The role of E-2-hexenal and γ-amino butyric acid in plant defense responses
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GENERAL DISCUSSION

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Two model organisms are better than one: *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *tomato*

The flowering plant *Arabidopsis thaliana* has been adopted as a model organism by the vast majority of plant biologists in the last three decades. It is not surprising that this small member of the *Brassicaceae* family became the model of choice of plant biologists if we mention some of its characteristics i.e. small size, simple growth requirements even under lab conditions, short life cycle of 8 weeks, self-fertilizing diploid, mutants are easily crossed, thousands of seeds are produced from a single plant, easily transformed with *Agrobacterium tumefaciens*, one of the smallest known plant genomes, and a large collection of T-DNA insertion lines with sequences of the flanking regions and DNA microarrays (Somerville and Koornneef, 2002). Another model organism used extensively is *Pseudomonas syringae* pv. *tomato* (DC3000), the first *Pseudomonas* plant pathogen to be sequenced (Buell et al., 2003), which causes disease not only on *Solanum lycopersicum* but also on *A. thaliana* (Cuppels, 1986; Whalen et al., 1991). The interaction between *Arabidopsis thaliana* and *Pseudomonas syringae* pv. *tomato* DC3000 has been widely studied in plant pathology for the plant immune system and the complex network of plant hormones (Jones and Dangl, 2006; Pieterse et al., 2009; Pieterse et al., 2012; Xin and He, 2013). With these premises it was an easy choice to study the role of Green leaf volatiles (GLVs) and
of γ-amino butyric acid (GABA) in plant defense responses using *Arabidopsis thaliana* and DC3000.

GLVs have received considerable attention for their ability to induce direct and indirect defense responses in plants and they can be considered important players in the already complex network of regulated responses during biotic stress. However the mechanisms by which GLVs influence pathogenesis and the signaling pathways involved are not well known. GABA has also raised a lot of questions in the early 2000s. This interest originated because, after being characterized as a neurotransmitter in animals (Tillakaratne et al., 1995), it emerged that in plants GABA is largely and rapidly produced in response to biotic and abiotic stresses and it has been linked to the regulation of cytosolic pH, carbon fluxes into the TCA cycle, nitrogen metabolism, deterrence of insects, protection against oxidative stress, osmoregulation and signaling (Bouché and Fromm, 2004). Despite the attention received, the role of GABA in plants has not been elucidated completely.

Several years ago a connection between GLVs and GABA was established: one of GLVs, E-2-hexenal, specifically caused root growth inhibition and, based on this phenotype, an EMS-mutagenized Arabidopsis population was screened, searching for mutants lacking this response. The first *hexenal response* (*her*) mutant analyzed, *her1*, was mutated in the gene coding for one of the GABA shunt steps i.e. *GABA TRANSAMINASE (GABA-*
This finding was the milestone for further research. In this mutant her1 (allelic with pop2, pollen pistil interaction 2, (Palanivelu et al., 2003)), GABA accumulated to high levels. Based on the observation that E-2-hexenal treatment induces GABA accumulation, and that high GABA levels confer resistance to E-2-hexenal, a role for GABA was proposed in mediating E-2-hexenal responses.

DC3000 also has GABA Transaminases (GabT), which transfer the amine group from GABA to acceptors, but it lacks Glutamate dehydrogenases (Gad) (Park et al., 2010). Since GABA is the main amino acid in the tomato apoplast (Solomon and Oliver, 2001; Rico and Preston, 2008) and DC3000 a tomato pathogen in the apoplast, it was decided to further investigate their relationship in Arabidopsis, especially in the GABA hyper accumulator her1 (pop2-1) mutant. Moreover a DC3000 mutant lacking all three GABA-T (ΔgabT) genes was constructed (Park et al., 2010). It was found that ΔgabT is unable to utilize GABA and, in complete minimal media supplemented with GABA, it grew less well than wild-type DC3000 and had reduced expression of hrpL and avrPto, encoding an alternative sigma factor and effector, respectively (Tang et al., 2006), associated with the type III secretion system (TTSS) necessary for the pathogenesis (Alfano and Collmer, 1996; Alfano and Collmer, 2004; Büttner and He, 2009). Indeed the ΔgabT mutant grew less than wt DC3000 in Arabidopsis ecotype Landberg erecta (Ler) and much
less in the pop2-1 (Ler) mutant that accumulates high levels of GABA. Interestingly the defense marker *PATHOGENESIS RELATED 1 (PR-1)* (Loake and Grant, 2007) was strongly expressed in the mutant pop2-1 upon DC3000 infection, indicating that increasing levels of GABA negatively influence DC3000’s ability to suppress PAMP triggered immunity (PTI). Moreover the ability of DC3000 to elicit the hypersensitive response (HR) in tobacco leaves, which is dependent on the type III secretion system, was reduced in the ΔgabT mutant compared to wild-type DC3000 when both bacteria were co-infiltrated with GABA, indicating that GABA may have various effects on DC3000 - plant interactions.

