Chapter 5

Analysis of wound stress response in transgenic plants with anti-jasmonate scFv antibodies

One of the main aims of this study was the investigation of wound-stress response in plants deficient in functional jasmonate. Four independent lines of both JAII and JAVII transgenic plants accumulating to a high level anti-JA scFv antibodies in the ER and in the cytosol, respectively, and three independent lines of both ST3 and OCM transgenic plants with a high level of anti-OPDA scFv in the stroma and in the outer membrane of chloroplasts, respectively, have been used for the investigation. Seedlings of F1 generation of transgenic plants were tested by Western blot analysis for their level of antibody expression and high expressing individuals were grown afterwards in the phytochamber under controlled and stable conditions to minimise other stress factors. Wild type grown under the same conditions together with the transgenic plants have been used as a control.

Two types of wounding have been performed (Fig. 5.1). Leaves of three months old transgenic and wild-type plants were wounded while attached to the plants (\textit{in situ}) by thorough perforation of whole leaf surface in direction across main veins, harvested after a defined time and immediately frozen in liquid nitrogen. In the other way of wounding, detached leaves of three months old transgenic and wild-type plants of the F1 generation were wounded by perforation, incubated for a defined time in a petri dish containing water to prevent desiccation stress and frozen in liquid nitrogen. Leaves cut of the plant and immediately frozen in liquid nitrogen were in both types of wounding used as an untreated control. Techniques that allow thorough, highly sensitive and quantitative analysis of plants on a level of their hormonal content (GC-MS/MS) and on a level of gene expression (macroarray analysis) have been adopted for the analysis of the wound response.

5.1 Macroarray analysis of gene expression pattern in response to mechanical wounding

The attack of diverse aggressors elicits induction of diverse specific sets of defence genes but the induction of individual genes may overlap between different defence responses.

A number of methods exist to measure the expression levels of specific genes or to screen for significant differences in mRNA abundance, such as northern blots, differential display, subtractive hybridisation or serial analysis of gene expression [Vedoy et al., 1999]. Recently a new technology has been invented enabling more complex analysis of gene expression – DNA array-based methods. Several reviews
provide a detailed description of these techniques [Carulli et al., 1998], [Lockhart and Winzeler, 2000].

Fig. 5.1 Schematic representation of mechanical wounding of attached and detached leaves.

The principle of nucleic acid array-based methods is simple. An array of DNA (plasmids, PCR products), each corresponding to a specific gene, is immobilised by
spotting in a certain pattern onto a nylon membrane (macroarray) or on a glass surface (microarray). Fluorescently- or radioactively-labelled probe – cDNA, cRNA or RNA – is hybridised with the DNA on the solid surface and the signal for each DNA, detected by high resolution scanner, reflects the abundance of the corresponding messenger RNA in the sample. Alternatively genes are represented by twenty specific oligonucleotides (usually 20-25 bp long) synthesised in situ on a glass surface using photolitography. The array technique is in principle reverse to a northern blot analysis, where immobilised RNA is hybridised with a labelled DNA probe. Factors like labelling method, hybridisation conditions or the sequence of the gene influence the intensity of the probe signal. Therefore, array-based methods reflect only the relative representation of RNA for each gene.

Based on the assumption that genes with similar expression behaviour are likely to be related functionally and on the finding that reliable predictions can only be based on the observation of more than one single gene, it is possible to read out the biological meaning of the expression profile revealed by array-based techniques.

A database of multiple microarray analysis in different organisms was already developed at Stanford University (http://genome-www4.stanford.edu/MicroArray/SMD/index.html). The macroarray technique has been, for instance, used for analysis of jasmonate-responsive genes [Sasaki et al., 2001] or the microarray for differential gene expression analysis upon wounding [Reymond et al., 2000], both in Arabidopsis thaliana. The analysis of expression profiling upon different elicitors revealed surprisingly large number of genes regulated by multiple treatments, indicating a high degree of coordination among different defence signalling pathways [Reymond, 2001].

