CONTROL OF ISOPRENE EMISSION FROM PLANTS AND BACTERIA

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ABSTRACT

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Isoprene is a volatile compound produced in large amounts by some, but not all, plants by the enzyme isoprene synthase. Plants emit vast quantities of isoprene, with a net global output of 600 Tg per year, and typical emission rates from individual plants are around 2% of net carbon assimilation in the light. Isoprene is also an important economic compound, as it is a precursor to synthetic fragrances, pharmaceuticals, and rubber. Despite being studied since the discovery of isoprene emission from plants in 1954, the reason why plants make so much isoprene is unknown. My graduate work presented in this thesis focuses on the joint control of isoprene emission by the methylerythritol 4-phosphate (MEP) pathway, which produces the precursor dimethylallyl diphosphate (DMADP), and by isoprene synthase. I found that the changes in isoprene emission from plants due to changes in CO_2 and temperature are not linked to electron transport and can occur independently of changes in carbon assimilation. This disproves several modern models of isoprene emission and my data predict that contrary to current models, isoprene emission will increase in future climate conditions. While changes in electron transport should lead to changes in the MEP pathway, there are changes in DMADP concentration that are not linked to electron transport. I propose that calcium signaling caused by wounding or high CO_2 leading to post-translational modification of isoprene synthase may explain some of the changes in isoprene emission. I report changes in isoprene synthase itself under increased temperature that are necessary to understand how isoprene emission will change under future atmospheric conditions. In collaboration with the Kerfeld lab, we encapsulated isoprene synthase

in bacterial microcompartments, which may improve synthetic isoprene production by channeling DMADP directly into isoprene synthase. We also produced a modified isoprene synthase and methylbutenol synthase that has improved activity. I profile MEP pathway metabolites and isoprene synthase under changing environmental conditions and in transgenic emitting and non-emitting species to further our understanding of the joint control of isoprene emission by the MEP pathway and isoprene synthase.

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KEY TO ABBREVIATIONS

Enzymes:

PEPC = phosphoenolpyruvate carboxylase

DXS = 1-deoxy-D-xylulose-5-phosphate synthase

DXR = 1-deoxy-D-xylulose-5-phosphate reductoisomerase

CMS/MCT = 4-diphosphocytidyl-2-C-methylerythritol synthase/2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase

CMK = 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase

MCS = 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase

HDS = 4-hydroxy 3-methylbut-2-enyl-diphosphate synthase

HDR = 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase

IDI = isopentenyl diphosphate isomerase.

ISPS = isoprene synthase

MBOS = methylbutenol synthase

LytB = 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase

Metabolites:

PEP = phosphoenolpyruvate

OAA = oxaloacetic acid

GAP = glyceraldehyde 3-phosphate

DXP = 1-deoxy-D-xylulose-5-phosphate MEP = methylerythritol 4-phosphate

MEP = methylerythritol 4-phosphate

CDP-ME = 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol

CDP-MEP = 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol phosphate

MEcDP = 2-C-methyl-D-erythritol-2,4-cyclodiphosphate

HMBDP = 4-hydroxy-3-methylbut-2-enyl-diphosphate

IDP = isopentenyl diphosphate

DMADP = dimethylallyl diphosphate.

Chapter 1 Literature review

Introduction

Isoprene (2-methyl-1,3-butadiene) is produced in large amounts by some, but not all, plants by the enzyme isoprene synthase (Sharkey et al., 2008). Isoprene is highly volatile and only tiny amounts exist in chloroplastic membranes (Harvey et al., 2015), and almost none in solution. Despite its small concentration, it improves plant resilience to thermal, oxidative, and biotic stresses. Plant-derived isoprene enters the atmosphere in vast quantities (600 Tg per year) (Guenther et al., 2006), which makes it an important player in atmospheric chemistry, contributing to ozone and aerosol production in the troposphere and increasing the lifetime of methane by reducing the amount of oxidative species that can react with it (Pike and Young, 2009; Young et al., 2009; Zhang et al., 2007). Isoprene synthase is also biochemically similar to other terpene synthases, which makes it an attractive candidate for testing novel synthetic biology techniques. While the control of isoprene emission under changing environmental conditions has been well-studied, the mechanisms behind the physiological responses have not been proven.

Isoprene emission is a substantial carbon and energy sink for emitting plants (typically 2% of photosynthesis). Isoprene affects gene expression (Harvey and Sharkey, 2016; Zuo et al., 2019), the proteome (Vanzo et al., 2016; Velikova et al., 2014), the metabolome and metabolic fluxes (Behnke et al., 2010a; Ghirardo et al., 2014; Way et al., 2013), and improve plant resilience to thermal, oxidative, and biotic stresses (Behnke et al., 2007; Laothawornkitkul et al., 2008; Sharkey et al., 2001; Singsaas and Sharkey, 1997; Velikova et al., 2011; Vickers et al., 2009). However the mechanism of this protection is not known (Harvey et al., 2015). It had been hypothesized that isoprene could intercalate into membranes and improve membrane stability, which could explain the thermal effects (Sasaki et al., 2007; Siwko et al., 2007; Velikova et al., 2011). The high concentration of isoprene necessary to produce this effect would allow it to react

with, and reduce, reactive oxygen species in the membrane, protecting chloroplasts from oxidative stress (Jardine et al., 2012; Sharkey, 2005; Velikova et al., 2012; Vickers et al., 2009). However, this does not explain the resistance to biotic stress seen by Laothawornkitkul et al. (2008). Furthermore, other studies have shown that isoprene typically exists at very low concentration in chloroplastic membranes (0.0044 mol% isoprene) (Harvey et al., 2015), and that leaf discs fumigated with isoprene do not exhibit thermal protection (Logan et al., 1999; Logan and Monson, 1999). Therefore, it is necessary to formulate new hypotheses that can adequately explain how isoprene protects plants from biotic and abiotic stresses.

While isoprene enters the atmosphere in vast quantities (600 Tg per year) (Guenther et al., 2006), there is no complete mechanistic model of isoprene emission (Arneth et al., 2008; Monson et al., 2012; Monson et al., 2007a). Three major factors will affect isoprene emission from plants in the future: leaf temperature, atmospheric CO_2 concentration, and changing land use. The effects of temperature and CO_2 are well understood, but the mechanistic models are based on assumptions that have not been proven, which leads to a gap between models of isoprene emission and reality (Morfopoulos et al., 2014; Rasulov et al., 2010; Rasulov et al., 2009b; Rasulov et al., 2016). While it is well-established that crop plants generally do not emit isoprene and forest plants often do, changes in environment may lead to the more resilient isoprene-emitting plants becoming dominant and may even stimulate plants currently classed as non-emitting to start to emit as isoprene synthase is induced by higher temperatures (Monson et al., 1994; Taylor et al., 2018; Wiberley et al., 2005). In this work, I approach the gap between models and reality in multiple ways, formulating new mechanistic hypotheses for the CO_2 effect on isoprene emission as well as modeling the effect of temperature on isoprene synthase activity.

Isoprene emission and control of its precursors is not only interesting for understanding plant responses to their environment, but also for developing this pathway for synthetic biology. Isoprene is itself a valuable economic compound that is currently obtained as a byproduct from petroleum refining (Zurbriggen et al., 2012). Because of isoprene synthase's similarities to other terpene synthases, and the ease of measuring isoprene production using the Fast Isoprene Sensor, it is also ideal as a model for improvements to enzyme activity that may be applicable to a wide variety of economically valuable enzymes (Chen et al., 2011; Degenhardt et al., 2009; Gao et al., 2012; Greenhagen et al., 2006). Research into biosynthetic isoprene is ongoing (Bentley et al., 2014; Chaves et al., 2016; Chaves et al., 2017; Cheng et al., 2017; Georgianna and Mayfield, 2012; Kim et al., 2016; Korman et al., 2014; Lindberg et al., 2010; Yang et al., 2016; Ye et al., 2016), but biosynthetic isoprene has not been commercialized, indicating that further optimization is necessary to make it economically viable. This work seeks to improve the understanding of the control of isoprene emission by plants, and to use that information to engineer better isoprene synthases for industrial production of isoprene in bacteria.

Biochemical control of isoprene emission

Isoprene synthase is a terpene synthase (TPS-b) with chloroplastic targeting that converts dimethylallyl diphosphate (DMADP) to isoprene (Sharkey et al., 2013; Silver and Fall, 1995). DMADP is produced in the chloroplast by the prokaryotic methylerythritol 4-phosphate (MEP) pathway, also called the non-mevalonate pathway (Figure 1.1). Carbon for the MEP pathway comes directly from photosynthesis. Some studies show that small amounts of carbon for isoprene emission can come from extrachloroplastic sources, and this amount increases under stress (Affek and Yakir, 2003; de Souza et al., 2018; Ferrieri et al., 2005; Kreuzwieser et al., 2002; Schnitzler et al., 2004). Delwiche and Sharkey (1993) found that when plants are fed

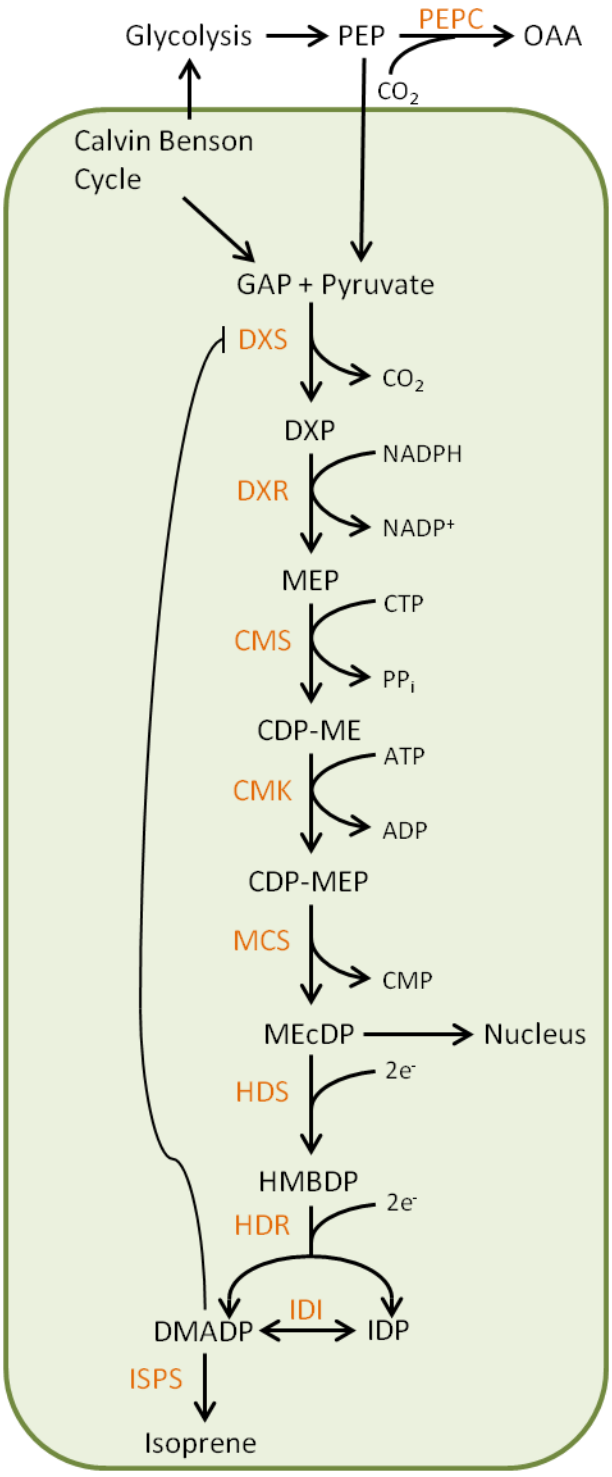

Figure 1.1: The MEP pathway in plants. Isoprenoids are produced by the MEP pathway under light-dependent reactions in the chloroplast. Abbreviations: Enzymes: PEPC = phosphoenolpyruvate carboxylase, DXS = 1-deoxy-d-xylulose-5-phosphate synthase, DXR = 1-deoxy-d-xylulose-5-phosphate reductoisomerase, CMS/MCT = 4-diphosphocytidyl-2-C-methylerythritol synthase/2-C-methyl-d-erythritol-4-phosphate

cytidylyltransferase, CMK = 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol kinase, MCS = 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase, HDS = 4-hydroxy 3-methylbut-2-enyl-diphosphate synthase, HDR = 4-hydroxy-3-methylbut-2-enyl-diphosphate reductase, IDI = isopentenyl diphosphate isomerase. In isoprene emitting plants the conversion of DMADP to isoprene is catalyzed by isoprene synthase (ISPS).
Metabolites: PEP = phospho*enol*pyruvate, OAA = oxaloacetic acid, GAP = glyceraldehyde 3-phosphate, DXP = 1-deoxy-d-xylulose-5-phosphate, MEP = methylerythritol 4-phosphate, CDP-ME = 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol, CDP-MEP = 4-(cytidine-5'-diphospho)-2-C-methyl-d-erythritol phosphate, MEcDP = 2-C-methyl-d-erythritol-2,4-cyclodiphosphate, HMBDP = 4-hydroxy-3-methylbut-2-enyl-diphosphate, IDP = isopentenyl diphosphate, DMADP = dimethylallyl diphosphate.
Two reducing equivalents are required to convert MEcDP into HMBDP and HMBDP into DMADP; these can be provided by NADPH or by two ferredoxin molecules.

$^{13}CO_2$, approximately 20% of isoprene remains unlabeled, however, 20% of photosynthetic intermediates are unlabeled, indicating that the carbon in isoprene is from photosynthesis. When plants are fed uniformly labeled ^{13}C glucose, isoprene will be labeled (Kreuzwieser et al., 2002). Bick and Lange (2003) proposed crosstalk between the MEP and mevalonic acid pathways, but these data could not be reproduced. Knockouts of DXS and DXR are lethal, which means that the crosstalk cannot be at such high levels as to rescue the pathway (Banerjee and Sharkey, 2014; Estévez et al., 2000; Mandel et al., 1996; Phillips et al., 2008). The MEP pathway requires one CTP (releasing CMP), one ATP, one NADPH and four ferredoxin molecules, which links it closely to electron transport (Niinemets et al., 1999; Rasulov et al., 2009b). Isoprene emission is therefore light-dependent (Behnke et al., 2013; Laffineur et al., 2013; Pallozzi et al., 2013; Sharkey et al., 1996; Singsaas et al., 1993).

Isoprene synthase is a protein unique to plants. It has evolved multiple times, most likely from closely related terpene synthases (Dani et al., 2014; Hanson, 1999; Harley et al., 1999; Li et al., 2017; Loreto and Fineschi, 2015; Monson et al., 2013; Sharkey et al., 2013; Sharkey et al., 2005). It has been hypothesized that this is because of the relative ease of converting terpene synthases to isoprene synthase via only a few amino acid mutations (Gao et al., 2012; Gray et al.,

2011; Kampranis et al., 2007; Koksal et al., 2011; Li et al., 2017; Sharkey et al., 2013), allowing plants to maintain a balance between the energetic demands of isoprene emission and the physiological benefits under different conditions (Monson et al., 2013). Isoprene emission has also been lost in several species; for example *Glycine max* (soybean) has two isoprene synthase pseudogenes while its close wild relative *Glycine soja* has an active isoprene synthase with a very high similarity to the coding parts of the soybean pseudogene (Sharkey et al., 2013). Isoprene synthesis is found in all divisions of Plantae and all climates; it is common in fast-growing plants, particularly fast-growing hardwood trees such as *Populus* spp, *Eucalyptus* spp; in mosses such as *Campylopus introflexus* and *Sphagnum* spp; as well as a wide variety of legumes such as *Peuraria montana* and *Mucunia pruriens*. While isoprene emission is very high in some monocot species, for example *Arundo donax* and *Phragmites australis*, only recently was a monocot isoprene synthase cloned (Li et al., 2017).

Ecosystem-level isoprene emission is highest in hot climates with short dry seasons, and these conditions may select for a higher percentage of isoprene emitting species (Taylor et al., 2018). Long dry seasons may reduce the number of isoprene emitting plants because isoprene synthesis is a larger burden under drought stress because drought does not decrease isoprene emission, while it does greatly decrease CO_2 assimilation (Fang et al., 1996; Fortunati et al., 2008; Guidolotti et al., 2011; Potosnak et al., 2014b; Ryan et al., 2014; Tani et al., 2011). Isoprene emission is significant even in cold climates, and these regions will become increasingly important to estimates of global isoprene production as global temperatures increase (Ekberg et al., 2011; Lindwall et al., 2016; Svendsen et al., 2016; Tiiva et al., 2007). Far northern and far southern climates are expected to change more drastically due to climate change, leading to a disproportionate response to global change (Pachauri et al., 2014; Peñuelas and Staudt,

2010). Furthermore, chilling disables isoprene emission in some species (Mutanda et al., 2016a; Mutanda et al., 2016b), and an increase in temperature may cause previously non-emitting plants to emit (Monson et al., 1994; Oku et al., 2014; Schnitzler et al., 1997; Sharkey et al., 1999).

Control of isoprene emission

The rate of isoprene emission is affected by environmental factors such as light, temperature, CO_2 concentration, and O_2 concentration. Isoprene emission is light-dependent and this light dependence is most likely due to the activity of photosystem II (Laffineur et al., 2013; Rasulov et al., 2016; Sharkey et al., 1996). The availability of DMADP depends on the availability of ferredoxin, NADPH, and ATP supplied by photosynthetic electron transport in the light (Mongelard et al., 2011; Rasulov et al., 2009a; Seemann et al., 2006). When plants are subjected to darkness, ATP, ferredoxin, and NADPH supply by photosynthetic electron transport ceases, stopping DMADP biosynthesis within seconds; all remaining DMADP is converted to isoprene within 4-5 minutes (Li et al., 2011; Monson and Fall, 1989; Rasulov et al., 2009a; Rasulov et al., 2011). After this, a second burst is seen that is correlated with the amount of MEcDP (Dani et al., 2017; Jud et al., 2016; Li and Sharkey, 2013). This suggests that HDS and HDR can change in activity some minutes after the imposition of darkness. These proteins usually use ferredoxin for reducing power but can also use NADPH in some cases (Seemann et al., 2006; Wolff et al., 2003; Xiao et al., 2009). After 10-30 minutes of darkness, the remaining MEcDP can be converted to HMBDP and then to DMADP/IDP, perhaps because HDR and HDS are using reducing power from alternative sources such as NADPH from catabolism (Li and Sharkey, 2013; Seemann et al., 2006; Wolff et al., 2003; Xiao et al., 2009).

Isoprene emission increases with temperature and decreases with increasing CO_2 (Monson et al., 2016; Possell and Hewitt, 2011; Rosenstiel et al., 2003; Scholefield et al., 2004; Sharkey et

al., 1991b). Increasing temperature has been shown to mitigate, and sometimes entirely abolish, the CO_2 inhibition of isoprene emission (Potosnak et al., 2014a; Rasulov et al., 2010; Sun et al., 2013). Isoprene emission is more sensitive to temperature than photosynthesis is based on the Q_{10} (change in rate as a consequence of a 10°C increase in temperature) between 25 and 35°C (Table 1.1) and has a higher optimum - above 45°C. The effect of temperature is caused by the combined effect of DMADP availability and isoprene synthase activity. The V_{max} of isoprene synthase has a temperature optimum of 42-45°C, while DMADP concentration peaks at 30-35°C (Lehning et al., 1999; Monson et al., 1992; Morfopoulos et al., 2013; Rasulov et al., 2010). The mechanism behind the effect of CO_2 on isoprene emission rate has not been proven, and will be covered in Chapter II.

Isoprene emission does not change when the stomata close (Fall and Monson, 1992; Sharkey et al., 1991a). Stomatal closure greatly increases the resistance to diffusion of isoprene from the leaf, which increases internal isoprene concentration (Fall and Monson, 1992). The increased concentration is proportional to the resistance such that emission rate remains constant when stomata close. Pallozzi et al. (2013) found that pure blue light (leading to stomatal closure) decreased isoprene emission compared to white light; however, the decrease was not as large as the decrease in photosynthesis in response to blue light, and the decline in isoprene emission rate may have been related to decreased photosynthesis. Isoprene emission remains high even when plants are severely drought stressed (Brilli et al., 2013; Fang et al., 1996; Fortunati et al., 2008). The reduced sensitivity to drought enhances the role isoprene could play in resilience to abiotic stress (Arab et al., 2016; Marino et al., 2017; Ryan et al., 2014; Tattini et al., 2014).

Table 1.1: Q_{10} values for isoprene emission and assimilation. Values were calculated at 400 ppm CO_2 for a temperature increase from 25 to 35°C. Data from Chapter 2.

Species	Isoprene	Assimilation
Tobacco	3.0	1.0
Phragmites	2.9	0.6
Sycamore	3.9	1.0
Poplar	5.7	0.7

Modeling isoprene emission rates

While it is important to understand the regulation of isoprene emission rate on a leaf scale for understanding plant resilience, it also enables accurate predictions of isoprene emission on a canopy, landscape, and global scale, which is crucial for accurate atmospheric models for human health. Isoprene emission can have negative impacts on human health in urban areas because it can react with nitrogen oxides in the presence of sunlight to produce tropospheric ozone (Cheung et al., 2014; Fiore et al., 2011; Han et al., 2013; Hellen et al., 2012; Sato et al., 2013; Wang et al., 2013; Young et al., 2009). Secondary reaction products, two diastereoisomeric 2-methyltetrols, can form organic aerosols, which produce a cooling effect by increasing the albedo and may increase rainfall (Claeys et al., 2004; Day and Pandis, 2011; Fuzzi et al., 2006; Hsieh et al., 2017; Kleindienst et al., 2007; Sato et al., 2013; Zhang et al., 2007; Zhu et al., 2017). Increased drought stress, CO_2, and temperature are all expected to have effects on isoprene emission (Hantson et al., 2017; Taylor et al., 2018; Vanzo et al., 2015), as will changing land use (Hantson et al., 2017). Most studies are based on the prediction that atmospheric CO_2 concentrations will increase to 800-1000 ppm by the year 2100 and temperatures will increase by 3°C globally, although larger temperature increases are expected for northern climates (Pachauri et al., 2014). Different studies have come to different conclusions, not only because of fundamental differences in their models, but also because of which primary research the models use as inputs. Arneth et al. (2007) modeled the effect of CO_2 on isoprene and showed that it would suppress the

increase otherwise expected from the temperature response of isoprene emission (Wilkinson et al., 2009; Young et al., 2009). Hantson et al. (2017) proposed a similar effect but also incorporated changing land use, which led to the conclusion that global isoprene emissions will greatly decrease in the next 100 years. However, other groups have concluded that isoprene emission will increase under future climates (Guenther et al., 2006; Han et al., 2013; Heald et al., 2009; Hsieh et al., 2017; Keenan et al., 2011; Keenan and Niinemets, 2012; Monson et al., 2012; Monson et al., 2007b; Pugh et al., 2013). This is because some data show that high temperature abolishes or reduces the CO_2 effect (Monson et al., 2016; Potosnak et al., 2014a; Rasulov et al., 2010; Rasulov et al., 2009b).

