of KIN10 and hence it is hypothesized that the stability of HMGR is lower resulting in a decreased sesquiterpene production.

Primers used to isolate the genomic sequences from Bos, ABI1-1, KIN10, tHMGR, HHMGR, RNAi KIN10_6, KIN10_8, and OtsA are shown in Supplemental Data table 1.

2 RESULTS

2.1 SESQUITERPENE PRODUCTION

In this section, headspace trapping and thermal desorption GC-MS were used to collect, identify and quantify the emission of sesquiterpenes, in particular α-bisabolol. All genes and gene combinations were transiently expressed in young leaves of *N. benthamiana* and their effect on sesquiterpene production was measured 3-5 days post inoculation. A part of the treatments was co-expressed with the p19 protein of tomato bushy stunt virus (p19). P19 suppresses the onset of posttranscriptional gene silencing in the infiltrated tissue (Voinnet et al., 2003), which allows higher expression levels of the infiltrated genes. All treatments and controls were co-expressed with α-bisabolol synthase, Bos, which converts the sesquiterpene precursor farnesyl diphosphate (FPP) to α-bisabolol. Volatiles were trapped for 1 or 24 hour(s) depending on the treatment, because it was observed that trapping the dexamethasone inducible constructs for 24 hours led to more consistent results. After trapping, the volatiles were analysed with thermal desorption GC-MS.

2.1.1 The effect of constitutive overexpressing of KIN10, silencing KIN10 and overexpressing HMGR

In this experiment I tested the effect of constitutive overexpression and silencing of KIN10, and constitutive overexpression of HMGR by tHMGR and endogenous HMGR. Thus, sesquiterpene emission was measured upon decreased and increased SnRK1 activity and increased HMGR activity. In Figure 2 three experiments are shown in which the treatments are represented relative to the control. As a control expression of empty pBIN vector (EV), tHMGR and Bos were used. In the first experiment KIN10, the silencing constructs of KIN10, RNAi KIN10_6, KIN10_8, and HMGR were separately expressed to investigate the effect of each gene on the α-bisabolol emission. In the second experiment the overexpression of tHMGR was compared to the overexpression of HMGR on α-bisabolol emission when co-expressed with p19. In the third experiment, tHMGR and HMGR were expressed simultaneous to investigate whether HMGR also has a similar effect on the α-bisabolol emission when combined with tHMGR.

Overexpression of KIN10 resulted in a 35% lower emission of α-bisabolol compared to the control (Fig. 2). Expression of the first silencing construct of KIN10, RNAi KIN10_6, resulted in a significant increase of 89% α-bisabolol emission ($P<0.05$, $N=3$). Expression of the second silencing construct, RNAi KIN10_8, resulted in a 39% higher α-bisabolol emission ($P>0.05$, $N=3$). To conclude, changing the SnRK1 activity by modulating its catalytic subunit KIN10, changes the α-bisabolol production.

In order to test the difference between overexpression of HMGR by the endogenous HMGR and the truncated Arabidopsis HMGR, HMGR and tHMGR were compared. As shown in Figure 2, overexpression of HMGR by HMGR resulted in a significantly lower emission of α-bisabolol of 93% when co-expressed with an EV, and 96% when co-expressed with p19 ($p<0.05$, $N=3$). Furthermore, when tHMGR and HMGR are expressed together, there is a reduction in emission of α-bisabolol of 72% ($P>0.05$, $N=3$). This shows that overexpression by full length endogenous HMGR leads to downregulation of the α-bisabolol production.

In summary, overexpression of KIN10 leads to a reduced α-bisabolol emission and both RNAi KIN10_6 silencing constructs led to higher α-bisabolol emission, which is in agreement with the hypothesis. Expression of HMGR, in contract, did not increase the α-bisabolol emission, but instead reduced it strongly. This is contrary to the hypothesis. In the following experiment the expression of KIN10 and otsB was tested to explore the hypothesis more thoroughly.

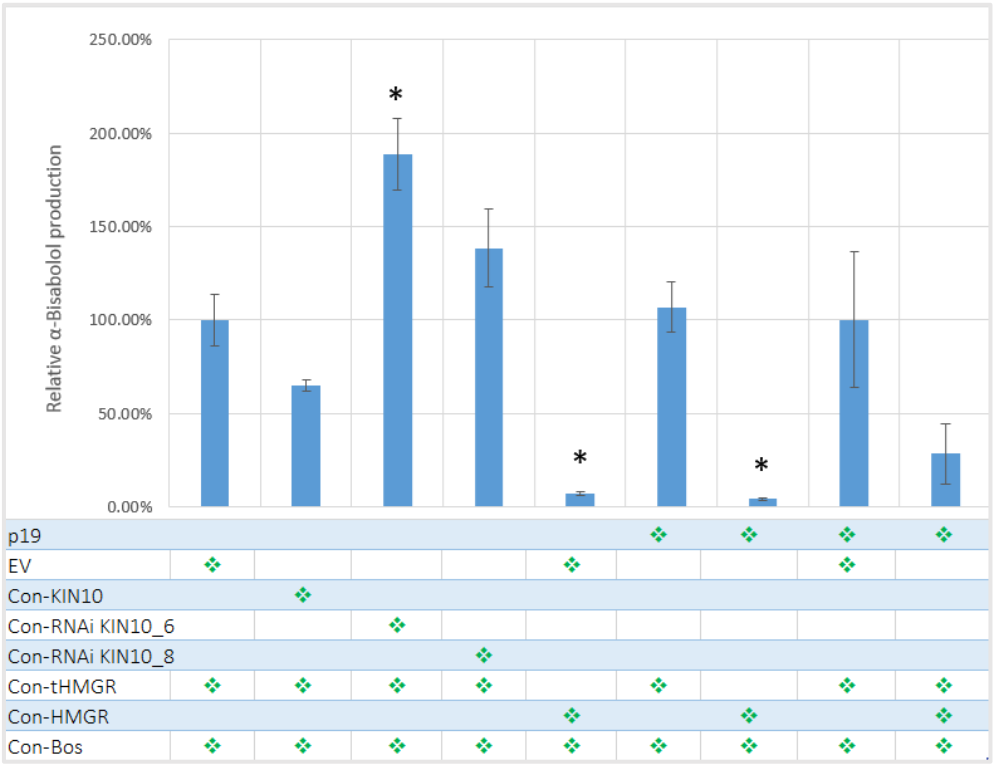

Figure 2. Relative α-bisabolol emission in *N. benthamiana* leaves upon constitutive expression of tHMGR, RNAi KIN10_6 and KIN10_8, KIN10 and HMGR. All treatments co-expresses Bos. Leaves were harvested three days post inoculation. Thereafter, the emitted volatiles were collected for one hour on Tenax-filled steel cartridges and analysed by thermal desorption GC-MS. p19, RNA silencing suppressor p19; EV, empty pBIN vector; KIN10, *A. thaliana* KIN10; RNAi KIN10_6 and KIN10_8, RNAi hairpin constructs *N. benthamiana* KIN10; tHMGR, truncated Arabidopsis HMGR; HMGR, *N. benthamiana* HMGR. Bos, *A. annua* α-bisabolol synthase. The mean ± SE of 3-4 independent measurements. The asterisks denote statistical difference between the EV+tHMGR infiltration and other treatments, analysed by a two-tailed Student's t-test: *p<0.05.

2.1.2 The effect of combining OtsB and KIN10

It was hypothesized that increased KIN10 activity would lead to lower stability of HMGR and would subsequently lead to lower production of sesquiterpenes. In this experiment I tested whether constitutive overexpression of KIN10 and dephosphorylation of KIN10 by constitutively expressing a TPP to reduce the T6P levels confirms this hypothesis (Fig. 3). KIN10 was overexpressed by expression of KIN10 and T6P levels were decreased by expression of otsB, which is a bacterial TPP. Both genes were expressed using a constitutive vector, and were with co-expression of p19, tHMGR and Bos.

As shown in Figure 3, simultaneous expression of KIN10 and otsB led to a decrease in α-bisabolol emission of 71% compared to the control. When KIN10 and OtsB were expressed separately, this led to a decrease in α-bisabolol emission of 55% and 32%, respectively (P>0.05, N=2-3). In conclusion, I observed an additive

effect for expression of KIN10 and otsB on decreasing α-bisabolol emission. Additionally, expression of KIN10 and otsB independently also led to a decrease of α-bisabolol emission, in which KIN10 is more effective than otsB. These results confirm the hypothesis that a higher activity of KIN10, directly by overexpression or indirectly by lower T6P levels, lead to a decrease in sesquiterpene production. In the next experiment, expression of several genes under an inducible system was tested to investigate the potential advantages of an inducible system over a constitutive system.

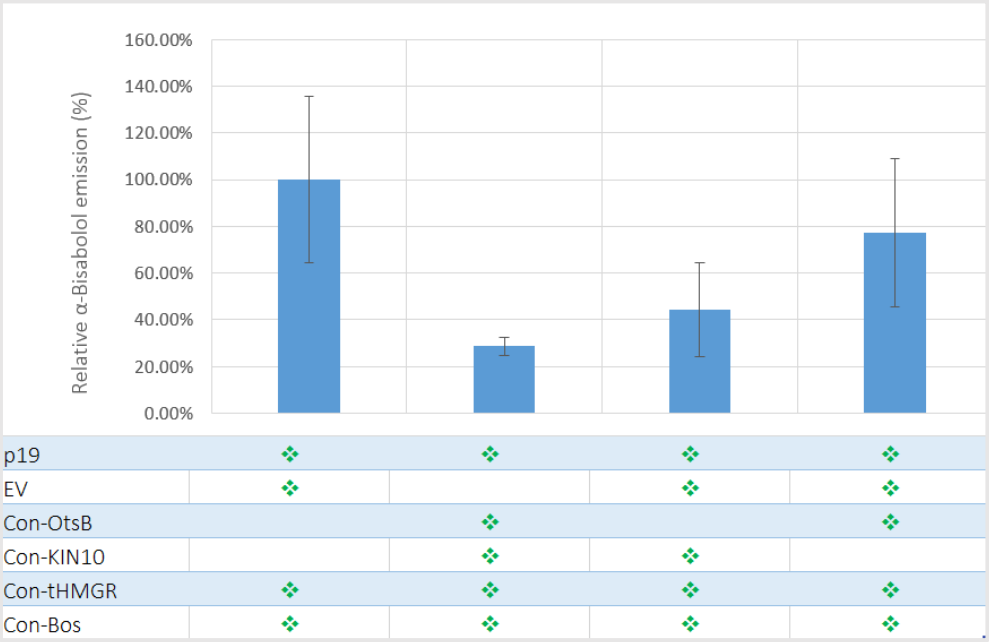

Figure 3. Relative α-bisabolol emission in *N. benthamiana* leaves upon constitutive expression of OtsB and KIN10, constitutively co-expressed with tHMGR and Bos. Leaves were harvested three days post inoculation. Thereafter, the emitted volatiles were collected for 24 hours on Tenax-filled steel cartridges and analysed by thermal desorption GC-MS. Bos, *A. annua* α-bisabolol synthase; Con, constitutively expressing vector; EV, empty pBIN vector; p19, RNA silencing suppressor p19; KIN10, *A. thaliana* KIN10; OtsB, trehalose-phosphate phosphatase; tHMGR, truncated Arabidopsis HMGR. The mean ± SD of 2-3 independent measurements. Results were statistically analysed using a two-tailed Student's t-test (P>0.05).

2.1.3 The effect of inducible silencing of KIN10, expressing ABI1-1 and Bos

In this experiment I investigated the effect of inducible expression of silencing construct KIN10_6, ABI1-1, and Bos (Fig. 4). These genes were cloned into the dexamethasone inducible expression vector, pTA7002. The aim of using an inducible vector is to reduce the pleiotropic effect of the transformed genes on the plant. ABI1-1 is known to dephosphorylate KIN10 and thereby decreases SnRK1 activity (Rodrigues et al., 2013). ABI1-1 has a similar effect to the silencing construct KIN10, the impairment of KIN10, however ABI1-1 dephosphorylates KIN10 instead of silencing it. These two treatments were compared to investigate which mechanism is most effective. The effect of ABI1-1 was compared to the most effective silencing construct for KIN10, RNAi KIN10_6 (Fig. 2). All treatments were compared relative to the control, in which p19, EV, tHMGR and Bos were constitutive expressed.

