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

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Characterization of Arabidopsis glutamate decarboxylase mutants during biotic and abiotic stress: unravelling the role of γ-amino butyric acid.

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Abstract:

γ-amino butyric acid (GABA) is a four carbon non-protein amino acid present in plants. Even though GABA was discovered more than half a century ago, its role in plants is not completely understood. This metabolite is known to be produced under stress conditions from glutamate by GLUTAMATE DECARBOXYLASE (GAD) and to be metabolized to succinate, feeding into the tricarboxylic acid cycle (TCA). In order to unravel functions of GABA in Arabidopsis, gad double and triple T-DNA mutants were generated and characterized their resistance to Pseudomonas syringae pv. tomato and analyzed their root architecture during salt stress. Interestingly, we found that low GABA levels influenced the accumulation of JA and ABA during the infection, resulting in a reduced growth to this pathogen. Moreover the different gad mutations influenced the root system architecture differently, suggesting that GADs are involved in plant’s shaping responses during abiotic and biotic stress.
INTRODUCTION

In plants GABA is made in the GABA shunt (figure 1a), whose first committed step is catalyzed by glutamate decarboxylase (GAD). GAD activity is highly regulated by calcium-calmodulin (Baum et al., 1996), except in rice (Akama et al., 2001), and by pH (Snedden et al., 1995; Gut et al., 2009) The Arabidopsis thaliana genome contains 5 genes that code for GADs. The basal expression of these Arabidopsis isoforms is highly organ specific (figure 1b): GAD1 (At5g17330) is expressed in the vegetative tissues, flowers and roots, GAD2 (At1g65960) in the roots, GAD3 (At2g02000) in siliques, GAD4 (At2g02010) in flowers and GAD5 (At3g17760) in stamen and mature pollen ((Miyashita and Good, 2008) and data from the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, (Winter et al., 2007)). The GABA shunt further consists of GABA-transaminase (GABA-T), which converts GABA to succinic semialdehyde (SSA) and succinic semialdehyde dehydrogenase (SSADH), which converts this to succinate (Fait et al., 2008). The Arabidopsis genome contains only one GABA-T encoding gene (At3g22200), named POP2 (Pollen-Pistil Incompatibility (Palanivelu et al., 2003), and one SSADH (At1g79440) (Bouché et al., 2003), which was also identified as hexenal response 1 (HER1) (Mirabella et al., 2008). SSA can also be converted to \( \gamma \)-hydroxybutyrate (GHB), and there are two isoform of glyoxylate reductase that can catalyse this reaction, GLYR1 (cytosol) and GLYR2 (plastid)
Glutamate decarboxylase mutants in biotic and abiotic stress (Simpson et al., 2008) (figure 1a). GABA is synthesized in the cytosol, but can also be found in chloroplasts, the vacuole, and in the apoplast (Tilsner et al., 2005) and has to be translocated into the mitochondria for conversion. GABA transporters identified so far are AtGAT1 an H⁺-driven, high affinity transporter located in the plasma membrane and AtGABP, a mitochondrial GABA permease (figure 1a).

Several roles for GABA and the GABA shunt have been postulated, overall connecting this metabolite to the protection against various stresses. Indeed the production of succinate and NADH through the GABA shunt bypasses two steps of the TCA cycle (succinyl-CoA ligase and the a-ketoglutarate dehydrogenase), which can be inhibited by oxidative stress (Sweetlove et al., 2002) and it is needed fully working for C skeletons for biosynthetic processes and reducing agents supply for ATP biosynthesis (Sweetlove et al., 2010). There is not so much evidence for the function of GABA during biotic interactions.

Since GABA is a neurotransmitter in vertebrates (Tillakaratne et al., 1995) and its accumulation is triggered by mechanical stimulation or damage in plants (Ramputh and Bown, 1996), even by insects crawling on leaves (Bown et al., 2002), it is plausible to suppose that the ingested GABA interferes with insect development as was shown in tobacco for root-knot
nematodes (McLean et al., 2003) and tobacco budworm larvae (MacGregor et al., 2003).