Several questions remain: what is the connection between E-2-hexenal and GABA in plant defense responses? How can we shed more light on this topic?

**Plant defenses are influenced by low GABA levels**

Our following research branch was aimed to investigate what happens to the DC3000-Arabidopsis interaction when GABA levels are low (*chapter 3*). The choice of *A. thaliana* as the model system helped us with the availability of T-DNA knock out lines. The *A. thaliana* genome has 5 *GAD* genes, which are expressed in different tissues and organs under normal conditions (Miyashita and Good, 2008). Among these 5 genes, the ones that are reported to be mainly involved in GABA
synthesis are $GAD1$ and $GAD2$ (Zik et al., 1998; Bouché et al., 2004), while $GAD4$ is induced during abiotic stress such as cold, hypoxia, drought and salt stress conditions (Kaplan et al., 2007; Miyashita and Good, 2008; Urano et al., 2009; Renault et al., 2010). Thus we decided to use T-DNA knock-out lines of these 3 genes to achieve double ($gad_{1,2}$ and $gad_{1,4}$) and triple ($gad_{1,2,4}$) mutants likely resulting in lower GABA levels. Our data confirmed that GAD1 and GAD2 are the main enzymes that synthesize GABA, that GAD4 is not induced and that GAD3 and GAD5 do not compensate the other $gad$ mutations under normal growing conditions (figure 4, chapter 3). The logic follow-up was to test the susceptibility to DC3000 of the $gad$ double and triple mutants with the hypothesis that they would be more susceptible. However, we found that the $gad_{1,2}$ double mutant and the $gad_{1,2,4}$ triple mutant showed increasing resistance to DC3000 with a trend correlating with the decreasing basal GABA levels (figure 5, chapter 3). During the DC3000 infection GABA levels were monitored as well, along with the major plant hormones i.e. SA, JA and ABA. First of all we found different patterns of GABA accumulation in the $gad_{1,2}$ and $gad_{1,2,4}$ mutants showing a similar degree of resistance to DC3000. Indeed, when infected, GABA levels did not increase in the double mutant, while the triple mutant had extremely low GABA levels, but still it managed to trigger a GABA increase. What is more intriguing is that these two very different patterns
for the GABA accumulation did not have a differential effect on the susceptibility of DC3000, indicating that the relationship between the bacteria and this metabolite is far more complex than our hypothesis. Conversely, the measurement of the plant hormones unravelled that GABA is not only a source of C/N, or that it only influences PR-1 and TTSS, as was published in 2010, but also it can change the plant hormone dynamics, especially JA and ABA, during Pseudomonas infection. This is a quite important finding which has to be supported by further experiments, for example by analyzing the expression of well-known JA and ABA marker genes during the Pseudomonas infection. In a recent paper it is shown, in a very smart way, that in Arabidopsis glutamate receptor like genes (GLRs), coding for putative cation channels, control the distal expression of JA-dependent genes (Mousavi et al., 2013). This is quite interesting because it has been speculated that GLRs, which mediate Ca^{2+} intake in the presence of the ligand glutamate, could be also activated by GABA (figure 1a and 1b) (Bouché and Fromm, 2004). Indeed GLRs have domains which share structural homology with domains of GABA receptor B in animals (Bouché and Fromm, 2004). This evidence might explain the different JA accumulation in gad mutants infected with DC3000. We hypothesize that in gad_{1,2} and gad_{1,2,4} mutants with GABA levels strongly reduced (figure 1c and 1d), the lower presence of this metabolite might reduce the Ca^{2+} influx. As a consequence the
expression of JAZ10 and the other JA-dependent genes might be reduced, likely leading to more resistant plants. Indeed is well known that DC3000 exploits JA-dependent gene expression through coronatine in order to inhibit SA-dependent plant defenses (Block et al., 2005; Koornneef et al., 2008).

