Molecular mechanisms involved in hyponastic growth of terpene overexpressor plants

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Table of contents

Introduction

Hyponasty

Nastic movements can be considered as an adaptation of the plant to the environment, an adaptation that is rapid and rhythmic because follows the light and dark alternance. These variations of leaf angle during the day and night have evolved in most of the species in order to ensure the best surface to the sun during the day and to protect the stem during the night and are mainly regulated by an asymmetric petiole growth. When the upper part of the petiole elongates faster than the lower side, the leaf angle will be modified. In certain species such as *Mimosa pudica* these movements are instead tightly regulated by an organ called pulvinus that depending on the water turgor and ionic pumps can lead to rapid and contact-induced movements.

In *A. thaliana* the pulvinus is not present and leaf movements are mainly regulated by three factors: heat (as a sudden shift from 20 to 38°C), low light and ethylene. (van Zanten, et al., 2009). On the other hand ethylene acts as inhibitor of the heat-induced hyponastic movement while low-light conditions trigger hyponasty independently from the presence of ethylene in the air.. (van Zanten, et al., 2009) (fig. 1)

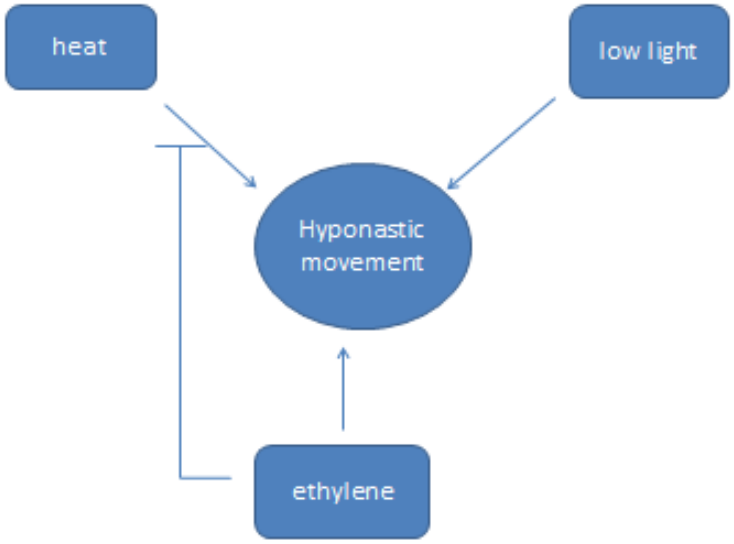

Figure 1: Schematic representation of the main factors involved in hyponastic movement. Heat, low light and ethylene induce hyponastic movement; ethylene inhibits heat-induced hyponasty (van Zanten, et al., 2009)

Ethylene

Ethylene, or ethene, is a hydrocarbon with the formula C_2H_4 that plays a plethora of roles as a plant hormone thanks to the ability of freely diffuse in the membranes and its activity also at nanomolar concentrations.

Its biosynthesis arises from the amino acid methionine, to which ATP is added and converted into SAM (S-Adenosyl methionine) by the enzyme methionine adenosyltransferase. SAM is further converted into the main ethylene precursor, ACC (1-aminocyclopropane-1-carboxylic-acid), by the action of ACS (ACC synthase). ACC oxidase finally converts ACC into ethylene. Through the so called "Yang cycle" the MTA (methylthioadenosine) regenerates the initial precursor methionine. (Yang & Hoffman, 1984)

One of the earliest described responses to ethylene is known as the "triple response" (Neljubov, 1901). This test is based on the natural behaviour of a seed germinating in the dark to elongate the hypocotyl in search of light. If the ethylene in the air, or in the plant, is artificially increased the plant will give rise to this "triple response" making its hypocotyl shorter, thicker and with a more evident apical hook. This phenotype is the plant response to external stimuli, such as a rock on top of the germinating seed. This response would be driven by an ethylene production to help the leaves in growing outside the soil and to prevent synthesis of chloroplasts in absence of light.
Another important role played by ethylene during plant development is the establishment of a correct gravitropism. A proper gravity driven path has to be followed by roots to ensure a correct anchoring to the soil and water/nutrients uptake. A negative gravitropism instead has to be followed by the shoot that has the need to maximize light exposure and to compete for light. The need of ethylene as a growth regulator is also shown by the roots of ethylene insensitive plants that are characterized by an irregular gravitropism. (Edelmann & Roth, 2006)

Terpenes

Part of the carbon that is assimilated by the plants is emitted in the air as volatile compounds. These molecules are grouped together under the name of BVOCS, biogenic, volatile organic compounds and represent approx. 20% of the carbon released in the atmosphere.

VOCs react with Nitrogen oxides (NOx) to form ozone (Atkinson, 2000), this indicates a role of these compounds in climate and nowadays each issue related with the ongoing climate change has to be properly considered.

In this regard the question if the BVOCS can constitute a defence of the plant against warm temperatures can be an intriguing link between these molecules and climate change (Peñuelas & Llusià, 2003)

Some terpenes are considered BVOCs (isoprene, monoterpene and sesquiterpene). Terpene are naturally found in the resin of most trees and in flowers to whose these molecules provide a characteristic scent and a bacterial defence (Huang, et al., 2012).

Terpenes are composed of multiple isoprene (C_5H_8) units bound together and their production can differ quantitatively and spatially according to the plant species. In some aromatic plants belonging to the family of Lamiaceae (e.g. mint, sage, oregano) terpenes are stored in glandular trichomes or, in the case of conifers, in the resin ducts and, more generally, quantities can vary in relation to exposure to wounding and herbivores (Loreto & Schnitzler, 2010).

As extensively reviewed by M. Ashour et al. (2010) MEP and MVA are the two metabolic pathways that lead to terpene biosynthesis, these abbreviations respectively stand for the Methylerythritol Phosphate and the Mevalonate-dependent pathways. MVA is the common route for most of the eukaryotes while MEP takes place mainly in plants' plastids and in most bacteria. Both pathways contribute to the formation of dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) which are the precursors for this large family of molecules (Figure 2a). Beside terpene biosynthesis, many cell/plant regulators such as hormones and steroids are the outcome of these pathways.

Hemi, mono and diterpenes are the products of the MEP pathway while most of the C_5 units for the sesquiterpene production derives from the cytosolic route. Surprisingly an interchange of precursors is known to occur between the two pathways and labelling experiments in snapdragon flowers showed a crucial role for the MEP pathway in the biosynthesis of both mono and sesquiterpenes (Dudareva, et al., 2005).

Caryophyllene

Particular scents such as the one from black pepper or the one from cloves are largely due to the presence of (E)-β-caryophyllene, a volatile sesquiterpene interestingly characterized by a cyclobutane ring (Figure 2a). Caryophyllene is emitted by a plethora of plant species in different tissues and expression levels of this sesquiterpene in the flowers are very high compared to the other plant organs and tissues (Figure 2b for *A. thaliana*). Similarly to many volatile compounds, this molecule can be involved in pollinators attraction but there is a growing evidence that it plays a role also in defence from biotic stresses supported by the presence of this molecule also in autogamous and anemophilous plants (Sabulal, et al., 2006; Köllnera, et al., 2008; Huang, et al., 2012).