Figure 1. The GABA shunt in Arabidopsis thaliana. Compartmentation of GABA metabolism. The key enzymes involved are in red. GAD, glutamate decarboxylase; GABP, GABA permease; GABA-T, GABA transaminase; GAT1, GABA transporter 1; SSADH, succinic semialdehyde dehydrogenase; GLYR1 and GLYR2, glyoxylate reductase isoforms 1 and 2; TCA, tricarboxylic acid cycle and (b) Expression of GAD homolog in different organs in normal conditions (black).
In the tomato apoplast, GABA is the most abundant amino acid (Solomon and Oliver, 2001) and some pathogens as *Pseudomonas syringae pv. tomato*, *Cladosporium fulvum* and *Botrytis cinerea* can use this metabolite as a sole source of carbon and nitrogen (Solomon and Oliver, 2001; Park et al., 2010; Seifi et al., 2013). GABA levels increase during the infection with apoplastic pathogens, such as *Cladosporium fulvum* in tomato and *Pseudomonas syringae pv. tomato* (DC3000) in Arabidopsis (Solomon and Oliver, 2001; Park et al., 2010; Solomon and Oliver, 2014) and probably also in wheat infected by *Puccinia triticina* where GAD is induced (Bolton et al., 2008). In Arabidopsis, the *gaba-t (pop2-1)* mutant, which has increased GABA concentrations (Mirabella et al., 2008), displayed higher resistance to *P. syringae pv. tomato*, particularly to GABA-T mutants of *P. syringae pv. tomato* that are unable to degrade GABA (Park et al., 2010). High concentrations of GABA suppressed Type III Secretion System (T3SS) expression, especially in the GABA transaminase-deficient mutant of *P. syringae*, suggesting that a high rate of GABA turnover is important for the growth and virulence of these bacteria *in planta* (Park et al., 2010).

GABA rapidly accumulates also to high levels under environmental stress conditions such as salt, hypoxia, heat, drought, cold and heavy metals in several plant species (Rhodes et al., 1986; Cholewa et al., 1997; Shelp et al., 1999; Kinnersley
and Turano, 2000; Kaplan and Guy, 2004; Kempa et al., 2008; Urano et al., 2009; Renault et al., 2010). Moreover, it has been demonstrated to participate in pH regulation (Carroll et al., 1994; Crawford et al., 1994), to modulate nitrate uptake (Beuve et al., 2004), to regulate 14-3-3 genes (Lancien and Roberts, 2006) and to be involved in pollen tube growth and guidance (Palanivelu et al., 2003). In Arabidopsis the effect of salt stress on GABA has been better characterized. Renault and colleagues found that NaCl increases the activity of enzymes involved in GABA metabolism and *gaba*-t mutants were hypersensitive to ionic stress and accumulated more amino acids, GABA included, and less carbohydrates, suggesting for the GABA shunt a central role for carbon and nitrogen metabolism regulation (Renault et al., 2010). Moreover mutation of *gaba*-t causes hypocotyl defects and alterations of cell wall composition during salt stress, due to imbalance in sugar composition in the mutant, indicating that GABA metabolism is needed for carbon adjustment during salt stress (Renault et al., 2013). Moreover the genes involved in carbon metabolism and starch catabolism were upregulated in this mutant under salt stress, while several cell wall related genes were differentially expressed, confirming the physical alterations in the cell wall and the central role of GABA in sustaining the TCA cycle and C/N metabolism during salt stress (Renault et al., 2010; Renault et al., 2013). Interestingly, the *gaba*-t (*pop2*-*1*) mutant is also associated with
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$E$-2-hexenal response (Mirabella et al., 2008), as we found that hexenal response mutant 1 (her1) is mutated in POP2 and is insensitive to $E$-2-hexenal root growth inhibition. Moreover her1 seedlings accumulate higher GABA levels upon $E$-2-hexenal exposure than wild-type seedlings. This phenotype suggests for GABA a role in mediating the $E$-2-hexenal signalling pathway. Since $E$-2-hexenal is a Reactive Electrophile Species, which can influence the redox state, one of the major claims about the GABA shunt was that it is necessary to cope with reactive oxygen intermediates (Bouché et al., 2003). However this hypothesis has been invalidated by Ludewig and colleagues who found that the ssadh dwarf phenotype (Bouché et al., 2003) is caused by the accumulation of SSA and/or GHB, which is toxic to the plants, resulting in higher peroxide content, and not by the lack supply of succinate and NADH to the TCA cycle (Ludewig et al., 2008).

To clarify the role of GABA in Arabidopsis we generated double ($gad_{1,2}$ and $gad_{1,4}$) and triple mutants ($gad_{1,2,4}$), since GAD1, 2 and 4 are reported to be mainly involved in sustaining GABA levels in normal and stressed conditions respectively (Bouché et al., 2004; Kaplan et al., 2007; Miyashita and Good, 2008; Urano et al., 2009; Renault et al., 2010). We subjected these mutants to a biotic stress (Pseudomonas syringae pv. tomato) and to an abiotic stress (NaCl) and measured GABA and plant responses.
RESULTS

