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

Scala, A.

Citation for published version (APA):
Scala, A. (2015). The role of E-2-hexenal and γ-amino butyric acid in plant defense responses
The Arabidopsis her2 mutant implicates an oxidoreductase in E-2-hexenal responsiveness.

Authors: Scala, Alessandra; Mirabella, Rossana; Goedhart, Joachim; de Vries, Michel; Haring, Michel A.; Schuurink, Robert C.
Abstract:

It is widely accepted that plants produce and respond to green leaf volatiles (GLVs), but the molecular components involved in transducing their perception are largely unknown. We have previously shown that treatment of Arabidopsis seedlings with the E-2-hexenal inhibits root elongation and, using this phenotype, we isolated E-2-hexenal response (her) Arabidopsis mutants. Here, we mapped the her2 mutation to the At5g63620 locus, which encodes a protein belonging to the GroES-like zinc-binding alcohol dehydrogenase family. The target peptide of HER2 directed mCherry to the mitochondria, confirming the predicted localization. In silico analysis of the protein sequence revealed that HER2 has homology to a succinic semialdehyde dehydrogenase acetylating (SSADH-Acetylating) of a thermoacidophilic bacterium, Metallosphaera sedula. The overexpression of HER2 resulted in a stronger root growth inhibition upon E-2-hexenal treatment than in wt. Furthermore we used redox sensitive GFP2 (roGFP2) to identify differences in the redox status of the root cells due to the her2 mutation. Since HER2 is localized in mitochondria, we targeted the expression of roGFP2 to this organelle or to the cytosol. We found that E-2-hexenal specifically induced an increase in the oxidation state of roGFP2 in the mitochondria, but not in the cytosol, indicating that signalling downstream this volatile occurs in this organelle, although we did not see a difference in the redox state of the roGFP2 in her2 compared to wild-type Arabidopsis. Still, this response in the mitochondria did not occur with Z-3-hexenol, one of the most abundant GLVs, suggesting also that this response is quite specific.

Since the redox status of the plant can influence its resistance to pathogen infection and in silico analysis showed that HER2 is induced by Pseudomonas syringae pv. tomato, we tested her2 for DC3000 resistance. The mutation resulted in an increased susceptibility to these bacteria, suggesting a role for HER2 during biotic responses.
INTRODUCTION

Plants are continuously challenged by a wide variety of biotic stresses which activate different signalling pathways orchestrated by e.g. jasmonate (JA), salicylate (SA), ethylene (ET) and abscisic acid (ABA), which all contribute to setting up the complex signalling network needed for counteract biotic attackers and induce the defense machinery (Pieterse et al., 2009; Pieterse et al., 2012).

During battles between plants and pathogens other important players are C$_6$ volatiles, which are produced in response to herbivory, wounding and environmental stress (Croft et al., 1993; Turlings and Loughrin, 1995; Fall et al., 1999; Shiojiri et al., 2000; Gouinguené and Turlings, 2002; Heiden et al., 2003; Shiojiri et al., 2006). Biosynthesis of these volatiles occurs mainly by the consequential action of two enzymes, C$_{13}$ lipoxygenase (LOX) and hydroperoxide lyase (HPL; (Hatanaka, 1993; Matsui et al., 2000; Matsui, 2006)), followed by the activity of an aldehyde dehydrogenase (ADH), an isomerase and acetyltransferase in order to produce the GLV bouquet. Although data recently recapitulated in a review (Scala et al., 2013a) clearly show that plants sense and respond to C$_6$-volatiles, almost nothing is known about the molecular mechanisms that mediate their perception in plants. To reduce complexity of studying this class of compounds, we decided to focus on E-2-hexenal, which has been shown to be one of the
most active volatile in triggering C$_6$-volatile-induced responses in Arabidopsis (Kishimoto et al., 2005; Kishimoto et al., 2006). This characteristic likely derives from the $\alpha,\beta$ unsaturated carbonyl moiety with its very high reactivity towards nucleophilic atoms that are widespread in biological molecules, such as cysteine, lysine and histidine in proteins (Esterbauer et al., 1991; Marnett et al., 2003; Farmer and Davoine, 2007; Mano, 2012). Due to this chemical structure, $E$-2-hexenal is grouped together with another big family of molecules i.e. Reactive Electrophile Species (RES) (Farmer and Davoine, 2007) which can influence gene expression massively (Alméras et al., 2003; Taki et al., 2005; Mueller et al., 2008). ROS generate RES and these compounds together effectively cause redox changes and oxidative stress (Potters et al., 2010; Suzuki et al., 2012; Farmer and Mueller, 2013). Being a RES, $E$-2-hexenal, can influence the redox status and consequently trigger downstream specific responses. The rapid production of ROS also takes place when plants respond to infections by using membrane-bound NADPH oxidases (Marino et al., 2012), secreted peroxidases or amine oxidases (Bolwell et al., 2002), as part of the general pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or effector-triggered immunity (ETI) responses (Yoshioka et al., 2009; Torres, 2010; Heller & Tudzynski, 2011; Thomma et al., 2011; Tudzynski et al., 2012).
To reveal the molecular mechanisms underlying \( E \)-2-hexenal response, we performed a mutagenesis screen to select Arabidopsis mutants with altered responses to this RES. In our previous paper (Mirabella et al., 2008) we developed an assay to screen for \( E \)-2-hexenal response (\( \text{her} \)) mutants in an EMS mutagenized Arabidopsis population. Here we describe the mapping of the mutation in \( \text{her}2 \) and its characterization with regard redox status and involvement in biotic stress.

**RESULTS**

The mutation in \( \text{her}2 \) maps to At5g63620.

In our previous paper (Mirabella et al., 2008) we described the isolation of 18 different hexenal response (\( \text{her} \)) mutants and we showed that the \( \text{her}1 \) mutation was in the GABA transaminase gene (At3g22200). Here we describe the mapping of the mutation in \( \text{her}2 \). The \( \text{her}2 \) mutant has the same phenotype as the \( \text{her}1 \) mutant, i.e. the growth of the root of young seedlings is less inhibited by \( E \)-2-hexenal while the wild type Arabidopsis ecotype Col-O stops growing (\textbf{figure 1c}).

Since the trait was segregating as single recessive mutation, we used positional cloning to map the \( \text{her}2 \) locus as we did for \( \text{her}1 \) (Mirabella et al, 2008), based on the methods developed by Lukowitz et al., (2000) and Jander et al., (2002) (Lukowitz and Gillmor, 2000; Jander et al., 2002). We created an F2 population from a cross between Ler and \( \text{her}2 \) and phenotyped 512 F2
plants. Bulk segregant analysis on 50 plants with the markers developed by Lukowitz et al (2000) put the mutation on the lower arm of chromosome 5 (ciw9 marker, data not shown). To further delimit the position of the her2 locus, all 512 phenotyped F2 plants were genotyped with markers in this area (Supplemental figure S6 and Supplemental table S1). For markers Mbk5c7 and Mbk5c8, spanning a region of 25 kbp, only three recombinants were identified. This interval contains 9 genes that were subsequently all sequenced. Only At5g63620 had a point mutation in an exon compared to the published genomic sequence. This lead to a single amino acid substitution in the predicted HER2 protein, from serine (S223) to phenylalanine (F223) (figure 2).