Rather than broad and global screening of gene expression, a small-scale macroarray for thorough analysis of the expression pattern of transgenic and wild-type tobacco plants in response to mechanical wounding was adopted in this study. Two hundred forty seven genes falling into few groups with different putative physiological functions have been used. The filters include genes with house-keeping functions, stomata specific genes, ABA-inducible genes, embryogenesis relevant genes, potassium channel relevant genes and above all stress-relevant genes of tobacco, potato and tomato. DNA, each corresponding to a certain gene, was spotted on nylon membrane in three different concentrations to exclude selection of false positive genes (Fig. 5.2).

Total RNA has been isolated from unwounded and wounded leaf of transgenic and wild-type plants and oligo (dT)25 Dynabeads have been used for mRNA purification. The principle of the purification is based on base pairing between the poly A residues at the 3’ end of messenger RNA and the oligo dT residues covalently coupled to the surface of the magnetic Dynabeads (dT)25. The mRNA attached to the oligo dT has been reverse-transcribed, cDNA labelled with $[^{33}\text{P}]$dCTP and after the elution from Dynabeads hybridised with the nylon filter. Radioactive images have been obtained with high-resolution scanner. As an example, results of the hybridisation are shown in the Fig. 5.2. Quantification of the signal intensity was carried out using an Array Vision software (Amersham Pharmacia Biotech, USA).

Global normalization has been adopted for normalizing the difference of signal intensity of each nylon filter.
Logarithmical value of ratio of each signal intensity on a filter A to a corresponding signal intensity on a filter B, chosen as the reference filter, has been calculated. The median of the ratios of all spots on the filter A has been determined and the antilogarithmic value represented the normalization factor of the filter A. The relative signal intensity of each spot of the filter A was then calculated as the ratio of each signal to the normalization factor of the filter A. The median of the relative signal intensities for three concentrations of each gene was determined and thus estimated value, called expression level, represents level of the gene expression under given experimental conditions.

![Diagram of macroarray process]

Fig. 5.2 The schematic representation of macroarray used in this study. Purified DNA was spotted on a nylon membrane in a pattern 3x3. DNA, each corresponding to a particular gene, was applied in concentrations 1500 ng, 750 ng and 375 ng in neighbouring patterns. Messenger RNA purified from total RNA via dynabeads was reverse transcribed and cDNA labelled with $[^{33}\text{P}]d\text{CTP}$. For more detailed description and hybridisation conditions see Chapter 7.

The gene expression ratio represents here the ration of expression level of treated to expression level of untreated leaf of the same plant, where treatment is either wounding or application of exogenous methyl jasmonate. Only these EL have been taken into account where the relative signal intensity grew linearly with the corresponding increasing concentration of spotted DNA.

Fig. 5.3 depicts, as an example, two-dimensional logarithmical representation of gene expression of wounded wild-type leaf plotted against unwounded wild-type leaf. The
same representation of wounded leaf of transgenic JAVII plant plotted against unwounded leaf of JAVII plant is also shown.

Fig. 5.3 Scattered plot of gene expression of unwounded and 24 hours wounded detached wild-type leaf (green) and JAVII transgenic leaf (blue). Lines show twofold gene induction (line +2) or repression (line -2).

5.1.1 Macroarray analysis of „in situ“ wounded plants

A wild-type plant of Nicotiana tabacum, v. Samsun NN, JAVII and ST3 transgenic plants have been wounded as illustrated in Fig. 5.1. Gene expression patterns in unwounded leaf and leaf 20 min, 2 hours, 8 hours and 24 hours after wounding have been investigated by macroarray analysis as described above. The comparison of the expression level of corresponding genes of wounded and unwounded leaf revealed unexpectedly no wound induction at any time point examined after wounding. This result has been obtained for wild-type plants as well as for transgenic plants and is in contrast to a strong wound induction in detached leaves (Chapter 5.1.2.). Wounding induction by treatment „in situ“ has been reported in other species, such as Lycopersicum esculentum [Farmer et al., 1992], Solanum tuberosum [Hildmann et al., 1992], Arabidopsis thaliana [Bell et al., 1995] or in other species of tobacco like Nicotiana plumbaginifolia [Tire et al., 1994]. In experiments with Nicotiana tabacum the wound induction has been obtained by slicing leaves (v. Xanti nc [Hirsinger et al., 1997]), punching out leaf discs (v. Samsun NN [Niki et al., 1998]) or treatment with hemostat (v. L.cv.Wisconsin 38 [Laudert et al., 2000]). In the variant Samsun NN of Nicotiana tabacum, known for its increased resistance, the perforation of leaf surface might be not sufficient to elicit a wound response to the extend needed for reproducible analysis.

