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# Long-Distance Wound Signalling In *Arabidopsis*

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# LONG-DISTANCE WOUND SIGNALLING IN ARABIDOPSIS

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# Abstract

The leaves of all plants use elaborate and inducible defence systems to protect themselves. A wide variety of such defences are known and they include defence chemicals such as alkaloids, phenolics and terpenes, physical structures ranging from fibre cells to silica deposits, and a wide variety of defence proteins many of which target digestive processes in herbivores. It has long been known that the defence responses of plants under attack by insects are not restricted to the site of attack. Instead, if a leaf is damaged, defence can be triggered in other parts of the plant body, for example in distal leaves or even in roots and flowers. This raises the question of what are the organ-to-organ signals that coordinate this process. Several hypotheses have been proposed. These include the long-distance transfer of chemical signals through the plant vasculature, hydraulic signals that may transit through the xylem, and electrical signals that would move through living tissues such as the phloem. Much evidence for each of these scenarios has been published. In this thesis we took advantage of the fact that many plant defence responses are regulated by a signal transduction pathway based on a molecule called jasmonic acid. We used this molecule, one of its derivatives (jasmonoyl-isoleucine), and some of the genes it regulates as markers. Using these we investigated the possible role of the electrical signals in the leaf-to-leaf activation of the jasmonate pathway. We found that feeding insects stimulate easily detected electrical activity in the leaves of *Arabidopsis thaliana* and we used non-invasive surface electrodes to record this activity. This approach showed that jasmonate pathway activity and the electrical activity provoked by mechanical wounding occurred within identical spatial boundaries. Measurements of the apparent speed of surface potentials agreed well with previous velocity estimates for the speed of leaf-to-leaf signals that activate the jasmonate pathway. Using this knowledge we were able to investigate the effects of current injection into Arabidopsis leaves. This resulted in the strong expression of many jasmonate-regulated genes. All these results showed that electrical activity and the activation of jasmonate signalling were highly correlated. In order to test for possible causal links between the two processes, we conducted a small-scale reverse genetic screen

on a series of T-DNA insertion mutants in ion channel genes and in other genes encoding proteins such as proton pumps. This screen, which was based on surface potential measurements, revealed that mutations in genes related to ionotropic glutamate receptors in animals had impaired electrical activity after wounding. Combining mutation of two of these glutamate-receptor-like genes in a double mutant reduced the response of leaves to current injection. When a leaf of this double mutant was wounded it failed to transmit a long-distance signal to a distal leaf. This result distinguished the double mutant from the wild-type plant and provides the first genetic evidence that electrical signalling is necessary to coordinate defence responses between organs in plants.

# Résumé

Les feuilles des plantes disposent de systèmes de défense inductibles très élaborés. Un grand nombre de ces systèmes de défenses sont connus et sont basés sur des composés chimiques comme les alcaloïdes, les composés phénoliques ou les terpènes, des systèmes physiques allant de la production de cellules fibreuses aux cristaux de silice ainsi qu'un grand nombre de protéines de défense ciblant le processus digestif des herbivores. Il est connu dépuis longtemps que la réponse défensive de la plante face à l'attaque  pas un insecte n'est pas seulement localisée au niveau de la zone d'attaque. A la place, si une feuille est attaquée, les systèmes de défense peuvent être activés ailleurs dans la plante, comme par exemple dans d'autres feuilles, les racines ou même les fleurs. Ces observations soulèvent la question de la nature des signaux d'organes à organes qui régulent ces systèmes. Plusieurs hypothèses ont été formulées; une ou plusieures molécules pourraient être véhiculées dans la plante grâce au système vasculaire, un signal hydraulique transmis au travers du xylème ou encore des signaux électriques transmis par les cellules comme dans le phloème par exemple. De nombreuses études ont été publiées sur ces différentes hypothèses. Dans ce travail de thèse, nous avons choisi d'utiliser à notre avantage le fait que de nombreuses réponses de défense de la plante sont régulées par une même voie de signalisation utilisant l'acide jasmonique. Nous avons utilisé comme marqueurs cette molécule, un de ses dérivés (le jasmonoyl-isoleucine) ainsi que certains des gènes que l'acide jasmonique régule. Nous avons alors testé l'implication de la transmission de signaux électriques dans l'activation de la voie du jasmonate de feuille à feuille.  Nous avons découvert que les insectes qui se nourrissent de feuilles d'*Arabidopsis thaliana* activent un signal électrique que nous avons pu mesurer grâce à une technique non-invasive d'électrodes de surface. Les enregistrements ont montré que la génération de signaux électriques et l'activation de la voie du jasmonate avaient lieu aux mêmes endroits. La mesure de la vitesse de déplacement des impulsions électriques correspond aux estimations faites concernant l'activation de la voie du jasmonate. Grâce à cela, nous avons pu tester l'effet d'injection de courant électrique dans les feuilles d'*Arabidopsis*. La

conséquence a été une forte expression de nombreux gènes de la voie du jasmonate, suggérant une forte corrélation entre l'activité électrique et l'activation de la voie du jasmonate. Afin de tester le lien de cause entre ces deux phénomènes, nous avons entrepris un criblage génétique sur une série de mutants d'insertion à l'ADN-T dans des gènes de canaux ioniques et d'autres gènes d'intérêt comme les gènes des pompes à protons. Ce criblage, basé sur la mesure de potentiels de surface, a permis de montrer que plusieurs mutations de gènes liés aux récepteurs au glutamate ionotropique présentent une baisse drastique de leurs activités électriques après une blessure mécanique des feuilles par rapport au type sauvage. Par la combinaison de deux mutations de ces récepteurs au glutamate en un double mutant, on obtient une réponse à la stimulation électrique encore plus faible. Quand une feuille du double mutant est blessée, elle est incapable de transmettre un signal à longue distance vers une feuille éloignée. Ce résultat permet de distinguer le double mutant de la plante sauvage et amène la première preuve génétique que l'activité électrique est nécessaire pour coordonner les réponses de défense entre les organes chez les plantes.

# Abbreviations

| Abbreviation | Name |
|---|---|
| WT | Wild-type |
| Col-0 | *Arabidopsis thaliana* accession Columbia-0 |
| Ler | Landsberg erecta |
| Ws | Wassilewskija |
| AGI | Arabidopsis Genome Initiative |
| NASC | Nottingham Arabidopsis Stock Centre |
| SALK | Salk Institute For Biological Studies |
| JA | Jasmonic acid |
| MeJA | Methyl jasmonate |
| JA-Ile | Jasmonoyl-L-isoleucine |
| qRT-PCR | Quantitative real time polymerase chain reaction |
| SP | Surface potentials |
| WASP | Wound activated surface potential change |
| ci | Current injection |
| COI1 | Coronatine insensitive 1 |
| JAZ | Jasmonate ZIM-domain proteins |
| VSP | vegetative storage protein |

| | |
|---|---|
| TPC1 | Two pore channel 1 |
| *fou* | Fatty acid oxygenation up-regulated |
| GLR | Glutamate receptor-like |
| AOS | Allene oxide synthase |
| AOC | Allene oxide cyclase |
| CNGC | Cyclic nucleotide-gated channel |
| VHA | Vacuolar ATP synthase |
| AHA | Plasma membrane $H^+$-ATPase |

# Chapter 1

# Introduction: Wound signalling and plant survival

Most leaves have large surface-to-volume ratios and they lack thickened protective barriers. This makes them particularly prone to wounding. But despite this, leaves are very abundant in nature. Why is this? Clearly there are several reasons. Firstly, the numbers of leaf-eating organisms (herbivores) are controlled by carnivorous organisms. Secondly, leaves defend themselves (Walters 2011). These are not the only two reasons for the success of the leaf, but they are both important factors in leaf survival. In this thesis I have studied wound signalling in leaves. Inducible wound responses, first discovered as damage-induced defence protein accumulation occurring in leaves (Green and Ryan 1972) are now known to involve much transcriptional reprogramming (Reymond et al. 2004). During the period of 1972 until 1990 the nature of the signal pathway that controls defence gene expression in plants was unknown but it was eventually shown that a small lipid called 'jasmonic acid' mediates some of the key molecular responses of leaves to wounding (Farmer and Ryan 1990, Farmer and Ryan 1992). Using developments based on this discovery the role of wound-activated defence gene expression, particularly which is regulated by jasmonate, has been investigated extensively. We now know that jasmonate signalling in response to herbivory is critical for plant survival against numerous arthropod herbivores (Kessler and Baldwin 2002, Howe and Jander 2008). Furthermore, there are

recent indications that the output of the signal pathway may also protect plants from vertebrate herbivores (Bricchi et al. 2013). Finally, the jasmonate pathway seems to protects plants from detritivores organisms that normally only feed on dead plants or animals. A crustacean that normally only eats dead or dying plant tissues ate a living jasmonate mutant (Farmer and Dubugnon 2009). Today we know that the process of perceiving and responding to wounding is important in plant survival and that the jasmonate pathway plays a major role in wound-activated plant defence. We also know much about jasmonate synthesis and signalling but we know relatively little about other wound signal pathways.

## Wound signal pathways

The wound response of plants involves the action of multiple signal pathways. Common sense tells us that wounding is expected to trigger touch gene expression (Braam 2005). It also leads to water loss and the activation of several associated pathways some of which involve the hormone abscisic acid (Urano et al. 2009). Not surprisingly, water-stress gene expression is indeed seen in wounded leaves (Reymond et al. 2004). There are yet other wound-response signal pathways. One recently described pathway requires the NADPH oxidase gene RBOH-D to transmit organ-to-organ signals in Arabidopsis. These signals lead to the expression of a reactive oxygen species (ROS)-regulated gene called *ZAT12* (Miller et al. 2009). Also, wounding causes the rapid activation of many genes that have promoters containing the 'rapid wound responsive' *cis*-regulatory element CGCGTT (Walley et al. 2007). None of these pathways has been investigated in the context of wounding as much as the jasmonate pathway which is based on the synthesis of the small fatty acid-derived regulator called jasmonic acid (JA), and is known to control and coordinate a particularly large number of responses to wounding (Browse 2009, Koo and Howe 2009). In Arabidopsis, the jasmonate pathway regulates the expression of over one thousand wound-response genes (Reymond et al. 2004). These genes are typically upregulated by various chewing insect herbivores (such as lepidopteran larvae) and by mechanical wounding.

## Jasmonate synthesis

Jasmonates (Wasternack 2007, Browse 2009, Schaller and Stintzi 2009) are derived from plastidial fatty acids, and include the powerful regulatory ligand jasmonoyl-isoleucine (JA-Ile) (Staswick and Tiryaki 2004, Fonseca et al. 2009b). A first striking feature of jasmonate synthesis is that it is complex, requiring at least three cellular compartments, at least 10 intermediates, and several inter-organellar transport steps (Wasternack 2007, Acosta and Farmer 2010). The process begins in plastids with the dioxygenation of fatty acids in reactions catalysed by 13-lipoxygenases. This is a crucial and potentially regulatory step in jasmonate synthesis (Chauvin et al. 2013), although it is not known whether or not the fatty acids that are oxygenated by all of the four jasmonate-producing 13-LOXs in *A. thaliana* are free or esterified *in vivo*. Arabidopsides, secondary metabolites that contain the jasmonate intermediates 12-oxophytodienoic acid (OPDA) and dinor OPDA, are made directly from galactolipids without prior de-esterification of the substrate fatty acids (Nilsson et al. 2012). But these two compounds are, to our knowledge (Glauser et al. 2009), not signalling molecules, so they will not be discussed again. In any case, the first steps of jasmonate biosynthesis start with the oxygenation of fatty acids and proceed from lipoxygenase action through a dehydration step catalysed by allene oxide synthase (AOS), followed by a cyclization step directed by the allene oxide cyclase (AOC) protein which produce OPDA and dinor OPDA in plastids. OPDA and its 16-carbon homolog dinor OPDA, both cyclopentenones, are reduced to cyclopentanones (by OPDA reductase 3; OPR3) and thereafter shortened by successive β-oxidation steps leading to the 12-carbon prohormone jasmonic acid (JA). Finally, the process of biosynthesis of regulators like JA-Ile is terminated when JA is exported from the peroxisome for conjugation to *L*-isoleucine as shown in figure 1.1 and 1.2.

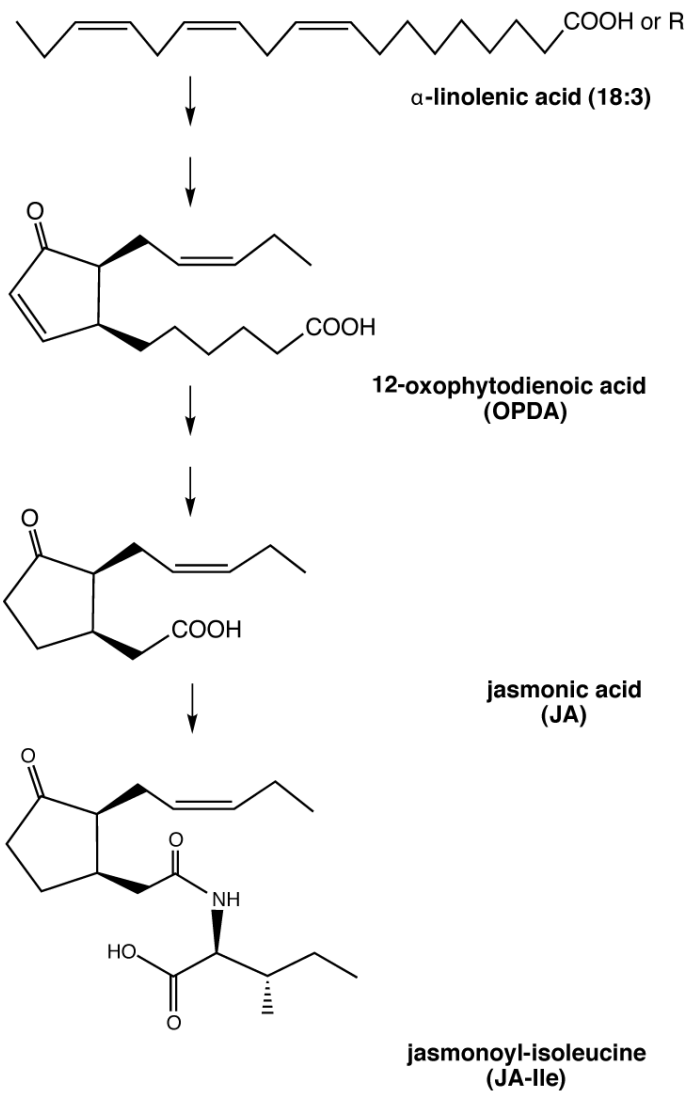


**Figure 1.1. Jasmonate biosynthesis.** Jasmonates are produced by oxygenation and cyclisation of triunsaturated fatty acids, chiefly α-linolenic acid (either in a free or bound form). The first steps leading to the production of 12-oxophytodienoic acid (OPDA) take place in plastids. OPDA is reduced and shortened to the prohormone jasmonic acid (JA) in the peroxisome and finally conjugated to amino acids such as isoleucine in the cytosol. The resulting product, JA-Ile, is a biologically active jasmonate.

## Jasmonate signalling

In the resting state, when JA-Ile concentrations are low, JASMONATE ZIM-DOMAIN (JAZ) proteins, of which there are 12 encoded in the Arabidopsis genome (Browse *et al*., 2009), act as repressors by binding transcription factors such as MYC2 and preventing their function as activators of jasmonate-responsive genes. Stimuli such as wounding

promote jasmonate biosynthesis leading to active (+)-7-*iso*-JA-Ile (Fonseca et al. 2009b). This hormone is then perceived by the protein CORONATINE INSENSITIVE1 (COI1) which is the F-box subunit of an E3 ubiquitin ligase of the type SKP1-CUL1-F-box (SCF). Hormone recognition by COI1 favours binding of COI1 to JAZ proteins via a conserved amino acid motif called the Jas motif. In this model which is supported by crystallographic data (Sheard et al. 2010) the JAZ proteins can be seen as co-receptors acting with COI1. This promotes ubiquitination of JAZ proteins and their subsequent degradation by the 26S proteasome. The transcription factors are now relieved from JAZ-mediated repression and free to recruit the RNA polymerase II transcriptional machinery to the promoter of jasmonate-responsive genes (Browse 2009, Acosta and Farmer 2010). All these processes take place in the nucleus and there are variations in the details depending on which of the 12 JAZ proteins is involved (Withers et al. 2012). Furthermore, this pathway is hardwired into other signal pathways such as a gibberellic acid-requiring signalling that helps to control plant growth. The interplay of these two pathways regulates the balance of growth and defence in response to attack (Yang et al. 2012).

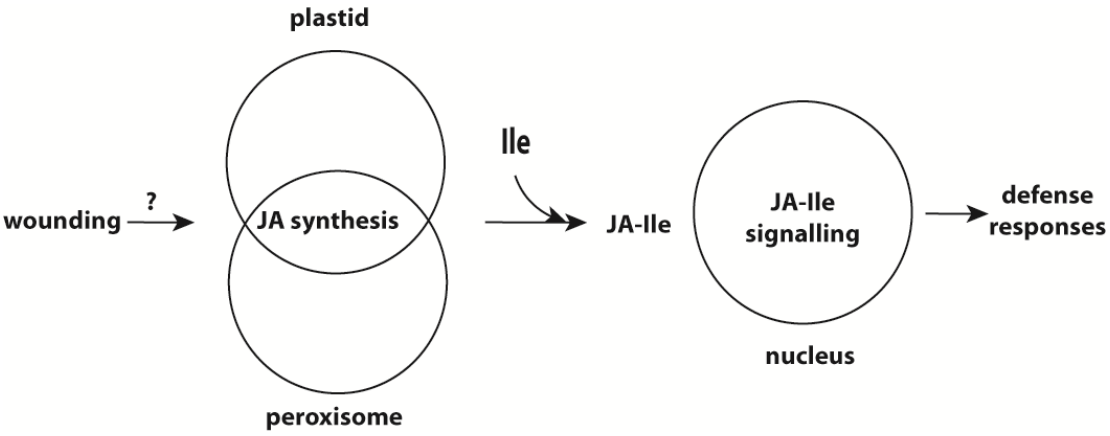


**Figure 1. 2. An overview of jasmonic acid (JA) synthesis and signalling after wounding in *Arabidopsis*.** The signals that lead from wounding to the activation of JA synthesis are unknown. JA synthesis is initiated in the plastid, and then continued in the peroxisome. In the cytoplasm, JA is conjugated to hydrophobic amino acids and in particular to isoleucine (Ile) to form JA-Ile. JA-Ile then initiates jasmonate signalling in the nucleus and this leads to defence responses. The question mark shows where the work in this thesis was focused.

## Long distance signals: spatial and temporal aspects

An additional notable feature of both wound-inducible jasmonate biosynthesis and jasmonate signalling is that these processes are not confined to the site of tissue damage (Koo and Howe 2009). The fact that the response of leaves to wounding is not restricted to the wound site has been known for a long time (Green and Ryan 1972). From the more recent literature we know that long-distance wound signalling from leaf to leaf depends, at least in some plants, on source-sink relationships.This was shown for the expression of the gene *WOUND-INDUCED3* in poplar (*Populus* sp.) (Davis et al. 1991). In adult-phase Arabidopsis (*Arabidopsis thaliana*), the plant used throughout this thesis, the wound-induced expression of the genes *JAZ10* and LIPOXYGENASE2 (*LOX2*) follows intervascular connections termed parastichies (Glauser et al. 2009, Chauvin et al. 2013). Previous work suggested that the approximate limits of wound-induced gene expression should, in theory, be predictable if a plant's vascular architecture is known. This past work also demonstrated that it is not the entire aerial parts of adult-phase plants that respond when one or even two leaves are wounded. The fact that wound-response domains are limited to certain parts of the plant probably reduces investment in defence to an extent that the plant does not expend all of its resources on defence and yet the response that is induced still has adaptive advantages.

Knowing that there are wound-response domains in plants has been useful in the determination of leaf-to-leaf signalling speeds after wounding. For example, when a single leaf, leaf 8, is wounded, jasmonate levels in this leaf as well as in distal leaf 13 on the same plant increase (Glauser et al. 2009). This is because these two leaves share a connected vasculature: they are part of the same parasthichy (Dengler 2006). Furthermore, previous research has shown that when leaf 8 is wounded, JA accumulates in this leaf and also in leaf 13, but to a far lesser extent in leaf 9 which is not directly coupled through the vasculature to leaf 8 (Glauser et al. 2009). This means that when leaf 8 is wounded leaf 9 can be used as a kind of negative control. In theory it should either not receive long-distance wound signals or it should receive different signals than does leaf 8.

Next there is the question of timing. How long does it take for jasmonate accumulation to start in wounded leaf 8 and in unwounded leaf 13? Can the answer to this be used to derive an estimate of long distance signal speed? We know that approximately 40 seconds after completion of wounding (a process that takes < 10 s; (Chauvin et al. 2013) statistically robust increases in JA concentrations occur in the wounded WT leaf 8. Significant JA accumulation began at c. 90 s in leaf 13. Taking into account the size of the Arabidopsis rosette the calculated speed of the signal(s) that triggers JA accumulation in the distal leaf 13 was 7.5 cm $min^{-1}$ (Chauvin et al. 2013). A previous estimate from experiments in which two leaves were wounded gave a slightly lower estimate (Glauser et al. 2009) raising the possibility that the signal speed might be somewhat variable but being in the range of 3 - 8 cm $min^{-1}$. When JA-Ile concentrations in leaves 8 and 13 were measured they were found to increase after 90 s in leaf 8 after wounding this leaf and at 190 s in distal leaf 13. Therefore, it takes 50 s to convert JA to JA-Ile in the wounded leaf, while it appears to take longer than 100 s in a distal connected leaf (Chauvin et al. 2013). These velocity estimates and a closely similar speed estimate from independent experiments on leaf-to-inflorescence stem signalling that may not be related to jasmonate pathway activation (Miller et al. 2009) provide a useful starting point with which to investigate the nature of the long distance wound signal(s). In the figure we use the term 'wound-activated surface potential change' (WASP). This term will be used later in the thesis when we use surface electrodes to record electrical activity both in wounded and in distal leaves.

## Possible long distance wound signals in jasmonate pathway activation

### Transport of jasmonates

One obvious way of activating the jasmonate pathway at a distance from a wound is to transport a jasmonate or a jasmonate precursor to distal sites. This has been one of the major hypotheses for long-distance wound signalling for many years and received strong experimental support from radiotracer experiments (Sato et al. 2011) and from grafting experiments in tomato (Li et al. 2005, Schilmiller and Howe 2005). Some of these grafting

experiments involved fusing WT tissues onto tissues of mutants that could not make jasmonates such as the *acx1* β-oxidation mutant in tomato that cannot complete jasmonate synthesis (Li et al. 2005). This mutant was used as either a rootstock or a scion and then plants were wounded and the expression of inducible jasmonate-regulated proteinase inhibitor (PI) genes was assessed. The outcome of these extensive experiments was that jasmonate synthesis was necessary for production of long-distance wound signals leading to PI gene expression. The authors concluded that a product of the jasmonate pathway was likely to be a component of the long distance signal (Schilmiller and Howe 2005). More recent experiments with inducible *OPR3* genes in *Arabidopsis* showed that events downstream of OPDA production were necessary for jasmonate signalling in distal leaves but not in the wounded leaf itself (Koo et al. 2009). This suggests that immediate jasmonate precursors or derivatives do not move from leaf to leaf to stimulate rapid jasmonate-regulated gene expression in *Arabidopsis*.

The authors of this work have recently proposed that the long-distance jasmonate transport route is only one route to the activation of jasmonate signalling far from a wound (Koo and Howe 2009). Their new model describes cell-autonomous and cell non-autonomous signal routes. The former comprises signals that do not move from cell to cell, while the latter can move over distances in the plant and might include jasmonate precursors. This perspective indeed does fit with much earlier data and many authors have always kept open the possibility that there is more than one mechanism involved in long distance wound signalling. Finally, regarding other chemicals that could activate jasmonate synthesis/signalling there is the peptide hormone systemin from tomato (McGurl et al. 1992). Despite its name, genetic experiments with a systemin perception mutant named *spr1* in tomato showed that systemin is likely to act locally in and near wounds to somehow amplify other long distance signals (Lee and Howe 2003).

**Hydraulic signals**

Much of the bodies of small plants like *Arabidopsis* is held up by the turgor pressure exerted by cells on their cell walls. Cells share connections between their cytoplasms (plasmodesmata) and they have two principle types of vascular cell that transport fluids over long distances: xylem and phloem. Any wound to a plant is expected to cause water loss from the wound site and to alter the pressure of water columns such as those in vascular tissues. Therefore, wounding could well lead to hydraulic pressure waves that would convey information about the wound to distal sites. Hydraulic signals do not need to pass through living tissue (Stahlberg et al. 2006). They could be transmitted through the xylem, for example, or could even transit through dead tissue. It is almost certain that any form of rapid physical damage to a plant will cause pressure changes and these could, in theory, be inseparably linked to the generation of electrical activity.

Among the most informative studies on the transmission of hydraulic signals is work on the effects of severing the roots and/or hypocotyls of cucumber (*Cucumis sativa*) by Stahlberg and Cosgrove (1997). These authors showed that the immediate effect of cutting cucumber tissues was to generate a hydraulic pressure drop in the xylem and that this proceeded any changes in electrical activity as recorded with surface electrodes. Wounding changes the turgor pressure of cells and leads to change the xylem tension and this is suggested to underpin the production of hydraulic signals that then induce a type of electrical activity to be discussed below: variation potentials (Malone and Stankovic 1991, Malone 1992). Additionally, the mass flow generated by wounding could theoretically transport chemical wound signals through xylem (Malone et al. 1994). Vodeneev et al. (2012) proposed that hydraulic signals intensify the propagation of variation potentials and these authors also concluded that the velocities of hydraulic signals are faster than those of variation potentials. Finally, there are already established links between hydraulic signals and hormone signalling in plants. Christmann et al. (2007) showed that hydraulic signals from roots caused the distal synthesis of a hormone that functions in water stress - abscisic acid.

## Electrical signals

Plants generate electrical activity in response to environmental changes including biotic and abiotic stresses. For instance, herbivores or mechanical wounding, heat or cold, touch or water stress can evoke electrical activity in plants. Starting well over a century ago, Burdon-Sanderson (1873), Darwin (1875) and Bose (1926) all recorded electrical signals from *Mimosa* or *Dionaea*. In plants, electrical signals are currently divided into three categories, action potentials (APs), variation potentials (VPs) and system potentials (SPs). It is believed that, in general, non-damaging stimuli favour the generation of action potentials, while damaging stimuli favours VP production (Fromm and Lautner 2007). Electrical signals might transmit information over long distances in the response to wounding. For example, tomato (Wildon et al. 1992), tobacco, barley and bean (Zimmermann et al. 2009), wheat (Vodeneev et al. 2012), *Mimosa* (Kaiser and Grams 2006) and maize (Grams et al. 2009) all generate readily detectable electrical activity after mechanical damage. But, so far, links between electrical signals and physiological events are poorly understood. However, electrical signals were proposed to underlie the induction of distal *PROTEINASE INHIBITOR II (PIN2)* gene activation after wounding tomato leaves (Wildon et al. 1992) and current injection stimulated the expression of this gene (Peña-Cortés et al. 1995). The main features of three categories of electrical activity in plants can now be summarized briefly.

***Action potentials***: APs are initiated when cell membrane potentials reach a certain threshold in plants and animals. In animals, APs carry information in the nervous system and in cells in muscles and other tissues such as heart tissue. $Na^+$ and $K^+$ are the two ions involved in animal cells in AP generation where $Na^+$ influx into the cytoplasm leads to depolarization of the plasma membrane. However, most plant cells cannot tolerate high levels of $Na^+$ and contain only low levels of this ion. In plants, $Ca^{2+}$ influx into the cytoplasm leads to small depolarisations that, above certain thresholds, are thought to initiate APs followed by anion (possibly $Cl^-$) efflux to enhance the depolarization phase. Subsequently, $K^+$ efflux leads to membrane repolarization (Fromm and Lautner 2007). The

velocities of APs in plants vary and are reported to be in the range of 20–400 cm $min^{-1}$ (Zimmermann et al. 2009). A feature of APs generated in response to a specific stimulus in a given plant species is constant speed and amplitude over distance (Malone and Stankovic 1991, Fromm and Lautner 2007).

***Variation potentials:*** VPs have only been observed in plants and their generation depends on the deactivation of plasma membrane $H^+$-ATPases (Fromm and Lautner 2007). It has been suggested that hydraulic waves are involved in VP propagation over long distances (Malone and Stankovic 1991, Stanković et al. 1998, Stahlberg et al. 2006, Vodeneev et al. 2012). Unlike APs, the velocities of VPs decrease with increasing the distance from the stimulus (Stahlberg et al. 2006). The electrical signals generated from cutting maize and barley roots were associated with hydraulic signals, and led to decreasing $H^+$ efflux and reduced $K^+$ influx (Shabala et al. 2009). In *M. pudica*, VPs were also generated after cutting the tips of leaf pinnae (Fromm and Lautner 2007). In this plant, electrical signals recorded after heating were associated with changes in photosystem II fluorescence (Koziolek et al. 2004). This means that electrical signals may affect plastids, the site of initiation of jasmonate synthesis.

***System potentials:*** Recently, 'system potentials (SPs)' were suggested by Zimmermann et al. (2009) as a new type of electrical signal. It was suggested that they propagate from leaf to leaf and they were proposed as long distance electrical signals in plant. SPs were observed in both monocots such as *H. vulgare* and *Z. mays* and in dicots including *N. tabacum* and the bean species *P. lunatus and V. faba*. In contrast to APs and VPs, system potentials involve plasma membrane hyperpolarisations and they are triggered by activation of the plasma membrane $H^+$-ATPase. Fusicoccin and orthovanadate, activators and inhibitors of plasma membrane $H^+$-ATPase, respectively, accentuated or suppressed the propagation of SPs (Zimmermann et al. 2009). Self-propagation of SPs is the only similarity that they share with APs. The speed of SPs is reported to be between 5–10 cm $min^{-1}$ which is slower than reported for APs (Zimmermann et al. 2009).

In summary, while we do not yet know the nature of the long distance wound-activated signals that lead to distal activation of the jasmonate pathway there is increasing evidence that long-distance wound signalling is complex and could involve multiple pathways. Initial experiments with tomato (Wildon et al. 1992), although they have not been extended to genetic approaches, imply a role for electrical signals in wounding. This in turn suggests roles for ion channels.

## Ion channels and pumps in the regulation of jasmonate synthesis and signalling

Prior to discussing membrane proteins that may regulate transmembrane ion balance during wounding it is necessary to highlight the fact that plant and animal cells differ greatly in the way they use ions to generate gradients across membranes. This is summarized in figure 1.3 which shows several features that distinguish animal and plant cells. Three key points are a) that plants cells rely heavily on ATP-driven pumps to establish proton gradients across membranes, b) that levels of sodium ($Na^+$) are normally low both inside and outside most plant cells, and c) the two major reservoirs of extracellular calcium in plants are the extracellular matrix and the vacuole-both of which are large compartments in most plant cells. There are of course many other differences to discuss but I will continue to focus on the plant cell rather than discuss electrical signal transmission in animals since it cannot be completely homologous between the two cell types. However, one parallel will be touched on briefly at the end of the thesis.

Damage-associated molecular patterns (DAMPs) are host-derived molecules that tell the organism that it is wounded. In plants such molecules include oligogalacturonides derived from the extracellular matrix, and a variety of peptide signals (Koo and Howe 2009). The best known example of a peptide DAMP is from plants in the Solanaceae family. This molecule, named systemin, is necessary for the activation of the jasmonate pathway after

wounding tomato leaves (McGurl et al. 1992, McGurl et al. 1994). The *Arabidopsis* genome does not encode systemin but it does encode 'plant elicitor peptides' (Peps) that, while they show no structural homology with systemin, cause the activation of defence gene expression (Ma et al. 2012). It is not yet clear which, if any, of the six putative Peps encoded in the *Arabidopsis* genome function in the activation of jasmonate synthesis but they are strong candidates, although they have not been investigated in this thesis.

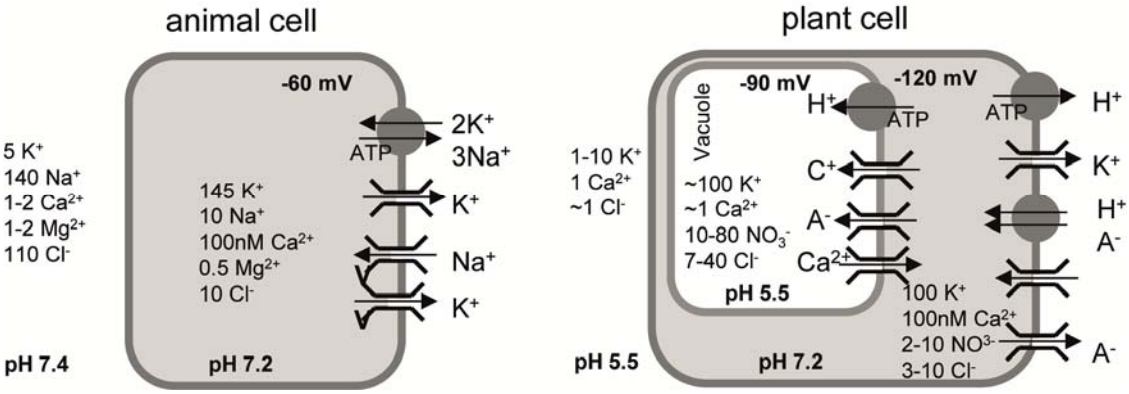


**Figure 1.3: Main ion channels and transporters involved in the control of the membrane potential in animal and plant cells and intra- and extracellular ion concentrations.** Ion concentrations are indicated in mM except for cytoplasmic $Ca^{2+}$ concentrations (in nM). $A^-$, anions; $C^+$, cations; V, voltage-dependent; ATP, ATP-dependent; resting membrane potential (in mV) is indicated relative to extracellular compartment.

Several other lines of evidence suggest that ion channels and proton pumps might regulate jasmonate synthesis or signalling. One such report concerns a vacuolar $H^+$-ATPase subunit a3 (VHA-a3). Mutations that inactive the gene encoding this proton pump subunit cause increased levels of the jasmonate synthesis intermediate OPDA (Brüx et al. 2008). Another line of evidence, perhaps more directly linked to wounding, came from a forward screen conducted previously in our laboratory. The *Arabidopsis fou2* (*fatty acid oxygenation upregulated 2*) mutant displayed a constitutively active jasmonate pathway (Bonaventure et al. 2007b, Bonaventure et al. 2007a). This mutant was found to have a G to A transition at the beginning of exon 14 of the *TWO PORE CHANNEL 1* (*TPC1*) gene which predicted a missense mutation in the putative voltage sensor of the gene. *TPC1* encodes a non-

selective cation channel and patch-clamp analysis of *fou2* vacuolar membranes showed faster time-dependent conductivity and activation of the mutated channel at lower membrane potentials than wild-type (Bonaventure et al. 2007b, Beyhl et al. 2009, Dadacz-Narloch et al. 2011). The dimerization of *TPC1* subunits most likely occurs in the endoplasmic reticulum. An N-terminal signal peptide is important for targeting to the vacuolar membrane (Larisch et al. 2012). The results of genetic studies suggest that cation fluxes from the vacuole may exert strong control over jasmonate synthesis and/or signalling. TPC1 has been placed in figure 1.4 which shows a GLR protein, along with other types of channels, pumps and transporters that have been implicated in wound responses.

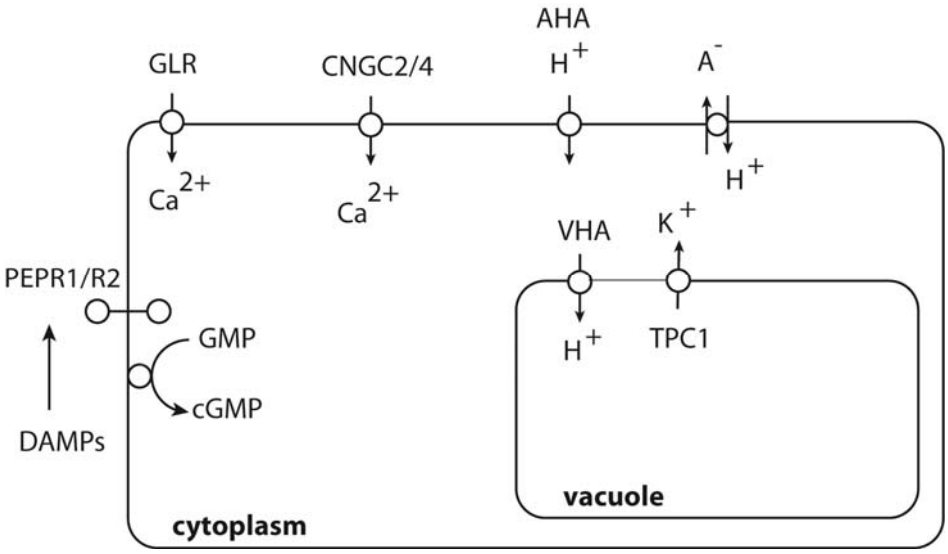


**Figure 1.4. Ion channels, transporters and pumps implicated in responses to wounding.** Surface receptors can activate ion channels in the plasma membrane. These include the receptor pair PEPR1/R2 that after binding to peptide DAMPs, activate cyclic nucleotide-gated channels (CNGCs) through generating cGMP. Elevation of $Ca^{2+}$ influx into the cytoplasm is regulated in part by ligand-gated cation channels such as glutamate receptor-like (GLRs) and CNGCs. Alkalization of the apoplast and acidification of the cytoplasm occurs through inactivation of plasma membrane and/or vacuolar $H^+$-ATPases or activation of proton/anion antiporters. The TWO PORE CHANNEL 1 (TPC1) protein is implicated in cation release from the vacuole. Our work has highlighted the importance of GLRs in the activation of jasmonate synthesis and signalling.