In order to corroborate the correlation between the genotype of her2, i.e. the mutation in the gene At5g63620, and its phenotype, we tested two Salk T-DNA lines (Alonso et al., 2003), SALK_072101 with an insertion in the fifth exon and SALK_079558 with an insertion in the first exon of At5g63620, for E-2-hexenal responsiveness together with the her2 mutant and Col-0. Three-day-old seedlings were exposed for 24 h to methanol (MeOH) or to 0.3 μM aerial E-2-hexenal. Root growth was measured 3 days after the treatment. Results are shown in the figure 1c. The extent of resistance to E-2-hexenal-induced root growth inhibition shown in the her2 mutant is also displayed in the SALK_072101 mutant, even to an higher extent
than in her2, whereas the other SALK line had a similar response as wt. This indicates that a T-DNA insertion close to the point mutation in HER2 results in a similar phenotype, but an insertion in the first exon not.

![Diagram](image)

Figure 1. Positional cloning of her2 location and response to E-2-hexenal. (a) Representation of chromosome 5 between nucleotides 25464351 and 25487847. Arrows denote the position of the genes present in this area. The red square indicates the At5g63620 locus. (b) Representation of the HER2 genomic region. Exons (white blocks) introns (black lines) and putative promoter (grey arched box) are shown. Black triangles represent T-DNA insertions for the Col-0 SALK_072101 (5th Exon) and SALK_079558 (1st Exon) lines, grey triangle represents the base substitution of C with T, 507bp downstream the start codon. (c) Three-day-old seedlings from SALK lines 072101 and 079558, her2 and wild-type Col-0 were exposed for 24 h to MeOH or to 0.3 µM aerial E-2-hexenal. Root growth was measured 3 days after the treatment. Values are the mean of at least 15 plants, distributed over three plates with standard error indicated.
HER2 participates to E-2-hexenal signalling

HER2 is shares similarity to a bacterial succinic semi-aldehyde dehydrogenase.

The TAIR database (www.arabidopsis.org) predicts that the gene At5g63620 encodes a GroES-like zinc-binding alcohol dehydrogenase family protein. Alcohol dehydrogenases (ADH, alcohol:NAD+ oxidoreductase, EC 1.1.1.1) are Zn-binding enzymes that act as a dimers and use NAD(P) as co-factor to convert e.g. ethanol in acetaldehyde (and other short linear alcohols in their related aldehydes) and vice versa. ADHs are members of the medium-length dehydrogenase/reductase (MDR) protein superfamily.

The predicted 427 amino acid (aa) sequence of HER2 was subsequently used as input sequence for a Basic Local Alignment Search Tool (BLAST) analysis and compared against a non redundant protein database at UniProt (www.ebi.ac.uk/uniprot). Among the subject sequences with highest homology with HER2 we found that the only protein similar to HER2 with a confirmed activity is a Succinic Semi Aldehyde Dehydrogenase Acetylating (A4YGN0, SSADH-Acetylating, EC 1.2.1.76) of a thermo-acidophile bacterium, i.e. Metallosophaera sedula (Berg et al., 2007), which has 42% aa identity with HER2. The reaction catalyzed by this enzyme is as follows:
Succinate semialdehyde + Coenzyme A + NADP⁺ = succinyl-CoA + NADPH

Interestingly the SSADH-Acetylation activity is not reported yet in plants, accordingly to Plant Metabolic Network (PMN, www.plantcyc.org) and MetaCyc (metacyc.org). However the product, succinyl-Coenzyme A, is a substrate for the TCA cycle, produced by 2-oxoglutarate dehydrogenase. There are many plant proteins at NCBI database (www.ncbi.nlm.nih.gov) that are highly similar to HER2, but although putatively annotated, their enzymatic function has not been confirmed yet.

The alignment (UniProtKB, www.uniprot.org) of the mutated protein HER2 with HER2 and SSADH of *M. sedula* is depicted in *figure 2*. The mutation in HER2 caused a substitution from a serine (S223), a polar amino acid to a phenylalanine (F223) a non-polar amino acid with a big aromatic group. This mutation is close to the putative NADH/Zn binding domain of the protein predicted by NCBI (www.ncbi.nlm.nih.gov, *supplemental figure S1 b*).
HER2 participates to E-2-hexenal signalling

Figure 2. Alignment of amino acid sequences of HER2, mutated HER2 with SSADH-Acetylating of Metallosphaera sedula. In red are underlined the small and hydrophobic, in blue the acidic, in magenta the basic amino acids except for H; in green hydroxyl, sulphydryl, amine plus G amino acids, * (asterisk) indicates positions which have a single, fully conserved residue, : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix, . (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix. Black square underlines the amino acid substitution from S to F.

The first 55 amino acids of HER2 sequence (figure 2), which do not align with the M. sedula enzyme, form a putative signal peptide for sorting to the mitochondria (Wolf PSORT, http://www.genscript.com/psort/wolf_psort.html). The mito-
Chapter 4

Mitochondrial localization is predicted with another signal peptide prediction server, SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) by using D-cutoff sensitive, with cleavage site between position 27 and 28: GSA-LS (supplemental figure s1 a).

**Phylogenetic analysis of HER2 and ADHs**

Given the predicted mitochondrial localization, the 42% sequence similarity with an enzyme that uses SSA as a substrate (figure 2) and the annotation as an ADH, we decided to use the sequences of *Arabidopsis thaliana* ADHs (Kirch et al., 2004) as well as the sequences of SSADH of *Oryza sativa* (UniProtKB entry B9F3B6) and *Arabidopsis thaliana* (Bouché et al., 2003) to calculate a phylogenetic tree (figure 3), accordingly to maximum likelihood algorithm (Felsenstein, 1981; Tamura et al., 2011) in order to gain information about the putative HER2 function. We also included GLYR1 and GLYR2 because they are respectively cytosolic and mitochondrial oxido-reductases, involved in the conversion of Succinic Semi Aldehyde (SSA) to γ-hydroxybutyrate (GHB)(Allan et al., 2009). The phylogenetic tree shows that the HER2 protein and the SSADH-Acetylating of *M. sedula* form a separate subcluster (figure 3).
HER2 participates to E-2-hexenal signalling

Figure 3. Maximum likelihood phylogenetic tree of amino acid sequences of with the A. thaliana ADH family and O. sativa SSADH (brown squares), GLYR1 and GLYR2 (yellow squares), HER2 (dark green square) and SSADH-Acetylating A4YGN0 from Metallosphaera sedula (light green square). ADH, aldehyde dehydrogenase; BADH, betaine aldehyde dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydroge-nase; MMSA, methylmalonyl semialdehyde dehydrogenase; P5CDH, D1-pyrroline-5-carboxylate dehydrogenase; SSADH, succinic semialdehyde dehydrogenase, GLYR, glyoxylate reductase; SSADH-AC, succinic semialdehyde dehydrogenase acetylating. Genetic distance scale is shown.

HER2 does not influence GABA levels

Since the phylogenetic tree and similarity with succinic semi aldehyde dehydrogenase (acetylating) indicates that succinic semialdehyde is a putative substrate of HER2, we investigated whether the her2 mutant had different GABA levels than wt.
Results showed that the levels of GABA in her2 under unstressed conditions are similarly low as the wt ones, while, as we previously published in 2008, GABA levels in her1 are much higher (Mirabella et al., 2008) (figure 4).

![Figure 4. GABA levels in wild type, her1 and her2 plants/seedlings under unstressed normal conditions. 3 pools from 3 different leaves from 3 weeks old plant were used. Error bars represent error standard. Bars annotated with different letters indicate significant differences between mutants and Col-0 wild type (P<0.05, according to ANOVA test analysis, followed by a least significant difference (LSD), n=1)](image)

Recombinant HER2 expression in Escherichia coli

Since the data gathered so far did not shed light on HER2 function, we decided to express HER2 in E. coli without the signal peptide and with GST tag at the N-terminus to purify it in order to perform in vitro activity assays. The recombinant protein was purified successfully as shown by the western blot (figure 5, b lane).
HER2 participates to E-2-hexenal signalling

![Western blot image]

**Figure 5. Western blot with anti-GST antibodies for GST-HER2 purified from E.coli.** In lane a, b and c are loaded different washes of protein fraction from E. coli culture carrying the vector pGEX-KG-HER2. Molecular weight marker is displayed on the left in kDa. HER2-GST is predicted to be 67kDa with HER2 41kDa and GST 26kDa.