Isoprene synthase levels and activity may change at high CO_2; a few studies looked at this effect but did not find statistically significant results. Scholefield et al. (2004) found that isoprene synthase activity decreased based on proximity to a natural CO_2 spring. Calfapietra et al. (2007) showed that expression of MEP pathway genes and isoprene synthase protein levels did not significantly decrease in plants grown under high CO_2, although there was a slight downward trend. Isoprene synthase activity and levels are primarily responsible for the temperature response, and most models take into account V_{max} of isoprene synthase, but do not modify equations based on the binding coefficient for DMADP, or substrate inhibition, even though DMADP levels can be sufficiently high to cause substrate inhibition in some isoprene synthases. Furthermore, while this model works well on a plant-level scale, enzyme activities can vary greatly between species and even between individual plants based on acclimation, which is difficult to model on a global scale.

Physiological role of isoprene

Isoprene has a number of physiological effects, which have been probed with a variety of techniques, leading to widely varying hypotheses for the mechanism of these effects. The effect

of isoprene on treated and untreated plants has been tested in three ways: **(1)** by removing isoprene emission by knocking down isoprene synthase or with treatment with fosmidomycin (which inhibits DXS, the enzyme that catalyzes the first committed step in isoprene emission, Figure 1.1); **(2)** by transgenically modifying non-emitting plants to emit isoprene constitutively; and **(3)** by fumigating non-emitting plants with physiologically relevant levels of isoprene. Sharkey et al. (2001) and Velikova and Loreto (2005) observed that in plants treated with fosmidomycin, thermotolerance was reduced and the effect was rescued by exogenous isoprene. In a later experiment, Velikova et al. (2011) used both transgenic, isoprene-emitting *Arabidopsis thaliana* as well as fosmidomycin-treated *Platanus orientalis* leaves to confirm that thermotolerance as measured by thylakoid membrane stability was improved by the presence of isoprene. Vickers et al. (2009) used transgenic, emitting *Nicotiana tabacum* plants to show that isoprene also improves plant responses to oxidative stress. Methylvinyl ketone found in plant cells under high ozone is produced by breakdown of linolenic acid, and not from isoprene; isoprene does not react with reactive oxygen species (Cappellin et al., 2019; Kai et al., 2012). Knocking down isoprene emission using RNAi in *Populus* × *canescens* also demonstrated the effect of isoprene on thermotolerance, but did not show any effect on herbivore behavior (Behnke et al., 2007; Muller et al., 2015; Velikova et al., 2015).

Isoprene emission improves tolerance of leaves exposed to rapidly changing temperatures, such as sunflecks or rapidly changing weather conditions (Behnke et al., 2013; Behnke et al., 2010b; Harley et al., 1999; Sharkey et al., 1991b; Singsaas et al., 1999; Singsaas and Sharkey, 1998; Vanzo et al., 2015). Harvey et al. (2015) showed that isoprene does not accumulate in thylakoid membranes at sufficiently high concentrations to produce the membrane stabilization effects shown by Siwko et al. (2007) and Velikova et al. (2012). An alternative hypothesis is that

isoprene acts as a heat dissipator - that is, emission acts as a form of evaporative cooling, reducing leaf temperature (Pollastri et al., 2014; Sanadze, 2017). However exogenous isoprene should not produce this effect, and the amount of isoprene released from the leaf relative to the amount of water transpired is so minute that it should not significantly affect cooling. At 30°C and 400 ppm CO_2 (basal conditions for isoprene emission), isoprene has a ΔH_{vap} of 26 kJ mol^{-1}, while water has a ΔH_{vap} of 43 kJ mol^{-1} (Reid and Zwolinksi, 1971). A high isoprene emission rate for isoprene under these conditions is 126 nmol m^{-2} s^{-1}; a typical conductance of water at 30°C is 21 mmol m^{-2} s^{-1} which is an emission of 9 mmol m^{-2} s^{-1} if the dew point is 25°C (Pollastri et al., 2014; Sharkey et al., 1996). This means that isoprene cools at a rate of 4.3×10^{-3} J m^{-2} s^{-1} compared to water's 3.7×10^{2} J m^{-2} s^{-1} or nearly 100,000 times less heat loss by isoprene evaporation than by water evaporation.

Changes in gene expression may be a better explanation for the mechanism of action of isoprene. The presence of isoprene was shown to increase expression of stress response genes, which may make the plants able to respond faster to stressful conditions and recover more quickly (Harvey and Sharkey, 2016; Mutanda et al., 2016b; Zuo et al., 2019). Xiao et al. (2012) showed that MEcDP can act as a retrograde signaling molecule, increasing salicylic acid concentration and expression of stress response genes. In non-emitting poplar, Ghirardo et al. (2014) measured the flux through the MEP pathway; their data suggest that increasing isoprene emission can lead to an overall increase in MEcDP. Mechanistically, this is because DMADP and IDP inhibit DXS, leading to decreased flux through the MEP pathway (Banerjee and Sharkey, 2014; Banerjee et al., 2013). Isoprene emission may decrease DMADP pools slightly and therefore increase pools of other MEP pathway metabolites. Rosenstiel et al. (2002) showed that native emitters of isoprene have much higher DMADP levels than species that do not emit

isoprene, which may be because isoprene emitters have adapted to the high flux through isoprene synthase. However, flux through the pahtway has not been measured. MEcDP levels need to be measured directly in native and transgenic emitters to determine if there is any change that could lead to differences in nuclear signaling. Harvey and Sharkey (2016) observed that exogenous isoprene alone can alter gene expression with similar patterns as constitutive expression of isoprene synthase. Fosmidomycin and fumigation experiments typically saw effects after the plants were exposed for an hour or more, which is sufficient time for changes in gene expression to take place. Other alkenes could produce a similar effect as fumigation with isoprene (Sharkey et al., 2001). These results suggest that there may be a receptor for isoprene; quite likely this receptor is not specific for isoprene but may bind many alkenes. This may be synergistic with the effect of increased MEcDP concentrations.

Engineering terpene synthases

Understanding the control of the MEP pathway and isoprene synthase is not only crucial to understanding plant resilience, but also to turning these pathways to commercial production of terpenoids. The MEP pathway produces precursors that are used for monoterpenes and diterpenes as well as isoprene; these can be precursors to important economic substances including medications. Isoprene itself is used as an industrial precursor and to make synthetic rubber, a fact which has led companies like Goodyear into investing heavily into isoprene synthase research and development. Furthermore, the MEP pathway may be used to produce any terpene compound including sesquiterpenes, which are suitable for use as diesel fuels. Production of valuable terpenoids can be done in plants as a valuable secondary product to pulp production or in fermentation in both photosynthetic and heterotrophic bacteria and yeast. The mechanism of isoprene synthase is similar to other Class I terpene synthases, but its product is easy to measure in real time, making it an ideal model for testing improvement of the MEP

pathway as well as the enzyme itself. It can also be used in bacteria or yeast to test output of the engineered mevalonic acid (MVA) pathway, which is commonly used to produce terpene precursors in fermentation.

Efforts in engineering terpene production in yeast, bacteria, or plant leaves (for example *Nicotiana benthamiana*) have focused on two major aspects. The first is developing our understanding of the variety of terpene synthases, and creating enzymes that can produce novel products or elucidating the pathways to new products. Terpene synthases are a family of closely related enzymes that produce a staggering array of secondary metabolites with related structures. Terpene synthases may accept a single substrate and produce a single product, or may be able to accept multiple substrates (for example both geranyl diphosphate [GPP] and farnesyl diphosphate [FPP]) and produce a product profile of over 50 compounds (Degenhardt et al., 2009; Koksal et al., 2011; Kumari et al., 2013; Vattekkatte et al., 2018; Vedula et al., 2005).

Class I terpene synthases, which are the majority of known plant proteins in this family, proceed via a carbocation intermediate, which can then be quenched by water or hydrogen abstraction to produce the terpene skeleton (Chen et al., 2011; Degenhardt et al., 2009). Because the carbocation can change structure prior to quenching, a vast number of products can result from a single substrate. Class II terpene synthases proceed through protonation-induced cyclization, which is generally more specific, but can also produce a variety of products. Specificity is defined by four to five amino acid differences between enzymes with different primary products, although product profiles can be greatly altered by changing just one amino acid (Cao et al., 2010; Greenhagen et al., 2006; Kampranis et al., 2007; Li et al., 2017; Sharkey et al., 2013; Xu et al., 2007). In addition, product profiles can be altered by environmental changes, including pH and particularly the presence of cations. Mn^{2+} and Mg^{2+} are required for

binding the substrate and many isoprene synthases also require K^+ for activity, however presence of the non-preferred cation can change product profile (Greenhagen et al., 2006; Richard et al., 2004; Tashiro et al., 2018; Vedula et al., 2005; Whittington et al., 2002). As new species that may express novel terpene synthases are sequenced, many economically useful terpene synthases, including novel isoprene synthases, have been discovered (Alquezar et al., 2017; Chen et al., 2011; Martin et al., 2010; Vezzaro et al., 2012). Synthetic biologists also create novel terpene synthases. Kampranis et al. (2007) converted a 1,8-cineole synthase from *Salvia fruticosa* to the more economically valuable enzyme sabinene synthase by exchanging a single amino acid using rational design. Yoshikuni et al. (2006) wer able to accomplish a similar task with a pair of sesquiterpene synthases. From these efforts there is a fairly good understanding of which residues and protein structures are critical for product and substrate specificity in terpene synthases, and engineering new product profiles or conversion of terpene synthases to produce other products is regularly successful (Greenhagen et al., 2006; Lopez-Gallego et al., 2010; Xu et al., 2007).

A second focus of engineering terpene production is increasing precursor supply. The key to high precursor supply seems to be careful tuning of gene expression in order to minimize toxic intermediates while maximizing yield of the final diphosphate (IDP, DMADP, GPP, or FPP) (Kang et al., 2017; Wang et al., 2017). Alper et al. (2005) used a promoter library to tune expression of critical MEP pathway enzymes in *E. coli*, producing a substantially higher yield of lycopene. Wang et al. (2017) used directed evolution to tune expression of the MVA pathway in *Saccharomyces cerevisiae* to produce a similarly high yield. Yields in *S. cerevisiae* using the MVA pathway are generally higher than in *E. coli*, so the majority of the research has focused on *S. cerevisiae* (Chaves et al., 2016; Fischer et al., 2011; Ignea et al., 2014; Wang et al., 2017;

Yang et al., 2012; Ye et al., 2016). Tuning the performance of the specific enzyme is the final step, and is where the least research has been done. Tashiro et al. (2017) used directed evolution to tune the expression of geraniol synthase to improve geraniol titers. Tashiro et al. (2016) were also able to evolve a pinene synthase to improve the activity of this enzyme directly, as well as modify its preferred cofactor for improved performance in *E. coli* (Tashiro et al., 2018). Pinene synthase is a terpene cyclase; I was unable to find if any researcher had improved the activity of a Class I terpene synthase using directed evolution or rational design. We describe the improvement of an isoprene synthase in Chapter IV.

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Chapter 2 Isoprene suppression by CO_2 is not due to triose phosphate utilization (TPU) limitation

The following published paper in its entirety is presented for Chapter 2:

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Isoprene suppression by CO_2 is not due to triose phosphate utilization (TPU) limitation

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Abstract

Isoprene is one of the most abundant volatile organic compounds produced by some, though not all, plant species. It confers stress tolerance in both emitting and non-emitting species and has large impacts on gene regulation as well as on atmospheric chemistry. Understanding the control of isoprene emission from plants is important to understanding plant responses to future atmospheric conditions. In this study we determined that suppression of isoprene emission from plants by high CO_2 concentrations is reduced but not eliminated by high temperature. We tested whether the CO_2 suppression is caused by the reduction in ATP or NADPH availability caused by triose phosphate utilization (TPU) limitation of photosynthesis at high CO_2. We measured CO_2 assimilation as well as several photosynthetic electron transport parameters under multiple atmospheric conditions in four plant species grown at ambient CO_2. While CO_2 sensitivity of isoprene emission was somewhat correlated with TPU in some species, in other species it was not. Poplar exhibited significant CO_2 suppression of isoprene emission but no evidence for TPU so we investigated further measuring the electrochromic shift that gives information on ATP synthesis and photosystem I oxidation state. In all cases photosynthetic parameters were

unchanged while isoprene emission dropped in response to increasing CO_2. Non-photorespiratory conditions (2% O_2) led to an increase in isoprene emission at low CO_2 but did not alleviate suppression by CO_2. In all measured species the combination of higher temperature along with higher CO_2 concentrations led to a net increase of isoprene emission in response to a moderate scenario for temperature and CO_2 concentration in 2100 in the upper Midwest.

Introduction

Isoprene is emitted in large amounts (typically 2% of photosynthesis) by some, but not all, plants (Sharkey et al., 2008). Exogenous isoprene changes gene expression and protects leaves from oxidative and thermal stress in non-emitting plants (Harvey and Sharkey, 2016; Velikova et al., 2014; Zuo et al., 2019), while knocking down emission in native emitters reduces thermal tolerance (Behnke et al., 2007; Sharkey et al., 2001). The large amount of isoprene emitted to the atmosphere (600 Tg/year) has significant atmospheric effects, contributing to ozone and aerosol production in the troposphere and increasing the lifetime of methane (Guenther et al., 2006; Pike and Young, 2009; Young et al., 2009; Zhang et al., 2007). For these reasons an accurate understanding of the physiological control of isoprene and its propensity for change as global temperatures and atmospheric CO_2 concentrations rise is essential for understanding plant health and stress tolerance as well as changing atmospheric chemistry.

Isoprene is produced by isoprene synthase, a TPS-b terpene synthase, from dimethylallyl diphosphate (DMADP) in the chloroplast (Sharkey et al., 2013; Silver and Fall, 1995). Chloroplastic DMADP is produced by the methylerythritol 4-phosphate (MEP) pathway, also called the non-mevalonate pathway (Figure 2.1). The MEP pathway consumes one pyruvate and one glyceraldehyde 3-phosphate molecule (derived from photosynthesis), one NADPH and four

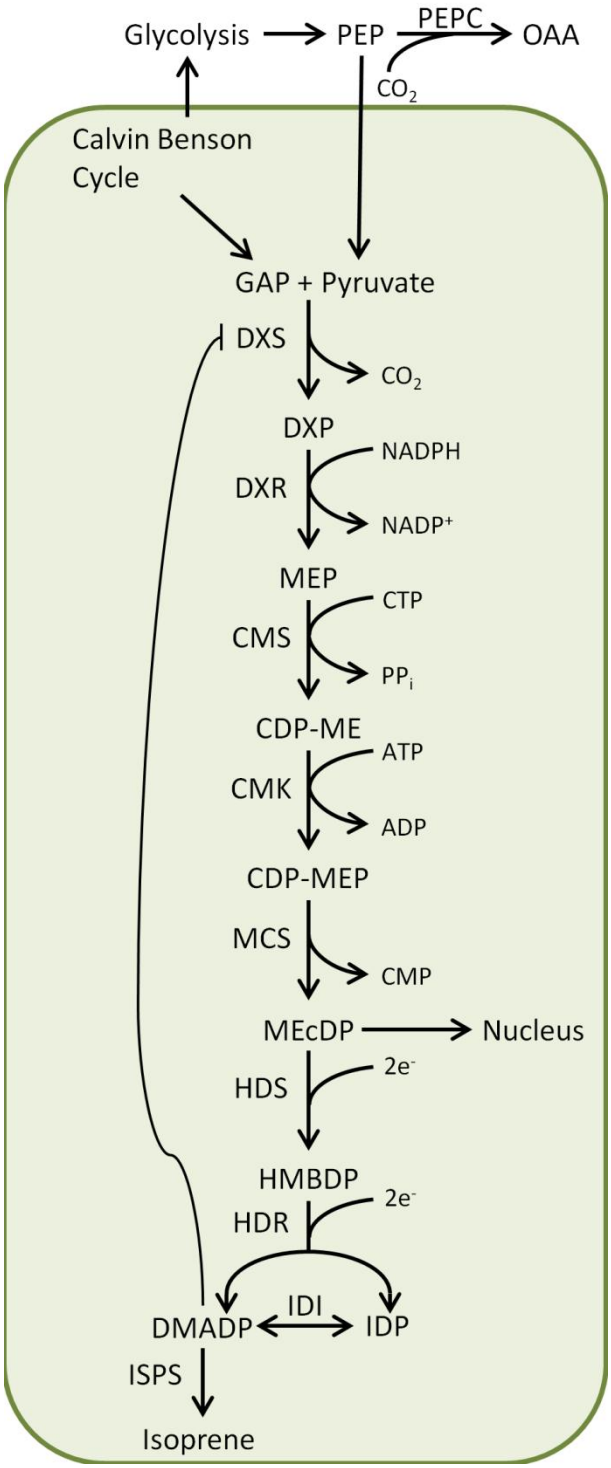

Figure 2.1: The MEP pathway in plants. Isoprenoids are produced by the MEP pathway under light-dependent reactions in the chloroplast. In isoprene emitting plants the conversion of DMADP to isoprene is catalyzed by isoprene synthase. Two reducing equivalents each are required to convert MEcDP into HMBDP and HMBDP into DMADP; these can be provided by NADPH or by two ferredoxin molecules.

ferredoxin molecules, one CTP, and one ATP, to produce a single DMADP or isopentenyl diphosphate (IDP) molecule (Figure 2.1). The final enzyme in the pathway, HMBDP reductase (HDR), produces more IDP than DMADP; IDP isomerase (IDI) converts this to a final ratio of two DMADP for every IDP (Zhou et al., 2013). DMADP production has a similar temperature sensitivity as photosynthesis, although isoprene emission has a much higher temperature maximum (Li et al., 2011).

The rate of isoprene emission is affected by environmental factors such as light, temperature, CO_2 concentration, and O_2 concentration. Isoprene emission increases with high temperature (Cole, 2016; Lindwall et al., 2016; Monson et al., 1992; Sharkey et al., 1999; Sharkey et al., 1996; Singsaas and Sharkey, 1998; Staudt et al., 2016; Wiberley et al., 2008) and decreases with increasing CO_2 (Centritto et al., 2004; Monson et al., 2016; Monson et al., 2007; Pegoraro et al., 2007; Pegoraro et al., 2005; Possell and Hewitt, 2011; Possell et al., 2005; Rosenstiel et al., 2003; Scholefield et al., 2004; Sharkey et al., 1991; Way et al., 2013), although some studies have reported that growth at high CO_2 does not decrease isoprene emission (Buckley, 2001; Calfapietra et al., 2007; Rapparini et al., 2004; Sharkey et al., 1991; Sun et al., 2012; Tognetti, 1998; Wilkinson et al., 2009); a summary of the CO_2 effects on isoprene can be found in Pacifico et al. (2009). CO_2 inhibition of isoprene seems to be oxygen-insensitive, although low O_2 increases isoprene emission at low CO_2 (Loreto and Sharkey, 1990). As future CO_2 levels have been predicted to double by the end of this century, a number of studies have been carried out to model the effect of rising CO_2 on future isoprene emission rates. Hantson et al. (2017) used a dynamic vegetation model taking into consideration changes in CO_2 levels and land use by natural and anthropogenic causes, to show that isoprene levels have steadily decreased over the 20^{th} century. This decrease was predicted to continue over the next century (Hantson et al.,

2017). Way et al. (2013) investigated physiology and metabolomics of poplar under three different CO_2 levels that reflected preindustrial, present, and future atmospheric conditions. This study also indicated that isoprene emission could decrease under future high CO_2 levels, and that such a decrease in isoprene emission would lead to a reduction in isoprene-mediated benefits to plants such as tolerance to abiotic stress. While both of these studies modeled the effect of temperature as well as CO_2, they did not model the combined effect of CO_2 and temperature. Increasing temperature has been shown to mitigate or entirely abolish the CO_2 effect (Potosnak et al., 2014a; Rasulov et al., 2010; Sun et al., 2013). The reduced effect of CO_2 on isoprene at higher temperature must be included in order to accurately predict future isoprene emission rates. In effect, there is a gap between models and reality (Arneth et al., 2008a; Monson et al., 2007), a gap which may be closed with a mechanistic understanding of physiological controls of isoprene emission (Morfopoulos et al., 2014; Rasulov et al., 2010).

The physiological response of isoprene emission to the combination of changing temperature and CO_2 has been well studied (Monson et al., 2016; Potosnak et al., 2014a; Rasulov et al., 2010; Rasulov et al., 2009b), as it is vital to modeling isoprene emission under future climate scenarios. Despite this, the mechanisms controlling emission rate are still under debate. Multiple hypotheses have been formulated to explain the decrease in isoprene emission under high CO_2. One hypothesis is that high CO_2 concentration stimulates phosphoenolpyruvate carboxylase (PEPC), which competes for the pyruvate required for the MEP pathway (Figure 2.1) (Rosenstiel et al., 2004; Rosenstiel et al., 2003; Wilkinson et al., 2009). However, competitive inhibitors of PEPC such as malate and diethyl oxalacetate do not impact the inhibition of isoprene emission by CO_2 (Rasulov et al., 2018). Furthermore, a study of the flux through PEPC found that PEPC activities are lower at high CO_2 (Abadie and Tcherkez, 2019). An alternative hypothesis is that

the physiological control of DMADP, and therefore isoprene emission, is based on energy status of the chloroplast (Morfopoulos et al., 2014; Niinemets et al., 1999; Rasulov et al., 2009b; Rasulov et al., 2016). Under conditions of high CO_2 where triose phosphate utilization (TPU) limits photosynthesis, both ATP and NADPH production are reduced, which under the energy hypothesis would reduce DMADP production.

TPU limitation occurs when downstream sinks of photosynthetic carbon (such as sucrose, starch, and amino acid synthesis) are unable to keep up with the flux of triose phosphates produced by the Calvin-Benson cycle, which leads to feedback control of photosynthesis (McClain and Sharkey, 2019; Sharkey, 1985). Under TPU limitation, buildup of phosphorylated intermediates lowers the free phosphate in the chloroplast, slowing ATP synthase, which in turn lowers ATP levels and decreases the pH of the lumen, leading to a broad alteration in chloroplastic conditions. Ultimately under TPU limitation, proton motive force increases, while carbon assimilation flattens and electron transport declines with increasing CO_2 (Yang et al., 2016). TPU limitation occurs at high CO_2 when rubisco and ribulose 1,5-bisphosphate (RuBP) regeneration are not limiting, and is more likely at low temperature where TPU capacity is reduced. Like the CO_2 sensitivity of isoprene emission, TPU limitation is temperature sensitive; it is rarely seen above 30°C (Harley and Sharkey, 1991). When photosynthesis is limited by TPU, it has no, or reverse, sensitivity to oxygen; that is, unlike under other limitations, photosynthesis is not increased, and sometimes is inhibited, by low oxygen (McClain and Sharkey, 2019; Sharkey et al., 1986). In this study we tested the hypothesis that changes in the energy status of the cell resulting from TPU limitation at high CO_2 concentrations drive the sensitivity of isoprene emission to CO_2.