Generation of double and triple gad mutants

The $gad_{1,2}$ double mutant was generated by crossing the respective single mutants (SALK_017810 and GABI_474 E05). The $gad1$ mutant has a T- DNA insertion in the 5\textsuperscript{th} exon (figure 2\textit{a}) and $gad2$ in the 6\textsuperscript{th} exon (figure 2\textit{b}). The $gad_{1,4}$ double mutant was generated by crossing the respective single mutants (SALK_017810 and SALK_106240). The $gad4$ mutant has the T-DNA insertion in the 2\textsuperscript{nd} exon (figure 2\textit{c}). For both crosses, the F1 plants were selfed to obtain homozygous mutants. T-DNA insertions were confirmed with PCR on gDNA, following the strategy depicted in figure 3\textit{c}. Gel pictures of the mutant genotype are depicted in the panels in figure 3. The triple $gad_{1,2,4}$ was obtained by crossing the obtained double mutants: the progeny, after one auto-pollination round, was genotyped, following the strategy depicted in figure 3\textit{c}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Figure 2. Representation of the intron (white bars)-exon (black line) structure of (a) GAD1, (b) GAD2 and (c) GAD4 with T-DNA positions.}
\end{figure}
Figure 3. Analysis of PCR of the gad mutants by agarose gel electrophoresis. Panel (a) gad\textsubscript{1,4}, (b) gad\textsubscript{1,2} and (d) gad\textsubscript{1,2,4}. Numbers indicate the following primer pairs: gad\textsubscript{1}\_RP-gad\textsubscript{1}\_LP (1), gad\textsubscript{1}\_RP-LB (2), gad\textsubscript{4}\_RP-gad\textsubscript{4}\_LP (3), gad\textsubscript{4}\_RP-LB (4), gad\textsubscript{2}\_RP-GABI\_LB (5), gad\textsubscript{2}\_RP-gad\textsubscript{2}\_LP (6). White continuous (cropped from a gel) and dotted line separate different PCR reactions loaded on the same gel. (c) genotyping strategy: LB is left border of T-DNA, RP is Right Primer, LP is Left Primer. Molecular marker (MM) in a, b and c is gene ruler 1kb plus (Thermo), in d is smart ladder (Eurogentec). On top of the loading wells are indicated the samples: mutant (m), Col-0 wt (c) and water (w).

Mutations in GAD\textsubscript{1}, GAD\textsubscript{2} and GAD\textsubscript{4} effect GABA levels

In order to check whether the various combinations of gad mutation resulted in an effect on GABA accumulation in the mutants, levels of this metabolite were measured in 10 day old seedlings, grown on agar plates, as shown in figure 4. The double mutants gad\textsubscript{1,4} had lower GABA levels than the wildtype.
Col-0, but these were not statistically significantly different from Col-0. The double mutant gad\textsubscript{1,2} and the triple gad mutant gad\textsubscript{1,2,4} had GABA levels that were significantly lower than the wild type (Col-0), indicating that GAD1 and GAD2 are the isoforms that mainly contribute to basal GABA levels in seedlings.

**Figure 4: GABA levels in 10 day old wild type, gad\textsubscript{1,2}, gad\textsubscript{1,4} and gad\textsubscript{1,2,4} seedlings. Values are the mean of 8 sets of 6 seedlings. Error bars represent standard error. Bars annotated with different letters indicate significant differences between mutants and wild type (P < 0.05, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test). The data presented are the mean of two experiments.**

GABA levels influence susceptibility to *Pseudomonas syringae* pv. *tomato* (DC3000)

Since high GABA levels have been shown to influence the susceptibility of Arabidopsis to DC3000 (Park et al., 2010), we now used the various gad mutants to determine the effect of lower GABA levels on DC3000 susceptibility. We infiltrated wt
Col-0, \(gad_{1,2}\), \(gad_{1,4}\) and \(gad_{1,2,4}\) plants with this pathogen and counted bacterial populations 72 hours post infection (hpi). As shown in figure 5, the DC3000 populations in the mutants were significantly smaller in the \(gad_{1,2}\) and \(gad_{1,2,4}\) plants than in the wt. Moreover GABA levels were monitored during the infection at 2, 24 and 48 hpi and compared with a mock inoculation. As depicted in figure 6, the levels of GABA measured in 4 weeks old leaves did not change much during the first 24h. At 48 hpi there is an increase in the GABA levels in wt and \(gad_{1,4}\) plants during infection, but not in the \(gad_{1,2}\) mutant. Interestingly, the triple \(gad_{1,2,4}\) mutant accumulated very low GABA levels compared to the double mutants, but GABA levels still increased during DC3000 infection.

Figure 5. Bacterial population size in DC3000-infected wild type Col-0, \(gad_{1,4}\), \(gad_{1,2}\) and \(gad_{1,2,4}\) plants measured 72 hpi. Values are the mean of 18 sets of two leaf disks from 12 plants. Error bars represent standard error. Bars annotated with different letters indicate significant differences between 72 hpi samples (\(P < 0.05\), according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test). The data presented are from a representative experiment that was repeated twice with similar results.
Figure 6. GABA levels in wild type Col-0, gad_{1,4}, gad_{1,2} and gad_{1,2,4} during inoculation with DC3000 or mock infiltration at 2, 24 and 48 hpi. Values are the mean of 6 different pools of 3 different leaves from 3 different plants. Error bars represent standard error. Bars annotated with different letters indicate significant differences between samples at 48hpi (P < 0.05, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test), n=2.