**In vitro assay of GST-HER2 biological activity**

In order to test the activity of GST-HER2 with different putative substrates, we decided to measure change in the absorbance spectrophotometrically at 340nm, the wavelength at which NADH/NADPH absorbs light maximally. HER2 belongs to oxidoreductase family and has putative NADH putative binding site (supplemental figure S1 b). As a positive control for enzymatic activity we used alcohol dehydrogenase (ADH, Sigma Aldrich, www.sigmaaldrich.com) from *Saccharomyces cerevisiae* in buffer TRIS at pH 9. To test the hypothesis that HER2 could be the one of ADHs that converts Z-3-hexenal and/or E-2-hexenal into their respective alcohols, we used also these molecules as substrates and used a reaction buffer close to the internal pH of the cell. In figure 6 the ADH and GST-HER2 activities are shown: it is clear that the recombinant HER2 is not functioning as an
ADH or alternatively the recombinant protein is not active at all. The second hypothesis we tested is that HER2 could be a SSADH and thus we added succinate/SSA as substrates but once again we could not measure any activity with NAD+/NADH or NADP+/NADPH absorbance (data not shown).

![Figure 6](image)

Figure 6. Enzymatic activity of GST-HER2 at pH 7.2 (PIPES buffer). ADH is used as positive control at pH 9 (TRIS buffer). NADH absorbance was measured at 340nm for 200sec. The enzymatic activity was measured in $\mu$mol/min/mg protein as follows: $(\Delta \text{Abs/min})/\varepsilon$ with $\varepsilon_{\text{mM(NAD)/cm}} = 6.2$. Values are reported for mg of protein used in the assay (n=2), error bars represent standard deviation; n.d, not detected.

In silico analysis of HER2 expression

In order to characterize more in depth the role of HER2 gene, we decided to collect information in widely used bioinformatics programs for Arabidopsis gene expression patterns such as GENEVESTIGATOR (www.genevestigator.com, (Hruz et al., 2008)) and e-FP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, (Winter et al., 2007)).
As shown in **Supplemental figure S2**, the expression of HER2 seems to be high in all the stages of *Arabidopsis thaliana* development, accordingly GENEVESTIGATOR. Subsequently we investigated the expression of HER2 in various organs and anatomical parts. As displayed in **Supplemental figure S3** the organ that recurs the most in the highest part of the heat-map, with the darker colour corresponding to the higher level of expression, is the root and protoplasts. The differentiated root cells in which HER2 is highly expressed are the pericycle, root phloem companion cell, quiescent center, root endodermis and root cortex cells (**Supplemental figure S3** with asterisks). Moreover if we compare *in silico* the absolute expression of HER2 in the root stele, HER2 is relatively higher expressed in the maturation zone and lower in the columella (**Supplemental figure S4**).

**HER2 overexpression leads to higher sensitivity to E-2-hexenal.**

In order to investigate the role of HER2 in the E-2-hexenal response further, we overexpressed HER2 cDNA under control of the 35S promoter in Arabidopsis. We obtained two independent lines that had higher HER2 expression, with a higher level in 4b4 than in 5la3 (**figure 7a and b**).
Figure 7. HER2 gene expression in overexpressing plants. HER2 transcript levels were measured by qRT-PCR in independent overexpressing lines, 4b4 and 5Ia3, and normalized for SAND transcript levels. a and b represent two independent experiments.

Four day-old seedlings of two independent HER2 overexpressing lines (HER2:OE), 4b4 and 5Ia3, were subsequently exposed for 24 h to E-2-hexenal or MeOH as negative control to test the hypothesis that overexpressing lines were more sensitive to the GLV tested. Root growth of the seedlings was measured 3 days after the exposure (figure 8 and 9).
HER2 participates to E-2-hexenal signalling

Figure 8. Three-day-old seedlings of the her2 mutant, two HER2 overexpression lines (a) 4b4, (b) 5la3 and wild-type plants (Col-0) were exposed for 24 h to MeOH or to aerial E-2-hexenal, 0.15μM for (a) and 0.2μM for (b). The image was taken 3 days after treatment.
Figure 9. Root growth of her2, 35S-HER2 (4b4 and 5ia3) and wt seedlings after 24 h treatment with MeOH or with 0.15 μM (a and c) or 0.2 μM (b and d) aerial E-2-hexenal. The measurement was taken 4 days after treatment on at least 30 seedlings (n=2). Bars represent standard error, bars annotated with different letters indicate significant differences between methanol or E-2-hexenal treatment (P<0.05, according to ANOVA test analysis, followed by a least significant difference (LSD) post hoc test). Percentage of the root growth with E-2-hexenal compared with root growth in MeOH is represented (c and d).

Since the standard concentration of 0.3 μM E-2-hexenal in these experiments stopped the growth of the her2, which is our control, we used lower concentrations. We used two different E-2-hexenal concentrations, 0.2 μM and 0.15 μM, since the two
independent HER2 overexpression lines were different in their response to the tested aldehyde. Indeed line 4b4 showed a root growth inhibition phenotype at 0.2 μM while the line 5la3 did at a lower dose (0.15 μM). In both cases the HER2 overexpression lines showed a stronger growth inhibition than the wt (figure 9c and 9d) while her2 grows still better than the wt at 0.15µM and 0.2µM concentrations.

**HER2 is localized in the mitochondria**

The HER2 protein is predicted to be localized in the mitochondrion (http://www.arabidopsis.org/). To confirm this localization we used Arabidopsis mesophyll protoplasts with 35S mito-GFP reporter (Nelson et al., 2007) with GFP localized in mitochondria (figure 10b) and we transfected them with pMON999-35S-mCherry with the coding sequence for the putative signal peptide of HER2 (55 amino acid, supplemental figure S1), cloned upstream of mCherry (figure 10a). overlapped, which definitely localize the HER2 protein in mitochondria.

A picture of chlorophyll autofluorescence (blue) was additionally taken (figure 10c) to show the chloroplasts. From the overlay picture in figure 10d it is clear the red and green channel, respectively HER2 signal peptide-mCherry and GFP expressed in mitochondria,
Figure 10. Confocal image of transfected protoplast with (a) HER2 signal peptide-mCherry, (b) Mito-GFP reporter and in (c) chlorophyll autofluorescence, (d) overlay.

**E-2-hexenal is sensed in the mitochondria**

Since HER2 is predicted to be a mitochondrial oxidoreductase, thus putatively involved in redox reactions, we set out to determine whether HER2 is involved in conditions with redox changes affected. To do so we used a redox-sensitive GFP2 (roGFP2, (Meyer et al., 2007), to monitor the redox state of the cell and in particular, we decided to check both mitochondria and cytosol. We thus transformed wt Col-0 and her2 with roGFP2 constructs driving constitutive expression of roGFP2 (35S promoter) and targeting roGFP2 either to the cytosol or mitochondria. In order to confirm the reliability of roGFP2 reporter we sought to measure the fluorescence lifetime in four day-old seedlings treated for 30 minutes with 20mM hydrogen peroxide (H$_2$O$_2$) (Wierer et al., 2012). The treatment was
HER2 participates to E-2-hexenal signalling

performed on her2 seedlings with the reporter expressed in the cytosol (her2 roGFP2_cyt) or in the mitochondria (her2 roGFP_mit) and on Col-0 seedlings with the reporter expressed in the cytosol (wt roGFP2_cyt) or in the mitochondria (wt roGFP_mit) as controls.