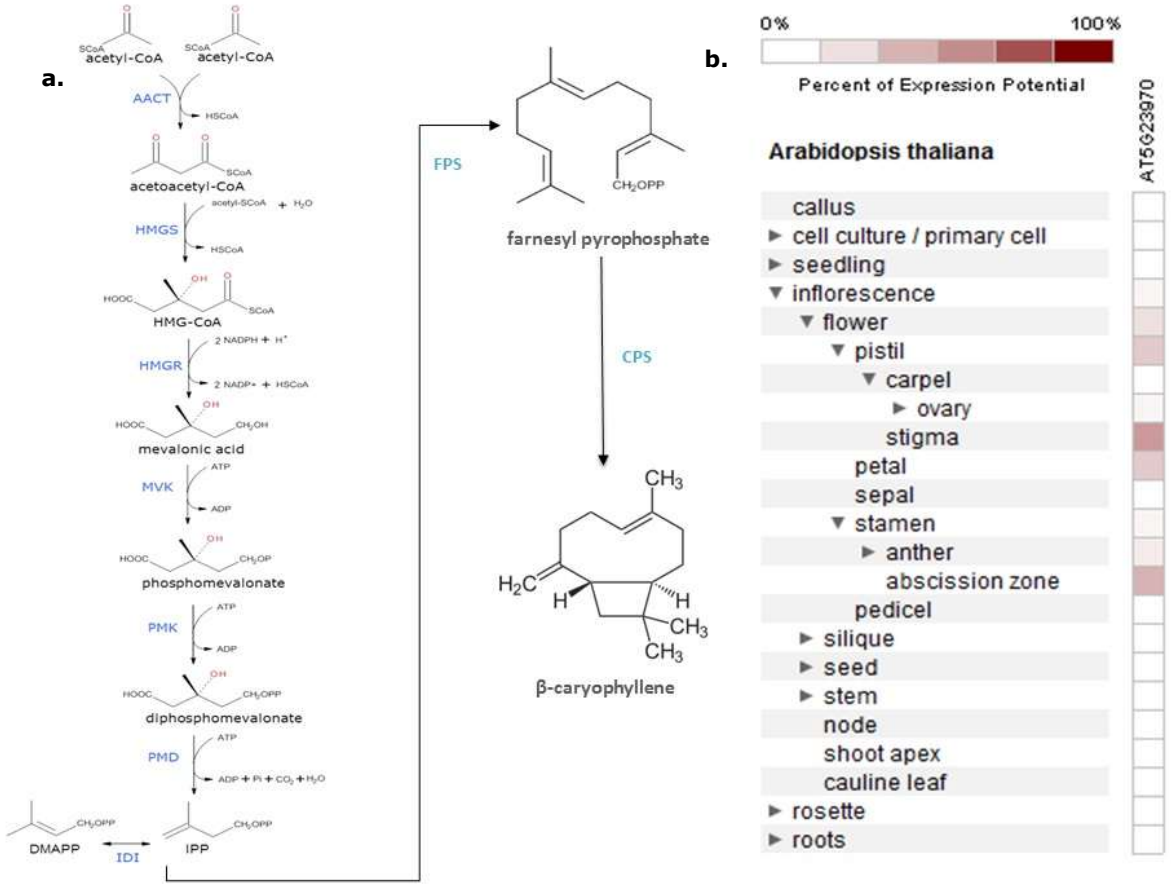

Figure 2 (a) Chemical structure of β-caryophyllene and the MVA pathway; (b)Heat map of the expression of the Caryophyllene synthase gene, At5g23960 (AtTPS21), in A. thaliana with focus on the flower zone. (Modified from Genevestigator 4.1)

The first link between caryophyllene and pathogens has been shown by Sabulal et al (2006) through an inhibition of bacterial and fungal growth on agar and test discs in a caryophyllene concentration dependent manner

In flowers, in fact, through the plant's natural openings such as the stigma and the stamen abscisic zone many bacterial pathogens can enter and infect the plant. Challenging *A. thaliana* mutants defective or overexpressing caryophyllene with a bacterial pathogen has been shown to produce different levels of virulence linearly dependent to the sesquiterpene storage and emission (Huang, et al., 2012).

Furthermore a signalling to herbivores predators as a form of indirect defence response has been hypothesized in maize measuring caryophyllene production in leaves and roots and its attraction respectively to parasitic wasps and entomophagous nematodes (Köllnera, et al., 2008).

An hypothetical link between a sesquiterpene and hyponastic movement.

The long list of known roles played by caryophyllene is likely to not to be complete. His chemical structure suggests a strong affinity for the membrane lipid layer where it could play a role in regulating membrane fluidity.

The caryophyllene molecule, synthesized in the plastids, has a strong lipophilic behaviour and if produced in excess is supposed to be expelled out of the cell after saturation of the cell membrane. The membrane, in fact, is mainly composed by lipids such as phospholipids, glycolipids and steroids, and a terpene like caryophyllene can fit and saturate the phospholipid layer (Zunino, et al., 2011) (Figure 3).

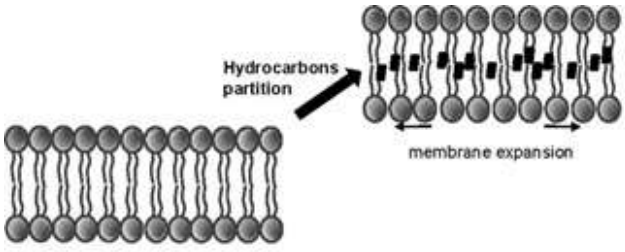

Figure 3 The addition of hydrocarbons modifies the membrane properties. This extra lipid loading of the plasma membrane is expected to interfere with the membrane properties especially increasing its fluidity. Adapted from Zunino et al.(2011).

An attempt of shedding light on sesquiterpene storage and excretion has been given in 1995 through electron microscopy on *Sauromatum guttatum* flowers showing a fusion event of a vesicle from the ER to the plasma membrane in correspondence of a heat-induced release of sesquiterpenes (Skubatz, et al., 1995).

Heat induction can overlap with the natural rhythm of terpene excretion that occurs according to the circadian rhythm and, interestingly, with the same pattern of hyponastic movement. In this regard a confirmation that heat alone can up-regulate terpenoid emission comes from the analysis of volatiles emission under different temperatures during night in *Populus tremula* and *Betula pendula* (Ibrahim, et al., 2010).

Moreover the gas exchange of the plant for its photosynthesis is sharply reduced by night when stomata are closed and this can reflect the rhythmicity of terpene emission which is characterized by a clear increase during the day. (Aharoni, et al., 2003) (Dudareva, et al., 2005) .

Another point of view on the movement of terpenes across membranes and cell walls comes from the hypothesis that wax precursors need a solvent to be carried until the cuticle layer (Shepherd & Griffiths, 2006) . The low but constant production of terpenes that have to be transported outside the plant can support this hypothesis but further research is needed.

As already mentioned, being ethylene and its pathway a key regulator of hyponastic movement it is likely to be one of the connections between terpenes and hyponastic movement.

The goal of this MSc thesis was to investigate the plasma membrane fluidity, ethylene sensitivity, rhythm of emission and all the possible scenarios involved in identifying the complicated but fascinating missing link between terpene overexpression and hyponastic movement. We used caryophyllene as a sesquiterpene model and we performed transient expression studies on *N. benthamiana* with caryophyllene synthase genes from *A. thaliana* and *Cucumis sativus* and their precursors to choose the best candidate. Thereafter caryophyllene overexpressor *A. thaliana* mutants were studied for possible membrane fluidity changes through a fluorescence recovery after photo-bleaching (FRAP) experiment carried out on YFP membrane-labelled protoplasts. Their ethylene sensitivity was investigated with the triple response test and, finally, root elongation studies together with headspace analysis gave insights on the terpene production rhythmicity and its effect on the plant growth.