To identify the underlying energetics and biochemical mechanisms responsible for the response of isoprene emission to high CO_2, we measured the combined effect of temperature and CO_2 concentration on isoprene emission, assimilation, and chlorophyll fluorescence in four species - *Nicotiana tabacum* cv. Samsun NN (tobacco, a non-emitter) genetically engineered to emit isoprene (Vickers et al., 2009), and three native isoprene emitters: *Phragmites australis* (phragmites grass, a high-emitting monocot), *Platanus* × *acerfolia* (sycamore, a dicot tree) and *Populus nigra* × *maximowiczii* NM6 (poplar, a dicot tree used for commercial biomass production). We hypothesized that changes in the energy status of the chloroplast would explain the changes in isoprene emission at high CO_2 and temperature. While previous studies have suggested this effect, they did not measure the energy status *in vivo,* and TPU limitation was not observed in all data sets. In fact, we observed that isoprene emission was not correlated with TPU limitation, and isoprene emission was highly sensitive to changing CO_2 even under conditions where TPU limitation was not occurring and photochemical electron transport was constant. Isoprene emission was more strongly affected by temperature than by CO_2. Therefore, in contrast to previous predictions, our data also support a significant increase in isoprene emission under future climate and CO_2 conditions.

Methods

Plant material

Nicotiana tabacum cv. Samsun NN (tobacco) seeds transformed with *Populus alba* (poplar) isoprene synthase under control of a CaMV35 promoter were obtained from Claudia Vickers, University of Queensland. The plasmid construction and generation of the isoprene emitting and azygous lines are described in Vickers et al. (2009). We used isoprene emitting line 32, which was reported to have the highest isoprene emission, and matching azygous line [see Figure 2.1 in

Vickers et al. (2009)]. Seeds were planted in Suremix growing medium (Michigan Grower Products, MI, USA) in 3 L pots and grown in a growth chamber (Big-Foot, BioChambers), under a 16 h photoperiod at a light intensity of 400 µmol m^{-2} s^{-1}, day/night temperature of 25°C/22°C, and humidity of 60% for bulk seed production. For experiments reported here, seeds were planted in Suremix growing medium (Michigan Grower Products, MI, USA) in 3 L pots and grown in a greenhouse with supplemental lighting to extend the daylength to 16 h with a mean daytime light intensity of 300 µmol m^{-2} s^{-1} PAR, day/night temperature of 27°C/22°C, and at least 60% relative humidity. Plants were watered every other day with half-strength Hoagland's solution (Hoagland and Arnon, 1938). Four to six-week-old plants were taken to the lab for experiments.

Phragmites australis (phragmites) was wild harvested from June to July from a colony on the campus of Michigan State University 42°43'13.5"N 84°28'22.6"W. Stalks were cut in the morning each day before the start of experiments. The cut ends were immediately placed in water, the stems were cut again under water to prevent air bubble formation in the xylem, and transferred to the lab, where they were kept in water throughout the day. Cuttings were discarded at the end of day.

Platanus × *acerfolia* (sycamore) was wild harvested from four trees outside the Plant Biology building, Michigan State University 42°43'22.9"N 84°28'28.8"W. Branches were cut in the morning each day before the start of experiments. The branches were immediately placed in water, the petioles were cut again under water, and transferred to the lab. Petioles were kept in water throughout the day. Cuttings were discarded at the end of day.

Populas nigra × *maximowiczii* NM6 (poplar) trees were grown from cuttings provided by the Great Lakes Bioenergy Research Center (GLBRC). Cuttings were grown in 11 L pots filled with

Suremix growing medium and watered daily with de-ionized water. Pots were kept under greenhouse conditions of 16-hour photoperiod with a mean daytime light intensity of 300 µmol m^{-2} s^{-1} PAR, and mean day/night temperature of 28°C/ 22°C. Figures 3, 5, and 6 represent data obtained from two, three, and five-month-old cuttings, respectively. Plants were taken to the lab to perform experiments on attached leaves.

Gas exchange and chlorophyll fluorescence studies

Gas exchange and chlorophyll fluorescence measurements were taken simultaneously on individual, fully mature leaves using a LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE) connected to a Multiphase Flash^{TM} Fluorometer and a 6 cm^2 chamber with controlled light and gas flow (6800-01A). Leaves were equilibrated at each temperature (25, 30, or 35°C), 420 ppm reference air CO_2 concentration, 1000 µmol m^{-2} s^{-1} actinic light (10% blue, 90% red LEDs), and a constant vapor pressure difference (VPD) of between 1.6 and 2 kPa, depending on temperature. For ambient oxygen experiments, room air was used and conditioned by the LI-6800. For the low oxygen experiment, pure oxygen and nitrogen gas from tanks (Airgas, Radnor, PA) were mixed at the desired ratio and provided to the LI-6800 for control of water and CO_2 concentration. Chlorophyll fluorescence measurements were taken using the multiphase flash setting as described by Avenson and Saathoff (2018). *A*-C_i curves were performed from low to high CO_2 concentration, leaving the leaf at each CO_2 concentration for 2-3 minutes before continuing to the next.

Real-time isoprene emission measurements were taken with a Fast Isoprene Sensor (FIS). The FIS uses the chemiluminescent reaction of isoprene and ozone to rapidly, sensitively, and specifically detect isoprene (Guenther and Hills, 1998). We used the instrument as described in Guenther and Hills (1998). Approximately 60% of the airflow coming over the leaf was

redirected from the LI-6800 output to the FIS for isoprene analysis. The air flow rate in the LI-6800 was set to 500 µmol s^{-1} and the FIS sample flow rate to 400 sccm (280 µmol s^{-1}). A 3.25 ppm isoprene standard (in nitrogen) purchased from Airgas was used for calibration of the FIS.

Leaf spectroscopy measurements

Electrochromic shift (ΔA_{520}, a measure of proton-motive force, PMF) and ΔA_{820} (a measure of photosystem I (PSI) oxidation measurements were taken with a modified IDEA spec (Hall et al., 2013). The IDEA spec described by Hall *et al*. (2013) was modified by addition of blue LEDs to supplement the red actinic light of the original spec. When the IDEA spec is set to 1000 µmol m^{-2} s^{-1} PAR, blue light represents less than 10% of the total light, similar to the LI-6800 fluorescence head. In addition, the light guide was adapted to fit the LI-6800 clear top chamber (6800-12A), a 9 cm^2 square chamber. Electrochromic shift measurements were taken as described in Kanazawa et al. (2017), except that the modified IDEA spec does not have far red capability and so the measurement was taken without the benefit of far red to fully oxidize PSI. As such, the measurements of oxidation state presented here are estimates based on measurements in which PSI may not have been fully oxidized. Electrochromic shift measurements were taken and fitted as described in (Takizawa et al., 2007) to determine PMF.

Metabolite measurements

The post-illumination method for quantification of methylerythritol cyclodiphosphate (MEcDP) and DMADP *in vivo* was performed as described in Li and Sharkey (2013). For post-illumination isoprene emission measurements, a poplar leaf was equilibrated in a LI-6800 as described above. Chamber clearing was determined by running isoprene standard into the LI-6800 fluorescence head via a needle, then removing the needle. After correcting for chamber clearing, the total isoprene emitted for the first 3 min after turning the light off (the initial decay

in isoprene emission) was calculated which corresponds with the amount of DMADP present. Between 12 and 25 min a post-illumination "burst" of isoprene is observed and the total isoprene emitted in this time frame corresponds with the amount of MEcDP present in the leaf (Li and Sharkey, 2013). A sample burst with chamber clearing is shown in Figure 2.2.

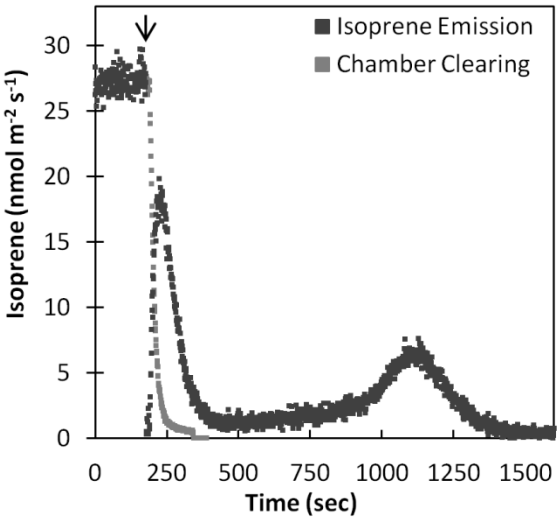

Figure 2.2: Sample post-illumination burst. A poplar leaf was equilibrated at 400 ppm CO_2 for 40 minutes prior to turning the light off (shown at 178 sec, marked with arrow). Chamber clearing (light grey squares) was determined by running an isoprene standard into the chamber, and then removing it; the time it takes the chamber to clear of isoprene is proportional to initial emission rate. Isoprene emission is shown after subtracting chamber clearing (dark grey squares). The peak in isoprene emission between 178 and 428 sec is proportional to the DMADP pool, which gets converted entirely by isoprene synthase post-illumination. The peak between 428 and 1500 sec is proportional to the MEcDP pool, which is slowly converted to DMADP in the dark.

Statistical analyses

Statistical analyses were performed using Excel 2007 (Microsoft, Redmond, WA). Averages and standard error for n = 3 plants (n = 5 plants for phragmites) are reported. For data presented in Figures 4 and 7, one-factor ANOVA followed by a t-test was used to identify differences between means for each metabolite and isoprene emission under varying CO_2 levels. For data presented in Figure 2.5, a representative sample is shown; this was selected from n = 4 plants. The raw spectroscopic data presented in Figure 2.5B was processed using an R script (R Foundation for Statistical Computing), which deconvoluted the changes in absorbance at 505 and

535 nm from the changes at 520 nm and fit the dark interval reaction kinetics to calculate PMF according to the principles reported in Kanazawa et al. (2017). R scripts are available upon request.

To compare the sensitivity of isoprene emission and assimilation rates to temperature, measurements taken at 400 ppm CO_2 and from 25 to 35°C temperature settings were used to calculate the temperature coefficient (Q_{10}) values for isoprene emission and assimilation. Q_{10} was calculated using the following equation: $Q_{10} = (R2/R1)^{(10/T2-T1)}$ where, T1 = 25°C, T2 = 35°C, and R1 and R2 are isoprene emission or assimilation rates measured at 25°C or 35°C, respectively. Isoprene emission rates collected at different CO_2 and temperature levels were used to predict the combined effect of CO_2 and temperature on isoprene emission under current climate conditions and in year 2100. Typical summer CO_2 and temperature highs in Michigan under current conditions were considered as 400 ppm CO_2 and 28°C, respectively. Based on the moderate climate scenario (RCP 6.0) proposed by the IPCC (Pachauri et al., 2014), a CO_2 level of 800 ppm and a 5°C increase in temperature was assumed for the year 2100. Estimates were performed in Excel.

Results

The combined effects of temperature and CO_2 on isoprene emission and assimilation

Regardless of the species, increasing temperature greatly increased isoprene emission at all CO_2 concentrations (Figure 2.3, top), but had a much smaller effect on assimilation (Figure 2.3, middle). A sharp decline in isoprene emission was observed under high CO_2 at 25°C and 30°C in all species. In phragmites and in tobacco, higher temperature (35°C) nearly abolished the CO_2 inhibition of isoprene emission, although a small, but not statistically significant (at $\alpha = 0.05$), decrease is still apparent. In poplar and sycamore, the CO_2 inhibition of isoprene emission was

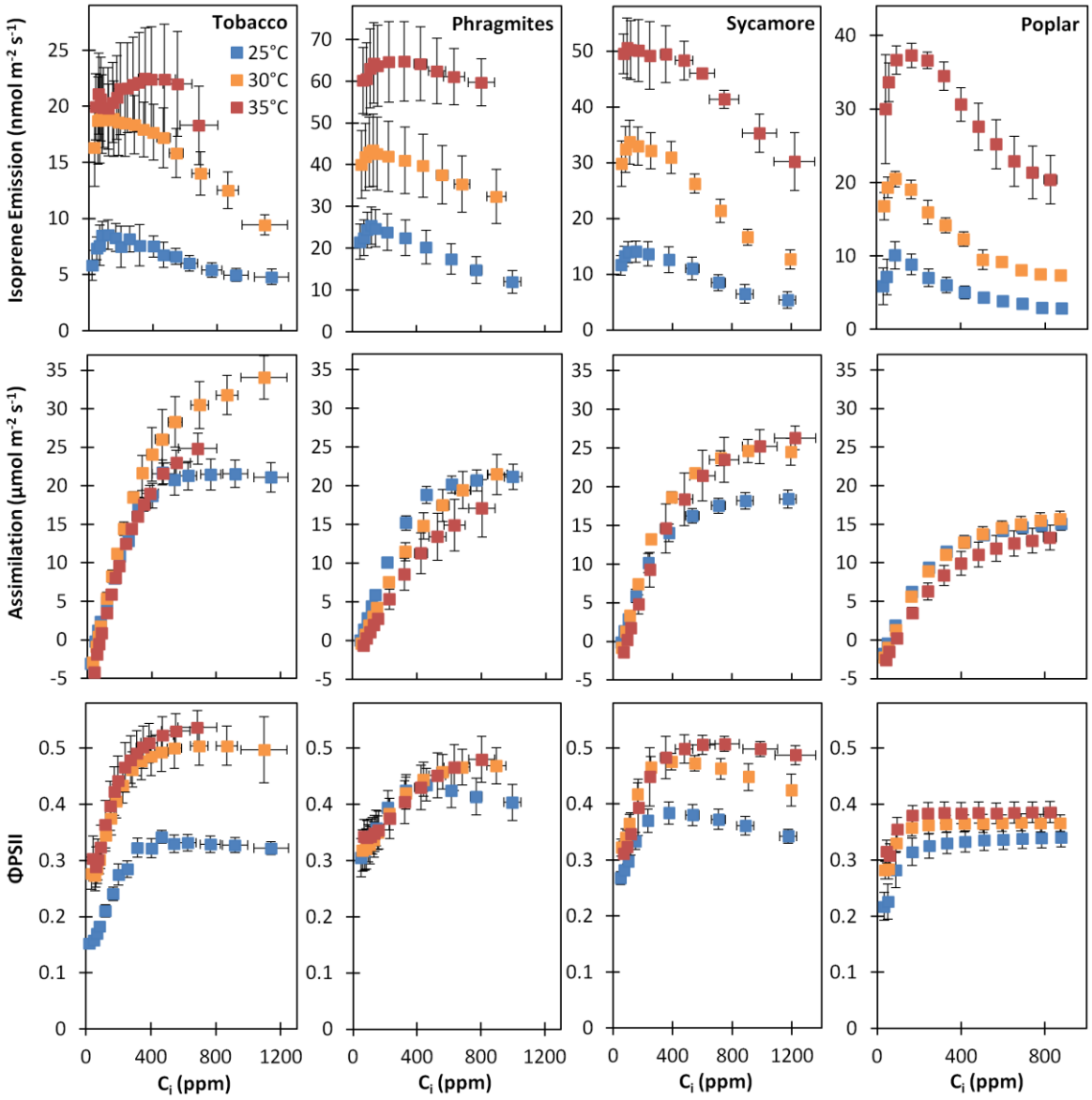

Figure 2.3: Comparison of isoprene emission, carbon assimilation, and electron transport under varying CO_2 and temperature levels. The combined effect of CO_2 and temperature on isoprene emission (top), carbon assimilation (middle), and ΦPSII (bottom) is presented for tobacco genetically engineered to emit isoprene, phragmites, sycamore, and poplar. Blue, orange, and red squares represent data at 25, 30, and 35°C respectively. For isoprene emission (top), different scales are indicated on the Y-axis for each species for clarity. Values represent the mean ± SE of n = 3 plants; n = 5 for phragmites. Poplar leaves were from two-month-old cuttings. Plants were allowed to equilibrate at 420 ppm CO_2 (incoming air CO_2 concentration) prior to beginning measurements for each A-C_i curve. Each point represents carbon assimilation or isoprene emission at the end of a 3-min time period at each CO_2 concentration. Adjustment of CO_2 concentration proceeded from low (25 ppm) to high (1500 ppm) reference concentration. ΦPSII was derived from chlorophyll fluorescence, which was measured using the LI-6800 multiphase saturating flash at the end of each 3 min period.

still strong even at 35°C. TPU limitation should cause assimilation to plateau or even decrease at high CO_2. We observed a flattening, although not a decrease, of the A-C_i curve in tobacco at 25°C, but not at higher temperatures or in the other plant species. This flattening suggests that tobacco plants had a mild TPU limitation under these conditions. The loss of TPU limitation at higher temperatures in some species is consistent with the previously characterized temperature sensitivity of TPU limitation (Harley and Sharkey, 1991). However, the inhibition of isoprene emission rate by increasing CO_2 concentration was seen at 30°C in all species even when TPU was not occurring. This is in contrast to previous studies that showed that the CO_2 sensitivity was temperature dependent and would not occur at high temperature (Potosnak et al., 2014a). In particular, we saw that poplar had no TPU limitation at any temperature and also showed strong suppression of isoprene by CO_2 at every temperature, indicating that TPU limitation is not required for suppression of isoprene by CO_2 in this species.

The Q_{10} values for isoprene emission and assimilation, summarized in Table 2.1, clearly show that isoprene emission is far more sensitive to temperature than is assimilation (Table 1). We also calculated isoprene emission rates for all four species under conditions of global warming (Figure 2.4). In our region (northern Midwest, North America), where all species except tobacco are common urban plants, current summer CO_2 and temperature conditions are estimated at 400 ppm and 28°C, respectively. Under the IPCC moderate climate scenario (RCP 6.0) (Pachauri et al., 2014), which predicts an increase in global CO_2 concentrations from 400 ppm to 800 ppm, temperature in our region is expected to increase by an average of 5°C, to new summer conditions of 33°C. We extrapolated data to these exact conditions from the data in Figure 2.3. Extrapolated C_i values are in Table 2. While the large change in CO_2 does suppress

isoprene emission somewhat, the change in temperature more than overcomes the suppression, leading to an increase of isoprene emission in all species (Figure 2.3, top, and Figure 2.4).

Table 2.1: Q_{10} values for isoprene emission and assimilation.

Species	Isoprene	Assimilation
Tobacco	3.0	1.0
Phragmites	2.9	0.6
Sycamore	3.9	1.0
Poplar	5.7	0.7

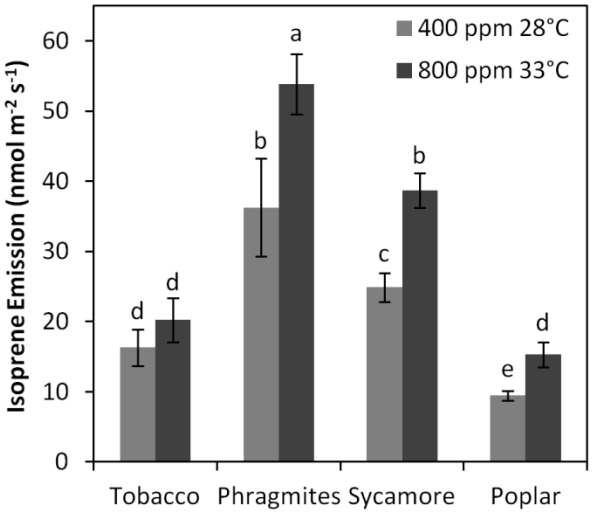

Figure 2.4: The combined effect of CO_2 and temperature on isoprene emission in current day climate and in year 2100 based on moderate climate change scenario. Current year is shown in light grey and year 2100 scenario in dark grey. 400 ppm CO_2 and 28°C temperature represents a typical summer high in northern climates under current conditions. The future isoprene emissions are based on a moderate climate change scenario for the year 2100, which predicts CO_2 concentrations at 800 ppm and a temperature rise of 5°C in northern climates (Pachauri et al., 2014). Values represent data extrapolated from Figure 3 shown as the mean ± SE of n = 3 plants; n = 5 for phragmites. Statistical differences at $\alpha = 0.05$ are marked with lower-case letters.

Table 2.2: C_i values for Figure 2.4. C_i values are extrapolated from the measured values in Figure 2.3.

Species	C_i at 400 ppm ambient, 28°C (ppm)	C_i at 800 ppm ambient, 33°C (ppm)
Tobacco	301 ± 17	580 ± 90
Phragmites	331 ± 21	660 ± 80
Sycamore	380 ± 30	700 ± 100
Poplar	330 ± 4	759 ± 28

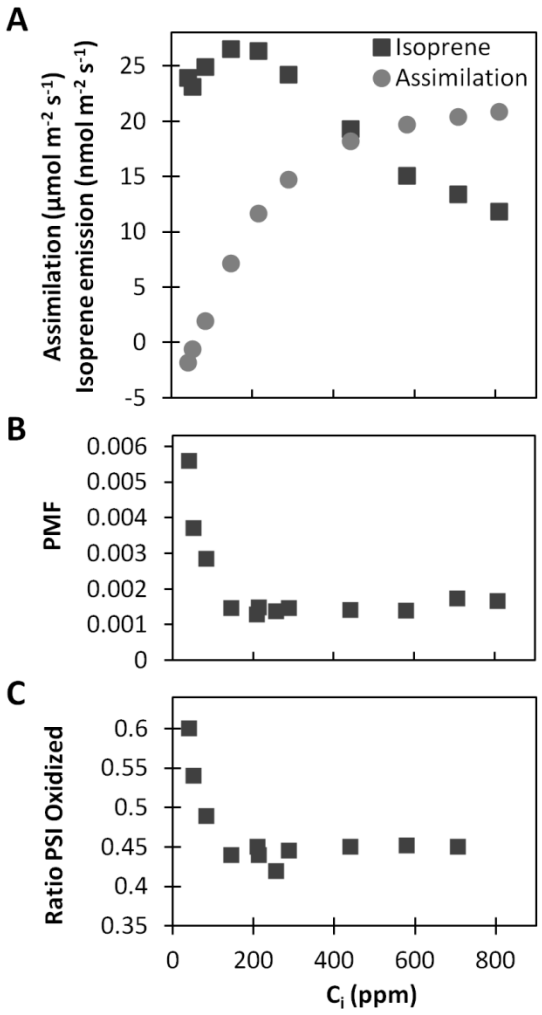

Figure 2.5: Effect of CO_2 on ATP synthesis and photosystem I in poplar. Isoprene emission (squares) and carbon assimilation (circles) (A), the proton motive force (PMF) (B), and the ratio of oxidized to reduced PSI (C) under varying CO_2 concentrations were measured in poplar leaves from three-month-old cuttings. Poplar leaves were placed in the 9 cm^2 leaf chamber of the modified Idea Spec which is a custom instrument that can measure ΦPSII, PSI oxidation (ΔA_{820}), and electrochromic shift (ΔA_{520}). Representative data is shown out of n = 4. Measurements were taken at 30°C and 1000 µmol m^{-2} s^{-1} PAR.

Effect of CO_2 on energy status of the leaf

It is possible for TPU limitation to affect the energy status of the chloroplast before changes in assimilation are observed. Previous studies concluded that energy status of the chloroplast is responsible for changes in isoprene emission due to CO_2 and temperature, but did not directly measure energy status. We measured energy status of the chloroplast using well established spectroscopic techniques. If high CO_2 concentrations altered energy status of the leaf through

TPU limitation or any other mechanism, ΦPSII, the ratio of excitons reaching photosystem II that are used for photochemistry, should decrease at high CO_2 concentrations. ΦPSII is derived from chlorophyll fluorescence analysis. In tobacco, phragmites, and poplar, ΦPSII remained constant at CO_2 concentrations that suppressed isoprene emission at 30°C and 35°C (Figure 2.3, bottom). However, in sycamore ΦPSII indicated there was a TPU limitation even at 35°C. The data from phragmites and sycamore is particularly interesting as at 25°C ΦPSII decreases slightly at high CO_2, while assimilation does not show TPU limitation. Therefore, we did observe changes in the energy status of the chloroplast that did not cause an obvious TPU behavior in assimilation.