The fluorescence lifetime $\tau(\phi)$ was measured in the root tip of seedlings as an indication of the oxidation state of the redox sensitive reporter, roGFP2. This reporter has disulphide bonds that react to the redox state of the cell surrounding, which results in an increase of the $\tau(\phi)$ in a augmented oxidised environment (Meyer et al., 2007; Wierer et al., 2012). Figure 11a shows that $\tau(\phi)$ of the reporter in H$_2$O$_2$ treated seedlings is statistically significantly higher (P<0.01, t-test) than $\tau(\phi)$ of untreated seedlings for both the mitochondrial and cytosolic compartments as has been reported for an oxidative treatment (Wierer et al., 2012). No differences were found between her2 and wt. We then tested methanol since we dissolve GLVs in it. As it is shown in figure 11b, MeOH has no effect on $\tau(\phi)$ in all plant lines. To assess further if the her2 mutation has an effect on the cellular redox state during GLV perception, we treated the seedlings for 1 hour with two different GLVs, 0.3µM aerial E-2-hexenal (figure 11c) as it is reported to be influencing gene expression (Farag et al., 2005; Kishimoto et al., 2005) and 0.3 µM aerial Z-3-hexenol (figure 11d) because it is one of the most
abundantly produced by Arabidopsis (Matsui et al., 2012) or MeOH as negative control.

Figure 11. Fluorescence lifetime measurement of wt roGFP2 cyt, her2 roGFP2 cyt, wt roGFP2 mit, her2 roGFP2 mit under different conditions: (a) 30 min 20mM H₂O₂ and untreated control, (b) untreated and 1 h MeOH, (c) 1 h MeOH and 0.3 μM aerial E-2-hexenal, (d) 1h MeOH and 0.3μM aerial Z-3-hexenol. Every experiment was repeated at least three times with similar results.

As shown in figure 11d Z-3-hexenol had no effect on the $\tau(\phi)$ of the roGFP2 mit expressed in her2 and wt plants while the E-2-hexenal increased $\tau(\phi)$ significantly ($P<0.01$, T-Test) (figure 11c). Even though we did not see differences between her2
HER2 participates to E-2-hexenal signalling

mutation and wt, we, interestingly, found that E-2-hexenal influenced the redox status in the mitochondria and not in the cytosol. It has to be noted that $\tau(\phi)$ in the cytosol is higher than in the mitochondria in figures 11b, 11c and 11d.

The her2 mutation enhances the susceptibility to Pseudomonas syringae pv. tomato

We previously published that the perception of E-2-hexenal is important during Pseudomonas syringae pv. tomato (DC3000) infections for the hormonal balance (Scala et al., 2013b). Thus we decided to test whether the hexenal response mutant her2 is affected in its susceptibility to pathogenic bacteria. Expression analysis done in silico with e-FP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) predicted that HER2 expression is induced in leaves by DC3000 infections, higher by virulent bacteria as shown in Supplemental data S5. In both HER2 overexpression lines, the bacteria population showed no differences compared to wt, whereas her2 was significantly more susceptible to DC3000 infection (figures 12a and 12b).
Figure 12. her2 mutation influences bacterial growth. Bacterial populations of DC3000 in infected her2, HER2:OE 4b4 (a) or 5la3 (b) and wt leaves 72 hpi. Values are the mean of 24 sets of two leaf disks from 15 plants. Error bars represent standard error. Bars annotated with different letters indicate significant differences among 72 hpi samples (P < 0.05, according to ANOVA followed by LSD post-hoc test). The data presented are from a representative experiment that was repeated three times with similar results.
DISCUSSION

In this study we describe the characterization of the Arabidopsis her2 mutant that has an altered response to the C6-aldehyde E-2-hexenal. HER2 codes for a Zinc-binding GroES-like alcohol dehydrogenase (ADH, At5g63620), predicted and confirmed to be located in the mitochondria. We show that this growth response of seedlings is directly proportional to the amount of HER2 transcript, that, as a consequence of the mutation in her2, plants are more susceptible to Pseudomonas syringae pv. tomato (DC3000).

E-2-hexenal was identified more than 25 years ago as a volatile that is released upon wounding by plants. It has received attention for its ability to induce defense-related responses in plants, but the mechanisms by which plants respond to E-2-hexenal, as well as the signalling pathways involved in these responses, are unknown. As an approach to answer these questions we isolated several E-2-hexenal response mutants (her mutants), as their characterization might help to elucidate the signalling pathways induced by this C6-aldehyde because her mutants are the only mutants so far known to be affected in a specific physiological response to E-2-hexenal.

Most of the responses of Arabidopsis to E-2-hexenal have been characterized in the Col-0 ecotype. Although Col-0 is a natural hpl mutant with impaired C6-volatile production, the her mutants were isolated in this ecotype because we wanted to
exclude the possibility to isolate mutants with altered responses to E-2-hexenal associated with altered E-2-hexenal production.

The mutation in the her2 mutant was mapped in the gene At5g63620 which is putatively assigned as an ADH. In order to confirm the mapping, we screened 2 different SALK lines with T-DNA insertion in the HER2 in different positions, SALK_072101 in the 1st exon and SALK_079558 in the 5th exon (figure 1b). The SALK_072101 showed the same response to E-2-hexenal on root growth as the mutation in her2, thus confirming involvement of this gene, while the other line SALK_079558 did not display the E-2-hexenal insensitivity, indicating that the mutation in this position does not influence the protein activity (figure 1c). However we must still investigate if the T-DNA insertions caused a complete knock out or only a knock down of the HER2 expression. Moreover we translated in silico the her2 cDNA sequence and we determined that the point mutation caused by EMS treatment resulted in the change of the amino acid in position 223 from a polar serine to a non polar phenylalanine (figure 2). According to the conserved domain detection by NCBI, amino acid 228 is at the end of the catalytic Zn binding site (Supplemental figure S1 b), close to the amino acid substitution. The phenylalanine has an aromatic group that has steric effect and possibly influence negatively the Zn binding. When HER2 was compared to a non-redundant database of protein sequences (UniProt), the only protein with reported
function that shared similarity was a succinic semialdehyde dehydrogenase acetylation (SSADH-Acetylating) of a thermophile archaea, *Metallosphaera sedula*. This bacterial SSADH-Acetylating shares 42% identity with HER2 (*figure 2*) and in Metallosphaera catalyzes the reduction of succinate semialdehyde (SSA) to succinyl-CoA. The bacterial enzyme is specific for succinate semialdehyde and succinyl-CoA and has no activity with NAD\(^+\) as co-substrate (Berg et al., 2007; Kockelkorn and Fuchs, 2009). Moreover it is involved in the 3-hydroxypropanoate/4-hydroxybutyrate cycle, an autotrophic CO\(_2\) fixation pathway that is present in some thermophile archaea (Berg et al., 2010). This enzymatic activity is not reported in plants so far (Metabolic Network, PMN, www.plantcyc.org and MetaCyc, metacyc.org). Succinate semialdehyde (SSA) is a toxic molecule and its accumulation negatively affects plant growth as it has been published by Ludewig and colleagues in 2008 (Ludewig et al., 2008). Since the *her2* phenotype in soil is not dwarf as *ssadh* we exclude that HER2 is coding for the same enzyme. This results is also confirmed by Maximum likelihood phylogenetic tree (*figure 3*), in which amino acid sequences of SSADH from Arabidopsis and rice as well as other Arabidopsis ADHs are on completely unrelated branches. SSA is formed in the mitochondrion so it is likely that in this organelle different enzymes are present that convert it into a non toxic molecule as succinyl-CoA. The fact
that SSA is toxic and highly reactive is proved by the presence of other two enzymes, GLYR1 and GLYR2, that convert SSA to GHB, which is less reactive than the aldehyde albeit still toxic (Allan et al., 2009). The SSADH-Acetylating enzyme from *M. sedula* produce succinyl-CoA which is an intermediate of Tricarboxylic Acid Cycle (TCA cycle) and it is produced by 2-oxoglutarate (α-ketoglutarate) dehydrogenase, which is a committed step of this metabolic route, because it is irreversible, producing NADH and CO₂ (*figure 13*). Then succinyl-CoA is used by succinyl-CoA ligase to produce succinate, though this step is reversible (Studart-Guimarães et al., 2005). In tomato the reduced expression of this enzyme caused little alteration of photosynthesis and plant growth (Studart-Guimarães et al., 2005). Further analysis unravelled that the reason for such a mild phenotype was the up-regulation of GABA shunt for succinate production. However the authors do not exclude others possible bypass of succinyl-CoA ligase. By following this hypothesis, HER2 could supply the TCA cycle with succinyl-CoA (*figure 13*), or alternatively might catalyze the opposite reaction starting with succinyl-CoA and giving SSA as product, which is then used by SSADH to provide succinate (*figure 13*). According to the first hypothesis, HER2 could be active in an alternative metabolic loop, from 2-oxoglutarate to succinyl-CoA. Indeed the reaction of 2-oxoglutarate dehydrogenase is irreversible and it is inhibited by oxidative stress (Sweetlove et
HER2 participates to E-2-hexenal signalling