Mutations in GAD1, GAD2 and GAD4 have different effect on hormone levels during Pseudomonas syringae pv. tomato (DC3000) infection

It is well known that the balance between JA and SA is crucial for the interaction that will be established between a pathogen and its host (Spoel and Dong, 2008; Grant and Jones, 2009; Pieterse et al., 2009). Emerging evidence has pointed out that the hormone crosstalk is more than just a mere antagonistic relationship between JA and SA, indicating that other
phytohormones, such as abscisic acid (ABA), are involved (Loake and Grant, 2007; Bari and Jones, 2009; Robert-Seilaniantz et al., 2011). We therefore monitored the changes in these major phytohormones in the gad mutants and wt at 2, 24 and 48 hpi with DC3000 or mock inoculation in order to check if they are associated with the lower resistance of the gad\textsubscript{1,2} and gad\textsubscript{1,2,4} mutants compared to wt. As expected, at 48hpi there was clearly an increase of SA levels compared to the mock when wt Arabidopsis Col-0 plants were infected by DC3000 (Figure 7a). The SA response was unaffected in all mutants upon DC3000 infection. ABA levels (figure 7b) increased in all plant-pathogen combinations after 48h compared to the mock. However, the increase in ABA levels in the triple gad mutant gad\textsubscript{1,2,4} during the infection was only half of the increase in the other double mutants and in the wt Col-0. Levels of JA were, as expected, higher at 48hpi in wt. Considering the mutants, JA levels were similar to wt Col-0 in the gad\textsubscript{1,4} plants infected by DC3000 at 48 hpi. However, JA levels were much lower in the gad\textsubscript{1,2} and gad\textsubscript{1,2,4} mutants 48hpi.
Figure 7. Hormone levels in wild type, gad\textsubscript{1,4}, gad\textsubscript{1,2} and gad\textsubscript{1,2,4} during inoculation with DC3000 or mock infiltrated at 2, 24 and 48 hpi. (a) SA, (b) ABA and (c) JA. Values are the mean of 6 different pools of 3 different leaves from 3 different plants. Error bars represent standard error. Bars annotated with different letters indicate significant differences between samples at 48hpi (P < 0.05, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test), n = 2.
Mutations in \textit{GAD1}, \textit{GAD2} and \textit{GAD4} have different effect on root architecture during salt stress

It has been established by several studies that GABA accumulates in plants during salt stress (Bolarín et al., 1995; Bor et al., 2008; Renault et al., 2010; Zhang et al., 2011; Akçay et al., 2012). In particular Renault and colleagues characterized \textit{pop2-1}, a GABA over-accumulator mutant in Arabidopsis, during different salt treatments. In order to investigate different \textit{gad} mutations on salt stress tolerance, we decided to measure the Root System Architecture (RSA), since a tight correlation between salt stress and root development has been established (Galvan-Ampudia and Testerink, 2011). In \textbf{figure 8} we measured \textbf{(a)} Total Root Size (TRS), \textbf{(b)} Main Root Length (MRL), \textbf{(c)} Number of Lateral Roots (NLR), \textbf{(d)} Lateral Root Length (LRL) because they are the main parameters for RSA. We decided to use 25mM, 50mM, 100mM and 125mM salt because these concentration were already used to study RSA in our group (Julkowska et al., 2014). However in 100mM and 125mM salt without sucrose in the medium plants had a stunted growth and high mortality so we decided not to use these conditions for further experiments.
Figure 8. (a) Total Root Size (TRS), (b) relative TRS, (c) Main Root Length (MRL), (d) relative MRL, (e) Number of Lateral Roots (NLR), (f) relative NRL, (g) Lateral Root Length (LRL), (h) relative LRL of 8 old days seedlings of wt Col-0, gad_{1,4},gad_{1,2}, gad_{1,2,4} after 4 days of salt treatment at 25mM and 50mM and 0mM as control. Error bars represent standard error. Bars annotated with different letters indicate significant differences (P < 0.05, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test), n = 2.
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In mild salt stress conditions (25 mM NaCl), the total root size (TRS) of wild type (Col-0) was lower while in the double mutants the TRS did not change compared to control conditions (0 mM NaCl) and even increased in the triple gad mutant (figure 8a and b). The MRL of gad1,2 and gad1,2,4 was higher than in the wt and gad1,4 during mild salt stress conditions (25 mM NaCl, figure 8c and d). In higher salt stress conditions (50mM) the double mutants displayed similar MRL as the wt, while in the triple mutant the MRL was bigger. The number of lateral roots (NLR) was strongly affected by gad mutations since gad1,4 did not respond to increasing salt stress by reducing the number of lateral roots as the Col-0 wt (figure 8e and f). The mutant gad1,2 had more lateral roots in the control conditions and in 25 mM salt it showed a reduction in NLR comparable to the wt in the same mild salt stress conditions. However at 50 mM salt gad1,2 did not show the reduction in NLR that was found in the wt in the same salt stress conditions. Strikingly, the gad1,2,4 mutant did not respond to increasing salt stress by reducing the NLR, as the wt did, but also it had a higher NLR in control conditions. The lateral root length (LRL) was reduced with increasing salt stress for the gad1,2 and gad1,2,4 as well as the wt, while gad1,4 was responding less to salt stress (figure 8g and h). Indeed in this mutant LRL did not change much by increasing the salt concentration.
GABA levels are modulated during salt stress