All other wounding experiments reported further have been performed with detached leaves.
5.1.2 Macroarray analysis of detached wounded tobacco leaves

Three months old F1 plants of each, wild type, JAI, JAVII, ST3 and OCM transgenic plants were wounded as illustrated in Fig. 5.1. Gene expression pattern of unwounded leaf and detached leaf 8 hours and 24 hours after wounding has been analysed by macroarray. Previous wound experiments with *Nicotiana tabacum* showed maximal levels of the investigated defence gene transcripts at time range 8 – 24 hours after wounding [Farmer et al., 1992], [Hirsinger et al., 1997] or [Wang et al., 1999]. Also in preliminary macroarray tests with wild-type detached leaves 20 min, 2 h, 8 h and 24 h after wounding highest levels of transcripts of genes included in this study were detected in range 8 – 24 hours. The only exception was the AOC gene from tobacco, which was maximally induced (about four fold) after two hours in wounded wild type and tested transgenic plants (data not shown). As described above, expression ratio of the expression level of wounded to expression level of unwounded leaf calculated for each corresponding gene represents the induction of the gene after wounding of the particular plant. For simplicity, only those genes for which the transcript level changed substantially as a result of wounding are included here. Among 247 genes tested, 8 genes were identified whose mRNA levels changed repeatedly more than threefold (induction) 8 or 24 hours following wounding. These genes are listed in table 5.4.

Table. 5.4 List of genes with substantially changed transcription abundance after mechanical wounding identified by macroarray analysis.

<table>
<thead>
<tr>
<th>number</th>
<th>gene</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nt-pin2</td>
<td><em>Nicotiana tabacum</em> proteinase inhibitor 2</td>
</tr>
<tr>
<td>2</td>
<td>osmotin</td>
<td>PR-5 – osmotin-like protein</td>
</tr>
<tr>
<td>3</td>
<td>prp1</td>
<td>pathogen-related protein 1</td>
</tr>
<tr>
<td>4</td>
<td>EAS</td>
<td>5-epi-aristolochene synthase</td>
</tr>
<tr>
<td>5</td>
<td>extensin</td>
<td>extensin-like cell wall protein</td>
</tr>
<tr>
<td>6</td>
<td>PR-1b</td>
<td>pathogenesis-related protein PR-1b</td>
</tr>
<tr>
<td>7</td>
<td>SAR 8.2</td>
<td>salicylic acid responsive protein</td>
</tr>
<tr>
<td>8</td>
<td>HMG CoAR</td>
<td>hydroxymethylglutaryl CoA reductase</td>
</tr>
</tbody>
</table>

Results, summarised in Fig. 5.5 and Fig. 5.6, are median of four independent experiments with each, wild type, JAI and JAVII plants, and median of three independent experiments with both ST3 and OCM plants.

Fig. 5.5(a) gives an overview of the induction of transcript level of the 8 identified genes in wild-type leaves (green bars), leaves of JAVII transgenic plants (light blue bars) and leaves of JAI transgenic plants (dark blue bars) 8 hours after wounding. The induction of the 8 genes in the same plants at the time point 24 hours after wounding summarises Fig. 5.5(b). Unlike the wild-type plants, transgenic plants with anti-jasmonic acid scFv
antibodies in the cytosol and in the ER show reduced expression of wound-inducible genes and this effect is more pronounced in plants with the cytosolic localization of anti-JA scFv than in plants with the ER-localised anti-JA scFv. The effect is more evident 24 hours after wounding than 8 hours after wounding.