However, most important were the cases where ΦPSII was not decreasing and therefore could not have been limiting for isoprene emission, even though strong suppression of isoprene emission was observed. This is clearest in the poplar data, where this was observed at all temperatures, although also in other species at the higher temperatures (Figure 2.3). For this reason we chose poplar for further experiments to look at the electrochromic shift and PSI oxidation. Electrochromic shift is the shift in absorbance of carotenoids resulting from an electric field across the molecule (Takizawa et al., 2007; Witt, 1979). As such, it serves as an *in vivo* spectroscopic measure of proton-motive force (PMF). Following the methods and models of Takizawa et al. (2007) as implemented in our modified IDEA spec, we determined the change in PMF of poplar leaves under increasing CO_2. While we continued to see the large decrease in isoprene emission under elevated CO_2 (Figure 2.5A), PMF remained constant (Figure 2.5B). This spec can also measure the change in absorbance at 820 nm under normal or fully oxidized conditions, which is a measure of the oxidation status of PSI (Kanazawa et al., 2017). PSI oxidation was also constant under high CO_2 that suppressed isoprene emission (Figure 2.5C).

The effect of oxygen on the isoprene response to CO_2

To test whether energy consumed by photorespiration can explain the CO_2 effect on isoprene, as photorespiration decreases at high CO_2, we measured the isoprene response to CO_2 at low (2%) as well as normal (21%) oxygen concentrations in poplar (Figure 2.6A). Interestingly, poplar leaves used for the oxygen sensitivity measurements were exhibiting signs of TPU limitation, even though the conditions (30°C and 1000 µmol m^{-2} light) used were the same as in Figures 3 and 5. At 21% oxygen, both assimilation (Figure 2.6B) and ΦPSII (Figure 2.6C) began to decrease with increasing CO_2 once past the rubisco limitation of photosynthesis, which is indicative of TPU limitation. Low (2%) oxygen increased isoprene emission at low CO_2, but as CO_2 concentration increased past 400 ppm, the inhibition of isoprene emission was sufficiently strong that the isoprene emission at 800 ppm and higher CO_2 were identical under both conditions (Figure 2.6A).

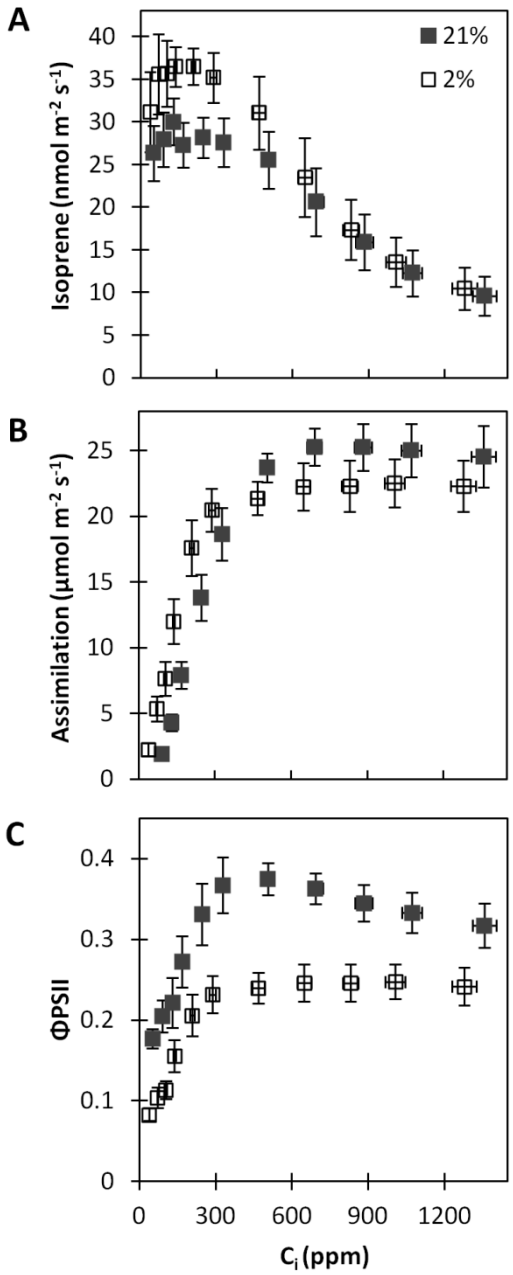

Figure 2.6: Effect of oxygen on the isoprene emission response to CO_2 in poplar. Isoprene emission (A), carbon assimilation (B), and ΦPSII (C) under varying CO_2, and 21% (filled squares) or 2% (empty squares) oxygen was measured simultaneously in poplar. Poplar leaves were from five-month-old cuttings. Values represent the mean ± SE and n = 3 plants.

The effect of CO_2 on MEcDP and DMADP

If either NADPH availability or ATP/CTP availability was responsible for the changes in isoprene emission under elevated CO_2, then distinct patterns in the MEP pathway metabolites should be visible. If reducing power (electron availability) is limiting, then MEcDP should build

up, as HDS and HDR are extremely dependent on reducing power availability. We measured MEcDP and DMADP in poplar leaves by measuring decay in isoprene emission immediately after turning off the light (which is proportional to the amount of DMADP present) and during the burst of isoprene 12 to 25 min after turning off the light (which is proportional to the amount of MEcDP present) (Li and Sharkey, 2013; Rasulov et al., 2009a). We saw a decrease in MEcDP and DMADP with increasing CO_2 (Figure 2.7), indicating that electron availability is not responsible for the reduction in isoprene emission at high CO_2 in poplar.

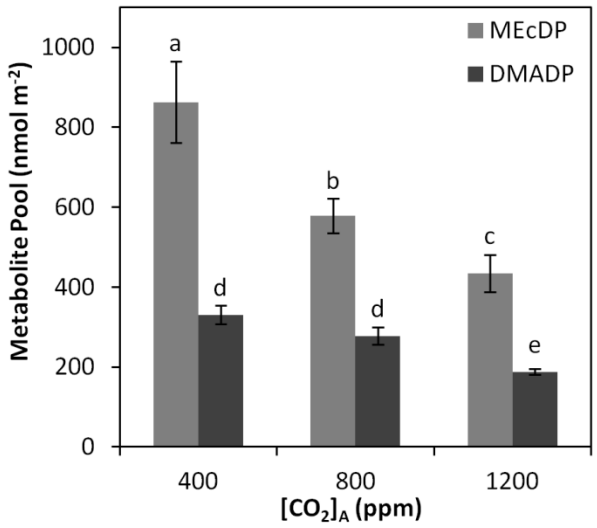

Figure 2.7: The effect of high CO_2 on downstream MEP pathway metabolite levels in poplar. MEcDP (light grey) and DMADP (dark grey) levels in poplar leaves were measured by the post-illumination method. Leaves were equilibrated in the LI-6800 for 40 minutes at 30°C, 420 ppm reference CO_2 concentration, and 1000 µmol m^{-2} s^{-1} actinic light. After turning off the light, isoprene emission continues for about 3 minutes. Between 3-12 minutes a burst of isoprene emission is observed. This continuing post-illumination emission directly correlates with DMADP and MEcDP pool sizes respectively (Li and Sharkey, 2013). Values represent the mean ± SE of n = 3 plants. Statistical differences at $\alpha = 0.05$ are marked with lower-case letters.

Discussion

We investigated the combined effects of temperature and CO_2 on isoprene emission and found that isoprene emission was more strongly affected by temperature than by CO_2. We hypothesized that TPU limitation would explain the changes in isoprene emission at high CO_2 and temperature. In fact, we observed that isoprene emission was not correlated with TPU

limitation, and that isoprene emission was highly sensitive to changing CO_2 even under conditions where TPU limitation was not occurring. We confirmed these measurements in four species, including a transgenic tobacco plant that has been widely used to probe the effects of isoprene emission on plants. While TPU limitation can occur at the same time as CO_2 suppression of isoprene emission, sufficient data show that TPU limitation is not necessary for this suppression to occur. Furthermore, the leaf energetic status remained constant, which indicated that isoprene is not solely controlled by the energy status of the leaf, but other drivers must be necessary in order to explain the changes in isoprene emission. The significance of above findings and alternative hypotheses that may explain the suppression of isoprene emission under high CO_2 are discussed below.

Suppression of isoprene emission by high CO_2 can occur in the absence of TPU limitation

Previous studies have indicated that the conditions under which decreased isoprene emission at high CO_2 occurs correlate with the conditions under which TPU limitation occurs (Monson et al., 2016; Rasulov et al., 2016). The CO_2 sensitivity of isoprene emission is temperature sensitive, and is abolished under high temperature, an effect we see in two species but not in data from all species. TPU limitation is also temperature sensitive and rarely seen above 30°C (Harley and Sharkey, 1991). TPU limitation is also oxygen insensitive and still occurs under low oxygen, unlike photorespiratory effects (Sharkey et al., 1986). Loreto and Sharkey (1990) observed that the effect of CO_2 on isoprene was also oxygen insensitive. We observed that at low CO_2, low O_2 increased isoprene emission, but isoprene emission rapidly decreased with increasing CO_2; that is, the CO_2 effect on isoprene is oxygen insensitive. Given the effect of TPU limitation on ATP synthesis and electron transport, previous studies concluded that the changes in energy status in

the chloroplast are responsible for the changes in isoprene emission in response to CO_2 and temperature (Monson et al., 2016; Rasulov et al., 2009a; Rasulov et al., 2016). However, none of these studies directly measured energy status in the chloroplast under high CO_2 when a decrease in isoprene emission was observed.

Using combined CO_2 and temperature treatments on four species representative of broad isoprene emitting capabilities, we clearly showed that suppression of isoprene emission under high CO_2 can occur when TPU limitation of assimilation and electron transport (ΦPSII) is absent. This was previously observed, but we hypothesized that changes in the energy status of the chloroplast can happen even when there are no indications of TPU limitation based on assimilation rate. Using several *in vivo* spectroscopic techniques we characterized in detail the energy status of poplar under high CO_2 and found that while PMF and photosystem I oxidation were constant, isoprene emission was still reduced under high CO_2. These both measure the ability of the plant to make ATP and if they are constant, the ATP available for isoprene emission is probably constant. In multiple species our data show that changes in ΦPSII are not necessary in order to see decreases in isoprene emission at high CO_2. We further confirmed this by measuring the MEcDP and DMADP pools, which showed that MEcDP was decreasing with increasing CO_2. If electron transport rate (as opposed to ATP) was limiting, we would expect MEcDP to build up as ferredoxin is required to convert it to DMADP. While in some conditions we did see ΦPSII decrease with increasing CO_2, it was not required for isoprene emission to decrease. Taken together, these data shows that the suppression of isoprene emission by CO_2 is not dependent on the energy status of the chloroplast.

Alternate hypotheses for the CO_2 effect on isoprene emission

Given that our data rule out TPU limitation and reduced energy availability as causes, we can speculate several possibilities to explain the observed reduction in isoprene emission under high CO_2. Our data are consistent with the carbon limitation (PEP carboxylase) hypothesis for the suppression of isoprene emission. However, this hypothesis was previously ruled out by Rasulov et al. (2018), and our data are not sufficient to re-open this hypothesis for investigation. CO_2 sensing, either through direct sensors in the stomata, or indirect sensing through a method such as calcium release from the apoplast, may lead to changes in the MEP pathway that would explain this response. Feeding EGTA, a specific chelator of Ca^{2+} has previously been shown to alter bursts or changes in isoprene emission due to wounding (Loreto and Sharkey, 1993). We attempted to test this hypothesis by feeding EGTA to detached poplar leaves; however, the EGTA led to such broad systemic changes, including a massive decrease in ΦPSII, that no conclusion could be drawn about isoprene emission specifically. Calfapietra et al. (2007) showed that expression of MEP pathway genes and isoprene synthase protein levels can decrease in plants grown under high CO_2 and Scholefield et al. (2004) saw a similar effect in extractable enzyme activity, although these differences were not significant. More research is required to determine if changes in isoprene synthase levels or activity can explain changes in isoprene emission at high CO_2. Even if this is a potential explanation for plants grown at high CO_2, we saw significant effects within 10 min (the length of a rapid A-C_i curve), which is too short a time scale for substantial changes in gene expression or protein levels to occur. Wiberley et al. (2009) calculated that the lifetime for isoprene synthase was 3.4 days at 30°C. Therefore, a metabolic change at high CO_2 is still necessary in order to explain these short-term changes.

Effect of CO_2 on isoprene emission models

Prior studies showed that the global increase in temperature due to climate change would not be sufficient to overcome the suppression of isoprene emission by increasing atmospheric CO_2 concentrations (Hantson et al., 2017; Possell and Hewitt, 2011). These models were based on data that showed both the CO_2 and temperature effects on isoprene, but not their combination, and showed an overall decrease in global isoprene emissions. We found no support for electron-transport based models of isoprene emission that predict decreased isoprene emission rates (Grote et al., 2014; Morfopoulos et al., 2014; Niinemets et al., 1999). Changing land use in the future will likely decrease the biomass of isoprene emitting species (Arneth et al., 2008b; Hantson et al., 2017); but increased temperature may select for isoprene emission on an ecosystem level (Mutanda et al., 2016; Purves et al., 2004; Taylor et al., 2018). Long term growth at high CO_2 leads to increased leaf area in *Populus* species (Murthy et al., 2005; Pegoraro et al., 2007; Rey and Jarvis, 1998); under drought stress or in *Eucalyptus* species leaf area may not increase (Duursma et al., 2016; Potosnak et al., 2014b). Pegoraro et al. (2005) concluded that the increased leaf area under high CO_2 conditions will lead to a smaller decrease in ecosystem isoprene emissions than would be indicated by leaf-level measurements corrected by leaf area. Another concern is that the overall emission rates used in these studies are lower under all conditions than is typically reported for these species, indicating that perhaps the conditions do not reflect real-world forest conditions (Hantson et al., 2017; Possell and Hewitt, 2011; Possell et al., 2005). Some studies showed that high temperature (35°C) abolishes the CO_2 effect (Monson et al., 2016; Potosnak et al., 2014a). We did not see that high temperature abolished the CO_2 effect in sycamore or poplar, although it did in phragmites and tobacco. We show that even small changes in temperature increase isoprene emission by far more than a doubling of CO_2 concentration would decrease it. We calculated isoprene emissions in all four species under

current-day summer conditions (400 ppm and 28°C) and predicted year 2100 conditions under an IPCC climate model (800 ppm and 33°C) based on our short-term data. While the global change in temperature is only 3°C in this scenario, data support larger changes in northern climates compared to the global average, up to 5°C in the northern United States. In contrast to the commonly accepted models, we predict that global isoprene emissions will increase greatly under future climate scenarios. This may help protect plants against increasing thermal stress as well as increases in ozone production and the lifetime of methane in the lower atmosphere.

Our data are based on very short-term changes, from ten minutes to an hour under high CO_2 conditions. Long term changes may lead to different effects. Long term effects like suppression of gene expression at high CO_2 may be offset by high temperature enhancement of gene expression and isoprene synthase protein accumulation (Wiberley et al., 2008). The delay between leaf emergence and isoprene emission could be significantly shortened at higher temperature (Monson et al., 1994). Plants grown under high CO_2 seem to have less strong suppression of isoprene emission than plants that are held at high CO_2 at short timescales (Calfapietra et al., 2007; Possell and Hewitt, 2011; Possell et al., 2005; Scholefield et al., 2004; Way et al., 2013), however suppression of isoprene emission is still evident. Long term temperature changes can also have different effects than short term ones (Arab et al., 2016; Centritto et al., 2011; Fares et al., 2011; Hanson and Sharkey, 2001; Rasulov et al., 2015). Rasulov et al. (2015) found that the isoprene emission rates of plants grown at high temperature were more responsive than that of plants grown at low temperature on a gram dry weight basis, but that the responsiveness was the same on a leaf area basis. Fares et al. (2011) found the opposite: the isoprene emission of plants grown at high temperature had a lower Q_{10} than those grown at low temperature. However, growing plants at high temperature still suppresses the

effect of CO_2, leading to overall higher isoprene emissions (Potosnak et al., 2014a). Short term changes in metabolism under high CO_2 are likely reflective of long term adaptation to high CO_2, as is evident in the TPU literature (McClain and Sharkey, 2019). It is reasonable to expect that the same holds true for isoprene emission, and that our predictions based on short term measurements of changes in CO_2 are reflective of what will hold true on longer time scales. There is likely to be more isoprene emission in the future in some, and perhaps many, terrestrial ecosystems.

Author Contributions

TDS conceived the experimental plan. CS and LG performed the assimilation, ΦPSII, and isoprene emission response to CO_2 and temperature experiments. ATL performed the IDEA spec (ECS and PSI oxidation), low oxygen, and post-illumination experiments. AMM wrote R scripts to analyze the IDEA spec data. ATL and CS performed the data analysis. ATL wrote the first draft of the manuscript and SMW assisted in production of the manuscript. All authors contributed to manuscript writing and revision, read and approved the submitted version.

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Chapter 3 The effect of temperature on isoprene emission rate

Abstract

Isoprene emission is highly temperature-sensitive and this effect is dependent in part on the temperature dependence of isoprene synthase. The effect of temperature on the K_M and substrate inhibition (K_I) of isoprene synthase has not been studied. In this study, we found that K_M of *Eucalyptus globulus* isoprene synthase is more sensitive to temperature than the turnover rate, k_{cat}, between 30 and 40°C, and has a temperature optimum of 33°C. K_I is only weakly sensitive to temperature and has an optimum of 32°C. Including K_M and K_I estimates in models of isoprene emission from plants based on substrate availability improves the match to empirical measurements of temperature sensitivity.

Introduction

Isoprene synthase is a strongly temperature sensitive enzyme that partially controls the response of isoprene emission to temperature (Monson et al., 1992; Rasulov et al., 2010). All enzymes are sensitive to temperature (Griffith, 2001; Somero, 1995). At low temperatures, increased temperature increases reaction rates dependent on the activation energy; at high temperatures, enzymes begin to unfold (depending on their thermal stability) and rapidly lose activity (Griffith, 2001; Somero, 1995). At a set measurement temperature, enzymes from cold-adapted species have higher k_{cat} values than those from warm-adapted species, however these enzymes also have a lower K_M (Somero, 1995). Enzyme concentrations may change in response to environmental conditions. In particular, isoprene synthase expression has been shown to shut down under very cold conditions, and be re-induced by high temperature conditions (Mutanda et al., 2016a; Mutanda et al., 2016b; Oku et al., 2014; Parveen et al., 2019; Wiberley et al., 2008). This response does not occur under conditions of drought, where isoprene emission is less highly regulated by temperature and CO_2 concentration (Arab et al., 2016; Fortunati et al., 2008; Tani et

al., 2011). Temperature control of isoprene emission is adaptive to have the highest production under high temperatures when it is most needed to improve plant resilience.

Table 3.1: Kinetic parameters and temperature optima of isoprene synthases from different species. k_{cat} (turnover number) and K_M (Michaelis-Menten constant) for the conversion of DMADP to isoprene are shown. Inhibition is seen at high DMADP concentrations.

Species	k_{cat} (s^{-1})	K_M (mM)	Inhibition	Thermal Maximum Rate (°C)	Reference
Quercus petraea	NA	0.97	Above 10 mM	35	Schnitzler et al., 1996
Populus × *canescens* (purified from plant material)	NA	2.45 ± 0.10	Above 7.5 mM	40	Schnitzler et al., 2005
Campylopus introflexus	NA	0.37 ± 0.28	None	40	Lantz et al., 2015
Populus × *canescens* (C-terminal His)	NA	1.20 ± 0.29	Above 5 mM	45	Schnitzler et al., 2005
Quercus Robur	NA	NA	NA	50	Lehning et al., 1999
Metrosideros polymorpha	0.35	8.11	None	50	Yeom et al., 2018
Pueraria montana	0.088	7.7	None	NA	Sharkey et al., 2005
Salix discolor (soluble)	NA	1-4 mM	Product Inhibition	NA	Wildermuth and Fall 1998
Eucalyptus globulus	0.195	0.16	0.9 mM	NA	Sharkey et al., 2012
Populus tremuloides	1.7	8	Above 10 mM	NA	Silver and Fall, 1995

Differences between species in temperature responsiveness of isoprene emission and in basal emission rate may be due to differences in isoprene synthases. For example, *Populus tremuloides* was shown to have a higher turnover number (k_{cat} = 1.7 s^{-1}) and Michaelis-Menten constant (K_M = 8 mM) than *Eucalyptus globulus* (k_{cat} = 0.195 s^{-1} and K_M = 0.16 mM respectively) (Sharkey et al., 2013; Silver and Fall, 1995). This may reflect fundamental differences in these enzymes as

they have adapted to different climates. A comparison of the turnover numbers (which is reflective of the activity of an individual enzyme molecule), K_M, and temperature optima of isoprene synthases from different species is shown in Table 3.1. Only relatively recently have all of these parameters been reported for different species; for many species one or more aspects of the isoprene synthase is not known. Differing responses of enzymes to temperature may reflect ecological adaptations (Somero, 1995); this should particularly be true for an enzyme that is both highly sensitive to temperature and itself confers physiological tolerance to high temperature.

Most isoprene synthases have a temperature optimum around 40°C, which is similar to the overall optimum of isoprene emission in most species and higher than the temperature optimum of DMADP production (Rasulov et al., 2010). The temperature optimum of isoprene synthase activity follows classic understanding of enzyme kinetics. As temperature increases, enzymatic rate increases exponentially, until the temperature at which enzymatic denaturation takes place. For isoprene synthase, unfolding begins around 45-50°C, although some enzymes are more stable and may retain activity up to 60°C (see Table 3.1). Denaturation is rapid and greatly decreases enzyme activity, creating a sharp optimum temperature. This is very different than the responses to other environmental variables such as pH, which have broad optima (particularly for isoprene synthase, which is active from 6.5 to 8.5 in all species) (Lantz et al., 2015; Lehning et al., 1999; Schnitzler et al., 1996; Schnitzler et al., 2005; Silver and Fall, 1995; Wildermuth and Fall, 1998; Yeom et al., 2018). Temperature may have different effects on the k_{cat} and K_M, as substrate binding and subsequent conversion require different changes in enzyme structure which may be more or less sensitive to temperature. Generally binding is considered to be much faster than conversion and this assumption is required for Michaelis-Menten kinetics (the activation energy of conversion is higher than that of binding). Binding is enthalpically driven and the

reverse (release of substrate) is entropically driven, but the level of enthalpy released by binding depends on the enzyme binding pocket, which itself may change with temperature. By comparison, conversion and release of product is often entropically driven (certainly in the case of isoprene synthase), in which case increased temperature always improves rate. A broad survey of enzymes from different species and metabolic pathways shows that K_M is typically less sensitive to temperature than k_{cat}, and both increase with temperature (meaning that substrates are bound less tightly at higher temperatures, but conversion takes place more rapidly) (Scopes, 1995). In some grass species the K_M's of the enzymes NAD-malate dehydrogenase and glutathione reductase display U-shaped response to temperature with an optimum that is dependent on their environment (Hakam and Simon, 2000; Simon, 1986). For the carboxylation reaction of Rubsico, the effect of temperature on k_{cat} and K_M is the same in the Arrhenius plot (which plots ln rate vs. 1/T), with a distinct breakpoint at 15°C (Badger and Collatz, 1997). When substrate concentration is low, Rate $\cong$ or $\propto \frac{V}{K_M}$. If V and K_M have the same temperature sensitivity, rate is linear with temperature, as can be seen with rubisco carboxylation (Badger and Collatz, 1997).