Since the different gad mutants seemed to be influenced differently by salt stress we decided to measure the GABA levels in the double and the triple mutants after 6 days of salt treatment at 25mM and 50mM and 0mM as control.

We used the same plant material from the RSA analysis in order to better correlate the amount of GABA with the root growth. It can be noticed in figure 9 that while the wild type induced GABA levels during salt stress as previously shown (Renault et al., 2010), in the mutants this response was lost and in particular gad$_{1,4}$ produced less GABA by increasing the salt concentration and gad$_{1,2}$ and gad$_{1,2,4}$ did not induce GABA at all.

Figure 9. GABA levels 10 day old seedlings of wild type, gad$_{1,4}$, gad$_{1,2}$ and gad$_{1,2,4}$ grown on 0, 25 and 50mM NaCl. Values are the mean of 2 different experiments of 4 different pools of 5 different seedlings. Error bars represent standard error. Bars annotated with different letters indicate significant differences in different plant lines ($P < 0.05$, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test), $n=2$. 
DISCUSSION

Arabidopsis thaliana genome has 5 GAD genes, which are expressed in different tissues and organs in normal conditions (Miyashita and Good, 2008) as depicted in figure 1b. GAD1 and GAD2 are reported to be the mainly responsible for the biosynthesis of GABA (Zik et al., 1998; Bouché et al., 2004), while GAD4 is induced during 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 and triple mutants likely resulting in lower levels of GABA.

Under normal, unstressed conditions (figure 4) GABA levels were the lowest in the gad_{1,2} seedlings as expected, since GAD1 and GAD2 are the more expressed isoforms in normal conditions (Zik et al., 1998; Bouché et al., 2004), while gad_{1,2,4} seedlings did not exhibit a further decrease in GABA levels. This means that GAD4 does not contribute to GABA levels during normal unstressed condition and that GAD3 and GAD5 do not compensate the mutations in the other GADs.

For biotic stress we chose bacterial infection with Pseudomonas syringae pv. tomato (DC3000), since we have already published the effect of GABA over-accumulation in pop2-1 mutant on this interaction (Park et al., 2010) and this bacterium is a model pathogen for probing disease susceptibility in Arabidopsis (Xin and He, 2013). We hypothesized that gad mutants with lower
GABA levels would be more susceptible than the wt because pop2-1 is reported to be more resistant to DC3000 (Park et al., 2010).

However we found the opposite result: \( g_{ad1,2} \) double mutants and the \( g_{ad1,2,4} \) triple mutant showed increasing resistance to DC3000 (figure 5) with a trend correlating with lower GABA levels under control conditions (figure 4). To further investigate the reason why \( g_{ad} \) mutants are more resistant to DC3000, we measured GABA levels at three different time points before the bacterial population scoring assay at 72hpi. As depicted in figure 6 GABA levels increased during the infection in wt and \( g_{ad1,4} \) and, to a lesser extent in \( g_{ad1,2,4} \), while in \( g_{ad1,2} \) the levels did not change. Mutants that are, to a similar degree, more resistant to DC3000, i.e \( g_{ad1,2} \) and \( g_{ad1,2,4} \), showed different patterns of GABA accumulation: GABA levels did not increase in the double mutant, while the triple mutant had extremely low GABA levels, but still it managed a GABA increase when infected (figure 6), implying a minor role for GAD3 and GAD5. These different patterns of GABA accumulation are really interesting because very low GABA levels, still increasing during the infection, and low GABA levels unchanged during the infection seem to determine the same effect on plant susceptibility. This could be explained by a GABA threshold which is needed to trigger downstream signalling and effects on plant defence response. Indeed if we look (figure 6), the wt and the \( g_{ad1,4} \)
have GABA level higher than about 1 ng/mg FW and are both more susceptible. However we cannot exclude that also the delta concentration (the difference between basal and induced level) reached during the infection plays a role, influencing the susceptibility to DC3000. GABA is the most abundant metabolite in the tomato apoplast (Solomon and Oliver, 2001; Rico and Preston, 2008), the place where Pseudomonas syringae pv. tomato multiplies (Hirano and Upper, 2000). Indeed DC3000 can use GABA as sole source of C/N, but the effect of this metabolite to the bacterial pathogenicity is far more complex. If we consider GABA only as a carbon source for DC3000, then the equal susceptibility of $gad_{1,2}$ and $gad_{1,2,4}$ cannot be explained by the GABA levels in figure 6. Moreover it is not known how much of this amino acid is used by DC3000 in the apoplast. In Park et al., 2010 we have published that higher GABA levels induced an higher expression of PR-1, repressed hairpin ($hrp$) gene expression in vitro and repressed tobacco HR, which overall suggest that the higher the concentration of this amino acid, the less DC3000 is able to suppress PAMP triggered immunity (PTI), possibly through impaired effector delivery or through priming of PTI. However we found that the effect of the GABA concentrations on DC3000 growth in the $gad$ mutants seem to allow other scenarios. We did not check the expression of PR-1 and $hrp$ genes, but we can assume that low GABA levels triggers
some other plant defence mechanisms during DC3000 infection.