t al., 2002). In this way 2-oxoglutarate is converted to glutamate by glutamine oxoglutarate aminotransferase (GOGAT, (Lancien et al., 2000)) and glutamate is used for GABA biosynthesis. Then in the GABA shunt, GABA is used by GABA-transaminase to form SSA, which could be a substrate for HER2 to provide succinyl-CoA to TCA cycle. Similarly SSADH is used to by-pass both succinyl-CoA ligase and 2-oxoglutarate dehydrogenase during oxidative stress conditions that could inhibit these two steps (Sweetlove et al., 2002). Thus in this prospective HER2 could be another component of the GABA shunt, linking this metabolic route to the TCA cycle and reducing SSA to a less reactive molecule (figure 13). This scenario should be further investigated non only in vitro, by giving to recombinant HER2 succinyl-CoA + NADPH or SSA + Coenzyme A, but also measuring succinyl-CoA and SSA in vivo as it has been reported (Ludewig et al., 2008; Perera et al., 2009). If HER2 belongs to GABA shunt has to be investigated, but its mutation did not influence the GABA levels in normal conditions as reported for her1 (figure 4 and (Mirabella et al., 2008)). However we cannot exclude that HER2 is induced under stress conditions, as reported in silico for biotic stress (Supplemental figure S5). To consider all the possible functions that HER2 could play, we tested if it could be an ADH, which converts the Z-3-hexenal /E-2-hexenal into Z-3-hexenol and E-2-hexenol respectively. From our preliminary result with the recombinant protein (figure 6) we can exclude
that HER2 is the ADH responsible for the conversion from Z-3-hexenal to E-2-hexenal. The other ADH reported so far in Arabidopsis responsible for the conversion of the aldehyde into an alcohol for GLVs, ADH1 (Dolferus et al., 1990; Bate and Riley, 1998), is predicted to be located in the cytosol (Scala et al., 2013a). We tested if HER2 protein could use SSA or succinate as a substrate in the same conditions as we tested the ADH activity without any result (data not shown). From these data and from the phylogenetic tree (figure 3) we can exclude that HER2 encodes a SSADH. From the high similarity shared with the SSADH-Acetylating enzyme of M. sedula, we hypothesize HER2 has the acetylating activity, although we did not try yet this reaction in vitro.

To investigate from another perspective the role of HER2, we decided to use two independent overexpression lines (HER2:OE) with different HER2 expression levels. As hypothesized OE lines were more susceptible to the GLV treatment and we needed to optimize the E-2-hexenal concentrations to having a working assay. Indeed the concentrations tested in our previous paper (Mirabella et al., 2008) were too high and resulted in the stop of the root growth for both wt and her2, with the latter being our positive control for the assay. This was likely caused by the change of the growth chamber in which the assay was performed. To be able to measure an effect of the E-2-hexenal concentration on both OE lines we decided to decrease the
voluntary concentration to the amount at which the root growth of the HER2- OE lines was inhibited while her2 was still growing (figure 8a and 8b). Although the final E-2-hexenal concentration used for the two independent OE lines was different, we did not find a correlation between the sensitivity and the transcript levels: the lines with the higher HER2 expression levels (4b4) showed the root growth inhibition at an higher concentration of the volatile (figure 7 and 9). For the control treatment the root of OE lines grew differently, with the 5la3 root being longer than wt root and 4b4 root being shorter than wt root (figure 9).

Another observation in seedlings is that her2 root growth is significantly shorter than in wt (figure 9), indicating that this could be, beside the E-2-hexenal response, another phenotype for this mutation. This is particular for the her2 mutant, since the root growth of her1 is the same as wt in methanol (Mirabella et al., 2008).

We recently published that the perception of E-2-hexenal is important during Pseudomonas syringae pv. tomato (DC3000) infections for the hormonal balance (Scala et al., 2013b). We tested her2 and OE lines for DC3000 susceptibility (figure 12) and found that her2 is more susceptible than wt while the OE lines were not different than wt, indicating that in this specific biotic response the different transcript levels do not influence the final result of the infection. From in silico analysis with eFP browser HER2 expression is induced by Pseudomonas syringae.
pv. tomato (Supplemental figure S5), indicating that it has a role in the defense responses of the plant against this pathogen.

Being annotated as an oxidoreductase, we can speculate that HER2 might have different roles during the DC3000 - Arabidopsis interaction. Indeed earlier than plant hormone signalling network, the perturbation of redox status is a pivotal element that enables the perception of a physiological change and starts downstream signalling events. For example it has been reported that the SA receptor NPR1 has a different conformation, accordingly to the redox status (Dutilleul et al., 2003; Desikan et al., 2005). Signalling events triggered by redox changes are also found during abiotic stress perception and on this topic Pham and Desikan recently presented a model which suggest a role for reactive oxygen species (ROS) and hormones in integrating abiotic and biotic stress responses (Pham and Desikan, 2012).

RES, as the similarity of the name recall, have been compared with ROS: both of them can be produced enzymatically or not enzymatically and influence massively gene expression (Alméras et al., 2003; Taki et al., 2005; Mueller et al., 2008). One of the major ROS detoxification mechanism is mediated by glutathione (GSH) (Dixon et al., 1998; Møller, 2001). Also RES-glutathione adducts are produced in vivo during the hypersensitive response in tobacco leaves (Davoine et al., 2006). Moreover we published that in Arabidopsis, during E-2-hexenal treatment, this RES is
HER2 participates to E-2-hexenal signalling

linked to GSH (Mirabella et al., 2008). In this context HER2, being an oxidoreductase, might act by integrating the signalling cascade activated by DC3000 (ROS-dependent) and E-2-hexenal (RES-dependent) perception.