Current estimates of isoprene emission from plants suffer from a lack of mechanical understanding of the effects of temperature and CO_2 on the MEP pathway and isoprene synthase, which leads to models that do not fully fit all situations, particularly at high temperature. One aspect of this is that the effect of temperature on isoprene synthase is not fully understood. V_{max} of isoprene synthase is often estimated in vivo, where the enzyme is assumed to be operating at maximum efficiency for a given substrate concentration - therefore only rate and apparent substrate concentration is measured under different conditions (Rasulov et al., 2010). In addition, extractable activity has been used to estimate isoprene synthase response to environmental

conditions (Lehning et al., 1999; Schnitzler et al., 1997; Scholefield et al., 2004). These studies did not estimate k_{cat}, that is, the activity of the protein independent of protein levels. It has been shown that protein levels of isoprene synthase may change in response to environmental conditions, and this change may be independent of changes in enzyme activity (Wiberley et al., 2008). Furthermore, they did not measure changes in substrate affinity; while many enzymes have constant affinity as temperature changes, this is not true for all enzymes(Blagodatskaya et al., 2016; Quinlan, 1980; Sorensen et al., 2015). In this work I used recombinant *Eucalyptus globulus* isoprene synthase to determine the effect of temperature on K_M and k_{cat} of isoprene synthases. I then used these to refine a model of isoprene emission based on empirical activities and substrate concentrations.

Materials and Methods

Expression and purification of recombinant isoprene synthase

For this work isoprene synthase genes from *Eucalyptus globulus, Robinia pseudoacacia,* and *Populus alba* were used to express isoprene synthase in *E. coli*. These genes were originally cloned by Dennis Gray in our lab (Sharkey et al., 2013). All plasmids were as described in Sharkey et al. (2013); the isoprene synthase had a 6xHis tag attached to the C terminus in the PJexpress 401 vector (DNA 2.0, Menlo Park, CA, USA). The molecular weight is 93 kDA. Recombinant isoprene synthase was expressed in *E. coli* BL21 (pLysS) and purified as in Weise et al. (2013), using a His-tag purification on a Qiagen nickel-nitrilotriacetic acid (Ni-NTA) column. Cells were grown in LB broth with 25 µg/mL kanamycin at 37°C to an OD_{600} of 0.8 and induced by 5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6-8 hr. Cells were harvested by centrifugation at 4500 g for 10 min at 4°C and resuspended in lysis buffer (50 mM Tris, pH 8, 300 mM NaCl, 10 mM imidizole, 250 units/mL benzonase, 1x SIGMAFAST™ Protease

Inhibitor Cocktail Tablets, EDTA-Free per 100 mL [all reagents from SIGMA, St. Louis, MO, USA]) to which was added 1 mg/mL lysozyme. Resuspended cells were incubated on ice for 30 min, followed by sonication with a Branson Sonifier 250 with a 3-mm ultrahigh-intensity tapered microtip (Branson Ultrasonics, Danbury, CT, USA). Sonication was carried out in six steps at a 50% duty cycle; each step was done for 15 pulses followed by cooling in ice water to prevent overheating. The lysate was then centrifuged again at 5000g for 30 min to remove insoluble proteins and lipids, followed by purification of His-tagged proteins using the Ni-NTA agarose according to manufacturer's directions. The column was washed with 50 mM imidizole lysis buffer and protein was eluted in 1-mL fractions with 250 mM imidizole lysis buffer. Isoprene synthase was detected using an activity assay containing 1 mM DMADP and purity was confirmed with a Coomassie-stained SDS-Page gel and protein concentration with a Lowry protein assay kit (Thermo Scientific, Waltham, MA).

Enzyme assays

Assays were performed as described in Weise et al. (2013). Isoprene synthase extracts were diluted such that approximately 0.25 nmol enzyme was used per assay, and mixed with assay buffer (50 mM HEPES, pH 8, 10 mM $MgCl_2$, 20 mM KCl, 10% glycerol v/v) to a final volume of 100 µL. DMADP was obtained from isoprenoids.com (Tampa, FL) and dissolved to make an 80 mM stock, from which a dilution series was made such that a range of DMADP concentrations from 0.01 to 2.5 mM could be added to the assay vials. Assays were mixed in 2 mL gastight vials and incubated in a waterbath at the set temperature for 15 min. After exactly 15 min, 1 mL headspace gas was removed from the vial and the isoprene concentration was measured on the Fast Isoprene Sensor (FIS). The FIS uses the chemiluminescent reaction of

isoprene and ozone to rapidly, sensitively, and specifically detect isoprene (Guenther and Hills, 1998). We used the instrument as described in Guenther and Hills (1998).

Statistical analysis

At each temperature, three independent (separate enzyme extraction) trials were performed; each trial had itself three technical replicates at each DMADP concentration. Averages, standard errors, and graphs were prepared with Excel 2007 (Microsoft Corporation). Non-linear curve fitting was performed using a least-squares regression using Origin 8 (OriginLab Corporation). Errors are determined from the average of the three independent trials.

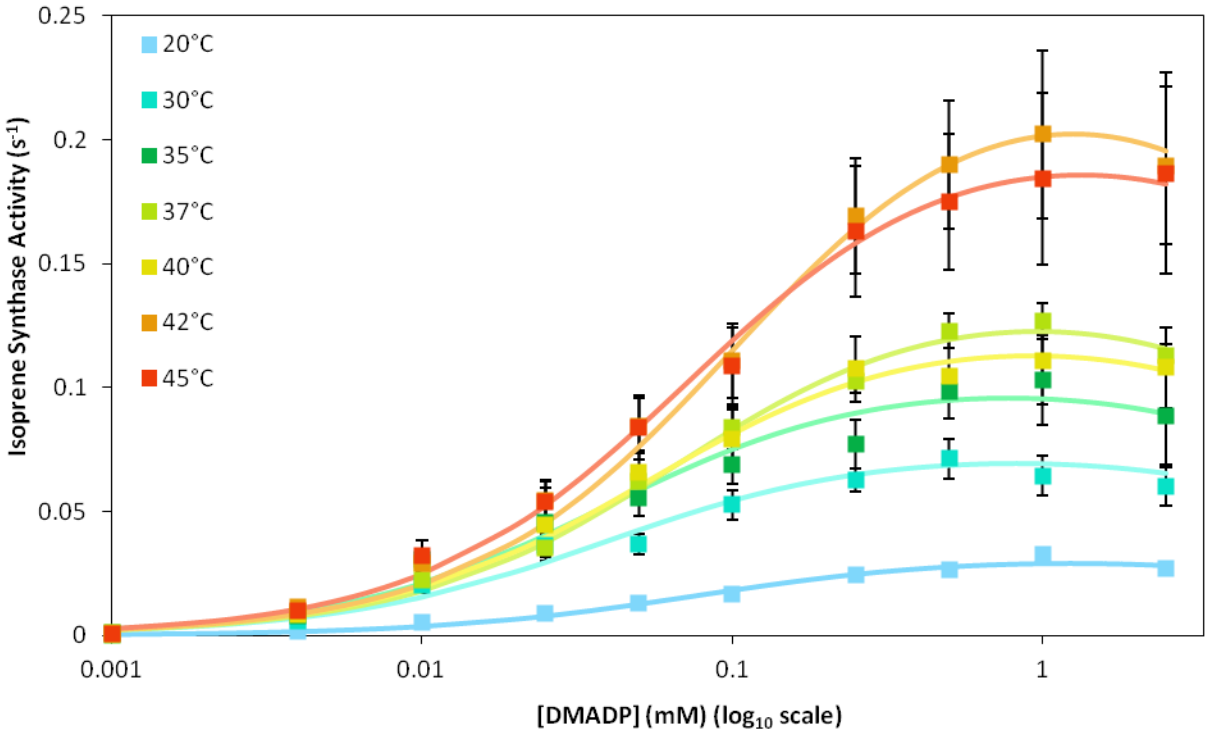

Figure 3.1: Temperature-dependent kinetics of *Eucalyptus globulus* isoprene synthase. The x-axis (DMADP concentration) is shown on a log scale to aid in seeing the differences between rates at low concentrations of substrate. Data points are averages of 3 independent trials (which themselves have 3 technical replicates at each DMADP concentration). Data are fit to modified Michaelis-Menten kinetics (Equation 3.1) using a least-squares regression.

Results

The reported measurements were made with *E. globulus* isoprene synthase. Isoprene synthases from *Robinia pseudoacacia* and *Populus alba* followed similar trends. *E. globulus*

isoprene synthase had faster turnover and a lower K_M than other isoprene synthases reported in the literature, which makes it interesting for industrial applications as well as for understanding the effects of temperatures on plants (Sharkey et al., 2013). As expected, the activity of isoprene synthase was highly temperature sensitive (Figure 3.1). At temperatures above 45°C, activity rapidly decreased, with almost no activity remaining at 50°C (data not shown). Therefore, we focused on

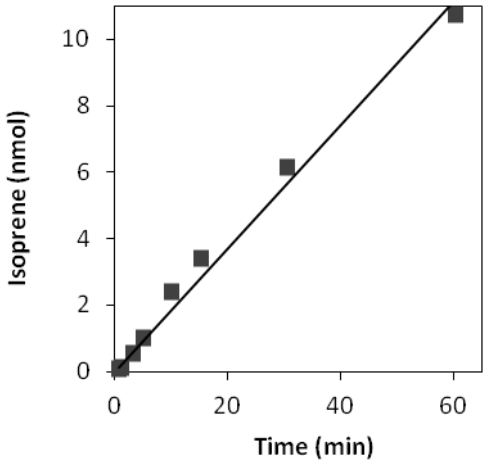

Figure 3.2: Activity of *Eucalyptus globulus* isoprene synthase over time. Activity assays were performed at 1 mM DMADP. Individual points are shown with a least-squares linear regression.

kinetics between 20 and 45°C. At all temperatures, the enzyme displayed mild substrate inhibition at 2.5 mM; this is consistent with past measurements of this enzyme, however the mechanism is unknown (Sharkey et al., 2013). To test if this effect was caused by accumulation of PP_i, a known inhibitor of isoprene synthase we considered the following. In 15-minute assays, the highest amount of isoprene released is 5 nmol. This means that the pyrophosphate concentration in solution could not be higher than 0.05 mM. Furthermore, activity is linear up to one hour at 1 mM DMADP (Figure 3.2). Therefore, it is unlikely that the observed decrease in activity at high DMADP is due to the pyrophosphate inhibition observed by Wildermuth and Fall (1998), as the concentration would be too low to cause inhibition. Because of this, the data was

fit to modified Michaelis-Menten kinetics, which includes substrate (S) inhibition (Equation 3.1) (Reed et al., 2010).

Equation 3.1 $$\textbf{Rate} = \frac{k_{cat}}{1+\frac{K_M}{[S]}+\frac{[S]}{K_I}}$$

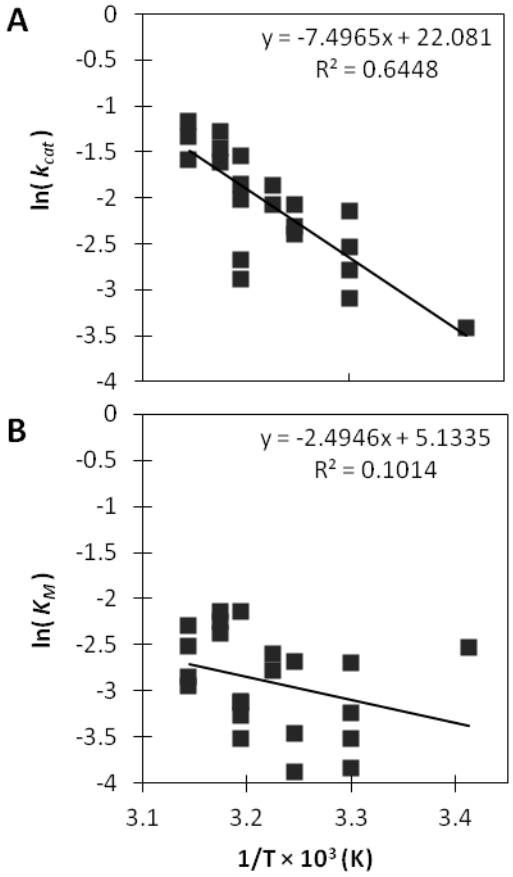

Figure 3.3: Arrhenius plots of k_{cat} and K_M. k_{cat} (A) and K_M (B) are plotted vs $\frac{1}{T}$ (in Kelvin). Individual points from each trial are shown. k_{cat} produces a linear plot that indicates it follows Arrhenius kinetics. K_M does not.

For some trials substrate inhibition was not observed at the highest concentration of DMADP used, in which case K_I would approach infinity. For these trials, the K_I was not used for further analysis. Between 20 and 45°C, k_{cat} was strongly temperature dependent and was well described by the Arrhenius equation (Figure 3.3A). Data were plotted as $\ln(k_{cat})$ vs. $\frac{1}{T}$ (Figure 3.3A). The slope and intercept of a linear regression of the data were used to calculate the activation energy

E_a = 62 kJ mol^{-1} and pre-exponential factor $A = 3.89 \times 10^9$ s^{-1}. The nonlinear fit of k_{cat} is shown in Figure 3.4A. K_M followed a surprising quadratic response to temperature (Figure 3.3B); at low temperature K_M is high and at 33°C it is at a minimum. It was not well described by the Arrhenius equation, which is different from prior studies that calculated Q_{10} for K_M (Figure 3.3B). A non-linear fit is shown in Figure 3.4B. Q_{10} values between 30 and 40°C were estimated and shown in Table 3.2. These show that K_M was more sensitive to temperature than k_{cat} for this enzyme. K_I was most weakly affected by temperature, but there was a temperature effect that

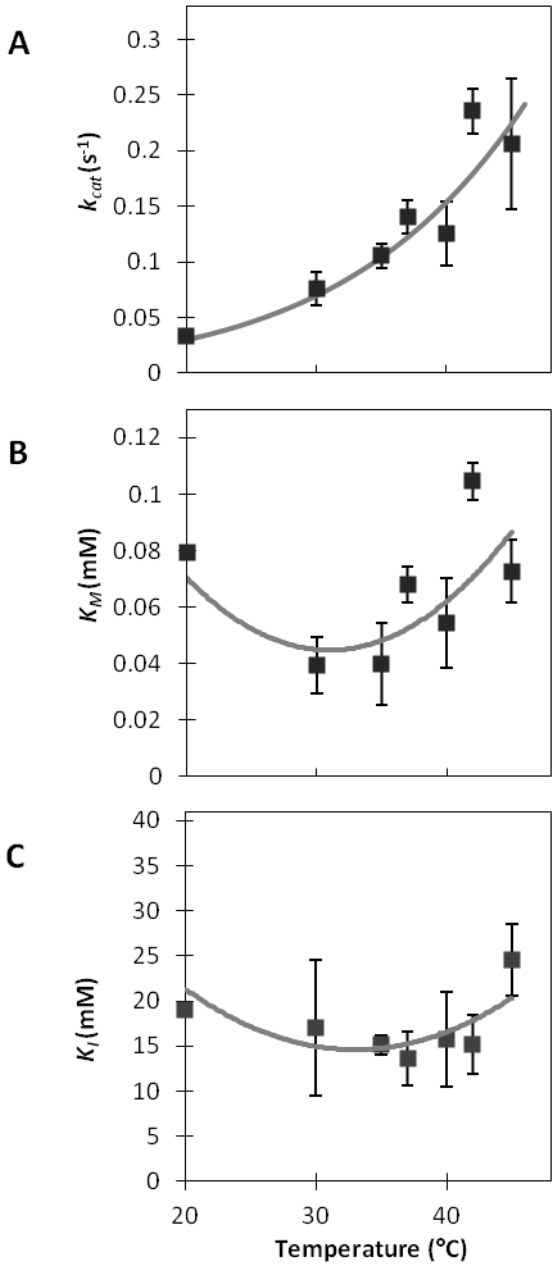

Figure 3.4: Curve fitting of modified Michaelis-Menten parameters. Averages of 3 independent trials are shown with standard error. k_{cat} (A) follows the Arrhenius equation and is shown with that fit (least-squares regression). K_M (B) and K_I (C) do not follow the Arrhenius model and are shown with a quadratic fit.

mostly mirrored the effect of K_M (Figure 3.4C). This is not surprising as these two parameters tend to be strongly correlated when fitting the data. Both K_M and K_I were fit to quadratic equation as no mechanistic equation exists to explain these responses. These equations were used to predict K_M and K_I for subsequent models.

Table 3.2: Q_{10} values for k_{cat}, K_M, and K_I between 30 and 40°C.

Parameter	Q_{10}
k_{cat}	1.66
K_M	1.38
K_I	0.93

DMADP concentration changes greatly with temperature and this change will interact with the effect of the enzyme on isoprene emission rate. There is currently no good model for how DMADP changes with temperature, and with no *in vivo* data for DMADP concentrations available for *E. globulus*, I used data from Rasulov et al. (2010) to estimate the concentration of DMADP in the chloroplasts of oak leaves. The post-illumination method is an efficient way to measure chloroplastic DMADP concentration (see Chapter II for further discussion of the post-illumination method). I converted the resultant DMADP concentrations to mM using leaf volume data from Marron et al. (2005), which included chloroplastic volume estimates. These estimates and the quadratic curve used to fit them are shown in Figure 3.5. Strictly speaking, an Arrhenius equation combined with enzyme denaturation above 35°C would be a better mechanistic fit of the data; however, because of the relatively high error of the points and the low number of data points, I was unable to make an accurate estimate using this method.

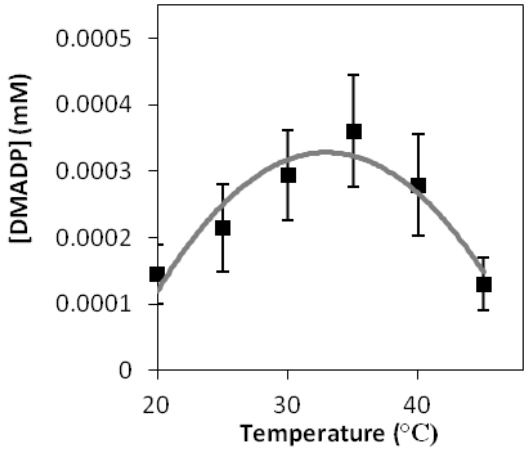

Figure 3.5: Concentration of DMADP in *Populus tremula* × *Populus tremuloides* leaves.DMADP concentrations were published in Rasulov et al. (2010) and converted to mM based on leaf volume data from Marron et al. (2005). Data are shown with a quadratic fit using least-squares regression.

The model resulting from combining estimates of DMADP, k_{cat}, K_M, and K_I is shown in Figure 3.6 (dark gray). For comparison, data from *E. globulus* and my own data from *Arabidopsis thaliana* expressing the *E. globulus* isoprene synthase are shown. Because the substrate concentration is so low, the $\frac{[S]}{K_I}$ term approaches zero. However, this is not true for K_M, which has a large effect on the modeled optimum of isoprene emission. When substrate concentration is at or less than K_M, the rate of the enzyme increases linearly with substrate concentration. In this situation, the activity is proportional to the rate k times the substrate concentration. A rough estimate of other models is possible by including only turnover rate and substrate concentration (Equation 3.2). Equation 3.2 is much better fit for the empirical isoprene emission rates shown in Figure 3.6. As such, I have included it for comparison.

Equation 3.2 $\mathbf{Rate} = \boldsymbol{k}[\boldsymbol{S}]$

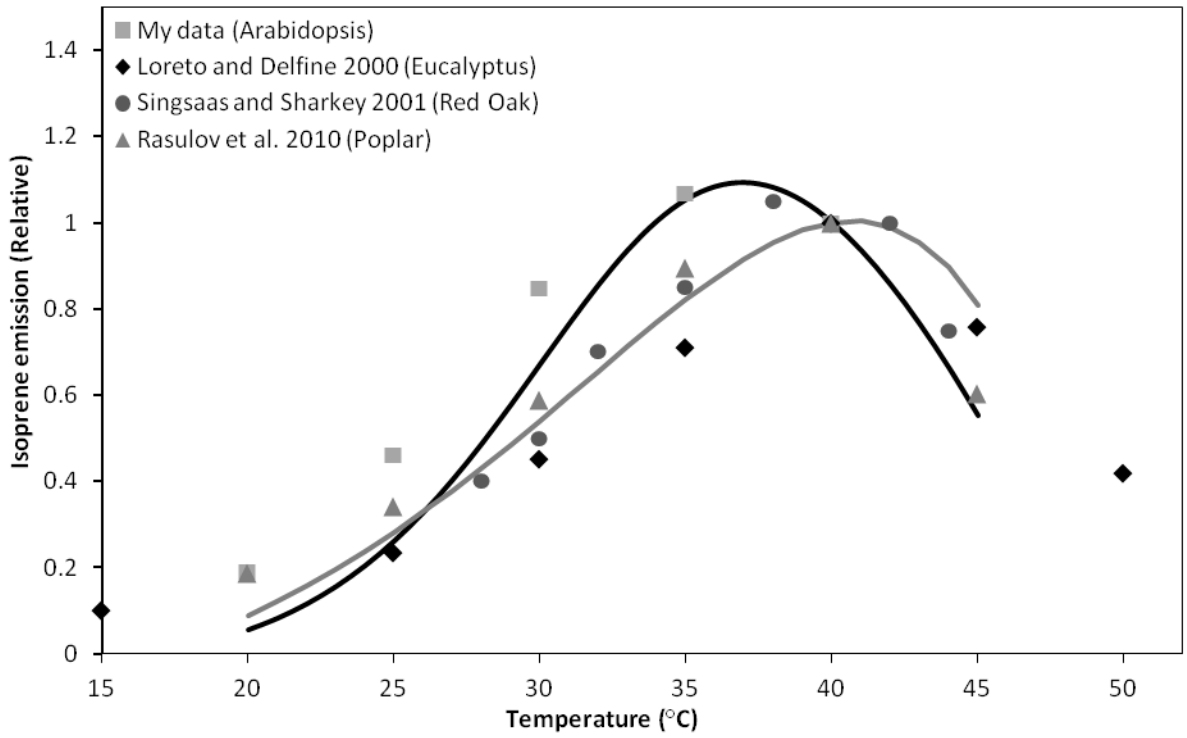

Figure 3.6: Model of isoprene emission rate based on estimated DMADP concentrations. Black line is equation 3.1 and grey line is equation 3.2. Shown for comparison are isoprene emission rates from Loreto and Delfine (2000) in *Eucalyptus globulus* (black squares), Singsaas and Sharkey (2000) in red oak (dark grey squares), and my own data from *Arabidopsis thaliana* expressing *E. globulus* isoprene synthase under the control of a RBCS promoter (light grey squares) (see chapter V). All values are shown relative to the rate at 40°C.