Materials and methods

Plant growth and conditions

Seeds of *A. thaliana* ecotype Col-0 and caryophyllene synthase (At5g23960) overexpressor mutant were surface sterilised, stratified 3-5 days in darkness in 0.1% agar at 4°C and sown on soil or alternatively on ½ MS agar in a nearly vertical position for root growth studies. Plants on soil were grown in a 12/12 (light/dark) regime. Plants on agar were grown in 8/16 or 16/8. For the triple response assay the plants were grown in the dark for 7 days on ½ MS agar + 1% of sucrose and 0, 1, 10 µM of ACC (1-aminocyclopropane-1-carboxylic-acid, Sigma Aldrich). All the plants were grown at 20°C except those kept in negative DIF (12°C during the day and 22°C during the night) .

For agroinfiltratin, wild type *N. benthamiana* plants were grown on soil in greenhouse (Unifarm, WUR) with 16 hours photoperiod and 28/25 °C day/night temperature.

Plasmid construction for transient and stable gene expression

The *A. thaliana* genes At5g23960 (caryophyllene synthase), AT1G76490 (HMGR), At5G47770(FPS), C62 (limonene synthase) (El Tamer, et al., 2003) and the *Cucumis sativus* gene Ay640155 (caryophyllene synthase) were cloned each in a pGM-Teasy vector (Promega) after T4 ligation. PCR primers containing SacI and NcoI were used to amplify the fragment, this was then double digested and T4 ligated into a pImpactVector pIV 1.1 (www.pri.wur.nl/UK/products/ImpactVector/). The gateway system has been used to transfer the construct in a pBINPlus vector by LR clonase (Gateway-LR Clonase TM II). Once the quality of the insertion was checked using colony PCR and sequencing the vector were transfected by electroporation in Alg0 *Agrobacterium tumefaciens*. Resulting colonies were used for stable transformation trough floral dipping or for transient expression in *N. benthamiana* (Liu, et al., 2011).

Transient expression in N. benthamiana

A. tumefaciens cultures were grown overnight in 250 ml of LB medium Kan 50mM, Rif 50mM, then pelleted through 4000 rpm centrifugation and resuspended in 10 ml of MES buffer containing 10 mM MgCl2 and 100 µM. Multiple constructs were coinfiltrated together to study the role of the MVA pathway in boosting caryophyllene production. Mixtures were infiltrated into 3 weeks old *N. benthamiana* leaves by gentle pressure through a 1ml without needle syringe on the abaxial surface.

Leaf movement imaging

The 11th and 12th rosette leaves movements have been recorded in time lapse every 20 minutes during day and night for one week. To allow imaging during the night with a correct exposure an infrared constant lighting that was shown to not affect plant growth has been used (Bours, et al., 2012) . The images acquired have been cropped in single stacks of photos using ImageJ (http://rsbweb.nih.gov/ij/). The manual tracking plugin has been used to follow the growth of the leaves by clicking at the edge of the leaf for each photo, this step has been speeded up using Standard Mouse Auto Clicker 2.8 (http://ljtab.com/autoclicker). The resulting output combined with the coordinates of the rosette centre have been converted into the final growth oscillation data by the OSCILLATOR script (Bours, et al., 2012) running into R 2.13.1 (http://www.r-project.org/).

Protoplast isolation and transfection

A. thaliana mesophyll protoplasts (pps) have been isolated using the "tape method" (Wu, et al., 2009). The use of two different types of tape to peel away the lower epidermis layer gives, in fact, the name to this so called "Arabidopsis tape sandwich method". The leaves have been incubated 20-40 minutes in a 1% cellulase, 3% viscozyme (Sigma Aldrich) solution to degrade cell walls and after two washing steps a clearly visible green pellet of protoplasts was achieved and diluted according to the needs in a isotonic "W5" solution (5 M NaCl, 1 M $CaCl_2.2H_2O$, 2 M KCl, 0.2 M MES, pH 5.7).

Protoplasts have been counted using a counting chamber (Labor OptiK Burker, Germany).

Transfection has been carried out adding to the protoplasts 80 mg of poly-ethylene glycol (PEG) and 20 µl of 1µg/µl plasmid DNA containing the CAAX-YFP construct within the pMON999 vector (van Bokhoven et.al., 1993) kindly provided by JW Borst, Biochemistry Department, WUR. The PEG was then removed from the solution by washing with W5 solution.
Samples were diluted in W5 solution with 8mM glucose to a final level of 150 pps/µl.
The protoplasts were then kept in the dark at 20 °C for 16 h before imaging.

Transfection efficiencies were higher with 5 weeks old leaves rather than younger ones hence this leaf age has been preferred. An evident quantity of debris was generally present but did not lead to problems for imaging or other analyses.

Confocal Microscopy and FRAP

To perform the FRAP experiment 300 µl of the protoplast solution was poured in a 8 chambers slide and analysed using a confocal microscope (Zeiss LSM 510 META). All the images were taken using the Apochromat lens 40X oil 1.2 numerical aperture. The argon ion laser was set for excitation at 514 nm with an output of 36% (6.0 A). The emission was simultaneously recorder on two channels: the "red" channel with a long pass filter (620-700nm) for visualizing chloroplasts through the autofluorescence of the chlorophyll, and the "yellow" channel to detect expression of the YFP with the band pass filter set at 530-600nm.

Image size was set at 215x215 pixels to maximize imaging speed (11/12) and obtain an average of 3 scans per second. 50 maximum intensity laser pulses were directed to a circular region of interest (ROI) along the membrane to perform the photo-bleaching. The fluorescence intensity was followed up for 120 scans in the bleached region and, as a control, in two same size regions, one in the background and one in a not bleached area of the membrane.

The output data (.txt) were then background corrected and normalized using the EASYFRAP software (University of Patras, http://ccl.med.upatras.gr). Manual corrections were made with Microsoft Excel 2010 (office.microsoft.com) to random samples to check the accuracy of the automatic software.

Headspace analysis

A comparison between day and night production of caryophyllene in A. thaliana was carried out placing a whole not flowering plant (three replicates per genotype) in a ½ litre glass jar closed with a Teflon-lined cap equipped with stainless steel in- and outlet. The incoming pumped air was filtered with Tenax tubes (Markes International Ltd, U.K.) and volatiles trapped in the same kind of tube placed on the outlet. The headspace has been sampled for 2 hours at 23°C starting at 12:00 and 10:00 pm. Samples were then desorbed using a thermo-desorption system (Ultra TD, Markes International) coupled with a GC-MS detector (Thermo Fisher Scientific; MA, U.S.A.)

The same setup has been used for volatile analysis of agroinfiltrated *N. benthamiana* leaves. In this case, though, the length of the sampling was reduced to 20 minutes to avoid saturation of the tubes.

GC-MS analysis

For the analysis of terpene production in WT and COE A. thaliana plants 300 mg of leaves were grinded in liquid nitrogen and extracted with 2 ml of hexane (C_6H_{14}). The extracts were sonicated 10 minutes using Branson 450 Sonifier (Branson, Danbury, CT) , dried using $MgSO_4$ and concentrated with a flow of nitrogen.