Fig. 5.6 shows induction of the same 8 identified genes in wild-type leaves (green bars), leaves of ST3 transgenic plants (light red bars) and leaves of OCM transgenic plants (dark red bars) 8 hours (a) and 24 hours (b) following mechanical wounding. The macroarray analysis revealed wild-type-like levels of wound inducible genes in transgenic plants with anti-OPDA scFv in the stroma and outer membranes of chloroplasts.

![Fig. 5.5 cDNA macroarray analysis of gene expression at 8 hours (a) and 24 hours (b) after mechanical wounding.](image)

Fig. 5.5 cDNA macroarray analysis of gene expression at 8 hours (a) and 24 hours (b) after mechanical wounding. Expression ratio (ER) represents the wound-induction of above listed genes in wild type (green), JAVII transgenic (light blue) and JAIL transgenic (dark blue) plants. Description of the genes is given in the Table. 5.4.

### 5.1.3 Northern blot analysis of detached wounded tobacco leaves.

Three genes from the 8 selected genes have been used as probes for northern blot analysis to verify the results of macroarray analysis. The expression profile of these three genes represented a wide range in transcript abundance between 24 hours-wounded wild type and transgenic plants. While Nt-pin 2 gene expression of wounded JAVII (or JAIL) plants was dramatically different from that of wounded wild type, the osmotin gene transcript levels differed only moderately and no dissimilarities in the extensin gene expression were found, Fig. 5.5 (b). Coding sequence of the genes Nt-pin 2, osmotin and extensin have been cleaved with the suitable restriction enzymes, labelled with \[\alpha^{-33}P\]dCTP and hybridised with 10 µg of total RNA transferred on nylon filter. The total RNA has been isolated from unwounded and 24 hours-wounded leaf of both wild type and transgenic plants. The northern blot analysis was performed for three independent lines of each, wild type, JAVII, JAIL, ST3 and OCM transgenic plants.
Fig. 5.6 cDNA macroarray analysis of gene expression at 8 hours (a) and 24 hours (b) after mechanical wounding. The expression ratio (ER) represents the wound-induction of the selected genes in wild type (green), ST3 transgenic (light red) and OCM transgenic (dark red) plants. Description of the genes is given in the Table 5.4.

The macroarray analysis of the three genes is presented in more details here for the comparison of the gene expression profile given by macroarray and by northern blot analysis.

Fig. 5.7(a), (b) and (c) shows the expression profile of the Nt-pin 2, osmotin and extensin gene, respectively, in wild type and transgenic plants after mechanical wounding revealed by macroarray. As shown already in Fig. 5.5 and Fig. 5.6, the expression ratio represents wound induction and it is a median value of four experiments with wild type, four experiments with JAVII and JAIL plants, and three experiments with each, ST3 and OCM transgenic plants. The median values ± standard deviation are also included.

Fig. 5.8 (a), (b) and (c) shows the expression profile of the Nt-pin 2, osmotin and extensin gene, respectively, in wild type and the transgenic plants after mechanical wounding obtained by northern blot.
Fig. 5.7 The expression profile of the Nt-pin 2 (a), osmotin (b) and extensin (c) gene in unwounded (U) and 8h or 24h wounded leaves of wild type and transgenic plants. Result of cDNA macroarray analysis. The y-axis on the left corresponds to the expression ration (ER) and the y-axis on the right to the median ± SE of 3-4 independent experiments.

Fig. 5.8(a) The expression profile of the Nt-pin 2 gene in wild type and transgenic plants after mechanical wounding. Result of northern blot analysis. Total RNA was isolated from unwounded leaf (U) or 24 hours-wounded leaf (W) of wild type (WT), transgenic plants with anti-JA scFv in the cytosol (JAVII) or in the ER (JAIi) and transgenic plants with anti-OPDA scFv in the stroma (ST3) or outer membrane (OCM) of chloroplasts.
Fig. 5.8(b) The expression profile of the osmotin gene in wild type and transgenic tobacco plants after wound induction. Result of northern blot analysis. For explanation of abbreviations see Fig. 5.8 (a).

Fig. 5.8(c) The expression profile of the extensin gene in wild type and transgenic plants after mechanical wounding. Result of northern blot analysis. For explanation of abbreviations see Fig. 5.8 (a).