To elucidate these mechanisms underlying the response of *gad* mutants to DC3000 infection, we measured the levels of SA, ABA and JA, the major phytohormones, because their crosstalk is crucial for the development of pathogenesis in Arabidopsis (Loake and Grant, 2007; de Torres-Zabala et al., 2007; Koornneef et al., 2008; Ton et al., 2009). SA levels were induced to similar levels in all infected plants, regardless of the mutation (*figure 7a*). This accumulation is expected since SA is used by the plant to switch on defences against (hemi)-biotrophs pathogens as DC3000 (Glazebrook, 2005).

Surprisingly we found major differences in the levels of ABA and JA in the mutants with the lowest GABA content i.e *gad*$_{1,2}$ and *gad*$_{1,2,4}$. Still, ABA levels were induced as well in infected plants, with a smaller accumulation in *gad*$_{1,2,4}$ (*figure 7b*) and JA levels were lower and not induced in the DC3000 infected *gad*$_{1,2}$ and *gad*$_{1,2,4}$ (*figure 7c*). This means that GABA levels influenced the phytohormone levels, especially ABA and JA.

In early plant defence ABA is known to be responsible for stomata closure and (Merlot et al., 2001; de Torres-Zabala et al., 2007; Melotto et al., 2008; Clay et al., 2009) but its role in later stages of plant defence is less established and it is dependent of the pathogen type (Ton et al., 2009). Application of ABA suppressed SA-inducible defences activation during DC3000
infection (Mohr and Cahill, 2007) and DC3000 exploited this signalling inhibition (de Torres-Zabala et al., 2007). *Pseudomonas syringae* pv. *tomato* strain DC3000 synthesizes coronatine (Mitchell, 1982) a phytotoxin that mimics JA-Ile (Thines et al., 2007; Yan et al., 2009), in order to antagonize the SA-dependent defences (Brooks et al., 2005; Glazebrook, 2005). *Pseudomonas syringae* not only boosted JA dependent responses, but also ABA dependent ones, for examples *NCED3*, encoding a key enzyme of ABA biosynthesis and abscisic acid insensitive 1 (*ABI1*) group of genes, involved in the regulation of ABA signalling (de Torres-Zabala et al., 2007). This stimulation of ABA biosynthesis and ABA dependent signalling is exerted through the type III secreted effector AvrPtoB (de Torres-Zabala et al., 2007; de Torres Zabala et al., 2009). Another example of ABA influencing JA signalling is reported during *Fusarium oxysporum* infection: ABA promotes the plant susceptibility by suppressing ORA59 and ERF1, which integrate JA and ET dependent defense signals, through MYC2 dependent signalling induction (Anderson and Badruzsaufari, 2004). The triple *gad* mutant is the most resistant (*figure 5*) to DC3000 in the later stage of the infection and its ABA levels were lowest. The higher resistance of the triple *gad* mutant is probably caused by less ABA-dependent inhibition of SA defences and is confirmed by higher susceptibility of double *gad* mutants, which synthesized higher levels of ABA (*figure 7b*). Although the SA levels seemed
unaffected by this ABA signalling inhibition (figure 7a), the JA accumulation is strongly reduced (figure 7c) in gad1,2 and gad1,2,4, compared to wt and gad1,4 and this is consistent with their lower susceptibility to DC3000 (figure 5) because in these conditions the JA antagonism on SA dependent defences is less active (Koornneef et al., 2008).