Alternatively, to confirm presence of caryophyllene in the protoplasts before the FRAP experiment 200µl of protoplast solution (150pps/µl, 5 M NaCl, 1 M $CaCl_2.2H_2O$, 2 M KCl, 0.2 M MES pH 5.7) was mixed with 1 ml of hexane by brief vortexing then sonicated, dried and concentrated as described above.

A gas chromatograph (7809A, Agilent, USA) was used for GC-MS analysis. Splitless mode was used for the injector at a temperature of 250 °C. The initial temperature was 45°C and was increased to 300°C at a rate of 10°C min^{-1} and held for 5 min at 300°C. Mass scanning was carried out from 33 to 280 m/z with a scan time of 4.2 scans per second. Xcalibur software (Thermo, USA) was used to compare the mass spectra of the eluted compounds with reference standards (Liu, et al., 2011).

Results

Caryophyllene synthase transient expression

Agrobacterium mediated transient transformation (ATTA) on *N. benthamiana* leaves was performed to test differences in volatile emissions using different combinations of two known caryophyllene synthases, HMGR, a crucial enzyme of the MVA pathway and FPS the enzyme that catalyse the formation of farnesyl diphosphate, the immediate precursor of caryophyllene. A silencing suppressor, P19 at OD_{600} 0.1 and pBIN empty vector until OD_{600} 0.6 have been added to all the combinations in order to avoid gene silencing and to keep constant the bacterial infiltration dosage.

All the headspace of the agroinfiltrated leaves after GC-MS analysis (TIC set at 133) originated clear peaks corresponding to caryophyllene and its by-products at the following retention times apexes: 19.27 (copaene), 20.14 (caryophyllene), 20.47 (copaene), 22.15 (caryophyllene oxide) and an unidentified compound shows a peak at retention time 20.30 only when the enzyme HMGR is present (Figure 4c). Production levels of the agroinfiltration mixtures containing the *Cucumis sativus* caryophyllene synthase were low and often not significantly different from the natural caryophyllene production by the *N. benthamiana* leaves (Figure 4b). The Arabidopsis gene under the same 35S promoter, instead, increased massively the caryophyllene production and when the HMGR enzyme (HMG-CoA reductase) is co-infiltrated the production is further boosted (Figure 4a). Co-infiltration with FPS (farnesyl diphosphate synthase) decreased significantly caryophyllene production in all the combinations. (Figure 4a and b).

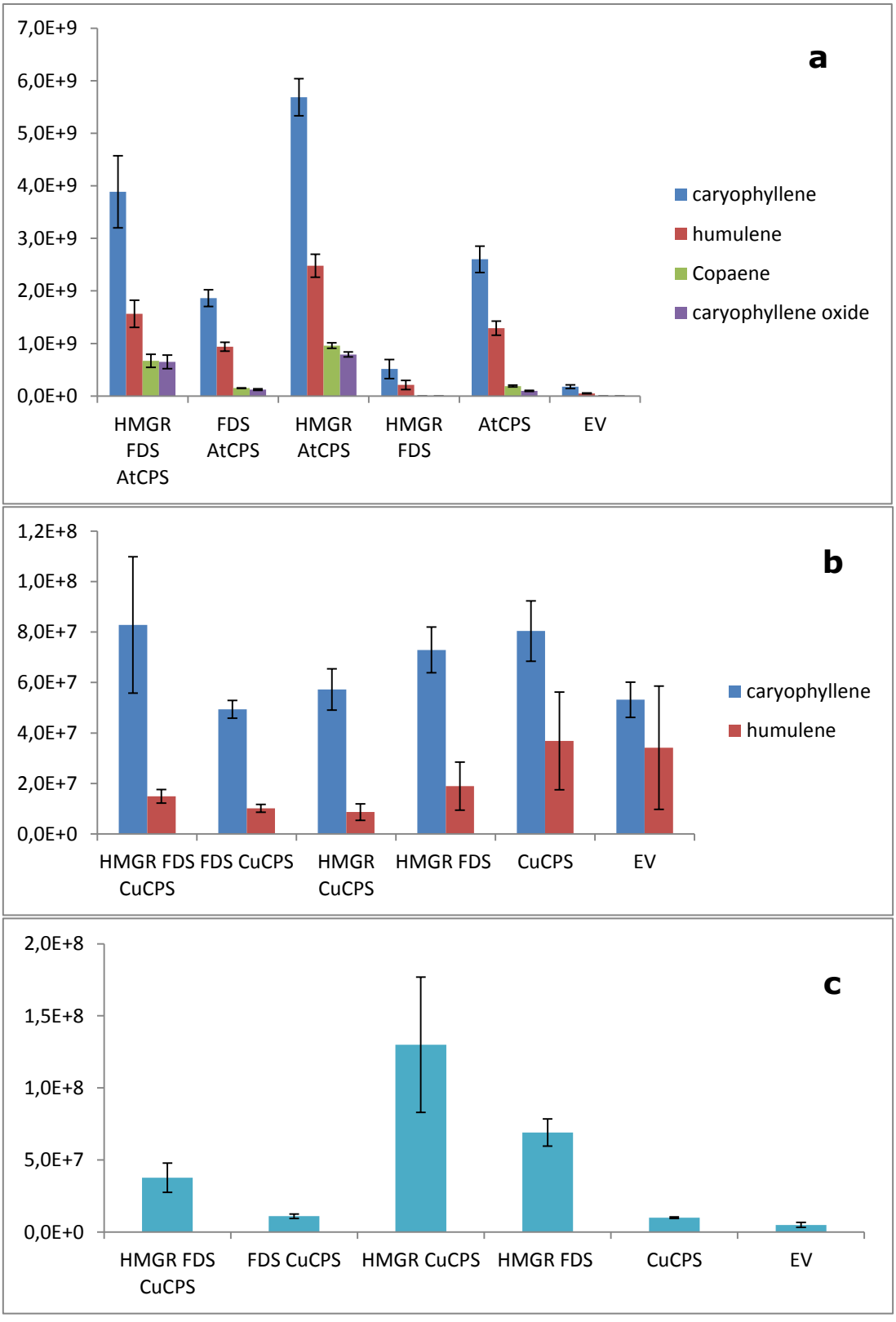

Figure 4 Relative peak intensities (TIC 133) of volatiles trapped through headspace of agroinfiltrated N. benthamiana leaves. (a)Headspace of leaves agroinfiltrated with the A.thaliana caryophyllene synthase. (b) Headspace of leaves agroinfiltrated with the Cucumis sativus caryophyllene synthase. (c) Peaks found at retention time 20.30. No reference compound was available for this peaks. A Abbreviations: HMGR:HMG-CoA reductase , FDS: farnesyl diphosphate synthase, AtCPS: A.thaliana caryophyllene synthase, CuCPS: Cucumis

sativus caryophyllene synthase, EV: empty vector. For combination of less than three DNA constructs EV was added to reach OD600 0.6 Each bar represents the average of three replicates ± SE.

Arabidopsis stable transformation

Based on the caryophyllene production levels observed between the *Arabidopsis* and the *Cucumis* genes we decided to proceed with the *Arabidopsis* gene AtTPS21. Three independent lines of caryophyllene overexpressors were generated in our lab by HM Ting and resulted in three different level of expression: low, medium and high (data not shown). All the further analysis, unless stated differently, have been carried out using the highest expressor line. Hereafter this line will be referred as COE.