There are many other strands of evidence leading to ion channels and transporters and potential roles in wounding. These include intriguing observations on heterologous expression of candidate ion channels. For example, the overexpression of a *GLUTAMATE RECEPTOR-LIKE* (*GLR*) gene from radish stimulated the expression of jasmonate-regulated genes including *VSP1* in *Arabidopsis* (Kang et al. 2006). Evidence supporting direct or indirect roles for such proteins in the control of jasmonate synthesis or signalling also comes from experiments with chemical inhibitors or activators of proton pumps and channels. For example, hyperpolarization of the plasma membrane by treating tomato cells with fusicoccin represses the expression of some genes known to be regulated by jasmonate (Schaller and Frasson 2001). This thesis examines the possible involvement of ion channels in mediating long distance wound responses and electrical events elicited by wounding.

# Hypotheses tested in the thesis

This work takes a new look at long-distance wound signalling in *Arabidopsis* thaliana. Several questions and experimental approaches are listed in below.

**Question 1:** Does herbivory or mechanical wounding generate surface potential changes (SPs) in *Arabidopsis*?

**Approach:** Recording SPs on *Arabidopsis* leaves upon caterpillar feeding or mechanical wounding (Chapter 3).

**Question 2:** What are the spatial and temporal patterns of wound associated surface potential changes (WASPs) in *Arabidopsis*? What are WASPs boundaries and speeds?

**Approach:** Recording SPs with multiple electrodes in all leaves of *Arabidopsis* after wounding (Chapters 3 and 4).

**Question 3:** Is there any relationship between WASPs and activation of JA pathway?

**Approach:** Analysis of transcript levels of JA-markers genes after wounding and comparison to WASPs recorded in all leaves, as well as disrupting WASPs movement in *Arabidopsis* (Chapter 4).

**Question 4:** Do WASPs directly induce activity of the jasmonic acid (JA) pathway?

**Approach:** Mimicking SP changes by current injection into leaves, then analysis of JA, JA-marker gene expression levels and microarrays (Chapters 5 and 6).

**Question 5:** Which genes regulate WASPs in *Arabidopsis*?

**Approach:** Screening of T-DNA insertion lines for SP changes after wounding (Chapter 5).

# Chapter 2

# Materials and methods

**Plant material and growth conditions:**

*Arabidopsis thaliana* accession Col-0 and mutants in this background, were soil-grown (one seed per 7 cm diameter pot) for 5 to 6 weeks with 10 h light (100 μE $sec^{-1}$ $m^{-2}$), 70% humidity; day 22°C, night 18°C. Prior to the experiments plants were moved into a Faraday cage (80 x 60 x100 cm in length, width and height) under the same conditions.

**Bioassay:**

For recording surface potential changes, two species of generalist and specialist caterpillars were used. *Spodoptera littoralis* (Egyptian cotton worm) and *Pieris brassicae* (cabbage white). *P. brassicae* was from P. Reymond and F. Schweizer. *S. littoralis* eggs were produced by Syngenta (Stein, Switzerland) and were stored at 10 °C until use. The eggs were placed in a beaker covered with plastic film in a growth chamber (28 °C, 65% relative humidity, light **(**100 μmol $m^{-2}$ $s^{-1}$**)**,10/14 h light/dark photoperiod) to allow hatching. The larvae (4th or 5th instar) of both caterpillar species were applied to the plants for electrophysiology. For feeding bioassays, *S. littoralis* larvae (2d old) were placed on 4 week-old plants, 4-5 larvae per plant, 11 plants per replicate, 4 replicates per genotype. After 7 d the larvae were recovered and weighed.

**Wounding**

Five-week old plants were wounded as described (Reymond et al. 2004). The apical parts of the leaves (40% surface area) were crushed with plastic forceps. A plastic support was used to stabilize the wounded leaf during the experiments.

**Surface potential recordings and current injection**

For surface potential recordings, silver electrodes 0.5 mm in diameter (World Precision Instruments, Sarasota, FL) were chloridized with HCl (0.1M), stored at room temperature and rechloridized after several uses. Experiments were conducted in an air-conditioned room without changing the growth conditions. Two 2-channel amplifiers (FD 223 and Duo 773, World Precision Instruments, Sarasota, FL) were simultaneously used to record the surface potential at 4 positions. The electrode-leaf interface was a drop (10 µl) of 10 mM KCl in 0.5% (w/v) agar placed so that the Ag electrode did not contact and damage the cuticle. The interelectrode distance was the distance between the nearest edges of these agar droplets. The ground electrode was placed in the soil. For experiments on interrupting signals, ceramic scissors (CS-250 Kyocera, Kyoto, Japan) were used. For current injection two platinum wire electrodes (Advent Research Materials, Oxford, UK), 0.1 mm diameter were inserted in the midrib 1 cm apart so that the end of the wire was visible from the abaxial leaf side but did not make contact with the soil (Fig. 3A). After insertion of the Pt wires the plants were rested for 24h prior to experiments. For current injection the two Pt wires were connected to a homemade current source that was controlled by the acquisition program. In the current injection experiments, surface potentials were recorded as described above. In these combined experiments, the Chartmaster program via the InstruTECH LIH 8+8 interface (HEKA Electronic, Lambrecht-Pfalz, Germany) was used to record the induced surface potential changes and to control the time and duration of current injection. In the experiments without current injection, surface potentials were recorded with Datatrax2 software via the LabTrax-4/16 interface (World Precisions Instruments, Sarasota, FL). The sampling interval was 10ms. Control plants were implanted with Pt wires in all current injection experiments. Trypan blue staining (van Wees 2008) was used to assess the extent of damage caused by implanting Pt wires in petioles.

**Numbering *Arabidopsis* leaves**

In order to experiment with defined leaves, we numbered them (excluding cotyledons) from old to young (Fig. 2.1). The angle between successive leaves was calculated as 137º. The direction of growth of *Arabidopsis* is clockwise or counter clockwise of which the ratio is around 50% (Dengler 2006). Adult rosette plants at 5 weeks old have 17-20 leaves. The old leaves are small, rounded and have less trichomes, later leaves in the juvenile phase are narrower and have more trichomes. Adult leaves have an intermediate shape, but they have more abaxial trichomes. These characteristics allow us to easily distinguish the leaf number.

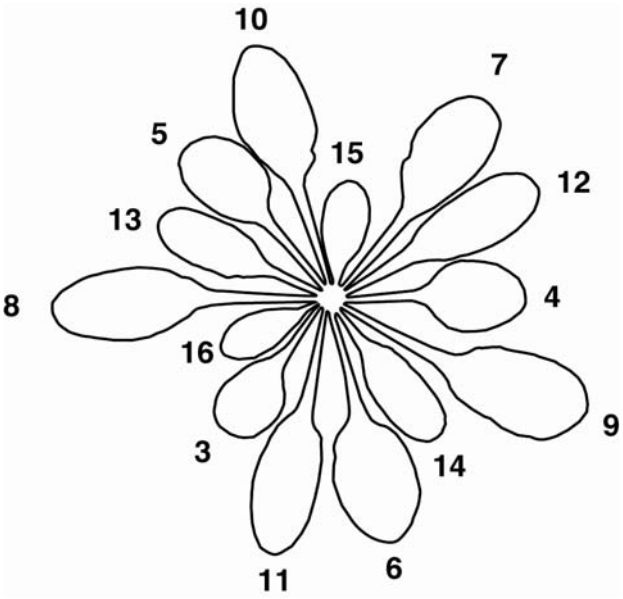


**Figure 2.1. *Arabidopsis* leaf numbering**. In *Arabidopsis*, the direction of growth is clockwise or counterclockwise (50% for both). Numbering starts from the first leaf emerging after the cotyledons. Cotyledon leaves are not counted in the leaf numbering.

**Calculation of speed of electrical activity**

Three electrodes were placed on the midrib, petiole/midrib junction and petiole and one electrode on the lamina (Fig 3.1). The distance between each electrode was 1 cm. The distance of the wound site to the electrode on the midrib was also 1 cm. Then, we wounded

40% of the apical part of the leaf containing electrodes. In experiments, we placed one electrode on wounded leaf 8, and three electrodes were placed on the petiole, the petiole/midrib junction and the midrib of leaf 13. The distance between electrodes on leaf 8 from the wound was 2 cm. For leaf 13, electrodes were placed 1 cm from each other and 1 cm from the centre of the plant rosette. Then, the data of the latency of surface potential changes for each electrode were used to calculate the speed of surface potential changes in the wounded leaf, within connected leaves or from wounded to connected leaves.

**Quantitative PCR**

Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagene, Germany) or with DNA-free RNA isolation protocols (Onate-Sanchez and Vicente-Carbajosa 2008). Total RNA (1 µg) was copied into cDNA with M-MLV Reverse Transcriptase, RNase H Minus, *Point Mutant* first strand synthesis system (Promega, Madison WI) and oligo(dT) primers according to the manufacturer's instructions. Quantitative real time PCR (qRT-PCR) analysis was performed on 100 ng of cDNA in a final volume of 20µl according to the FullVelocity SYBR Green instruction manual (Stratagene, La Jolla, CA) or with a home-made master mix containing GoTaq polymerase (Promega, WI) and its buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, ROX dye and SYBR green in a final volume of 20µl. qRT-PCR was performed in an Mx3005P spectrofluorometric thermal cycler (Stratagene, La Jolla, CA). The data were standardized in relation to ubiquitin-conjugating enzyme (UBC21) At5g25760(Czechowski et al. 2005). The thermal cycle conditions were: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 20 s at 95 °C, 30s at 60 °C and 45s at 72 °C. Three or four biological replicates were used for each experiment. Primers used were: *UBC21* (At5g25760), 5′-CAGTCTGTGTGTAGAGCTATCATAGCAT and 5′-AGAAGATTCCCTGAGTCGCAGTT; *JAZ10* (At5g13220), 5′-ATCCCGATTTCTCCGGTCCA and 5′-ACTTTCTCCTTGCGATGGGAAGA; *VSP2* (At5g24770), 5′-CCGTGTGCAAAGAGGCTTA and 5′-CACAACTTCCAACGGTCAC.

## GUS staining

Transgenic *Arabidopsis* plants expressing β-glucuronidase (GUS) were stained as described by Jefferson (Jefferson et al. 1987). The leaves of 5-week old plants were collected after treatment. The leaves were stained with 1 mM X-Gluc (X-Gluc Direct, from x-gluc.com, http://www.x-gluc.com) in a pH 7.0 phosphate buffer containing 10 mM EDTA, 0.1 mM potassium ferricyanide, 10% (v/v) Triton X-100 at 37 °C overnight. The tissue was destained in 70% (v/v) ethanol.

## Transcriptomics

Total RNAs from leaves were isolated and purified with RNeasy Plant Mini Kit (Qiagene, Germany). All RNA quantities were assessed with a NanoDrop®ND-1000 spectrophotometer and the RNA quality was assessed using RNA 6000 NanoChips with the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA). For each sample, 300ng of total RNA were amplified using the MessageAmp™ II-Biotin Enhanced Single Round aRNA Amplification Kit (AM1791, Ambion). 12.5µg of the resulting biotin-labelled cRNA was chemically fragmented. Affymetrix ATH1 (batch 1211501) arrays (Affymetrix, Santa Clara, CA, USA) were hybridized with 11µg of fragmented target, at 45°C for 17 h and washed and stained according to the protocol described in Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol FS450_0007). The arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix) and raw data was extracted from the scanned images and analyzed with the Affymetrix Power Tools software package (Affymetrix). Statistical analysis was performed using the free high-level interpreted statistical language R and various Bioconductor packages (http://www.Bioconductor.org). Hybridization quality was assessed using the Expression Console software (Affymetrix). Normalized expression signals were calculated from Affymetrix CEL files using the RMA normalization method. Differential hybridized features were identified using the Bioconductor package "limma" that implements linear models for microarray data (Smyth Gordon 2004). The *P* values were adjusted for multiple testing with Benjamini and Hochberg's method to control the false discovery rate (FDR) (Benjamini and Hochberg

1995). Probe sets showing at least 2-fold change and a FDR < 0.05 were considered significant. The microarray data with Affymetrix chips have been deposited in the Gene Expression Omnibus (GEO), (GEO accession; GSE41779).

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rlwndeamikqiaxc&acc=GSE41779

**JA and JA-Ile quantification**

Isopropanol and methanol were obtained from VWR Prolabo (Leuven, Belgium) and used for extraction analysis. Liquid chromatography mass spectrometry-grade acetonitrile and water from Biosolve (Dieuze, France) were used for the high-performance liquid chromatography. The internal standards used were $[^{18}O]_2$-jasmonic acid synthesized with the method of Mueller et al.(Mueller et al. 2006) and $[^{13}C]_6$-jasmonoyl L-isoleucine synthesized according to Kramell et al.(Kramell et al. 1997). Jasmonates were extracted according to Glauser et al.(Glauser et al. 2008). Frozen leaves (200mg, from 5 week-old plants) were ground in a ball mill extractor with internal standards (40ng/ml) prior to extraction with isopropanol. Chlorophyll was removed with a C18 solid-phase extraction cartridge using MeOH:$H_2O$ (85:15, v/v) for elution. The eluate was concentrated and dissolved in 100µl MeOH:$H_2O$ (85:15, v/v). Separation was carried out on a Phenomenex Kinetex (Torrance, CA) 2.6mm C18 100A column (100 x 3.0mm). A gradient elutation was run at a flow rate of 0.4mL/min with the following solvent system: A= 0.1% formic acid / water, B= 0.1% formic acid/ acetonitrile; 5% B for 3min, 5-75% B in 11min, 75-95% B in 2min, 95% B for 2min and 95-5%B in 2 min. The electrospray ionisation conditions were as follows: capillary voltage 3300V; cone voltage 24V; extractor 3V; RF Lens 0V; source temperature 120°C; desolvation temperature 350°C; cone gas flow 900L/h and desolvation gas flow 27L/h. Jasmonates were monitored with quantitative multiple reaction monitoring (MRM) in a Quattro micro™ API mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization interface coupled with the Agilent LC system (Hewlett Packard). Detection was performed in negative ion mode over an m/z range of 100-1000. The MRM transitions were: JA: 209.1 > 58.7, $^{18}O_2$JA: 213.1 > 62.8,

JA-Ile: 322.2 > 130.0 and JA-[$^{13}$C]$_6$Ile: 328.2> 136.0 (parent> daughter). The limit of quantification (LOQ = 3x limit of detection) could reach up to 9.2pmol/g FW for JA and 4.5pmol/g FW for JA-Ile. Data below LOQ were considered as non-informative.

### *VSP2$_{Pro}$:GUSPlus* plant transformation

The *VSP2* (At5g24770) promoter, amplified using 5'-TTC TCT CTG GTT ATA TTT TGT TGC TG-3' and 5'-TGT TTA TAT GTG TGA CGC AAA GG -3' primers) was cloned with *XmaI* and *KpnI* (New England Biolabs, Ipswich, MA, USA) into the pUC57-L4-*KpnI/XmaI*-R1 plasmid producing a pEN-L4-*VSP2$_{Pro}$*-R1 as an pENTRY clone. The pUC57-L4-*KpnI/XmaI*-R1 plasmid was generated by Joop Vermeer (DBMV, University of Lausanne) by introducing L4-*KpnI/XmaI*-R1 *att* recombination and restriction sites into pUC57 (Invitrogen, Paisley, UK). pEN-L1-*GUSPlus*-L2 plasmids were obtained with Gateway technology according to manufacturer instructions (Invitrogen) with *GUSPlus* cDNA (amplified from pCAMBIA1305.2 (CAMBIA, Australia) and pDONR$^{TM}$/ZEO (Invitrogen). The final *VSP2$_{Pro}$:GUSPlus* constructs were generated by using a double Gateway reaction into pEDO097pFR7m24GW. pEDO097pFR7m24GW was generated by inserting the FAST (fluorescence-accumulating seed technology) cassette(Shimada et al. 2010) into pH7m24GW (Invitrogen) by Ester M.N. Dohmann (DBMV, University of Lausanne). WT plants were transformed using *Agrobacterium tumefaciens* cells as described by Berberich et al.(Berberich et al. 2008). Transformed seeds expressing red fluorescence protein (RFP) were selected by florescence microscopy. The $T_1$ generation was used for experiments.

### Genotyping of T-DNA insertion lines

T-DNA insertion lines were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Homozygosity was confirmed prior to experiments. For genotyping, 5 mg fresh leaf samples were placed into 96-well microtiter plates and tissues were ground using a Qiagen TissueLyser II (Retsch Technology GmbH, Haan, Germany). Then, 60 µl of

extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM $Na_2EDTA$, 0.5 % SDS) was added to each well and the samples were centrifuged at 14000 g for 10 min. The supernatants were transferred into new microtiter plates and the same volume (50 µl) of isopropanol was added. The plates were then centrifuged at 14000 g for 5 min. The resultant pellets were washed with 70% ethanol (150 µl) and centrifuged at 14000 g for 5 min. Finally, DNA was resuspended in 50 µl deionized water. 2 µl of this extracted DNA was used as template for each final 20 µl PCR reaction. The sequences of forward or reverse primers pairs used for genotyping are shown in Table 2.1.

**Table 2.1. Sequences of primer pairs used for genotyping of T-DNA insertion lines.**

| Locus | Gene | Stock name | Left primer (LP) | Right primer (RP) |
|---|---|---|---|---|
| At3g04110 | *glr1.1* | salk_057748 | ACCTCTTGACGCGTATGAAAG | GTGAAAAAGAAAAGCCAAGGG |
| At3g07520 | *glr1.4* | salk_129955 | TATATTTGGCCAAGCTCAACG | CTTATAGTGCGGGCTTTGTTG |
| At2g24710 | *glr2.3* | salk_113260 | TATTTGCGGAAGTTCCATTTG | AGAGCGACAAGAAACAGAACC |
| At2g29120 | *glr2.7* | salk_121990 | GGAAATCTTGCCGGTTAAAAG | ACAAATTTGGGGACATTAGGG |
| At2g29110 | *glr2.8* | salk_111695 | GAGTACCTTTCCCTGACCCTG | GAAGGGAGGAGAAGAATGGTG |
| At2g29100 | *glr2.9* | salk_125496 | TGACAAGGTGCTCCCATTATC | AGAAATTCATGGTGACGGTTG |
| At2g17260 | *glr3.1* | salk_063873 | AGATGAACAAACGTGACCACC | TGGCTTTTTGTGGTTCTGATC |
| At4g35290 | *glr3.2* | salk_150710 | TTTTGGATCCAGCATTAGTCG | TTTTGCGGTTTTGTTTGTAGG |
| At4g35290 | *glr3.2* | salk_133700 | TCCATTACTCAATTTCGGTGG | AAACCCAAACCAAAATCATCC |
| At1g42540 | *glr3.3* | salk_077608 | TGCTGTTGATCTCTTGCAATG | CACACAACCATATGCAGCATC |
| At1g42540 | *glr3.3* | salk-099757 | GATGCTGCATATGGTTGTGTG | GTTGAACGATAAGCTTGCGAG |
| At1g05200 | *glr3.4* | salk_079842 | GGGTTAATCCGGCTTATGAAG | GAAGTGAGACTGGCCGTGTAG |
| At2g32390 | *glr3.5* | salk_035264 | TGAAGTTGCTGCAAATGTGAG | TGTCGACATGTCCACAGCTAG |
| At3g51480 | *glr3.6* | salk_091801 | TTCGTTCAAAGGTGGCATAAC | CGACTATGAGGAAAGACGCAG |
| At2g46450 | *cngc12* | salk_092622 | ATTGATGCATTGAAGTCAGGG | TACTTTGGTTTCGAAGCTTGC |
| At5g14870 | *cngc18* | sail_191_H04 | GTTTATCGCCAAGACTGCTTG | TAGCATCTCATTCACCGGATC |
| At3g17700 | *cngc20* | salk_129133 | AAAACAGTTACCTGGAAGCCC | TGCCTTTACACCACCTTTTTG |
| At3g57330 | *aca11* | salk_121482 | TTGCCTCACAAATTACGTTTTG | ACAAACTCCCACGTTTGACAG |
| At3g27170 | *clc-b* | salk_027349 | TCAACCCGTGGAGTTCTGTAG | GGAATTCTTGGGAGCCTGTAC |
| At4g35440 | *clc-e* | salk_142812 | ACAAAGAACAAAAATTGGCCC | CTCAACCAATCTGAGGAGCTG |
| At1g04690 | *kab1* | salk_030039 | GAGGGAATAGCTCCCTTGTTG | GATGTGAAAGAAGCGAAATCG |
| At2g25600 | *akt6* | salk_136050 | GAGAGGAAGAAGAAGCCTTGC | ATGGTCAGCAACATCATCCTC |
| At3g02850 | *skor* | salk_097435 | CCCATATCTCACTGGTTCACC | CCAAACTTCAGCGAAACAGAG |
| At5g55630 | *tpk1* | salk_146903 | AAATGTCGAGTGATGCAGCTC | TCAAGTTGCTCGAACTCATCC |
| At4g18160 | *tpk3* | salk_049137 | ATTGATTACAGCCATTGCTGG | CCGTATATCTCCATTCGGAAC |
| At5g10220 | *annat6* | salk_043207 | TTCTATCCACTGTAGACAGCCTG | AATACGCATCTCTCTCCGTTG |
| At1g59870 | *pen3* | salk_110927 | GCGAGAGTTGGACTCACTTTG | TCACCCAACTAAATCCTCACG |
| At4g11150 | *vha-e1* | salk_019365 | AAGAGTTGGTCCTTGGAAAGC | GTAGATCGGATTTTCACGACG |
| At3g01390 | *vha-g* | salk_087613 | GCTGTTACAATCGCTGAAAGC | TTGAGCTTCTACCTCAGCAGC |
| At2g21410 | *vha-a2* | salk_142642 | ACCTCTGGCTCAAAATTGTCC | TCCACATGAATATAGCCCGAG |
| At2g18960 | *aha1* | salk_118350 | TTCGATTCTCCCACACAGATC | ACGGATTGTGATTGAGACTGC |

| | | | | |
|---|---|---|---|---|
| At4g30190 | *aha2* | salk_073730 | GCGAAAACATATGAACTTTCGAC | CTTAGGGAGCTGCACACACTC |
| At5g57350 | *aha3* | sail_810_C08 | GTAGATTGCAACGGCTATTGC | TTGTCGTGAAGAAGCTATGGC |
| At5g62670 | *aha11* | salk_152723 | ATGACAGCGATTGAGGAAATG | GGCAAAACAACATCATTGATG |
| At5g12080 | *msl10* | salk_076254 | GTTGGTTTCTGGGTTTAAGCC | TACTTGGAGTAACCGGTGCTG |

# Chapter 3

# Insect feeding and mechanical wounding induce surface potentials in *Arabidopsis thaliana* leaves

## Introduction

During their lives plants have to combat environmental stresses and predators. Jasmonic acid (JA) and its derives regulate a wide range of stress responses, including defence responses against insect herbivores, necrotrophic pathogens, wounding, and ozone exposure etc., (Browse 2009, Acosta and Farmer 2010). Additionally jasmonates have roles in development, fertility, water deficiency, and senescence (Fonseca et al. 2009a). Plants activate jasmonate synthesis upon perceiving damage. Successful defence depends on these fast responses to stress, as well as on signalling from damaged tissues to distal organs.

The level of induced defence depends on the strength of stress. Some stresses have a gradual effect on the plant and act slowly to reduce its vigour. This can occur in pathogenesis or water deficit. In contrast, herbivory and mechanical wounding destroy plant tissues and cause immediate effects on plants. In severe stress many plant tissues are involved in the synthesis of defensive products. Rapid reaction is crucial to minimize

damage. In *Arabidopsis*, JA is first produced after less than 30 s in the wounded parts of leaves and at less than 2 min in certain leaves distal to wounds (Glauser et al. 2009). In spite of our understanding of many details of JA biosynthesis and signalling, the early events after wounding that lead to JA biosynthesis in wounded and unwounded leaves are largely unknown. However, the velocity of long distance signalling leading to JA accumulation in *Arabidopsis* is estimated to be in the range of 4-8 cm $min^{-1}$ (Glauser et al. 2009, Chauvin et al. 2013) and less than 2 cm/min for JA-isoleucine accumulation (Koo et al. 2009). Miller et al. (2009) also showed that long distance signals propagate at 8.4 cm/min along *Arabidopsis* stems after wounding leaves. Due to the fast speed of accumulation of JA in distal leaves, Glauser et al (2008) proposed that the long distance signal was unlikely to be JA or JA derivatives. Koo et al. (2009) experimentally confirmed this.

Currently an interesting question concerns the nature of the long distance signals that lead to JA accumulation in unwounded parts of the plant. In other words, how are wound signals generated, transmitted and decoded? In water stress a hydraulic signal is the root-to-shoot signal that stimulates abscisic acid (ABA) synthesis and stomata closure (Christmann et al. 2007). It has long been known that plants respond electrically to many environmental changes, including touch, darkness (Gurovich and Hermosilla 2009), cold (Minorsky 1989, Krol et al. 2004, Carpaneto et al. 2007), water deficiency, burning, heat, salt stress (Fromm and Lautner 2007), insect damage (Maffei et al. 2004, Bricchi et al. 2013), wounding (Wildon et al. 1992, Favre et al. 2001, Favre and Agosti 2007, Zimmermann et al. 2009, Oyarce and Gurovich 2011), and that electrical activity can affect respiration and photosynthesis (Fromm and Lautner 2007, Grams et al. 2009). However, it remains unclear how electrical signals are generated and transmitted and what their physiological consequences are. The speeds of electrical signals in plants are in the range of 1-2 mm $s^{-1}$ and 1-20 cm $s^{-1}$ for variation potentials (VP) and action potentials (AP) respectively (Fromm and Lautner 2007). Wound-induced electrical potentials were reported to induce tomato *proteinase inhibitor2* gene expression (Wildon et al. 1992,

Stanković and Davies 1996). This work suggested that electrical signals could regulate gene expression.

The two commonly used methods for recording electrical activity in plants are intracellular and extracellular recording. In intracellular recording, a small glass microelectrode penetrates inside the plant cell or aphid stylets are used as electrodes. Alternatively whole aphids are used as electrodes (Tjallingii 1988). All intracellular electrodes record events in only one cell. Alternatively, in extracellular recording an electrode (Ag/AgCl) is placed on the surface of plant to record events from cell populations. We chose extracellular recording to monitor electrical activity upon wounding in *Arabidopsis*. Extracellular recording permits the recording of electrical activity for long durations without damaging cells and without interactions of electrode electrolytes with cell contents.

This chapter describes the use of extracellular recording to measure electrical activity in wounded *Arabidopsis* leaves. First, we measured wound-activated surface potential changes (WASPs) induced by caterpillar feeding and then we used mechanical wounding to induce JA synthesis and activation of its signalling pathway. We also characterized surface potentials produced in response to touching and cutting. Furthermore, we calculated the velocities of surface potentials generated by wounding. The results show that wounding can generate surface potentials in *Arabidopsis* leaves and that these potentials are transmitted all over the wounded leaf and beyond.

## Experimental design

### Electrode placements

Surface potential (SP) activity was recorded on different parts of leaves with electrodes placed in four different positions. The electrodes were placed on the adaxial surface of the lamina (eL), midrib (e1), petiole/midrib junction (e2) or petiole (e3) as shown in Fig. 3. 1. The distance between electrodes was 1 cm, except for lamina to midrib electrodes which were 3 mm apart. The distance from the wound to the electrodes on the lamina and midrib was 1 cm (Fig. 3. 1).

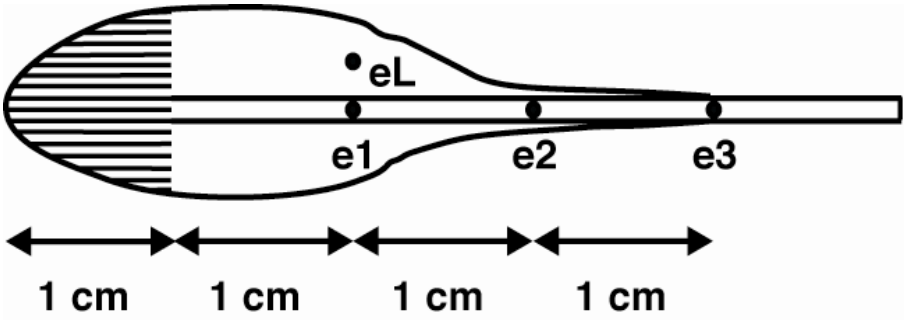


**Figure 3. 1. Experimental design for detecting surface potential changes on leaf 8.** The approximate length of leaf 8 (petiole included) from 5 week-old in *Arabidopsis* plants was 5 cm and 1.5 cm width. Measuring electrodes were placed at 1 cm intervals on this leaf: midrib (e1), petiole/midrib junction (e2), petiole (e3). The lamina electrode (eL) was 3 mm from e1. The apical part of the leaf was wounded with forceps, except for experiments with insect larvae in which only the insects damaged the leaves.

### Quantification analysis of electrical activity induced by mechanical wounding

Due to the fact that a trend of change of the electrical signal was slow, it was difficult to determine the exact point of change. We defined the mid-amplitude points as the transitions in potential change. The time between damage and mid-point of amplitude

change was termed as latency. Duration was the time between the mid points of depolarization or hyperpolarization and the mid points of repolarization phase (Fig. 3.2).

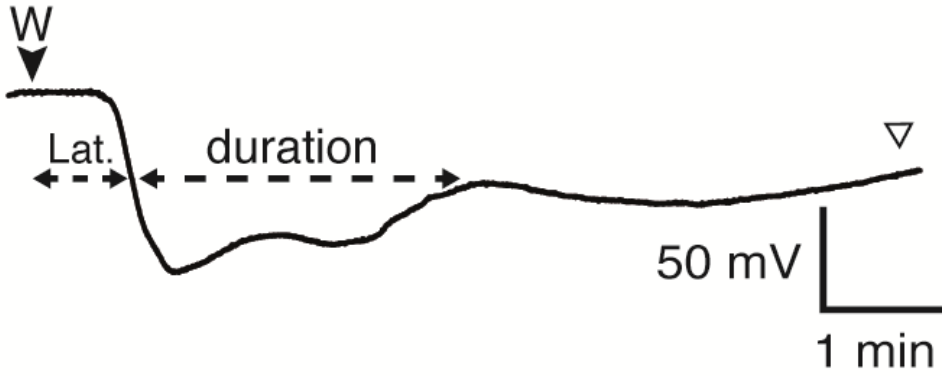


**Figure 3. 2. Quantification of wound-associated surface potential changes (WASPs).** Three distinct variables, latency, duration and amplitude of WASPs were analysed. Duration is the time between amplitude change midpoints. Latency (Lat.) is the period between wounding and WASP detection. The signal, recorded from the e2 position, typically did not recover to baseline during recording (unfilled arrowhead). Time of wounding is indicated with a filled arrowhead.

## Results:

### *Spodoptera littoralis* induces SP changes on *Arabidopsis* leaves

In order to understand insect feeding-induced surface potential changes in *Arabidopsis*, three 1 cm long larvae of the generalist caterpillar *Spodoptera littoralis* were placed in a small plastic cage around leaf 8. The plastic cage did not damage the leaf. The *S. littoralis* had been starved for one day before the experiment. Two recording electrodes were placed on the leaf at distal (e2) and proximal (e3) positions (Fig. 3.1 and 3.3). Electrical recording was started before placing the insects. Results showed that when caterpillars walked on the surface of the leaf there were no detectable SP changes (Fig. 3.3).

Caterpillars started eating from the leaf edge or the inside of lamina a few min after placement on leaves. The insects did not feed on the main veins or midrib. Feeding led to changes in SP within the damaged leaf. The signals did not move to the other leaves unless the caterpillars damaged the midribs. Putting two electrodes 1 cm apart on the petiole allowed us to calculate the speed of SP changes generated by the caterpillars. The speed of SP changes caused by caterpillar feeding was 3.5 ± 0.3 cm $min^{-1}$(n=5), although the amplitude and pattern of SP changes generated by caterpillars was varied (Table 3.1).

**Table 3. 1. WASPs generated by feeding *Spodoptera littoralis*.** *S. littoralis* placed on leaf 8 for 6 independent replicates. Two recording electrodes (e2 and e3) were placed on leaf 8.

| **Replicate** | | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|---|
| **e2** | Latency (s) | 1700 | 508 | 651 | 382 | - | 810 |
| | Amplitude (mV) | -37 | -80 | -15 | -97 | - | -52 |
| **e3** | Latency (s) | 1720 | 524 | 647 | 547 | - | 827 |
| | Amplitude (mV) | -35 | -55 | -12 | -63 | - | -19 |

Feeding on leaves by *Pieris brassicae*, a specialist caterpillar, also generated SP changes (not shown). A typical pattern of insect-induced SPs is shown in figure 3.3.

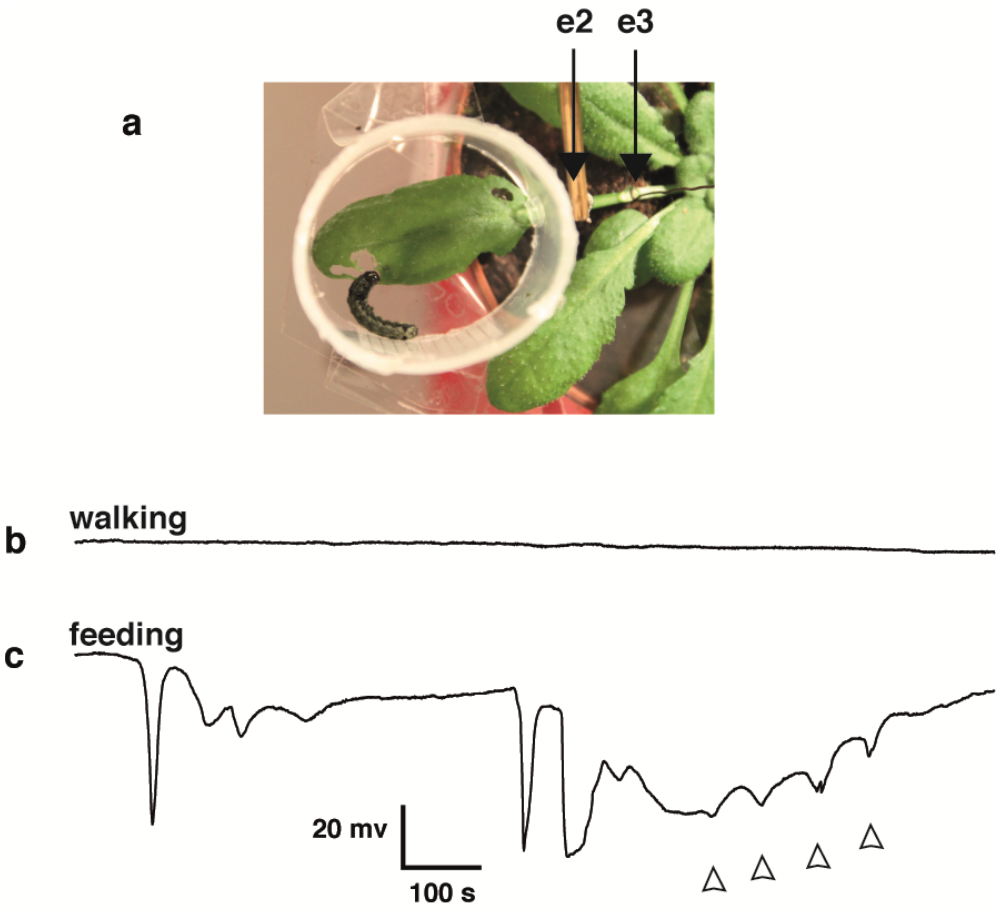


**Figure 3. 3. Insect-induced electrical activity. a**. The setup showing the ring cage around the insect (*S. littoralis*) and the position of the recording electrodes (e2 and e3) on leaf 8. **b**. Surface potential recording from electrode e2 while *S. littoralis* walked on the leaf. **c**. Typical surface potential changes recorded on electrode e3 during *S. littoralis* feeding. The arrowheads indicate periodicity in the signal.

Since insects might affect plants in a variety of ways other than wounding, such as triggering touch responses or releasing chemical factors from the body or saliva, we tried to recapitulate the events we monitored using mechanical wounding. For this we used plastic forceps and routinely wounded 40 % of the laminar surface.

**Plastic and metal forceps have similar effects on the *JAZ10* expression**

Metal forceps are routinely used to damage leaves to stimulate the JA activity pathways. We used homemade plastic forceps inside the Faraday cage to avoid electrical interference.

In initial experiments we compared gene expression levels after wounding leaves with plastic and metal forceps. 40% of the apical part of the leaf was wounded with metal forceps outside of the Faraday cage or plastic forceps inside of the Faraday cage. Leaf 8 was wounded and harvested 1 hour after wounding. This showed that there is no significant difference between plastic and metal forceps for induction of *JAZ10* as a primary jasmonate-responsive gene (Fig. 3.4).

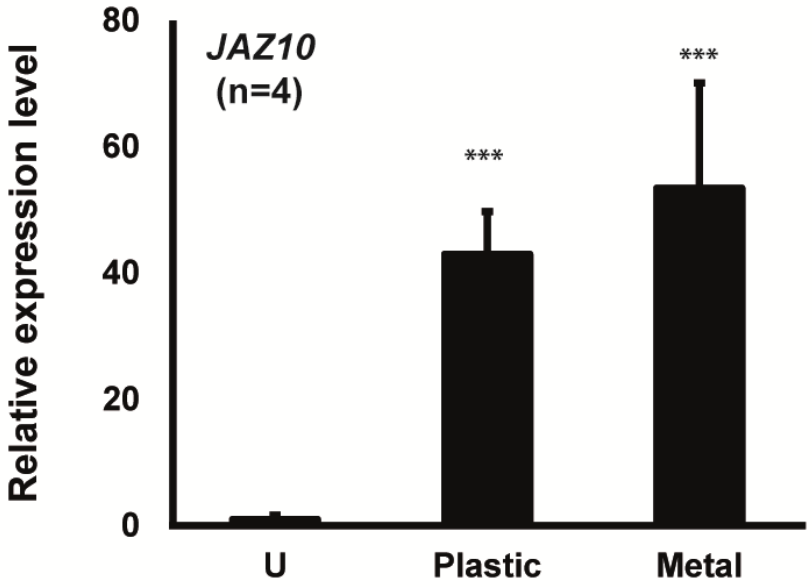


**Figure 3. 4. Expression levels of *JAZ10* 1 h after wounding with plastic or metal forceps.** Unwounded (U). The data are from four independent replicates ± S.D.

