Immunomodulation of jasmonate functions

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Immunomodulation of jasmonate functions

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Our freedom extends only as far as our consciousness reaches.

Carl Gustav Jung

List of abbreviations

ALP ..... alkaline phosphatase

ABA ..... abscisic acid

amp^r ..... ampicilin resistance

anti-JA scFv ..... anti-jasmonic acid single-chain Fv antibodies

anti-OPDA scFv ..... anti-12-oxo-phytodienoic acid single-chain Fv antibodies

AOC ..... allene oxide cyclase

AOS ..... allene oxide synthase

BSA ..... bovine serum albumin

c-myc ..... c-myc tag sequence

CaMV35S ..... Cauliflower mosaic virus 35S promoter

CDR ..... complementary determining region

diHZR ..... dihydro-zeatin riboside

E.coli ..... *Escherichia coli*

ELISA ..... enzyme-linked immunosorbent assay

ER ..... endoplasmic reticulum

EREBP ..... ethylene-responsive element binding protein

FITC ..... fluorescein isothiocyanate

GC ..... gas chromatography

IAA ..... indole-3-acetic acid

JA ..... (3R,7R)-jasmonic acid

JAME ..... (3R,7R)-jasmonic acid methyl ester

JAII ..... transgenic plants with anti-JA scFv targeted into the ER

JAVII ..... transgenic plants with anti-JA scFv targeted into the cytosol

KDEL ..... retention sequence

LeB4SP ..... legumin B4 signal sequence

LOX ..... lipoxygenase

MAPK ..... mitogen-activated protein kinase

MS ..... mass spectrometry

OCM ..... transgenic plants with anti-OPDA scFv targeted into the

outer chloroplast membrane

OCMTP ..... outer chloroplast membrane transit sequence

OPDA ..... (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid

OPR ..... OPDA-reductase

ori ..... origin of replication

PCR ..... polymerase chain reaction

Pin 2 ..... proteinase inhibitor 2 protein

poly A ..... polyadenylation signal

PR ..... pathogenesis-related

PUFA ..... polyunsaturated fatty acid

ROS ..... reactive oxygen species

rpm ..... revolutions per minute

SA ..... salicylic acid

SAR ..... systemic acquired resistance

scFv ..... single-chain variable fragment

ST3 ..... transgenic plants with anti-OPDA scFv targeted in the stroma of chloroplasts

ST3TP ..... chloroplastic stroma transit sequence

TSP ..... total soluble protein

WT ..... wild type

Contents

Chapter 1

Plant defence mechanisms

Every organism has developed during evolution a range of mechanisms against adverse environmental conditions or infectious organisms. There are intriguing parallels between animal and plant defence responses as demonstrated by the structural and functional conservation of some of their signal transduction processes.

Plants have to cope with various forms of stress stimuli, and these are biotic stresses and abiotic stresses. Biotic stresses include bacterial, viral or fungal attack. The causes of the abiotic stress can be mechanical wounding, wounding by insects or herbivores, high salinity, high or low osmolarity, extreme temperature, drought, ozone, reactive oxygen species or UV illumination. Recent studies have revealed strong parallels in the signalling machineries plants use to respond to different forms of stress stimuli [Farmer, 2000]. The defence response is a both time and space controlled complex signalling network leading to host defence-genes expression. The nature of the physical injury and the nature of pathogen-encoded molecules – elicitors – are two variables that modulate which set of defence-related genes will be expressed and to which extend. The defence response occurs not only in local attacked tissues but is triggered also in other healthy tissues as systemic response.

Fig. 1.1 summarises known defence signalling pathways [Pieterse and Van Loon, 1999]. Mechanical wounding or wounding by insects or herbivores triggers the wound response pathway where the signalling is mediated by plant regulators – jasmonic acid and ethylene. Also in induced systemic resistance the response is mediated by jasmonic acid and ethylene but, unlike the wound response pathway, the induced systemic resistance seems to be associated with an increase in sensitivity to jasmonic acid and/or ethylene rather than an increase in their production. Moreover the induced systemic resistance signalling pathway is dependent on NPR1 protein, which regulates also pathogen elicited responses. Thus the induced systemic resistance might activate a different set of defence genes than the wound response pathway [Pieterse and Van Loon, 1999], [Pieterse et al., 1998]. Pathogen elicitors initiate the induction of systemic acquired resistance where the key secondary signal is salicylic acid.