These new findings about GABA also raise some questions, first of all: what is the dynamic range of GABA concentrations that triggers a certain response? In Park et al, it was hypothesized that DC3000 might use GABA to down-regulate its Hrp system and effectors delivery during later stages of infection. What is the effect of very low GABA levels on the expression of the Hrp system and what is the effect of very high GABA levels on the JA and ABA levels? In addition it is known that a pre-treatment with E-2-hexenal increases the GABA levels in pop2-1 mutant (Mirabella et al., 2008). What will be the effect on DC3000 susceptibility of this additional GABA increase and on hormone levels? Moreover, we showed that an E-2-hexenal pre-treatment is able to increase the susceptibility through the ORA59-dependent JA signaling pathway (chapter 2).
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Figure 1. Glutamate receptor-like (GLRs) working hypothesis with E-2-hexenal and GABA. GLRs are located in the plasma membrane. a) GABA acts as a ligand for GLRs and b) triggers the cation intake. The calcium flux can activate downstream signaling events (black arrows) such as the induction of JA-dependent genes in the nucleus, for example JAZ10. In a) E-2-hexenal can induce an increase in GABA levels. In b) calcium intake activate (white arrow) calcium/calmodulin dependent Glutamate Decarboxylases (GAD) and synthesize (grey arrow) new GABA, which could be transported outside through GABA transporters (GT). c) With low GABA levels, for example in gad mutants, the GLRs do not mediate a calcium intake and d) neither JA-dependent genes neither GADs are activated. In a-b is represented the control condition, in c-d is represented the condition with gad mutants.
Another research line that should be explored is performing the experiments done in chapter 3 with the DC3000 mutant $\Delta g_{abT}$, which is less virulent than wt DC3000 and cannot metabolize GABA. A possible result would be that the $\Delta g_{abT}$ mutant is able to grow better on $gad$ mutants, with a degree proportional to the reduction of GABA levels. Additionally the $\Delta g_{abT}$ mutant might result in different in the hormone levels in the $gad$ mutants pending on the GABA levels.

As shown in figure 2b, where we tried to summarize the behaviour of mutants with altered levels of GABA and wild type during the interaction with DC3000, it is noticeable that steady GABA levels correlate, to a certain extent, with the susceptibility to DC3000, being the latter higher when GABA reaches levels in the range of wt. This observation made us think that, maybe, there was a certain difference in GABA concentration, which is given by the difference of the concentrations of the induced state and the control state, $\Delta [\text{GABA}] = [\text{GABA}]_{\text{induced state}} - [\text{GABA}]_{\text{control state}}$, which is needed to have downstream GABA-dependent responses. This situation applies for $gad_{1,4}$ which can still induce, to a certain extent, an increase in the GABA levels during DC3000 infection and it is as susceptible as wt. For the other $gad$ mutants the relationship between GABA levels and susceptibility changes. Indeed we found that the $gad_{1,2}$ mutant, which has GABA levels similar to $gad_{1,4}$ when mock infiltrated, does not trigger a GABA accumulation and is more resistant.
than wt. On the other hand the \textit{gad} triple mutant, which has very low levels of GABA, succeeded to induce a small significant increase in the GABA levels during DC3000 infection. Moreover the susceptibility of the \textit{gad} triple mutant is equal to the one displayed by \textit{gad}_{1,2} mutant. In addition we noticed that in the induced state, at 48hpi with DC3000, GABA levels are about double compared to the control state in wt, \textit{gad}_{1,4} and \textit{gad} triple mutant. Also in pop2-1 the increase in the GABA levels triggered by DC3000 infection are doubled compared to mock inoculated control (Park et al., 2010). Then another question raised: are, in the triple \textit{gad} mutant, the doubled GABA levels reached during the infection, enough to compensate a response which might not be triggered otherwise, given the extremely low GABA levels?