### Touching does not change the SP

Mechanical wounding with forceps is a combination of touching and cell disruption. In order to understand whether touching also generates SP changes, an experiment was designed to record the SP while the leaf was touched. Leaf 8 was touched gently 3-4 times by moving it up and down with plastic forceps. The results show that touching leaves with forceps did not induce SPs unless the recording electrodes were moved (Fig 3.5 and Fig 3.7).

**Surface potentials in mechanically wounded leaves**

In unwounded plants, spontaneous surface potential changes were not observed in recording that lasted for more than 1 h (n=10) and simply touching of leaf 8 with plastic forceps did not generate surface potential changes (n=10). Wounding 40% of a leaf leads to changes in surface potentials in *Arabidopsis*. Such recordings made when leaf 8 was wounded are shown in Figure 3.5. We found that wounding leaf tips generated a large change in surface potential approximately 10 s after wounding the leaf (Fig. 3.5). The amplitude of this potential change ranged from -71 to -73 mV as shown in Table 3.2. The surface potential then recovered slowly to approach that of the resting state which was not reached within the time frame of our measurements (triangle in Fig. 3.2).

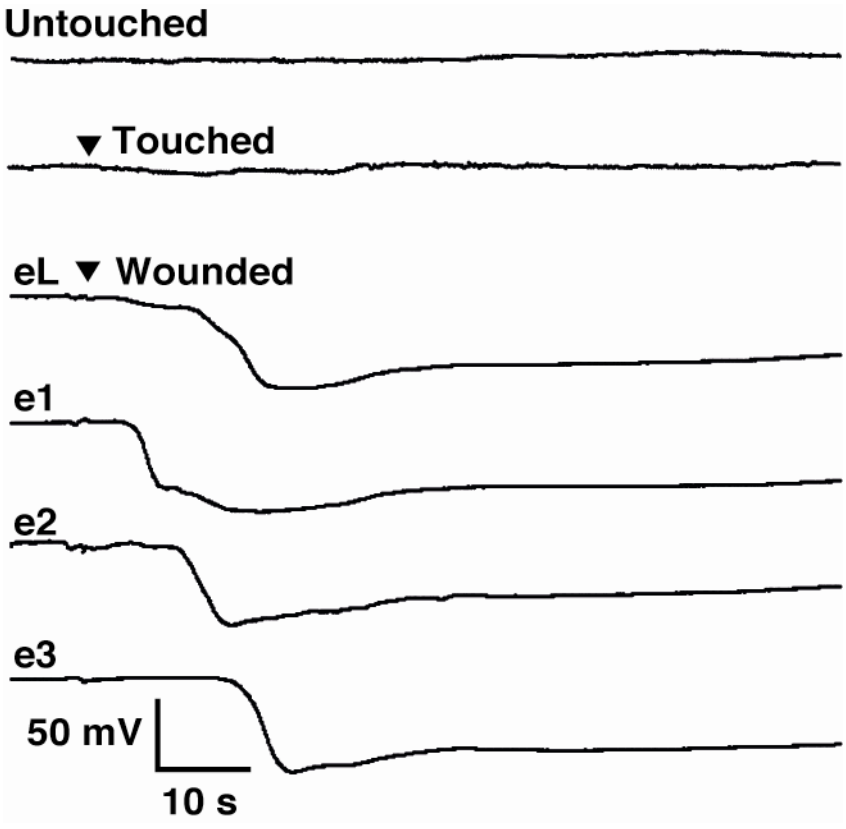


**Figure 3. 5. Examples of typical surface potential changes recorded on leaf 8.** The arrowheads indicate when the leaf was either touched or wounded. For electrodes placement see Fig. 3.1.

The duration of WASPs was different from 96-164s. Electrode placed on the lamina showed shorter duration than those placed on midrib. SP changes first recorded in e1 with latency of 9 ± 3s, then e2 at 15 ± 3s. The latency of SP changes on the lamina was 24s while the electrode placed on midrib with same distance was 10s (table 3.2).

**Table 3. 2. Characterization of wound-induced surface potentials (SPs) in *Arabidopsis* leaves in the wounded leaf.** x/n= number of experiments in which EPs were > -10 mV / total number of experiments. Values are means ± SD. See Fig. 3.1 for details of electrode placement.

| Electrodes | Latency (s) | Amplitude (mV) | Duration (s) | Velocity (cm/min) | x/n |
|---|---|---|---|---|---|
| **Lamina (eL)** | 24 ± 7 | -73 ± 23 | 96 ± 14 | 2.6 ± 0.6 | 13/15 |
| **Midrib (e1)** | 9 ± 3 | -71 ± 15 | 143 ± 22 | 7.1 ± 1. 5 | 13/15 |
| **Petiole/midrib junction (e2)** | 15 ± 3 | -73 ± 14 | 164 ± 32 | 8.2 ± 1.4 | 15/15 |
| **Petiole (e3)** | 23 ± 5 | -71 ± 13 | 160 ± 28 | 7.9 ± 1.4 | 15/15 |
| **Average** | | -72 ± 16 | | | |

## Depolarization vs. hyperpolarization

To investigate whether the WASPs generated after wounding in *Arabidopsis* were due to depolarization or hyperpolarization, we recorded surface potentials of leaves under cold shock. Cold shock treatment was carried out by putting 150 µl of 0 ºC of water on the surface of the lamina of leaf 8 and recording the SP before and after this treatment in this leaf. In this case the recording electrode was placed on the petiole (Fig. 3.1, position e3). Three out of 7 plants showed SP changes after cold treatment. In addition, the timing of SP changes was variable among plants. Figure 3. 6a shows a typical SP changes after cold shock. Figure 3. 6b shows the amplitude changes after wounding. Figure 3.6c shows the polarity and amplitude of SP changes after cold shock or wounding.

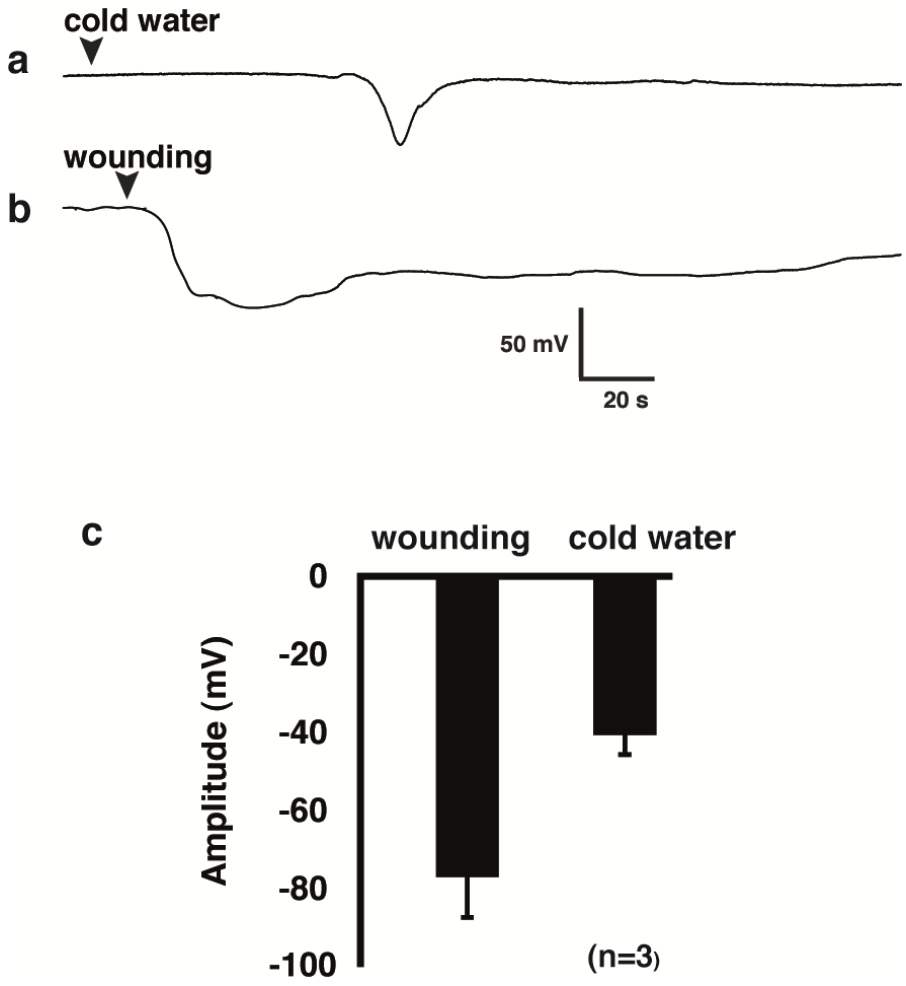


**Figure 3. 6. Establishment of signal polarity using chilling-induced depolarization**. **a**. Chilling-induced depolarization generated by gently placing water (150 µl, 0°C) onto leaf 8 at the time indicated with the arrowhead. Chilling induced a change in surface potential in 3 out of 7 recordings. **b**. Typical WASP of the same polarity. In both cases the recording electrode was on leaf 8 at position e3 (Fig. 3.1). **c**. Amplitude of the change in surface potential induced by wounding or by cold water.

**Artefacts due to mechanical wounding**

To avoid moving electrodes during the experiment, the wounded leaf was stabilized with a plastic sheet before the experiment. Due to the small distance between recording electrodes and the surface of the plant (1-2 mm), mechanically damaging leaves with forceps sometimes led to leaf movement. Furthermore, the position and size of leaves in the pot

affected leaf movement during wounding. The artefacts generated by moving electrodes caused a sharp amplitude change with an irregular pattern. Figure 3. 7 shows the typical artifacts generated by electrode movement or touching the electrodes by forceps.

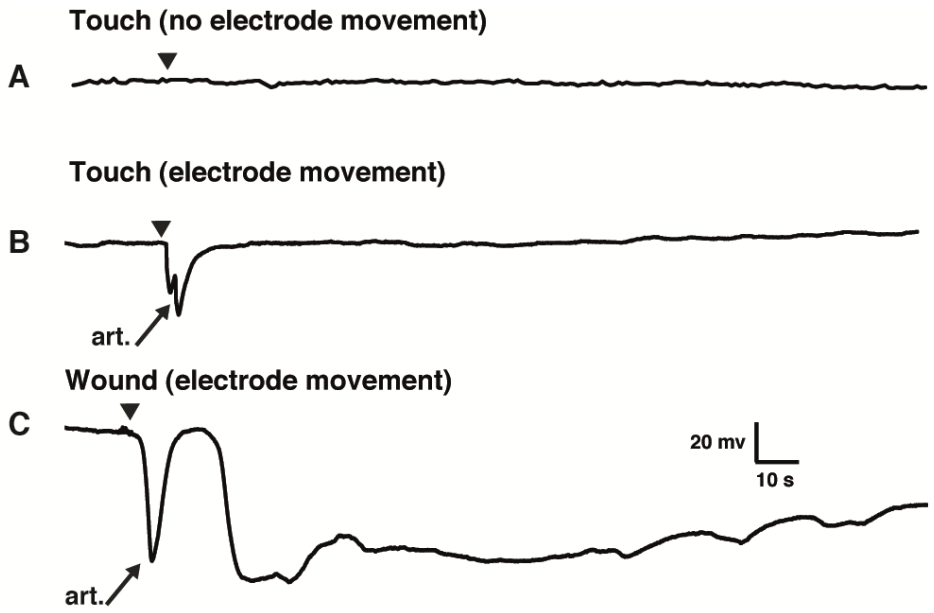


**Figure 3. 7. Electrode movement creates artifacts.** An electrode was placed at position e3 (shown in Fig. 3.1) on leaf 8. **A**. Recordings were stable when leaves were touched without moving the electrode. **B.** Artefactual signal generated by touching the leaf abruptly to cause electrode movement. **C**. Electrode disturbance causes artefact generation prior to detection of the wound-induced SP change. Art = artefact.

**Periodicity of WASPs**

Figure 3.8 shows WASPs generated after wounding. In most cases we observed an irregular pattern of SP repolarization in leaves 8 and 13 which we termed as periodicity. This periodicity was observed in 63 % (n=110) of experiments. The amplitude of this phase was became smaller over time (Fig 3.8).

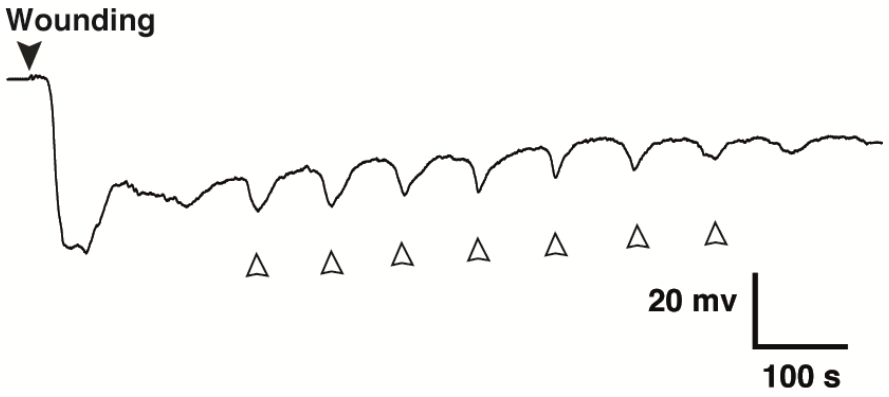


**Figure 3.8. A proportion of WASPs induced by mechanical damage show periodicity.** The recording electrode was placed on leaf 8 at position e3 (shown in Fig. 3.1). The apical 40% of leaf 8 was wounded with forceps. Periodicity was seen in 61% (n=110) of experiments. The filled arrowhead indicates time of wounding. The unfilled arrowheads indicate periodicity.

**Cutting leaves lead to SP change**

In order to find out whether cutting induced SPs, leaves were cut with ceramic scissors in different ways. For example, leaf 8 was cut at 0.5 cm from the tip, or else the edge of leaf 8 was cut in parallel or perpendicular to the midrib. Figure 3. 9 shows the effect of different types of cutting on SP changes. The results show that cutting of the end of leaf tip did not generate WASPs while cutting through the midrib generated large WASPs.

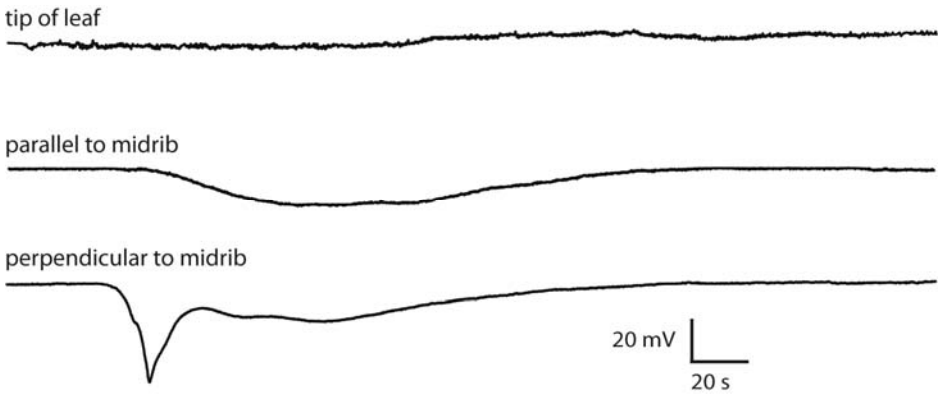


**Figure 3. 9. Leaf excision generates surface potentials in *Arabidopsis*.** Typical pattern of surface potentials recorded on leaf 8 after cutting the leaf tip, parallel or vertical to midrib. Recording electrode was placed at position e3 (shown in Fig 3.1).

**Jasmonate biosynthesis mutant displays similar WASPs to the WT**

In order to know if WASP generation was independent of the JA pathway the *allene oxide synthase (aos)* mutant (Park et al. 2002) in the JA biosynthetic pathway was examined. Figure 3.10 shows the WASPs recorded in the WT and *aos* mutant. The results show that there were no significant differences in amplitude changes between the WT and *aos*.

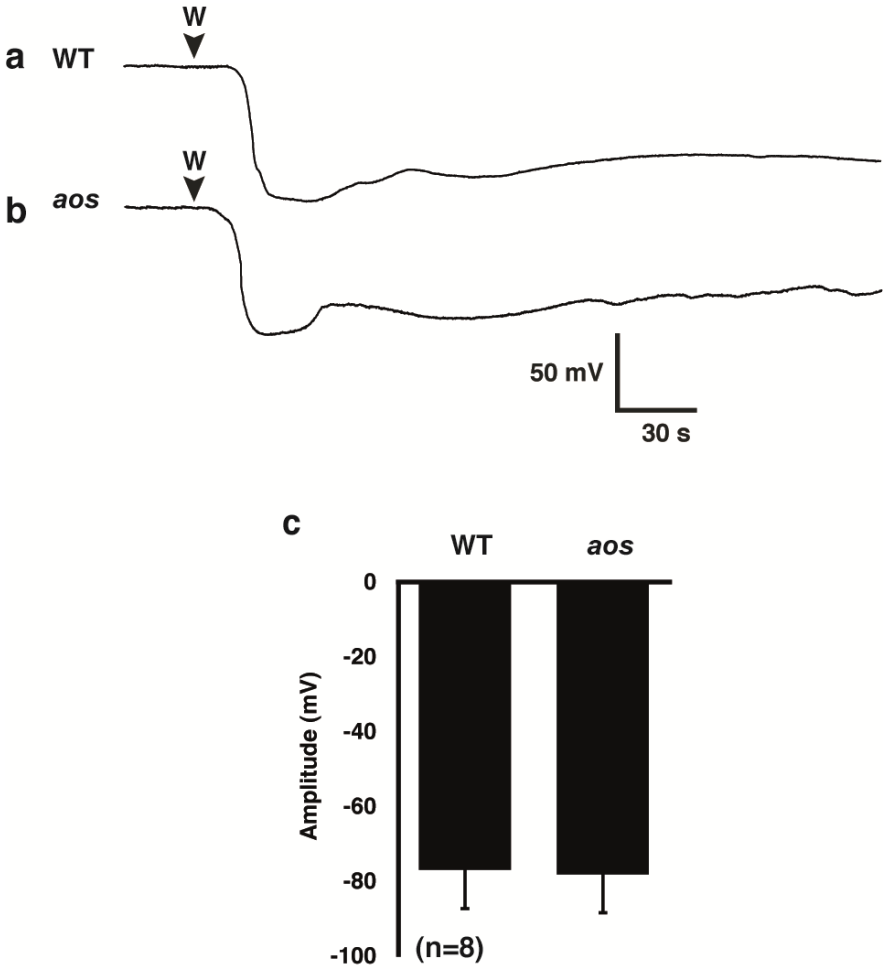


**Figure 3. 10. WASP generation in a jasmonate biosynthesis mutant. a**. Typical recording from leaf 8 of the WT after wounding the leaf tip. **b**. A typical recording from leaf 8 of the *allene oxide synthase* **(***aos*) mutant after similar mechanical damage. In both cases the recording electrode was placed at position e3 (shown in Fig. 3.1) prior to wounding the apical 40 % of leaf 8. Arrowheads indicate the time of wound infliction. **c**. WASP amplitude in WT and *aos* plants. W = wounded.

## Discussion

Herbivory and mechanical wounding induce electrical events in the undamaged part of plants (Wildon et al. 1992, Favre et al. 2001, Bricchi et al. 2013). We used extracellular recording to measure WASPs in leaves of *Arabidopsis thaliana* at the rosette stage after herbivory or mechanical wounding (Figs. 3.3 and 3.5). Surface potential changes were generated in response to insect feeding with velocity of 3.5 ± 0.3 cm $min^{-1}$. As shown in figures 3.2, 3.3 and 3.5, WASPs of the same polarity were generated by mechanical wounding and caterpillar feeding. Caterpillars cause both mechanical damage and they also release oral secretions. Although oral secretions can affect defence pathways in damaged plant (Mattiacci et al. 1995, De Vos and Jander 2009, Consales et al. 2012), there is a strong overlap of genes that respond to herbivores and to mechanical wounding (Reymond et al. 2000).

The depolarization of membranes is an early step in many stress responses (Schaller and Frasson 2001, Maffei et al. 2006, Maffei et al. 2007, Maischak et al. 2007). In the resting state, plant plasma membrane potentials are between -80 to -200 mV (Fromm and Lautner 2007). Knight et al. (1991) showed that cold shock increased the level of free $Ca^{2+}$ in the cytosol (Knight et al. 1991), and this is thought to lead to membrane depolarization (Tracy et al. 2008, Meimoun et al. 2009, Jeworutzki et al. 2010). It has been already shown that cold treatments induce depolarization in *Arabidopsis* (Krol et al. 2004, Carpaneto et al. 2007). The same polarity of WASPs and SP changes generated by chilling (Fig. 3.6) suggests that WASPs are due to membrane depolarization.

Zimmerman et al. (2009) found that electrical signals generated from wounding in barley and bean reflect hyperpolarisations (Zimmermann et al. 2009), while our finding showed that WASPs are due to membrane depolarisations. This suggests that WASPs might be due to action potentials (APs) or variation potentials (VPs) both of which have been proposed as two type of electrical signals in plants (Homann and Thiel 1994, Fromm and Lautner 2007). APs have been generated in response to wounding small parts of *Arabidopsis* leaves

and applying KCl (> 0.25 M) to the wounded part (Favre and Agosti 2007). Given that our findings are based on extracellular recording without analysing ion exchange across membranes, further data collection is required to know whether the SPs we detect are APs or VPs.

It has been reported that touching can induce electrical signals in plants (Fromm and Lautner 2007). *Mimosa pudica* and *Dionaea muscipula* are good examples of plants that are sensitive to mechanical stimulation or touching (Koziolek et al. 2004, Escalante-Pérez et al. 2011). In these plants, fast leaf movement is initiated by electrical signals (Volkov et al. 2008). However, touching *Arabidopsis* leaves did not generate detectable SPs, while cutting leaves did produce SPs (Fig. 3.9). Cutting leaves of *Hordeum vulgare* or *Vicia faba* also led to electrical potential changes in distal tissues (Zimmermann et al. 2009).

Having four recording electrodes allowed us to calculate the velocity of WASPs in the wounded leaf. Electrodes were placed on the lamina, midrib, midrib/petiole junction and petiole of wounded leaf. SPs were first observed in the position of e1 which was the closest electrode to the wound. The SPs then reached the other more distal electrodes. The speeds of SPs were calculated from data in table 3.2. These calculated speeds are in agreement with the results of Favre et al. (2007) who reported the speed of electrical signals generated in response to current injection in *Arabidopsis* as 4.6-6.9 cm $min^{-1}$ (Fromm and Lautner 2007). We found WASP velocities were slower in the lamina with a mean of 2.6 ± 0.6 cm $min^{-1}$ than in the midrib with a mean of 8.2 ± 1.4 cm $min^{-1}$. This suggests that the fastest route of WASPs transmission is through major veins. Indeed, Grams et. al. (2009) showed that electrical signals moved faster within the veins than through the intervein region of the lamina of maize leaves.

Previous work showed that JA or its active derivative jasmonoyl-isoleucine are mobile signals in tomato (Howe 2004, Matsuura et al. 2012). It has been demonstrated that JA is not likely to be a highly mobile long distance wound signal in *Arabidopsis* (Glauser et al.

2009, Koo et al. 2009). Wounding leaves leads to accumulation of JA within about 30 s (Glauser et al. 2009). However, WASPs were detected less than 10s after wounding in the wounded leaf. Production of WASPs earlier than JA accumulation would be consistent with WASPs being wound signals in *Arabidopsis.* In addition, the similarity of WASP patterns between WT and *aos* demonstrates that JA synthesis is not required for WASP generation. In conclusion, our results indicate that WASPs generated in *Arabidopsis* leaves lead to plasma membrane depolarisations. Touching does not induce SPs while mechanical wounding or herbivory generates reproducible WASPs in *Arabidopsis* leaves.

# Chapter 4

# Leaf surface potential changes in wounded *Arabidopsis*

## Introduction

All multicellular organisms need fast and reliable routes to distribute information concerning the health status of different parts of their bodies. Unicellular organisms use intracellular and interorga nismal communication while multicellular organisms additionally need intercellular and interorgan communication. Unlike animals that have a well-developed nervous system including central and peripheral elements, plants do not appear to have specific structures dedicated to exclusively transferring information over long distances. Instead, vascular tissues that conduct fluid and nutrients are a feature of vascular plants (ferns, gymnosperms, angiosperms, etc) and can serve as long distance information transfer networks. Vascular tissue cells are typically long and slender and connected end to end to make the equivalent of cellular pipe lines. In *Arabidopsis*, parastichies connecting leaves are a critical factor. In the adult phase a major parastichy follows an n+5 and n+8 rule. For example, leaf 10 is connected directly through vascular flow to leaves 15 and 18 and to leaves 5 and 2 (Dengler 2006). Consistent with this, when

leaves 5 and 8 of adult phase *Arabidopsis* plants were wounded, the expression of the *JASMONATE-ZIM DOMAIN 10* (*JAZ10*) gene was stronger in leaf 13 than in the non-connected leaves 9 and 12, as was the accumulation of JA (Glauser et al. 2009).

The velocity of wound-induced long distance signals leading to the accumulation of jasmonate has been estimated in *Arabidopsis thaliana* in several experiments. By combining JA accumulation measurements with a knowledge of vascular connections in *Arabidopsis* provided by Dengler (2006), it has been possible to refine the speed measurements and to set temporal boundaries. Such experiments led to speed estimates ranging from 3-8 cm $min^{-1}$ for the JA accumulation-inducing signal moving from wounded leaves to distal parastichious leaves (Glauser et al. 2009, Chauvin et al. 2013). In independent experiments a slightly slower speed was estimated for wound-stimulated signalling leading to jasmonoyl-isoleucine (JA-Ile) accumulation in leaves distal to wounded (Koo and Howe 2009). Additionally, a different experimental setup involving a reporter gene construct that responds to the wound-stimulated accumulation of reactive oxygen species, indicated the speed of (a) signal(s) travelling from wounded leaves to floral stems in *Arabidopsis* at 8.4 cm $min^{-1}$ (Miller et al. 2009).

In this chapter, our goal was to better understand wound-associated surface potential changes (WASPs) in distal leaves to mechanical wounding. To test possible correlations with the activation of jasmonate signalling, we mapped the expression of the jasmonate-responsive gene *JAZ10* (Yan et al. 2007) throughout the rosette after wounding leaf 8 and compared this to a spatial map of surface potential changes (SPs). These two maps correlated well and revealed new information on parastichies followed by wound signals. Using these two methods we were able to solve the issue of whether signal displacement is source-sink dependent. Further investigation of wound-to-distal leaf signalling showed that interrupting the signal necessary to generate surface potentials also interrupted *JAZ10* expression.

## Results

### Parastichious leaves display surface potential changes (SPs) different to those from nonparastichious leaves

The pattern of vascular connections between leaf primordia is conserved within species but can depend on developmental stages and can differ between juvenile and adult phases. A knowledge of phyllotaxis helps to predict the vascular pattern in plants and to understand long distance transport of water, minerals or signalling molecules. In addition, it is usually easy to predict the position of the next leaf primordium. In *Arabidopsis*, there are more than 20 leaves in the rosette phase of 5-week old plants. The leaves are contacted in the pattern of n+5 or n+8 as connected parastichies and n+3 as contact parastichies (Dengler 2006). For example, the vascular system of leaf 13 is connected to that of leaf 5 (n+8) and leaf 8 (n+5). Figure 4.1 shows the mathematical prediction of leaf placement in *Arabidopsis thaliana* in the rosette stage.

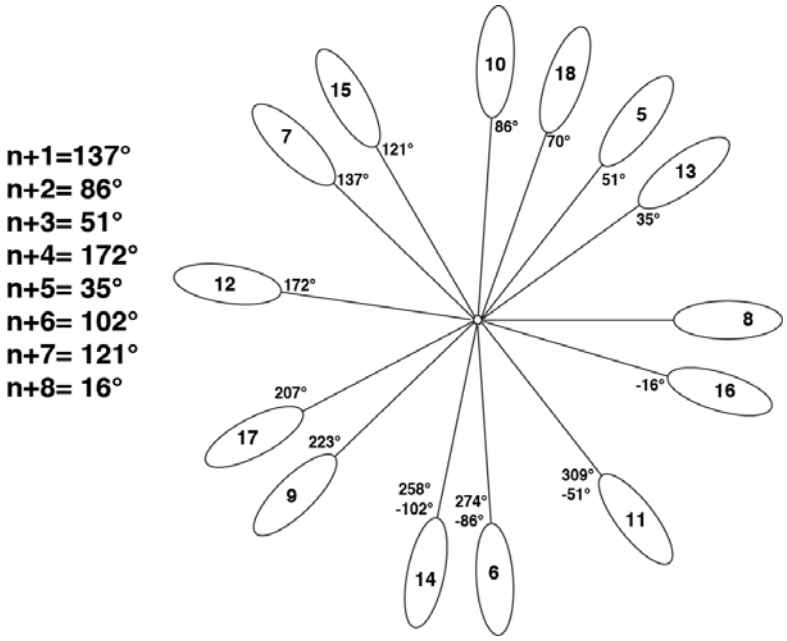


**Figure 4.1. Leaf placement in the *Arabidopsis* rosette.** The angles were calculated between leaves from leaf 5 to leaf 18. Note, the angles for n+3, n+5 and n+8 are closest. Leaf 8 is in position 0°.

## Vascular connections and jasmonate signalling in distal tissues

To assess the role of vascular connections in *Arabidopsis* rosettes, jasmonyl isoleucine (JA-Ile) and jasmonic asid (JA) were measured.[1] Leaf 8 and leaf 13 were harvested 20 and 60 min after wounding leaf 8. To get enough material for JA quantification, five leaves of control or wounded plants were pooled as one biological replicate. High-performance liquid chromatography-tandem mass spectrometry in the multiple reactions monitoring (MRM) mode was used for JA and JA-Ile quantification. The results showed that at both time points, the levels of JA and JA-Ile increased in the wounded leaves and leaves 13 in respect to unwounded leaves (Fig. 4.2).

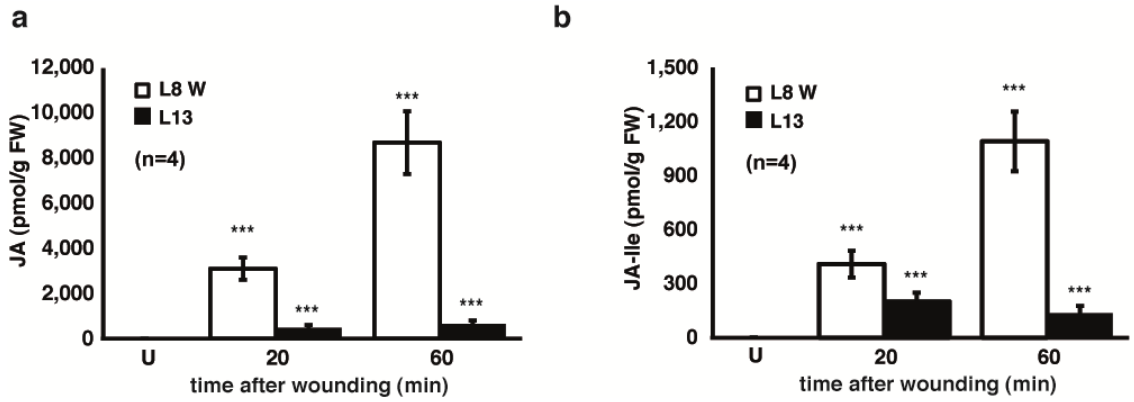


**Figure 4. 2. Levels of JA and JA-Ile after wounding.** In each case leaf 8 was wounded and this leaf as well as leaf 13 was harvested for jasmonate analysis 1h after wounding. **a**. JA level in leaves 8 and 13. **b**. JA-Ile level in leaves 8 and 13. U = unwounded . W = wounded. *** = $P<0.001$ (n=4 ± SD).

## WASPs are limited to a part of the *Arabidopsis* rosette

With a knowledge of leaf placement and vascular connections between leaves, we attempted to record WASPs in all leaves of *Arabidopsis* after wounding 40% of the apical part of leaf 8. Leaf 8 was chosen for wounding due to the leaf that it is the first adult leaf in the rosette and it allowed us to conduct experiments aimed at studying signalling from

[1]- This experiment was performed by Adeline Chauvin, University of Geneva.

younger or older leaves. At 5-weeks old in short day growth conditions, *Arabidopsis* usually has approximately 20 leaves.

Measurements to map surface potentials throughout the rosette were then performed with four electrodes placed on different parts of plant. One electrode was always on the wounded leaf 8 at position of e2 (Fig. 3.1), an electrode was placed on leaf 9, 1 cm from the centre of rosette as non-connected leaf. The two other electrodes were placed on different leaves from leaf 5 to leaf 18 on 1 cm from the centre of rosette. Characteristic surface potential patterns for each of these leaves are shown in Figure 4. 3. Leaves 5, 11, 13 and 16 showed responses similar to those in the wounded leaf (Fig. 4.3, Table 4.1). For example, after wounding leaf 8, a WASP with a duration of 78 ± 20 s and a peak amplitude of -51 ± 9 mV was reached in leaf 13 after a latency of 66 ± 13s (n=61). Other leaves (7, 9, 10, 12, 14, 15 and 18) showed responses of opposite polarity responses as small positive changes in surface potential. For example, leaf 9 showed a 20 ± 5 mV change in surface potential with a latency of 54 ± 12 s (n=46). WASP values for leaf 16 (n+8) were 56 ± 14 s duration, -45 ± 10 mV for amplitude with a latency of 73 ± 14s (n=27). We also noted large changes in surface potential for leaf 11 (n+3). Additionally, we recorded changes in surface potentials in the n-2 leaf (leaf 6) that were similar to those in wounded leaf 8 in 63% (13 out of 19) recordings. The remaining recordings from leaf 6 (Fig. 4.3) resembled traces from leaves such as leaf 9. In contrast to leaf 6, variable wound-stimulated electrical activity was not observed in leaf 10 (the n+2 leaf relative to wounded leaf 8). Initial hypothesis that proved to be wrong was SPs travels to leaf n+5+5. In other words, signals from wounding leaf 8, first go to leaf 13 and then go to leaf 18. However, as mentioned previously, the changes of surface potentials in leaf 18 was similar to those in non-connected leaves (Fig. 4.3).

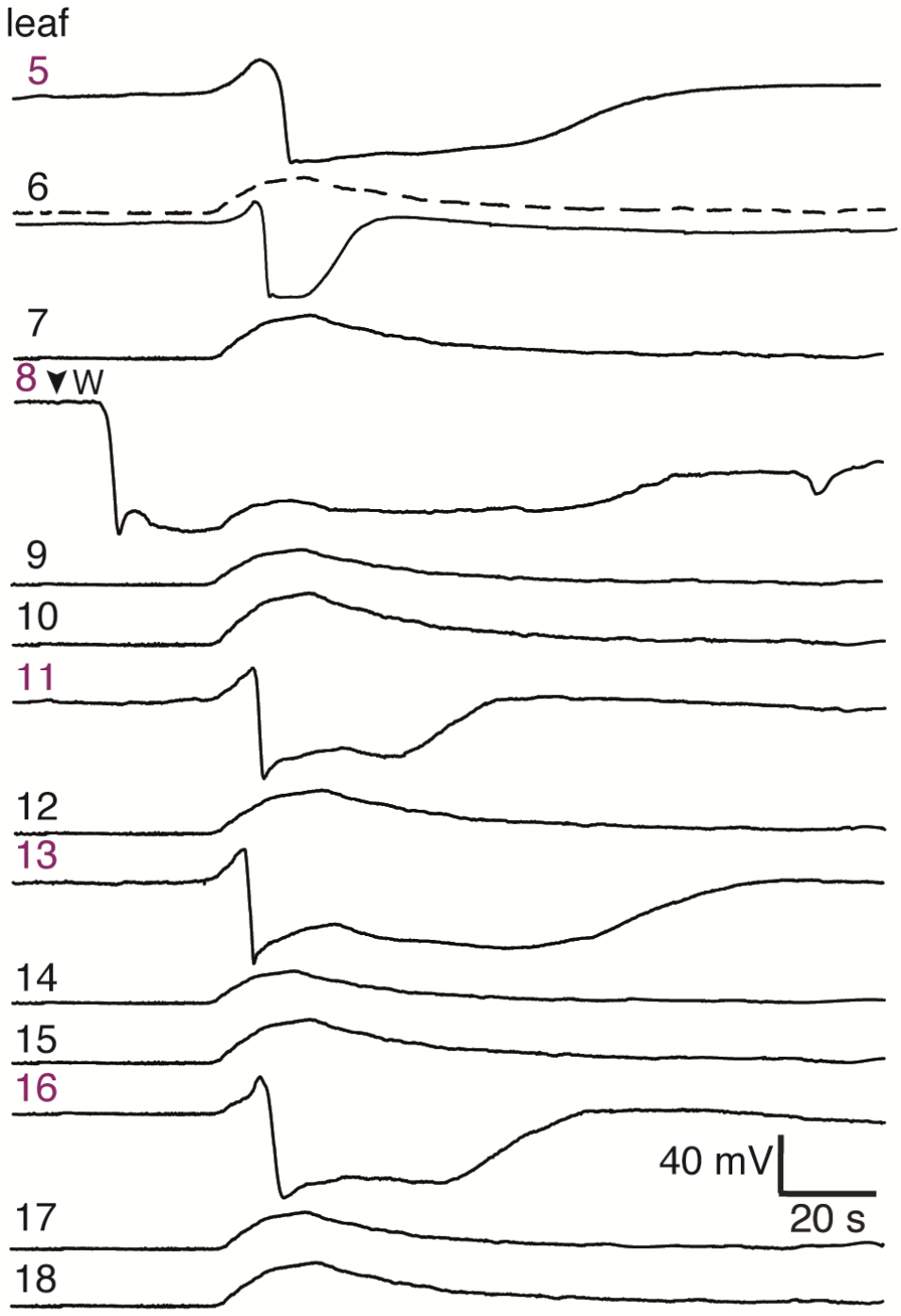


**Figure 4. 3. Representative surface potential changes generated on distal leaves after wounding leaf 8.** One electrode was located on the petiole (2 cm from wounded part) of wounded leaf 8 (W). The other electrodes were on the petioles of the non-wounded leaves 1 cm from centre of plant. The arrowhead shows when leaf 8 was wounded. The data are representative of 8-10 independent replicates. Two types of surface potential change were observed on leaf 6. The solid line shows a representative trace from 63% (n=19) of events and the dashed line indicates an opposite polarity change in surface potential seen in 37% of cases.