To expand the investigation on the effect of terpenes more in general a monoterpene limonene overexpressor line previously generated in our lab by I. Kappers has been used. Hereafter this line will be referred as LIM.

GC MS analysis of protoplasts and leaf extract

The extraction from rosette leaves analysed with GC MS resulted in the presence of a clear peak corresponding to caryophyllene. The peak reaches a relative abundance comparable with the internal standard nerolidol (Figure 5). The extraction from protoplasts yielded a peak at same retention time but its intensity was highly variable depending on many factors such as the protoplast concentration, vitality and the age of the leaves from which they have been isolated. (data not shown)

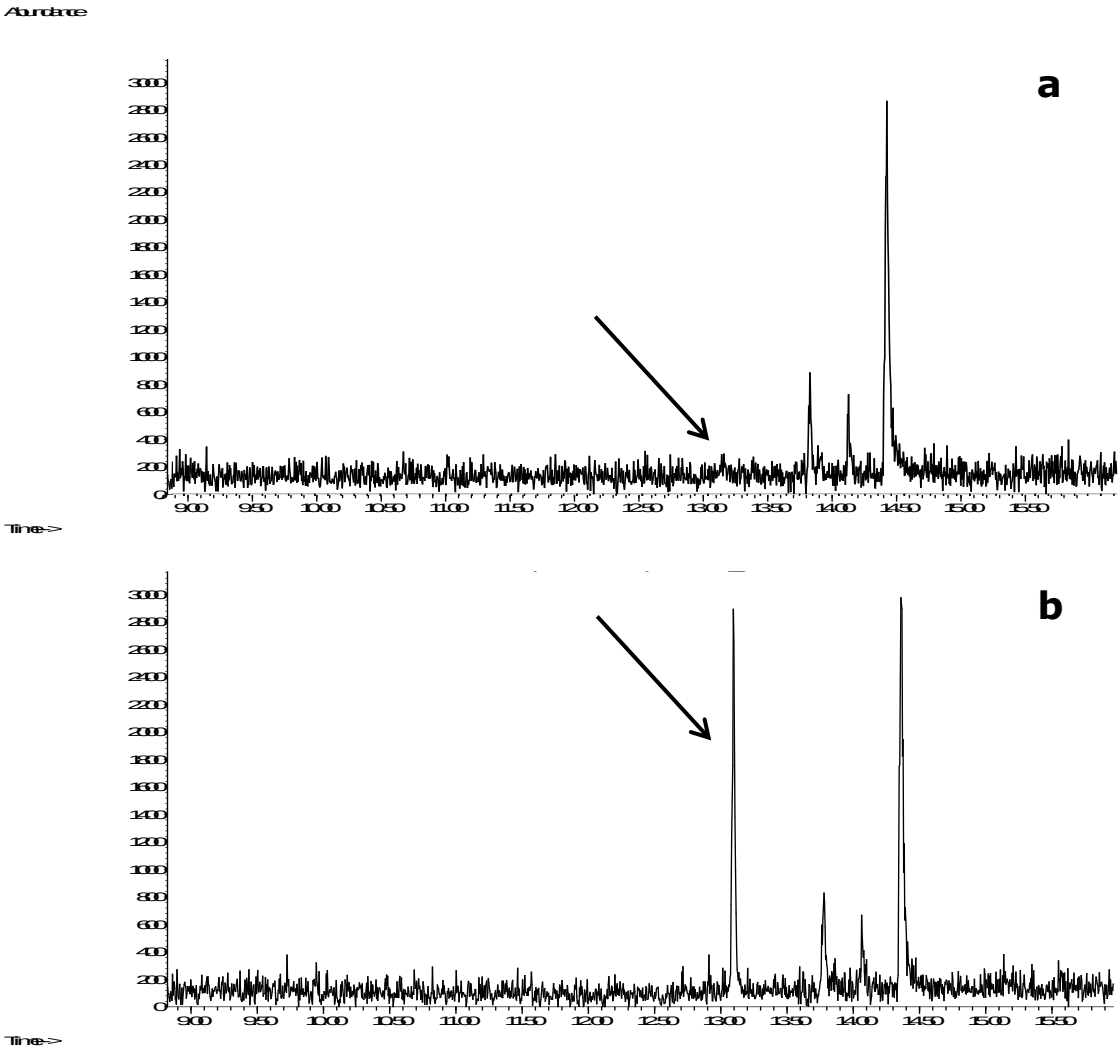

Figure 5 GC MS analysis of leaf extract in WT (a) and COE (b). The caryophyllene peak indicated by the arrows is absent /very low in the WT.

Leaf movement tracking

The hyponastic movement has been accurately tracked to reveal differences between the caryophyllene overexpressor and WT as initially suggested by a naked eye observation of the COE plants put for 10 minutes in darkness. Time lapse imaging of Arabidopsis rosette in normal growth conditions showed a moderate increase of leaf movement for the COE compared to the WT. The difference was 30% more evident for the plants grown in negative DIF environment even if the overall amplitude of all these plants was reduced by 50% (Figure 6). Caryophyllene synthase KO mutants behaved indistinguishably from the WT (data not shown).

Other terpene overexpressors have been analysed for leaf movement and although they did show a higher hyponasty compared to WT plants the amplitude was from 5 to 40% lower than the COE .

(data not shown). Terpene overexpression did not influence the phase of hyponastic movement(Figure 6).

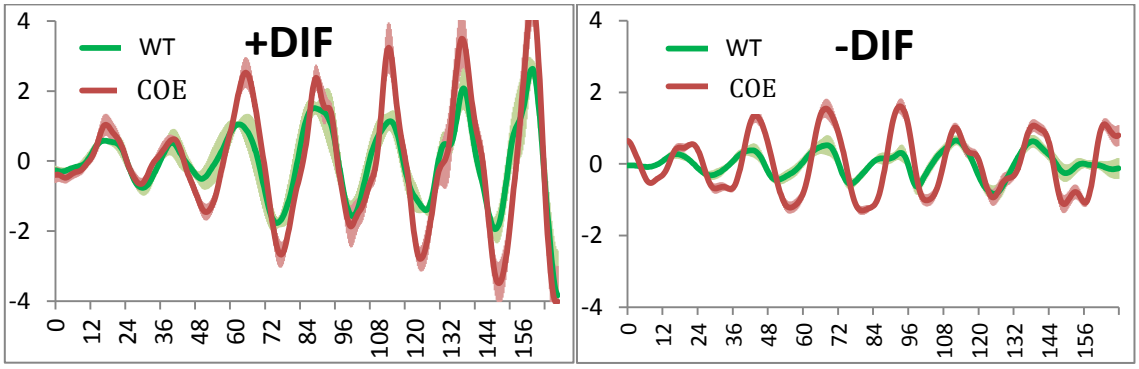

Figure 6: Leaf movement amplitude under normal growth conditions (left) and negative DIF (right). Each bar represents the average of three replicates, two leaves per plant ± SE.

Protoplast isolation and FRAP

Such a high production of caryophyllene in the headspace made us hypothesize a change in the membrane properties due to the flow of molecules crossing the membrane. To investigate differences in membrane fluidity we performed a FRAP Experiment on fluorescently labelled protoplasts.

Healthy protoplasts from WT and COE *A. thaliana* plants have been successfully isolated in quantities reaching approx. 150 pps/µl using the "tape method" (Wu, et al., 2009).