Results of the northern blot analysis of wounded transgenic and wild-type plants confirmed the expression profile of the selected genes revealed by the macroarray. In comparison to wild type, the expression level of the Nt-pin 2 gene was significantly reduced, the expression of the osmotin gene slightly reduced and of the extensin gene was rather alike in 24 hours-wounded leaves of transgenic plants with the intracellular accumulation of anti-jasmonic acid scFv. Wounded leaves of plants accumulating anti-12-oxo-phytodienoic acid scFv showed wild-type-like levels of the Nt-pin 2 gene and the osmotin gene. The expression of the extensin gene was moderately higher in ST3 plants compared to the wild type.

5.1.4 Normalization of the wild-type-like levels of gene expression by exogenous application of methyl jasmonate

Transgenic plants accumulating anti-jasmonic acid scFv antibodies in the cytosol and in the ER of tobacco cells exhibit reduced expression of several wound-inducible genes in comparison to wild-type plants (Fig. 5.5, 5.7, 5.8). In order to determine whether this
effect is a consequence of jasmonate deficiency in subcellular compartments caused by the ectopic expression of anti-JA antibodies, a normalization experiment has been performed. Detached leaves of JAVII and JAII transgenic plants and wild-type plants were incubated for 24 hours and 48 hours in petri dishes containing either 200 µM concentration of methyl jasmonate or containing only water. Untreated leaves were also harvested from the same plants. Total RNA has been isolated from these leaves and used for both macroarray analysis (only transgenic plants) and northern blot analysis (transgenic plants and wild type).

The macroarray has been done as described above and in Chapter 7. For each plant calculated expression ratio of the expression level of JAME-treated (or water-treated) to the expression level of untreated leaf for each corresponding gene represents the induction of the gene after JAME (or water) treatment. Only those genes for which the transcript level changed at least threefold as a result of JAME (or water) treatment are included here.

Fig. 5.9 summarises the macroarray of two JAVII lines (a) and two JAII lines (b) after JAME (or water) treatment for 24 hours and 48 hours. Fig. 5.10 shows in more detail the expression profile of the Nt-pin 2 gene in JAVII and JAII transgenic plants obtained by the macroarray. In both Fig. 5.9 and Fig. 5.10, the expression ratio represents the median values of two experiments with each, JAVII and JAII transgenic plants.

Fig. 5.9 cDNA macroarray analysis of JAME-treated and water-treated leaves of transgenic plants. Expression ratio (ER) represents the median of two experiments with JAVII plants (a) and two experiments with JAII plants (b). Description to the numbers of genes is given in the Table. 5.4.
The northern blot analysis has been done as described in Chapter 7. Labelled DNA of the Nt-pin 2 gene has been used as a probe. Three independent lines of each, JAVII transgenic plants, JAII transgenic plants and wild type were analysed. Fig. 5.11 shows the expression profile of the Nt-pin 2 gene in wild type and transgenic plants revealed by northern blot.

![Northern blot analysis](image)

**Fig. 5.11** The expression profile of the Nt-pin 2 gene in wild type and transgenic plants after JAME (or water) treatment. Results of northern blot analysis. Analysis of three wild-type plants (WT) and three transgenic plants accumulating anti-JA scFv either in the cytosol (JAVII) or in the ER (JAII) are presented. Leaves of each plant were treated for 24 hours and 48 hours in jasmonic acid methyl ester or in water, as a control.

The correlation between results obtained by macroarray and by northern blot analysis of the Nt-pin 2 gene expression profile after JAME treatment can be read from the comparison of the Fig. 5.10 and Fig. 5.11. Like in wild-type plants, treatment of JAVII transgenic plants with JAME causes strong induction of the Nt-pin 2 gene expression. In contrast, in JAME-treated JAII transgenic plants the Nt-pin 2 gene was always induced.
only to half level of that of WT and JAVII plants. The Nt-pin 2 transcript abundance in water- and JAME-treated JAI leaves was rather similar.