\textbf{Root architecture during salt stress is influenced by different GADs.}

Since GABA has been shown to accumulate upon salt stress, we decided to use the \textit{gad} mutants to explore the role of GABA during this environmental stress further. The response to this kind of abiotic stress has already been characterized in the GABA over accumulator \textit{pop2-1} (Renault et al., 2010; Renault et al., 2013) and based on these data, our hypothesis was that \textit{gad} mutants would display higher resistance to NaCl. Plants can adjust their root system architecture (RSA) in response to salt
concentrations and the degree of this complex response is easily measurable by dissecting single components with EZ-Rhizo (Armengaud et al., 2009). Surprisingly we found that different GADs influence the different components of the RSA, indicating that all the isoforms we analyzed have a different role in shaping the root during control and salt stress conditions. This is likely realised through different GABA gradients in different cell root layers, gradients that are achieved through tissue specific expression of different isoforms. Although specific GABA transporters identified so far are few (Meyer et al., 2006; Michaeli et al., 2011), we can speculate that during salt stress GABA might influence auxin levels and ABA during root growth, thus contributing to regulate the complex responses of this organ. Moreover it is known that the Arabidopsis roots respond to glutamate, the GABA precursor. Indeed external presence of very low concentration of glutamate triggers the inhibition of primary root growth and induces the proliferation of the lateral roots, while glutamate signaling changes root architecture via a kinase dependent pathway (Walch-Liu et al., 2006; Forde et al., 2013), indicating that roots respond to this amino acid modifying their growth pattern. Interestingly among the gene classes downregulated by phytoprostane A₁, also a reactive electrophile species as E-2-hexenal, are PIN FORMED 1 and other auxin-dependent signaling genes (Mueller et al., 2008). This indicate that E-2-hexenal might have a double influence on
the root, one through GABA the second through transcriptional reprogramming of auxin related genes.

Figure 2. Summary of GABA responses during biotic and abiotic stress. a) untreated gad mutants, wt and gaba-t (pop2-1) mutants and their root length under normal conditions are shown, b) gad mutants, wt and gaba-t (pop2-1) mutants with their susceptibility (S) to DC3000 compared to wt and the amount of GABA in response to DC3000 infection compared to mock are shown, c) gad mutants, wt and gaba-t (pop2-1) mutants are shown with their responses to salt stress (50mM NaCl) for what concerns the main root length and the amount of GABA accumulated in response to salt stress compared to control. The increasing GABA concentration in the plant lines is shown with increasing grey shades i.e. gad triple mutant and gad1,2 white, gad1,4 light grey, wt grey, gaba-t (pop2-1) mutant dark grey. For pop2-1 data we used data published in Renault et al., 2010.
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Also in this case we tried to summarize the behavior of mutants with altered levels of GABA and wild type in control conditions and during salt stress (figure 2a and 2c). I focused on the main root length parameter and we choose 50mM NaCl because we could compare our results with the one published by Renault et al., 2010.

The results obtained open up further investigations about the role of GABA during root growth in general, for example it would be interesting to measure the phenotype of all quadruple mutants of GADs, all of them with only one but different active isoform. We speculate, due to the importance of GABA, that a quintuple gad mutant would be lethal.

Again as suggested by the results of the plant hormones measurements during biotic stress (figure 7, chapter 3), it is straightforward to think about an interaction between ABA and GABA that might also occur during the response to salt stress.

As was mentioned above, it was found that the treatment with E-2-hexenal triggers an increase of GABA levels (Mirabella et al., 2008). Thus it would be interesting to test if this also occurs in the gad mutants. E-2-hexenal treatment, beside its effect on GABA, is reported to be involved in plant defense responses such as inducing defense related genes (Bate and Rothstein, 1998; Kishimoto et al., 2005).
**E-2-hexenal promotes susceptibility through JA-dependent signaling pathway.**

We decided also to develop another research branch by investigating the effect of a fully working GLV pathway and, in detail, the effect of E-2-hexenal on the interaction between DC3000 and Arabidopsis (chapter 2). Our data pointed out that a functional HPL in Arabidopsis promotes susceptibility to DC3000 through increased JA levels, an effect that is partially mediated by ORA59 in the plant and by COR in the bacteria. On the other side when HPL is present and active, as it is in Ler ecotype, it might be a target for DC3000 effector arsenal to be manipulated and increase the susceptibility to the bacteria by inducing higher levels of JA and ORA59-dependent signalling pathway which inhibit the SA-dependent defenses, effective against the bacterial invader (Glazebrook, 2005). This scenario opens the research to further investigation, for example testing if there are single nucleotide polymorphisms (SNPs) in the *HPL* gene (At4g15440) that are associated with DC3000 resistance or susceptibility. Only a preliminary analysis on the 1001 Genomes project website (1001genomes.org), whose aim is to discover the whole-genome sequence variation in Arabidopsis accessions, confirmed the presence of several SNPs in both the *HPL* exons.
**E-2-hexenal-dependent signaling is upstream GABA.**

From data previously published, the treatment with E-2-hexenal induced an increase of GABA levels in both wt and her1 mutant (Mirabella et al., 2008). We confirmed this result by measuring the GABA synthesized after the infection with DC3000 in Ler and in the introgression line hpl1(Shiojiri et al., 2012, chapter 3, figure 10)). Indeed in hpl1, which produces only GLV traces, the GABA amount triggered by the bacterial infection at 48hpi is lower than the one measured in the control Ler, which possesses active HPL.