In order to make them express a fluorescent protein and to target it to the membrane the CAAX-YFP construct has been used. The CAAX domain is needed to trigger protein prenylation. This means that when an hydrophobic group is added to the C-terminus of the protein, in this case of the Yellow Fluorescent Protein, YFP, it will mediate association to the plasma membrane (Wright & Philips, 2006). Transfection with the CAAX-YFP construct resulted in 0.2% of protoplasts strongly expressing membrane-localized YFP. Seldom protoplasts with an incorrect YFP localization such as in the nucleus and cytosol were found but they have not been used for the FRAP experiment to avoid artefacts in recovery of fluorescence.

In both the genotypes analysed the laser pulse was clearly photo-bleaching the selected area and the fluorescence recovery was rapid (Figure 7) .

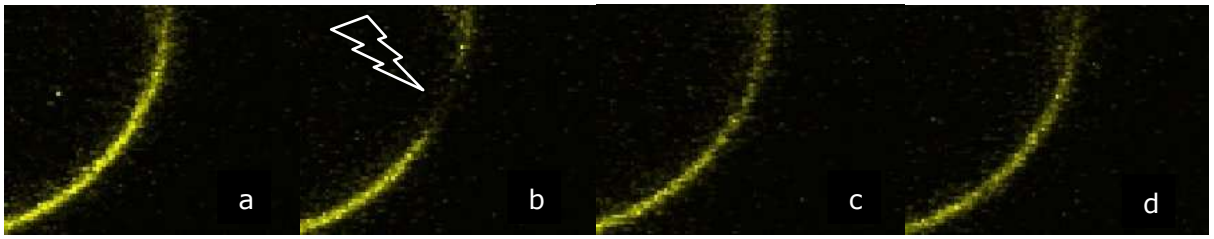

Figure 7. Time-lapse imaging of the fluorescence recovery during the FRAP experiment. The YFP is localized on the plasma membrane of an *A. thaliana* protoplast (a); a manually selected region is photo-bleached with 50 laser pulses (b); after 9 seconds the fluorescence is partially recovered(c) and after 40 seconds the recovery reaches its maximum (d)

The Fluorescence recovery after photo-bleaching has been corrected for the background and normalized showing a good robustness of the data. Eight scans were acquired before the bleach pulse (data not shown) and 112 scans were acquired after it to track the recovery rate until a plateau level. Each batch of replicates has been analysed independently to check the reliability of the data then all the results have been averaged together. In both of the cases the experiment did not result in any significant difference between the WT and COE fluorescence recovery curves (Figure 8).

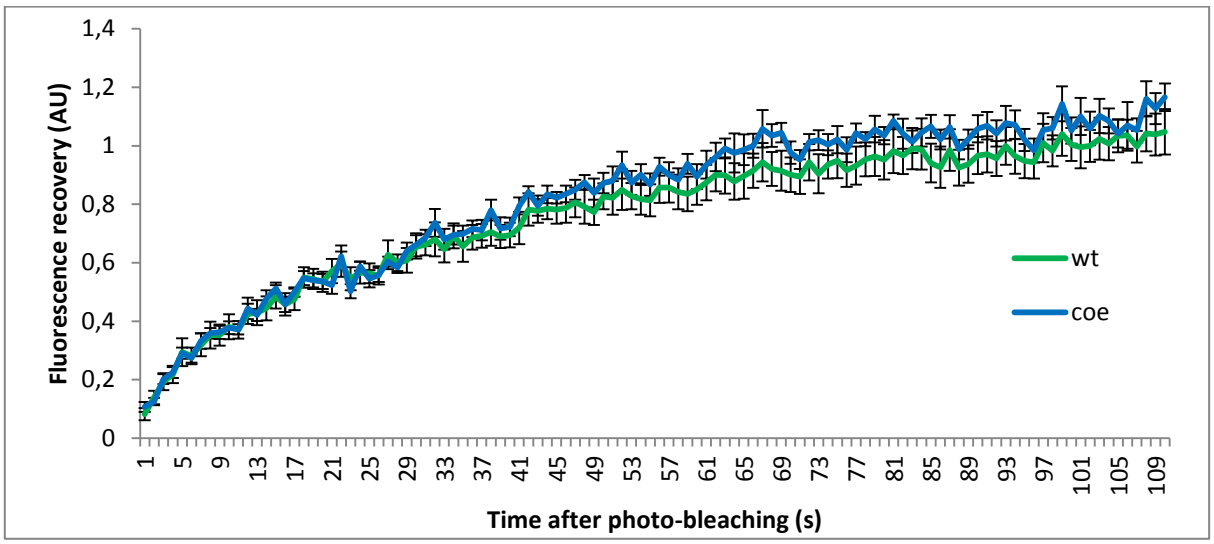

Figure 8. FRAP results averaged after normalization. WT: green line (n=17); COE: blue line (n=15) Error bars represent the average of all the replicates ± SE.

Triple response

Once excluded the involvement of membrane fluidity our attention went to the ethylene signalling, hence we performed the triple response assay (Neljubov, 1901). This test is the ideal platform to visualize differences among mutants in ethylene signalling. All the seedlings were white, with a very scarce or null root formation and an elongated hypocotyl. Without ACC in the medium the first difference that was found was the significantly shorter hypocotyl length of the COE seedlings(Figure 9a). The test showed also a small but significant degree of ethylene insensitivity for these seedlings since, in proportion, their growth was less affected than the WT or LIM seedlings. (Figure 9b).