5.1.5. Expression profile of the PR-1b gene in detached tobacco leaves.

The pathogenesis-related protein PR-1b, believed to be induced by salicylic acid, was found to be synergistically induced by ethylene and methyl jasmonate [Xu et al., 1994] and induced by wounding [Wang et al., 1999], see also Chapter 6. Rather weak wound induction of the PR-1b gene in detached leaves of wild-type tobacco plants and transgenic plants with anti-12-oxo-phytodienoic acid scFv antibodies in the outer chloroplastic membrane was observed in this study (Fig. 5.6). No wound-induction of PR-1b gene in plants accumulating anti-jasmonic acid scFv antibody in the cytosol and in the ER could be detected (Fig.5.5). However, transgenic plants expressing anti-OPDA recombinant antibodies in the stroma of chloroplasts had increased levels of the PR-1b gene in unwounded leaves and wound induction caused a decrease in the PR-1b transcript level. This is in contrast with an absence of the PR-1b transcript in healthy tobacco tissue [Sano et al.,1996]. Fig. 5.12 shows the induction of the PR-1b gene during 24 hours after wounding in transgenic tobacco plants with anti-OPDA scFv in the stroma of tobacco chloroplasts in comparison to wild-type plants, detected by macroarray. Northern blot analysis of unwounded wild type and ST3 transgenics is also shown in the Fig. 5.12. The macroarray has been done as described above and in Chapter 7, with the following exception. Instead of expression ratio defined at the beginning of Chapter 5.1, the expression level of transgenic and wild-type plant was compared. The northern blot analysis was performed as described in Chapter 7.

![Fig. 5.12 Kinetics of the induction of the PR-1b gene in unwounded (U) and wounded detached leaves of transgenic tobacco plants with anti-12-oxo-phytodienoic acid antibodies in the stroma of chloroplasts (ST3) compared to wild-type plants (WT). Results of macroarray analysis (a). The northern blot (b) shows the PR-1b gene expression of unwounded detached leaves of three wild type and three ST3 transgenic plants.](image-url)
5.2 Analysis of fatty acids by gas chromatography

It is generally assumed that octadecanoids are produced from α-linolenic acid. Linolenic acid is a polyunsaturated fatty acid released from membrane-bound lipids probably by enzymatic activity of phospholipase A$_2$, which is induced by stimuli like wounding, systemin or oligosaccharides [Naváez-Vásquez et al., 1999]. Dioxygenation of linoleic and α-linolenic acids by lipoxigenase creates hydroperoxy polyunsaturated fatty acids, which can be further converted in different reactions of the LOX pathway (Fig. 5.13).

The question emerged whether the immunomodulation of the end product of one of the LOX-dependent metabolic rout (the JA biosynthetic pathway) would influence the overall balance in the distribution of PUFAs, leading to the changed content of PUFAs. To answer this question the analysis of fatty acids was performed.

The highest wound-induced JA accumulation in wild-type *Nicotiana tabacum* plants should occur around 2 hours after wounding [Wang et al., 1999] and jasmonate accumulation was expected to have a „spike“ profile [Farmer, 1994], [Prof. C. Wasternack, pers. comm.] indicating decrease of JA to basal levels safely at 24 hours after wounding. Thus content of PUFAs and levels of phytohormones were determined at 2 hours and 24 hours after mechanical wounding.

Analysis of fatty acids was carried out by gas chromatography. Three individual lines of each, wild-type plant, JAII, JAVII, ST3 and OCM transgenic plants were used for the analysis. Untreated leaves and 2h- and 24h-wounded detached leaves of each plant have been extracted (HIP extraction – Chapter 7) with a presence of 10 µg / µl of heptadecanoic acid as an internal standard. Membrane-bound and free fatty acids were transmethylated (Dr. C. Göbel) and methylated, respectively, to increase the volatility of the compounds. Levels of palmitic acid, hexadecenoic acid, stearic acid, oil acid, linoleic acid and linolenic acid were determined.
The ratio of signal intensity of fatty acid of interest to signal intensity of internal standard fatty acid of known concentration represents the amount of fatty acid of interest calculated in nmol per 1g of fresh weight (FW).