Another hypothesis for HER2 function could link the HER2 involvement in the redox status and its similarity to the bacterial SSADH-Acetylating of M. sedula (figure 2): indeed we can speculate that this enzyme could help the plant to support energetical needs during infection, when the activation of plant defences is costly and redox state of the cell is altered by the ROS production. A similar scenario has been recently published for tomato mutant sitiens which is more resistant to Botytis cinerea because the GABA shunt enzymes provide more succinate for the TCA cycle (Seifi et al., 2013). Indeed we found that the mutant her2 is more susceptible to Pseudomonas syringae pv tomato (figure 12).

HER2 is referred to be an oxidoreductase thus it was straightforward to determine whether HER2 is involved in redox changes, especially the ones that E-2-hexenal, being a RES, could trigger into the plant cell. We decided to take a targeted approach for the mitochondria because we proved HER2 is located in this organelle (figure 10) but also decided to check the cytosol. To be able to determine possible differences in the redox status in vivo we used a redox sensitive GPF (roGFP2) (Meyer et al., 2007) and decided to measure the fluorescence
lifetime with Fluorescence Lifetime Imaging Microscopy (FLIM) because this technique: i. enables to measure samples by taking only one image (not two as needed with confocal laser scanning microscopy), ii. Fixation of the samples is not necessary, iii. it is photobleaching independent (Nakabayashi et al., 2008) and iv. it has twofold-higher sensitivity than confocal microscopy with roGFP2 reporter (Meyer et al., 2007; Wierer et al., 2012).

We focused our measurement in the root because the chlorophyll is absent, it is the organ where the growth inhibition occurs upon E-2-hexenal treatment and in silico analysis showed higher HER2 expression in root tissues (Supplemental figure S3). First of all we set as experimental conditions several controls, beside the E-2-hexenal as treatment for seedlings wt and her2 both having the roGFP2 expressed or in the mitochondria or in the cytosol. We used as first control treatment H₂O₂ to test whether the roGFP2 in the seedling would sense it and MQ water as negative control. Indeed Wierer and colleagues determined that 20mM H₂O₂ treatment for half an hour was enough to see roGFP2 become oxidized (Wierer et al., 2012). We confirmed the reproducibility of this method and decided to test volatile treatments with the same conditions as we used to test the root growth phenotype. We tested methanol as a treatment because we use it to dissolve GLVs and we wanted to exclude any effect on the redox potential. As it is shown in figure 11b the methanol treatment did not change the
HER2 participates to E-2-hexenal signalling

fluorescence lifetime $\tau(\phi)$ thus also not the oxidation state. We noticed that lines with roGFP2 expressed in the cytosol had a basal $\tau(\phi)$ higher than the one of the lines with roGFP2 expressed in the mitochondria, meaning that they had a basal more oxidised redox state. This could be due to the different pH of the compartments with mitochondria (pH about 8.4) and cytosol (pH 7.3) (Shen et al., 2013).

Being methanol treatment a good negative control, we used it to compare it with the E-2-hexenal treatment (figure 11c). Surprisingly we found that E-2-hexenal specifically increased $\tau(\phi)$ in the mitochondria, indicating that roGFP2 is oxidized and that this aldehyde affects specifically the mitochondria redox state. To the best of our knowledge, this finding has never been reported before in vivo and adds a piece to the puzzle of the E-2-hexenal signalling pathway. We cannot exclude completely that the increase of oxidation in the mitochondria is caused by a cytotoxic effect as the one reported for another RES, hydroxy-2-(E)-nonenal (HNE), on Arabidopsis mitochondria (Winger et al., 2007). However in this experiment 700 $\mu$M HNE was added directly to mitochondria, while we treated the seedlings with aerial concentrations of 0.3 $\mu$M E-2-hexenal, which are far more diluted compared to HNE and more similar to natural occurring plant to plant communication events (Engelberth et al., 2004).

We did not see a difference between the wt and her2, which could be due to the sensitivity of the roGFP2. In the
mitochondria the redox conditions are tightly kept balanced so is it possible to speculate that if the mutant had some small differences under E-2-hexenal treatment in the redox potential, they could be buffered by the mitochondria and lost. This explanation is supported by the fact that roGFP2 expressed in the mitochondria has a lower degree of oxidation by H₂O₂ than of roGFP2 in cytosol (Schwarzländer et al., 2009) and that the E-2-hexenal oxidation of roGFP2 is lower than the one given by H₂O₂ (figure 11a and figure 11c). Despite roGFP2 and FLIM technique have proven to be very useful and reproducible, in the future we could use other redox sensitive markers, such as roGFPiE, which sense the reduction of the redox state in the compartment of interest (Avezov et al., 2013). Another option could be to express the roGFP2 targeted to the mitochondria in HER2 overexpressing lines and treat them with E-2-hexenal (or methanol as control) to achieve a boost in the phenotype, since the reduction in root growth with this treatment is quite drastic in the HER2 overexpressing lines (figure 9).

In order to prove the specificity of E-2-hexenal redox response in the mitochondria, we decided to test another GLVs, i.e. Z-3-hexenol which is reported to induce several defense associated genes (Farag et al., 2005; Engelberth et al., 2013). Most importantly Z-3-hexenol is involved in plant to plant communication (Engelberth et al., 2004; Farag et al., 2005; Heil and Silva Bueno, 2007) so it would have been interesting to
HER2 participates to E-2-hexenal signalling

check if this GLVs was able to trigger a plant response that could be the start for a transduction signalling event. The increase in \( \tau(\phi) \) induced in the mitochondria by E-2-hexenal was not detected with Z-3-hexenol treatment, indicating that the oxidation of the mitochondria is rather specific for this aldehyde.

**Figure 13. TCA cycle and GABA shunt.** TCA cycle enzymes (capital letter, black): citrate synthase, CS; isocitrate dehydrogenase, IDEH; 2-oxoglutarate dehydrogenase, 2-ODEH; succinyl-CoA synthetase, SCoAS; succinate dehydrogenase, SDH; malate dehydrogenase, MDH. GABA shunt enzymes (capital letter, grey): glutamate decarboxylase, GAD; GABA-transaminase (GABA-T); succinate semialdehyde dehydrogenase, SSADH; glyoxylate reductase, GLYR; \( \gamma \)-aminobutyric acid, GABA; \( \gamma \)-hydroxybutyrate, GHB; glutamine oxoglutarate amino transferase, GOGAT. HER2 (in red) is depicted as SSADH-Acetylating, connecting GABA shunt and TCA cycle.
Overall our data suggest that we have identified a putative SSADH-Acetylating enzyme involved in coping with biotic stress (*Pseudomonas syringae pv. tomato*) and by *E*-2-hexenal (being a RES), stress that possibly inhibits some important steps of the TCA cycle, which is needed fully working to supply the cell for NADH, ATP and carbon skeletons and to cope with demanding energetic needs during plant defense responses (Sweetlove et al., 2002; Sweetlove et al., 2010)

**MATERIALS AND METHODS**

**Mapping**

The Arabidopsis mutagenized population was provided by Dr G-J. de Boer (http://www.enzazaden.nl). In brief, Arabidopsis seeds, ecotype Col-0, were mutagenized by soaking about 50 000 seeds in 0.2% (v/v) EMS, as previously described by Weigel and Glazebrook (2002). M2 seeds were collected from pools of 1000 plants. For the mutagenesis screen, approximately 35 000 M2 seeds were screened from 12 independent pools. The *her2* mutant was outcrossed to wild-type ecotype Ler. F1 plants were allowed to self-fertilize, and the F2 progeny was screened for sustained root growth in the presence of aerial 0.3 μM *E*-2-hexenal. Mapping was done accordingly to what we previously published (Mirabella et al., 2008).
**Plant lines**

SALK-generated T-DNA insertion lines 072101 and 079558 were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA, (Alonso et al., 2003)).