There is some evidence that ABA influence GABA signalling and vice versa: GABA down-regulates the expression of 14-3-3 gene family members in Arabidopsis seedlings in an ABA-dependent manner (Lancien and Roberts, 2006) and ABA-deficient tomato sitiens, which displays a stronger induction of GABA-shunt enzymes, displays higher resistance to Botrytis cinerea (Seifi et al., 2013). The explanation of the possible crosstalk between GABA and JA arises from the GABA measurement during DC3000 infection of Landsberg erecta (Ler) Arabidopsis ecotype and the introgression line hpl1 (Shiojiri et al., 2012) which has a 10nt mutation in the HPL gene from Col-0 (Duan et al., 2005). Ler with a functional HPL, synthesized more GABA than hpl1 at 48 hpi (figure 10) and accumulates more JA than hpl1 at 24 hpi (Scala et al., 2013). It is thus possible to speculate that GABA and JA are downstream in the signalling pathway of GLVs through HPL, with JA-signalling upstream of GABA since the GABA peak is later than JA one. Moreover our hypothesis is supported by the fact that GABA has a role in mediating the E-2-hexenal signalling pathway (Mirabella et al., 2008). In this
context it would be interesting to check the $E$-2-hexenal responsiveness of \textit{gad} mutants as root growth inhibition as we did for pop2-1/\textit{her1}. Overall we showed that GABA participates in the hormone signalling crosstalk by influencing ABA and JA levels during DC3000 infection and we suggest it has an important role in establishing the final result of such a kind of plant-pathogen interaction.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{GABA accumulation in Ler and hpl1 during \textit{Pseudomonas syringae pv. tomato} infection or mock inoculation at 2,24,48 hpi. Error bars represent standard error. Bars annotated with different letters indicate significant differences between samples ($P < 0.05$, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test), $n=2$.}
\end{figure}

For abiotic stress we chose NaCl treatment, since Renault and colleagues also characterized \textit{pop2-1} responses during salt stress (Renault et al., 2010; Renault et al., 2013). In Arabidopsis
during salt stress GABA accumulates in shoot and root, with a higher increase in the former, mainly due to the action of GAD2 and GAD4 (Renault et al., 2010). Growth of pop2-1 roots is hypersensitive to ionic (LiCl and NaCl) stress, but not to osmotic stress (Renault et al., 2010). The inhibition of primary root growth, triggered by NaCl treatment in pop2-1 mutant, is associated with a drastic change in root metabolite profiles, especially in the accumulation of amino acids and a decrease in carbohydrates (Renault et al., 2010), which cause root and hypocotyl developmental defects and alterations of cell wall composition (Renault et al., 2013). Thus our hypothesis was that gad mutants would display lower sensitivity to NaCl. We decided to dissect the salt responses of these mutants by measuring several RSA parameters, such as TRS, MRL, NLR and LRL (figure 8). We chose not to add sucrose in the media because there is some evidence that it affects ABA content and the osmotic response (Verslues et al., 2006). In this case our hypothesis was confirmed by experimental results. As hypothesized, GABA levels affected root length and size. For example the total root size (TRS) is inversely proportional to GABA levels in control conditions because the gad1,2 and gad1,2,4 with less GABA have a larger TRS (figures 4 and 8a). Moreover TRS at 25mM NaCl follows the same trend, while at the higher salt concentration only the triple gad mutant has larger TRS (figure 8a and 8b). Overall the triple gad mutant has a larger
TRS in control and in different salt conditions, probably due to the lower ability to synthesize GABA. Further analysis of RSA led us to dissect the role of different gad mutations during salt stress. For example the mutation of gad4 together with gad1 highly influenced the emergence of lateral roots, since the mutant gad1,4 did not develop less NLR in response to increasing salt stress (figure 8e and 8f). This effect in seen as well in the triple gad mutant (figure 8f). GAD4 and GAD1 not only influenced the number, but also the length of lateral roots during salt treatment. Indeed gad1,4 the LRL did not drastically become smaller in response to increasing salt concentrations as with the wt, gad1,2 and gad1,2,4 (figure 8g and 8h). Interestingly the triple mutant did show the LRL decrease with increasing salt concentration, response that seemed to be restored by the mutation of gad2 in combination with gad1, although to a lesser extent (figure 8h). Also for LRL the triple gad mutant has longer lateral roots than the wt for control conditions and mild salt stress. For what concerns the main root length (MRL) it seems that in mild salt stress the combination of gad1 and gad2 mutations triggers a bigger main root length growth than in wt and in gad1,4. This enhanced growth of the main root in 25mM NaCl has already been reported also in media with sucrose (Zolla et al., 2010; Julkowska et al., 2014).