Fig. 5.14 and Fig. 5.15 show the levels of free fatty acids and membrane-bound fatty acids, respectively, in wild-type plants and JAVII transgenic plants in unwounded leaf and leaf 2 hours and 24 hours after mechanical wounding. Each bar represents the median of three independent experiments. The Median ± standard deviation is also included.

Fig. 5.14 Levels of free fatty acids (palmitic acid, hexadecaenoic acid, stearic acid, oil acid, linoleic acid and linolenic acid) in wild type (a) and in JAVII transgenic plants (b) measured by gas chromatography. The y-axis on the left corresponds to the levels of free fatty acids and the y-axis on the right to the median ± standard deviation of 3 independent experiments.
Fig. 5.15 Levels of membrane-bound fatty acids in wild type (a) and JAVII transgenic plants (b) measured by gas chromatography. The y-axis on the left corresponds to the levels of membrane-bound fatty acids and the y-axis on the right to the median ± standard deviation of 3 independent experiments.

As seen from Fig. 5.14 and Fig. 5.15 no significant differences in free fatty acid levels and bound fatty acid levels between transgenic plants and wild type were detected. The same result has been obtained also for JAIL, ST3 and OCM transgenic plants (data not shown). The intracellular expression of anti-jasmonate scFv in transgenic tobacco plants does not change the overall content of PUFAs.
5.3 Analysis of phytohormones by mass spectrometry

The inductive control of the jasmonate biosynthetic pathway (Fig. 1.5) and also the results of macroarray analysis (Chapter 5.1) raise questions, such as which mechanisms regulate the jasmonate induction and control its temporal character, and what is the contribution of other phytohormones to the processes.

Mass spectrometry coupled to gas chromatography became in recent years a powerful probe into a black box of endogenous hormonal state of plant tissue under various developmental and environmental conditions. Gas chromatography ion-trap mass spectrometry (GC-MS/MS) has been used in this study for quantitative reproducible analysis of levels of phytohormones in transgenic and wild-type plants and thereby for better insight into events taking place in transgenic tobacco cells expressing anti-jasmonate scFv antibodies.

The analysis of endogenous levels of jasmonic acid, 12-oxo-phytodienoic acid, salicylic acid, abscisic acid and indole-3-acetic acid has been done as described in details in [Müller et al., 2002]. About 0.5 – 1g of leaf tissue was extracted in methanol in the presence of the following internal standards: 10 pmol of $[^2H]_4$-salicylic acid, 30 pmol of $[^{13}C]_2$-jasmonic acid, 25 pmol of $[^2H]_2$-indole-3-acetic acid, 25 pmol of $[^2H]_6$-abscisic acid and 10 pmol of $[^2H]_5$-12-oxo-phytodienoic acid. Samples were cleaned on silica-based aminopropyl matrix to remove lipids and chlorophyll, methylated with ethereal diazomethane and applied on Varian Saturn 2000 ion-trap mass spectrometer connected to a Varian CP-3800 gas chromatograph. Mass spectrometer was working in mode of chemical ionisation – multi reaction monitoring (CI-MRM) with methanol as reactant gas and positive ion detection.

MS/MS-analysis allowed the detection of full scan daughter ions from the characteristic most intense parent ions and thus identifying safely the compounds of interest. Even though MS enables to distinguish between cis- and trans-isomers of JA and OPDA, the sum of the cis- and trans-isofoms of JA and OPDA has been calculated, because the conversion between cis- and trans- isoforms taking place in tissue and during extraction procedure would not allow to determine the exact ratio between both isoforms “in situ” in plants.

The GC-MS/MS analysis was performed with detached leaves of four plants of each, wild type, JAVII, JAI and ST3 plants and three OCM transgenic plants. Levels of JA, OPDA, SA, ABA and IAA were determined in each plant in unwounded leaf and in leaf wounded for 2 hours and 24 hours. Those time points were chosen for the same reason as discussed in Chapter 5.2.