**Table 4. 1. Wounding leaf 8 causes surface potential changes in connected leaves 5, 11, 13 and 16.** Leaf 8 was wounded and surface potentials were monitored in distal leaves with electrodes placed on these leaves at position e3' (see Fig. 3.1 for details of electrode placements). For leaf 8 the monitoring electrode was at position e2. W = wounded, x = number of experiments in which amplitudes of surface potentials exceeded -10 mV. Values are means ± SD.

| Electrodes | Latency (s) | Amplitude (mV) | Duration (s) | x/n |
|---|---|---|---|---|
| Leaf 5 | 66 ± 14 | -57 ± 14 | 64 ± 16 | 26/27 |
| Leaf 8 W | 22 ±3 | -76 ± 11 | 163 ± 30 | 33/33 |
| Leaf 9 | 54 ± 12 | 20 ± 5 | 21 ± 9 | 46/46 |
| Leaf 11 | 69 ± 14 | -56 ± 13 | 36 ± 18 | 41/48 |
| Leaf 13 | 66 ± 13 | -51 ± 9 | 78 ± 20 | 61/61 |
| Leaf 16 | 73 ± 14 | -45 ± 10 | 56 ± 21 | 24/27 |

### Mapping *JAZ10* expression levels in distal leaves

To better understand the role of WASPs in the activation of the jasmonate pathway, quantitative electrophysiological data (Table 4.1) were compared with transcript levels for *JASMONATE-ZIM DOMAIN 10* (*JAZ10*), a robust marker for activity of the jasmonate pathway (Yan et al. 2007). We studied *JAZ10* expression levels for 14 leaves (leaves 5-18) of 6-week *Arabidopsis thaliana* that were either unwounded or wounded on leaf 8. To do this, 40% of apical part of leaf 8 was wounded and then, after 1 h, leaves (5-18) were harvested separately. Leaves from 5-18 in the unwounded plant were also harvested separately as controls. Four biological repeats and three technical repeats were used for both unwounded and wounded plant. One h after wounding leaf 8 we detected >100 fold increases in *JAZ10* transcript levels in leaves 5, 8, 11, 13 and 16. In contrast, at this time leaves 7, 9, 10, 12, 14, 15, 17 and 18 showed levels of expression similar to or only slightly higher than those in the cognate leaves of the unwounded plants (Fig. 4.4). *JAZ10* transcript induction in leaf 6 was variable and below the significance threshold ($p>0.05$)

and, as for WASP generation, we did not observe increases in *JAZ10* transcripts in leaf 10. Heat maps from quantitative data show that *JAZ10* expression at 1h postwounding and WASP durations covered identical territories, spanning 137° of the rosette when variable leaf 6 (n-2) is included (Fig. 4.5). In addition, we found that leaves 5 and 11 (n+3) which are apparently contact parastichy and not connected directly by the vascular system (Dengler 2006) showed a high level of *JAZ10* expression which indicates that n±3 is another route for transmitting long distance signal in *Arabidopsis* rosette.

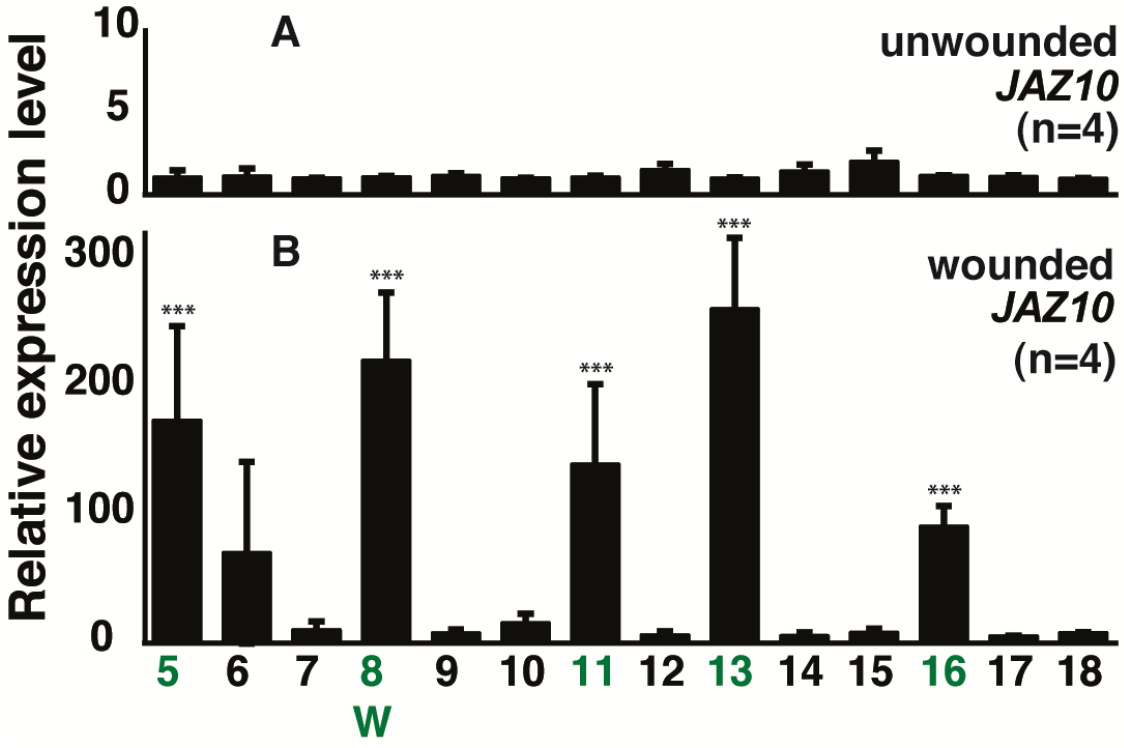


**Figure 4. 4. Relative expression levels of *JAZ10* 1h after mechanical wounding of leaf 8.** The data are from four independent replicates ± SD. ***, $P \leq 0.001$. W=wounded.

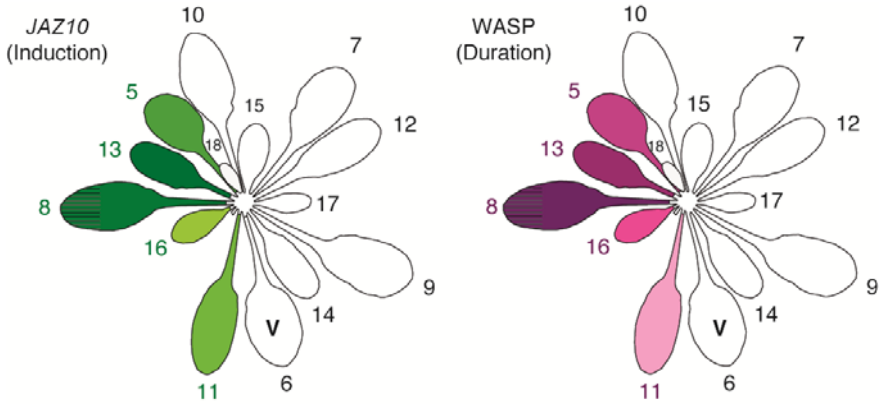


**Figure 4. 5. Heat maps representing the level of *JAZ10* expression and the duration of WASPs produced after wounding leaf 8.** Data for *JAZ10* levels are from figure 4. 4. WASP durations are from Table 4.1. V = variable in leaf 6.

### N+3 is a new long distance route in *Arabidopsis*

To investigate whether there were direct n+3 vascular connections between leaves, we employed the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) which moves through the vascular system and causes chlorosis in the leaves (Kiefer and Slusarenko 2003). The abaxial surface of leaf 8 of four week-old plants was infiltrated with an insulin syringe with 10 µl of 2 mM of 3-AT dissolved in water. For control plants, 10 µl of water were infiltrated into leaf 8. Figure 4.6 shows that leaf 11 (n+3) did not develop chlorosis symptoms. This indicates that leaf 11(n+3) might not be connected directly to leaf 8.

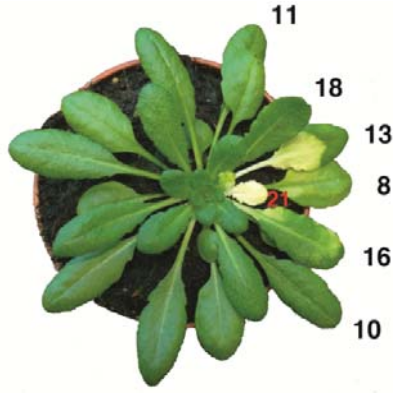


**Figure 4.6. The morphological effect of 3-amino-1,2,4-triazole (3-AT) on *Arabidopsis* leaves after 2 weeks.** The abaxial of leaf 8 was infiltrated with 2 mM of 3-AT at 4-week old plant.

**New estimations of wound-induced signal speed**

The speed of signals leading to surface potential changes after wounding was estimated by placing electrodes e1', e2' and e3' on leaf 13 and e2 on leaf 8 (Fig. 4.7). The distance between electrodes on leaf 8 from the wound was 3 cm. In leaf 13, electrodes were placed 1 cm from each other and 1 cm from the centre of the plant. Table 4. 2 presents data for speed estimation in a quantitative form. An average velocity of 8.5 cm $min^{-1}$ was found between midrib and petiole of leaf 13 when leaf 8 was wounded. The amplitude changed in leaf 8 to -73 ± 14 mV at 15 ± 3 s after wounding. The electrical potentials in leaf 13 were -53 ± 17 mV at 70 ± 16 s after wounding, -58 ± 15 mV at 63 ± 15s and -56 ± 18 mV at 53 ± 15 s for electrodes e1', e2' and e3' respectively (n=17) (Fig. 4.7). The distance from the electrode placed ion the petioles of the wounded leaf 8 to the electrode placed on petiole of leaf 13 was 3 cm. Therefore, the velocity of signals that lead to changes in surface potential moved from the wounded leaf to the unwounded, connected leaf at 5.4 ± 1.5 cm $min^{-1}$.

**Table 4.2. Characterization of surface potentials (SPs) in *Arabidopsis* leaves in parastichious leaf 13 after wounding leaf 8.** x/n= the number of experiments in SPs were > -10 mV / total number of experiments. Values are means ± SD. See Fig. 4.7 for details of electrode placement.

| Electrodes | Latency (s) | Amplitude (mV) | Duration (s) | Velocity (cm/min) | x/n |
|---|---|---|---|---|---|
| **Midrib (e1')** | 70 ± 16 | -53 ± 17 | 76 ± 15 | 5.8 ± 1.1 | 17/17 |
| **Petiole/midrib junction (e2')** | 63 ± 15 | -58 ± 15 | 77 ± 25 | 5.7 ± 1.1 | 16/17 |
| **Petiole (e3')** | 53 ± 15 | -56 ± 18 | 65 ± 17 | 5.5 ± 1.2 | 17/17 |
| **Average** | | -56 ± 15 | | | |

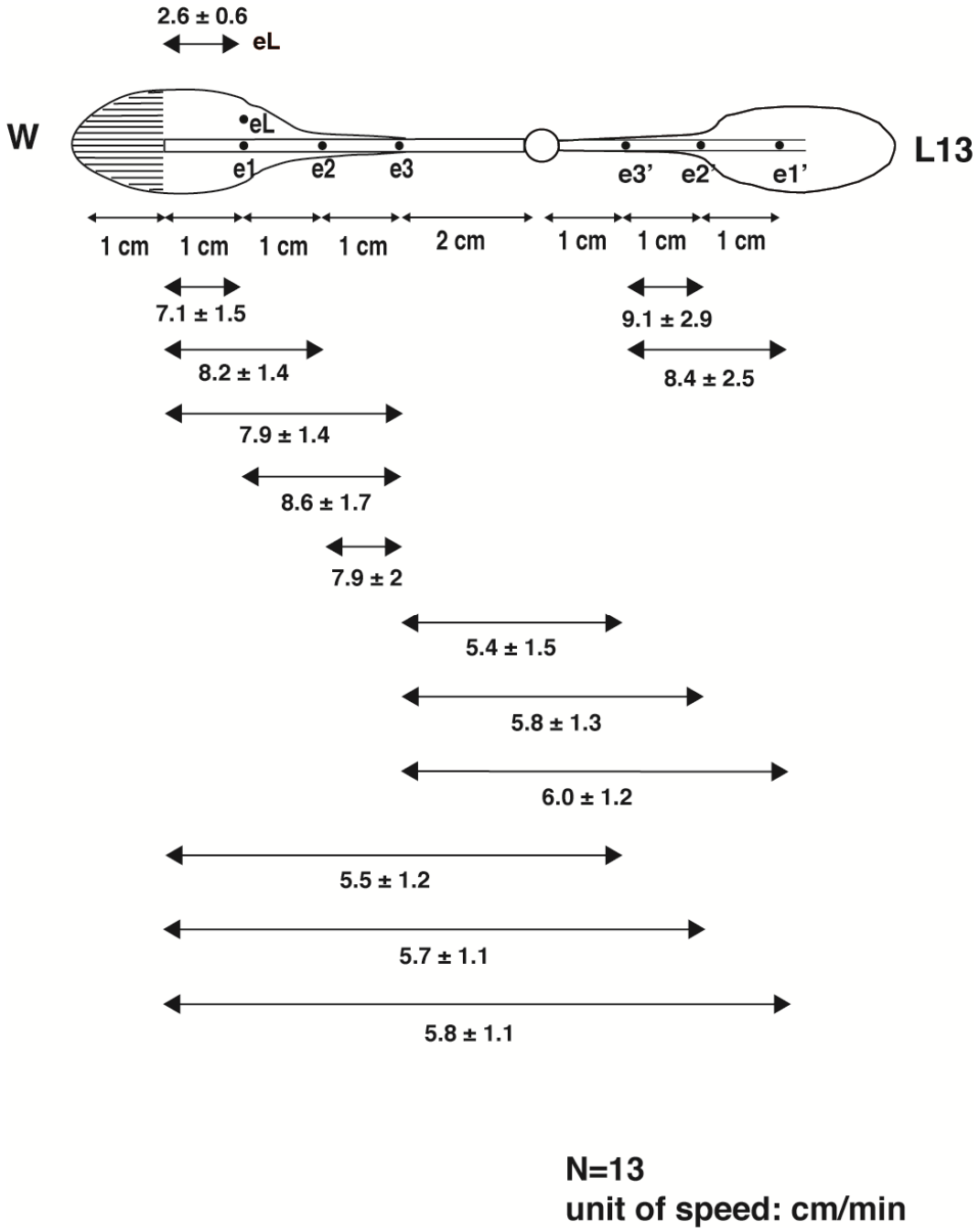


**Figure 4.7. Estimation of apparent WASP velocities.** Leaf 8 or 12, the largest rosette leaves in 6 week-old plants, were chosen for estimating velocities within a wounded leaf. For estimating the apparent velocities of signals that travel from leaf to leaf, leaf 8 was wounded and recordings were made both on this leaf and on leaf 13. ANOVA, followed by Bonferroni post-hoc test showed that the WASP speed indicated in cm/min along the midrib and petiole within a leaf was not significantly different between leaves 8, 12 and 13, but was faster than the overall signalling speed from leaf 8 to leaf 13, and the speed of the signal from the wound to the electrode placed in the lamina of the wounded leaf. All data are from 13 replicates (± SD). W = wounded.

**Leaf 8 is not unique: Long distance signal transfer patterns are conserved in other leaves**

In order to understand whether parastichies function similarly in WASP propagation in leaves other than leaf 8, leaf 10 was wounded. The electrodes were placed on leaves 10 (wounded leaf), the non-connected leaf 11 and leaf 15 as a connected leaf. The pattern of WASPs generated from wounded leaf 10, leaf 11 and leaf 15 were similar to wounded, non-connected and connected leaves when leaf 8 was wounded (data not shown). Moreover, the *JAZ10* expression levels in leaf 15 1 h after wounding leaf 10 were similar to *JAZ10* levels in leaf 13 when leaf 8 was wounded (Fig. 4.8).

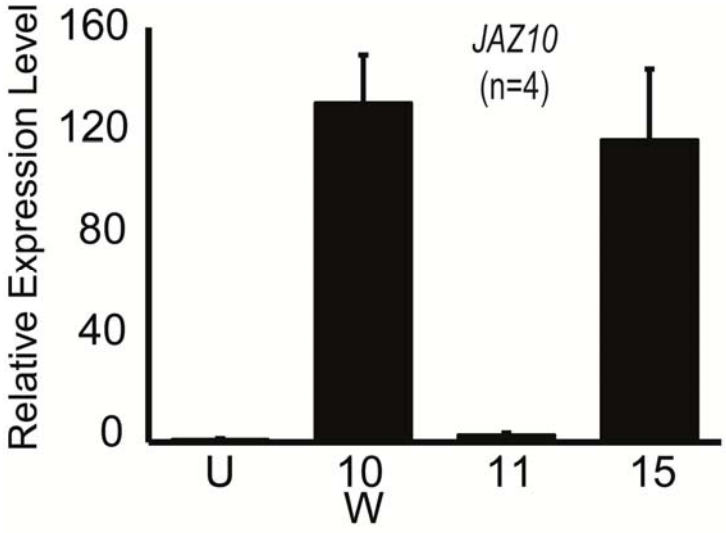


**Figure 4.8. Relative expression level of *JAZ10* 1 after wounding leaf 10.** The data are from four independent replicates ± SD. *, $P \leq 0.05$. U= unwounded, W=wounded.

**The accessions Ws (Wassilewskija) and Ler (Landsberg erecta) have similar wound SPs to Col-0 (Columbia-0)**

In order to investigate the electrical activity in different accessions of *Arabidopsis*, Ws (Wassilewskija) and Ler **(***Landsberg erecta)* were also assessed for the ability to produce measurable surface potential changes after leaf wounding. Two electrodes were placed on leaf 8 at position of e2 and e3 (Fig 3.1), one electrode on leaf 9 and one on leaf 13 1cm from the rosette centre. The results showed that both accessions were similar in WASP duration and amplitude in the wounded leaf 8, leaf 9 and leaf 13 with respect to Col-0. Moreover, there was no significant difference of velocities of WASPs among these accessions (data not shown).

**WASPs are bidirectional**

Next we asked the question of whether wound-stimulated signals can travel downwards from younger to older leaves. In other words, if we wounded leaf 13 (Fig. 4.9a), would we find the highest induction of *JAZ10* in leaf 8? We wounded leaf 13 and recorded surface potentials on leaves 8 (connected), 9 (non-connected) and 13 (wounded). The duration and amplitude of WASPs in leaf 8 when leaf 13 was wounded was similar to duration of leaf 13 when leaf 8 was wounded (Fig. 4. 9 b). WASPs duration and amplitude in the wounded leaf 13 were similar to wounded leaf 8. In addition, in the connected leaf 8 when leaf 13 was wounded, WASP duration and amplitudes were similar to leaf 13 when leaf 8 was wounded (Fig 4.9 c and d). Leaves 8, 9 and 13 were harvested 1 h after wounding leaf 13 for *JAZ10* expression level measurement. Figure 4.9e shows the high expression of *JAZ10* in the connected leaf 8 when leaf 13 was wounded.

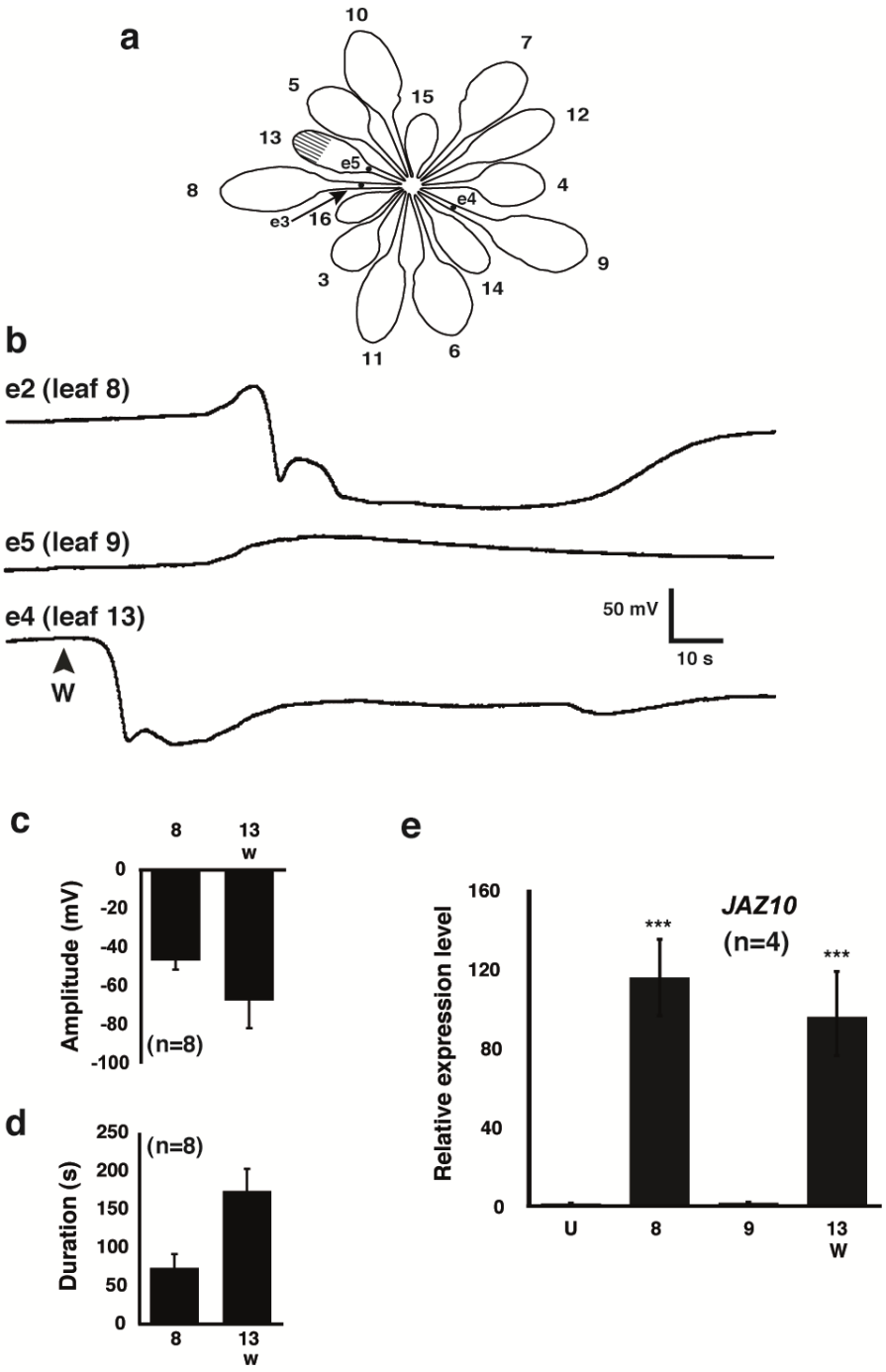


**Figure 4. 9. Wounding young leaves triggers WASPs and *JAZ10* expression in older leaves. a**. Electrode placement positions on leaves 8 (e3), 9 (e4) and 13 (e5). **b**. Typical changes in surface potential in leaves 8, 9 and 13 after wounding leaf 13. The arrowhead shows the time of wounding. **c**. WASP amplitudes after wounding of leaf 13 (n=8). **d**. WASP durations after wounding of leaf 13 (n=8). **e**. *JAZ10* expression 1h after wounding leaf 13 (n=4 ± S.D). Unwounded leaves (U), wounded leaf 13 (W). *** = $P<0.001$ (n=4, ± SD).

**WASPs are not transmitted to distal leaves after limited leaf damage**

Crushing leaves leads to breakage and destruction of cells. An experiment was designed to access the effect of wound size on WASP generation and *JAZ10* expression. 10%, 40% and 80% of apical part of leaf was wounded with forceps. Wounding 80% of leaf 8 showed similar patterns of WASPs duration and amplitude to wounding 40 % in both leaves 8 and 13. But, when 80% of leaf 8 was wounded, wounding leaf 9 showed depolarization about -23±8 mv and duration of 11±5 s in 40% (4 out of 9) of replicates. In 10 percent wounding of leaf 8, WASPs was similar to 40% wounding for the electrode which placed on e2 (Fig. 3.1) in leaf 8, but WASPs was not recorded in leaf 13. The samples of 10% and 40% wounding were harvested for 1 h after wounding to measure the *JAZ10* transcript level. Figure 4.10 shows that the leaf 13 that did not receive WASPs after wounding 10% of leaf 8 also did not display an increased level of *JAZ10* expression (Fig. 4.10).

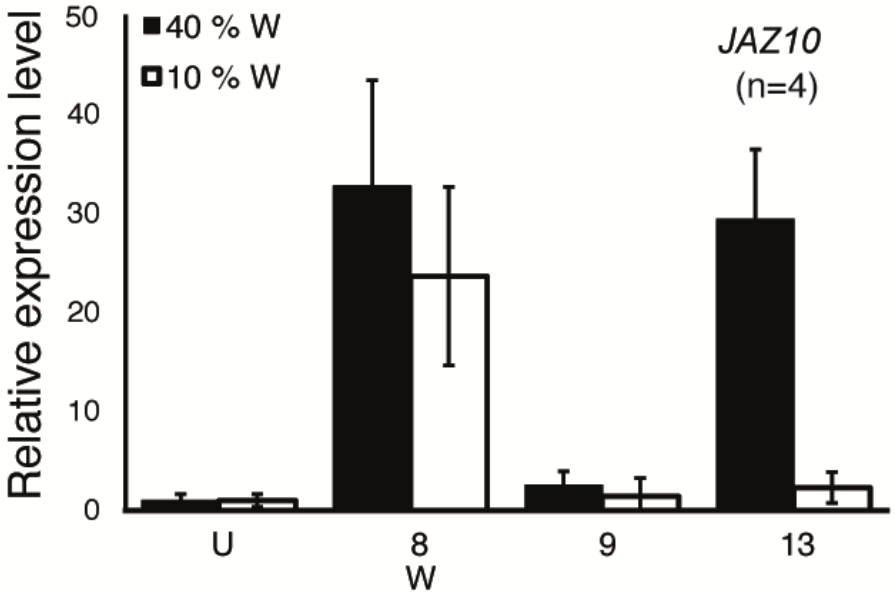


**Figure 4. 10 *JAZ10* expression level 1h after wounding of leaf 8.** Leaf 8 was wounded 10 or 40 percent of apical part. The data are from four independent replicates ± S.D. Unwounded leaves (U), wounded leaves (W).

**Experiments to interrupt surface potential generation**

To test the relation between surface potential generation and *JAZ10* expression, the petiole of the wounded leaf 8 was cut with zirconia ceramic scissors (CS-250 Kyocera, Japan) which do not act as strong capacitors and therefore do not interfere with surface potential measurements. Two electrodes were placed on the petiole of wounded leaf 8; e2 was placed on the midrib and e3 was closer to centre of plant as shown in Figure 4. 11A. Two other electrodes were placed on leaf 9 (e4) and 13 (e5). 40% of the apical part of leaf 8 was wounded by crushing. The petiole of leaf 8 was cut immediately when a surface potential change was recorded in e2 but before it reached e3 (Figure 4.11B). At this time the recording was stopped due to disconnecting of wounded part with e2 to ground and surface potential decline sharply (due to cut). No surface potential changes were recorded on leaves 9 (E4) and 13 (E5) after cutting petiole of leaf 8 (cut no WASP, Fig 4.11B) and shown the pattern of SP changes similar to control unwounded plants (cut no WASP). Likewise, when leaf 13 did not receive any wound signal from leaf 8, the expression level of *JAZ10* was low and similar to that recorded from an unwounded plant (Fig 4.11C). Due to the speed of signals resulting in surface potential changes within wounded *Arabidopsis* leaves (8.4 cm $min^{-1}$) and the short distance between e2 and e3 (less than 2.5 cm) the time of cutting was critical, although this procedure took less than 5s. Sometimes, a delay in cutting did not prevent surface potential migration and we recorded surface potential change of e3, thus the pattern of surface potential recorded of e4 and e5 was similar to non-cut plants (cut WASP) in Fig. 4.11B). In other words, cutting after surface potential changes were monitored at e3 did not inhibit their appearance in distal leaves. In these cases leaf 13 showed greater *JAZ10* inductions than did leaf 9. In control experiments, leaf 8 was wounded without cutting its petiole and leaves 8, 9 and 13 showed their characteristic electrical behaviour and *JAZ10* expression levels.

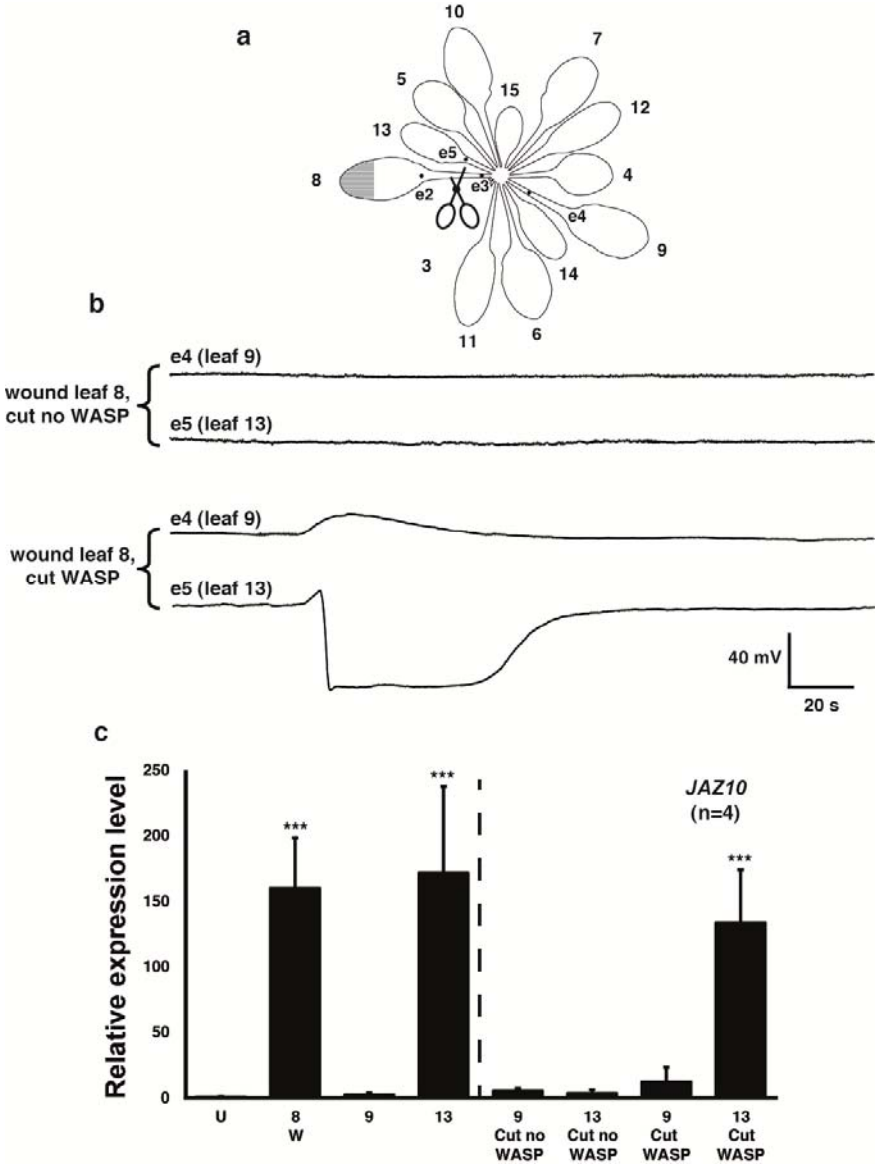


**Figure 4.11. Effects of interrupting WASP propagation on *JAZ10* expression. a**. Experimental design: two electrodes were placed on leaf 8, one of these (e2) was placed on the midrib and the second electrode (e3) was at the base of the petiole. Electrodes were also placed on leaf 9 (e4) and on leaf 13 (e5). 40% of leaf 8 was wounded. **b**. WASP traces for leaves 9 (non-parastichious) and leaf 13 (connected) that were provoked by wounding leaf 8. The first pair of traces was recorded when leaf 8 was severed upon detection of a signal at e2 and before a WASP was detected at e3. The second pair of traces was recorded when the WASP generated by wounding leaf 8 was allowed to reach e3 and the leaf was then severed immediately. **c**. *JAZ10* expression in unwounded leaves (U), wounded leaf 8 (W) and leaves 9 and 13. The left side of the dashed line shows *JAZ10* transcript levels in leaves 8, 9 and 13 of intact control plants 1 h after wounding leaf 8. Data on the right side of the dashed line are from plants in which the wounded leaf 8 was severed when WASPs were detected at e2 but were not allowed to reach electrode e3 (cut no WASP) or when WASPs were allowed to reach e3 prior to severing leaf 8 (cut WASP). *** = $P<0.001$ (n = 4, ± SD).

## Discussion:

In this chapter, we have demonstrated the correlation between WASP generation and activation of the JA pathway. In response to wounding leaf 8 an increased expression of *JAZ10* was observed in leaves 5, 8, 11, 13, and 16. This was consistent with the pattern of WASP generation in the rosette and indicated that WASPs and *JAZ10* expression are highly correlated. Accumulation of JA after 120 s in vascularly interconnected leaves (Glauser et al. 2009) is comparable with the change of surface potential that we monitored in such leaves within 60-90s of wound infliction to leaf 8.

There are many reports of mobile electrical signals generated in response to wounds. These include action potentials as well as variation potentials, wound potentials and system potentials (Wildon et al. 1992, Stahlberg et al. 2006, Favre and Agosti 2007, Zimmermann et al. 2009). However, it is important to note that we have no evidence that the surface potential changes we monitored correspond to signals that move through the plant. Instead, they might reflect the arrival of other types of signals that then cause ion fluxes in populations of cells in the leaf. Additionally, multiple different long distance signals may be generated in response to a wound and among the evidence that is consistent with this is the fact that genes regulated through multiple signal pathways can be activated in tissues distal to wounds (Onkokesung et al. 2010, Walley and Dehesh 2010). What we measure may, like the accumulation of JA, or the expression of genes like *JAZ10*, be the consequence of arrival of such unknown signals. The events we record with surface electrodes probably reflect the behavior of populations of cells in which events are summed to produce the output which we have exploited in connection with gene expression, to map wound-related events in the rosette.

From measurements in which electrodes were placed out on the wounded leaf and on distal leaves we were able to look at surface potential changes throughout the rosette. These correlated well with the n+5, n+8 parastichies (Dengler 2006) that we previously reported to be relevant to the spatial pattern of JA accumulation in rosettes of wounded plants

(Glauser et al. 2009). A new finding to emerge from the present study is that n+3 signal transfer was elicited on wounding. The n+3 parastichy was defined as a non-contact parastichy by Dengler (2006). These type of contact parastichies are defined by interleaf vascular strand proximity rather than vascular strand interconnection. Therefore, the wound signal(s) that gives rise to changes in surface potential in n+3 leaves may be able to 'jump' between vascular strands. Alternatively, the n+3 parastichy might be a truly connected parastichy, although this could not be demonstrated in experiments using 3-AT to bleach leaves. Also related to the spatial distribution of surface changes following wounding is directionality. Signals generated by wounding leaf tips move basipetally to the centre of the rosette and then disperse acropetally into certain distal leaves to activate JA accumulation and *JAZ10* expression (Glauser et al. 2009).

Now, the question arises as to whether signal transfer is only from older leaves to younger leaves (e.g from wounded leaf 8 to unwounded leaves 11, 13 and 16). To test this we monitored *JAZ10* gene expression in leaves preceding and anteceding leaf 8. The pattern was clear, strongest *JAZ10* expression took place in both leaf sets, i.e. the response corresponded to n± 3, 5 and 8 parastichies. Similarly, surface potential changes followed these same parastichies i.e. *JAZ10* expression corresponds to surface potential changes. This result is consistent with the literature. Firstly, plasma membrane depolarization causes the expression of genes known to be regulated by the jasmonate pathway (Schaller and Oecking 1999, Frick and Schaller 2002) whereas hyperpolarization of cells by fusicoccin treatment repressed the expression of some of these genes (Schaller and Frasson 2001). Secondly, the activation of proteinase inhibitor genes in tomato that are regulated by jasmonates (Farmer and Ryan 1992) is reported to correlate with strong depolarizations of the plasma membrane of phloem sieve tube and companion cells (Rhodes et al. 1996). We also note that both slow wave potentials (SWPs) and wound potentials (WPs) are bidirectional i.e. moving both basipetally and acropetally away from a wound (Stahlberg et al., 2006). Summarizing, severely wounding one leaf in the rosette leads to the activation of *JAZ10* in both older and younger leaves in parastichies that, spatially, correspond to a

sector covering approximately (137º) 38 % of the rosette area. A similar coverage is observed from changes in surface potential. It would be interesting if insects avoid repeated feeding in these sectors.

Additionally, we recorded changes in surface potentials in the n-2 leaf (leaf 6) that were similar to those in wounded leaf 8 in 63% recordings. The remaining recordings from leaf 6 (Fig. 4.3), resembled traces from leaves such as leaf 9. In contrast to leaf 6, variable wound-stimulated electrical activity was not observed in leaf 10 (the n+2 leaf relative to wounded leaf 8). We termed leaf 6 the variable leaf and noted that we could increase the proportion of large depolarisations in this leaf if a greater surface area of leaf 8 was wounded.