Additionally, two variants of the inducible vector were compared, one with a cauliflower mosaic virus 35S (35S) promoter and one with an ubiquitin 10 (UB10) promoter, to test which promoter induces the highest α-bisabolol emission. Under those promoters ABI1-1 and RNAi NKIN10 were expressed. In this experiment

N. benthamiana leaves were induced with DEX directly after harvesting, and four hours after induction the volatile emission was measured for one hour.

As shown in Figure 4, expression of the genes under the inducible vector led to a reduction of 10-49% α-bisabolol relative to the control. Expression of ABI1-1 led to higher emission of α-bisabolol compared to RNAi KIN10_6, for both the vector under 35S promoter and UB10 promoter, 14% and 19% respectively. Inducible expression of Bos under the 35S promoter led to a 73% lower α-bisabolol emission compared to the control. Furthermore, the difference between the vectors containing the 35S or UB10 promoter was tested. As shown in Figure 4, both for RNAi KIN10_6 and ABI1-1 expressed with the vector containing the UB10 promoter resulted in a higher α-bisabolol emission, 20% and 25% respectively (P>0.05, N=4).

Thus, it can be concluded that expression of ABI1-1 leads to a higher α-bisabolol emission than the silencing construct KIN10_6. Furthermore, expression of the genes under the UB10 promoter is more efficient than under the 35S promoter when enhancing the sesquiterpene production. However, expression of ABI1-1 and RNAi KIN10_6 led to lower α-bisabolol emission than the control. Additionally, when Bos is expressed under an inducible system instead of a constitutive system, this resulted in a substantially lower emission of α-bisabolol. Therefore, it was hypothesized that a more extensive measurement period would result in more reliable results. In the following experiment, the leaves were measured for 24 hours instead of 4 hours and different combinations of the genes in the inducible vector were tested.

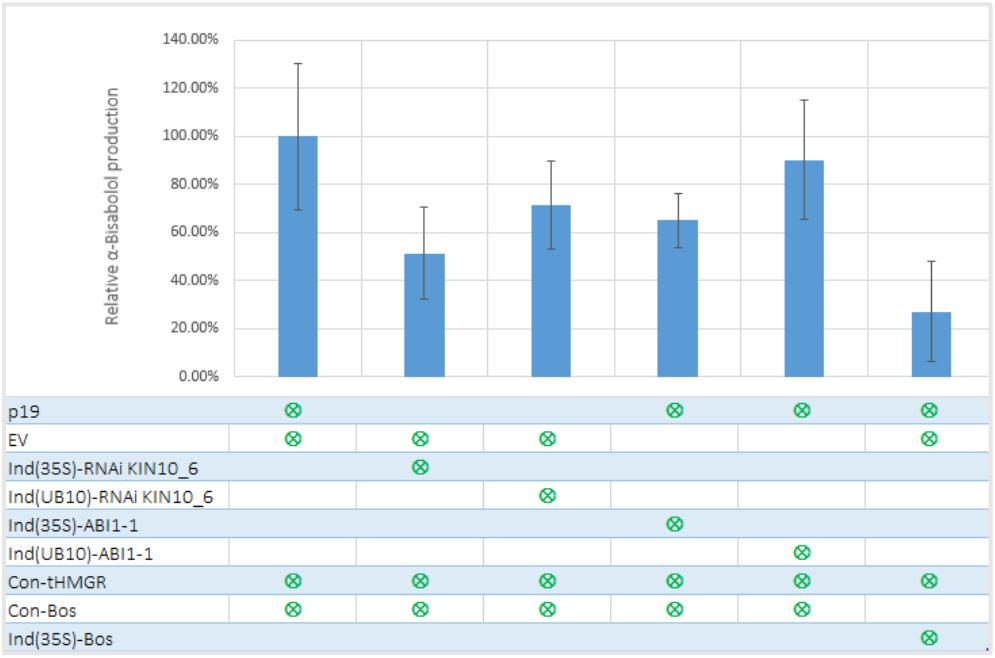

Figure 4. Relative α-bisabolol emission in *N. benthamiana* leaves upon inducible expression of silencing construct KIN10_6, ABI1-1 and Bos, constitutively co-expressed with tHMGR. Leaves were harvested three days post inoculation. Thereafter, the vectors were induced with dexamethasone. Four hours after induction, the emitted volatiles were collected for one hour on Tenax-filled steel cartridges and analysed by thermal desorption GC-MS. ⊗: dexamethasone induced treatment. Ind(35S), inducible vector with the 35S promoter; Ind(UB10), inducible vector with ubiquitin 10 promoter; Con, constitutively expressing vector; EV, empty pBIN vector; p19, RNA silencing suppressor p19; RNAi KIN10_6 and KIN10_8, silencing constructs RNAi hairpin *N. benthamiana* KIN10; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; tHMGR, truncated Arabidopsis HMGR; Bos, *A. annua* α-bisabolol synthase. The mean ± SD of 4 independent measurements. Results were statistically analysed using a two-tailed Student's t-test (P>0.05).

2.1.4 The effect of combining OtsA, ABI1-1 and RNAi KIN10_6

It was hypothesized that a combination of genes that impair KIN10, directly or indirectly, and genes that overexpress HMGR would lead to a substantial higher production of α-bisabolol. Therefore, based on the results of the previous experiments the potentially most optimal α-bisabolol producing system was designed to test this hypothesis. Here, I show two experiments in which different combinations of genes were tested (Fig. 5). OtsA increases the T6P levels and thereby inhibits KIN10, ABI1-1 dephosphorylates KIN10 and silencing construct NKIN10 silences KIN10, thereby also impairing the activity of SnRK1. This was hypothesized to enhance the sesquiterpene production. In the first experiment, inducible expression of otsA and ABI1-1 were tested separately and combined. In the second experiment, inducible expression of the combination of otsA, ABI1-1, and silencing constructs RNAi KIN10_6, and otsA and RNAi KIN10_6 was tested. For the first experiment, as a control non-induced leaves were taken from each treatment. In the second experiment the leaves infiltrated with EV, tHMGR and Bos served as a control. In all experiments Bos was co-expressed. In this experiment *N. benthamiana* leaves were induced with DEX directly after harvesting, and subsequently the volatile emission was directly measured for 24 hours.

As shown in Figure 5, combining otsA and ABI1-1 resulted in a 44% lower α-bisabolol emission compared to the control. Expressing otsA and ABI1-1 separately caused an increase of α-bisabolol emission of 41% and 83%, respectively ($P>0.05$, N=2-3). The combination of otsA, ABI1-1, and silencing construct KIN10_6 resulted in an increase of α-bisabolol emission by 6%. Combining otsA and silencing construct KIN10_6 caused a decrease in α-bisabolol emission of 6% ($P>0.05$, N=4). These results show that separate modification of T6P levels and SnRK1 activity can enhance α-bisabolol, however an additive effect is not observed.

For the combination of otsA and ABI1-1 the same effect was observed as described in section 2.1.3, which is a decrease in α-bisabolol emission. However, when otsA or ABI1-1 are expressed separately there is a higher α-bisabolol emission. For the combinations of otsA, silencing construct KIN10_6, ABI1-1, and otsA and silencing construct KIN10_6, there is neither a substantial increase nor decrease when compared to the control. Thus, in this experiment I observed an increase in α-bisabolol emission for ABI1-1 relative to the control. It was hypothesized that DEX induction takes more time than 4 hours to induce the vectors sufficiently, and that a longer measurement period shows the accumulation effect better than measuring the sesquiterpene emission for 1 hour. Additionally, in this experiment a different control is used than in the previous experiment. This possibly explains the difference between expressions relative to the control. In the next section the HMGR activity was assessed to investigate whether the change in sesquiterpene production can be explained by change in HMGR activity.

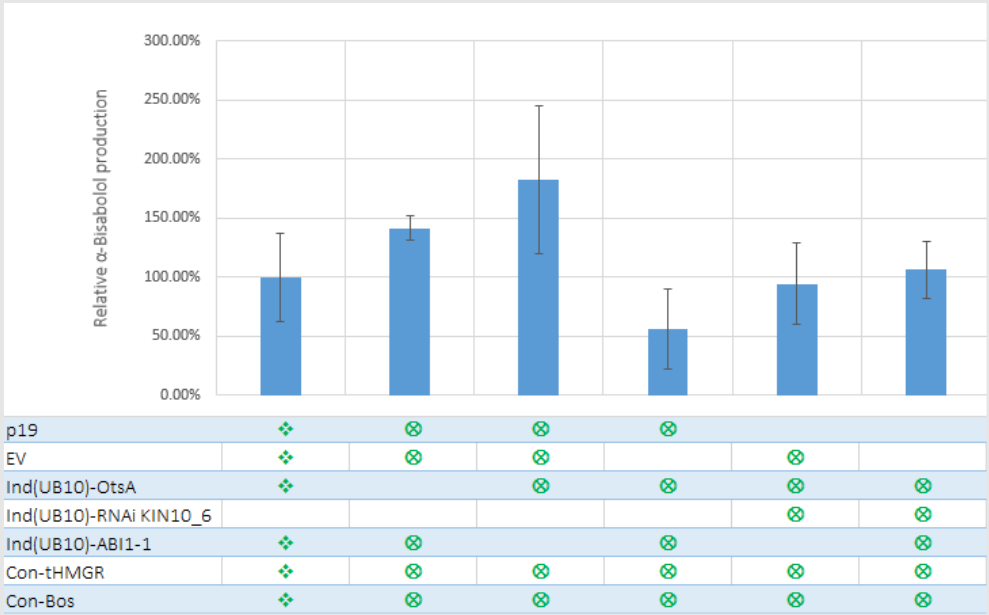

Figure 5. Relative α-bisabolol emission in *N. benthamiana* leaves upon constitutive expression of otsA, silencing construct KIN10_6 and ABI1-1, constitutively co-expressed with p19, tHMGR and Bos. Leaves were harvested three days post inoculation. Thereafter, the vectors were induced with dexamethasone. The emitted volatiles were collected for 24 hours on Tenax-filled steel cartridges and analysed by thermal desorption GC-MS. ❖, non-induced treatment; ⊗, dexamethasone induced treatment; Con, constitutively expressing vector; EV, empty pBIN vector; p19, RNA silencing suppressor p19; OtsA, trehalose-phosphate synthase; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; RNAi KIN10_6; RNAi hairpin silencing constructs *N. benthamiana* KIN10; tHMGR, truncated Arabidopsis HMGR; Bos, *A. annua* α-bisabolol synthase. The mean ± SD of 2-3 independent measurements. Results were statistically analysed using a two-tailed Student's t-test (P>0.05).

2.2 HMGR ACTIVITY

In this section the total HMGR activity of the agroinfiltrated *N. benthamiana* leaves was investigated. As described before, it was hypothesized that expression of the genes of interest would influence the sesquiterpene production, which is tested by measuring the α-bisabolol emission. Because HMGR is the rate limiting step in the MVA pathway, it was hypothesized that the effect on the production of α-bisabolol could be explained by a change in HMGR activity. In order to investigate this an HMGR activity assay was performed using an HMGR-CoA Reductase Assay kit (Sigma-Aldrich CS1090) as described in section 5.6. In advance to the experiments, the methods for extraction and measurement were optimised as described in section 5.6.3. For the following experiments, the same plant material that was measured with headspace trapping in sections 2.1.1, 2.1.3 and 2.1.4 was used to assess the total HMGR activity. From this plant material a crude extract was taken and subsequently the HMGR activity was measured. The HMGR activity assay measures the absorbance at 340 nm, representing the oxidation of NADPH by the catalytic subunit of HMGR in presence of the substrate HMG-CoA. Using a conversion formula, the HMGR activity was calculated in units per µg fresh weight tissue.

2.2.1 The effect of constitutive overexpressing of KIN10 and OtsB

In this section the HMGR activity was assessed from the leaves that were measured in section 2.1.2. Leaves were snap frozen five hours after induction with dexamethasone (DEX). Figure 6 shows the HMGR activity of the leaves treated with KIN10 and otsB, separately and combined. In this experiment three controls were used. For the first control HMGR activity of a wild type *N. benthamiana* leaves was measured, to obtain insight in the total HMGR activity of non-infiltrated plants. For the second control leaves infiltrated with p19, EV, and Bos, were measured to assess the effect of agroinfiltration on the HMGR activity. As a third control leaves infiltrated with p19, EV, Bos, and tHMGR was used, to investigate the effect of the additionally infiltrated genes on the HMGR activity, likewise to the controls used in section 2.1.