The chromatogram in Fig. 5.16 shows, as an example, relative signal intensities of characteristic ion fragments selected for each phytohormone analysed in 2 hours wounded leaf of wild-type plant (a) and transgenic JAVII plant (b). In Fig. 5.17 (c) relative signal intensities of characteristic ion fragments of corresponding internal standard compounds, obtained in the same analysis, are plotted. The white and grey columns determine retention time and selected mass-to-charge ratio (m/z) of the characteristic daughter ion fragments. In each column, the lower m/z value belongs to
the daughter ion of a particular endogenous compound and the higher m/z value belongs to the labelled internal standard of the same compound.

Fig. 5.16 Chromatograms obtained from GC-MS/MS phytohormone analysis of wild type and transgenic tobacco plant. Relative signal intensities of characteristic daughter ions of five endogenous compounds detected in 2h-wounded leaf of wild-type plant (a) and JAVII transgenic plant (b) are plotted. Relative signal intensities of characteristic ion fragments of internal standard compounds, obtained in the same analysis are shown as well (c). The relative signal intensity was calculated as percent of the measured signal intensity. The grey and white columns determine retention time and selected mass-to-charge ratio (m/z) of the characteristic daughter ion fragments. In each column, the lower m/z value belongs to the daughter ion of a particular endogenous compound and the higher m/z value belongs to the labelled internal standard of the same compound. 1 – SA, 2(a) – unnatural 9E-jasmonic acid detected only in standard samples, 2(b) – (3R,7R)-jasmonic acid, 2(c) – (3R,7S)-jasmonic acid, 3 – IAA, 4 – ABA, 5(d) – (9S,13R)-12-oxo-phytodienoic acid, 5(e) – (9S,13S)-12-oxo-phytodienoic acid.

The ratio of signal intensity of unlabelled compound to signal intensity of recovered labelled compound of known molarity represents the amount of endogenous compound of interest calculated per 1g of fresh weight.

Fig. 5.17 summarises the kinetic of 12-oxo-phytodienoic acid levels (a) and jasmonic acid levels (b) during 24 hours following wounding (at time points 0 h, 2 h and 24 h.
after wounding) of leaves of wild type, JAVII, JAI, ST3 and OCM transgenic plants. Similarly, Fig. 5.18 gives an overview of kinetic of salicylic acid levels (a), abscisic acid levels (b) and indole-3-acetic acid levels (c) in time range 24 hours after mechanical wounding of wild type and the transgenic plants.

Different behaviour of each analysed phytohormone has been observed in wounded tobacco plants. Mechanical wounding causes always a transient increase in 12-oxo-phytodienoic acid levels, which are, in comparison to wild type, slightly higher in JAVII, JAI and ST3 transgenic plants and slightly lower in OCM plants (Fig. 5.17(a)). A transient increase in jasmonic acid levels in wild-type plants 2 hours after wounding is negligible in comparison to the dramatic increase in JA levels in JAVII, JAI, ST3 and OCM transgenic plants. OCM plants have a moderate wound-inducible induction of JA (Fig. 5.17(b)).

The pattern of wound-induced changes in endogenous levels of salicylic acid, Fig. 5.18(a), is approximately opposite to the kinetic of jasmonate levels. Wounding causes a significant increase in SA levels above basal levels only 24 hours after wounding, when jasmonate levels decrease to basal levels. Interestingly the kinetic of salicylic acid following wounding is also opposite to changes of abscisic acid levels,
which are very high in untreated plants and decrease after mechanical wounding (Fig. 5.18(b)). Finally, endogenous levels of indole-3-acetic acid show a transient decrease in correlation with the increase in the jasmonate levels at 2 hours after wounding (Fig. 5.18(c)).

To summarize, the wound-induction of jasmonic acid and 12-oxo-phytodienoic acid differ between wild type and transgenic plants, accumulating anti-jasmonate scFv antibodies, significantly. The kinetic of salicylic acid, abscisic acid and indole-3-acetic acid levels is approximately similar between wild-type plants and all transgenic plants.

Fig. 5.18 Kinetic of salicylic acid levels (a), abscisic acid levels (b) and indole-3-acetic acid levels (c) at time points 0 h, 2 h and 24 h after mechanical wounding of wild type, JAVII, JAII, ST3 and OCM transgenic plants. The y-axis on the left corresponds to levels of the endogenous phytohormones and y-axis on the right to standard deviation of 3-4 independent experiments.