From these preliminary analyses it seems that different GADs influence differently the RSA components in salt stress.
conditions. It is difficult to correlate the GABA levels measured in the mutants (figure 9) and the effect on the RSA we found. By grinding whole seedlings together we have not been able to measure GABA concentrations differences in different root tissues. However, we can observe that as occurred with gad1,2 infected with DC3000, this gad mutant did not change the GABA amounts during abiotic and biotic stress (figure 6 and 9). This supports our hypothesis that the delta inducible GABA concentration (the difference between basal and induced level) could be important in order to trigger GABA dependent responses. We further hypothesize, based on the effect of different mutations on RSA, that not only different amounts/grads of GABA in different parts of the root, but also tissue-specific expression differences between GADs might contribute to shape the this organ in response to salt stress. We cannot exclude that, as showed by the phytohormone analysis during DC3000 infection, ABA is influenced by or acts together with GABA in shaping the salt response. However our data did not reveal any difference (data not shown). Even so additional analysis is needed to clarify if GAD3 and GAD5 are also involved or compensate the effect of the other GADs knock-out. Overall is clear that that GABA is a key molecule and this is underpinned by the presence of 5 different genes that are likely differently expressed in diverse plant organs in different stress or growth conditions. Indeed our data pointed out new roles for GABA, i.e
the ability to influence phytohormone crosstalk during pathogenesis and the root system architecture.

MATERIAL AND METHODS
Isolation of gad mutants
The Arabidopsis thaliana (ecotype Columbia 0) T-DNA knockout lines were characterized based on the information provided by the Salk Institute Genomic Analysis Laboratory (http://www.signal.salk.edu). The single mutants gad1 (SALK_017810), gad2 (GK_474E05) and gad4 (SALK_106240) seeds were screened for homozygosity by genotyping with primers on genomic DNA (supplemental table 1). For the generation of the double mutants gad1,2 and gad1,4 the respective single mutants were crossed by emasculating the mother plant, followed by pollination with the pollen from the male parent. F1 was selfed and PCR was performed on gDNA of F2 plants to find the homozygous double mutant. The gad1,2 and gad4 double mutants were crossed and the progeny, after one selfing round, was genotyped. The generated triple mutant was termed gad1,2,4.

For gDNA isolations, leaf tissue was ground with 900μl extraction buffer (2% SDS, 10 mM EDTA, 100mM Tris-Cl pH 7.5) and incubated at 65°C for 25min. Once cooled at RT, 300μl of 10M ammonium acetate was mixed in. Samples were spun down at 12000g for 10min, and 750μl of supernatant
transferred to a new tube. 750µl of 2-propanol was added and samples were spun down at 12000g for 10min. Pellet was washed with 70% ethanol and subsequently dried at RT. 100µl of MQ water was used for re-suspension and 2µl used as template for PCR. The PCR program was as follows: 94°C x 1min, (94°C x 30", 56°C x 1', 72°C x 1'30") for 40 cycles and final extension of 72°C x 5'. The strategy used is depicted in figure 2c.

Root architecture analysis
Seeds were vapour sterilized in a desiccator of 1.6L volume using 20ml household bleach and 0.6ml 40% HCl for 3 hours. The seeds were stratified in 0.1% agar at 4°C in the dark for 48h and sown on square Petri dishes containing 50ml of control growth medium consisting of ½ concentration Murashi-Skoog plus 0.1% MES buffer pH 5.8 (KOH) and 1% daishin agar, and then dried for 1 h in a laminar flow. Plates were placed under an angle of 70 degrees. Seeds were germinated under long day conditions (21°C, 70% humidity, 16/8h light/dark cycle). Four days old seedlings were transferred to square Petri dishes containing control medium supplemented with 0mM, 25mM, 50mM or 100mM NaCl. Next, plates were transferred to a growth chamber 21°C, 70% humidity, 16/8h light/dark cycle. Pictures were taken 4 days after the treatment and used as input files for EzRHIZO software (Armengaud et al., 2009). Six
days after treatment the seedlings were harvested and frozen in liquid N\textsubscript{2} for GABA extraction.

**Bacterial population counts**

Bacteria were grown overnight at 28°C in liquid King’s broth KB medium (King et al., 1954) containing rifampicin (50µg/ml) for the *Pseudomonas syringae* pv. *tomato* DC3000 strain. Plants were inoculated with either a low dose (OD\textsubscript{600} of 0.0007), for bacterial growth assays, or a high dose (OD\textsubscript{600} of 0.007), for JA, SA and ABA quantification, of the bacterial suspension, and bacteria (colony forming units, cfu) were counted as reported in Park et al. (2010).

**GABA measurement**

GABA measurement was done as we previously published by Park et al., 2010.

**JA, SA, ABA measurement**

Phytohormones were measured as previously published by Scala et al., 2013

*Author contributions: A.S did and designed the research; R.M. crossed gad single mutants, F.L provided additional set of gad double mutants, M.A.H. revised the chapter; R.C.S. advised the research and edited the chapter.*
Chapter 3

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