In Figure 6 is shown that the non-infiltrated plant had a 22% higher HMGR activity compared to the second control, which was infiltrated with p19, EV, and Bos. Furthermore, the non-infiltrated plant had a 12% higher activity when compared to the third control which additionally expressed tHMGR ($N=1\text{-}2$, $P>0.05$). Compared to the second control which is expressing p19, EV, and Bos, the third control had an 11% increase in HMGR activity ($P>0.05$, $N=1\text{-}2$). The treatments were compared to the third control. Combined expression of otsB and KIN10 combined led to a decrease of 28% in HMGR activity ($P>0.05$). Expression of otsB and KIN10 separately resulted in a significant decrease of 42% and 55% in HMGR activity, respectively ($P<0.05$, $N=2\text{-}3$).

In summary, when KIN10 and otsB are expressed separately, the HMGR activity is significantly lower than the control, with otsB construct showing the strongest effect on HMGR activity. When KIN10 and otsB are combined, the HMGR activity is lower than the control as well, however, the additive effect observed in section 2.1.2 was not shown for the HMGR activity. This indicates that expression of KIN10 and otsB simultaneously does not only affect the sesquiterpene production through HMGR, but also trough additional mechanisms. To further investigate the link between sesquiterpene production and HMGR activity, the HMGR activity of the treatments with inducible gene expression were assessed.

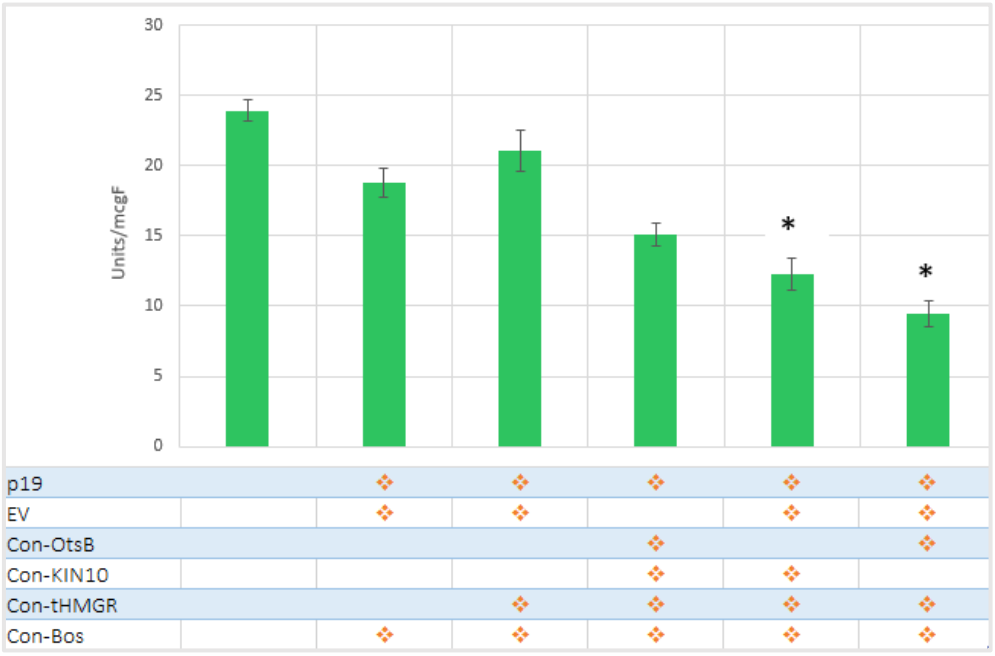

Figure 6. HMGR activity measured in *N. benthamiana* leaf crude extract upon constitutive expression of otsB, KIN10, co-expressed with P19, tHMGR and Bos. Leaves were flash frozen after headspace measurement, 24 hours after harvesting. Then, a crude extract was taken and directly used for measuring the HGMR activity. Con, constitutively expressing vector; p19, RNA silencing suppressor p19;EV, empty pBIN vector; OtsB, trehalose-phosphate phosphatase; KIN10, *A. thaliana* KIN10; tHMGR, truncated Arabidopsis HMGR; Bos, *A. annua* α-bisabolol synthase. Units/mcgF, one unit will convert 1.0 mmol of NADPH to NADP+ per 1 minute at 37 °C. The unit specific activity is defined as mmol/min/mcg fresh weight. The mean ± SD of 3-9 measurements. The asterisks denote statistical difference between the EV+tHMGR infiltration and other treatments, analysed by a two-tailed Student's t-test: *p<0.05.

2.2.2 The effect of combining OtsA, ABI1-1 and RNAi KIN10_6 and expression of HMGR

In this section I carried out two experiments that demonstrate the HMGR activity from treatments described in section 2.1.4 and one treatment described in 2.1.1. Leaves were snap frozen 24 hours after induction with DEX. Figure 7 shows the treatments with otsA, ABI1-1, and the combination of those two. Figure 8 shows the treatments combining otsA, ABI1-1, and silencing construct KIN10_6, and the expression of HMGR. In these two experiment three different controls are used. In the first experiment, one plant that was not infiltrated, the WT, is used. As a control in both experiments *N. benthamiana* infiltrated with p19, EV and Bos is used. As a third control for Figure 7, non-induced leaves were taken from each treatment.

As shown in Figure 7, the non-infiltrated plant had a 22% higher HMGR activity compared to plants infiltrated with p19, EV, and Bos, and 23% higher HMGR activity when compared to the control co-expressed with tHMGR (N=1-2, P>0.05). Plants infiltrated with p19, EV, and Bos had 1% higher HMGR activity compared to the control that additionally co-expressed tHMGR (P>0.05, N=1-2). The treatments were compared to the non-induced leaves infiltrated with constitutively expressed p19, EV, tHMGR, and Bos, and inducible otsA and ABI1-1. Compared to this control, inducible expression of otsA and ABI1-1 combined led to a 4% lower HMGR activity. Inducible expression of otsA and ABI1-1 separately resulted both in a 5% lower HMGR activity (P>0.05, N=2-3).

Altogether, the treatments have a similar total HMGR activity as the control. This shows that upregulation of HMGR activity is not observed when it is measured 24 hours after the induction of the genes. This indicates that either the effect on HMGR activity should be measured on a different time point, or that total HMGR activity has reached it optimum, and is not further upregulated by inhibition of SnRK1 or increased T6P levels. Additionally, the untransformed leaves had higher HMGR activity than all treatments and controls. This indicates that total HMGR activity is not further upregulated when HMGR is overexpressed with tHMGR, SnRK1 is inhibited or T6P levels are increased. Additionally, agroinfiltration possibly has a negative effect on the HMGR activity of the plant. In the following graph a similar experiment is conducted (Fig. 8), showing the effect of combinations with otsA, ABI1-1, and additionally silencing construct KIN10_6, and overexpression of HMGR by endogenous HMGR.

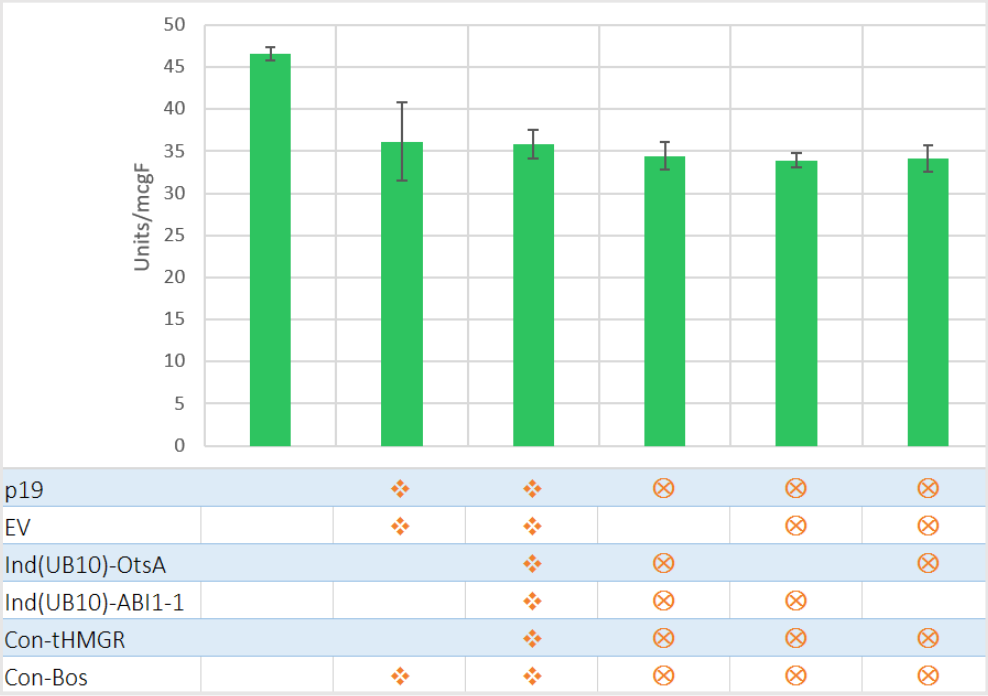

Figure 7. HMGR activity measured in *N. benthamiana* leaf crude extract upon inducible expression of otsA and ABI1-1, co-expressed with P19, tHMGR and Bos. Leaves were flash frozen after headspace measurement, 24 hours after dexamethasone induction. Then, a crude extract was taken and directly used for measuring the HGMR activity. ❖, non-induced treatment; ⊗, dexamethasone induced treatment; Con, constitutively expressing vector; p19, RNA silencing suppressor p19;EV, empty pBIN vector; OtsA, trehalose-phosphate phosphatase; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; tHMGR, truncated Arabidopsis HMGR; Bos, *A. annua* α-bisabolol synthase. Units/mcgF, one unit will convert 1.0 mmol of NADPH to NADP+ per 1 minute at 37 °C. The unit specific activity is defined as mmol/min/mcg fresh weight. The mean ± SD of 3-9 measurements. Results were statistically analysed using a two-tailed Student's t-test (P>0.05).

In Figure 8 leaves infiltrated with p19, EV, tHMGR, and Bos were used as a control for the treatments. Also as an extra control the leaves from a plant infiltrated with p19, EV, and Bos were assessed. In this experiment, the plant which was not infiltrated with tHMGR had a 40% lower HMGR activity compared to the plant that co-expressed tHMGR additionally to expression of p19, EV, and Bos (P>0.05, N=1-2) (Fig. 8). Compared to the second control, inducible expression of otsA, ABI1-1, and silencing construct KIN10_6 combined led to 8% lower HMGR activity. Inducible expression of otsA and silencing construct KIN10_6

combined resulted in a 5% reduction in HMGR activity (P>0.05, N=2-3). Constitutive expression of HMGR instead of tHMGR led to 40% lower activity of HMGR (P>0.05, N=1-2).

In summary, in this experiment I show that inducible expression of combinations of otsA, ABI1-1, and silencing construct KIN10_6 does not lead to a significant higher total HMGR activity. This is a similar trend to the previous experiment, where constitutive expression of otsA and ABI1-1 did not result in a significant change in total HMGR activity. Therefore, it can be concluded that, when measured 24 hours after induction, those genes do not have a clear impact on the total HMGR activity. On the other hand, constitutive expression of HMGR instead of tHMGR does result in a lower total HMGR activity. This indicates that total HMGR activity can be decreased upon lower T6P levels and overexpression of HMGR by endogenous HMGR, but conversely, not increased upon higher T6P levels and a lower activity of SnRK1.