By measuring the speed it took for surface potential changes to occur in parastichious leaves we were able to provide new indirect estimates for the speed of signal displacement in the wounded *Arabidopsis* rosette. These similar apparent velocities for surface potential changes in the midribs of wounded leaves and distal leaves suggest that related mechanisms control electrical signalling in these leaves. Previous estimates based on this approach and using JA accumulation as output placed the signal velocity in the range of 4-8 cm $min^{-1}$ (Glauser et al. 2009, Chauvin et al. 2013). This fits with the observations of the present study. However, signals from the wounded leaf appeared to slow to 5.4±1.5 cm $min^{-1}$ at the centre of the plant prior to accelerating again in the distal leaf that 8.4±1.5 cm $min^{-1}$, bringing the average signal speed from wounded leaf 8 to receiver leaf 13 to 5.8±1.1 cm $min^{-1}$ (n=13). This overall velocity estimate is concordant with conservative estimates of signal speeds that were based on JA accumulation within parastichies in *Arabidopsis* (Glauser et al. 2009).

# Chapter 5

# Glutamate Receptor-Like genes mediate long distance electrical signalling after wounding

## Introduction

Wounding the leaves of *Arabidopsis* induces the expression of a wide range of genes and the synthesis of many chemical compounds. These events take place both close to and distal to the wound. Jasmonic acid (JA) levels also increase upon wounding in the local and distal part of plants. JA regulates a broad variety of physiological responses and plays central roles in wounding responses and in defence (Browse 2009, Acosta and Farmer 2010). At present the nature of the long distance signal(s) needed to activate distal JA synthesis and defence gene expression is/are unknown. Two sets of hypotheses have emerged. Firstly, jasmonates themselves may be translocated from leaf to leaf or their synthesis may be required throughout the path of signal propagation (Schilmiller and Howe 2005). Alternatively, the long distance signal(s) may be independent of jasmonate synthesis (Koo et al. 2009, Koo and Howe 2009). Several models for long distance

signalling have emerged with the serial production of reactive oxygen species along the path of signalling leaves being a recent model (Miller et al. 2009). However, a larger body of literature makes reference to various types of electrical signalling (eg. Wildon et al., 1992).

Environmental stresses largely alter plant physiology and metabolism and they can also trigger the production of electrical signals. The effects of wounding on the generation of electrical activity have been widely studied among others species such as tomato (Wildon et al. 1992), tobacco, barley and bean (Zimmermann et al. 2009), *Mimosa pudica* (Kaiser and Grams 2006) and maize (Grams et al. 2009). Wildon et al. (1992) found a correlation between electrical signal production and *proteinase inhibitor II (Pin2)* gene activation after wounding tomato (Wildon et al. 1992). Moreover, electrical signals generated by current injection activated JA accumulation and *Pin2* expression in tomato leaves (Peña-Cortés et al. 1995). However, in *Arabidopsis*, the generation of electrical signal following wounding is the subject of only a few studies (Favre et al. 2001, Favre and Agosti 2007). So far, there is no evidence that electrical activity and jasmonate-regulated gene expression are causally linked in *Arabidopsis*. Moreover, no genes reported to regulate electrical activity have been found to effect wound physiology. The identification of channel or transporters genes shown to have a role in wounding would be a crucial step in proving the relevance of electrical signals.

Twenty genes encoding glutamate receptor-like (GLR) non-selective cation channels are annotated in the *Arabidopsis* genome. These fall into three distinct clades (Lacombe et al. 2001). The function and physiology of GLR genes is poorly understood, however, it is already reported that they regulate photomorphogenesis, calcium homeostasis, aluminum toxicity, root elongation hypocotyl elongation, floral stem and vascular bundle development, senescence, carbon and nitrogen metabolism, and salt and cold stress (Gilliham et al. 2006). GLRs are thought to be localized in the plasma membrane. However, *GLR3.4* localized to both plasma membrane and to plastids (Teardo et al. 2011).

It has been shown that some GLRs regulate $Ca^{2+}$ influx to the cytoplasm (Qi et al. 2006, Michard et al. 2011). For instance, glutamate application transiently increases the level of $Ca^{2+}$ in WT but not the *glr3.3* mutant (Qi et al. 2006). Furthermore, depolarization phase of electrical signals was reduced as compared to WT in the *glr3.4* mutant after application of either of the 6 amino acids glutamate, glycine, alanine, serine, asparagine, and cysteine (Stephens et al. 2008). Overexpression of *glr3.2* from radish in *Arabidopsis* increased JA and *VSP1* expression levels (Kang et al. 2006). There is very little known about the subunit composition of GLR channels in plants. Stephans et al. 2008 concluded that GLR channels may form heteromers and each GLR might be activated by a different amino acid

In this chapter we show that wound-associated surface potential changes (WASPs) are essential for long-distance wound signalling in *Arabidopsis* leaves. JA and JA-Ile levels and *VSP2* and *JAZ10* transcripts levels were increased strongly in response to surface potential changes generated by current injection. In addition, several mutants in which WASPs were altered upon wounding were isolated through the reverse screening of T-DNA mutant lines. Furthermore, a double mutant derived from crossing two *glr* single mutants was unable to propagate WASPs from leaf 8 to leaf 13. Consequently, transcript levels for *JAZ10* in this *glr3.3glr3.6* mutant were not increased in leaf 13 when leaf 8 was wounded. Finally, *glr3.3glr3.6* mutant might have a higher susceptibility to *Spodoptera litteralis* than the WT.

## Experimental design:

### Electrode placement and current injection:

Platinum (Pt) wires were inserted into the petiole of leaf 8. The distance between these wires was 1 cm and the distance to the proximal (eP) recording electrode was also 1 cm (Fig. 5. 1). Surface potential activity was recorded on different parts of leaves with electrodes placed in three different positions. The recoding electrodes were placed on the proximal (eP), distal midrib (eD) and lamina (eL) of leaf 8 as shown in Fig. 5. 1. The distance between electrodes eP and eD was 1 cm.

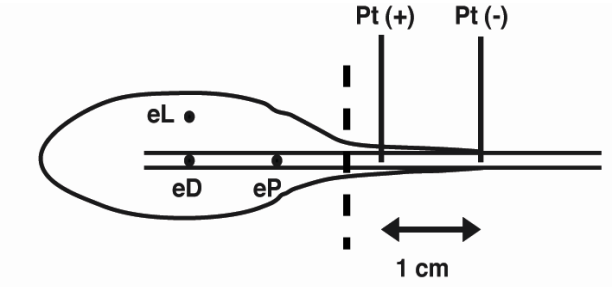


**Figure 5. 1. Experimental design for current injection (ci) into leaf 8.** Placement of Pt wires, proximal electrode (eP), distal electrode (eD) and laminar electrode (eL) for ci experiments. The leaf blade to the left of the dashed line was used for transcript measurements and quantitation of jasmonate levels.

## Results:

### Ionophores and inhibitor infiltration

The expression of JA-inducible genes can be affected by ionophores and ion channel blockers (Schaller and Frasson 2001). We applied several ionophores and ions channel inhibitors to *Arabidopsis* leaves in order to attempt to disrupt surface potential changes after wounding, or to generate surface potential changes without wounding. 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), tetraethylammonium (TEA), orthovanadate, fussicoccin, alamathicin, gramadicin were infiltrated into the lamina or injected into the petioles of leaves in different concentrations. These compounds and ionophores which have effects on the activity of ion channels and proton pumps did not eliminate SPs after wounding in the wounded or connected leaves. However, some of them had minor effects on the amplitude or duration of SPs in the connected leaves. Moreover, the *JAZ10* transcript level was not significantly different in the leaves that had shown SP changes (data not shown).

### Current injection induces surface potentials in *Arabidopsis* leaves

In order to generate electrical signals in *Arabidopsis* leaves and to monitor their effects in plant function, we injected current into leaves and recorded SPs. To do this, we inserted two platinum wires in the petiole of leaf 8 and we injected current the following day. We found that 40 μA current for 10 s could reproducibly generate surface potential changes in leaf 8 (Table 5.1). Surface potentials generated by current injection were detected by all three electrodes (eP, eD and eL) were placed on leaf 8 (Fig 5.2). SPs were first detected in the proximal electrode with an amplitude of -79 ± 12 mV and duration of 75 ± 20s (Table 5.2). The speeds of SPs generated by current injection were calculated between eP and eD. Duration of SPs were decreased with increasing distance from platinum wires.

During current injection a large amplitude (5-9 V) voltage spike was observed at all electrodes placed on leaf 8. This artefact corresponded to the time of starting and finishing

of current injection (Fig. 5.2, 'art'). For further experiments on leaf 8, the leaf lamina was harvested after current injection starting at a distance of 3-4 mm from the platinum wires. The harvested area is indicated with a vertical dashed line in figure 5.1.

**Table 5.1. Optimization of current injection to generate surface potential changes.** The experimental setup is shown in Fig. 5.1. Current was injected into the petiole region and surface potentials were measured with an electrode placed on the midrib in position eD (see Fig. 5.1). n = number of experiments.

| Current (µA) | Time (s) | Surface potential change/n |
|---|---|---|
| 0 | 0 | 0/5 |
| 10 | 1 | 0/3 |
| 10 | 10 | 0/3 |
| 20 | 1 | 1/3 |
| 20 | 10 | 0/3 |
| 40 | 1 | 1/3 |
| 40 | 10 | 5/5 |

**Table 5.2. Characterization of surface potential changes in different parts of leaf 8 generated by current injection.** Current (40 µA, 10 s) was injected into the petiole of leaf 8 (see Fig. 5.1 for electrode placements). eL, laminar electrode; eP, proximal electrode and eD, distal electrode. x/n= the number of experiments in which signal amplitudes exceeded -10 mV / total number of experiments. Values are means ± SD.

| Electrodes | Latency (s) | Amplitude (mV) | Duration (s) | x/n |
|---|---|---|---|---|
| Lamina (eL) | 22 ± 9 | -87 ± 21 | 47 ± 21 | 33 /47 |
| Midrib (eP) | 6 ± 3 | -79 ± 12 | 75 ± 20 | 44/47 |
| midrib (eD) | 15 ± 3 | -80 ± 24 | 52 ± 23 | 43/47 |
| Average | | -81 ± 19 | 59 ± 25 | |

**Note:** An apparent velocity of surface potential displacement of 6.4 ± 1.9 cm/min$^{-1}$ was estimated from recordings at eP and eD.

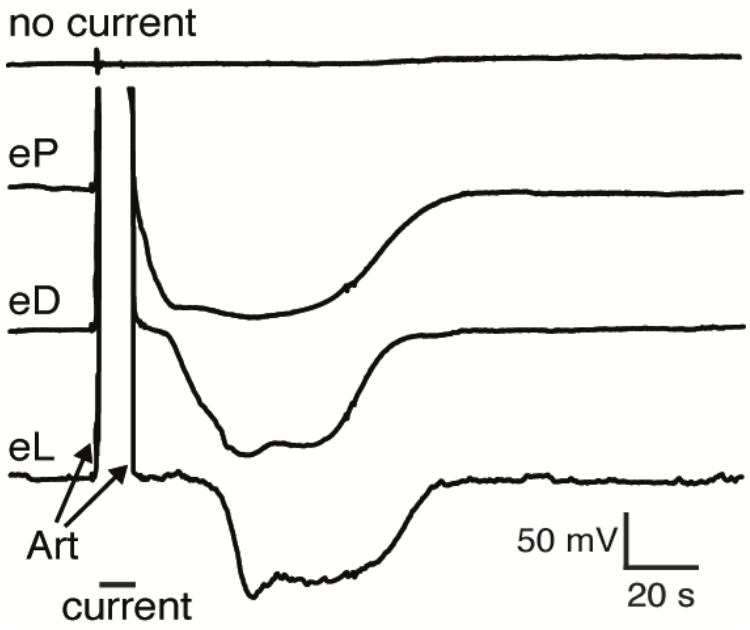


**Figure 5. 2. Current injection (ci) induces surface potentials.** Surface potential generation following ci (40 µA for 10 s). Art = artefacts recorded in the leaf during ci. (10 s, indicated with a bar). See fig. 5.1 for electrode placements. Note that the signal amplitude at eP reaches a maximum before that at eD and eL.

**Current injection induces JA, JA-Ile and JA-responsive genes**

To confirm that SPs generated after ci could induce jasmonate synthesis, we measured the levels of both JA and JA-Ile in leaf 8 at 20 min and 1 h after ci. Figure 5.3 shows that current injection stimulated JA and JA-Ile accumulation at both time points (Fig. 5.3)[2]. Two jasmonate-responsive genes, the regulatory gene *JAZ10* (Yan et al. 2007) and *VEGETATVE STORAGE PROTEIN 2* (*VSP2*), an anti-insect defence gene (Liu et al. 2005), were also monitored after ci. Leaf samples were harvested 1h after ci or wounding for *JAZ10* transcript measurement, and 4h after stimulation or wounding for *VSP2* measurements. Transcript levels for both genes increased strongly in response to ci (Fig. 5.4).

[2] This experiment was performed by Adeline Chauvin, University of Geneva.

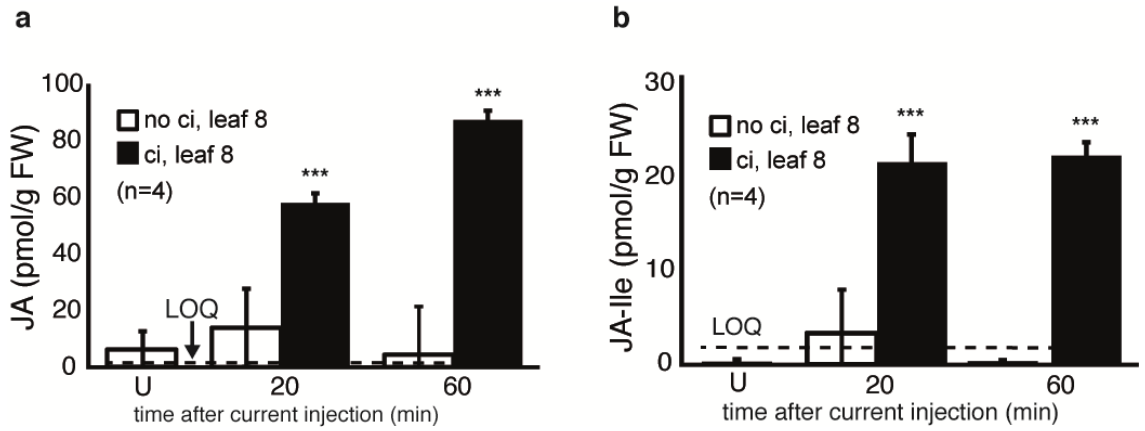


**Figure 5. 3. Current injection (ci) induces jasmonate accumulation. a.** Levels of JA 20 min and 1 h after ci. **b**. Levels of JA-Ile 20 min and 1 h after ci (40 µA, 10s). Limits of quantitation (LOQs) are indicated with dashed lines. U=unwounded. Significant differences from the unwounded WT are indicated, *** = P<0.001. Error bars indicate standard deviation.

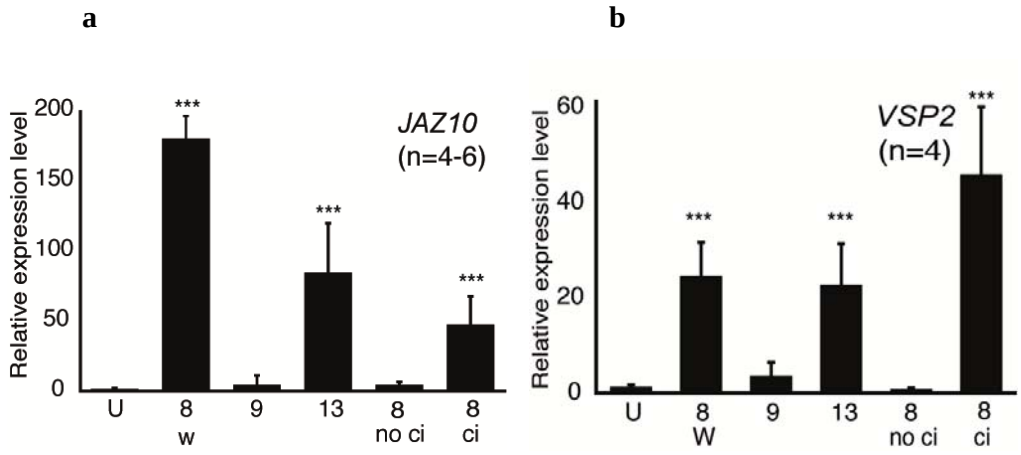


**Figure 5. 4. Levels of *JAZ10* and *VSP2* transcripts in leaves after wounding or ci.** For wounding, leaf 8 was wounded and leaves 8, 9 and 13 were harevested. **a.** Relative expression levels of *JAZ10* in the wounded and current injected leaves (40µA, 10s). In all cases leaves were harvested 1h after wounding or current injection. **b**. Relative expression levels of *VSP2* in the wounded and current injected leaves (40µA, 10s). Leaves were harvested 4h after wounding or current injection. U=unwounded; W=wounded; ci=current injection. Significant differences from the unwounded WT are indicated, *** = *P*<0.001. Error bars show standard deviation.

**Current injection induces GUS reporter plants**

We used the *JAZ10* and *VSP2* promotors to control GUS expression in *Arabidopsis*. *VSP2:GUSPlus* plants were generated by Stephanie Stoltz and the *JAZ10:GUSPlus* plants were generated by Dr. Ivan Acosta. These transgenic *Arabidopsis* plants were injected with 40 µA current for 10s. Leaf 8 was also wounded as control for current injection. Figure 5.5 shows the expression pattern of *VSP2:GUSPlus* 4 h after current injection or wounding. We did not detect GUS activity in the non-current injected leaves or in the unwounded leaves. But there was a strong activation of *VSP2:GUSPlus* in the wounded and ci leaves. Figure 5.6 shows the pattern of *JAZ10:GUSPlus* expression 4 h after wounding or current injection. The unwounded plant and the no ci plant (control) showed no *JAZ10* expression. Similar to *VSP2:GUSPlus*, a strong activation of *JAZ10:GUSPlus* was detected in the wounded and ci leaves.

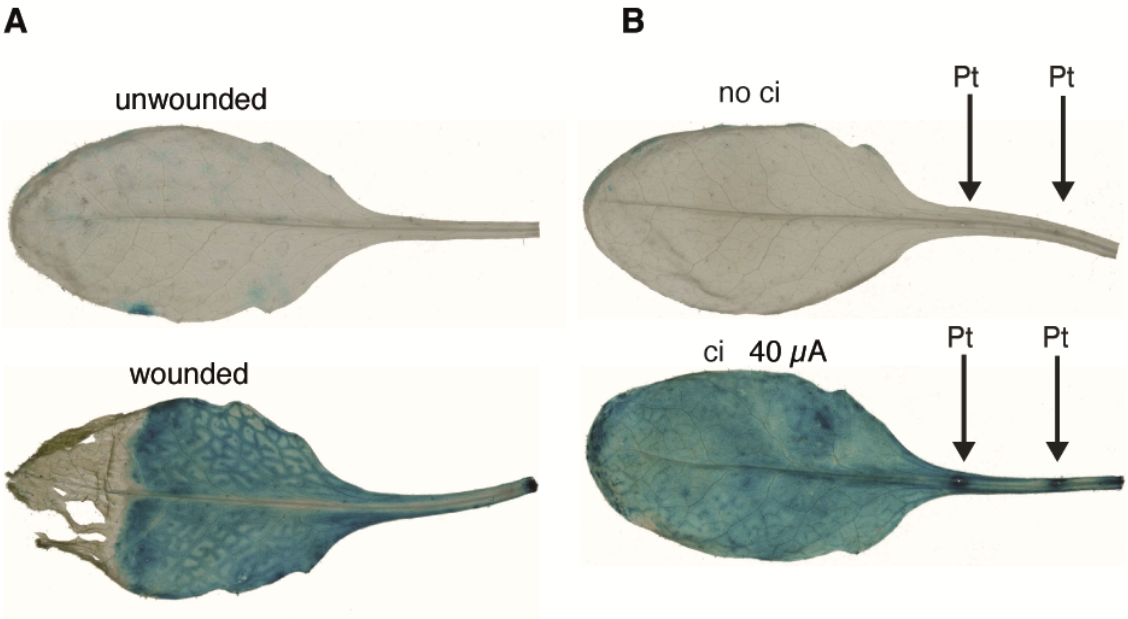


**Figure 5. 5. Histochemical staining of GUS in *Arabidopsis* (Col-0) plants under control of *VSP2*-GUSplus. a**. Expression pattern in *VSP2:GUSPlus* reporter line in leaf 8 from an unwounded *Arabidopsis* plant, and 4h after wounding. **b.** *VSP2:GUSPlus* activity in leaf 8 of control plants (no ci) and 4h after ci.

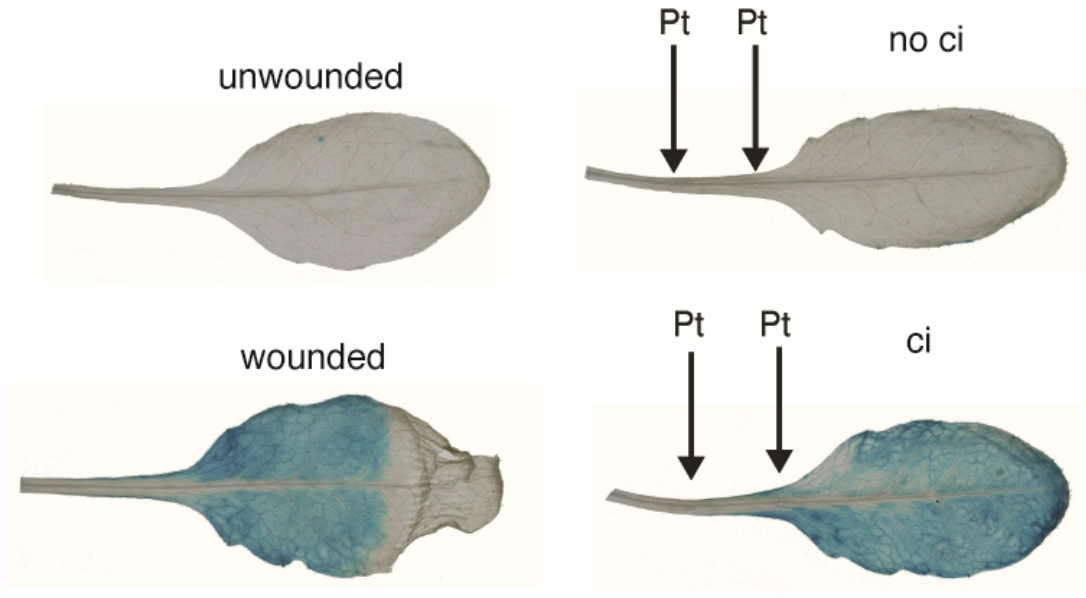


**Figure 5. 6. Histochemical GUS staining of *Arabidopsis* (Col-0) plants under control of *JAZ10:GUSplus*. a**. Expression pattern of a *JAZ10:GUSPlus* reporter line in leaf 8 from an unwounded *Arabidopsis* plant, and 4h after wounding. **b**. *JAZ10:GUSPlus* activity in leaf 8 of control plants (no ci) and 4h after ci.

**No cell death detected after current injection**

High electrical currents can cause cell and organ damage. In order to ensure that the surface potentials generated by ci were not due to burned cells or to heat generated during ci, we used two approaches; first, plants expressing β-glucuronidase under the promoter of small heat shock protein (HSP) 18.2 (At5g59720) (Takahashi et al. 1992) were used to control for leaf heating during current injections. The positive control was treated for 1.5 h at 37 °C and then rested at 22 °C for 7.5 h. The current injected plants were maintained throughout at 22 °C for 7.5 h after current injection. One leaf of each control plant and the current-injected leaves were harvested for GUS staining for 15 h at 37 °C and the tissue was then destained in 70% ethanol (Jefferson et al 1987). Figure 5. 7 show that current injection did not activate the heat shock gene.

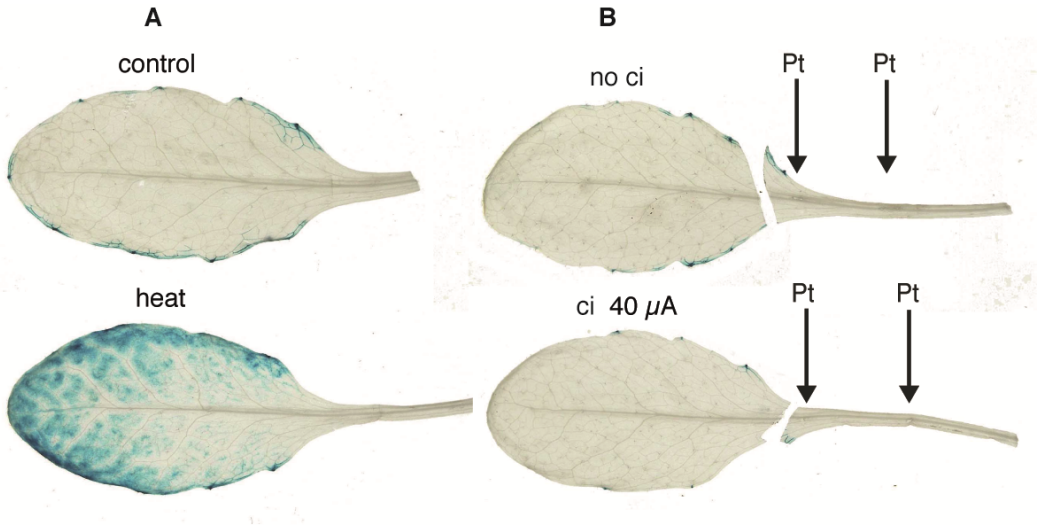


**Figure 5.7. Histochemical GUS staining of Arabidopsis (Col-0) plants under control of *HSP18.2: GUS*. a**. Expression pattern of a *HSP18.2: GUS* reporter line in leaf 8 from an unwounded *Arabidopsis* plant, and in leaf 8 4h after heat treatment. **b**. *HSP18.2: GUS* activity in leaf 8 of control plants (no ci) and 4h after ci.

Second, we used trypan blue staining for detecting cell death after current injection. No current injected (but harbouring Pt wires as a control), current injected or unwounded-leaves were stained with trypan blue 1h after stimulation. Figure 5.8 shows that there was a slight blue colour throughout the unwounded leaf 8. However, the midrib and petiole of unwounded leaf 8 stained blue with respect to lamina. In the 'no ci' leaves into which platinum wires had been inserted, a few cells around the wires showed a strong blue colour which indicated cell death due to the insertion of the wires (Fig 5.8b). A similar pattern of trypan blue staining was detected in the current-injected leaves (Fig 5.8c).

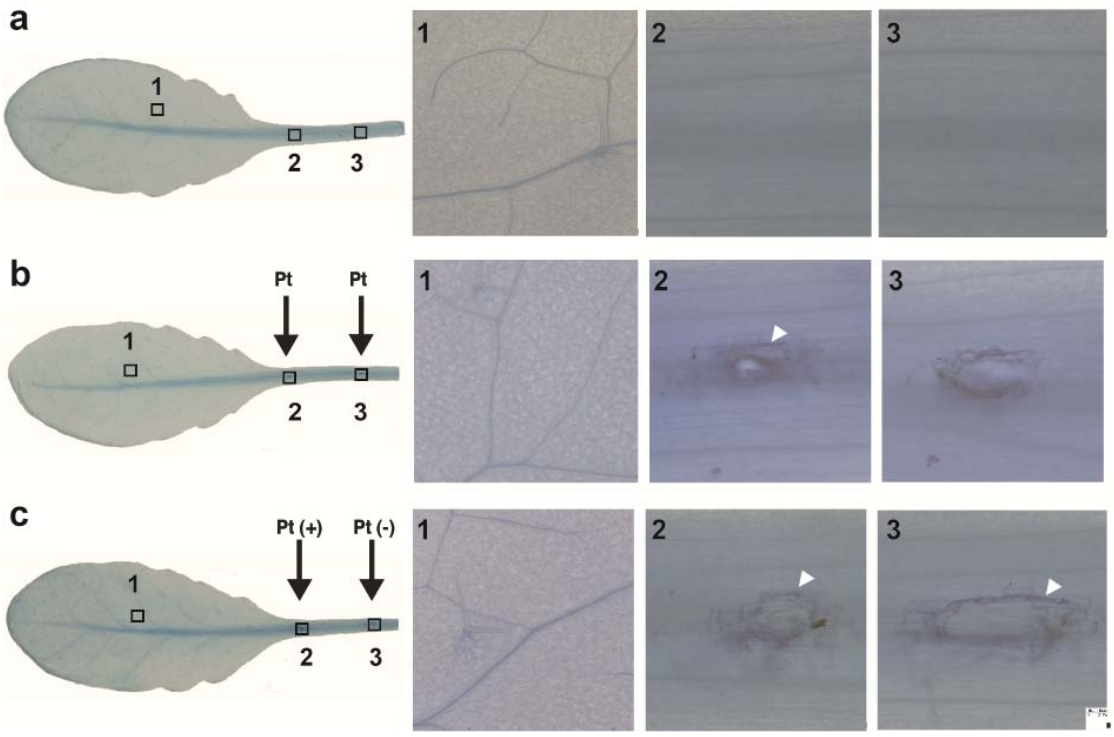


**Figure 5.8. Current injection does not cause cell death as detected by trypan blue (TB) staining. A.** Undamaged leaf. **B.** Leaf in which Pt wires were inserted but no current was injected. **C.** current injected leaf. Lefthand side: overview of leaves with placement of Pt wire electrodes. Righthand side: enlargements showing damage where Pt wires were inserted. Leaves were harvested 1h after current injection. Note that a small area of cells was killed around the Pt wires (see arrows). Current injection did not cause addition TB staining.

### Current injection does not induce *JAZ10* in *coi1-1*

*The coronatine-insensitive1 (COI1)* gene plays a central role in the JA signalling pathway (Xie et al. 1998). To test the hypothesis that SPs generated by current injection were independent of JA perception, we injected current into the homozygote *coi1-1* mutant. Platinum wires were first inserted in the petiole of leaf 8 and recording electrodes were placed in positions eP and eD on the midrib and lamina of leaf 8 (Fig. 5.1). 40 µA current injected over 10 s generated surface potentials that were similar to those in the WT (Fig. 5.9a). The 'no ci' and 'ci' leaves were harvested 1 h after current injection to measure *JAZ10* induction. Unwounded and wounded leaf 8 were harvested 1h after wounding this

leaf in both the WT and *coi1-1*. Figure 5.9b shows that *JAZ10* expression was not induced by ci in the *coi1-1* mutant as the JAZ10 expression was no significantly differenct between 'no ci' and 'ci'. The expression of *JAZ10* was slightly increased in the wounded *coi1-1* leaf with respect to unwounded *coi1-1* leaf. However, the expression level of *JAZ10* in the wounded leaf 8 of *coi1-1* was still much lower than in unwounded WT.

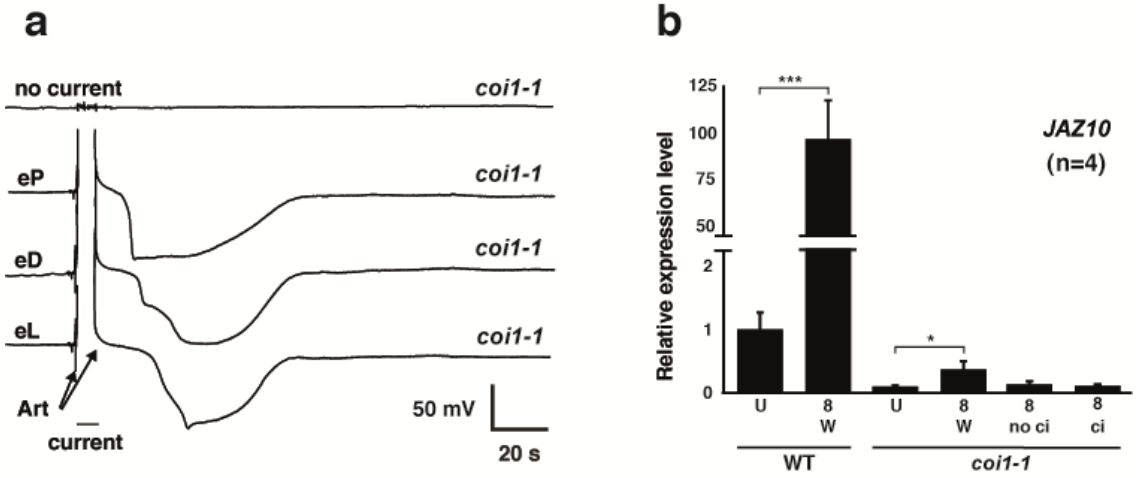


**Figure 5. 9. Current injection does not induce *JAZ10* transcript accumulation in the *coronatine-insensitive 1-1 (coi1-1)* jasmonate perception mutant.** a. Surface potential changes following ci (40 µA for 10 s) in the *coi1-1* mutant. Art = artefacts recorded in the leaf during ci (10 s, indicated with a bar). Note that the signal amplitude at eP reaches a maximum before that at eD and eL. For electrode placements see Fig. 5.1. **b**. Relative expression levels of *JAZ10* in the wounded WT and in the *coi1-1* mutant that had been wounded or into which current (40µA, 10s) had been injected. In all cases leaves were harvested 1h after wounding or current injection. U=unwounded; W=wounded; ci=current injection. Significant differences from the unwounded WT are indicated, * = $P<0.05$, *** = $P<0.001$.

### WASPs move slower in the *fou2* mutant

A gain-of-function mutant, *fatty acid oxygenation upregulated 2 (fou2)* in the two pore channel 1 *(TPC1)* gene has higher amounts of JA in the resting state (Bonaventure et al. 2007b). This plant therefore resembles a wounded plant. Having higher JA levels and a mutation in an ion channel made this mutant a useful tool for the study of WASPs after

wounding. *fou2* has small and epinasic leaves with shorter petioles and more anthocyanin than the Col-0 WT. Epinastic leaves in *fou2* appear after three weeks of growth, about the time of emergence of leaves 7 to 9. Surface recordings were made on the wounded leaf 8 and the parastichous leaf 13 in *fou2*. We also tested the effects of different leaf morphology in *fou2*. In order to assess the possible effect of epinastic leaves on WASP generation and *JAZ10* transcript level, we recorded WASPs in leaves 8 and 13 of *fou2* from both epinastic and normal leaves. Three possible combinations of epinastic and regular leaves 8 and 13 were investigated: 1) leaf 8 and 13 were both epinastic. 2) Leaf 8 and 13 were both normal. 3) Leaf 8 was normal and leaf 13 is epinastic. Furthermore, WASP generation and *JAZ10* expression levels were also analysed in the *fou2* heterozygote. *FOU2/fou2* has a phenotype between Col-0 and *fou2* and leaf epinasty in these plants develops later than in *fou2*, therefore the number of epinastic leaves in the heterozygote is lower than in the *fou2* homozygote. *FOU2/fou2* was used when leaves 8 and 13 had normal phenotype and leaf 8 was normal and leaf 13 was epinastic. Figure 5.10 shows the phenotype of the *fou2* homozygote and heterozygotes relative to the WT.

In *fou2* and *FOU2/fou2*, the amplitudes and durations of WASPs were similar to WT for leaves 8 and 13 after wounding leaf 8 (data not shown). However, in *fou2*, the speed of WASP movement within the wounded leaf was 2.6 ± 0.6 cm $min^{-1}$and from wounded leaf to connected leaf 13 was 2.1 ± 0.5 cm $min^{-1}$ . This was 2-3 times slower than in WT. The velocities of WASPs movement in FOU2/fou2 in the wounded leaf 8 and in the connected leaf 13 were similar to those in WT (data not shown). Table 5.3. shows the characteristics of wound-activated surface potential changes in *fou2* leaves.

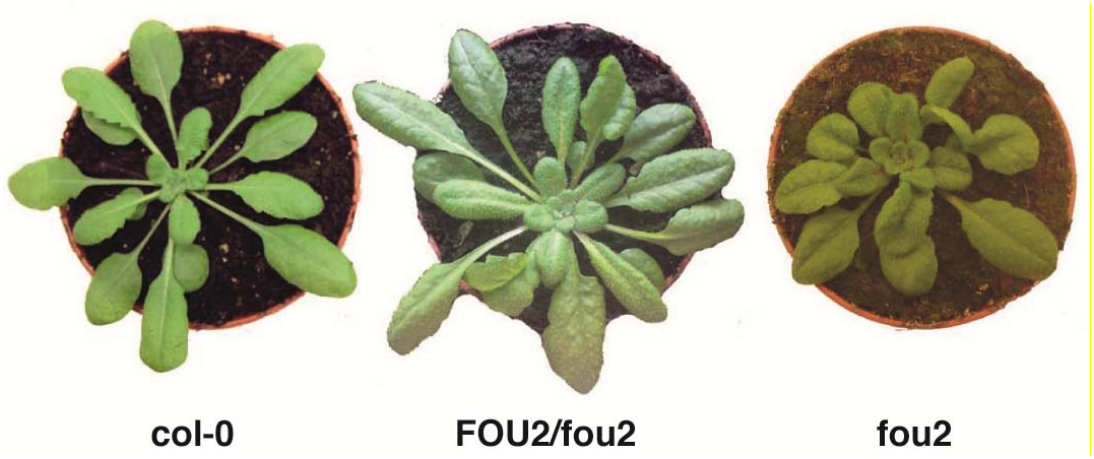


**Figure 5. 10. Rosette morphology of WT, *FOU2/fou2* (heterozygote) and *fou2*.** Notice that *fou2* has more epinastic leaves than *fou2* heterozygote. WT does not have epinastic leaves.