As we already suggested before, it would be interesting to investigate what would be the effect of the E-2-hexenal treatment on both gad double and triple mutants not only for what concerns the GABA amount, but also for hormone levels and plant susceptibility to DC3000. This approach could enlighten the more aspects of the link between E-2-hexenal and GABA.

**E-2-hexenal induces redox changes in mitochondria.**

In this thesis we characterized another hexenal response mutant, her2, which helped us to understand how E-2-hexenal is perceived by plants (chapter 4). In tomato E-2-hexenal, Z-3-hexenal, Z-3-hexenyl acetate were found to exert membrane depolarization with depolarization increasing with GLVs concentration. Z-3-hexenyl acetate was found to induce a strong
cytosolic Ca\textsuperscript{2+} increase (Zebelo et al., 2012). The induction of a Ca\textsuperscript{2+} flux could also trigger GABA synthesis, since GAD is regulated by Ca\textsuperscript{2+}/Calmodulin (Gut et al., 2009). Thus the calcium flux induced by the perception of GLVs might cause downstream the activation of the GABA biosynthesis. Moreover we found that E-2-hexenal exerts a specific effect on the mitochondria, which are the cell compartments that modulate whole cell redox homeostasis (Dutilleul et al., 2003) and where enzymes of GABA shunt are localized. Indeed we found that Arabidopsis seedlings, expressing a redox sensor roGFP2 in the mitochondria, specifically showed an increased oxidation of the redox state of these organelle, only when treated with E-2-hexenal (figure 11, chapter 4). While we do not have previous indications that the E-2-hexenal is perceived in the mitochondria, we can find an analogy in an animal system: in mice a small volatile molecule, 2-amino acetophenone (2-AA), produced by Pseudomonas aeruginosa, triggers mitochondrial dysfunction and is responsible for the quorum sensing for these bacteria (Tzika et al., 2013). Since GLVs and E-2-hexenal are able to induce membrane depolarization (Zebelo et al., 2012) and glutamate receptors-like (GLRs) are responsible for membrane depolarization in response to wounding (Mousavi et al., 2013), it would be interesting to test if the glr mutants also have an altered pattern of JAZ10 expression or wound-activated surface potentials (WASP) in response to E-2-hexenal or others
GLVs. As we discussed previously, GABA could be a ligand for these cation channels and in such way we can connect in another way $E$-2-hexenal and this non-protein amino acid. $E$-2-hexenal is a special GLV because its structure has an $\alpha,\beta$-unsaturated carbonyl moiety which binds nucleophilic atoms and makes it belong to Reactive Electrophile Species (RES) (Farmer and Davoine, 2007). As we discussed in the chapter 4, ROS generate RES, together they influence the redox state of the cell (Farmer and Mueller, 2013). Interesting data that can indicate that HER2 might be involved in coping with oxidative stress comes from evolutionary studies: in *Physcomitrella patens* the homolog of *HER2* (*PHYPADRAFT_132331*) has been inserted into moss genome through horizontal gene transfer (HGT) from gram positive bacteria (Yue et al., 2012). The hypothesis beyond this acquisition is that an oxidoreductase is an useful tool to survive and adapt to land colonization, which happened about 450 million years ago (Selosse and Le Tacon, 1998). Plants, as multicellular photothrophs, that started to colonize land had to deal with higher concentrations of oxygen thus with ROS. Indeed ever since the introduction of molecular oxygen ($O_2$) into our atmosphere by photosynthetic cyanobacteria about 2.7 billion years ago (Canfield, 2005), ROS have been new sources of stress for living organisms. The putative bacterial origin of the HER2 homolog gene supports somehow the high similarity we found between HER2 with the SSADH-Acetylating enzyme of
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Metallosphaera sedula (chapter 4). Overall our research elucidated some new aspects about GLVs, especially E-2-hexenal, and GABA and opened new interesting scenarios that are worth to follow for further research and in depth-analysis.

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