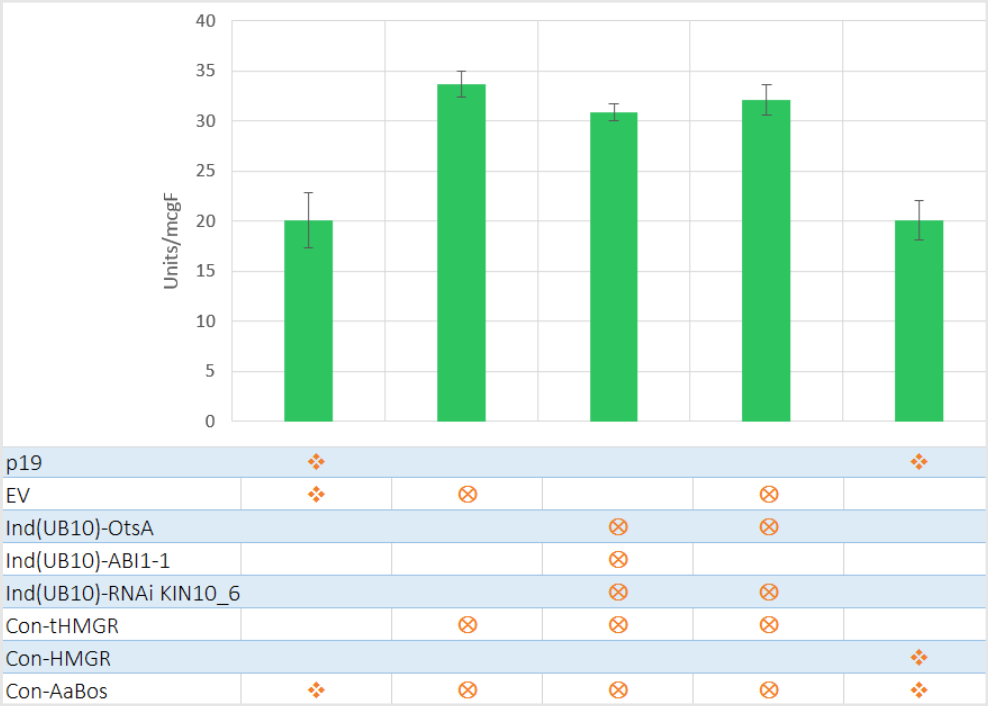

Figure 8. HMGR activity measured in *N. benthamiana* leaf crude extract upon inducible expression of otsA, ABI1-1 and silencing construct KIN10_6, endogenous HMGR, several treatments co-expressed with p19, tHMGR and Bos. Leaves were flash frozen after headspace measurement, 24 hours after dexamethasone induction. Then, a crude extract was taken and directly used for measuring the HGMR activity. ❖, non-induced treatment; ⊗, dexamethasone induced treatment; Con, constitutively expressing vector; p19, RNA silencing suppressor p19; EV, empty pBIN vector; OtsA, trehalose-phosphate phosphatase; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; RNAi KIN10_6; RNAi hairpin silencing construct *N. benthamiana* KIN10; tHMGR, truncated Arabidopsis HMGR; Bos, *A. annua* α-bisabolol synthase. Units/mcgF, one unit will convert 1.0 mmol of NADPH to NADP+ per 1 minute at 37 °C. The unit specific activity is defined as mmol/min/mcg fresh weight. The mean ± SD of 3-9 measurements. Results were statistically analysed using a two-tailed Student's t-test (P>0.05).

3 DISCUSSION

In this research I have investigated ways to enhance sesquiterpene production by modifying endogenous energy signalling pathways in *Nicotiana benthamiana*. Previous research has shown that trehalose-6-phosphate (T6P) has a major function in regulating growth and development, and one of its modes of action is inhibiting SnRK1 (Nunes et al., 2013; Y. Zhang et al., 2009). Furthermore, in literature it is shown that KIN10, a catalytic subunit of SnRK1, deactivates HMG-CoA reductase (HMGR) (Robertlee et al., 2017). Because HMGR is the rate limiting step in the mevalonate (MVA) pathway, this affects the production of sesquiterpenes. This link between the T6P pathway, the key sensor of energy availability and stress signalling, SnRK1, and the MVA pathway was investigated in order to optimise heterologous sesquiterpene production, in particular α-bisabolol.

3.1 Modification of KIN10 and HMGR by constitutive expression

It was observed that silencing of KIN10 by an RNAi silencing hairpin construct under a constitutive promoter led to an increase in α-bisabolol production (Fig. 2). The opposite, overexpression of KIN10, led to a decrease in production of α-bisabolol (Fig. 2 and 3). Additionally, this was observed likewise for the production of β-farnesol for overexpression of KIN10, and silencing of KIN10 by silencing construct RNAi KIN10_6 (SD3, Fig. 2). These results indicate that modification of SnRK1 activity affects the productivity of the MVA pathway, confirming the hypothesis that SnRK1 activity and the MVA pathway are linked. Furthermore, I constitutively expressed otsB, an *E.coli* trehalose phosphate phosphatase (TPP), which has previously been shown to reduce T6P levels in plants (Schluepmann et al., 2003). Expression of otsB caused a substantial decrease in sesquiterpene production, and an additive effect was observed when otsB and KIN10 were expressed simultaneously (Fig. 3). Additionally, HMGR activity was decreased for the treatments with otsB, overexpression of KIN10 and the combined treatment (Fig 6). However, there was no additive effect observed for HMGR activity for these treatments, showing that sesquiterpene production and HMGR activity are not linearly linked. This further confirms that SnRK1 influences the MVA pathway, but it additionally indicates that this effect is not completely due to alteration of the HMGR activity.

Because modifying the sugar signalling pathway and the stress signalling hub of the cell lead to a plethora of effects, it is expected that other processes will have an affect the MVA pathway efficiency. The MVA pathway, and sesquiterpene production, are modulated by many endogenous and external stimuli (Leivar et al., 2011), and it is likely that these stimuli are affected by changes in SnRK1 activity and T6P levels. Hence, it is hypothesized that changes in these stimuli affect the MVA pathway and the sesquiterpene productivity by alternate mechanisms. For example, the availability of the sesquiterpene precursor FPP can be limited because it is used by other endogenous pathways, such as the 5-epi-aristolochene synthase and squalene synthase (Cankar et al., 2015). Furthermore, HMGR activity is not completely dependent on its expression levels and protein accumulation (Nieto et al., 2009), suggesting that HMGR activity is also dependent on various other mechanisms. This confirms the hypothesis that other stimuli affect HMGR activity, the MVA pathway and the sesquiterpene production.

IN SUMMARY, these results show that changing T6P levels and SnRK1, separately or simultaneously, affects the output of the MVA pathway. Silencing of KIN10 led to upregulation of the MVA pathway. Likewise, the opposite effect is demonstrated with higher KIN10 activity, either by less inhibition through expression of

a TPP or by overexpression, led to downregulation of the MVA pathway. However, HMGR activity did not correlate linearly with these results. These results confirm the hypothesis that stated that KIN10 activity regulates the MVA pathway, however it is observed that this effect is not completely due to the HMGR activity.

Expression of truncated Arabidopsis HMGR (tHMGR) has been shown to increase MVA pathway end products significantly (Cankar et al., 2015; van Herpen et al., 2010; Wu et al., 2006). tHMGR is expressed in the cytosol instead of in the endoplasmic reticulum (ER), and it was hypothesized this causes HMGR to escape regulation by KIN10. Expression of full length *N. benthamiana* HMGR (HMGR) was hypothesized to have a similar effect as tHMGR, increasing HMGR activity by overexpressing endogenous HMGR. However, expression of HMGR, and the combination of HMGR and tHMGR led to great reduction in sesquiterpene production (Fig. 2). This was observed both with and without co-expression of p19, which supresses gene silencing. Therefore, it was concluded that this effect was not caused by gene silencing due to agroinfiltration. Additionally, the HMGR activity assay shows a strong reduction in HMGR activity when HMGR is expressed (Fig. 8), further confirming that expression of HMGR decreases the total HMGR activity. However, HMGR activity was not reduced as much as it was in sesquiterpene production, indicating that other factors than only HMGR activity play a role. This reduction can be explained by the presence of the N-terminal membrane binding domain in HMGR. The membrane domain has a variety of effects, such as influencing posttranscriptional and posttranslational regulation of HMGR (Robertlee et al., 2017) and functioning as an ER morphogenic signal (Ferrero et al., 2015). Overexpression of the membrane domain has shown to cause hypertrophy of the ER. Furthermore, it was hypothesized that the lack of the membrane domain escapes SnRK1. Therefore, I hypothesized that the membrane domain causes greater regulation because of multiple reasons, such as SnRK1 targeting and by regulatory mechanisms that detect ER hypertrophy. In conclusion, expression of the full length HMGR, in contrast to tHMGR, causes a significant reduction in HMGR activity and sesquiterpene production.

In Figure 9 the new hypothesis is represented based on the results described in this section. In the following section is discussed what the effect on the sesquiterpene production and HMGR activity is when the genes are expressed under an inducible promoter.

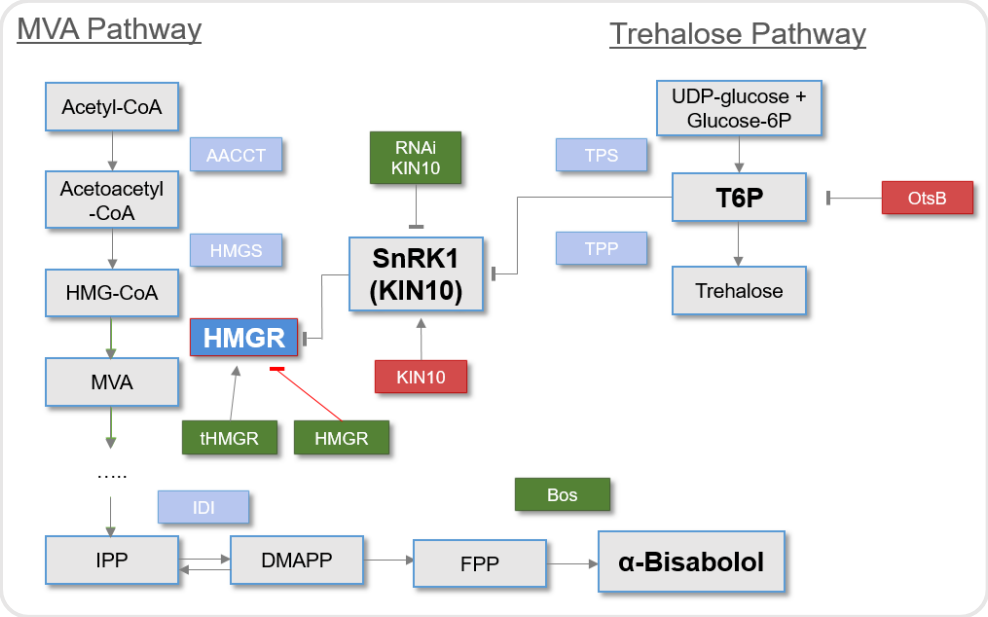

Figure 9 The hypothesized link between the trehalose pathway, SnRK1 and MVA pathway based on the consititutive expression experiments. The red line shows the difference compared to the initial hypothesis. Gene overexpression, inactivation and silencing are schemetically shown for all genes used in this study. Grey boxes, actors in the MVA and T6P pathways; Dark blue and light blue boxes, enzymes affecting the actors; Green boxes, infiltrated genes with a hypothesized positive effect on the α-bisabolol production and HMGR activity; Red boxes, infiltrated genes with a hypothesized negative effect on the α-bisabolol production and HMGR activity. Genes used: OtsB, trehalose-phosphate phosphatase; RNAi KIN10, RNAi hairpin silencing construct *N. benthamiana* KIN10; KIN10, *Arabidopsis thaliana* KIN10; tHMGR, truncated *A. thaliana* HMGR; HMGR, *N. benthamiana* HMGR; Bos, *A. annua* α-bisabolol synthase. Steps in the patway: Acetyl-CoA, Acetyl coenzyme A; KIN10, SNF1 kinase homolog 10; DMAPP, dimethylallyl; FPP, farnesyl diphosphate; HMG-CoA, hydroxymethyl-glutaryl coenzyme A; IPP, isopentenyl diphosphate; MVA, mevalonic acid; SnRK1, sucrose non-fermenting-1-related protein kinase 1; T6P, trehalose-6-phosphate. Enzymes are indicated in blue: AACT, acetoacetyl CoA thiolase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IDI, IPP isomerase; TPS, trehalose phosphate synthase; TPP, T6P phosphatase. (MVA pathway derived from Rodríguez-Concepción (2006), trehalose pathway derived from Schluepmann et al. (2003)).

3.2 Modification of T6P, KIN10 and HMGR by inducible expression

In this section, I will discuss the effect of the genes when expressed under an inducible promoter. Sesquiterpene production and HMGR activity were assessed to investigate the potential of these genes for enhancing sesquiterpene production. In Figure 5, the α-bisabolol emission is shown upon inducible expression of otsA, ABI1-1, and silencing construct KIN10. When otsA or ABI1-1 were expressed, sesquiterpene production was enhanced. However, when otsA was co-expressed with ABI1-1, or silencing construct KIN10_6, or with both, sesquiterpene production was lower to or similar as the control. This shows that the combination of changed T6P levels and KIN10 activity does not agree with the hypothesis, in which was suggested that this combination would enhance the sesquiterpene production most optimally. Therefore, it was concluded that different mechanisms play a role when both T6P levels and KIN10 activity are altered simultaneously. Lunn et al. (2014) suggested that SnRK1 possibly is not directly affected by T6P, but T6P can have overlapping targets with SnRK1 instead. Therefore, it can be argued that changing T6P levels has multiple effects when it acts through mechanisms other than only through SnRK1. For example, as demonstrated by Kolbe et al. (2005), T6P stimulates starch synthesis by acting on the plastidial metabolism. In this pathway, T6P promotes a thioredoxin-mediated redox transfer to ADP-glucose pyrophosphorylase (AGPase) when cytosolic sugar levels are perceived as high, which results in starch synthesis. Increased starch synthesis subsequently diverts the carbon flux from the MVA pathway. Altogether, it is hypothesized that increased T6P levels not only affect the MVA pathway through inhibition of SnRK1, but additionally affects the MVA pathway by competing for the carbon availability in the cell.