**Table 5.3. Characteristics of wound-activated surface potential changes (WASPs) in *fou2* leaves. a**. WASPs in the wounded leaf. **b**. Signals recorded in leaf 13 after wounding leaf 8. x/n= the number of experiments in which surface potential amplitude changes exceeded -10 mV / total number of experiments. See Figs. 3.1 for details of electrode placement and measurement of signal parameters. Velocity was calculated from the wounding site to the respective electrode. Values are means ± SD.

| **Electrodes** | **Latency (s)** | **Amplitude (mV)** | **Duration (s)** | **Velocity (cm/min)** | **x/n** |
|---|---|---|---|---|---|
| **a. wounded leaf 8** | | | | | |
| **midrib (e2)** | 24 ± 5.8 | -49 ± 26 | 125 ± 58 | 2.6 ± 0.6 | 5/5 |
| **Petiole (e3)** | 47 ± 7.5 | -50 ± 17 | 167 ± 20 | 2.6 ± 0.6 | 5/5 |
| **Average** | | -49 ± 21 | 146 ± 47 | 2.6 ± 0.6 | |
| **b. From leaf 8 wounded to leaf 13** | | | | | |
| **Petiole (e2)** | 128 ± 30 | -22 ± 19 | 83 ± 23 | 2.1 ± 0.5 | 4/5 |

### *JAZ10* expression levels in *fou2*

In order to assess *JAZ10* expression levels, unwounded leaf 8 and leaves 8, 9 and 13 of the WT and *fou2* mutants were harvested at 1 h after wounding. Figure 5.11 shows the relative expression level of *JAZ10* in the WT, *FOU2/fou2* (heterozygote) and *fou2/fou2*. The expression level of *JAZ10* was found to be significantly increased 5-fold in both the epinastic and the normal leaves of unwounded *fou2* compared to the WT and *FOU2/fou2*. The level of *JAZ10* in the wounded leaf 8 was found to be similar between WT and the *fou2* mutant. Interestingly, in *fou2,* the expression level of *JAZ10* in leaf 13 was independent of epinastic or normal shape of the leaf and the *JAZ10* expression level was lower in comparison to WT or *FOU2/fou2*. *FOU2/fou2* showed similar behaviour of *JAZ10* induction to the WT in unwounded leaves, in wounded leaf 8 and in leaf 13 when leaf 8 was wounded.

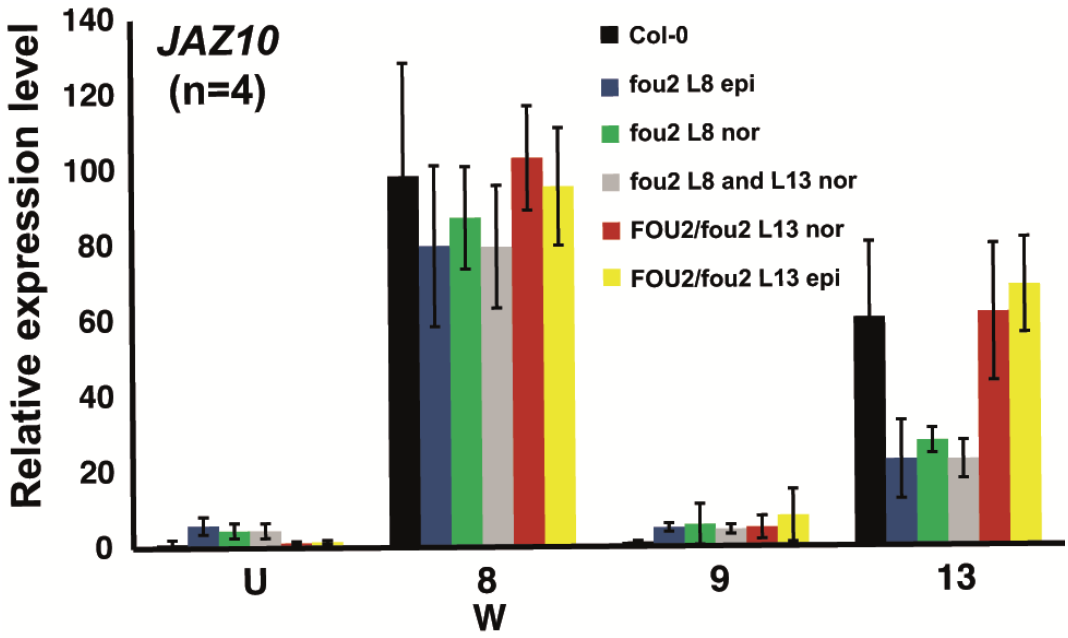


**Figure 5. 11. *JAZ10* expression level in *fou2*.** The expression level of *JAZ10* 1h after wounding of leaf 8 in the WT, *FOU2/fou2* (heterozygote) and *fou2*.U= unwounded, W= wounded, epi=epinastic and nor= normal. Error bars show standard deviation for 4 independent replicates.

## Reverse genetic screening of T-DNA insertion lines

In order to identify genes that are required for the generation and propagation of electrical potentials in plants, we used a reverse genetic approach based on monitoring SPs after wounding of leaf 8. WASPs were recorded on a total of 32 T-DNA insertion lines annotated for ion channels, proton pumps and aquaporins. Prior to experiments, the homozygosity of these T-DNA lines was confirmed by PCR. Two recording electrodes were placed on the wounded leaf 8 (Fig. 3.1) and two other electrodes were placed on the petioles (1cm from plant centre) one each on leaves 9 and 13 to monitor SPs events in the distal leaves. Table 5.4 presents WASP durations and amplitudes from the screening of the homozygote T-DNA insertion lines.

**Table 5.4 . Characterisation of wound-activated surface potential changes (WASPs) in homozygous T-DNA insertion lines.** In all experiments leaf 8 was wounded and the surface potential was monitored in leaf 8 and distal leaf 13. For leaf 8, an electrode was placed 2 cm from the leaf apex wound (Fig. 3.1, position e2). All measurements for leaf 13 were from electrodes placed on the petiole 1 cm from the centre of the rosette (position e3' in Fig. 4.7). n = the number of experiments. Values are means ± SD. Mutants displaying reduced WASP durations of <60s in leaf 8 or <40s in leaf 13 are highlighted.

| Locus | annotation | Stock name | Leaf 8 | | Leaf 13 | | |
|---|---|---|---|---|---|---|---|
| | | | Amplitude | Duration | Amplitude | Duration | n |
| - | WT | Col-0 | -76±11 | 163±30 | -51±9 | 78±20 | 33 |
| At3g04110 | glr1.1 | salk_057748 | -78±12 | 459±42 | -46±5 | 63±18 | 6 |
| At3g07520 | glr1.4 | salk_129955 | -81±23 | 149±66 | -54±14 | 81±9 | 5 |
| At2g24710 | glr2.3 | salk_113260 | -78±20 | 140±12 | -32±11 | 79±8 | 7 |
| At2g29120 | glr2.7 | salk_121990 | -96±12 | 213±34 | -43±10 | 74±11 | 5 |
| At2g29110 | glr2.8 | salk_111695 | -84±17 | 272±32 | -55±8 | 92±15 | 5 |
| At2g29100 | glr2.9 | salk_125496 | -59±14 | 219±29 | -43±4 | 103±23 | 4 |
| At2g17260 | glr3.1 | salk_063873 | -81±18 | 110±25 | -48±13 | 10±8 | 9 |
| At4g35290 | glr3.2 | salk_150710 | -87±7 | 37±6 | -46±11 | 34±4 | 8 |
| At4g35290 | glr3.2 | salk_133700 | -86±10 | 99±26 | -29±14 | 33±23 | 8 |
| At1g42540 | glr3.3 | salk_077608 | -71±15 | 36±6 | -40±18 | 23±5 | 9 |
| At1g42540 | glr3.3 | salk_099757 | -91±11 | 51±10 | -47±10 | 36±18 | 9 |
| At1g05200 | glr3.4 | salk_079842 | -75±16 | 70±9 | -36±10 | 59±16 | 7 |
| At2g32390 | glr3.5 | salk_035264 | -83±9 | 94±15 | -58±16 | 63±13 | 7 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At3g51480 | glr3.6 | salk_091801 | -73±12 | 116±18 | -44±6 | 16±7 | 9 |
| At2g46450 | cngc12 | salk_092622 | -69±16 | 299±31 | -36±4 | 87±14 | 6 |
| At5g14870 | cngc18 | sail_191_H04 | -87±6 | 430±14 | -46±6 | 74±12 | 6 |
| At3g17700 | cngc20 | salk_129133 | -70±8 | >400 | -40±10 | 88±11 | 4 |
| At3g27170 | clc-b | salk_027349 | -91± 3 | 309±11 | -42±12 | 55±6 | 5 |
| At4g35440 | clc-e | salk_142812 | -86±18 | 148±31 | -50±9 | 48±4 | 6 |
| At1g04690 | kab1 | salk_056819 | -83±15 | 156±25 | -47±10 | 78±27 | 7 |
| At2g25600 | akt6 | salk_136050 | -84±16 | 144±24 | -52±10 | 93±25 | 6 |
| At3g02850 | skor | salk_097435 | -71±16 | 209±56 | -47±12 | 74±20 | 7 |
| At5g55630 | tpk1 | salk_146903 | -81±11 | 125±12 | -33±12 | 45±35 | 8 |
| At4g18160 | tpk3 | salk_049137 | -82±14 | 117±13 | -51±12 | 48±9 | 9 |
| At5g10220 | annat6 | salk_043207 | -81±12 | 146±21 | -39±12 | 64±21 | 7 |
| At1g59870 | pen3 | salk_000578 | -91±10 | 147±25 | -53±12 | 80±8 | 7 |
| At4g11150 | vha-e1 | salk_019365 | -81±7 | >400 | -42±4 | 81±15 | 5 |
| At3g01390 | vha-g | salk_087613 | -87±10 | 232±76 | -71±9 | 87±18 | 5 |
| At2g21410 | vha-a2 | salk_142642 | -79±9 | 177±17 | -53±9 | 118±19 | 7 |
| At2g18960 | aha1 | salk_118350 | -63±20 | 109±31 | -36±19 | 70±28 | 9 |
| At4g30190 | aha2 | salk_073730 | -76±19 | 118±38 | -46±9 | 78±35 | 8 |
| At5g57350 | aha3 | sail_810_08 | 71±17 | 127±38 | -58±10 | 79±19 | 7 |
| At5g62670 | aha11 | salk_152723 | -77±15 | 101±33 | -53±14 | 87±20 | 9 |
| At5g12080 | msl10 | salk_076254 | -81±12 | 243±44 | -52±7 | 28±10 | 7 |
| At5g47910 | rbohD | salk-070610 | -92±9 | 98±36 | -53±12 | 61±13 | 9 |
| At2g18960<br>At5g57350 | aha1aha3 | salk_118350<br>sail_810_08 | -79±13 | 202±47 | -41±11 | 126±29 | 7 |
| At4g30190<br>At5g57350 | aha2aha3 | salk_073730<br>sail_810_08 | -82±10 | 128±33 | -44±15 | 72±30 | 8 |
| At4g35290<br>At1g42540 | glr3.3glr3.6 | salk_099757<br>salk_091801 | -55±18 | 8.5±1.7 | 0±0 | 0±0 | 11 |

## Glutamate Receptor-Like genes mediate WASPs in *Arabidopsis*

Twenty glutamate receptor-like (*GLR*) genes are predicted in *Arabidopsis* genome and they fall into three clades (Lacombe et al. 2001). In this study reverse genetic screening showed that the third clade of *GLRs* plays an important role in surface potential generation/propagation after wounding *Arabidopsis* rosette. *glr3.1, glr3.2* (two alleles), *glr.3.3* (two alleles) and *glr3.6* showed lower WASP duration in the leaf 8 or 13 or both

than did the WT (Fig. 5.13 and table 5.4). For instance, the durations of WASPs in leaf 13 when leaf 8 was wounded were decreased in *glr3.1* and *glr3.6* to 10±8 s and 16±7 s respectively. For *glr3.2* (Salk_150710) and *glr3.3* (two alleles), the duration of WASPs was decreased in both of wounded leaf 8 and leaf 13. In order to assess the effects of WASP duration on *JAZ10* expression, leaves 8, 9 and 13 were harvested 1 h after wounding leaf 8. Fig. 5.12 shows that the expression level of *JAZ10* in leaf 13 for both *glr3.1* and *glr3.2a* (Salk_150710) single mutants were significantly decreased with respect to WT.

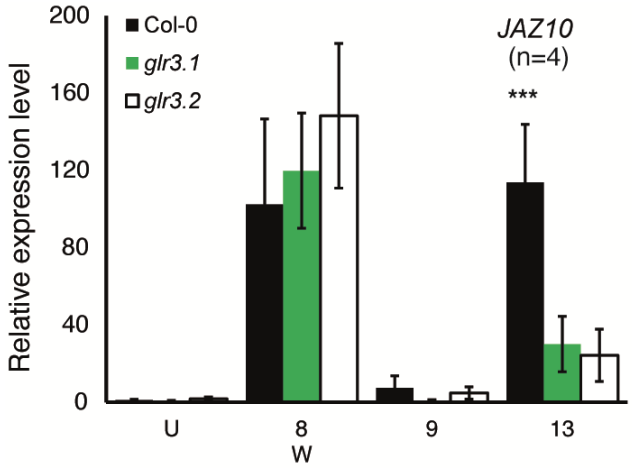


**Figure 5.12. *JAZ10* expression after wounding leaf 8 in the WT, *glr3.1* (Salk_salk_063873) and *glr3.2* (Salk_150710)**. For RNA samples were collected 1 h after stimulation and levels of *JAZ10* transcripts were normalized to the resting WT control.

WASPs and *JAZ10* expression levels were not fully suppressed in *glr* single mutants. Therefore, we attempted to make double mutants in all combinations of *glr* single mutants that had shown reduced WASP durations in leaf 8 or 13 after wounding leaf 8. In the first attempt, I could only produce a *glr3.3a glr3.6a* double mutant. The process of making the other of double mutants is in progress. *glr3.3* showed a decrease of WASP duration in both leaf 8 and leaf 13 and *glr3.6* showed a decrease duration in leaf 13 (Table 5.4). The *glr3.3glr3.6* double mutant showed reduced WASP durations in leaf 8 and prevented WASP production in leaf 13 after wounding of leaf 8 (Fig. 5. 13).

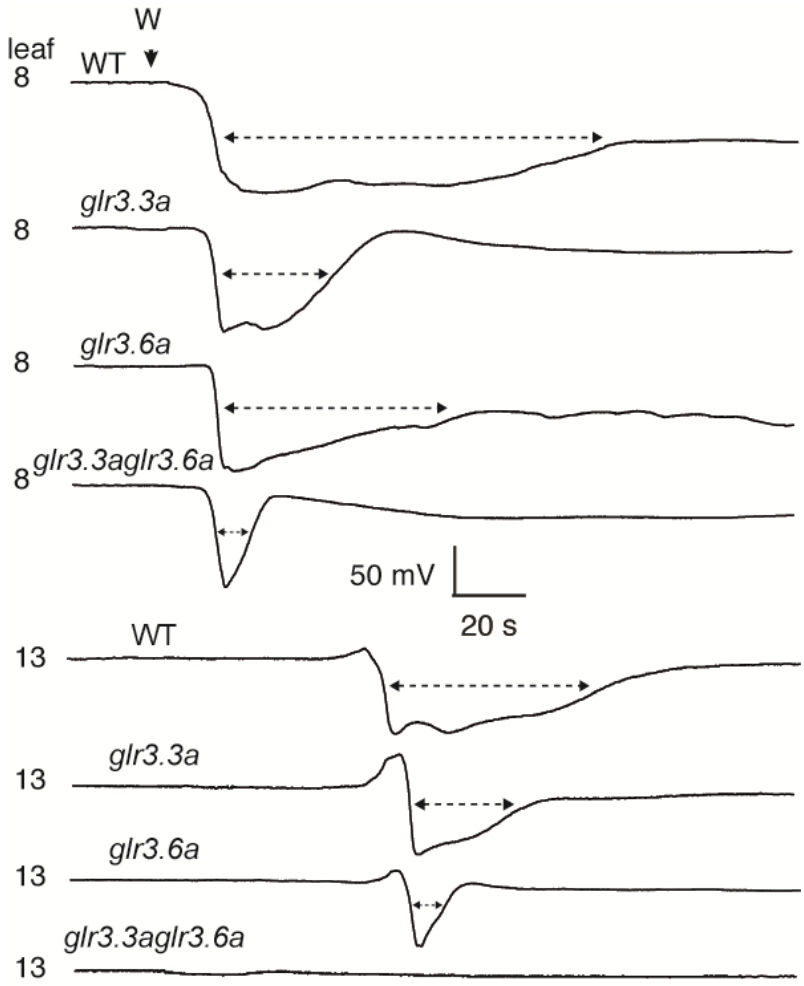


**Figure 5. 13. *glr* mutants reduce the duration of WASPs. A**. Typical WASPs from the wounded leaf 8 of the WT, *glr3.3a* (At1g42540, Salk line 099757), *glr3.6a* (At3g51480, Salk line 091801) and the double mutant *glr3.3a glr3.6a*. **B.** Surface potential recordings from leaf 13 of the same genotypes wounded on leaf 8. For leaf 8, an electrode was placed 2 cm from the leaf apex wound (Fig. 3.1, position e2). All measurements for leaf 13 were from electrodes placed on the petiole 1 cm from the centre of the rosette (position e3' in Fig. 4.7).

Leaves 8, 9 and 13 were harvested 1 h after wounding leaf 8 to measure *JAZ10* transcript level. The expression level of *JAZ10* in the unwounded and wounded leaf 8 were similar among the WT, the *glr3.3a* and *glr3.6a* single mutants and the double mutant *glr3.3a glr3.6a*. Interestingly, the transcript level of *JAZ10* in leaf 13 when leaf 8 was wounded was reduced for the single mutants of *glr3.3* and *glr3.6* and almost abolished for the double mutant (Fig. 5.14).

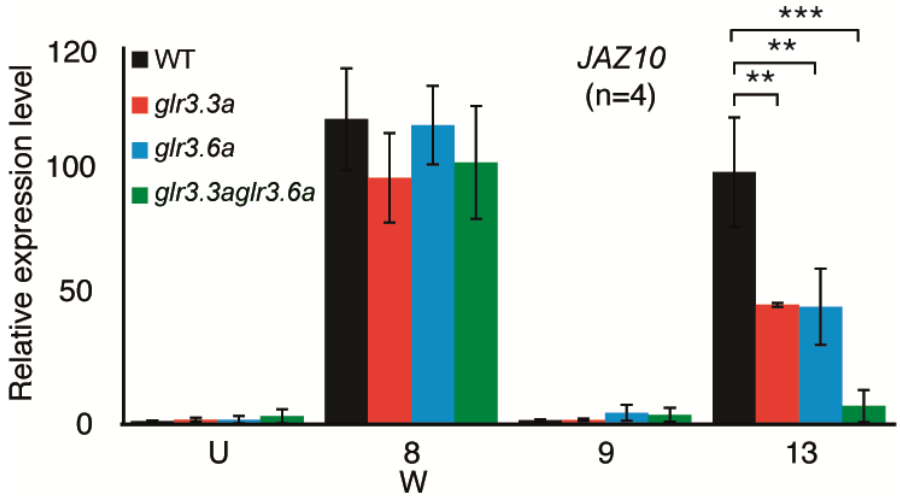


**Figure 5.14. *JAZ10* expression after wounding leaf 8 in the WT, *glr* single mutants, and the *glr3.3a glr3.6a* double mutant.** For RNA samples were collected 1 h after stimulation and levels of *JAZ10* transcripts were normalized to the resting WT control. ** = $P<0.01$, *** = $P<0.001$.

### The *glr3.3b* phenotype

No different phenotypes were observed for *glr3.1, glr3.2, glr3.3a, glr3.6a or glr3.3a glr3.6a* double mutants in comparison with the WT. However, relative to these plants, the petioles of the *glr3.3b* (Salk_077608) were longer and the leaves were curled in short day growth condition (Fig. 5.15). We assume that this may be due to secondary mutations in this mutant. In order to test the level of *JAZ10* transcript leaves 8, 9 and 13 were harvested 1 h after wounding leaf 8. Figure 5.16 shows that the expression levels of *JAZ10* in unwounded and wounded leaf 8 were not significantly different between *glr3.3b* and the WT. But, as we predicted, the level of *JAZ10* transcript in leaf 13 were between those of leaf 8 and unwounded leaves. The expression levels of *JAZ10* increased 20-fold in leaf 13 of *glr3.3b* which is 4 times lower than leaf 13 in WT (Fig. 5. 16).


**Figure 5.15. Rosette morphology of *glr3.3b* mutant (salk_077608) of 5 week old plant**. Plants were grown in short day conditions.

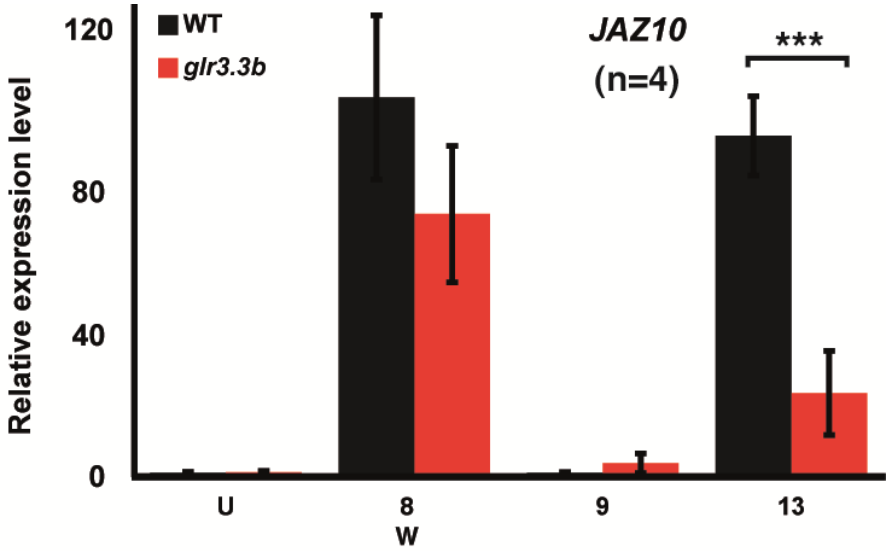


**Figure 5.16. A second *glr3.3* allele reduces wound-stimulated *JAZ10* expression**. Plants (WT or *glr3.3* mutant salk_077608, *glr3.3b*) were wounded on leaf 8 (W). One h later leaves 8 and 13 were harvested and *JAZ10* expression measured by PCR. ***=$P<0.001$ (n=4 ± SD).

**Current injection did not generate SPs in a *glr3.3a glr3.6a* double mutant**

40 µA of current for 10 s were applied to leaf 8 through platinum wires that were inserted into the petiole of this leaf (see Fig. 5.1 for electrode placement). Figure 5.17a shows that, for this genotype, there were no SP changes detected after current injection (n=11). The artefact change in amplitude was however detected in the *glr3.3a glr3.6a* double mutant as it was in the WT. Then, current injected and non-current injected leaves were harvested 1 h later to measure *JAZ10* transcript levels. Figure 5.17b shows that there was a slight induction (5-fold) of *JAZ10* transcript level after ci in the double mutant which is substantially lower than in WT in which *JAZ10* levels were increased 38-fold.

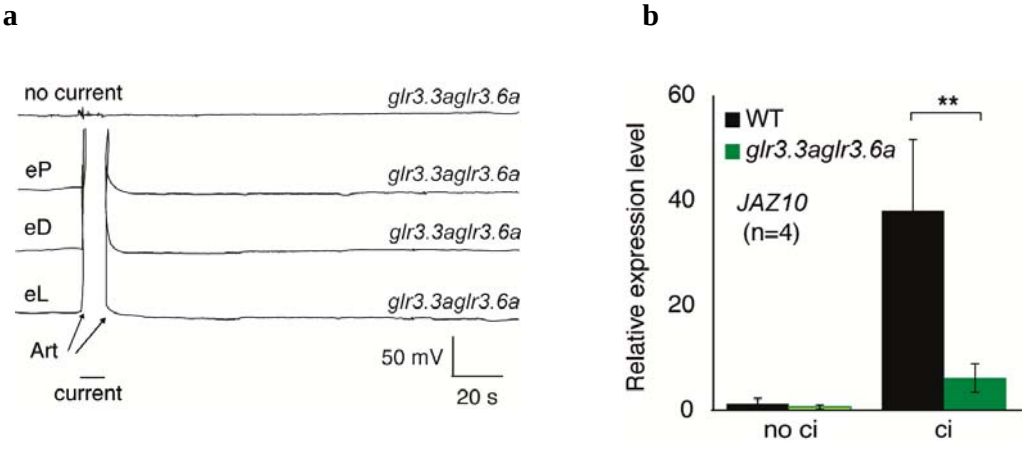


**Figure 5. 17. Electrical activity and *JAZ10* expression in leaf 8 of *glr33.a glr3.6a* double mutant after current injection**. **a**. typical Surface potential changes following ci (40 µA for 10 s) in the *glr3.3a glr3.6a* double mutant. **b.** *JAZ10* expression after current injection (ci) in the WT and the *glr3.3a glr3.6a* double mutant. For RNA samples were collected 1 h after stimulation and levels of *JAZ10* transcripts were normalized to the resting WT control. For electrodes placement see figure 5. Art = artefacts recorded in the leaf during ci. (10 s, indicated with a bar). X. ** = $p<0.01$. Error bars indicate standard deviation.

***glr3.3a glr3.6a* shows increased susceptibility to a chewing herbivore**

Four 2nd instar *S. littoralis* larvae were placed on the rosette leaves of the *glr3.3a glr3.6a* double mutant and WT of 4-week old *Arabidopsis*. Weight gain of the larvae was

measured 7 days later. The weight of *S. littoralis* caterpillars fed on *glr3.3a glr3.6a* double mutant leaves was increased by 43% ($P < 0.05$) in comparison to WT (Fig. 5.18). This was a single experiment and therefore needs repetition.

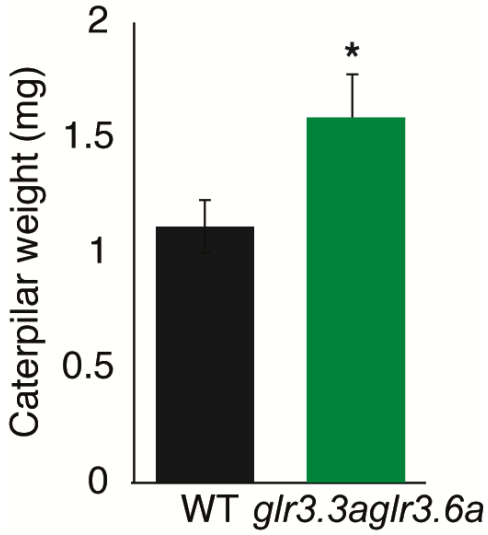


**Figure 5. 18. *Spodoptera littoralis* larval weight after feeding on WT, *glr3.3a glr3.6a*.** Four freshly hatched *S. littoralis* larvae were placed on WT and *glr3.3a glr3.6a* and weight after 7 days of feeding. Error bars shows standard deviation for 4 biological replicates. * $P<0.05$.

## Discussion

This study, is as far as we aware, the first to identify genes that regulate wound-induced electrical activity in *Arabidopsis* were identified. In addition, we demonstrated that surface potential changes (SPs) generated by current injection could activate many regulatory genes in the JA pathway. The activation of defence pathways in undamaged parts of plants is key to defending against further attacks. Therefore, having a long distance signalling pathway that is able to transmit signals between leaves is necessary. It has already been suggested that long distance wound signalling could be chemical, hydraulic or electrical (Malone and Stankovic 1991, Malone 1992, Wildon et al. 1992, Rhodes et al. 1996, Howe 2004). Several lines of evidence and chiefly the speed of the putative leaf-to-leaf signal which are estimated to be in the order of 4-8 cm $min^{-1}$ (Glauser et al. 2009, Chauvin et al. 2013) led us to test the hypothesis that electrical activity is linked to jasmonate signalling. Two of the approaches to find causal links between electrical signals and their effects on wound response gene expression are: to generate electrical activity without wounding or to find mutants in which wounding does not generate electrical activity. In plant electrophysiology, most studies have focused on pharmaceutical approaches in order to alter SPs.

### JA pathway activation by surface potential changes

Current injection was shown previously to activate JA and abscisic acid accumulation and *Pin2* expression in tomato leaves (Herde et al. 1996). Low voltage also led to movement of petioles and pinnae in *Mimosa pudica* (Yao et al. 2008, Volkov et al. 2010). Favre et al. (2007) showed that applying electrical current into *Arabidopsis* leaves can generate electrical activity in other parts of leaves distal to the site of current injection. We found that low current (40 µA for 10s) was able to trigger reproducible SP changes in parts of the leaf distal to the platinum current injection wires. SPs through ci were not due to heat produced during ci or to cell death. This was confirmed by the absence of activation of a GUS reporter gene in plants that expressed this gene under the promoter of a small heat

shock protein. In addition, only a few cells were damaged around the platinum wire implantation site as detected by trypan blue staining. The amplitude changes with a mean of -81±19 mV and duration a mean of 59±25 s of SP due to ci were similar to those generated in leaf 13 when leaf 8 was wounded. From these data we estimated the apparent velocity of the surface potentials resulting from ci to be 6.4±1.9 cm $min^{-1}$. This was close to an average velocity of 7 cm $min^{-1}$ that has been observed after ci in *Arabidopsis* (Favre and Agosti 2007) and was similar to unrelated signal velocity estimats from our laboratory (Glauser et al. 2009, Chauvin et al. 2013).

The most striking result to emerge from current injection is the activation of the JA pathway as evidenced by the measured accumulation of JA and JA-Ile in current injected leaves. In addition, the levels of *JAZ10* and *VSP2* transcripts were increased after ci. Plasma membrane depolarization is an early step in the signalling defence pathway (Maffei et al. 2004, Stahlberg et al. 2006, Fromm and Lautner 2007). Plasma membrane depolarization generated by current injection may induce the cascades that lead to accumulation of JA. This result is consistent with Herde et al. (1996) who showed that ci increased the level of JA in tomato leaves. Furthermore, the induced expression of GUS in *JAZ10:GUSPlus* and *VSP2:GUSPlus* transformed plants demonstrated that ci induces early and late JA-responsive genes. The insensitivity of *JAZ10* expression to ci in the *coi1-1* mutant demonstrated that SP changes can only lead to the expression of this gene through the canonical JA signalling pathway.

**Ion channels regulate the JA pathway**

The *fou2* mutant is a wound mimic mutant which has higher levels of JA than WT and displays a $K^+$ starvation phenotype (Bonaventure et al. 2007a). We investigated SP changes generation in this mutant. Leaves 7-9 in *fou2* display a transition of epinasty (Bonaventure et al. 2007b). We showed that leaf epinasty did not affect *JAZ10* expression levels and WASP generation after wounding in leaf 13 in both homozygote and heterozygote *fou2*. In *fou2,* the velocity of WASPs in the wounded leaves and from

wounded leaf to connected leaf 13 were decreased 2-3 times in respect to WT. This lower velocity might be due to the lower vacuolar $K^+$ levels known in *fou2* (Beyhl et al. 2009). This could indicate that ion channels in the tonoplast may play roles in WASP propagation.

**Proton pump deactivation does not affect WASP changes after wounding**

Action potentials (APs) are generated in animal and plants cells but variation potentials (VPs), a another type of electrical signal, are known only from plants and are transmited through the xylem as hydraulic pressure waves (Fromm and Lautner 2007). It is believed that plasma membrane proton pumps are involved in VP generation (Stahlberg et al. 2006). Fussicoccin and orthovandate, an activator and an inhibitor of plant plasma membrane $H^+$-ATPase s respectively, altered electrical activity in barley and bean (Zimmermann et al. 2009). However, fussicoccin, a compound of fungal origin, permanently activates plant plasma membrane $H^+$-ATPases, and this chemical can induce JA pathway activity (Schaller and Frasson 2001). We found that, applying fussicoccin and other ion channel activity modifying chemicals did not abolish SP changes after wounding nor did they generate SP changes without wounding. Additionally, we found that WASPs, were not eliminated in four T-DNA insertion lines in plasma membrane $H^+$-ATPase (*aha1, aha2, aha3, and aha11*) all of which were highly expressed in shoots and in the vascular system of *Arabidopsis.* Moreover, the *aha1aha3* and *aha2aha3* double mutants also did not affect WASPs. Several attempts to make a double mutant of *aha1aha2* failed. This double mutant is lethal (Haruta et al. 2010). Since there is a high redundancy in plasma membrane $H^+$-ATPase s, we cannot conclude whether WASP changes are VPs, APs or other type of electrical signals.

**Glutamate receptor-like (GLR) proteins mediate leaf-to-leaf signalling**

The *GLR* genes we studied encode putative cation channels, and *GLR3.3* has been implicated in agonist-stimulated plasma membrane depolarization (Qi et al. 2006, Stephens et al. 2008). This gene, as well as several *GLRs* expressed in pollen (Michard et al. 2011), can control cytosolic $Ca^{2+}$ influxes, and GLRs have also been implicated in mediating

calcium influxes in response to the perception of microbe-associated molecular patterns (Kwaaitaal et al. 2011). Cold and mechanical stresses including wounding and touching induced *GLR3.4* expression (Meyerhoff et al. 2005). Reduction of WASP duration in the wounded leaf 8 and and elimination of WASPs in leaf 13 when leaf 8 was wounded in the *glr3.3glr3.6* double mutant suggested that these genes play an essential role in WASP propagation. However, detection of WASPs in the wounded leaf 8 of the double mutant suggests that there are still other ion channel(s) involved in WASP propagation in the wounded leaves.

In plants, action potentials are generated after depolarization of cell membranes by $Ca^{2+}$ influx into the cytoplasm and subsequent $Cl^-$ efflux (Fromm and Lautner 2007). With regard to the suggested roles of plant GLR channels in $Ca^{2+}$ movement across the plasma membrane (Qi et al. 2006, Michard et al. 2011) it is possible that WASPs might reflect APs. Our results are consistent with a previous report by Favre et al. (2007) that showed wounding-generated APs in *Arabidopsis.* The induction of JA-responsive genes like *JAZ10* and *VSP2* indicates the activation of JA signalling. The low level of *JAZ10* transcripts in leaf 13 when leaf 8 was wounded in *glr3.3glr3.6* showed that JA-Ile synthesis far from a wound depends on GLR-mediated signals. The low level of *JAZ10* transcript of leaf 13 in *glr3.3glr3.6* is comparable to the *JAZ10* expression level in leaf 9 of WT which also did not receive WASPs. Taken together, these results support a role of electrical signals as major elements in the long distance wound signalling neccesary to activate JA synthesis and signalling.

**Electrical signals are part of the long distance wound signalling mechanism in plant**

Due to the fast accumulation of JA in tissues distal to wounds, this molecule was considered as a mobile wound signal for many years (Li et al. 2002, Howe 2004). Evidence for roles of other possible long distance wound signals like hydraulic or electrical signals has not been supported genetically. For example, a reported correlation between electrical

signal and *proteinase inhibitor II (Pin2)* (Wildon et al. 1992), has not been followed up. The present study has now allowed us to reinvestigate the phenomenon of organ-to-organ wound signalling.

The *glr3.3glr3.6* double mutant we produced provides a powerful tool for the study of electrical signals in plants. We believe that our results may improve the knowledge of the role of ion channels in the process of generation, propagation and translation of electrical signals in development and in stress responses. We are in the process of investigating the roles of other double mutants and triple mutants from the combination of *glr* single mutants shown in table 5.4. Finally, GLRs are related to ionotropic glutamate receptors (iGluRs) that are important for fast excitatory synaptic transmission in the vertebrate nervous system (Traynelis et al. 2010). It has been proposed that iGluRs and their plant relatives may control signalling mechanisms that existed prior to the divergence of animals and plants (Chiu et al. 2002). If so, a deeply conserved function for these genes might be to link the perception of damage to distal protective responses.

# Chapter 6

# Transcriptme of current injected and wounded leaves

## Introduction

Wounding elicits massive changes in the cellular, physiological, biochemical and genetic activities of plant tissues both near the wound itself and also distal from the wound. Many of these responses are controlled by jasmonates, phytohormones that have major roles in plant defence against herbivores (Browse 2009). Jasmonate biosynthesis and signalling has been investigated for several decades. However, the more we know about jasmoante function, the less we appear to know about early steps that lead to induction of jasmoante synthesis. Physical events like hydraulic or electrical signals along with jasmonic acid (JA) or jasmonayl isoleucine (JA-Ile) have been proposed as long distance wound signals (Wildon et al. 1992, Stanković and Davies 1996, Howe 2004, Matsuura et al. 2012). It is not yet known which of the proposed signals can activate JA synthesis in the wounded or distal parts of *Arabidopsis*. It is also not known whether one or multiple signals are involved in activating JA synthesis near the wound and distal parts of plant.

Many genes are induced upon herbivory or mechanical damage in *Arabidopsis* leaves (Reymond et al. 2000, Kilian et al. 2007, Walley et al. 2007, Yan et al. 2007, Miller et al. 2009). Hundreds of these genes have been found to be regulated by jasmonates (Reymond

et al. 2000, Reymond et al. 2004, Walley et al. 2007, Yan et al. 2007), and also through the ethylene (Van Zhong and Burns 2003) and ABA pathways (Yang et al. 2008). Wound-induced genes regulated by jasmonates have been shown to have peaks of expression that differ in timing. For instance, transcripts for the *JAZ10* gene, an early jasmonate-responsive gene reaches a peak of expression 1h after wounding (Glauser et al. 2009). *VSP2,* a late wound-responsive gene, produces maximum transcripts levels at 4 h after wounding leaves (Wang et al. 2008). Previous studies have overlooked the genes that regulate long distance wound signalling. Moreover, for years, experiments did not take into account vascular connections in leaves. This is important in leaf-to-leaf signalling.

The aim of this chapter is to understand how transcript levels in leaves are regulated distal to a wound. We first analysed transcript levels in parastichious leaf 13 when leaf 8 was wounded. Transcript levels in leaf 8 were also analysed after generation of surface potentials (SPs) with current injection (ci). Furthermore, we compared our results to the expression profiling data from independent microarray data from 18-day old wounded *Arabidopsis* (Kilian et al. 2007).