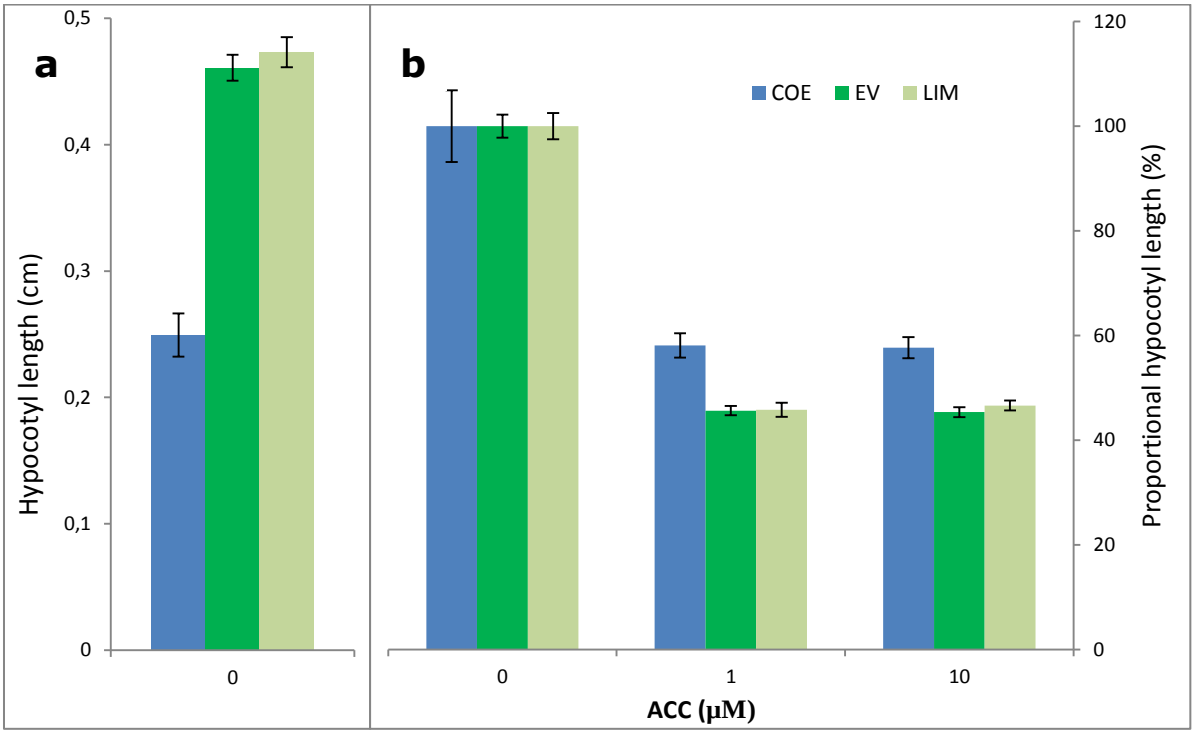

Figure 9 Triple response. (a)Hypocotyl length after 7 days in the darkness without ACC(1-aminocyclopropane-1-carboxylic-acid). (b)Data normalized on the length of the hypocotyl in absence of the ethylene precursor ACC. COE shows a moderate ethylene sensitivity. Each bar represents the length average of 80 seedlings ± SE.

Root length

To investigate the role of circadian rhythm of the terpene emissions in the overall growth of the plant we analysed the root growth after 7 days in normal light conditions (12/12 hours day/night) of COE, LIM and WT plants. This was then compared with the growth under short and long day conditions, respectively 8/16 and 16/8 hours day/night

Root length comparison in different day-length conditions did not show significant differences between the COE and the WT. The limonene overexpressor instead showed a significant increase under all the light conditions. (Figure 10)

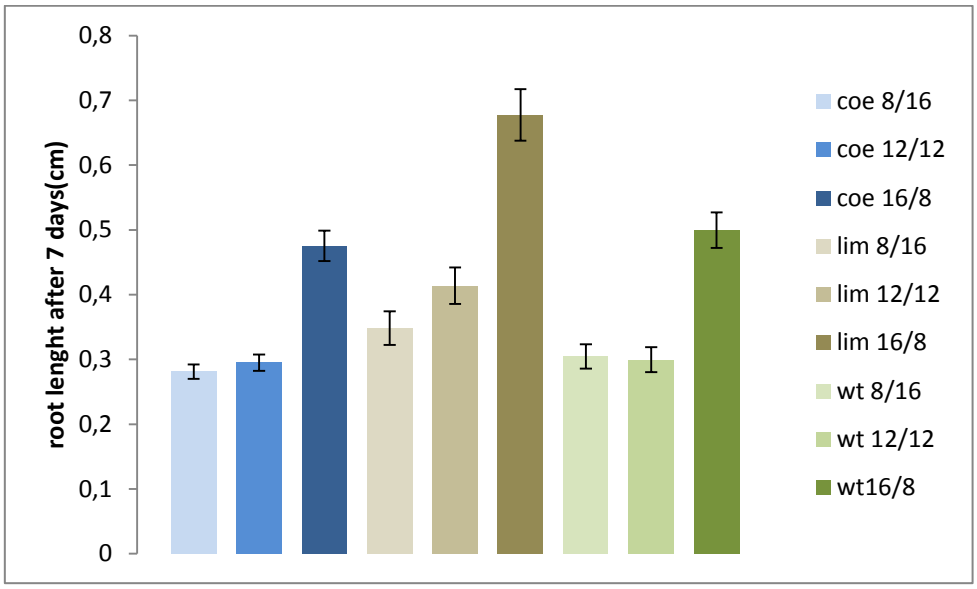

Figure 10 Root length analysis in 8/16, 12/12 and 16/8 hours day/night. Each bar represents the length average of 30 roots ± SE.

Head space analysis

To further investigate the role of the pathway in the production increase an analysis of the headspace for three COE plants and three WT controls has been carried out during two hours during the day and two hours during the night. The level of expression are remarkably different from the WT but they show a quite high variation among them. Beside this, the headspace measurement confirmed the diurnal activity of the caryophyllene synthase showing a 2.5 fold increase between the night and the day clearly visible in both genotypes (Figure 11)

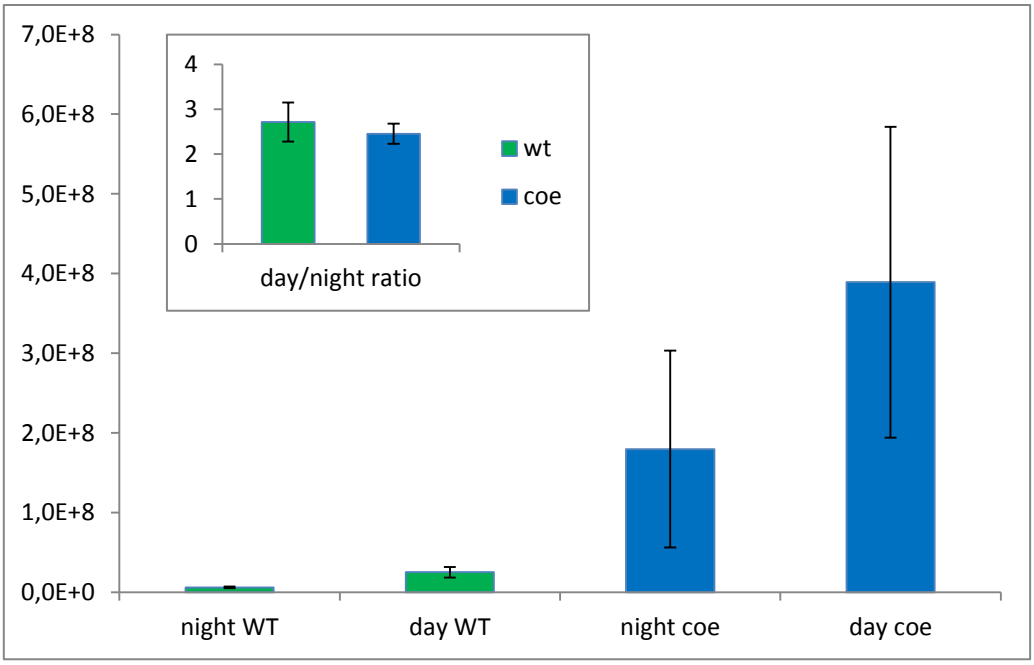

Figure 11 Head space trapping of volatile caryophyllene during day and night in WT and COE *A. thaliana* plants. Inner graph shows the ratio between day and night constant in both genotypes. Each bar represents the average of three replicates ± SE.

Discussion

The molecular mechanisms underlying the hyponastic movement in this terpene overexpressor resulted to be more complex than at first thought and many of the initial hypotheses had to be revised. This is often the case with strong overexpressors in which pleiotropic effects are hard to quantify.

The role of the MVA pathway and its intermediates is one of the main research topics in our lab and the boosting a terpene production is intended to give rise to a deeper knowledge of the mechanisms involved. In this regard, the co-infiltration of the HMGR enzyme showed a clear enhancement of caryophyllene production in all the replicates and combinations confirming the crucial role of this enzyme in the biosynthesis of a plethora of compounds including the caryophyllene precursors. One aspect to be considered though, is that we infiltrated a truncated version of this enzyme lacking the membrane binding peptide. This means that the increase of caryophyllene production is indeed caused by the presence but not necessarily by the expression of HMGR. The unidentified compound that we found after HMGR infiltration further shows the presence of this enzyme biosynthetic activity Even if, in this case, we lack the reference compound to precisely link the GC-MS peak with a known metabolite, we can imagine, according to its retention time and mass, we are dealing with a caryophyllene-like molecule such as farnesene or germacrene.