Furthermore, as shown in Figure 7 and 8, sesquiterpene production is not directly linked to HMGR activity, confirming that enhanced T6P levels and inhibition of SnRK1 affect the MVA pathway not only through HMGR activity. This can be explained by the many changes metabolic pathways undergo when the cell is made to believe it is in an energy rich state, and in absence of stress. For example, SnRK1 deactivates sucrose phosphate synthase, nitrate reductase, trehalose-phosphate synthase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (Halford & Hey, 2009), besides inactivating HMGR. Furthermore, overexpression of KIN10 has shown to cause abscisic acid (ABA) hypersensitivity, which results in inhibited growth (Cho et al., 2012; Jossier et al., 2009). Therefore, it can be reasoned that suppression of KIN10 leads to insensitivity to abscisic acid (ABA). ABA responses include stomatal closure, growth inhibition, and other stress related responses. Thus, when those responses are downregulated, more other pathways involved in growth are upregulated, resulting in an increased competition for available carbons in the cell. Hence, it is hypothesized that besides HMGR activity, the carbon flux in the cell is changed when both energy and stress signalling are modified. Figure 10 illustrates the new hypothesized relations between the energy pathways and genes.

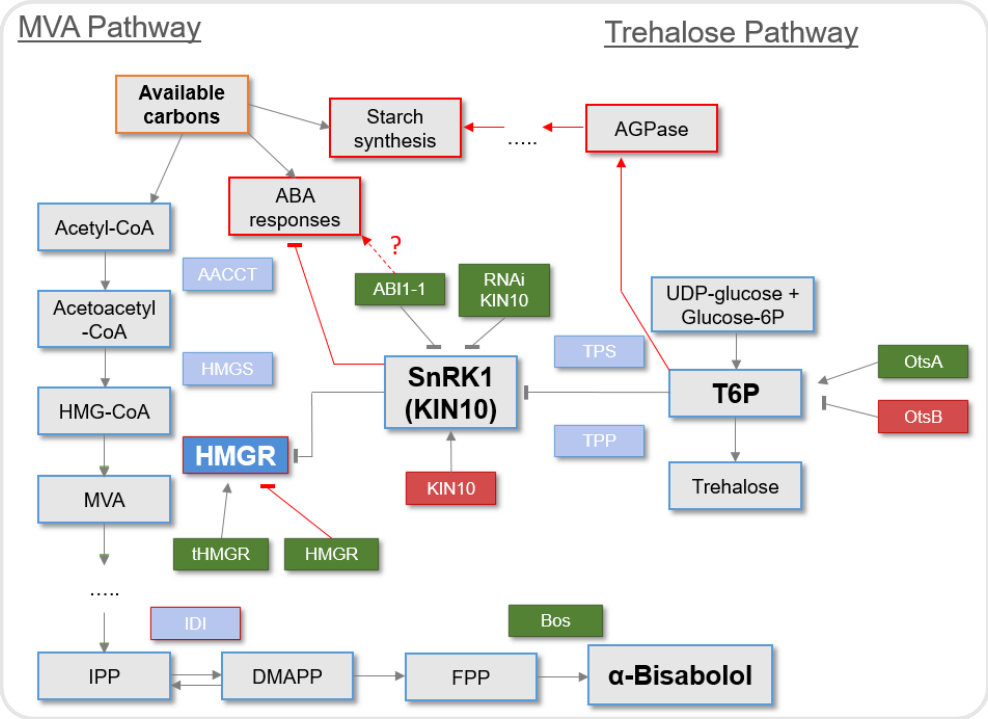

Figure 10 The hypothesized link between the trehalose pathway, SnRK1 and MVA pathway based on the consititutive expression experiments. The red lines show the difference compared to the initial hypothesis. Gene overexpression, inactivation and silencing are schemetically shown for all genes used in this study. Grey boxes, actors in the MVA and T6P pathways; Dark blue and light blue boxes, enzymes affecting the actors; Green boxes, infiltrated genes with a hypothesized positive effect on the α-bisabolol production and HMGR activity; Red boxes, infiltrated genes with a hypothesized negative effect on the α-bisabolol production and HMGR activity. Genes used: OtsA, trehalose-6-phosphate synthase; OtsB, trehalose-phosphate phosphatase; ABI1-1, *A. thaliana* abscisic acid insensitive 1-1; RNAi KIN10, RNAi hairpin silencing construct *N. benthamiana* KIN10; KIN10, *Arabidopsis thaliana* KIN10; tHMGR, truncated *A. thaliana* HMGR; HMGR, *N. benthamiana* HMGR; Bos, *A. annua* α-bisabolol synthase. Steps in the patway: ABA responses, abscisic acid responses; Acetyl-CoA, Acetyl coenzyme A; AGPase, ADP-glucose pyrophosphorylase; KIN10, SNF1 kinase homolog 10; DMAPP, dimethylallyl; FPP, farnesyl diphosphate; HMG-CoA, hydroxymethyl-glutaryl coenzyme A; IPP, isopentenyl diphosphate; MVA, mevalonic acid; SnRK1, sucrose non-fermenting-1-related protein kinase 1; T6P, trehalose-6-phosphate. Enzymes are indicated in blue: AACT, acetoacetyl CoA thiolase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IDI, IPP isomerase; TPS, trehalose phosphate synthase; TPP, T6P phosphatase. (MVA pathway derived from Rodríguez-Concepción (2006), trehalose pathway derived from Schluepmann et al. (2003)).

In Figure 4, it is shown that expression of ABI1-1 and silencing construct KIN10_6 under the inducible promoter results in lower sesquiterpene production than the control. However, in Figure 5, is shown that the sole expression of ABI1-1 leads to a higher sesquiterpene production. The difference between those graphs is the method of collecting the volatiles: in Figure 4 the leaves are induced four hours before

measurement, and volatiles were trapped for one hour. In Figure 5 the leaves were induced and immediately measured for 24 hours. As shown by Rossignol et al. (2014), expression of genes under the dexamethasone inducible systems is fluctuating the first 24 hours after induction. Therefore, it was hypothesized that measuring the leaves for a longer time period after induction gives more consistent results and a more accurate representation of the effect of expressed genes.

Furthermore, it is shown in Figure 4 that the inducible system was more efficient under the ubiquitin 10 (UB10) promoter than under the Cauliflower mosaic virus 35S (35S) promoter. For expression of both genes, silencing construct KIN10_6, and ABI1-1, the UB10 promoter showed higher sesquiterpene production. Expressing the genes under the UB10 promoter leads to a lower expression of the genes compared to the 35S promoter (Grefen et al., 2010). However, it was hypothesized that gene silencing under this promoter is less compared to the 35S promoter, because the UB10 promoter is less leaky. Besides gene silencing, lower expression of the genes possibly leads to less side effects on the energy metabolism of the cell, making genes expressed under the UB10 promoter more effective in upregulating the MVA pathway. Thus, expression of the genes under the UB10 promoter is more suitable for enhancement of sesquiterpene production.

IN CONCLUSION, the results show that ABI1-1 and otsA play a role in sesquiterpene production. Inhibition of KIN10, either by dephosphorylation or inactivation, led to upregulation of the MVA pathway, which is in agreement with the hypothesis. However, when T6P levels are elevated in combination with decreased KIN10 activity, this is effect is not observed. Therefore, it was hypothesized that competition for carbons and sesquiterpene precursors changes when stress and sugar signalling are modified simultaneously. More research is recommended to investigate which pathways compete with the MVA pathway by knocking down essential enzymes in those pathways, e.g. AGPase in the starch synthesis pathway. More recommendations are made in the next section. Overall, it was concluded that changing the T6P pathway, SnRK1, and the MVA pathway simultaneously can enhance sesquiterpene production, however more regulation is required to optimise this.

4 RECOMMENDATIONS

Based on the results of this thesis several recommendations can be made for future research. The aim of this research was to achieve further optimisation for sesquiterpene production, and the results gave more insights into the mechanisms behind this. However, there are still many processes that remain unknown, and in the following section options are given to unravel the mechanisms behind sesquiterpene production more extensively.

qPCR It is recommended to perform a quantitative polymerase chain reaction (qPCR) to test how gene expression of the target genes is influenced when the genes of interest are expressed. Quantifying the gene expression would confirm that overexpression, silencing, and inhibition of the genes was successful. This would prove that gene expression levels are affected as hypothesized, and that the change in sesquiterpene production and HMGR activity is due to modification of the expression of these genes, and not due other processes. This would elucidate the relation between the T6P pathway, SnRK1, and MVA pathway on a more genetic basis.

HMGR ACTIVITY Overexpression of HMGR by truncated Arabidopsis HMGR (tHMGR) has shown to be an effective way of upregulating the MVA pathway (Cankar et al., 2015). Because tHMGR lacks the membrane domain, it is expressed in the cytosol, in contrast to endogenous HMGR that is embedded in the membranes of the ER and spherical, vesicular structures (N Campos & Boronat, 1995; Leivar et al., 2005). Measuring the HMGR activity in the membranes and cytosol separately would elucidate the effect of tHMGR has on the endogenous HMGR. This would provide a better understanding of the molecular mechanisms behind HMGR activity, which could be used in further optimisation in upregulating the MVA pathway.

REDIRECTING ISOPRENOID PRECURSORS Further redirection of the carbon flow in the cell can lead to a higher sesquiterpene precursor production, and subsequently sesquiterpenes. Available carbons are used by a plethora of pathways, such as in the MEP pathway in the plastids, starch synthesis, and other metabolic pathways. Wu et al. (2006) demonstrated that overexpression of an avian farnesyl diphosphate synthase (FDS) in the cytosol, reduces carbon usage in the plastids. They showed that sesquiterpene synthesis can be increased up to 1,000-fold when co-expressed with a suitable terpene synthase. Therefore, it is suggested to co-express FDS with the α-bisabolol synthase to achieve a higher α-bisabolol production.

REDIRECTING FPP USAGE Besides competition for carbons, there is also competition for FPP by other endogenous pathways. To decrease this competition, these pathways can be shut down by silencing. As shown by Cankar et al. (2015), silencing of the endogenous 5-epi-aristolochene synthase and squalene synthase leads to increased sesquiterpene production. Therefore, it is hypothesized that incorporating this strategy with the method described in this study could further optimise α-bisabolol production. Also investigation of other pathways using FPP could potentially uncover new ways to enhance α-bisabolol production.

INCREASING FPP Additionally, FPP quantities could be increased by expression of a FPP synthase (FPS). Cytosolic FPP synthesis is argued to be under strong regulatory constraints due to its role in protein farnesylation and hence signalling, biosynthesis of brassinosteroid hormones and membrane sterols

(Sallaud et al., 2009). A higher level of FPP, created by expression of FPS, could therefore circumvent the negative effect of regulation of the FPP pool. Expression of an FPS was also adopted by Cankar et al. (2015), and has shown to increase sesquiterpene production when co-expressed with tHMGR. Using this enzyme could potentially further increase production of α-bisabolol.

EXPRESSION OF THE MVA PATHWAY IN THE CHLOROPLASTS An alternative approach in preventing regulatory mechanisms was tested by Kumar et al. (2012). They inserted the entire MVA pathway into the tobacco chloroplast genome, so that the MVA pathway was expressed in the chloroplasts instead of in the cytosol. This resulted in a higher accumulation of mevalonate, carotenoids, squalene, sterols, and triacyglycerols. It was hypothesized that the increased MVA pathway products were due to higher availability of the IPP monomer, leading to a higher flux through the downstream biosynthetic pathway or enhanced expression or activity of the pathway. This new metabolic engineering strategy could be used to further enhance sesquiterpene production in plants and thereby increase the α-bisabolol production.

EMISSION TIMELINE When using an inducible gene expression system it is beneficial to measure the α-bisabolol emission at different times during the day. This would give more insight in the optimal timing to collect sesquiterpenes. For both research and commercial purposes, this can be a great advantage, since obtaining the highest level of α-bisabolol is the main goal. Discovering the optimal collection timing is especially recommended when genes are expressed using an inducible system, because the induction time influences the sesquiterpene emission greatly. Additionally, the α-bisabolol emission can be tested by comparing the expression of the genes in an inducible system versus a constitutive system, to verify whether inducible expression is more efficient.