## Results

### Transcriptional changes due to long distance wound signalling

To investigate whether SPs produced following current injection and wounding had similar effects on gene expression, transcript levels were assessed in current injected leaves and also in connected leaf 13 when leaf 8 was wounded. Leaf samples from five weeks-old plant were harvested 1h after wounding, a time at which the *JAZ10* expression level is high (Yan et al. 2007, Glauser et al. 2009). Gene expression was analysed with ATH1 Affymetrix GeneChips. To reduce plant-to-plant variation we pooled RNA from three different plants for each triplicate sample. We then compared our results with published data from 18 day-old plants that had been wounded 1h prior to harvest (Kilian et al. 2007). To facilitate comparison, normalization of our experiments and published data were done in the same way. Genes were selected on the basis of a *P*-value < 0.05 and a mean induction ratio greater than 2-fold. We compared transcript levels between control leaves and current injected leaf 8 and leaf 13 from a plant on which leaf 8 had been wounded. Figure 6.1 shows a Venn diagram of genes that were up-regulated.

A total of 1960 genes were found to be significantly differentially expressed ($P \leq 0.05$) and a mean induction ratio greater than 2-fold in current injected leaf 13 when leaf 8 was wounded and wounded leaf. We identified 1153 (59%) genes that were significantly upregulated, and 807 (39%) genes that were significantly downregulated (Fig. 6.1). In leaf 13, 755 genes were significantly upregulated, and 402 genes were significantly downregulated (Fig. 6.1 and 6.2). A total of 379 genes were found to be significantly differentially expressed ($P \leq 0.05$) 1 h after 40μA current injection (Table 6.1 and 6.2). We identified 313 genes that were significantly upregulated, and 66 genes that were significantly downregulated (Fig. 6.1). A large number of upregulated genes (218 genes; 70% of genes activated by ci) were common among the three treatments. Moreover, 76 upregulated genes (24% of genes activated by ci) were also common to ci and leaf 13 (Fig 6.1). The expression levels of the genes common to all samples are shown in Table 6.1. In

contrast to the overlap of transcript populations for genes unregulated wounding and current injection, the downregulated genes showed less overlap among the three experiments. Of 66 genes that were downregulated following current injection only 20% of genes activated by ci (13 genes) were common to the three experiments (Fig 6.2 and Table 6.2). Current injection down regulated 3 genes between wounded rosette and ci and 26 genes which were exclusively downregulated in the current injected leaf.

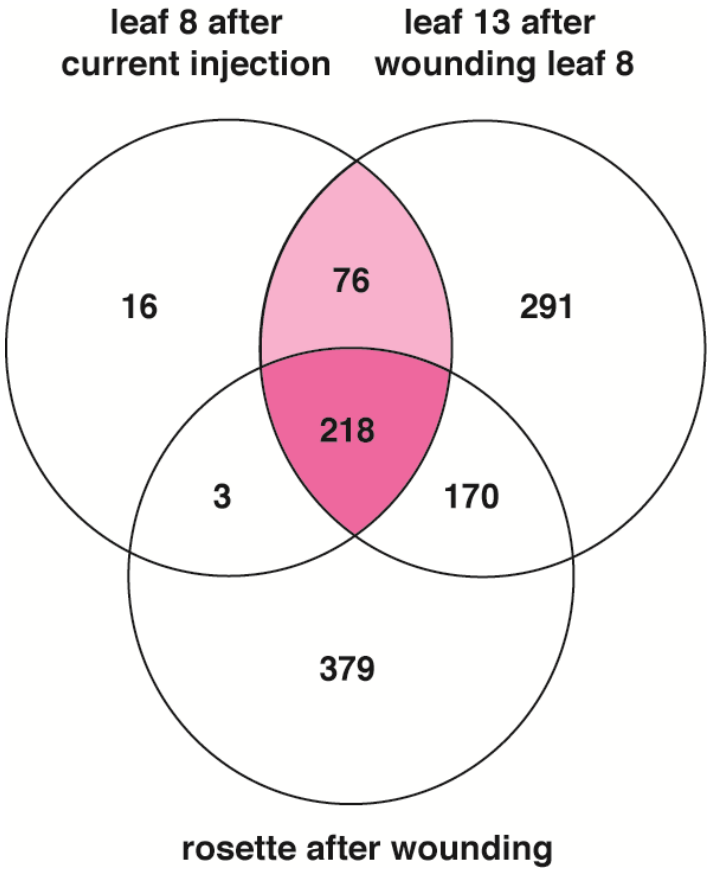


**Figure 6. 1. Current injection and wounding elicit overlapping transcript expression patterns.** Venn diagram illustrating the number of transcripts upregulated >2-fold compared to unstimulated plants ($P \leq 0.05$) for current injected leaf 8 (this study), for leaf 13 of plants that had been wounded on leaf 8 (this study), and for wounded *Arabidopsis* rosettes (from Kilian et. al, 2007).

**Table 6. 1. Gene expression in response to current injection or wounding.** List of genes that were upregulated more than 2-fold ($P \leq 0.05$) in leaf 8 1h after injecting current into leaf 8 (this study), in leaf 13 1h after wounding leaf 8 (this study), and in wounded leaves of 18 d-old plants 1 h after wound infliction (Kilian et. al, 2007), FC=fold change (upregulated), ci = current injection, no ci = no current injection, W= wound.

| locus | annotation | ci/ no ci | | Leaf 13/control | | W/control | |
|---|---|---|---|---|---|---|---|
| | | FC | *P*-value | FC | *P* -value | FC | *P* -value |
| At1g43160 | RAP2.6 (related to AP2 6); DNA binding / transcription factor | 39.6 | 2.4E-10 | 177.3 | 2.7E-12 | 100.7 | 6.6E-07 |
| At5g13220 | JAZ10 (JASMONATE-ZIM-DOMAIN PROTEIN 10) | 28.2 | 1.9E-10 | 123.1 | 1.5E-12 | 28.8 | 8.4E-06 |
| At5g63450 | CYP94B1 | 27 | 5.2E-10 | 59.3 | 3.1E-11 | 32.5 | 1.4E-07 |
| At2g34600 | JAZ7 (JASMONATE-ZIM-DOMAIN PROTEIN 7) | 25.9 | 9.0E-12 | 143 | 3.2E-14 | 18.6 | 7.5E-04 |
| At1g17420 | LOX3 | 24.8 | 1.2E-10 | 72.9 | 2.7E-12 | 68.4 | 7.5E-08 |
| At1g61120 | TPS04 (TERPENE SYNTHASE 04) | 20.1 | 7.9E-09 | 49.8 | 2.7E-10 | 11.4 | 2.1E-05 |
| At1g17380 | JAZ5 (JASMONATE-ZIM-DOMAIN PROTEIN 5) | 13.6 | 6.4E-09 | 102.8 | 3.6E-12 | 22 | 4.7E-06 |
| At3g25780 | AOC3 (ALLENE OXIDE CYCLASE 3) | 11.8 | 2.7E-08 | 42.2 | 1.3E-10 | 59.5 | 3.2E-06 |
| At4g10390 | Protein kinase superfamily protein | 11.4 | 1.0E-08 | 40.9 | 4.5E-11 | 5.6 | 1.2E-05 |
| At2g27690 | CYP94C1 | 10.9 | 5.1E-10 | 38.2 | 2.0E-12 | 86.1 | 3.1E-08 |
| At1g72520 | LOX4 (LIPOXYGENASE 4) | 10.8 | 1.0E-08 | 81.6 | 3.4E-12 | 27.9 | 7.1E-06 |
| At4g34410 | RRTF1 (REDOX RESPONSIVE TRANSCRIPTION FACTOR 1) | 10.2 | 1.6E-08 | 59.4 | 1.1E-11 | 18.9 | 2.2E-07 |
| At3g25180 | CYP82G1 | 10 | 3.0E-07 | 51.2 | 3.6E-10 | 6.7 | 1.2E-06 |
| At2g29440 | ATGSTU6 (GLUTATHIONE S-TRANSFERASE TAU 6) | 10 | 3.7E-09 | 32.9 | 1.7E-11 | 20.1 | 2.1E-05 |
| At2g24850 | TAT3 (TYROSINE AMINOTRANSFERASE 3) | 9.6 | 2.8E-05 | 38.6 | 1.3E-07 | 13 | 7.5E-04 |
| At3g48520 | CYP94B3 | 9.4 | 3.5E-06 | 42.8 | 7.6E-09 | 54.6 | 1.2E-07 |
| At5g65280 | GCL1 (GCR2-LIKE 1); catalytic | 8.2 | 1.1E-08 | 21.2 | 8.8E-11 | 10.2 | 4.7E-05 |
| At1g51780 | ILL5; IAA-amino acid conjugate hydrolase | 7.9 | 5.2E-09 | 30 | 8.0E-12 | 14 | 8.5E-06 |
| At1g44350 | ILL6; IAA-amino acid conjugate hydrolase | 7.5 | 1.1E-08 | 30.3 | 1.2E-11 | 22.2 | 8.7E-06 |
| At2g06050 | OPR3 (OPDA-REDUCTASE 3) | 7.5 | 2.8E-10 | 22 | 9.8E-13 | 25.6 | 8.5E-08 |
| At5g05600 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase | 7.3 | 1.7E-08 | 55.8 | 1.8E-12 | 42.4 | 2.5E-07 |
| At1g70700 | JAZ9 | 7 | 1.1E-07 | 24.8 | 2.0E-10 | 6.1 | 1.6E-05 |
| At1g30135 | JAZ8 (JASMONATE-ZIM-DOMAIN PROTEIN 8) | 6.9 | 1.6E-07 | 51 | 1.9E-11 | 16.8 | 5.8E-06 |
| At3g51450 | Calcium-dependent phosphotriesterase family | 6.8 | 7.3E-07 | 27.3 | 9.0E-10 | 30.3 | 1.1E-05 |
| At1g19670 | CORI1 (CORONATINE-INDUCED PROTEIN 1) | 6.8 | 1.1E-08 | 8.2 | 3.3E-09 | 7.4 | 1.3E-05 |
| At1g53885 | Protein of unknown function (DUF581) | 6.7 | 8.5E-08 | 23.5 | 1.4E-10 | 25.2 | 1.7E-07 |
| At4g01080 | member of the TBL (TRICHOME BIREFRINGENCE-LIKE) | 6.7 | 1.2E-08 | 12.9 | 2.6E-10 | 7.2 | 1.4E-04 |
| At5g56980 | unknown protein | 6.4 | 4.0E-09 | 27.4 | 2.1E-12 | 10.8 | 3.7E-07 |
| At2g22860 | ATPSK2 (PHYTOSULFOKINE 2 PRECURSOR) | 6.3 | 1.1E-08 | 11.1 | 3.3E-10 | 11.4 | 3.4E-05 |
| At1g52890 | ANAC019 (NAC domain containing protein 19) | 6.2 | 7.3E-07 | 44.6 | 7.2E-11 | 8.8 | 8.4E-07 |
| At2g22880 | VQ motif-containing protein | 6.2 | 7.2E-06 | 24.5 | 9.4E-09 | 9 | 1.5E-03 |
| At1g61610 | S-locus lectin protein kinase family protein | 6.2 | 5.6E-07 | 10.5 | 2.7E-08 | 3.1 | 8.9E-05 |
| At1g16370 | OCT6 (ORGANIC CATION/CARNITINE TRANSPORTER 6) | 6.1 | 3.1E-06 | 16.2 | 1.8E-08 | 18 | 7.4E-06 |
| At3g09940 | MDHAR (MONODEHYDROASCORBATE REDUCTASE) | 6.1 | 3.3E-05 | 6.4 | 2.4E-05 | 2.1 | 2.1E-02 |
| At3g44860 | Encodes a farnesoic acid carboxyl-O-methyltransferas | 6 | 1.3E-07 | 15.9 | 5.3E-10 | 65.4 | 2.9E-07 |
| At2g34930 | disease resistance family protein | 6 | 1.5E-10 | 13.5 | 1.1E-12 | 63.1 | 7.3E-08 |
| At1g80840 | WRKY40; transcription factor | 5.7 | 7.2E-06 | 31.6 | 2.1E-09 | 3.8 | 6.6E-03 |

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|---|---|---|---|---|---|---|---|
| At2g38750 | ANNAT4 (ANNEXIN ARABIDOPSIS 4) | 5.7 | 1.8E-08 | 7.6 | 2.7E-09 | 5.1 | 1.8E-05 |
| At1g32640 | MYC2; transcription factor | 5.5 | 1.0E-08 | 21.6 | 5.0E-12 | 8 | 8.0E-07 |
| At1g74950 | JAZ2 | 5.5 | 5.1E-08 | 19 | 4.6E-11 | 8 | 1.6E-06 |
| At5g60890 | MYB34 (MYB DOMAIN PROTEIN 34) transcription factor | 5.5 | 5.7E-10 | 8.2 | 3.7E-11 | 8.6 | 7.6E-06 |
| At5g54170 | Polyketide cyclase/dehydrase and lipid transport superfamily | 5.3 | 4.4E-08 | 13.5 | 1.6E-10 | 3 | 4.1E-04 |
| At2g42760 | unknown protein | 5.3 | 5.5E-08 | 12.1 | 3.6E-10 | 7.6 | 1.8E-06 |
| At4g39950 | CYP79B2 | 5.3 | 2.0E-10 | 5.5 | 1.6E-10 | 4.3 | 3.6E-03 |
| At2g46370 | JAR1 (JASMONATE RESISTANT 1) | 5.3 | 1.5E-08 | 5.4 | 1.4E-08 | 7.6 | 2.5E-05 |
| At1g74430 | MYB95 (myb domain protein 95) | 5.2 | 1.5E-07 | 28.4 | 2.0E-11 | 12.8 | 2.4E-06 |
| At4g15210 | BAM5 (BETA-AMYLASE 5) | 5.1 | 7.5E-05 | 5.4 | 5.2E-05 | 38.5 | 1.3E-06 |
| At1g61890 | MATE efflux family protein | 4.9 | 2.5E-09 | 21.5 | 4.9E-13 | 13 | 5.1E-07 |
| At2g39420 | alpha/beta-Hydrolases superfamily protein | 4.8 | 1.2E-05 | 11.6 | 7.2E-08 | 12.8 | 4.6E-06 |
| At1g19180 | JAZ1 (JASMONATE-ZIM-DOMAIN PROTEIN 1) | 4.7 | 8.4E-07 | 61.5 | 3.6E-12 | 22.7 | 5.3E-04 |
| At5g38120 | AMP-dependent synthetase and ligase family protein | 4.7 | 1.6E-08 | 23.2 | 1.6E-12 | 3.3 | 3.3E-03 |
| At2g38760 | ANNAT3 (ANNEXIN ARABIDOPSIS 3); calcium ion binding | 4.7 | 6.1E-08 | 15.5 | 4.2E-11 | 5.3 | 2.4E-05 |
| At1g52720 | unknown protein | 4.7 | 3.7E-07 | 13.7 | 5.5E-10 | 3.1 | 1.6E-04 |
| At1g64200 | VHA-E3 (VACUOLAR H+-ATPASE SUBUNIT E ISOFORM 3) | 4.7 | 4.1E-07 | 7.7 | 1.4E-08 | 2 | 5.8E-04 |
| At1g10585 | basic helix-loop-helix (bHLH) DNA-binding | 4.7 | 7.6E-07 | 3.3 | 1.3E-05 | 19.4 | 4.5E-06 |
| At1g28480 | GRX480; electron carrier/ protein disulfide oxidoreductase | 4.6 | 1.7E-05 | 15.7 | 1.9E-08 | 3.5 | 5.3E-04 |
| At2g22330 | CYP79B3 | 4.6 | 2.4E-09 | 7.1 | 9.4E-11 | 4.9 | 1.4E-05 |
| At1g06620 | protein is similar to a 2-oxoglutarate-dependent dioxygenase | 4.5 | 1.1E-06 | 15.2 | 7.1E-10 | 6.8 | 1.3E-04 |
| At5g38130 | HXXXD-type acyl-transferase family protein | 4.5 | 8.0E-06 | 11.5 | 2.9E-08 | 2.2 | 5.5E-03 |
| At3g19970 | alpha/beta-Hydrolases superfamily protein | 4.5 | 2.1E-06 | 7.1 | 9.8E-08 | 3.9 | 4.5E-06 |
| At5g27520 | PNC2 (PEROXISOMAL ADENINE NUCLEOTIDE CARRIER 2) | 4.4 | 5.3E-07 | 19.9 | 7.4E-11 | 12.9 | 4.9E-06 |
| At5g67210 | Encode a DUF579 | 4.4 | 7.6E-08 | 13.8 | 5.1E-11 | 6.7 | 2.5E-05 |
| At4g24350 | Phosphorylase family protein | 4.4 | 4.6E-07 | 8.5 | 5.2E-09 | 2.9 | 4.8E-04 |
| At5g24780 | VSP1 (VEGETATIVE STORAGE PROTEIN 1) | 4.3 | 5.4E-03 | 33.8 | 1.8E-06 | 12.6 | 1.1E-04 |
| At1g66760 | MATE efflux family | 4.3 | 2.7E-07 | 14.4 | 1.5E-10 | 4.5 | 3.3E-05 |
| At1g72450 | JAZ6 (JASMONATE-ZIM-DOMAIN PROTEIN 6) | 4.3 | 3.9E-08 | 12.8 | 2.7E-11 | 5 | 5.7E-06 |
| At5g24420 | 6-phosphogluconolactonase 5 (PGL5) | 4.3 | 2.1E-07 | 4.7 | 9.2E-08 | 2.3 | 2.4E-02 |
| At2g38240 | Oxidoreductase, 2OG-Fe-oxygenase | 4.2 | 2.0E-05 | 34 | 4.7E-10 | 44.2 | 4.2E-07 |
| At5g47220 | ERF2 (ETHYLENE RESPONSIVE ELEMENT BINDING | 4.2 | 4.1E-07 | 31.6 | 6.3E-12 | 2.5 | 1.4E-03 |
| At4g15440 | HPL1 (HYDROPEROXIDE LYASE 1) | 4.2 | 2.9E-07 | 23.5 | 1.3E-11 | 12.2 | 1.0E-05 |
| At5g10300 | MES5 (METHYL ESTERASE 5); hydrolase | 4.2 | 2.5E-06 | 14.5 | 1.5E-09 | 4.7 | 7.7E-05 |
| At2g30360 | SIP4 (SOS3-INTERACTING PROTEIN 4); kinase/ protein kinase | 4.2 | 2.7E-07 | 12.4 | 2.6E-10 | 4 | 5.5E-05 |
| At3g23250 | MYB15 (MYB DOMAIN PROTEIN 15) | 4.2 | 1.9E-04 | 9.2 | 2.1E-06 | 5 | 1.5E-03 |
| At5g38710 | Methylenetetrahydrofolate reductase family protein | 4.2 | 1.3E-05 | 7.4 | 3.1E-07 | 10.6 | 4.5E-06 |
| At5g44050 | MATE efflux family protein | 4.1 | 1.1E-06 | 10.1 | 3.1E-09 | 6.9 | 8.5E-04 |
| At5g06870 | PGIP2 (POLYGALACTURONASE INHIBITING PROTEIN 2) | 4.1 | 3.1E-08 | 9.2 | 8.6E-11 | 6.5 | 2.7E-05 |
| At2g29450 | ATGSTU5 (GLUTATHIONE S-TRANSFERASE TAU 5) | 4.1 | 3.5E-09 | 7.9 | 2.5E-11 | 3.3 | 3.3E-05 |
| At4g24340 | Encodes a F-box protein induced by various biotic or abiotic stress | 4.1 | 1.3E-07 | 7.6 | 1.4E-09 | 2.7 | 2.7E-04 |
| At1g54020 | GDSL-like Lipase/Acylhydrolase superfamily protein | 4 | 2.6E-09 | 7.6 | 1.7E-11 | 8.9 | 6.7E-03 |
| At4g18440 | L-Aspartase-like family protein | 4 | 4.2E-07 | 3.8 | 6.4E-07 | 2.6 | 5.3E-05 |
| At4g08170 | Inositol 1,3,4-trisphosphate 5/6-kinase | 3.9 | 4.1E-07 | 14 | 1.1E-10 | 14.3 | 2.9E-07 |

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|---|---|---|---|---|---|---|---|
| At5g47240 | atnudt8 (Arabidopsis thaliana Nudix hydrolase homolog 8) | 3.9 | 9.1E-07 | 13.3 | 3.2E-10 | 11.3 | 3.8E-05 |
| At5g63970 | Copine (Calcium-dependent phospholipid-binding protein) family | 3.9 | 7.0E-08 | 12.1 | 2.8E-11 | 2.7 | 2.8E-04 |
| At3g47960 | Major facilitator superfamily protein | 3.9 | 2.5E-07 | 6.6 | 5.1E-09 | 3.5 | 9.8E-06 |
| At5g43180 | Protein of unknown function, DUF599 | 3.9 | 2.2E-07 | 2.6 | 1.2E-05 | 2.1 | 6.4E-03 |
| At5g67300 | MYBR1 (MYB DOMAIN PROTEIN R1); transcription factor | 3.8 | 6.3E-09 | 7.7 | 2.7E-11 | 3.8 | 3.7E-05 |
| At5g46590 | anac096 (NAC domain containing protein 96); transcription factor | 3.8 | 2.7E-08 | 3.7 | 3.2E-08 | 3.7 | 3.8E-04 |
| At3g21890 | B-box type zinc finger family protein | 3.8 | 1.0E-04 | 3 | 6.3E-04 | 4.3 | 5.3E-06 |
| At3g55970 | jasmonate-regulated gene 21 (JRG21) | 3.7 | 3.4E-04 | 15.3 | 1.4E-07 | 10.9 | 3.3E-04 |
| At5g55120 | VTC5 (VITAMIN C DEFECTIVE 5) | 3.7 | 6.8E-07 | 12.6 | 1.6E-10 | 2.6 | 3.9E-04 |
| At1g20510 | OPCL1 (OPC-8:0 COA LIGASE1); 4-coumarate-CoA ligase | 3.6 | 2.0E-07 | 12.5 | 3.3E-11 | 10.2 | 1.3E-05 |
| At5g12340 | unknown protein | 3.6 | 2.2E-03 | 11.3 | 5.8E-06 | 5.1 | 1.9E-05 |
| At1g31550 | GDSL-like Lipase/Acylhydrolase superfamily protein | 3.6 | 8.9E-07 | 6.7 | 7.0E-09 | 3.2 | 7.6E-05 |
| At4g39980 | DHS1 (3-DEOXY-D-ARABINO-HEPTULOSONATE 7- | 3.6 | 9.2E-09 | 4.5 | 1.2E-09 | 2 | 8.3E-04 |
| At1g29330 | ERD2 (ENDOPLASMIC RETICULUM RETENTION | 3.5 | 1.9E-07 | 10.8 | 6.5E-11 | 4.1 | 5.6E-05 |
| At1g73080 | Encodes a leucine-rich repeat receptor kinase | 3.5 | 1.5E-07 | 6.9 | 6.7E-10 | 5.3 | 5.3E-06 |
| At5g22630 | ADT5 (arogenate dehydratase 5) | 3.4 | 8.4E-08 | 13.6 | 4.8E-12 | 5.5 | 4.7E-05 |
| At2g46510 | ATAIB (ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR) | 3.4 | 4.8E-07 | 10.5 | 1.2E-10 | 4.4 | 3.1E-06 |
| At1g12240 | ATBETAFRUCT4 | 3.4 | 9.7E-08 | 5.4 | 1.9E-09 | 2.7 | 8.1E-04 |
| At2g44840 | ERF13 (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13) | 3.3 | 8.3E-04 | 17.2 | 9.0E-08 | 3 | 4.6E-04 |
| At4g17230 | SCL13 (Scarecrow-like 13); transcription factor | 3.3 | 5.6E-06 | 9.5 | 3.1E-09 | 2.5 | 8.6E-04 |
| At1g19640 | JMT (JASMONIC ACID CARBOXYL | 3.3 | 2.5E-08 | 8.7 | 1.3E-11 | 3.8 | 7.9E-03 |
| At2g22200 | member of the DREB subfamily A-6 of ERF/AP2 transcription factor | 3.3 | 5.4E-05 | 8.5 | 8.5E-08 | 2.3 | 1.9E-04 |
| At1g60190 | Encodes PUB19, a plant U-box armadillo repeat protein | 3.3 | 9.9E-04 | 6.9 | 1.2E-05 | 2.3 | 2.2E-04 |
| At5g19110 | Eukaryotic aspartyl protease family protein | 3.3 | 1.2E-06 | 6.5 | 5.3E-09 | 3.6 | 1.9E-03 |
| At5g05730 | ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1) | 3.2 | 1.2E-07 | 5.8 | 5.8E-10 | 2.2 | 1.1E-02 |
| At3g50260 | CEJ1 (COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE 1) | 3.2 | 4.5E-04 | 5.4 | 1.2E-05 | 3.6 | 1.7E-04 |
| At2g20340 | Tyrosine decarboxylase, putative | 3.1 | 1.7E-06 | 10 | 3.0E-10 | 2.4 | 1.7E-04 |
| At5g02940 | unknown protein | 3.1 | 8.7E-07 | 6.4 | 2.2E-09 | 4.1 | 3.4E-05 |
| At5g36220 | CYP81D1 (CYTOCHROME P450 81D1) | 3.1 | 7.1E-07 | 6.4 | 1.9E-09 | 2.4 | 2.7E-02 |
| At3g21230 | 4CL5 (4-coumarate:CoA ligase 5); 4-coumarate-CoA ligase | 3.1 | 6.8E-06 | 5.8 | 4.0E-08 | 4 | 3.2E-04 |
| At4g27654 | unknown protein | 3.1 | 3.3E-04 | 5.4 | 5.9E-06 | 5.6 | 3.9E-06 |
| At2g46270 | GBF3 (G-BOX BINDING FACTOR 3) | 3.1 | 9.7E-05 | 2.2 | 1.9E-03 | 3.1 | 7.9E-04 |
| At4g17470 | alpha/beta-Hydrolases superfamily protein | 3 | 3.4E-04 | 25.3 | 1.8E-09 | 10.2 | 5.4E-07 |
| At4g02360 | unknown protein | 3 | 3.0E-05 | 18.1 | 2.9E-10 | 2.3 | 2.7E-04 |
| At4g27570 | UDP-Glycosyltransferase superfamily protein | 3 | 1.8E-06 | 9.6 | 2.8E-10 | 5.7 | 9.1E-06 |
| At1g52000 | Mannose-binding lectin superfamily protein | 3 | 3.5E-05 | 8.1 | 2.4E-08 | 3 | 2.5E-04 |
| At5g59730 | ATEXO70H7 (EXOCYST SUBUNIT EXO70 FAMILY PROTEIN H7) | 3 | 2.3E-07 | 7.4 | 1.1E-10 | 2.4 | 8.8E-05 |
| At1g24070 | ATCSLA10; cellulose synthase | 3 | 1.0E-05 | 5.1 | 1.1E-07 | 2.4 | 3.4E-03 |
| At3g50280 | HXXXD-type acyl-transferase family protein | 2.9 | 1.3E-06 | 11.6 | 3.7E-11 | 2.3 | 3.7E-03 |
| At4g29700 | Alkaline-phosphatase-like family protein | 2.9 | 4.9E-06 | 9.2 | 7.1E-10 | 3.6 | 1.9E-03 |

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|---|---|---|---|---|---|---|---|
| At1g69370 | CM3 (chorismate mutase 3); chorismate mutase | 2.9 | 1.2E-06 | 5.9 | 2.1E-09 | 3.2 | 9.4E-06 |
| At1g23850 | unknown protein | 2.9 | 4.8E-07 | 4 | 1.9E-08 | 2.8 | 4.7E-03 |
| At1g18710 | AtMYB47 (myb domain protein 47) | 2.8 | 7.9E-06 | 43.6 | 5.8E-13 | 11.4 | 1.1E-06 |
| At5g66650 | Protein of unknown function (DUF607) | 2.8 | 5.3E-06 | 12.3 | 8.6E-11 | 2 | 2.3E-03 |
| At1g77450 | anac032 (Arabidopsis NAC domain containing protein 32) | 2.8 | 1.2E-05 | 8.7 | 1.8E-09 | 5.3 | 5.6E-05 |
| At2g47180 | AtGolS1 (Arabidopsis thaliana galactinol synthase 1) | 2.8 | 5.9E-06 | 7.4 | 2.1E-09 | 3 | 1.0E-04 |
| At5g52320 | CYP96A4 | 2.8 | 3.3E-08 | 6.8 | 1.4E-11 | 12.9 | 2.5E-06 |
| At4g29740 | CKX4 (CYTOKININ OXIDASE 4) | 2.8 | 3.9E-05 | 6.5 | 5.0E-08 | 5.3 | 1.4E-05 |
| At2g34810 | FAD-binding Berberine family protein | 2.8 | 8.8E-06 | 3.9 | 4.5E-07 | 2.8 | 5.1E-05 |
| At5g52050 | MATE efflux family protein | 2.8 | 8.1E-06 | 3.1 | 2.2E-06 | 3.9 | 1.2E-04 |
| At1g14130 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily | 2.7 | 2.4E-05 | 13.7 | 2.2E-10 | 2.8 | 1.5E-03 |
| At5g14700 | NAD(P)-binding Rossmann-fold superfamily protein | 2.7 | 2.2E-07 | 12.4 | 1.8E-12 | 2.5 | 1.2E-03 |
| At1g09970 | LRR XI-23 | 2.7 | 4.1E-06 | 9.2 | 2.6E-10 | 4.2 | 3.9E-03 |
| At5g54300 | Protein of unknown function (DUF761) | 2.7 | 2.3E-05 | 6.7 | 1.1E-08 | 6.2 | 4.8E-06 |
| At5g01850 | Protein kinase superfamily protein | 2.7 | 8.8E-06 | 4.9 | 3.8E-08 | 2.1 | 1.8E-04 |
| At4g30530 | Encodes a gamma-glutamyl peptidase, outside the GGT family | 2.7 | 1.6E-06 | 3.8 | 4.3E-08 | 2.6 | 1.4E-03 |
| At5g61780 | Involved in the regulation of AtGA20ox3 expression | 2.7 | 2.2E-06 | 3.4 | 1.6E-07 | 3.5 | 3.8E-04 |
| At1g15010 | unknown protein | 2.7 | 3.2E-05 | 2.1 | 5.4E-04 | 61.5 | 8.6E-07 |
| At5g17490 | RGL3 (RGA-LIKE PROTEIN 3); transcription factor | 2.6 | 3.3E-04 | 18 | 1.6E-09 | 9.9 | 5.3E-06 |
| At2g33380 | RD20 (RESPONSIVE TO DESSICATION 20); calcium ion | 2.6 | 6.9E-05 | 15.7 | 2.3E-10 | 10 | 9.6E-05 |
| At1g75960 | AMP-dependent synthetase and ligase family protein | 2.6 | 2.8E-05 | 12.7 | 2.5E-10 | 2.4 | 2.4E-04 |
| At1g75230 | DNA glycosylase superfamily protein | 2.6 | 6.7E-06 | 10.7 | 1.1E-10 | 2.1 | 1.0E-04 |
| At1g76650 | calcium-binding EF hand family protein | 2.6 | 5.4E-04 | 8.6 | 8.5E-08 | 4.7 | 2.7E-05 |
| At3g27170 | CLC-B (CHLORIDE CHANNEL B); anion channel | 2.6 | 2.9E-04 | 5.6 | 6.7E-07 | 3.3 | 2.4E-05 |
| At4g36900 | RAP2.10 (related to AP2 10); DNA binding / transcription factor | 2.6 | 2.0E-05 | 5.1 | 3.8E-08 | 2.2 | 4.2E-04 |
| At5g42650 | AOS (ALLENE OXIDE SYNTHASE) | 2.6 | 2.8E-06 | 4.5 | 1.6E-08 | 9.6 | 3.0E-06 |
| At2g46520 | cellular apoptosis susceptibility protein | 2.6 | 6.3E-06 | 3.6 | 2.5E-07 | 2.7 | 8.7E-05 |
| At2g32210 | unknown protein | 2.6 | 3.3E-04 | 2.7 | 2.3E-04 | 5.6 | 2.3E-03 |
| At1g45145 | ATTRX5; oxidoreductase | 2.5 | 2.5E-04 | 8.1 | 2.6E-08 | 4.1 | 6.8E-03 |
| At3g47420 | Encodes a Pi starvation-responsive protein AtPS3 | 2.5 | 2.2E-04 | 8 | 2.8E-08 | 2.6 | 2.5E-03 |
| At1g61340 | Phosphorylase superfamily protein | 2.5 | 8.9E-04 | 7.6 | 2.8E-07 | 2.8 | 2.5E-04 |
| At1g60270 | BGLU6 (BETA GLUCOSIDASE 6) | 2.5 | 2.4E-06 | 7.5 | 1.3E-10 | 3.4 | 1.1E-03 |
| At5g53050 | alpha/beta-Hydrolases superfamily protein | 2.5 | 1.3E-06 | 6.3 | 2.1E-10 | 3.5 | 1.9E-05 |
| At5g53760 | MLO11 (MILDEW RESISTANCE LOCUS O 11) | 2.5 | 6.1E-06 | 5 | 6.8E-09 | 2.9 | 4.6E-04 |
| At1g80820 | CCR2 (CINNAMOYL COA REDUCTASE) | 2.5 | 9.1E-04 | 4.4 | 7.6E-06 | 3 | 3.8E-03 |
| At3g49620 | DIN11 (DARK INDUCIBLE 11); iron ion binding | 2.5 | 3.3E-05 | 4 | 3.5E-07 | 3.4 | 4.4E-02 |
| At1g74100 | SOT16 (SULFOTRANSFERASE 16) | 2.5 | 7.3E-07 | 3.5 | 1.5E-08 | 2.8 | 2.1E-03 |
| At4g31500 | CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1) | 2.5 | 5.6E-08 | 3.1 | 4.2E-09 | 4 | 3.1E-03 |
| At4g31800 | WRKY18; transcription factor | 2.4 | 1.2E-03 | 33.4 | 2.5E-10 | 11.4 | 1.3E-06 |
| At4g39030 | EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5) | 2.4 | 1.5E-06 | 6.4 | 1.6E-10 | 4.1 | 1.5E-05 |
| At3g17860 | JAZ3 (JASMONATE-ZIM-DOMAIN PROTEIN 3) | 2.4 | 1.8E-06 | 5.9 | 3.9E-10 | 5.5 | 4.8E-06 |
| At3g06500 | Plant neutral invertase family protein | 2.4 | 1.2E-05 | 5.7 | 5.0E-09 | 5.4 | 2.0E-06 |
| At3g16470 | JR1 | 2.4 | 1.4E-04 | 5.7 | 6.0E-08 | 2.1 | 2.9E-03 |
| At1g19380 | Protein of unknown function (DUF1195) | 2.4 | 1.0E-04 | 5.5 | 7.2E-08 | 2.8 | 1.2E-02 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At3g25760 | AOC1 (ALLENE OXIDE CYCLASE 1) | 2.4 | 4.7E-06 | 5.2 | 1.8E-09 | 8.3 | 6.5E-07 |
| At4g32800 | member of the DREB subfamily A-4 of ERF/AP2 transcription | 2.4 | 6.7E-07 | 4.4 | 9.0E-10 | 11.4 | 2.1E-06 |
| At1g58200 | MSL3 (MscS-LIKE 3) | 2.4 | 5.7E-06 | 4.4 | 1.0E-08 | 3 | 1.3E-04 |
| At5g48880 | PKT2 (PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 2) | 2.4 | 1.4E-06 | 4.1 | 5.0E-09 | 2.2 | 3.4E-03 |
| At1g11580 | PMEPCRA (METHYLESTERASE PCR A) | 2.4 | 2.7E-05 | 2.6 | 9.5E-06 | 3 | 5.6E-04 |
| At3g22740 | HMT3; homocysteine S-methyltransferase | 2.3 | 8.5E-05 | 9 | 1.2E-09 | 3.3 | 1.3E-03 |
| At5g13200 | GRAM domain family protein | 2.3 | 2.4E-05 | 6.3 | 1.7E-09 | 16.2 | 5.4E-05 |
| At2g39330 | JAL23 (JACALIN-RELATED LECTIN 23) | 2.3 | 8.2E-03 | 6 | 1.0E-05 | 4.3 | 7.0E-04 |
| At3g17120 | unknown protein | 2.3 | 4.4E-06 | 5.9 | 3.5E-10 | 3 | 4.9E-05 |
| At4g21850 | methionine sulfoxide reductase domain-containing protein | 2.3 | 8.4E-03 | 5.9 | 1.3E-05 | 2.5 | 3.3E-02 |
| At4g29780 | unknown protein | 2.3 | 1.4E-03 | 3.9 | 1.1E-05 | 2.8 | 2.2E-05 |
| At3g15210 | ERF4 (ETHYLENE RESPONSIVE ELEMENT BINDING | 2.3 | 5.1E-07 | 3.7 | 1.8E-09 | 3.3 | 1.0E-05 |
| At1g12610 | DDF1 (DWARF AND DELAYED FLOWERING 1) | 2.3 | 1.2E-04 | 3.4 | 2.1E-06 | 7.6 | 5.2E-06 |
| At5g56760 | ATSERAT1;1 (ARABIDOPSIS THALIANA SERINE | 2.3 | 3.0E-06 | 3.3 | 4.6E-08 | 3.5 | 5.8E-05 |
| At1g49530 | GGPS6 (geranylgeranyl pyrophosphate synthase 6) | 2.3 | 4.7E-05 | 3.3 | 9.2E-07 | 2.2 | 1.6E-03 |
| At3g02230 | RGP1 (REVERSIBLY GLYCOSYLATED POLYPEPTIDE 1) | 2.3 | 3.7E-06 | 2.6 | 8.3E-07 | 2.6 | 2.8E-03 |
| At4g36010 | Pathogenesis-related thaumatin superfamily protein | 2.2 | 3.0E-03 | 24.2 | 1.1E-09 | 27.5 | 9.8E-07 |
| At2g36590 | ProT3 (PROLINE TRANSPORTER 3) | 2.2 | 9.2E-05 | 10.4 | 3.9E-10 | 5.1 | 2.9E-04 |
| At3g55640 | Mitochondrial substrate carrier family protein | 2.2 | 3.6E-05 | 8.5 | 2.5E-10 | 2.4 | 4.4E-05 |
| At2g43520 | ATTI2; serine-type endopeptidase inhibitor | 2.2 | 2.8E-03 | 5.2 | 3.2E-06 | 3.2 | 1.6E-03 |
| At1g60260 | beta glucosidase 5 (BGLU5) | 2.2 | 2.6E-05 | 4.9 | 8.1E-09 | 3 | 4.2E-04 |
| At4g05100 | AtMYB74 (myb domain protein 74); transcription factor | 2.2 | 5.4E-03 | 4.8 | 1.9E-05 | 2.7 | 3.0E-04 |
| At3g28220 | TRAF-like family protein | 2.2 | 4.1E-03 | 4.8 | 1.3E-05 | 2.4 | 1.4E-02 |
| At2g43530 | Encodes a defensin-like (DEFL) family protein | 2.2 | 5.0E-06 | 4.7 | 1.6E-09 | 5.2 | 3.8E-05 |
| At2g32140 | transmembrane receptors | 2.2 | 1.1E-03 | 3.7 | 1.1E-05 | 2.9 | 3.3E-05 |
| At3g57450 | unknown protein | 2.2 | 1.1E-05 | 3.7 | 3.6E-08 | 2.6 | 1.7E-03 |
| At3g13110 | ATSERAT2;2 (SERINE ACETYLTRANSFERASE 2;2) | 2.2 | 2.9E-07 | 3.4 | 1.0E-09 | 2.2 | 1.6E-04 |
| At5g52120 | AtPP2-A14 (Phloem protein 2-A14); carbohydrate binding | 2.2 | 1.2E-03 | 3.3 | 2.7E-05 | 3.4 | 1.0E-05 |
| At5g48450 | sks3 (SKU5 Similar 3); copper ion binding | 2.2 | 4.4E-05 | 3.2 | 7.8E-07 | 2.7 | 4.8E-05 |
| At4g35110 | Arabidopsis phospholipase-like protein (PEARLI 4) family | 2.2 | 3.7E-06 | 3 | 7.3E-08 | 3.8 | 4.1E-04 |
| At5g60300 | Lectin Receptor Kinase involved in protein-protein interactions | 2.2 | 1.3E-06 | 2.8 | 7.1E-08 | 2.8 | 4.3E-05 |
| At1g69260 | AFP1 (ABI FIVE BINDING PROTEIN) | 2.2 | 7.5E-03 | 2.7 | 1.3E-03 | 3.3 | 5.4E-05 |
| At4g15660 | Thioredoxin superfamily protein | 2.2 | 2.7E-03 | 2.6 | 8.1E-04 | 2.4 | 2.7E-04 |
| At1g76390 | ARM repeat superfamily protein | 2.1 | 1.1E-05 | 7.7 | 4.7E-11 | 3 | 1.1E-05 |
| At5g53750 | CBS domain-containing protein | 2.1 | 1.0E-04 | 6.3 | 4.5E-09 | 3.2 | 2.7E-05 |
| At1g52410 | TSA1 (TSK-ASSOCIATING PROTEIN 1); calcium ion binding | 2.1 | 1.1E-04 | 5.9 | 6.6E-09 | 3.3 | 3.5E-03 |
| At4g34710 | ADC2 (ARGININE DECARBOXYLASE 2) | 2.1 | 1.8E-05 | 5.2 | 1.1E-09 | 4.7 | 1.7E-04 |
| At1g75450 | CKX5 (CYTOKININ OXIDASE 5); cytokinin dehydrogenase | 2.1 | 4.5E-04 | 4.8 | 1.2E-07 | 3.5 | 6.5E-06 |
| At5g61810 | Encodes a mitochondrial ATP-Mg/Pi transporter | 2.1 | 5.9E-07 | 4.4 | 1.0E-10 | 7 | 1.3E-05 |
| At5g41600 | BTI3 (VIRB2-INTERACTING PROTEIN 3) | 2.1 | 2.0E-07 | 4.4 | 3.3E-11 | 3.5 | 2.0E-05 |
| At4g31780 | MGD1 (MONOGALACTOSYL DIACYLGLYCEROL | 2.1 | 2.5E-05 | 3.7 | 3.5E-08 | 2.4 | 1.6E-04 |
| At3g08720 | S6K2 (ARABIDOPSIS THALIANA SERINE/THREONINE | 2.1 | 2.0E-04 | 3.6 | 4.3E-07 | 3 | 1.4E-04 |
| At3g17130 | Plant invertase/pectin methylesterase inhibitor superfamily protein | 2.1 | 3.5E-05 | 3.3 | 2.5E-07 | 6.6 | 6.3E-05 |
| At3g50910 | unknown protein | 2.1 | 1.8E-04 | 3.3 | 1.2E-06 | 2.6 | 6.8E-05 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At4g22620 | SAUR-like auxin-responsive protein family | 2.1 | 2.4E-03 | 3.1 | 4.8E-05 | 6.6 | 2.6E-05 |
| At1g76040 | CPK29 | 2.1 | 3.9E-07 | 3.1 | 3.7E-09 | 2.3 | 2.2E-03 |
| At1g09070 | SRC2 (SOYBEAN GENE REGULATED BY COLD-2) | 2.1 | 2.0E-05 | 2.5 | 2.1E-06 | 3.1 | 1.2E-05 |
| At4g18950 | Integrin-linked protein kinase family | 2.1 | 3.9E-05 | 2.3 | 1.4E-05 | 4.1 | 8.0E-05 |
| At1g50460 | HKL1 (HEXOKINASE-LIKE 1) | 2 | 2.2E-05 | 3.1 | 1.3E-07 | 2.1 | 1.6E-02 |
| At4g39940 | AKN2 (APS-kinase 2) | 2 | 1.1E-05 | 2.6 | 2.8E-07 | 2.1 | 3.7E-04 |
| At2g28550 | RAP2.7 (RELATED TO AP2.7); transcription factor | 2 | 6.6E-06 | 2.1 | 4.2E-06 | 3.8 | 6.2E-06 |
| At2g45680 | TCP family transcription factor | 2 | 4.0E-04 | 2.1 | 2.1E-04 | 2.4 | 2.3E-03 |
| At3g58790 | GAUT15 (Galacturonosyltransferase 15) | 2 | 5.5E-06 | 2 | 6.0E-06 | 4.5 | 2.9E-06 |