For HER2 overexpressing lines (HER2:OE), total RNA was isolated using Trizol from Col-0 leaves treated with TurBo DNA-free (Ambion®, http://www.lifetechnologies.com/) to remove DNA. cDNA was synthesized from 1µg of total RNA using M-MuLV reverse transcriptase (Fermentas, http://www.thermoscientificbio.com/fermentas/), as described by the manufacturer, in a 20-µl reaction. HER2 cDNA was cloned in pGreen0229 under the 35S promoter. The recombinant gene was subjected to sequencing to confirm the sequence of the insert and Col-0 plants were transformed and selected on BASTA (50µg ml⁻¹) till homozygosis was reached.

For redox sensitive GFP lines, Col-0 and her2 plants were transformed with the plasmid pBinCM-SHMT-roGFP2-GRX1 for expression of roGFP2-GRX1 into mitochondria and pBinCM-roGFP2-GRX1 for cytosolic expression (Jiang et al., 2006; Meyer et al., 2007; Meyer and Brach, 2009). Selection was done on kanamycin (15mg ml⁻¹) till homozygosis was reached.
Confocal microscopy measurement

Protoplast transfection was done as reported in (Vermeer et al., 2004) and confocal microscopy measurements were done as published by (Vermeer et al., 2009).

Quantitative RT-PCR

For analysis of transcript levels, total RNA was isolated using Trizol from 10 different plants, in three independent experiments and treated with TurBo DNA-free (Ambion®, http://www.lifetechnologies.com/nl/en/home/brands/ambion.html) to remove DNA. cDNA was synthesized from 1µg of total RNA using M-MuLV reverse transcriptase (Fermentas, http://www.thermoscientificbio.com/fermentas/), as described by the manufacturer, in a 20-µl reaction that was diluted to 50 µl prior to using it for the real-time PCR. This was performed in a 20-µl volume containing 2 µl of cDNA, 0.4 pmol of specific primer sets for each gene and 10 µl of Taq™ SYBR Green Supermix with ROX (Bio-Rad, http://www.bio-rad.com/). PCR conditions were as follows: 95°C for 2 min 30 s (first cycle), 95°C for 15 s and 60°C for 30 s (40 cycles). To ensure amplification of a single product during the qRT-PCR reactions, a dissociation protocol was performed in which samples were slowly heated from 55 to 95°C. qRT-PCR was performed using the ABI Prism 7000 real-time PCR detection system (Applied Biosystems, http://www.appliedbiosystems.com/) and the data were
HER2 participates to E-2-hexenal signalling

collected using software (ABI 7000 SDS version 1) provided by
the supplier. Transcript levels were normalized to the levels of
the SAND gene (At2g28390; (Hong et al., 2010)) and
quantification was performed as described in previous work
(Pfaffl, 2001). Primer sequences were qHER2fw
TGGAGGAAGAGCAAGACAGG, qHER2rev GAGACTGCGTTTGTGA
GATTG, SAND primer sequences were as reported in (Park et al.,
2010).

Volatile treatments
Seeds were surface sterilized and planted into horizontally slit
cuts in 1.5% agar plates containing half-strength MS salt, pH 5.8.
In this way, roots emerging from the germinated seeds grew on
the surface and did not penetrate in the agar. The plates were
chilled for 2 days at 4ºC before being placed vertically in an
environmental growth chamber with a light (100 μE sec⁻¹ m⁻²)/dark cycle of 16/8 h at 21°C and 70% relative humidity. Plants
were grown for 4 days under these conditions before being
exposed to volatiles. For the volatile treatment, two Petri
dishes, after being lid removed, were placed vertically into
airtight glass desiccator (22 l). Technical replicates were done in
different desiccators. E-2-hexenal was diluted to 0.09 M or
0.065 M in methanol, and 50 μl of the diluted solutions were
applied to a sterile cotton swab, placed in an Erlen- meyer flask,
between the plates in the desiccators. For the control treatment only methanol (MeOH) was applied.

For FLIM measurements plants were treated with 0.13M E-2-hexenal, M Z-3-hexenol or methanol as control. Plates were incubated in the desiccators with the volatiles for 24 hours, for root length measurement or 1 hour for FLIM measurement, and were subsequently removed and placed vertically under the growth conditions described above. For each measurement at least 8 seedlings 5 day-old for each plant line, distributed over four different plates were used. All experiments were done at least 3 times. After volatile exposure, root growth was measured 3 days later. For each treatment, the root length of at least 10 seedlings for each plant line, distributed over eight plates, was determined with ImageJ software (http://rsbweb.nih.gov/ij/).

**Hydrogen peroxide (H₂O₂) treatment**

For each plant line at least 8 seedlings 5 day-old were treated with 20 mM H₂O₂ for 30 min (Wierer et al., 2012) and then fluorescence lifetime was measured and compared with untreated ones.

**FLIM measurements**

Fluorescence lifetime imaging was performed using the wide-field frequency domain approach on a home-build instrument.
HER2 participates to E-2-hexenal signalling

(Van Munster and Gadella, 2004) using a RF-modulated image intensifier (Lambert Instruments II18MD) coupled to a CCD camera (Photometrics HQ) as detector. A 40X objective (Plan NeoFluar NA 1.3 oil) was used for all measurements. The modulation frequency was set to 75.1 MHz. Eighteen phase images with an exposure time of 20-80 ms were acquired in a random recording order to minimize artifacts due to photobleaching (van Munster and Gadella, 2004). An argon-ion laser was used for excitation at 488 nm, passed onto the sample by a 495 nm dichroic mirror and emission light was filtered by a 515/30 nm emission filter.

Pseudomonas syringae pv. tomato infection

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 OD$_{600}$ of 0.0007 of the bacterial suspension, and bacteria (colony forming units, cfu) were counted as reported in (Park et al., 2010))

Expression in E. coli

HER2 was amplified from pGreen0229 with the following primers HER2_XBAI FW CCTCTAGAGGATTCTCAATCGATGGGTG and HER2_XHOI_R CCCTCGAGTTATAGTATCTC AACACACCTGC with the cycle 96°C x 30", 57°C x 30", 72°C x 1'30" repeated 30
times. 1mM MgSO$_4$ was added to the PCR mix. The expression vector for *Escherichia coli* expression, pGEX-KG (ref), was cut, as well as the PCR product with XbaI and XhoI, and ligated into the vector pGEX-KG. *E. coli* was then transformed and the recombinant gene was subjected to sequencing to confirm the sequence of the inserts. The constructs harbouring GST-HER2 was transformed to *E. coli* strain BL21-DE3 and expression of the fusion proteins was induced using IPTG for 6 h at 20°C. The GST fusion protein were purified using affinity chromatography on glutathione agarose as described before (Testerink et al., 2007). Bound protein was eluted from the glutathione agarose resin using elution buffer containing 20 mM reduced glutathione in 50 mM TRIS-HCl pH 8.0. The elution fraction was used as input for SDS-PAGE, Western Blot analysis and enzymatic activity assay.

**Western blot**

After separation on an 12% SDS–polyacrylamide gel, proteins were electrotransferred onto a nitrocellulose membrane (GE Healthcare). Immunoblotting was performed with anti-GST peptide monoclonal antibody produced in mouse (GST(b-14), Tebu-bio, www.tebu-bio.com).