STABLE TRANSFORMANTS Using stable transformant lines over transient transformants is preferred for various reasons. Stable transformants provide a more homologous expression of the genes of interest. Furthermore, transient expression leads to a high transformation frequency compared to stable transformation, which leads to a higher regulatory response by the plant. Implementing the dexamethasone inducible system in stable transformant would enable α-bisabolol production when desired, with minimized regulatory cell responses. The ability of selectively turn genes on and off is hypothesized to reduce unwanted effects of these genes on the plant, such as lethality, gene silencing, and other phenotypical deviations.

STORAGE Sesquiterpenes are volatile compounds and therefore emitted in the headspace by the plant. This complicates harvesting and leads to a lower yield of the sesquiterpene of interest. Glycosylation of metabolites increase their stability and solubility, which can lead to accumulation of metabolites (Ohgami et al., 2015). Therefore, it is hypothesized that glycosylation of α-bisabolol could lead to α-bisabolol accumulation in the plants and thereby improve its method of harvesting.

RNASEQ Ultimately, RNA sequencing (RNAseq) is highly recommended to analyse expression of the complete transcriptome. This would give insight into the genes that are influenced by the modification of the pathways. Comparing those results to non-transformed plants will elucidate which other genes and pathways are involved. This would greatly improve the understanding in the underlying mechanisms involved in the MVA pathway, SnRk1, and T6P pathway. Subsequently, this information can be used to further redirect the carbon flow and improve the α-bisabolol production.

5 MATERIALS AND METHODS

5.1 Genetic material

The genetic material used in this study is obtained from different organisms. Bos (GenBank: JQ717161.1) was previously isolated from *Artemisia annua*. KIN10 (AT3G01090) and tHMGR (AT1G76490) were isolated from wild type *Arabidopsis thaliana*. RNAi KIN10 (AY919676.1) fragments and HMGR (LC015758.1) were isolated from *Nicotiana benthamiana*. ABI1-1 (AT1G08810) was isolated from a mutant *A. thaliana* plant carrying the dominant mutation. OtsA and OtsB were previously isolated from *Escherichia coli*, and are described in patents WO95/01446 and WO97/42326, respectively. All genes isolated in this study were obtained by first isolating the total DNA from the plant material, using a DNeasy Plant Mini Kit (Qiagen 69104). From the total DNA, the genes were subsequently isolated by polymerase chain reaction (PCR) with the enzyme Q5 High-Fidelity DNA Polymerase (NEB M0491S). Primers were specifically designed for isolation of the target genes and order at Macrogen Europe (The Netherland). The primer sequences are shown in Supplemental Data Table 1. Next, the isolated genes were used for cloning into different constructs.

5.2 Cloning

The genes Bos, tHMGR and OtsB were previously cloned into pBIN, a vector with a constitutive promoter. KIN10, silencing constructs RNAi KIN10_6, NKIN_6 and HMGR were isolated and cloned into the entry vector pCR8/GW/TOPO (Invitrogen K250020). Thereafter, KIN10 and HMGR were inserted into pB7WG2(II), also vector with a constitutive promoter. The transfer of the genes to the entry vector was done by recombination between the entry clone and the destination vector, pB7WG2, using the Gateway® LR Clonase® II enzyme mix (Invitrogen 11791100). The two silencing constructs, RNAi KIN10_6 and KIN10_8, were inserted into the expression vector pB7GWIWG2(II) also by recombination (Cankar et al., 2015). This vector produces an RNAi hairpin construct of the gene under a constitutive promoter, which leads to silencing of the endogenous variation of this gene. A graphical overview of the vectors is represented in Figure 12.

In later experiments I made use of the dexamethasone inducible expression vector pTA7002. Two variations of this vectors were used, one with a cauliflower mosaic virus 35S (35S) promoter and an ubiquitin 10 (UB10) promoter (patent US 09/438,392) (Aoyama & Chua, 1997; Schluepmann et al., 2003), as shown in Figure 12. All genes were inserted into this expression vector using digestion by the restriction enzymes XhoI and SpeI. The restriction site XhoI was added to the 5' end of each gene, the restriction site SpeI was added to the 3' end (NEB R0146L and R0133L) (Primers are shown in Supplemental Data table 1). All genes and vectors were digested by XhoI and SpeI and thereafter ligated with T4 DNA ligase (Promega M1794).

After recombination of the genes into pB7WG2 and ligation into pTA7002, the vectors were heat shocked into chemically competent *Escherichia coli* cells, strain DH5α. Those cells were grown for 24 hours at 37 °C on LB agar plates containing 100 µg/ml spectinomycin for pB7WG2 or 100 µg/ml kanamycin for pTA7002. Thereafter, colonies were tested using colony PCR with the detection primers shown in Supplemental Data table 1. The colonies containing the correct vector were grown for 24 hours/37 °C/250rpm in LB containing 100 µg/ml spectinomycin or kanamycin, depending on the resistance of the plasmid. Plasmid DNA was

isolated using the QIAprep Spin Miniprep Kit (Qiagen 27106). Isolated vectors were subsequently used for agroinfiltration.

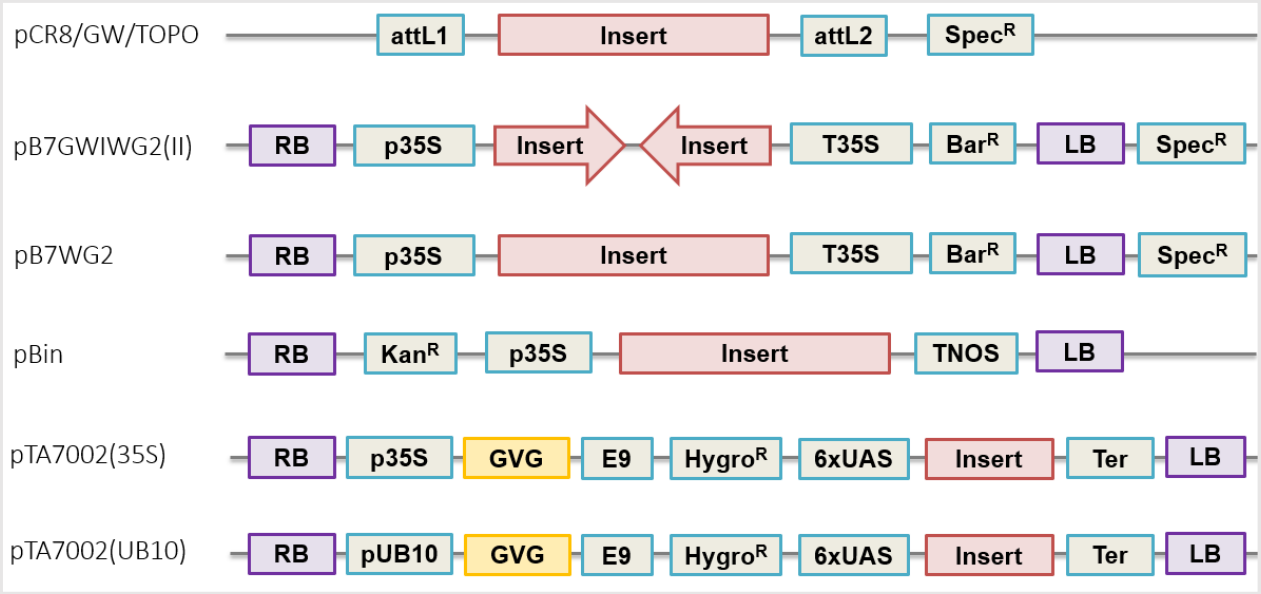

Figure 12. Plasmids used in this study. pCR8/GW/TOPO is the entry vector, pB7GWIWG2(II) is the constitutive silencing vector that forms hairpin construct from the inserts, pB7WG2 and pBin are constitutive vectors. pTA7002(35S) and pTA7002(UB10) are dexamethasone inducible vectors. AttL1 and attL2, recombination sites; $Spec^R$, spectinomycin resistance gene, RB, right border; p35S, CaMV 35S promoter; Insert, place of insertion of target gene; T35S, CaMV terminator; Bar^R, Bialaphos Resistance Gene; LB, left border; Kan^R, kanamycin resistance; TNOS, Nopaline synthase terminator; pUB10, ubiquitin 10 promoter; GVG, glucocorticoid-regulated transcription factor; E9, E9 terminator; $Hygro^R$, hygromycin-resistance gene; 6xUAS, promoter containing six copies of the GAL4 upstream activating sequence; Ter, terminator gene.

5.3 Agroinfiltration

Cells of *Agrobacterium tumefaciens* strain AGL0 were electroporated with 1 µl of plasmid DNA and grown for 48 hours at 28 °C on LB agar plates. The plates contained the vector specific antibiotic and 25 µg/ml rifampicin to select for *A. tumefaciens* containing the correct vector. Thereafter, colonies were picked and grown in LB containing 25 µg/ml rifampicin for 48 hours/28 °C/250rpm. Then, the cell suspension was centrifuged at 4000*g* for 20 minutes at 20 °C and the supernatant was decanted. The cells were resuspended in 10 ml agroinfiltration buffer (Supplemental Data table 2). Next, the *Agrobacterium* suspension was incubated for three hours at room temperature and the suspension was brought to a total optical density of 0.5 measured at a wavelength of 600 nm (OD_{600}). To achieve this, the *Agrobacterium* suspension was measured in a 1:10 dilution using a spectrophotometer and diluted in agroinfiltration buffer. P19 was co-expressed at an OD_{600} of 0.1 with a part of the treatments. Subsequently, the suspension was infiltrated into the abaxial side of leaves from 6 weeks old *Nicotiana benthamiana* plants as described by Cankar et al. (2015). Leaves were harvested 3 to 5 days post agroinfiltration and put on water directly after harvesting.

5.4 Headspace trapping

To investigate the sesquiterpene emission of agroinfiltrated *N. benthamiana* leaves, the emitted volatiles were collected. I used a method similar as described by Cankar et al. (2015). The leaves were detached

using a razor blade and directly put on 10 or 20 ml plastic vials filled with tap water. Each vial was separately placed under a 1 L glass jar. The emitted volatiles were collected on stainless steel cartridges filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech). Prior to collection of the volatiles the cartridges were cleaned by passage of helium at a flow rate of 200 ml/min at 250 °C for 1 hour. The volatiles were then collected for 1 hour or 24 hours, using an air inflow of 300 ml/min and an air outflow of 100 ml/min. The air inflow passed through clean cartridges to filter out all potential contaminants in the air. Air outflow passed through the cartridges to collect to volatiles. During trapping a GreenPower LED research module was used (Philips, The Netherlands) and light was set on 100% white, 10% deep red, 50% far red, and 5% blue. The light regime was 12 h of light and 12 h or darkness. After collection of the volatiles, the fresh weight of the leaves was determined. The cartridges were subsequently used for thermal desorption GC-MS to quantify the amount of emitted volatiles, including α-bisabolol.

5.5 Thermal desorption gas chromatography-mass spectrometry

Thermal desorption GC-MS was used to analyse the collected volatiles on the cartridges. The results in Figure 2 were obtained using a method similar as described by Cankar et al. (2015). Differences were that the cartridges were not flushed before analysation and the split ratio was 1:10. A-bisabolol was identified based on an external calibration curve. The total ion mass was used to quantify the α-bisabolol emission.

The results in Figure 3, 4 and 5 were obtained using the following method. Thermal desorption GC-MS analysis was performed with an Agilent GC 7890B series (Agilent Technologies, The Netherlands) coupled to an Agilent 7200 series Q-TOF MS (Agilent Technologies, The Netherlands). The headspace samples were desorbed from the cartridges at 240 °C (Ultra; Markes) for 5 min with a trap flow of 20 ml/min. Used split ratios used varied between 1:5 and 1:10, optimized for the concentration of volatiles in each experiment which was greatly influenced by the trapping time. The volatiles were thereafter focused on an electrically cooled sorbent trap (Unity; Markes) that was cooled to 0 °C. Subsequently, the sorbent trap was heated to 260 °C for 3 minutes and the released volatiles were injected on the analytical column (DB-5MS, 30 m x 0.25 mm ID, 1.0 μm – film thickness, Agilent Technologies, The Netherlands) with a split flow of 40 to 200 ml/min, depending on quantity of volatiles in the samples. The GC temperature program started at 40 °C, with a 2 min hold, and then linearly rose with 10 °C/min to 280 °C with a 4 min hold. There was a constant helium flow of 1.2 ml/min. Mass scanning was done from 50 to 350 m/z with 5 scans/s. The temperature of the ion source and transfer line were 230 °C and 280 °C, respectively. Results were qualitatively and quantitatively analysed using MassHunter Acquisition Data B.07.00. The retention time and mass spectrum were used to identify the constituents, using the NIST library version 2.2, 2014. The component area was used to quantify the sesquiterpenes.