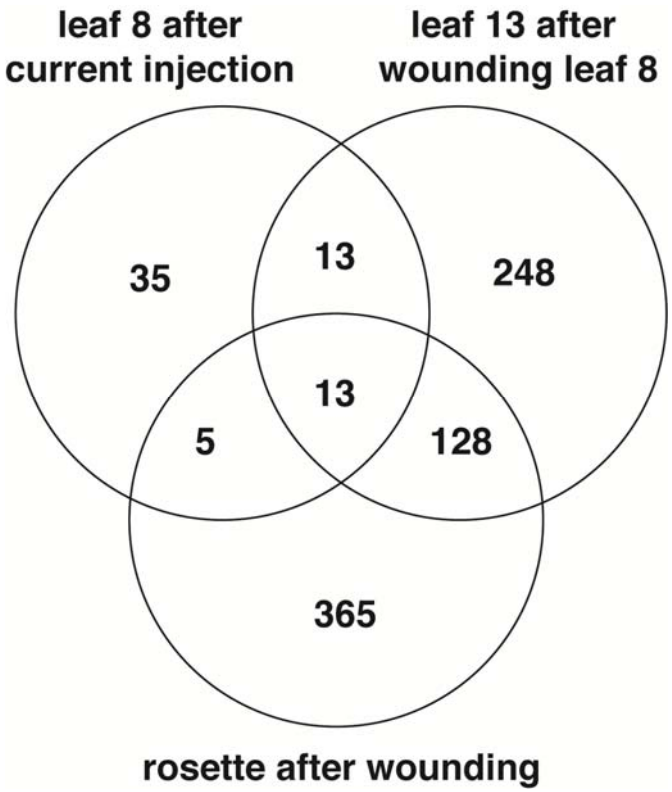


**Figure 6. 2. Genes downregulated in response to current injection and wounding.** Venn diagram illustrating the number of downregulated (>2-fold, $p<0.05$) genes for current injected leaf 8 (this study), for leaf 13 from plants wounded on leaf 8 (this study), and for wounded rosette leaves ('wounded plant', from Kilian et. al, 2007 Plant J. 50, 347-363.).

**Table 6. 2. Downregulation of gene expression in response to current injection and wounding.** List of common genes that were downregulated more than 2-fold ($P \leq 0.05$) 1h after current injection into leaf 8 (this study), in leaf 13 1h after wounding leaf 8 (leaf 13, this study), and in wounded leaves of 18 d-old plants (Kilian et. al, 2007), FC=fold change (downregulated). ci= current injection. no ci= no current injection. W= wound.

| Locus | annotation | ci/ no ci | | Leaf 13/control | | W/control | |
|---|---|---|---|---|---|---|---|
| | | FC | *P*-value | FC | *P* -value | FC | *P* -value |
| At2g24762 | AtGDU4 (GLUTAMINE DUMPER 4) | -3.1 | 2.0E-06 | -6.7 | 4.5E-09 | -3.2 | 2.1E-03 |
| At1g80440 | Member of the GDU (glutamine dumper) family | -2.8 | 2.6E-06 | -6.9 | 1.3E-09 | -14.1 | 2.7E-07 |
| At5g02760 | Protein phosphatase 2C family protein | -2.4 | 2.7E-02 | -11.1 | 7.9E-06 | -2.2 | 1.4E-04 |
| At5g22920 | RING-type Zinc finger protein | -2.3 | 1.6E-05 | -4.8 | 1.3E-08 | -11.6 | 7.4E-05 |
| At1g12200 | zinc finger (C3HC4-type RING finger) family | -2.3 | 6.9E-05 | -2.1 | 2.4E-04 | -2.2 | 8.2E-04 |
| At1g73830 | BEE3 (BR ENHANCED EXPRESSION 3) | -2.2 | 3.9E-03 | -2.7 | 5.3E-04 | -5.8 | 5.7E-05 |
| At2g44130 | Galactose oxidase/kelch repeat superfamily | -2.2 | 1.7E-08 | -2.3 | 8.1E-09 | -7.1 | 7.8E-06 |
| At2g40610 | ATEXPA8 (EXPANSIN A8) | -2.1 | 8.8E-03 | -5.4 | 5.9E-06 | -3.8 | 2.4E-03 |
| At4g30110 | HMA2; cadmium-transporting ATPase | -2.1 | 5.2E-06 | -4.7 | 9.8E-10 | -2.2 | 5.7E-03 |
| At2g15890 | MEE14 (maternal effect embryo arrest 14) | -2.1 | 1.4E-03 | -3.0 | 3.7E-05 | -4.0 | 1.9E-05 |
| At3g46130 | MYB111 (MYB DOMAIN PROTEIN 111) | -2.1 | 1.4E-07 | -2.3 | 2.2E-08 | -2.0 | 3.5E-03 |
| At1g23390 | Kelch repeat-containing F-box family protein | -2.0 | 3.0E-05 | -4.9 | 2.0E-09 | -12.3 | 5.1E-07 |
| At5g51560 | Leucine-rich repeat protein kinase family protein | -2.0 | 2.6E-05 | -2.5 | 1.2E-06 | -2.2 | 5.3E-03 |

## JA pathway activation through current injection

Among significantly upregulated genes, we found a high proportion of JA-responsive genes in the samples produced after current injection (Table 6.1). Several genes in the JA biosynthetic pathway including *LOX2, LOX3, LOX4, OPR3, AOC* and *AOS* and in the jasmonate signalling pathway (e.g. *MYC* and *JAZ* genes) were found to be significantly upregulated in all three treatments. Notably, 10 of the 12 *JAZ* genes in the JA signalling pathway were upregulated in all three stimuli (Table 6.3). For example, *JAZ10* which is an early responsive gene after wounding (Yan et al. 2007) was induced 28-fold compared to the non-current injected plant (Table 6.3).

**Table 6.3. Nine of the 12 *Arabidopsis JAZ* transcripts are upregulated by current injection and wounding.** List of the *JAZ* genes that were upregulated in leaf 8 1h after current injection (this study), in leaf 13 1h after wounding leaf 8 (this study), or in wounded leaves of 18 d-old plants (Kilian et. al, 2007), FC=fold change in transcript level. ci=current injected leaf

| locus | annotation | ci/ no ci | | Leaf 13/control | | W/control | |
|---|---|---|---|---|---|---|---|
| | | FC | *P*-value | FC | *P* -value | FC | *P* -value |
| At1g19180 | *JAZ1* | 4.7 | 8.4E-07 | 61.5 | 3.6E-12 | 22.7 | 5.3E-04 |
| At1g74950 | *JAZ2* | 5.5 | 5.1E-08 | 19 | 4.6E-11 | 8 | 1.6E-06 |
| At3g17860 | *JAZ3* | 2.4 | 1.8E-06 | 5.9 | 3.9E-10 | 5.5 | 4.8E-06 |
| At1g17380 | *JAZ5* | 13.6 | 6.4E-09 | 102.8 | 3.6E-12 | 22 | 4.7E-06 |
| At1g72450 | *JAZ6* | 4.3 | 3.9E-08 | 12.8 | 2.7E-11 | 5 | 5.7E-06 |
| At2g34600 | *JAZ7* | 25.9 | 9.0E-12 | 143 | 3.2E-14 | 18.6 | 7.5E-04 |
| At1g30135 | *JAZ8* | 6.9 | 1.6E-07 | 51 | 1.9E-11 | 16.8 | 5.8E-06 |
| At1g70700 | *JAZ9* | 7 | 1.1E-07 | 24.8 | 2.0E-10 | 6.1 | 1.6E-05 |
| At5g13220 | *JAZ10* | 28.2 | 1.9E-10 | 123.1 | 1.5E-12 | 28.8 | 8.4E-06 |
| At5g20900 | *JAZ12* | 1.8 | 1.9E-05 | 2.9 | 2.0E-08 | 2.5 | 6.2E-05 |

## Electrical signal-induced transcripts

A total of 379 genes were found to be differentially expressed more than 2-fold ($P \leq 0.05$) 1 h after current injection (40μA for 10s). We detected 300 genes that were significantly upregulated (Fig. 6.1), and 66 genes were significantly downregulated (Fig. 6.2). 76 upregulated genes overlapped between leaf 13 and the current injected leaf which indicated that the expression of these genes was unlikely to be affected by chemical components released from crushed tissues. Interestingly, genes known to be induced by crushing were not expressed in current injected leaf; *RNS1* is one such example (Reymond et al. 2000). Surprisingly, 60% of current injection - downregulated genes (40 genes) were not common to leaf 13 or the wounded leaf. Seventeen genes were upregulated by current injection alone.

**WASP-independent transcript changes:**

About 61% (461 out of 755) of upregulated transcripts in leaf 13 when leaf 8 was wounded were not significantly affected by current injection. Among them, 291 genes (39% of genes) were specifically induced in leaf 13, and 170 genes (23% of genes) were differentially regulated in common with genes in the wounded rosette (Kilian et al. 2007). Table 6.4 shows the list of the genes that exclusively upregulated in leaf 13. For example, *TPS10,* a gene that encodes a putative terpene synthase, is the most upregulated transcript with a 25-fold change. An ERF/AP2 transcription factor and *ATCSLA15*, a cellulose synthase, were also highly upregulated in leaf 13 when leaf 8 was wounded.

**Table 6.4. Exclusive upregulation of gene expression in leaf 13 after wounding leaf 8.** List of the 25 genes that were most highly upregulated (> 2-fold; $P \leq 0.05$) specifically in leaf 13 1h after wounding 8 from a 5 weeks-old plant wounded on leaf 8. FC=fold change (upregulated).

| locus | annotation | W/control | |
|---|---|---|---|
| | | FC | *P* -value |
| At2g24210 | TPS10 (terpene synthase 10) | 25.3 | 1.4E-12 |
| At4g13410 | ATCSLA15; cellulose synthase/ transferase | 14.7 | 4.3E-09 |
| At4g11310 | cysteine proteinase precursor-like protein | 8.9 | 2.5E-12 |
| At3g53600 | C2H2-type zinc finger family protein | 8.4 | 3.3E-07 |
| At4g28140 | member of the DREB subfamily A-6 of ERF/AP2 transcription factor | 7.2 | 1.3E-06 |
| At1g76640 | Calcium-binding EF-hand family protein | 6.9 | 9.5E-11 |
| At3g57520 | AtSIP2 (Arabidopsis thaliana seed imbibition 2) | 6.9 | 7.5E-08 |
| At4g16590 | ATCSLA01; cellulose synthase/ glucosyltransferase | 6.5 | 7.7E-08 |
| At4g25780 | CAP (Cysteine-rich secretory proteins | 6.5 | 1.7E-10 |
| At2g27310 | F-box family protein | 6.3 | 7.7E-10 |
| At2g15760 | Protein of unknown function (DUF1645) | 5.8 | 4.4E-09 |
| At3g15720 | Pectin lyase-like superfamily protein | 5.8 | 4.9E-06 |
| At1g59640 | ZCW32; DNA binding / transcription factor | 5.4 | 2.0E-10 |
| At3g55110 | ABC-2 type transporter | 5.2 | 6.3E-10 |
| At3g50930 | BCS1 (CYTOCHROME BC1 SYNTHESIS) | 5.1 | 2.6E-07 |
| At5g64120 | encodes a cell wall bound peroxidase | 5.1 | 8.0E-08 |
| At2g44580 | zinc ion binding | 5.1 | 3.5E-09 |
| At4g29950 | Ypt/Rab-GAP domain of gyp1p superfamily | 5 | 2.7E-10 |
| At3g51440 | Calcium-dependent phosphotriesterase superfamily | 4.8 | 3.0E-08 |
| At3g59710 | NAD(P)-binding Rossmann-fold superfamily | 4.8 | 7.3E-07 |
| At5g50335 | unknown protein | 4.8 | 1.3E-05 |
| At2g44500 | O-fucosyltransferase family protein | 4.8 | 5.4E-08 |
| At1g74420 | FUT3 (FUCOSYLTRANSFERASE 3) | 4.8 | 1.8E-10 |
| At5g07440 | GDH2 (GLUTAMATE DEHYDROGENASE 2) | 4.8 | 6.9E-09 |
| At2g20880 | Encodes ERF53 | 4.8 | 2.7E-09 |

### Genes induced by long distance wound signals

A total of 1216 genes were found to be significantly differentially expressed more than 2-fold ($P \leq 0.05$) 1 h after current injection (40μA for 10s) or in leaf 13 after wounding leaf 8 (Fig. 6.1 and 6.2). We identified 774 genes that were significantly upregulated, and 442 genes that were significantly downregulated by these two experiments. A large number of upregulated genes (294 genes) were in common between current injected and leaf 13 that include of 94% of genes activated by ci (Fig. 6.1). The expression levels of the 30 most upregulated genes common for current injection and leaf 13 are shown in Table 6.5. Surprisingly, only 19 genes (6 %) were downregulated in common between current injection and leaf 13.

**Table 6. 5. Upregulation of gene expression in response to current injection or leaf 13 when leaf 8 was wounded.** List of the 30 genes that were most highly upregulated in leaf 13 1h after wounding for leaf 8 from 5 week-old plants, and were also upregulated in current injected leaf 8 of a 5 week-old plant. FC=fold change (upregulated), ci=current injected leaf.

| locus | annotation | leaf 13 | | ci/ no ci | |
|---|---|---|---|---|---|
| | | FC | *P*-value | FC | *P* -value |
| At1g56650 | PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1) | 5.3 | 1.3E-05 | 11.7 | 1.5E-07 |
| At4g16740 | ATTPS03; (E)-beta-ocimene synthase | 4.5 | 1.8E-04 | 3.9 | 4.3E-04 |
| At2g39030 | GCN5-related *N*-acetyltransferase (GNAT) | 4.4 | 8.3E-04 | 12.3 | 5.3E-06 |
| At2g30830 | protein whose sequence is similar to 2-oxoglutarate-dependent | 4.2 | 3.2E-06 | 30.2 | 7.4E-11 |
| At4g17500 | ATERF-1 (ETHYLENE RESPONSIVE ELEMENT BINDING | 4.2 | 2.0E-06 | 26.2 | 7.7E-11 |
| At5g20230 | ATBCB (ARABIDOPSIS BLUE-COPPER-BINDING PROTEIN) | 4 | 3.1E-07 | 11.2 | 2.6E-10 |
| At4g27860 | vacuolar iron transporter (VIT) family protein | 3.7 | 3.8E-06 | 3.9 | 2.5E-06 |
| At5g67080 | MAPKKK19 | 3.6 | 1.2E-03 | 20.8 | 2.1E-07 |
| At1g06000 | flavonol-7-O-rhamnosyltransferase involved in the formation of | 3.6 | 1.1E-06 | 6.2 | 1.5E-08 |
| At2g25820 | member of the DREB subfamily A-4 of ERF/AP2 transcription | 3.6 | 3.8E-09 | 5.6 | 7.9E-11 |
| At1g70130 | Concanavalin A-like lectin protein kinase family protein | 3.6 | 4.8E-04 | 3.3 | 8.6E-04 |
| At4g04840 | methionine sulfoxide reductase domain-containing protein | 3.6 | 1.2E-05 | 2.4 | 4.9E-04 |
| At1g74930 | ORA47; DNA binding / transcription factor | 3.5 | 2.4E-06 | 10.2 | 1.5E-09 |
| At5g23820 | MD-2-related lipid recognition domain-containing protein | 3.4 | 3.2E-05 | 6.5 | 2.9E-07 |
| At3g61890 | ATHB-12 (ARABIDOPSIS THALIANA HOMEOBOX 12) | 3.2 | 3.5E-04 | 3.4 | 2.1E-04 |
| At4g27410 | RD26 (RESPONSIVE TO DESICCATION 26) | 3.1 | 2.1E-05 | 26.7 | 5.2E-11 |
| At1g28370 | ERF11 (ERF DOMAIN PROTEIN 11) | 3 | 2.4E-05 | 7.1 | 3.2E-08 |
| At5g64260 | EXL2 (EXORDIUM LIKE 2) | 3 | 2.1E-07 | 7.1 | 1.5E-10 |
| At4g20860 | FAD-binding Berberine family protein | 3 | 3.1E-05 | 6.8 | 6.8E-08 |
| At5g59760 | Protein of unknown function (DUF1635) | 3 | 2.6E-04 | 3.8 | 5.1E-05 |
| At2g32150 | Haloacid dehalogenase-like hydrolase | 2.9 | 2.6E-06 | 9.6 | 2.1E-10 |
| At5g52570 | BETA-OHASE 2 (BETA-CAROTENE HYDROXYLASE 2) | 2.9 | 1.1E-05 | 7.2 | 7.2E-09 |

| | | | | | |
|---|---|---|---|---|---|
| At5g52410 | CONTAINS InterPro DOMAIN | 2.9 | 1.3E-05 | 6.8 | 1.1E-08 |
| At1g65890 | AAE12 (ACYL ACTIVATING ENZYME 12) | 2.9 | 9.3E-05 | 4 | 7.4E-06 |
| At2g30040 | MAPKKK14 serine/threonine kinase | 2.8 | 2.6E-05 | 9 | 3.2E-09 |
| At2g28400 | unknown protein | 2.8 | 3.2E-05 | 3.4 | 4.9E-06 |
| At5g40350 | MYB24 (myb domain protein 24) | 2.7 | 3.3E-04 | 22.9 | 8.9E-10 |
| At1g76380 | DNA-binding bromodomain-containing protein | 2.7 | 1.6E-05 | 8.3 | 2.0E-09 |
| At3g22160 | VQ motif-containing protein | 2.7 | 3.6E-05 | 7.5 | 8.3E-09 |
| At4g08870 | (ARGAH2) ARGININE AMIDOHYDROLASE 2 | 2.7 | 2.1E-04 | 4.2 | 5.7E-06 |

## Discussion

This study provides support for wound activated surface potential changes (WASPs) as long distance wound signals that activate JA synthesis. Studies performed so far have focused mainly on mechanically damaged leaves (Reymond et al. 2000, Yan et al. 2007, Glauser et al. 2008, Miller et al. 2009) or damage induced by insect herbivores (Reymond et al. 2000, Reymond et al. 2004) without regard to vascular connections. Glauser et al. (2009) and Chauvin et al. (2012) did, however examine the importance of interleaf connections. It is critical to take these into account in long distance signalling studies performed on rosettes. In the present work we focused on the analysis of the transcriptome of a current injected leaf and of a leaf that was connected through vasculature to a wounded leaf. We also compared our work to the wounded rosette transcriptome from Kilian et al 2007.

Leaf 13, which is connected through the vasculature to leaf 8, receives WASPs from leaf 8 when this leaf is severely damaged. Consistent with this, leaf 13 is known to make higher amounts of JA and JA-Ile than do non-connected or unwounded leaves (Glauser et al. 2009). The expression level of JAZ10 in leaf 13 after wounding leaf 8 was found to be increased by more than 100-fold with respect to unwounded leaves (Fig. 4. 4 in chapter 4). In addition, both the wounded leaf 8 and leaf 13 displayed strong depolarisations after wounding (Fig. 4. 3 chapter 4). However, in the wounded leaf, many additional factors are released from broken cells, and lysed vacuoles or cell wall components could, in theory

influence wound responses. Reactive oxygen species (ROS) and ions from the extracellular matrix (apoplast) might also have an impact the wound response in the vicinity of damaged cells (Maffei et al. 2007). Therefore, in the wounded leaf, wound responses are probably not exclusively due to the wound long distance signalling.

Among the 196 transcripts that exhibited upregulation in all three treatments, (Table 6.1), were those encoding JA biosynthesis genes, such as OPDA-reductase (OPR3), lipoxygenase2 (LOX2) and LOX3 and LOX4, allene oxide cyclase 3 (AOC3) and allene oxide synthase (AOS). The upregulation of these genes is consistent with the known activation of JA biosynthesis distal to the wound. In addition, the expression of 9 out of 12 JAZ genes in response to wounding and current injection indicates that WASPs are likely to be long distance wound signals in Arabidopsis.

Cytochrome P450 transcripts are induced by wounding and can be jasmonate regulated (Koo et al. 2011). CYP94B1, and CYP94C1 showed high expression among the treatments we examined (current injection, leaf 13 and wounded rosette). Koo et al. (2011) showed that CYP94B3 has a physiological role in downregulating jasmonate responses in vegetative tissues. CYP94B3 controls JA-Ile levels through converting JA-Ile to 12-hydroxy-JA-Ile in *Arabidopsis* (Kitaoka et al. 2011). Overexpression of CYP94B3 decreased the level of JA-Ile in the wounded leaves and these plants display phenotypes indicative of JA-Ile deficiency (Koo et al. 2011). Additionally, CYP94C1 is reported to be involved in JA-Ile oxidation in *Arabidopsis* (Heitz et al. 2012).

Current injection stimulated the expression of a large number of genes encoding transcription factors (Table 6.1). These were from the AP2 family, the DREB family, the MYC family, the ERF family, the zinc finger family, the WRKY family, the MYB family and two bHLH families. There were also upregulated members of the bZIP family and NAC family among the three treatments. However, some zinc finger family transcription factors showed downregulation. ANAC019 from the NAC family regulates JA-induced

expression of defence genes (Bu et al. 2008). MYB transcription factors regulate hair differentiation and trichome initiation and also regulate anthocyanin biosynthesis (Zhu et al. 2009, Qi et al. 2011). SA, JA, ethylene, and pathogen attack can induce AP2 transcription factor gene expression (Maleck et al. 2000, Schenk et al. 2000). *RAP2.6* showed a high induction after current injection as well as in leaf 13 of plants wounded on leaf 8. *RAP2.6*, an AP2/ERF family member, is responsive to abscisic acid (ABA) and has important roles in many biological functions including response to biotic stress such as high salt, osmotic stress, and cold (Zhu et al. 2010). RAP2.6 is also induced upon pathogens infections (Chen et al. 2002).

The SPs recorded in the current-injected leaf were similar to those recorded in leaf 13 when leaf 8 was wounded (Table 5.2 and Fig. 5.2). While, in leaf 13, 473 genes were upregulated that are not in common with current injected leaf. Just 4% (19 genes) of downregulated genes were in common between leaf 13 and current injected leaf. These differences of unregulated genes can be explained by; 1) the smaller amplitude of SPs generated in the current injected leaf in compared to WASPs recorded in leaf 13 after wounding leaf 8. 2) Other signals in addition to WASPs are probably involved in long distance wound signalling. 3) WASPs may be the first long distance signal after wounding to reach connected leaves, then other signals might amplify WASP effects.

We have devised strategies with which we can investigate the role of electrical signals in controlling defence gene expression in *Arabidopsis*. Our results indicate that the electrome produced in response to WASPs and SPs generated in response to current injection are highly similar. Furthermore, a large proportion of transcripts induced by these stimuli are known to participate in JA synthesis and signalling. We have confirmed that WASPs have pivotal roles in long distance wound signalling in *Arabidopsis*.

# Chapter 7

# General Conclusions and perspectives

The nature of the long distance signals that activate defence responses after wounding in plants is largely unknown. Signalling substances such as hormones can act in parts of the plant other than where they are synthesized. Either they themselves move through the plant or they are made in response to the arrival of other signals. Among the possible long distance signal mechanisms in plants, electrical and hydraulic signals are potentially the fastest. The transport of active chemical signals or their precursors is a slower means of information transfer from leaf to leaf. Our evidence from this study points towards the idea that long distance wound signalling over centimetre distances in plants depends on electrical activity as it does in animals. However, many questions remain regarding the initiation, propagation and decoding of electrical activity in plants.

The non-invasive measurement of surface potential changes is suitable to follow wound-associated electrical activity after herbivory or mechanical wounding. The advantage of this technique that we used in our work is its simplicity and the feasibility of recording electrical activity in plants without wounding. However, this technique clearly has some limitations. Recording of electrical activity from the surface of leaf does not reflect what happens in single cells. Moreover, the surface covered with surface potential recording consists of different cell types including mesophyll, parenchyma, guard cells, phloem or

xylem cells. Single-cell recording with aphids for example using electrical penetration graphs (EPG) (Tjallingii 1988) or glass microelectrodes could disclose the type of cells that are involved in the process of generation, propagation or decoding of electrical activity. In addition, the main route of signal transmission could be investigated with these techniques. For example, do WASPs move through the apoplast or symplast, the phloem or xylem?

Wounding initiates complex reactions in the wounded zone including membrane depolarization and electrical activity. Wounding even a few cells triggers JA synthesis and defence responses. Small wounds only influence the neighbouring cells, while severe wounds have effects over much of the plant. Severe leaf damage by herbivores induces electrical activity while, in nature, most insect herbivores feed only on small parts of the leaf then move to other leaves. This is in part due to toxic defence compounds produced in and near the wound which prohibit insect feeding or at least reduce feeding duration. During evolution these compounds have probably reduced the occurrence of big wounds and long distance signalling to stimulate defence responses in distal leaves. Further investigation of surface potential changes (SPs) in response to herbivore wounding and its consequences for the activation of the JA pathway should help to better understand how plants defend themselves against attack. Furthermore, the possible effects of insect saliva compounds on ion channels and electrical activity should be investigated in order to reveal more about plant-herbivore interactions. Finally, it would also be interesting to know whether the depolarization phase of SPs is a stimulant that causes insects to move from one leaf to another.

Membrane depolarization is one of the earliest events after wounding in plants (Maffei et al. 2007). We found that in A*rabidopsis* wounding alters surface potentials in the wounded leaf, likely due to depolarization of the plasma membrane. Distal leaves that are connected through the vasculature to damaged leaves also receive SPs with the same polarity, while membranes in non-connected leaves are slightly hyperpolarized. The leaves that showed depolarization or hyperpolarization after wounding showed correspondingly large or small

increases in *JAZ10* expression levels respectively. In the non-connected leaves it was not clear whether the slight increases in *JAZ10* transcript levels that we detected were caused by hyperpolarization or by other unknown signals such as diffusion of JA or its precursors, hydraulic signals or ROS. It was shown that leaf-to-inflorescence stem wound signalling occurs (Miller et al. 2009). The present study has only investigated WASP changes in the rosette of *Arabidopsis*. Therefore, a study of the spatial and temporal dynamics of changes in the inflorescence stem and measuring JA and the expression of JA-responsive genes like *JAZ10* could expand our knowledge of long distance wounding signalling.

The velocity of WASPs in the wounded leaf was 8.5 cm $min^{-1}$, and from wounded to connected leaves was 5.5 cm $min^{-1}$. The speed of the long distance signal from the wounded leaf to connected leaves that lead to the activation of JA synthesis was shown to be in the range of 3-8 cm $min^{-1}$ (Glauser et al. 2009, Chauvin et al. 2013). This velocity was decreased to 2.1 cm $min^{-1}$ in *fou2*, the ion channel mutant in *TPC1*. Therefore, it is possible that *TPC1* plays a role in WASP propagation. This would need to be investigated in a *tpc1* null mutant.

Vascular connections play important roles in the transportation of photosynthetic products, water, nutrients and hormones in plants. The importance of n+5 and 5+8 vascular connections in the induction of JA synthesis was demonstrated previously (Glauser et al. 2009). Furthermore, we found that an n+3 contact parasthichy can also transmit long distance electrical signals in *Arabidopsis*. The current study did not investigate the structural architecture of this new transmission route in *Arabidopsis*. In addition, we found that an n-2 connection in *Arabidopsis* which has not previously been defined in this plant can be active in severe damage, while n+2 did not show changes either WASP or *JAZ10* transcript levels after wounding.

Our study has uncovered the casual relation between surface potential changes (SPs) and the activation of the JA pathway. Generation of SPs by current injection (ci) and induction

of *JAZ10, VSP2* and JA and JA-Ile reveal a fascinating relationship between physical signals and physiological function in plants. Furthermore, the induction of many genes by ci, many of them in the JA biosynthetic or signalling pathways by supported the role of WASPs as long distance wound signals in plants. In addition, genes which are involved in stress responses such as transcriptional factors or in ethylene response were regulated by ci. Finally, our results suggested that the electromes that resulted from ci and wounding are essentially identical. However, the functions of some ci-inducible genes remain unknown. Therefore, it will be important to analyse their function. They might play roles in other physiological responses. Besides global gene analysis after ci, it will be worth conducting global metabolite analysis after this procedure.

We show that genes annotated as calcium channels, Glutamate Receptor-Like (*GLR3.1, GLR3.2, GLR3.3* and *GLR3.6*) are involved in long distance electrical signalling in *Arabidopsis* and that the combined mutation of both *glr3.3* and *glr3.6* can almost abolish WASP propagation to organs distal to wounds. GLR proteins can now be indicated in our general model of wound signalling in *Arabisopsis* as shown in figure 7.1.We hope that further tests on other alleles in these mutants and additional double mutants will reconfirm and extend the roles of these channels in WASP generation and propagation. Further studies are needed to determine whether these genes are involved in $Ca^{2+}$ movement across the membrane. To expand our knowledge we are planning to use a triple mutant that we made from the combination of *glr3.1, glr3.3* and *glr3.6* or *glr3.2, glr3.3* and *glr3.6*. In addition, measuring the level of JA and JA-Ile in the wounded and connected leaves at early and late stages after wounding could allow us to establish the kinetics of JA accumulation in relation to WASPs. Furthermore, the transcriptome of wounded leaf 8 and connected leaf 13 could reveal the genes that are specifically regulated by WASPs. Finally, slight induction (2-3 fold) of *JAZ10* in a connected leaf of *glr3.3glr3.6* which did not receive WASPs suggests that there is at least another long distance wound signal. However, here we showed that an electrical signal is the major long distance signal after wounding.

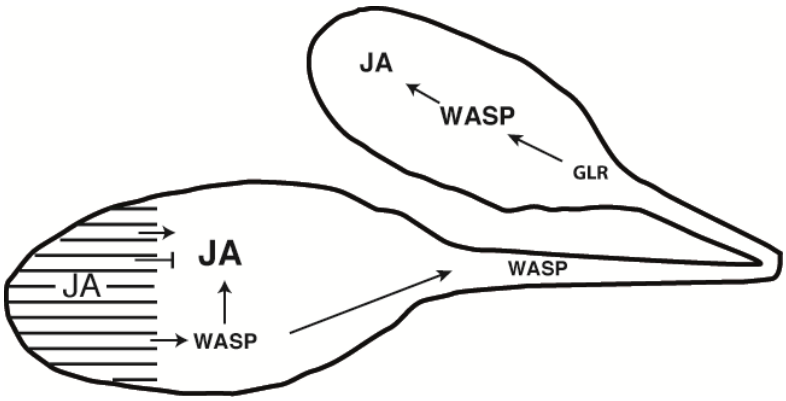


**Figure 7. 1. GLR proteins in the general scheme of events leading to the distal activation of the JA pathway after wounding.** WASPs are wound-associated surface potential changes. We have placed GLRs in the propagation route of the long distance wound signal. More work is needed to clarify if GLRs also contribute to the initiation of long distance wound signalling.

Electrical signals in plants are mainly categorized as action potentials (APs) or variation potentials (VPs). The surface potentials that we recorded after wounding were similar to VPs which are proposed to be conducted via plasma membrane proton pumps and hydraulic pressure changes (Fromm and Lautner 2007). However, we found that WASPs of T-DNA insertion lines in plasma membrane (PM) $H^+$-ATPases were not different from those of WT. Infiltration of the lamina or injection into petioles of PM proton pump inhibitors or activators also did not have detectable effects on WASPs. The similarity of amplitude and duration of WASPs from those T-DNA lines to the WT therefore suggested that WASPs might not be variation potentials. In addition, the influence of *GLR* genes in WASP propagation and the fact that they are annotated as $Ca^{2+}$ channels indicates that WASPs might result from APs. However, without identification of function of those GLR gene products it is too early to confirm that WASPs results from AP detection. Chloride and potassium channels might also be involved in AP generation. But, our WASP screening in T-DNA insertion lines did not reveal any differences in $K^+$ and $Cl^-$ channels

mutants. Further work needs to be done to test other T-DNA insertion lines for $K^+$ and $Cl^-$ channels to test the possible role of these ions in WASP generation.

In conclusion, our research showed that electrical activity can influence gene expression and metabolite levels after wounding. We are confident that our research will serve as a base for future studies not only in long distance wound signalling, and it might help in other domains of plant physiology and stress responses.

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