Unexpectedly the agroinfiltration of FPS did not further boost the caryophyllene production, but actually showed a detrimental effect in combination with both AtCPS and CuCPS, with or without HMGR. An improperly cloned gene can indeed act as an empty vector and dilute the infiltration of the other genes but such a clear decrease in production, constant for all the samples, and stronger than EV-diluted combinations, is indicating that the enzyme is present and expressed but likely subtracting precursors and directing them toward other compounds with retention times beyond our analysis. The proof for this hypothesis comes for example by the fact that leaves in which AtCPS has been infiltrated together with FPS yield significantly less caryophyllene than others in which AtCPS is coinfiltrated with EV only. Another possibility is that the gene we are infiltrating is actually silencing the endogenous caryophyllene synthase even if a suppressor of silencing (P19) has been added to all of the agroinfiltration solutions.

The cucumber-isolated gene instead, showed a very weak expression. In this case the actual presence of the transgene is uncertain and a clear correlation is absent but a moderate increase of the caryophyllene production compared to the empty vector is visible. The background signal was quite high, also in empty tubes used as controls (data not shown) this indicates that more replicates are needed to visualize a clear difference between the normal production of caryophyllene by the *N. benthamiana* leaves and a real expression of the agroinfiltrated *Cucumis sativus* gene.

As hypothesized by Zunino et. al (2011) a membrane structural change after terpene overexpression was supposed to occur and that was the first hypothesis of the project. The idea behind it would be an expansion of the membrane in which the terpenes accumulate between the phospholipids leading to a membrane curvature or expansion. In protoplasts the membrane reaches its maximum expansion due to the lack of cell wall hence differences in protoplast size are unlikely to be seen. A difference in membrane fluidity instead should be seen because the presence of hydrocarbons among the lipids should interfere with the microviscosity within the lipid layer. Now, if this will lead to a higher or a lower fluidity depends on the actual interaction. If we aim to find a link with the hyponastic movement a more "oiled" environment should facilitate the natural leaf movements thus increasing its amplitude. On the other hand it can also be argued that the presence of extra molecules among the phospholipids will actually decrease fluidity and add pressure on the cell walls. In this regard we might speculate that this can stimulate elongation linearly with terpene production and asymmetrically if the side of the leaf exposed to the light is actually producing more caryophyllene.

To test if light can have an impact on terpene production we showed that the terpene production is almost 3 fold increased during the day compared to the night but further tests are needed to discriminate variations in terpene production in different light conditions and their effect on (hyponastic) growth.

Our results confirmed the diurnal rhythmicity of caryophyllene emissions (Figure 11) as previously claimed by others (Aharoni, et al., 2003) (Dudareva, et al., 2005) and the constant ratio of increase from day to night indicates that the light activation of the 35S promoter is not playing a role in our overexpressor lines. Anyway no difference between WT and COE in growth speed have been found through root elongation studies. This was also expected because the overexpression of the caryophyllene and limonene synthase is not supposed to involve other metabolites upstream in the pathway.

The FRAP technique has been a robust, but time consuming, method to investigate all this fine tuning of membrane fluidity. At first obtaining good quality protoplasts is often labour intensive and the tape method in these regards indeed speeded up the process and avoided the vacuum infiltration step. A high quantity of debris was always present but a filtration step has not been added to the protocol because the confocal imaging sorted out all the autofluorescence from dead cells. Secondly, transfection efficiencies are highly variable due to many factors that is not always possible to consider. For example young leaves (2-3 weeks old) have a less strong cell wall hence they give a higher quantity of protoplasts but they are also more recalcitrant in incorporating external DNA. Anyhow, with 4-5 weeks old plants the transfection never went beyond the 1% of the protoplasts while often no transfection at all occurred. The transfected protoplasts were occasionally displaying YFP in all cellular compartments (hence not used for the FRAP ,due to the obviously higher recovery rate of free floating YFP). This could indicate that the CAAX peptide was lost during the transfection or other factors impeded a correct prenylation of the YFP (Zhang & Casey, 1996). This reduced further the number of protoplasts that could be reasonably used for the FRAP experiment. The settings of the confocal microscope needed lot of attention and each single parameter had to be kept constant for all the measurements and all the replicates to avoid differences between an experiment and the other. The fluorescence recovery was very fast thus the resolution had to be kept not higher than 512x512 pixels to maintain the speed of imaging as fast as possible without losing good image quality. A cooling device applied to the confocal might have helped in keeping the fluorescence recovery less fast and hence better see possible differences. On the other hand a too low temperature might interfere with the vitality of the protoplasts and hence decrease the significance of the measurements. The use of dyes might simplify the transfection procedure but, again, it can severely affect the membrane properties and different dye concentrations will give rise to incorrect results if the number of protoplasts is not equally divided among samples. A cold maintained glass slide and dyes have not been used in this project to avoid all the just mentioned possible artefacts.

One more thing to take into account is that the caryophyllene molecules might have crossed the membrane by simple diffusion dissolving in the W5 solution and if these cells in normal conditions were having a different membrane fluidity it might not be the case when their cell wall is artificially removed. Caryophyllene, though, is not water soluble at room temperature and at normal atmospheric pressure but to isolate protoplasts several centrifugation steps at 500 g might have

increased sensibly its solubility in water. The 16 hours in darkness before imaging might hence not be enough to re-establish an accumulation of terpenes leading to a membrane fluidity change. To control this factor, the presence of at least small amounts of caryophyllene production in protoplasts has been checked with GC-MS after each imaging.

Artefacts can occur measuring membrane in an unnatural model like protoplasts but maximum care has been given to minimize interferences with the measurements. Moreover the number of replicates from three independent transfections realized with three different batches of plants can let us conclude that no evident change in plasma membrane fluidity is occurring because of a high production of caryophyllene (Figure 8).

At this point an interaction with ethylene, the main trigger of hyponastic movement is highly expected. As a matter of fact the ethylene triple response assay gave a promising result. COE seedlings grown in the dark are considerably shorter and thicker than WT ones. Moreover when an ethylene precursor is added to the media the effect is opposite, being the COE seedlings proportionally less responsive to this plant hormone (Figure 9). This clearly suggests that the overexpression of caryophyllene is interfering with the ethylene signalling. This sesquiterpene is synthesized on the ER (Lichtenthaler, 1999) where also the ethylene receptor are localized (Guo & Ecker, 2004). This co-localization further suggests a link between ethylene signalling and terpene overexpression.

If ethylene, heat and darkness are known triggers of hyponastic growth(Figure 1), ethylene is on the other hand an inhibitor of the heat induced hyponasty (van Zanten, et al., 2009) and this, combined with the ethylene insensitivity phenotype in our mutants can explain their higher amplitude of leaf movements under negative DIF conditions(Figure 6).

In this MSc thesis project the hypothesis of a membrane fluidity change leading to an increase of hyponastic movement has been rejected, while a correlation with circadian rhythm of terpene emissions and ethylene insensitivity has been found.

Further investigation on the interference between the terpene overexepression and clock regulation together with the analysis of ethylene production and sensitivity will give new insights to the "missing link" between terpene overproduction and hyponastic movement.

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