Optimal dilution of the antibody at 1:2000 (v:v) in antisera buffer [0.05% (w/v) skim milk, 0.1% (v/v) 10× BS, and 0.001% Tween-20] was used. Peroxidase-conjugated anti mouse IgG
**HER2 participates to E-2-hexenal signalling**

(Fisher, www.thermofisher.com) diluted at 1:3300 (v:v) was used as secondary antibody for detection with ECL Plus (GE Healthcare).

**GST-HER2 enzymatic assay**

The fraction containing purified GST-HER2 (see above, 100ng) was used for various enzymatic assays in 10 mM PIPES pH 7.2 containing 2 mM ZnCl$_2$ in a final volume of 1 ml. As substrates 0.1 mM NAD$^+$, NADH, NADP$^+$, or NADPH, and 0.5 mM Z-3-hexenol, E-2-hexenal, Z-3-hexenol or E-2-hexenol were used and the activity was monitored spectrophotometrically at 340 nm. Additionally, sodium succinate or succinic-semialdehyde were used as substrates. For the positive control yeast ADH (220 units, Sigma) was used in 10 mM Tris.Cl pH 9.0 plus 2 mM ZnCl$_2$. 
Supplemental figure S1. *in silico* a) analysis of the cellular localization with SignalP 4.1 of amino acid sequence of HER2. C-score in red (raw cleavage site score) distinguish signal peptide cleavage sites from everything else. C-score is high at the position immediately after the cleavage site (the first residue in the mature protein). S-score in green (signal peptide score) distinguish positions within signal peptides from positions in the mature part of the proteins and from proteins without signal peptides. Y-score in blue (combined cleavage site score) is a combination (geometric average) of the C-score and the slope of the S-score, resulting in a better cleavage site prediction than the raw C-score alone. b) predicted conserved domains and binding sites of HER2 with NCBI conserved domain software (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The black triangle shows the position of the point mutation (S223).
**HER2 participates to E-2-hexenal signalling**

Supplemental figure S2. Developmental expression of At5g63620, HER2, during different developmental stages with GENEVESTIGATOR (www.genevestigator.com).

As shown in Supplemental figure S3 the expression of HER2 seems to be high in all the stages of *Arabidopsis thaliana* development, accordingly to GENEVESTIGATOR analysis. Then we investigated if the expression of our gene of interest is differentiated in diverse organs and anatomical parts. To do so we chose the *Anatomy* in *Conditions search tools* to have displayed 105 different biological parts where At5g63620 is differentially expressed, and we selected $\log_2$ as scale. For a given gene and sample selection, the level of expression within a tissue type is the average expression across all samples from
that particular tissue type. As displayed in Supplemental figure S2 the organ that recurs the most in the highest part of the heat-map, with the darker colour corresponding to the higher level of expression, is the root. Many hits between the ones underlined by the green box belongs to protoplast stage. The root parts in which HER2 is highly expressed is pericycle, root phloem companion cell, quiescent center, root endodermis and root cortex cells (Supplemental figure S3 with asterisks). Moreover if we check in silico the absolute expression of HER2 in the root stele, HER2 is relatively expressed higher in the maturation zone and it is repressed in the columella (Supplemental figure S4).
Supplemental figure S3. Heat map of At5g63620 (HER2) expression in 105 different biological parts of Arabidopsis thaliana, accordingly to GENEVESTIGATOR (www.genevestigator.com). Green box underlines the cell types where HER2 is expressed the most, asterisks point out the first differentiated ones.
Supplemental figure S4. Absolute expression of HER2 (At5g63620) with e-FP browser. Different cell layers are represented with heat-map, with orange/red expression induced and blue expression repressed. (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi)
Supplemental figure S5. Relative expression of HER2 compared to SAND (At2g28390) during mock, virulent and avirulent infection of Pseudomonas syringae pv. tomato at 2, 6 and 24 hpi with eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).
Supplemental figure S6. Chromosome 5 markers and respective position in Megabases (Mb). Next to the marker is indicated the recombination percentage. HER2 is located between Mbk5c7 and Mbk5c8 markers (grey square). Genes present in this area were sequenced and the point mutation was found in At5g63620.
HER2 participates to E-2-hexenal signalling

<table>
<thead>
<tr>
<th>marker</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciw9-f</td>
<td>CAGACGTATCAAATGACAAAT</td>
</tr>
<tr>
<td>ciw9-r</td>
<td>GACTACTGCTCAAACATTATCG</td>
</tr>
<tr>
<td>Mle2-f</td>
<td>GTTAAGTGGTCTCTCATCAG</td>
</tr>
<tr>
<td>Mle2-r</td>
<td>CATCACCTTATGTACATCACG</td>
</tr>
<tr>
<td>Mbk5d-f</td>
<td>CCATGCTTTTGAAAAGCACC</td>
</tr>
<tr>
<td>Mbk5d-r</td>
<td>CCGAATACGAAAGTCAAGTC</td>
</tr>
<tr>
<td>Mbk5c3-f</td>
<td>GTCTTTCTGATAACCATTCC</td>
</tr>
<tr>
<td>Mbk5c3-r</td>
<td>GTGGATCATCTTTTCTACC</td>
</tr>
<tr>
<td>Mbk5c7-f</td>
<td>ACGTACATAGGATAGGAGC</td>
</tr>
<tr>
<td>Mbk5c7-r</td>
<td>GAAGATCTTCTGTCTACTG</td>
</tr>
<tr>
<td>Mbk5c1-f</td>
<td>CCACTTGCTCAACTACGC</td>
</tr>
<tr>
<td>Mbk5c1-r</td>
<td>CACCTGTTCTACTGAGGTC</td>
</tr>
<tr>
<td>Mbk5e-f</td>
<td>GTGTAAGACTCTTAACACTGA</td>
</tr>
<tr>
<td>Mbk5e-r</td>
<td>ATGTATCTGCTCGAGGAG</td>
</tr>
<tr>
<td>Mbk5f-f</td>
<td>TTACACAACAGAGTGAGA</td>
</tr>
<tr>
<td>Mbk5f-r</td>
<td>AAGATGTTGAGGACGAG</td>
</tr>
<tr>
<td>Mbk5c8-f</td>
<td>GTGTACGTGACTATCTCC</td>
</tr>
<tr>
<td>Mbk5c8-r</td>
<td>CACAGTTGTGGTGGACG</td>
</tr>
<tr>
<td>Mgi19-f</td>
<td>GAGAGACAAAGATAAGACACC</td>
</tr>
<tr>
<td>Mgi19-r</td>
<td>CCAACTTGACCTTATAACC</td>
</tr>
<tr>
<td>MGI19c1-f</td>
<td>AGTACCGGAAGTGCAGC</td>
</tr>
<tr>
<td>MGI19c1-r</td>
<td>TGTTTAATTTCTAGACCTGTC</td>
</tr>
</tbody>
</table>

Supplemental table S1. Primer sequences for the markers used for mapping the point mutation in HER2, At5g63620.

Author contributions: A.S did most of the research and wrote the chapter; R.M. mapped the mutation, made 35S:HER2 mutants, and confocal measurements; J.G advised about FLIM, M.D.V selected transgenic lines, expressed HER2 in E.coli and measured
activity in vitro, M.A.H. revised the chapter; R.C.S. advised the research and edited the chapter.

BIBLIOGRAPHY


HER2 participates to E-2-hexenal signalling


HER2 participates to E-2-hexenal signalling


Pham J, Desikan R (2012) Modulation of ROS production and hormone levels by AHK5 during abiotic and biotic stress signaling. Plant Signal Behav 7: 893–897


HER2 participates to E-2-hexenal signalling