5.6 Determination of 3-Hydroxy-3-methylglutaryl CoA Reductase Activity

5.6.1 Isolation of membrane bound enzymes

In order to determine the activity of 3-Hydroxy-3-methylglutaryl CoA reductase (HMGR), the enzyme has to be extracted from the plant material. The harvested leaves were snap-frozen after headspace measurement and stored at -80 °C, and thereafter used for making a crude extract. HMGR was extracted similar to the method described by Campos et al. (2014). Samples were grounded with mortar and pestle, and subsequently the cells were lysed by using a glass homogenizer. 2 µl homogenization buffer (Supplemental Data table 2) per mg fresh tissue was added to the lysed cells, and the samples were kept

at room temperature until the plant material thawed. After thawing the samples, they were centrifuged at 3000*g* at 2 °C for 10 minutes. The supernatant was recovered after centrifugation and centrifugation was repeated. The then recovered supernatant was stored on ice until all samples were ready, after which they were directly used for measuring the HMGR activity.

5.6.2 Measuring the HMGR activity

HMGR activity was measured using an HMGR-CoA Reductase Assay kit (Sigma-Aldrich CS1090). The assay is based on spectrophotometric measurements of the decrease in absorbance at 340 nm. This represents the oxidation of NADPH by the catalytic subunit of HMGR in the presence of the substrate HMG-CoA. HMGR catalyses the following reaction: HMG-CoA + 2NADPH + $2H^+$ → mevalonate + $2NADP^+$ + CoA-SH. The samples are measured in a 96 well plate, and for each sample 134 µl 1x assay buffer, 4 µl NADH, 12 µl HMG-CoA and 50 µl crude extract was mixed directly before measuring. As a blank the reaction mix without the sample is used, with 50 µl more 1 x assay buffer. The samples were shaken for 10 seconds and measured for 10 minutes at 37 °C with a SpectraMax® Plus 384 absorbance microplate reader (Molecular Devices, US). Results were analysed with SoftMax Pro 7 software. The difference in absorbance was used to calculate the units per mg fresh weight, using the following equitation:
$\text{Units/mgF} = \frac{(\Delta A340/\text{minsample} - \Delta A340/\text{minblank}) \text{ x TV})}{\varepsilon\text{mM x V x LP}} = \frac{(\Delta A340/\text{minsample} - \Delta A340/\text{minblank}) \text{ x } 0.2)}{12.44 \text{ x } 0.025 \text{ x } 0.55}$. In which ε^{mM} = 12.44, the extinction coefficient for NADPH at 340 nm is 6.22 $mM^{-1}cm^{-1}$. 12.44 represents the 2 NADPH consumed in the reaction. TV = Total volume of the reaction in ml, V = volume of crude extract in the assay (mg), LP = Light path in cm. One unit will convert 1.0 mmol of NADPH to $NADP^+$ per 1 minute at 37 °C. The unit specific activity is defined as mmol/min/mg fresh material (Units/mgF). The calculated HMGR activity is shown in section 2.2.

5.6.3 Optimization extraction and enzymatic assay

Before starting the HMGR activity experiments, the HMGR-CoA Reductase Assay kit and its supplied enzymes were tested. Next, I investigated whether cytosolic and membrane bound HMGR could be measured separately, by using two different buffers. Furthermore, the NADPH usage was measured upon inhibition of HMGR activity by a statin, to confirm that NADPH was solely used by HMGR. Thereafter, the extraction method and the enzymatic assay were optimized.

The kit was tested by comparing a blank sample, an activity sample, and an inhibition sample. The blank sample contained 1x assay buffer and the substrates, NADPH and HMG-CoA. The activity sample contained purified HMGR in addition to the blank sample, and the inhibition sample contained an HMGR inhibitor, Prevastin, in addition to the activity sample. The activity and inhibition sample were corrected for the blank. The activity sample showed high NADPH usage, whereas the inhibition sample showed no NADPH usage. This proves that NADPH usage is due to HMGR activity and that Prevastin is efficacious in inhibiting HMGR activity.

As described in 5.6.1, a crude extract of the infiltrated leaves was used, in which all membrane bound enzymes were solubilized. However, first I tested whether I could separately measure tHMGR and the endogenous HMGR activity. Separation was based on the fact that tHMGR is expressed in the cytosol, whereas endogenous HMGR is embedded in the ER membranes. Therefore, the tissue was first lysed and then added to homogenization buffer without a nonionic detergent, which releases the membrane bound and so endogenous HMGR. The complete procedure described in 5.6.1 was followed, first with the buffer

lacking the nonionic detergent and thereafter with the buffer containing the nonionic detergent. Then, the centrifugation procedure was repeated after vortexing the pellet. It was hypothesized that cytosolic tHMGR would be extracted in the first treatment and endogenous HMGR in the second treatment. Treatments used for these steps were *N. benthamiana* leaves infiltrated with tHMGR, Bos and p19, and leaves infiltrated with endogenous HMGR, Bos and p19, all under a constitutive promoter. Because in section 2.1.1 I observed that expression of endogenous HMGR caused a dramatic decrease in sesquiterpene production I hypothesized that the HMGR activity in this treatment would be greatly reduced as well.

In this experiment, NADPH and the obtained samples were added to 1x assay buffer, and subsequently NADPH usage was measured for 5 minutes. Next, the substrate HMG-CoA was added and again the samples were measured for 5 minutes. Lastly, pure HMGR or Prevastin was added to the samples. The tHMGR sample extracted without a nonionic detergent showed high HMGR activity before the substrate HMG-CoA was added, indicating that NADPH was used by other constituents in the extract. The NHMGR sample extract without a nonionic detergent showed very low NADPH usage, however the highest before the substrate HMG-CoA was added. This indicates that NADPH usage also in this sample is reduced by other constituents in the sample. The tHMGR sample extracted with a nonionic detergent showed only high NADHP usage when the substrate HMG-CoA was added, indicating that NADPH usage was due to endogenous HMGR activity. The HMGR sample extracted with a nonionic detergent showed a low NADPH usage when HMG-CoA was added, indicating that HMGR activity is lower when endogenous HMGR is expressed. In summary, it was shown that endogenous HMGR activity can be determined by NADPH usage when the crude extract is made using a homogenization buffer with a nonionic detergent. Additionally, overexpression of endogenous HMGR by endogenous results in a lower HMGR activity.

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8 SUPPLEMENTAL DATA

SD1: Table 1. Primers

Supplemental Data Table 1. Primers used for isolation, detection and cloning of all genes used in this study. Nucleotides that are marked blue are the recognitions sites of the restriction enzymes used. Primers were ordered at Macrogen Europe (The Netherlands).

Cloning primers		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Bos	GTGTTCACCCAGAGCCATCT	GCCCAGAAGTAGCACTCCAC
ABI1-1	GAAGCAATTGTTGCATTAGCC	GTATGTAAAGTTTTGAAAGTAATTGCA
KIN10	ATGTTCAAACGAGTAGATGAGTTTAATT	TCAGAGGACTCGGAGCTGA
tHMGR	ACCGAATCGCTTCCTGAG	TGTTGTTGTTGTTGTCGTTGTC
HMGR	ATGGACGTTCGCCGGA	TTAGGAGGATGCCTTTGTGAC
RNAi KIN10_6	ATGCATCCAGCAGAAAGCG	CATACCATCTGCGCTGCT
RNAi KIN10_8	CATTTGTCTGTTAACAAATTCCAC	TACTAATAGTTCGAGGGTCAAAGAGAG
OtsA	ATGAGTCGTTTAGTCGTAGTATCTAACC	CTACGCAAGCTTTGGAAAGG
Detection primers		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
ABI1-1	GAATGGAGCTCGTGTTTTCGG	GTTCAAGGGTTTGCTCTTGAGT
KIN10	ATGCATCCAGCAGAAAGCG	CATACCATCTGCGCTGCT
tHMGR	TGCAGGCTCTCTAGGTGGAT	TGAGATGCAAGCTGTGTTCC
HMGR	CAGGGGAAAACACCGTCTTA	CCATTGGCACCGAATACTCT
RNAi KIN10_6	ATGCATCCAGCAGAAAGCG	CATACCATCTGCGCTGCT
RNAi KIN10_8	CATTTGTCTGTTAACAAATTCCAC	TACTAATAGTTCGAGGGTCAAAGAGAG
OtsA	TGACACCTTGCTTGAACAGC	GAGAAAACGCTCTGGCAAAC
Restriction site primers		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Bos	TATACTCGAGGGATCCAATGAGCCTGACC	TATATGATCAGCGGCCGCTCAGAT
ABI1-1	TATACTCGAGGAAGCAATTGTTGCATTAGCC	TATATGATCAGTATGTAAAGTTTTGAAAGTAATTGCA
KIN10	TATACTCGAGATGTTCAAACGAGTAGATGAGTTTAAT	TATATGATCATCAGAGGACTCGGAGCTG
tHMGR	TATACTCGAGACCGAATCGCTTCCTGAG	TATATGATCATGTTGTTGTTGTTGTCGTTGTC
HMGR	TATACTCGAGATGGACGTTCGCCGGA	TATATGATCATTAGGAGGATGCCTTTGTGAC
RNAi KIN10_6	TATACTCGAGGTTGCAACAAATTGATGAGCAATGC	TATATGATCAGTTGCAACAAATTGATGAGCAATTA
RNAi KIN10_8	TATACTCGAGGTTGCAACAAATTGATGAGCAATGC	TATATGATCAGTTGCAACAAATTGATGAGCAATTA
OtsA	TATACTCGAGATGAGTCGTTTAGTCGTAGTATCTAACC	TATATGATCACTACGCAAGCTTTGGAAAGG

SD2: Table 2. Buffers

Supplemental Data Table 2. Buffers used in this study.

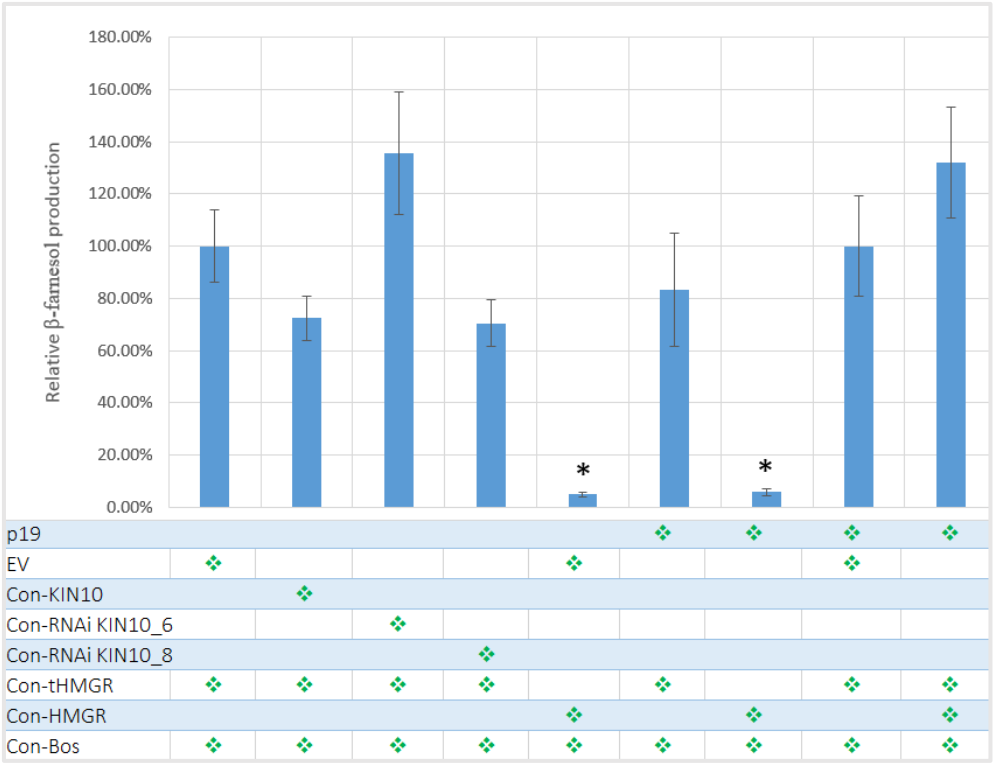
Agroinfiltration buffer	5 mM $MgCL_2$, 5 mM MES, 0.1 mM acetosyringone.
Homogenization buffer	40 mM Hepes-KOH, pH 7.2, 50 mM KCl, 100 mM sucrose, 16 mM EDTA, 0.2 % (v/v) Triton X-100, 10 mM DTT, 15 µg/mL aprotinin, 20 µg/mL leupeptin, 4.2 µg/mL E-64, 1.8 µg/mL pepstatin A, and 100 µg/mL PMSF.
1X Assay Buffer	5X Assay Buffer (Sigma-Aldrich A5981) 5 times diluted in ultrapure water.