Discussion

Because DMADP concentrations are very low, a few hundred nanomolar, whereas K_I is very high, above 20 mM, K_I has no effect on the shape of the curve and can be safely discarded. Substrate inhibition of isoprene synthase is not significant when estimating isoprene emission rates. For most enzymes, the effect of temperature on binding coefficient is somewhat exponential - that is, the mechanism of the change in K_M is similar to the effect on k_{cat}, with low temperatures leading to an exponential increase probably related to diffusion while very high temperatures lead to denaturation (Somero, 1995). I observed a very different effect: K_M was relatively high at lower temperatures, decreased with a minimum at 33°C, and then increased again, continuing to increase past 45°C (the point at which k_{cat} and isoprene emission rates start

to decline). This means that K_M is at its optimum (the lowest point) approximately when DMADP concentration is highest (also 33°C based on my fit). At this minimum, activation energy of binding and of release of substrate must be the same. This can lead to a much higher effective isoprene emission rate than the temperature effect on k_{cat} alone would indicate. Small changes in DMADP concentration can move up this portion of the curve very quickly, greatly changing the rate. In addition, small changes in binding efficiency could potentially have a large effect, which the model of Equation 3.1 shows.

Some studies have suggested that the substrate inhibition observed in isoprene synthase is in fact product inhibition (inhibition by inorganic pyrophosphate, PP_i) (Silver, 1994; Wildermuth and Fall, 1998). To my knowledge these are the only papers looking at the mechanism of inhibition of isoprene synthase. Since the inhibition was ultimately not necessary to accurately estimate isoprene emission rates, I did not examine whether DMADP itself or PP_i was causing the inhibition observed in my assays. Furthermore only 0.05 mM PP_i builds up under my assay conditions, which is much smaller than the amount Wildermuth and Fall (1998) tested, yet I still observed inhibition, which suggests that DMADP itself is inhibiting isoprene synthase. Because PP_i is rapidly broken down in the chloroplast and has concentrations less than 1 μM (Takeshige and Tazawa, 1989), inhibition by PP_i likely does not occur *in vivo*.

If K_M and DMADP concentration alone determined the emission rate, the optimal rate would be at 33°C. The optimal rate is approximately 37 - 40°C depending on species.

Conclusion

Equation 3.2 - where rate is assumed to be completely linear with substrate concentration, but not with temperature - fits the empirical data much better than any equation that includes K_M. This is true for the native emitters, although not transgenic *Arabidopsis*, which is more similar to Equation 3.1 model. This work has confirmed that the literature based on V_{max} alone is a valid

way to model isoprene emission rates. This is much simpler, particularly when it comes to *in vivo* measurements or attempting to model a large number of species, which is necessary for accurate global models of isoprene emission. Modeling K_M and K_I is not necessary in order to understand isoprene emission, and developing a better understanding of k_{cat} and DMADP concentrations in response to different environmental conditions will greatly further modeling efforts.

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Chapter 4 Engineering isoprene synthase for improved activity

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Abstract

Terpene synthases are economically valuable enzymes that catalyze an immense array of unique chemistries. However, the carbocation translocation mechanism that makes them so versatile leads to slow catalytic rates with high sensitivity to perturbation by metal ion concentration or attempts to modify activity through synthetic biology. In this work, we seek to improve a TPS-b terpene synthase, isoprene synthase (ISPS), in two ways: by fusing it to an additional protein, and by encapsulating it in a bacterial microcompartment. These approaches are potentially synergistic methods to harness increased local substrate concentrations through substrate channeling and to improve enzymatic stability and solubility under varied conditions. Inserting GFP into the middle of the ISPS sequence had a mild positive impact on catalytic turnover (0.37 s^{-1} over 0.29 s^{-1}).

Introduction

Terpene synthases (TPS) are a broad family of enzymes that produce a wide range of economically important compounds that are used for fragrances, flavorings, medicines, and biofuels. Terpene synthases produce the carbon backbones of terpenoids from allylic diphosphate compounds dimethylallyl diphosphate (DMADP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranyl geranyl diphosphate (GGPP), which are in turn produced by the mevalonic acid (eukaryotic/plant cytosolic) or MEP (prokaryotic/chloroplastic) pathways. While all species produce terpenoids, plants produce the largest quantities and variety. Plant terpene synthases are an area of active study in synthetic biology due to their wide range of functions and potential applications. About half of terpene synthases produce a single product, whereas others produce multiple products, potentially 50 or more products in different reaction cycles (Degenhardt et al., 2009).

Terpene synthases are often characterized as "bad enzymes". They have a low k_{cat}, often less than 1 s^{-1}, and high K_M, around 0.001 mM for monoterpene synthases but as high as 20 mM for methylbutenol synthase (MBOS) (Gray et al., 2011; Hyatt et al., 2007; Silver and Fall, 1995). Terpene synthases are often inhibited by the substrate DMADP or the product pyrophosphate, as well as excess Mg^{2+} or Mn^{2+} (Gray et al., 2011; Sharkey et al., 2013; Silver, 1994; Wiberley et al., 2005; Wildermuth and Fall, 1998; Wolfertz et al., 2003). All of these characteristics are inherent in the reaction mechanism. Class I terpene synthases, which are the majority of known plant enzymes in this family, proceed via a carbocation intermediate, which can then be quenched by water or hydrogen to produce the terpene skeleton (Chen et al., 2011; Degenhardt et al., 2009). The substrate can also react with the enzyme itself, deactivating it (Kersten et al., 2015). Class II terpene synthases proceed through protonation-induced cyclization, which has a faster reaction rate and tighter binding. Characteristics of Class I and II terpene synthases were described previously in Chapter I. While modification of the active site did result in altered product specificity or products (Xu et al., 2007; Yoshikuni et al., 2006), improvement of the enzyme activity or a reduction of substrate inhibition has not been achieved so far. In particular, even if a large amount of substrate is produced, the substrate inhibition and low activity mean that the terpene synthase itself remains a substantial bottleneck for terpenoid production.

One method for improving efficiency of “bad enzymes” is substrate channeling, or using a synthetic scaffold to bring proteins into close quarters in order to improve diffusion-limited kinetics or increase concentration of substrate for enzymes with a low K_M (Dueber et al., 2009; Gonzalez-Esquer et al., 2016). This involves placing two proteins involved in adjacent steps of a biosynthetic pathway on the same scaffold or even combining them into a single peptide strand. This method has not been applied in terpene synthases. In particular, modifying either end of a

terpene synthase (particularly the N terminus) greatly reduces activity by interfering with the RRSANY active site motif of TPS-b's (Sharkey et al., 2005). We have found that even placing short peptide tails to the C terminus reduces activity and solubility. Therefore, combining entire proteins using protein-protein interaction peptides or expressing them as a single fusion peptide would not be effective.

Bacterial microcompartments (BMCs) are protein capsules expressed in many bacterial species, including cyanobacteria and some *Escherichia coli* and *Bacillus subtilis* strains (Yeates et al., 2010). BMCs are used to concentrate substrates, as in the case of the cyanobacteria carbon concentrating carboxysomes, to protect the cell from toxic intermediates, and to prevent volatile products from leaving the cell (Kerfeld and Erbilgin, 2015; Kerfeld et al., 2010; Yeates et al., 2010). They are common in aldehyde metabolism (Kerfeld and Erbilgin, 2015). BMCs are highly stable, modular, and have a known assembly, which makes them ideal as scaffolds for synthetic biology (Aussignargues et al., 2015b; Cai et al., 2016; Cai et al., 2015; Cameron et al., 2013; Gonzalez-Esquer et al., 2016; Gonzalez-Esquer et al., 2015; Lassila et al., 2014; Lawrence et al., 2014). Compartments can be modified to produce different structures or to control what can pass through the pores (Aussignargues et al., 2015b; Cai et al., 2015). Novel pathways can be localized to the compartments using known peptide targeting sequences, and the compartments can be expressed in *E. coli*, cyanobacteria, and possibly even in plant cells for production of industrially important compounds (Aussignargues et al., 2015a; Gonzalez-Esquer et al., 2016; Hagen et al., 2018; Lawrence et al., 2014; Quin et al., 2016). We have encapsulated ISPS in a simplified *Haliangium ochraceum* BMC using a split bacterial adherin targeting approach (Hagen et al., 2018; Zakeri et al., 2012).

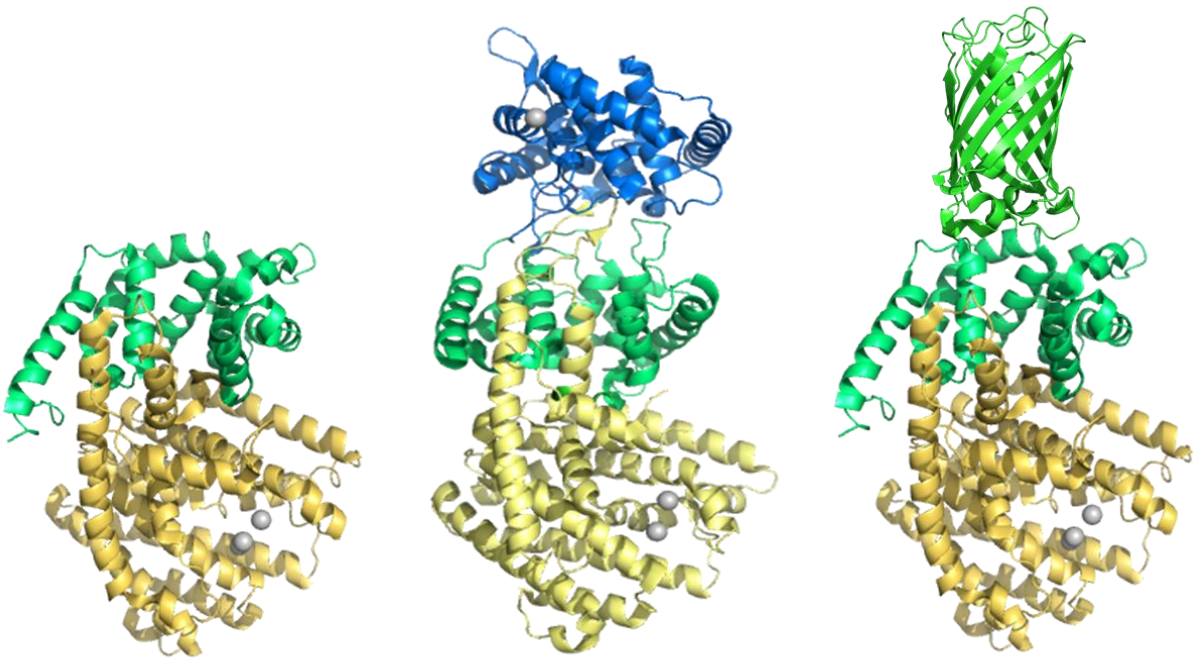

Figure 4.1: Structures of the isoprene synthase constructs. Shown are the two-domain isoprene synthase (Koksal et al., 2010), three-domain taxadiene synthase (McAndrew et al., 2011), and our novel ISPS-GFP construct (from homology modeling). The active site is denoted by the bound magnesium ions, in grey.

Class II terpene synthases are three domain proteins (Figure 4.1), described as αβγ with the cyclase active site in the βγ fold (McAndrew et al., 2011). However, class I TPS-b, TPS-d, and TPS-a terpene synthases are only two domain proteins, having lost the third cyclase domain and having the active site in the αβ fold. These enzymes produce a large range of terpene products, including isoprene, methylbutenol, and many monoterpenes, sesquiterpenes, and diterpenes. We inserted the sequence of Green Fluorescent Protein (*GFP*) and the *Escherichia coli* HMBDP reductase (*LytB*) at the position of the γ domain in the ISPS and methylbutenol synthase (*MBOS*) sequences to test whether it is possible to introduce a fusion at this position without disrupting the activity of the terpene synthases. ISPS is in the TPS-b subfamily and MBOS is in the TPS-d subfamily. We tested the activity of the novel constructs and the response of the ISPS construct to various physiological parameters.

Methods

Plasmid construction

Codon optimized genes were ordered from Genscript for *Populus tremuloides ISPS* , *Escherichia coli LytB*, and *Picea abies MBOS*. *LytB* codes for an enzyme involved in DMADP production in *E. coli* (Altincicek et al., 2002). Using Gibson Assembly, the genes for *ISPS* and *MBOS* were cloned into the pD881 plasmid. The genes were then further modified by placing the sequence of *GFP*, with a 5x GS linker on each side at the position of the missing third domain after amino acid 47 (Figure 4.2). The constructs were also modified with a C-terminus cleavable sumo tag, to improve solubility and ease of purification. A second ISPS construct was made by inserting the *LytB* sequence, also with a 5x GS linker on each side. ISPS-GFP was also modified with the addition of a Spy tag, which allows it to bind to Spycatcher (Zakeri et al., 2012). The Kerfeld lab had previously modified *Haliangium ochraceum* (HO) bacterial microcompartment BMC-T2 component to have the Spycatcher sequence in the compatible pET11n plasmid (Hagen et al., 2018).

Purification of untagged proteins

Separate BL21 *E. coli* strains were transformed with each plasmid created above. An additional strain was transformed with both the modified spycatcher HO shell proteins and Spy-ISPS-GFP. The *E. coli* was grown at 37°C to an OD of 0.6-0.8, induced with 1 mM rhamnose and continued growing at 18°C overnight. Cells were suspended in purification buffer A (50 mM Tris, 10% glycerol) and lysed with a French press. The clarified supernatant was eluted from a QHD anion exchange column followed by further purification on a size exclusion column. Protein purity was determined using SDS-PAGE and protein concentration was determined using the Lowry Assay.

```
ISPS-GFP MARRSANYEP NSWDYDYLLS SDTDESIEVY KDKAKKLEAE VRREINNGSG 50
    ISPS MARRSANYEP NSWDYDYLLS SDTDESIEVY KDKAKKLEAE VRREINN--- 47

ISPS-GFP SGSGSGSSKG EELFTGVVPI LVELDGDVNG HKFSVSGEGE GDATYGKLTL 100
    ISPS ---------- ---------- ---------- ---------- ---------- 47

ISPS-GFP KFICTTGKLP VPWPTLVTTL TYGVQCFARY PDHMKQHDFF KSAMPEGYVQ 150
    ISPS ---------- ---------- ---------- ---------- ---------- 47

ISPS-GFP ERTIFFKDDG NYKTRAEVKF EGDTLVNRIE LKGIDFKEDG NILGHKLEYN 200
    ISPS ---------- ---------- ---------- ---------- ---------- 47

ISPS-GFP YNSHKVYITA DKQKNGIKVN FKTRHNIEDG SVQLADHYQQ NTPIGDGPVL 250
    ISPS ---------- ---------- ---------- ---------- ---------- 47

ISPS-GFP LPDNHYLSTQ SALSKDPNEK RDHMVLLEFV TAAGITLGMD ELYKGSGSGS 300
    ISPS ---------- ---------- ---------- ---------- ---------- 47

ISPS-GFP GSRSEKAEFL TLLELIDNVQ RLGLGYRFES DIRGALDRFV SSGGFDAVTK 350
    ISPS --RSEKAEFL TLLELIDNVQ RLGLGYRFES DIRGALDRFV SSGGFDAVTK 95

ISPS-GFP TSLHGTALSF RLLRQHGFEV SQEAFSGFKD QNGNFLENLK EDIKAILSLY 400
    ISPS TSLHGTALSF RLLRQHGFEV SQEAFSGFKD QNGNFLENLK EDIKAILSLY 145

ISPS-GFP EASFLALEGE NILDEAKVFA ISHLKELSEE KIGKELAEQV NHALELPLHR 450
    ISPS EASFLALEGE NILDEAKVFA ISHLKELSEE KIGKELAEQV NHALELPLHR 195

ISPS-GFP RTQRLEAVWS IEAYRKKEDA NQVLLELAIL DYNMIQSVYQ RDLRETSRWW 500
    ISPS RTQRLEAVWS IEAYRKKEDA NQVLLELAIL DYNMIQSVYQ RDLRETSRWW 245

ISPS-GFP RRVGLATKLH FARDRLIESF YWAVGVAFEP QYSDCRNSVA KMFSFVTIID 550
    ISPS RRVGLATKLH FARDRLIESF YWAVGVAFEP QYSDCRNSVA KMFSFVTIID 295

ISPS-GFP DIYDVYGTLD ELELFTDAVE RWDVNAINDL PDYMKLCFLA LYNTINEIAY 600
    ISPS DIYDVYGTLD ELELFTDAVE RWDVNAINDL PDYMKLCFLA LYNTINEIAY 345

ISPS-GFP DNLKDKGENI LPYLTKAWAD LCNAFLQEAK WLYNKSTPTF DDYFGNAWKS 650
    ISPS DNLKDKGENI LPYLTKAWAD LCNAFLQEAK WLYNKSTPTF DDYFGNAWKS 395

ISPS-GFP SSGPLQLVFA YFAVVQNIKK EEIENLQKYH DTISRPSHIF RLCNDLASAS 700
    ISPS SSGPLQLVFA YFAVVQNIKK EEIENLQKYH DTISRPSHIF RLCNDLASAS 445

ISPS-GFP AEIARGETAN SVSCYMRTKG ISEELATESV MNLIDETWKK MNKEKLGGSL 750
    ISPS AEIARGETAN SVSCYMRTKG ISEELATESV MNLIDETWKK MNKEKLGGSL 495

ISPS-GFP FAKPFVETAI NLARQSHCTY HNGDAHTSPD ELTRKRVLSV ITEPILPFER 800
    ISPS FAKPFVETAI NLARQSHCTY HNGDAHTSPD ELTRKRVLSV ITEPILPFER 545
```

Figure 4.2: Peptide sequence of *Populus tremuloides* ISPS and ISPS-GFP.

Purification of sumo tagged proteins

Cells were suspended in purification buffer B (5 mM KCl, 50 mM $MgCl_2$, 5% glycerol, 0.02% sodium azide, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM tris(2-carboxyethyl)phosphine) and lysed with a French press. The clarified supernatant of the sumo-tagged proteins were run through a strep tag affinity column followed by cleavage overnight with Ubl-specific protease 1 (ULP1) at 4°C (Thermo-Fisher Scientific). The purified protein was then run on the strep tag affinity column a second time to remove any remaining ULP1 or sumo tag in

order to give completely pure protein. Protein purity was determined using SDS-PAGE and protein concentration was determined using the Lowry Assay.

Purification of intact bacterial microcompartments

Bacterial microcompartment shells were purified according to Hagen et al. (2018). In brief, a construct containing HO shells comprising a single hexamer and three trimer homologs (HT1T2T3 shells) was expressed in *E. coli* with or without the Spy-ISPS-GFP construct. Clarified lysates were produced as above, and mixed with lysates of a separate strain expressing P_{SII} (the pentamer with strep tag). Shells were then pulled down using Strep-Trap followed by anion exchange to remove any impurities. The Spycatcher was present on only T2. This corresponds to approximately Spy-ISPS-GFP being attached on approximately 1/13 of the shell proteins.

Measurements of activity

Protein solutions were diluted such that approximately 10 pmol of protein were used per assay. DMADP was obtained from isoprenoids.com and dissolved in 2 mM ammonium bicarbonate, pH 7.8. Assays were performed by mixing 10 µL protein solution with assay buffer 1 (50 mM HEPES pH 8, 10 mM $MgCl_2$, 20 mM KCl, 10% glycerol v/v) and DMADP to a final volume of 100 µL in a 2 mL gastight vial. Extracts were then incubated for 15 minutes in a 40°C waterbath (except where stated otherwise). One mL of headspace was pulled from the vial simultaneously as 1 mL of water was injected (in order to prevent creating a vacuum). The amount of isoprene in the headspace sample was immediately measured on the Fast Isoprene Sensor (FIS) (Hill Scientific). For measurements where DMADP was held constant, a final concentration of 1 mM DMADP was used as this concentration yielded the highest activity for most extracts. When testing the effect of pH on activity of the novel ISPS constructs, assay

buffer was made up with MES (pH 6 and 6.5) or HEPES (pH 6.5 to 8.4). To test the activity of the novel ISPS constructs under different metal ion concentrations, assay buffer was made up without metal ions (the untagged proteins purified without metal ions present were used for these assays) and the necessary concentration of metal salts was added directly to the assay mixture. For measurements of isoprene, the Fast Isoprene Sensor was used as described in Guenther and Hills (1998). For measurement of methylbutenol, the temperature of the reaction cell was increased to 70°C. At this temperature the instrument is more sensitive to methylbutenol. The MBOS extracts were also tested using the standard instrument configuration to ensure that they were not making isoprene.

Statistical analysis

For each construct three independent (separate enzyme purifications) trials were performed to determine activity and response to DMADP; each trial had itself three technical replicates at each DMADP concentration. A representative trial is shown. For response to temperature, pH, and ion concentration only one trial was performed. Averages, standard errors, and graphs were prepared with Excel 2007 (Microsoft Corporation). Non-linear curve fitting was performed using a least-squares regression using Origin 8 (OriginLab Corporation). DMADP data is fit to equation 3.1.

Results

Insertion of GFP into the ISPS sequence

To test whether it is possible to introduce a sequence at the position of the missing third domain without disrupting the activity of ISPS, we spliced GFP in to this location of *Populus tremuloides ISPS*. This *ISPS* construct had previously had its chloroplast targeting peptide removed and was known to express well in *E. coli*. One downside of this construct was

decreased solubility. This is likely because GFP itself is highly hydrophobic. We overcame the solubility issues by combining the *ISPS* construct with a sumo-tag purification system, which greatly improved solubility. While the sumo tag did not modify ISPS activity (data not shown), to ensure that the results were biochemically identical to the native enzymes, we removed the tag from ISPS and ISPS-GFP constructs using ULP1, which leaves a scarless cleavage site. Across all trials we found that the modified ISPS-GFP had a k_{cat} of 0.37 s^{-1} and a K_M 0.18 mM, which is superior to the unmodified ISPS values of 0.29 s^{-1} and 0.19 mM (Table 4.1). When testing the unpurified enzymes, ISPS displayed severe substrate inhibition and ISPS-GFP displayed no substrate inhibition (data not shown); however, this effect disappeared after the enzymes were prepared using the sumo purification, despite the fact that they should be biochemically identical. In the final trials, ISPS and ISPS-GFP showed identical mild substrate inhibition effects (Figure 4.3). Because substrate inhibition is closely tied with the Michaelis-Menten equation, it is difficult to say that the k_{cat} and K_M reported here are the "true" values. In fact, when varying substrate inhibition was observed, the apparent K_M of ISPS-GFP was 0.08, and that of ISPS was 0.36, although the turnover rate could not be determined due to the uncertainty in the true protein concentration.

Table 4.1: Kinetic parameters of ISPS and ISPS-GFP

	ISPS	ISPS-GFP
K_M (mM)	0.19	0.18
k_{cat} (s^{-1})	0.29	0.37
K_I (mM)	9.03	11.12

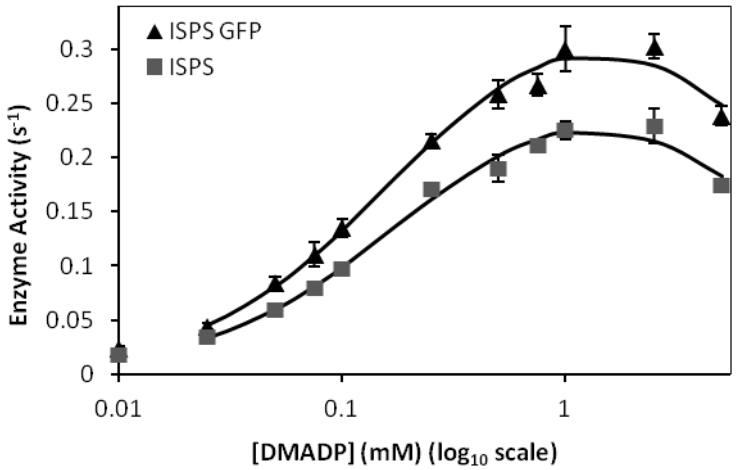

Figure 4.3: Activity of the ISPS-GFP construct compared to the unmodified ISPS. ISPS and ISPS-GFP were sumo tagged purified proteins. Due to low solubility of untagged isoprene synthase and ISPS-GFP protein, isoprene synthase and ISPS-GFP with the sumo tag were used in this experiment, however in crude extracts it showed similar activity (concentration estimated by western blot) and no substrate inhibition. Averages with standard error shown (n=3).