SD3: Emission of endogenous sesquiterpenes

In this section the emission of β-farnesol is shown, to give insight in the effect of changing the MVA pathway on an endogenous sesquiterpene of *N. benthamiana*. Measurements were done on the same treatments as shown in Figure 2, section 2.1.1. β-farnesol emission is comparable to α-bisabolol emission, for the treatments that overexpressed KIN10, silenced KIN10 by silencing construct RNAi KIN10_6 and overexpression of HMGR by full length endogenous HMGR. The treatments that showed different results are silencing KIN10 with RNAi KIN10_8 and the overexpression of endogenous HMGR by full length HMGR and tHMGR together.

Overexpression of KIN10 resulted in a 28% lower emission of β-farnesol compared to the control (Supplemental Data Fig. 1). Expression of the silencing construct RNAi KIN10_6 resulted in an increase of 35% β-farnesol emission ($P>0.05$, $N=3$). However, expression of the silencing construct RNAi KIN10_8 resulted in a 30% lower β-farnesol emission ($P>0.05$, $N=3$). It was concluded that the changes in KIN10 had a similar effect on β-farnesol emission compared to α-bisabolol emission, except when RNAi KIN10_8 was expressed. This confirms the hypothesis that silencing construct RNAi KIN10_8 is less effective in silencing KIN10 than construct RNAi KIN10_6.

The difference between overexpression of HMGR by the endogenous HMGR and the truncated Arabidopsis HMGR was tested. As shown in Supplemental Data Fig. 1, overexpression of HMGR by HMGR resulted in a significantly lower emission of α-bisabolol, 95% when co-expressed with an EV, 94% when co-expressed with p19 ($p<0.05$, $N=3$). In contrast, when tHMGR and HMGR are co-expressed together, there is an enhancement in β-farnesol emission of 32% ($P>0.05$, $N=3$). This shows that overexpression by full length endogenous HMGR leads to similar effect on the β-farnesol emission compared to the α-bisabolol emission, however this effect was not observed when tHMGR and full length HMGR were expressed simultaneously. This indicates that tHMGR can reverse the effect of full length HMGR, at least partly.



Supplemental Data Figure 1. Relative β-farnesol emission in *N. benthamiana* leaves upon constitutive expression of tHMGR, silencing construct KIN10_6 and KIN10_8, KIN10 and HMGR. All treatments co-expresses Bos. Leaves were harvested three days post inoculation. Thereafter, the emitted volatiles were collected for one hour on Tenax-filled steel cartridges and analysed by thermal desorption GC-MS. p19, RNA silencing suppressor p19; EV, empty pBIN vector; KIN10, *A. thaliana* KIN10; RNAi KIN10_6 and KIN10_8, RNAi hairpin silencing constructs *N. benthamiana* KIN10; tHMGR, truncated Arabidopsis HMGR; HMGR, *N. benthamiana* HMGR. Bos, *A. annua* α-bisabolol synthase. The mean ± SE of 3-4 independent measurements. The asterisks denote statistical difference between the EV+tHMGR infiltration and other treatments, analysed by a two-tailed Student's t-test: *p<0.05.

SD4: Sequence genes

In this section the assemblages of the sequences obtained by sequencing with Macrogen Europe (The Netherlands) are shown.

> ABI1-1 (2146 bp)

ACTACTTCTTTTACAACTTTTTTAGGTGTATATAGATGAATCTAGAAATAGTTTTATAGTTGGAAATTGATTGAAGAG
AGAGAGATATTACTACACCAATCTTTTCAAGAGGTCCTAACGAATTACCCACAATCCAGGAAACCCTTATTGAAATT
CAATTCATTTCTTTCTTTCTGTGTTTGTGATTTTCCCGGGAAATATTTTTGGGTATATGTCTCTCTGTTTTTGCTTTCCT
TTTTCATAGGAGTCATGTGTTTCTTCTTGTCTTCCTAGCTTCTTCTAATAAAGTCCTTCTCTTGTGAAAATCTCTCGAA
TTTTCATTTTTGTTCCATTGGAGCTATCTTATAGATCACAACCAGAGAAAAAGATCAAATCTTTACCGTTAATGGAG
GAAGTATCTCCGGCGATCGCAGGTCCTTTCAGGCCATTCTCCGAAACCCAGATGGATTTCACCGGGATCAGATTGG
GTAAAGGTTACTGCAATAACCAATACTCAAATCAAGATTCCGAGAACGGAGATCTAATGGTTTCGTTACCGGAGAC
TTCATCATGCTCTGTTTCTGGGTCACATGGTTCTGAATCTAGGAAAGTTTTGATTTCTCGGATCAATTCTCCTAATTTA
AACATGAAGGAATCAGCAGCTGCTGATATAGTCGTCGTTGATATCTCCGCCGGAGATGAGATCAACGGCTCAGAT
GTTACTAGCGAGAAGAAGATGATCAGCAGAACAGAGAGTAGGAGTTTGTTTGAATTCAAGAGTGTGCCTTTGTAT
GGTTTCACTTCGATTTGTGGAAGAAGACCAGAGATGGAAGATGCTGTTTCGACTATACCAAGATTCCTTCAATCTTC

TTCTGGTTCCATGTTAGATGGTCGGTTTGATCCTCAATCCGCCGCTCATTTCTTCGGTGTTTACGACGGCCATGACG
GTTCTCAGGTAAAAGATTGGATCTTTTGATTAGGGTTGTTTACAGTTTGCAGAATCTGATTTGGTTGTTGTTGTGTA
GGTAGCGAACTATTGTAGAGAGAGGATGCATTTGGCTTTGGCGGAGGAGATAGCTAAGGAGAAACCGATGCTCT
GCGATGGTGATACGTGGCTGGAGAAGTGGAAGAAAGCTCTTTTCAACTCGTTCCTGAGAGTTGACTCGGAGATTG
AGTCAGTTGCGCCGGAGACGGTTGGGTCAACGTCGGTGGTTGCCGTTGTTTTCCCGTCTCACATCTTCGTCGCTAAC
TGCGGTGACTCTAGAGCCGTTCTTTGCCGCGGCAAAACTGCACTTCCATTATCCGTTGACCATAAAGTAAGCATATA
TAGACTCAAGATCTATAGTTGGATTGGTTGTGAACTTGTGATAGGTTTTTAGTTTTCAAGATGTTGAGTTAGAGAGG
TTTATGAAACCATTTCTGATGTGTGCTTGTGTTATGTGTGTAGCCGGATAGAGAAGATGAAGCTGCGAGGATTGAA
GCCGCAGGAGGGAAAGTGATTCAGTGGAATGGAGCTCGTGTTTTCGGTGTTCTCGCCATGTCGAGATCCATTGGT
AAGCTTTCATTATATTTCTCCATAAAGCTTCTCAATGGTTCTTGAAATAGAAGAAACCTTTCTTCTTACAAACATTTAT
CATTGTGACAGGCGATAGATACTTGAAACCATCCATCATTCCTGATCCGGAAGTGACGGCTGTGAAGAGAGTAAAA
GAAGATGATTGTCTGATTTTGGCGAGTGACGGGGTTTGGGATGTAATGACGGATGAAGAAGCGTGTGAGATGGC
AAGGAAGCGGATTCTCTTGTGGCACAAGAAAAACGCGGTGGCTGGGGATGCATCGTTGCTCGCGGATGAGCGGA
GAAAGGAAGGGAAAGATCCTGCGGCGATGTCCGCGGCTGAGTATTTGTCAAAGCTGGCGATACAGAGAGGAAGC
AAAGACAACATAAGTGTGGTGGTGGTTGATTTGAAGCCTCGGAGGAAACTCAAGAGCAAACCCTTGAACTGAGGC
AGAGAGGGTCCTTTTTTTTAATTTTTAAAATGAATATGGGTCTCTCCAAGAAAAAGTATTTACTATTATTAATTTGTG
CTTATTTTTTTAACTAACAAGTTATAACCATATGGAGATAATGAAGCTTAATGTGTTAAGCTCTTTTGTCTTGACTAC
CTTC

> AtHMGR (1135 bp)

GGCGAAAAAATGGTGGATCGGTTATCGACGGAGTTATTCCATCGTACTCGCTTGAATCTCGTCTCGGTGATTGCAA
AAGAGCGGCGTCGATTCGTCGTGAGGCGTTGCAGAGAGTCACCGGGAGATCGATTGAAGGGTTACCGTTGGATG
GATTTGATTATGAATCGATTTTGGGGCAATGCTGTGAGATGCCTGTTGGATACATTCAGATTCCTGTTGGGATTGCT
GGTCCATTGTTGCTTGATGGTTATGAGTACTCTGTTCCTATGGCTACAACCGAAGGTTGTTTGGTTGCTAGCACTAA
CAGAGGCTGCAAGGCTATGTTTATCTCTGGTGGCGCCACCAGTACCGTTCTTAAGGACGGTATGACCCGAGCACCT
GTTGTTCGGTTCGCTTCGGCGAGACGAGCTTCGGAGCTTAAGTTTTTCTTGGAGAATCCAGAGAACTTTGATACTTT
GGCAGTAGTCTTCAACAGGTCGAGTAGATTTGCAAGACTGCAAAGTGTTAAATGCACAATCGCGGGGAAGAATGC
TTATGTAAGGTTCTGTTGTAGTACTGGTGATGCTATGGGGATGAATATGGTTTCTAAAGGTGTGCAGAATGTTCTT
GAGTATCTTACCGATGATTTCCCTGACATGGATGTGATTGGAATCTCTGGTAACTTCTGTTCGGACAAGAAACCTGC
TGCTGTGAACTGGATTGAGGGACGTGGTAAATCAGTTGTTTGCGAGGCTGTAATCAGAGGAGAGATCGTGAACAA
GGTCTTGAAAACGAGCGTGGCTGCTTTAGTCGAGCTCAACATGCTCAAGAACCTAGCTGGCTCTGCTGTTGCAGGC
TCTCTAGGTGGATTCAACGCTCATGCCAGTAACATAGTGTCTGCTGTATTCATAGCTACTGGCCAAGATCCAGCTCA
AAACGTGGAGAGTTCTCAATGCATCACCATGATGGAAGCTATTAATGACGGCAAAGATATCCATATCTCAGTCACT
ATGCCATCTATCGAGGTGGGGACAGTGGGAGGAGGAACACAGCTTGCATCTCAATCAGCGTGTTTAAACCTGCTC
GGAGTTAAAGGAGCAAGCACAGAGTCCCCGGGAATGAACGCAAGGAGGCTAGCGACGATCGTAACCCGGAA

> KIN10 (2627 bp)

CACGGATCGATCACAGAATCTTCAAAAGTGTAAAAATTACTTGCTTTTTGTTCCTATTACTCTTTTGCAGTTTGTTTTG
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AGTGGGGTAGAATCGATTCTACCAAATTACAAGCTTGGGAGAACTCTTGGTATTGGTTCCTTTGGTAGGGTGAAGA
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TC

> HMGR (2184 bp)

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TGGTCAGGACCCAGCTCAGAACATAGAGAGCTCTCATTGTATCACTATGATGGAGGCTGTAAATGATGGCAAGGA
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> RNAi KIN10_6 (251 bp)

GACTGATGGAATATCAAGGAGTTGGCTTGAGATCTCAATACCCTGTTGAGAGAAAATGGGCTCTTGGACTTCAGGT
TTTGATATTTTCTGTATTTATGGTATAAAGACTTCAGCTTTTGATATATATGTGATTAATATTGTAACAATTTGATCGC
ACAGTCTCGGGCTCATCCCCGTGAAATAATGACGGAAGTCCTGAAAGCCCTGCAAGATTTGAATGTATGTTGGAAG
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> RNAi KIN10_8 (134 bp)

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