High levels of Na^+ and K^+ had an inhibitory effect on the ISPS-GFP fusion protein (Figure 4.4A), which has been reported for ISPS in the past (Lantz et al., 2015; Schnitzler et al., 1996; Silver and Fall, 1995). The enzyme was able to use Mg^{2+} or Mn^{2+}, although highest activity was detected at 10 mM Mg^{2+} (Figure 4.4B); the native *P. tremuloides* ISPS is able to use either but is reported to have higher activity with Mn^{2+} (Silver and Fall, 1995). High concentrations of Mn^{2+} or Mg^{2+} did not inhibit ISPS-GFP activity (Figure 4.4B), contrary to what was previously reported for the native enzyme (Silver and Fall, 1995). ISPS-GFP was more sensitive to pH than ISPS (Figure 4.4C), with higher activity between 7.5 and 8 and lower activity at low pH values. ISPS-GFP was less sensitive to temperature than ISPS (Figure 4.4D), with better activity at low temperatures.

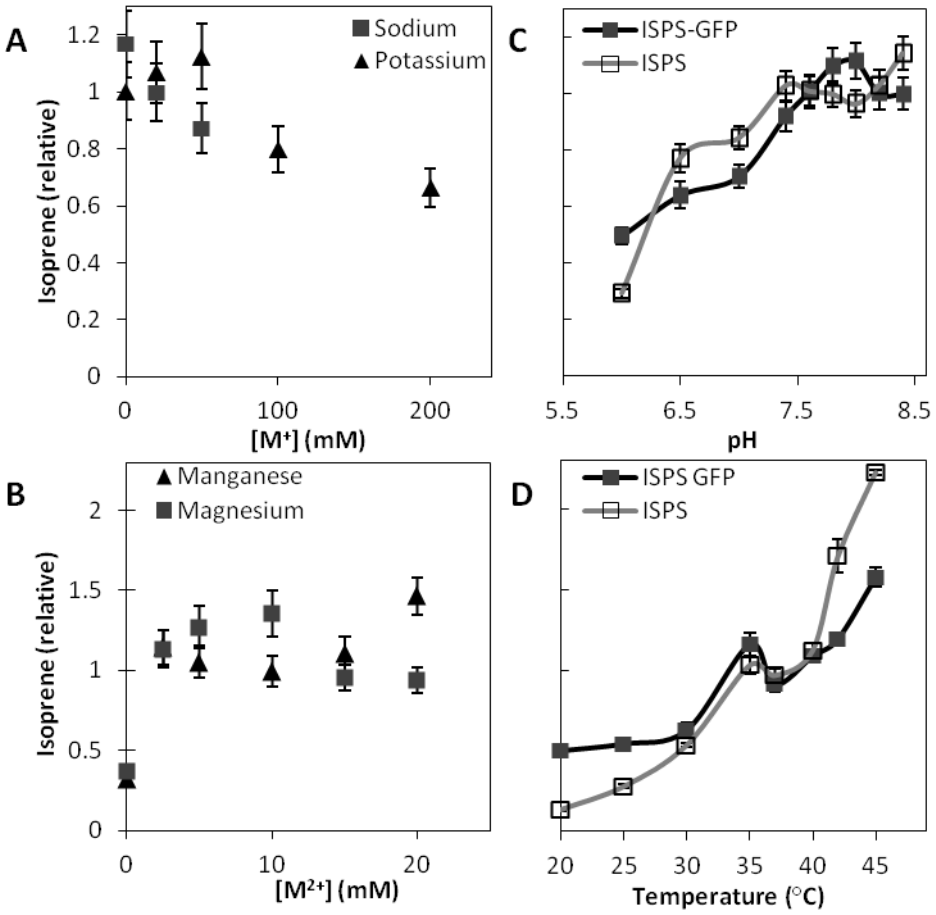

Figure 4.4: Response of the novel ISPS-GFP construct to various physiological parameters. Response of ISPS-GFP to increasing sodium and potassium ion concentrations (relative to 0 mM) (A) and manganese and magnesium ion concentrations (relative to 0 MM) (B), response of ISPS-GFP and ISPS to varying pH (relative to 8) (C) and temperature (relative to 40) (D) is presented. Data were collected with untagged, unpurified ISPS and ISPS GFP. Averages with standard error shown (n=3).

Additional constructs

In addition to ISPS and ISPS-GFP constructs, we created similar constructs with other combinations. We combined GFP with *Picea abies* MBOS and using the Fast Isoprene Sensor confirmed that this construct was able to produce methylbutenol - the counts per second was the same as unmodified MBOS under the same conditions and four-fold higher than a bacterial lysate containing no terpene synthase genes. However, MBOS-GFP was highly insoluble and we were unable to purify and further characterize it. In addition, the response of the FIS is non-linear

to MBOS so the actual rate of MBO production is unknown. We also combined ISPS with LytB. LytB produces DMADP, the precursor to isoprene, from HMBDP (Altincicek et al., 2002). The ISPS-LytB construct was able to produce isoprene at similar rates as ISPS alone, although LytB was inactive. A longer linker may have allowed LytB to fold and retain activity.

Encapsulation of ISPS in a BMC

Bacterial microcompartments that were expressed in a strain that did not contain the *ISPS* gene produced no isoprene. The modified spycatcher BMCs were expressed alongside spy-tagged ISPS-GFP construct in *E. coli* and purified. The resulting suspension produced isoprene in a DMADP-dependent manner (Figure 4.5). We estimated that there were between 60 and 70 ISPS proteins per BMC; this means that approximately 1/13 of the total protein is ISPS by mass. We used this to estimate k_{cat} for the encapsulated ISPS as well as for the unencapsulated Spy-ISPS-GFP. The apparent activity of the encapsulated protein was substantially lower than the unencapsulated protein, and it was not as sensitive to substrate inhibition (Table 4.2). We were unable to determine the actual concentration of DMADP inside the shell, or even whether ISPS was oriented towards the inside of the shell or the outside. The kinetic parameters listed in Table 4.2 are assuming that the DMADP concentration inside the shell is the same as the concentration in the assay solution; however, this is likely not the case.

Table 4.2: Kinetic parameters of Spy-ISPS-GFP (unencapsulated and unencapsulated)

	Unencapsulated	Encapsulated
K_M (mM)	0.3	0.08
k_{cat} (s^{-1})	0.9	0.05
K_I (mM)	0.3	21

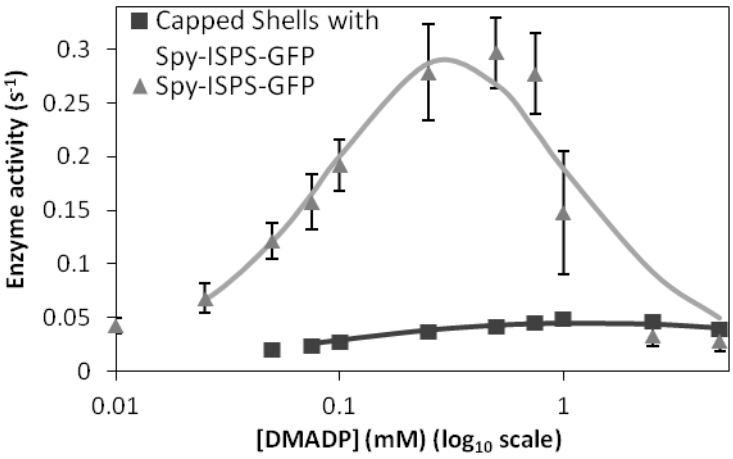

Figure 4.5: Substrate dependence of ISPS encapsulated in BMCs. Shells containing Spy-ISPS-GFP (dark grey) and substrate dependence of Spy-ISPS-GFP alone (light grey) are shown. Average of 3 technical replicates with standard error is shown. Data are normalized to the estimated concentration of Spy-ISPS-GFP.

Discussion

Engineering a three-domain Class I TPS

Re-introducing a third domain to ISPS and MBOS did not decrease activity, but marginally improved the turnover rate of ISPS. This makes it a viable approach for engineering terpene synthases even if a third domain is not desired for its other biochemical utility. Interestingly, we found the K_M of the unmodified enzyme to be much lower than that reported in the literature for protein extracted from leaf tissue, although similar to other TPS expressed in *E. coli* (Sharkey et al., 2013). This effect has been seen in the past, and may indicate that there is post-translational modification of ISPS in the plant leaves that decreases substrate affinity (Schnitzler et al., 2005). Because we are comparing enzymes that were produced and purified with identical methods at the same time, the conclusions about the effect on activity should still hold true for other extracts prepared in *E. coli*. However, if it is desired to move to a different platform such as plant leaves, the activities should be measured again to ensure that the observed increase in activity remains true under varying environmental conditions. As for the difference in the substrate inhibition between purified and unpurified extracts, there is a potential explanation. Some researchers have

proposed the substrate inhibition observed in ISPS is in fact product inhibition by pyrophosphate (PP_i) (Silver, 1994; Wildermuth and Fall, 1996). PP_i is rapidly broken down in chloroplasts, but was not removed from our assays. Without sumo, ISPS-GFP is very insoluble and the true protein concentration and apparent activity (total isoprene production) was lower than in the ISPS extracts. This means that the total concentration of PP_i may have been lower than in the ISPS or sumo-purified extracts, which is why less "substrate" inhibition was observed.

Rational engineering of terpene synthases is a relatively new field, despite decades of effort into elucidating pathways for, and improving production of, terpenoids in synthetic biology. To date most efforts have been in adjusting the expression levels of terpene synthases and the MEP or MVA pathway for optimum production (Tashiro et al., 2017; Tashiro et al., 2016; Wang et al., 2017; Ye et al., 2016). While the gains in activity were very slight, this research has a wide variety of potential applications in synthetic biology. The modifications shown here could potentially be used with any TPS-a, TPS-b, or TPS-d1 or d2 proteins, which encompass a large portion of terpene biochemistry. The inserted protein can be any protein where both tails are on the same side of a globular protein, like with GFP, or even when this is not the case when adapted with longer linkers. This could be used to combine biosynthetic steps in multiple ways, potentially opening up new chemistries by channeling substrates in directions that would otherwise be unfavored. It can also improve existing reaction rates for higher production of valuable compounds.

Encapsulation of ISPS in a BMC

We were able to encapsulate ISPS in a BMC using a spy-tag/spycatcher split bacterial adhesin approach. The encapsulated ISPS was able to produce isoprene and increased DMADP increased the production of isoprene. We were not able to determine the orientation of ISPS,

whether inside or outside the compartment, nor the DMADP concentration inside the compartment, which is an area for further study. However, the low activity and lack of substrate inhibition may indicate that ISPS was in the interior of the compartment and that DMADP flowed into the compartment, but at a very low rate. Due to the high electropositivity of the pores in the BMC structure we chose for this experiment, we would expect DMADP to enter the compartment freely, but a low effective concentration inside the compartment may still occur. In future work we would like to also encapsulate the precursor protein LytB to take advantage of substrate channeling and further improve activity.

Author Contributions

TDS and CK conceived the experimental plan. RG and AT produced the constructs. AT and ATL purified protein. ATL performed assays and data analysis. ATL wrote the chapter.

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Chapter 5 Measurements of MEP pathway metabolites and effect of calcium on isoprene emission

Abstract

A mechanistic understanding of isoprene emission requires understanding how both the methylerythritol 4-phosphate (MEP) pathway and isoprene synthase change in response to changing environmental conditions. This understanding can improve models of isoprene emission, lead to new hypotheses about the physiological role of isoprene in plants, and inform efforts to produce isoprene in plants and bacteria. I report the possibility that calcium signaling plays a role in control of isoprene emission under high CO_2. I show that the concentrations of early MEP pathway metabolites (1-deoxy-D-xylulose-5-phosphate [DXP] and MEP) do not change under changing conditions or between transgenic emitting and non-emitting plants.

Introduction

The significant effect of isoprene on atmospheric chemistry and plant resilience to changing environmental conditions makes it critical that we understand how isoprene emission changes in response to those conditions (Lantz et al., 2019). Increased CO_2 and temperature and fluctuations in light and temperature due to sunflecks or other mechanisms may overall increase isoprene emission, but to fully understand these changes and the difference between long-term and short-term changes, a mechanistic understanding is required. In Chapter 2 I showed that the sensitivity of isoprene emission to CO_2 is not caused by changes in electron transport or ATP production. While electron transport still explains the change in isoprene emission in response to other stimuli, such as changing light conditions (Laffineur et al., 2013; Morfopoulos et al., 2013; Rasulov et al., 2009a; Rasulov et al., 2009b; Way et al., 2011; Wildermuth and Fall, 1996), a new hypothesis is necessary in order to explain the response to CO_2.

The difference between isoprene synthase activities in protein extracted from plant leaves and versus that extracted from *E. coli* (see Chapter 3) may also indicate some post-translational modification. Scholefield et al. (2004) showed that an increase in growth CO_2 concentration

decrease isoprene synthase activity, which may also indicate modification of the enzyme. Alternatively, isoprene synthase may exist in different pools, membrane bound and soluble (Wildermuth and Fall, 1998; Wildermuth and Fall, 1996). Release from one pool to another in response to changes in environmental conditions (that may alter membrane stability) could lead to the apparent change in isoprene synthase activity.

Isoprene emission changes very rapidly in response to wounding, changes in CO_2, and temperature, with short-term responses occurring within seconds. Loreto and Sharkey (1993) showed that isoprene emission can be induced or lowered by transmissible wound signals. We found that *Glycine soja* has a putative isoprene synthase that matches all known characteristics for isoprene synthases (Sharkey et al., 2013). However, under growth conditions of 25°C day temperature, *G. soja* does not emit isoprene. When a remote leaf or section of a leaf is burned, the unburned leaves produce a burst of isoprene. This was previously seen in *Peuraria montana* (Joshua Allman, unpublished data). In *P. montana*, the effect was reduced by feeding 5 mM EGTA, but not 5 mM EDTA. EGTA specifically chelates calcium, while EDTA chelates other metal ions but not calcium. This may indicate that calcium signaling is involved in these isoprene bursts, and calcium signaling could lead to modification of isoprene synthase or release of isoprene synthase from insoluble pools. Increased temperature could also cause this change, which could explain why the kinetics of the enzyme and concentration of substrate dimethylallyl diphosphate (DMADP) alone were not a perfect fit to the temperature sensitivity of isoprene emission in chapter 3.

The data presented in Chapter 2 only indicates the size of the 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP) and DMADP pools, so it may have missed a change in a metabolite pool that could cause the rapid changes in isoprene emission because the post-illumination

technique. Changes early in the methylerythritol 4-phosphate (MEP) pathway would support the hypothesis that carbon or ATP is limiting. If the MEP pathway metabolites do not change significantly, it may be that the control is at isoprene synthase itself. Rapid changes in metabolite concentration could also explain the post-burning burst in *G. soja*.

If either NADPH availability or ATP/CTP availability is responsible for the changes in isoprene emission under elevated CO_2, then distinct patterns in the concentrations of MEP pathway metabolites should be visible. If reducing power (electron availability) is limiting, then MEcDP should build up, as 4-hydroxy 3-methylbut-2-enyl-diphosphate (HMBDP) synthase (HDS) and HMBDP reductase (HDR) are extremely dependent on reducing power availability. It is also possible to see DXP build up and all downstream metabolites decrease in concentration as the first committed step requires NADPH. If ATP or CTP is limiting then an increase in MEP and depletion of downstream metabolites would be expected. Therefore, measuring the concentration of MEP pathway metabolites can help explain what mechanism limits flux through isoprene synthase.

In this chapter, I present the concentration of MEP pathway metabolites under different CO_2 and temperature conditions, during the post-burning burst of *G. soja*, and in the transgenic emitters *Nicotiana tabacum* and *Arabidopsis thaliana*. I also report the effect of EGTA on the CO_2 sensitivity of isoprene emission. I show that it is not necessary for the concentrations of the MEP pathway metabolites to change in order for isoprene emission to change, and I propose that post-translational modifications in isoprene synthase activity may explain these results.

Materials and Methods

Plant material

Nicotiana tabacum cv. Samsun NN (tobacco) was grown as described in Chapter 2.

Glycine soja seeds were obtained from Felix Fritchi (University of Missouri). Seeds were planted in Suremix growing medium (Michigan Grower Products, MI, USA) in 10 L pots and grown in a growth chamber (Big-Foot, BioChambers), under a 16 h photoperiod at a light intensity of 400 µmol m^{-2} s^{-1}, day/night temperature of 25°C/22°C, and humidity of 60%. Plants were watered every other day with half-strength Hoagland's solution (Hoagland and Arnon, 1938). Four to six-week-old plants were taken to the lab for experiments.

Populas nigra × *maximowiczii* NM6 (poplar) trees were grown from cuttings provided by the Great Lakes Bioenergy Research Center (GLBRC). Cuttings were grown in 30 L pots filled with Suremix growing medium and watered daily with de-ionized water and twice weekly with half-strength Hoagland's solution (Hoagland and Arnon, 1938). Pots were kept under greenhouse conditions of 16-hour photoperiod with a mean daytime light intensity of 300 µmol m^{-2} s^{-1} photosynthetically active radiation, and mean day/night temperature of 28°C/ 22°C. Six month old trees were used for the experiments reported here. Leaves were detached in the greenhouse and immediately placed in water.

Arabidopsis thaliana isoprene emitting lines B2 and C4 along with empty vector line B3 were generated as reported in (Zuo et al., 2019). The data presented here are also published in (Zuo et al., 2019). Seeds were planted in Suremix growing medium (Michigan Grower Products, MI, USA) in 2" square pots and grown in a growth chamber (Big-Foot, BioChambers), under a 12 h photoperiod at a light intensity of 100 µmol m^{-2} s^{-1}, day/night temperature of 23°C/20°C, and humidity of 60%. Plants were watered every other day with half-strength Hoagland's solution (Hoagland and Arnon, 1938). Ten-week-old plants were cut from their roots and immediately frozen in liquid nitrogen in the growth chamber.

Metabolite measurements

Liquid chromatography–mass spectrometry (LC-MS) was performed as described in Li and Sharkey (2013). Leaves were rapidly frozen under controlled conditions using a "fast kill" gas exchange apparatus that had a 12.6 cm^2 leaf chamber with clear plastic (Saran wrap) windows attached to a LI-6800. The leaf was maintained in the “fast kill” chamber under high or low CO_2 controlled by the LI-6800 and high or moderate temperature controlled by a water bath. After 40 min of incubation under each condition, the leaf was rapidly killed by freeze-clamping between liquid-nitrogen-cooled pneumatically driven copper dies. Leaf tissue was stored at -80°C prior to quantification. Frozen leaf tissue was ground to a fine powder and extracted in 80:20 acetonitrile:water. Samples were then filtered and separated on an Acquity UPLC BEH Amide Column (1.7 μm particle size, 2.1 × 100 mm) and select ions were quantified on the Acquity TQD - tandem quadrupole mass spectrometer. Mass spectrometry metabolite standards were purchased from Echelon (DXP, MEP, CDP-ME, and MEcDP) or Isoprenoids.com (HMBDP and DMADP).

EGTA feeding

Leaves were cut from poplar trees and immediately placed in water. A leaf was placed in the LI-6800 6 cm^2 chamber under 400 ppm CO_2, 1000 μmol m^2 s^{-1} PAR, and 30°C and allowed to equilibrate in water for 1 hour, then an A-C_i curve was measured using the LI-6800. The leaf was then transferred to 5 mM EGTA and allowed to take up the EGTA solution for 1 hour under the same conditions, at which point approximately 1 mL of solution had been taken up. A second A-C_i curve was measured to determine if the EGTA had altered the leaf's response to CO_2.

Burning

G. soja leaves are compound, each leaf having three leaflets. A single *G. soja* leaflet was placed in a LI-6800 400 ppm CO_2, 1000 µmol m^2 s^{-1} PAR, and 30°C. The two adjacent leaflets were left under room conditions (420 ppm CO_2, 8 µmol m^2 s^{-1} PAR, and 25°C). A butane lighter was then used to burn an outside leaflet for 5 seconds (crisping the edge). For high CO_2 experiments, only the leaflet in the LI-6800 was placed under high CO_2 conditions. For mass spectrometer measurements, the same arrangement was used. A light was placed over the plant such that the entire plant was also under 1000 µmol m^2 s^{-1} PAR and the leaflet inside the LI-6800 was also held at 25°C to match conditions. A leaflet from elsewhere on the plant was taken as the pre-burn sample. The unburnt exterior leaflet was assumed to respond the same as the leaflet inside the measuring chamber. This leaflet was taken at the peak of the isoprene emission burst. The leaflet that was in the LI-6800 was taken for the post-burn sample. All leaflets were cut and immediately frozen in liquid nitrogen.

Results

The effect of CO_2 on MEcDP and DMADP

We found that CO_2 caused no significant difference ($\alpha = 0.05$) in metabolite concentrations between 150 ppm and 1200 ppm (Figure 5.1). At 25°C, when isoprene is highly suppressed by CO_2 in tobacco, we saw an increase in HMBDP and a decrease in MEcDP. The decrease is approximately the same as seen using the post-illumination method in Chapter 2, however using this method the decrease was not observed to be statistically significant. However, at 35°C, all compounds increased or remained the same between low and high CO_2 treatments. Due to the high levels of a compound with similar mass fragmentation to DMADP, DMADP concentrations could not be determined with this method.

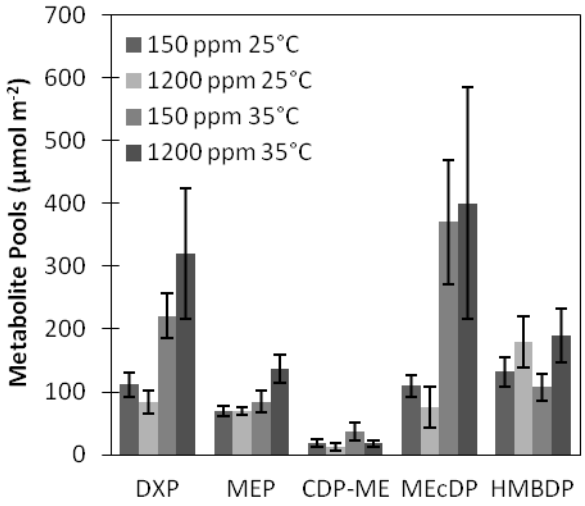

Figure 5.1: The effect of CO_2 and temperature on MEP pathway metabolite levels in tobacco. Averages (n = 5) with standard error are shown. There was no significant difference (α = 0.05) in any metabolite pool between low and high CO_2. Temperature significantly increased DXP and MEcDP concentrations.

Effect of EGTA on CO_2 sensitivity of isoprene emission

In the majority of the trials, cut poplar leaves treated with water displayed extremely limited sensitivity to CO_2, in contrast to the data presented in Chapter 2. Different trees were used for the current experiment than in Chapter 2, and these trees were older and under a different fertilization regimen, although leaves at the same height along the stem (12-15) were taken; data presented in Chapter 2 were taken from younger plants. It is unclear why this would have an impact on CO_2 sensitivity of isoprene emission. For these reasons, the single leaf shown in Figure 5.2 cannot be called a representative sample, but is instead the one case where the leaf was CO_2-sensitive when fed water. EGTA lowered photosynthesis greatly and removed TPU limitation entirely (Figure 5.2). Isoprene emission had no response to CO_2 in the EGTA-fed leaf. However, because of the large effects on photosynthesis, it is impossible to unravel whether this is due to the effects of calcium signaling on isoprene emission directly, or an indirect effect due to the overall changes in leaf conditions.

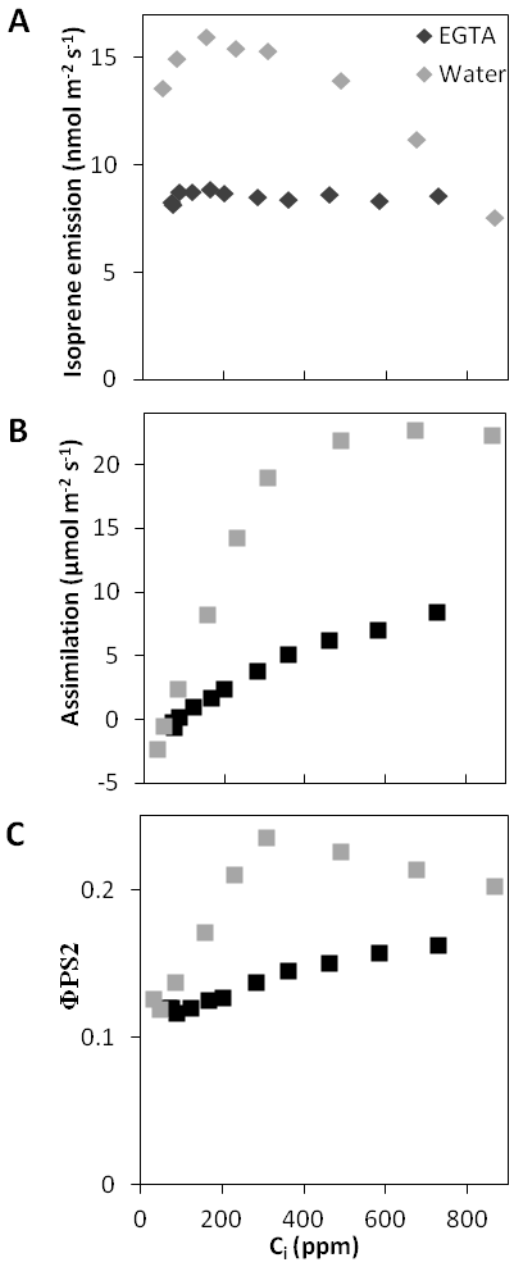

Figure 5.2: Effect of feeding 5 mM EGTA on isoprene emission, assimilation, and ΦPSII. A single leaf is shown fed first with water, then 5 mM EGTA for one hour prior to measuring an *A*-C_i curve. Isoprene emission (A) became insensitive to CO_2, but carbon assimilation (B) and ΦPS2 (C) maintained CO_2 sensitivity although were overall decreased by EGTA feeding. A single trial is shown.

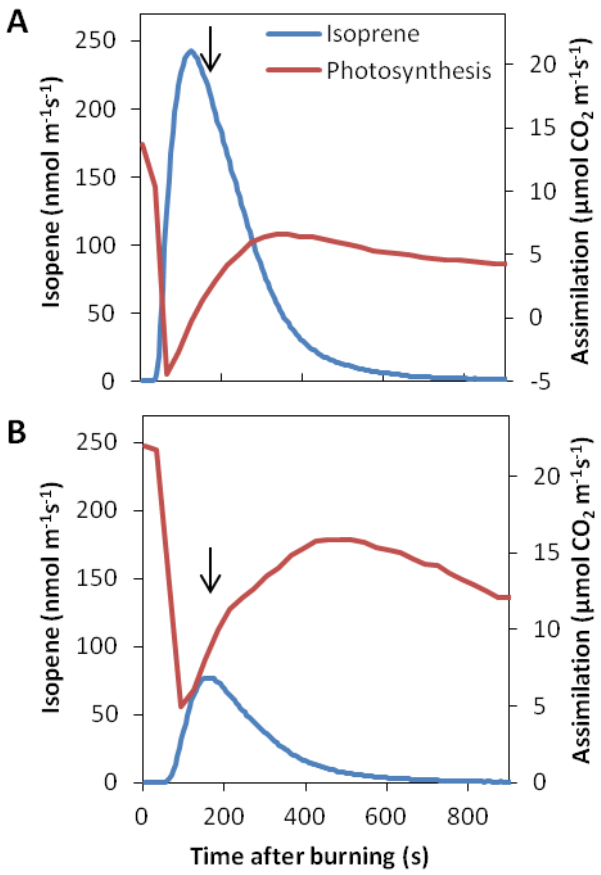

Figure 5.3: The post-burning burst of isoprene from *Glycine soja*. At time 0 leaves were burned for 5 seconds with a butane lighter. Assimilation and isoprene emission were tracked. The effects of 150 ppm ambient CO_2 (A) and 1200 ppm ambient CO_2 (B) are shown. The "Peak" time for Figure 5.4 is marked with an arrow; "Pre" was taken just before burning and "Post" was taken after 15 minutes.

Post-burning isoprene emission in Glycine soja

While *G. soja* did not constitutively emit isoprene under their growth conditions, isoprene emission could be induced for a brief period of time by burning an adjacent leaflet for five seconds (Figure 5.3). Cutting, puncturing, crimping, or otherwise damaging the leaf had no effect and did not trigger isoprene emission. This effect had been seen by our lab previously in *P. montana* (Sharkey lab, Unpublished results). The emission is sensitive to CO_2, similar to constitutive emission in other species (Figure 5.3 A compared with B). I was interested if the observed emission could be explained by changes in the MEP pathway. Therefore, I measured the metabolite pool sizes pre-burning, at the peak of isoprene emission, and after all isoprene

emission had dissipated (Figure 5.4). I found that the metabolite pools were not significantly different between pre, peak, and post conditions.

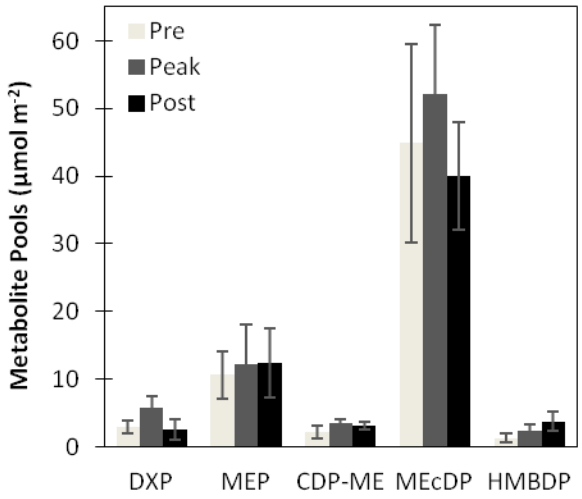

Figure 5.4: MEP pathway pool sizes during the post-burning burst. Samples were taken just prior to the burst, at the highest emission rate (peak, about 150 seconds) and after the burst had completed (15 min) at 400 ppm CO_2. Average (n = 7) with standard error are shown. There is no significant difference between time points ($\alpha = 0.05$).

Metabolite concentration in transgenic isoprene emitters

There was no significant difference ($\alpha = 0.05$) in the MEP pathway metabolite pools between transgenic emitting or wild type non-emitting *Arabidopsis* and *Nicotiana tabacum* (Figure 5.5). DMADP is not shown as it could not be estimated using LC-MS and the post-illumination method would not allow estimation of pool size in the non-emitting plants. Tobacco had much higher concentration of MEcDP and HMBDP than *Arabidopsis*, but lower concentration of MEP and DXP.

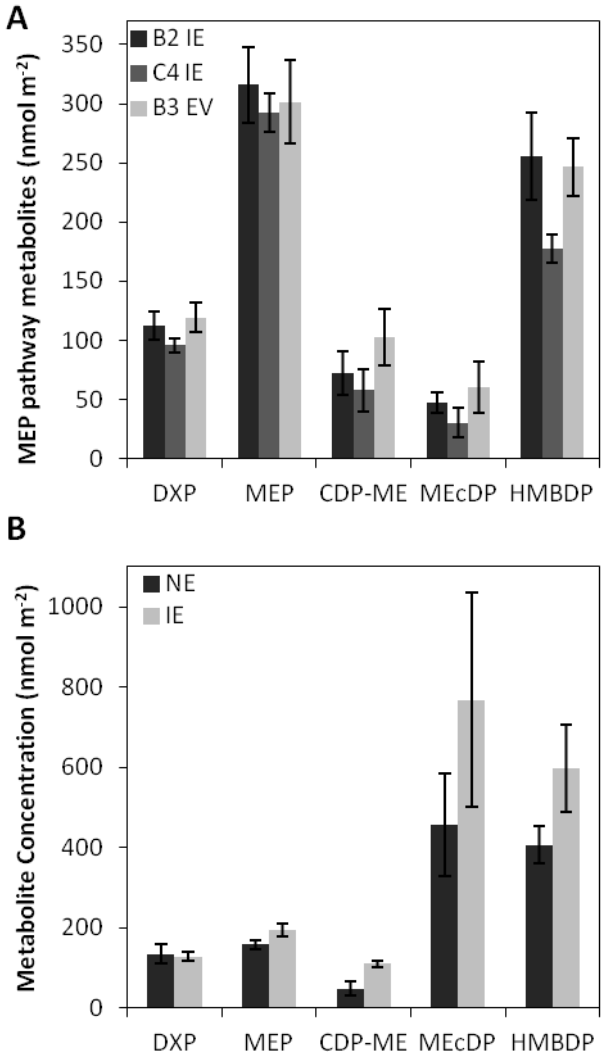

Figure 5.5: MEP metabolite concentrations in emitting and non-emitting plants.
Arabidopsis thaliana Col-0 transgenic isoprene emitting lines B2 and C4 and empty vector line B3 are shown in A. Averages are shown with standard deviation (B2 n = 7, C4 n = 5, B3 n = 10). *Nicotiana tabacum* transgenic isoprene emitting line and wild type line are shown in B. Averages are shown with standard deviation (NE n = 7, IE n = 4). There is no significant difference between lines in any metabolite pool ($\alpha = 0.05$).

Discussion

Effect of calcium on isoprene emission

Understanding the mechanism behind isoprene emission is critical to understanding its impacts on the atmosphere and plant physiology. The published work presented in Chapter 2 was unable to identify a mechanism for the response of isoprene emission to elevated CO_2. In this Chapter, I have described the current understanding of this mechanism and furthered our

understanding of how isoprene responds to changing environmental conditions. Past work from the Sharkey lab determined that isoprene emission is responsive to wounding, in particular burning, and that this response could be transmitted from leaf to leaf (Loreto and Sharkey, 1993). This transmission was blocked by EGTA, a specific chelator of calcium, but not by EDTA, which chelates magnesium and manganese (which are known to be required for isoprene emission and photosynthesis) (Sharkey Lab, Unpublished Data). I showed that isoprene emission has an extremely rapid response to burning in *G. soja*, with burning an adjacent leaflet inducing isoprene emission within 30 seconds. This is much faster than changes in gene expression leading to actual differences in proteins (typically an hour or more) could take place, and the MEP pathway metabolite pools did not change, so a rapid change in DMADP levels inducing emission seems unlikely. Post-translational modification of isoprene synthase, perhaps modulated by calcium signaling (which travels through the phloem at the rate of approximately 0.5 mm s^{-1}) (Choi et al., 2016; Choi et al., 2014; Gilroy et al., 2014; Toyota et al., 2018) is possible in this time frame.

Calcium signaling may also lead to the decrease in isoprene emission at high CO_2, as high CO_2 can acidify cell walls and lead to release of Ca^{2+} (Dani Way, personal communication). This may stimulate PEPC, further decreasing the supply of carbon to the MEP pathway, or may alter the activity of isoprene synthase directly (Bailey et al., 2007; Rosenstiel et al., 2003). However, DXP and MEP pools do not change significantly in response to high CO_2, which is counter to the PEPC hypothesis. I found that while EGTA did have some effect on the response of isoprene emission to CO_2, it altered photosynthesis directly, making it impossible to determine if there was any effect on isoprene emission directly. Further biochemical study on flux into the MEP pathway and whether isoprene synthase is modified directly under different environmental

conditions is necessary in order to complete our mechanistic understanding of the effect of CO_2 on isoprene emission and wounding.

Difference in MEP pathway metabolites between species

The control of isoprene emission may vary between species. Rosenstiel et al. (2002) found that different species accumulate different levels of DMADP, but did not measure the other metabolite pools. In poplar, Ghirardo et al. (2014) found that RNAi knockdown of isoprene emission modified flux through the MEP pathway. We did not find that MEP metabolite pool size was affected by adding isoprene emission to non-emitting plants, but did not measure flux. We found that relative pool sizes were different between *Arabidopsis*, tobacco, and what had previously been reported for poplar (Li and Sharkey, 2013). The metabolite pools were overall much lower in *Arabidopsis* which may explain its low isoprene emission rate. Tobacco had comparable amounts of MEcDP to poplar, but much higher HMBDP and lower MEP. The low MEP may indicate that overall flux through the pathway is lower, but more information is required to fully understand these differences between species and what regulates them. Zuo et al. (2019) did not find that there were differences in the expression of MEP pathway genes between emitting and non-emitting plants, which is consistent with the similar pool sizes.

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Chapter 6 Conclusions and future directions

Control of isoprene emission from plants

This work rules out the hypothesis that control of isoprene emission from plants is due entirely to changes in electron transport, with isoprene synthase (ISPS) playing a role only under changing temperature conditions. Isoprene emission can fluctuate significantly under conditions when electron transport is constant. There was no apparent change in the MEP pathway under different CO_2 concentrations. Therefore, models based entirely on electron transport are not mechanistic and do not adequately explain isoprene emission rates under changing conditions (Arneth et al., 2007; Grote et al., 2014; Morfopoulos et al., 2013; Morfopoulos et al., 2014; Niinemets et al., 1999; Rasulov et al., 2009; Rasulov et al., 2015). Isoprene synthase gene expression, protein levels, and activity may change under changing CO_2, which could explain this effect (Brilli et al., 2007; Calfapietra et al., 2007; Scholefield et al., 2004; Wiberley et al., 2008). I proposed that the changes are too rapid for changes in gene expression or protein levels to occur, with changes in isoprene emission due to CO_2 occurring in under 3 minutes. Isoprene synthase is slow to degrade even under changing temperature conditions (Wiberley et al., 2008). Post-translational modification, possibly regulated by Ca^{2+}, may explain the rapid changes in isoprene emission not only due to changing CO_2 but also wounding.

Isoprene synthase activity changes in response to temperature *in vivo* and *in vitro*, with these changes following classic Arrhenius predictions for thermal control of enzymes. I probed whether the change only affected the catalytic activity or whether it also affected binding. Temperature decreases the Michaelis-Menten constant below 33°C, but increases it after that, leading to a U-shaped temperature response. This alters the response of isoprene emission to temperature, as slow binding decreases the apparent rate even when the turnover constant remains high.

Despite having drastically different isoprene emission rates, *Nicotiana tabacum* has similar levels of MEcDP and DMADP as previously reported from *Populus tremula* × *alba*. The levels in *Arabidopsis thaliana* are much lower, but not ten-fold lower as the lower isoprene emission rate might suggest, given that the concentration is far below the K_M of isoprene synthase. Furthermore, the MEP pathway apparently plays no role in the wounding response observed in *G. soja*. New hypotheses must be formulated and tested in order to explain these fast changes in isoprene emission without measurable changes in MEP pathway pool size.

Because isoprene emission rate is not dependent on electron transport, current models predicting a decrease in isoprene emission rate as CO_2 concentration and temperature increase greatly over the next century are inaccurate. Based on my data, isoprene emission rate should increase over the next century. Not only does increasing temperature increase emission rate directly, but it also decreases the sensitivity of isoprene emission to CO_2. Furthermore, while current models take into account changing land use selecting for non-emitting crops, they do not predict that in non-cultivated areas, higher temperature may select for isoprene emitting species (Hantson et al., 2017; Taylor et al., 2018). My work on the CO_2 response and temperature should lead to improved mechanistic models of isoprene emission.

Engineering isoprene emission in plants and bacteria

Current work on engineering terpene production from bacteria focuses primarily on tuning expression of the mevalonic acid (MVA) or methylerythritol 4-phosphate (MEP) pathways. We determined that modifying the terpene synthase itself is not only possible, but may improve activity. Furthermore, compartmentalizing proteins in bacterial microcompartments (BMCs) may lead to new opportunities in synthetic biology. While we are not the first to encapsulate new chemistries in a BMC, there are only a few examples in the literature (Giessen and Silver, 2016; Lawrence et al., 2014; Liang et al., 2017). If we are able to extend this encapsulation to LytB, it

would be a significant advance in synthetic biology, as it would be the first time a redox-active enzyme would be synthetically encapsulated.

Zuo et al. (2019) showed that isoprene emission can improve plant growth in *Arabidopsis*. As such, it may be beneficial to express isoprene synthase in crop plants in order to improve resilience. However, this has several potential drawbacks. Notably, no modern crop plant releases isoprene - in fact, *Glycine max* actually lost isoprene emission sometime between becoming cultivated and the modern day (Sharkey, 2013; Sharkey et al., 2013). This may be because the cost of isoprene emission is not justified under the relatively stable, safe conditions of a tended field. In *Nicotiana tabacum*, isoprene emission decreased growth. Therefore, more research needs to be done to determine if this would be a net positive or net negative for crop plants under higher temperature conditions. There is an atmospheric cost as well. Increasing isoprene emissions can alter the local climate and lead to production of photochemical smog (Behnke et al., 2012; Beltman et al., 2013; Han et al., 2013; Wang et al., 2013).

Species that are engineered to emit isoprene do not have as high emission rates as native emitters. We observed that transgenic *Arabidopsis* plants emit around 3 nmol m^2 s^{-1}, while *Nicotiana tabacum* emits up to 10 nmol m^2 s^{-1}. Native emitters such as *Phragmites australis* and *Populus tremuloides* emit 30-60 nmol m^2 s^{-1}, and under some conditions emitting species, particularly tropical or subtropical species, can emit over 100 nmol m^2 s^{-1} isoprene. This may be because the MEP pathway in emitting species is upregulated to adapt to the high demand of isoprene synthase; while we saw no difference between the non-emitting and transgenic plants in MEP pathway metabolites, Rosenstiel et al. (2002) showed that native emitters such as *Quercus rubra* have as much as 300 times the DMADP concentration of naturally non-emitting species,

with large differences even within the same genus. Lower emission rates due to lack of DMADP in species that do not normally emit isoprene may limit the benefits of engineered emission.

Future Directions

Flux of the MEP pathway

MEP pathway metabolite pools provide no insight into what is altering isoprene emission under high temperature and CO_2. Measuring flux through PEPC may answer whether it can explain the change in isoprene emission, despite the constant pool size of DXP. Measuring flux in response to changing calcium may help our understanding of whether calcium plays a role in altering the MEP pathway.

Further understanding of isoprene synthase itself is required in order to fully explain these results. The rapid response to burning and changes in isoprene emission in response to CO_2 can only be explained by decreased substrate supply or by modifications on the proteins themselves. Carbon, ATP, and electrons from photosynthesis remain constant under conditions where isoprene emission is changing rapidly. In addition, MEcDP and DMADP concentrations change at similar rates in response to CO_2 using the post-illumination method in chapter II, which suggests that the control is most likely not at 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBDP) reductase (HDR). This means that there must be some post-translational modification on isoprene synthase (or possibly isopentenyl diphosphate isomerase (IDI), which converts IDP to DMADP) that can explain these rapid changes. Currently there is no evidence of any site for post-translational modification of isoprene synthase. If we were to find changes in isoprene synthase activity under these different conditions, it may lead to discovery of a novel, CO_2-sensitive post-translational modification mechanism.

Role of isoprene emission in plants

Isoprene emission can improve plant stress resilience by altering the chloroplastic structure and gene expression (Harvey and Sharkey, 2016; Vanzo et al., 2016; Velikova et al., 2014; Velikova et al., 2015; Zuo et al., 2019). We believe isoprene acts by constitutively increasing expression of genes linked to stress response, essentially preparing the plant to respond more rapidly to temperature, oxidative, and even biotic stresses, which in turn prevents damage from occurring and allows the plant to recover more quickly. Our lab intends to collaborate with a lab with expertise in plant pathogens to stress isoprene emitting plants using a bacterial infection; if isoprene emitting plants are more resilient than non-emitting plants, it shows that the upregulation of stress response genes shown in Harvey and Sharkey (2016) and Zuo et al. (2019) actually improves plant stress resilience. We previously tried to use thermal stress (40°C for one hour) but it did not consistently trigger lesions in non-emitting species, so was an insufficient test of these hypotheses.

Future work also involves determining what isoprene binds to. In order to cause changes in gene expression that lead to the observed stress tolerance, the plant must be detecting isoprene somehow. There is no known receptor for isoprene, but it may be possible to find one by using forward genetics. If a protein is necessary for isoprene fumigation to produce changes in gene expression, it may be a target of isoprene directly. Expression of the phenylalanine ammonia lyase (*PAL*) gene may be a potential output, as it is known to respond to both fumigation and constitutive expression of isoprene synthase (Harvey and Sharkey, 2016; Zuo et al., 2019). A luciferase reporter under control of the *PAL* promoter would serve as a useful output for a high-throughput forward genetic screen. An alternative is to visualize where isoprene is binding in the cell using click chemistry. Isoprene is ideal for this due to its double bonds, which makes it a natural reactant in click chemistry.

Engineering isoprene emission for industrial use

The work with bacterial microcompartments (BMCs) and ISPS-GFP shows that isoprene synthase is a promising candidate for synthetic biology. An important future step is to combine LytB with isoprene synthase within a scaffold. This could be accomplished by loading it into the BMC using the spy-tag/spycatcher system, although this would necessarily reduce the loading of both enzymes and would not ensure equal loading. A better possibility is to modify the linker region of ISPS-LytB such that LytB can fold correctly and the protein can produce isoprene directly from HMBDP. This could then be further modified with the spy tag and loaded into BMCs directly. While some pathways that natively occur in BMCs do require NADPH (Jakobson et al., 2015; Yeates et al., 2010), an alternative is to use a modified BMC that has an iron-sulfur cluster in some of the pores (Aussignargues et al., 2015). This would be a perfect test of both the synthetic biology potential of chimeric bacterial microcompartments as well as improving isoprene production.

Expressing isoprene synthase in non-emitting species

In order to further test whether isoprene emission can improve growth in a crop plant, Dr. Sharkey collaborated with Dr. Felix Fritchi (University of Missouri) to place the *Eucalyptus globulus* isoprene synthase in *Glycine max*. We plan to test whether these plants have improved growth and thermal tolerance, similar to the results reported by (Zuo et al., 2019). If this holds true, this may be a significant boost to crop plants in a warming global environment.

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