The terminal step in the defence signalling cascade is the activation of defence genes, called pathogenesis-related genes, that encode pathogenesis-related proteins. There are several groups of PR-proteins and their expression can be induced by different signalling molecules, such as salicylic acid, jasmonic acid or ethylene. Generally, genes encoding acidic PR-proteins (acidic PR-1, PR-2 and PR-3 gene families) are induced by salicylic acid while genes encoding basic PR-proteins (basic PR-1, PR-2, PR-5 and PR-6 gene families) are induced by jasmonic acid. Moreover, other plant hormones are involved. While ethylene could work co-operatively with jasmonic acid in activation of basic PR-genes, cytokinines and auxines inhibit the expression of basic PR-genes and activate acidic PR-genes, as salicylic acid does [Niki et al., 1998].

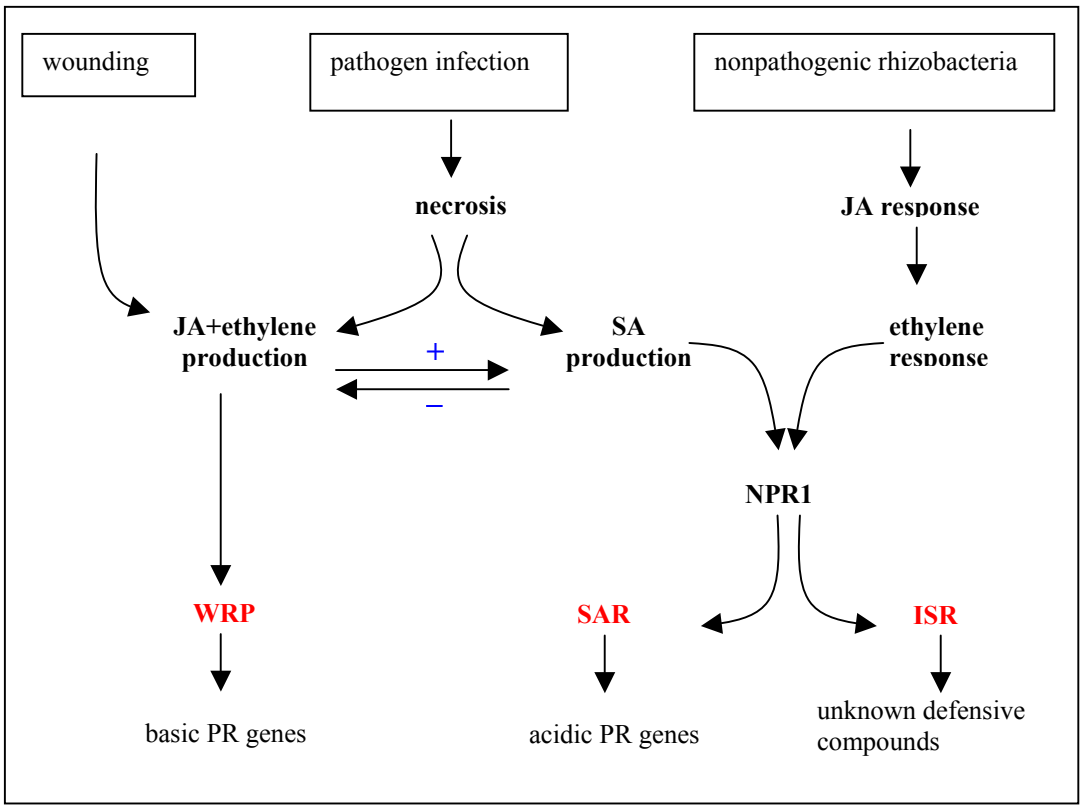


Fig. 1.1 Distinct systemic signalling pathways that can be induced in plants in response to various elicitors. Modified from [Pieterse and Van Loon, 1999]. WRP – wound response pathway, SAR – systemic acquired resistance, ISR - induced systemic resistance. For details see text above.

The differential regulation of a varying subset of PR-genes by different signalling molecules is dependent on the elicitor of the defence response and is mediated by the interaction between activated transcription factors and responsive *cis* elements in the promoters of defence genes. A single pathogen elicitor may activate multiple transcription factors that interact with different *cis* elements in the same or different promoters, leading to induction of many defence genes. Some of these transcription factors are activated by pathogen infection, wounding or by secondary messengers, like SA, JA or ethylene. Promoter regions of many PR-genes contain the GCC-box, which is recognized by members of the EREBP family of DNA-binding proteins. Another *cis* acting element is the W-box containing hexamer TTGACC sequence or two tetramer sequences TGAC-N_x-GTCA. These W boxes are binding sites for members of the WRKY family of transcription factors. Another class of *cis* elements are the P-box, L-box or H-box, recognized by MYB-like proteins. Finally, G-boxes (CACGTG), which are often in promoter region of genes regulated upon environmental cues like wounding or upon ABA-treatment, and as-1-like elements, which confers responsiveness to SA, JA, auxin and hydrogen peroxide, are bound by bZIP transcription factors (for review see [Rushton and Somssich, 1998]).
The signalling cascade upon pathogen attack (systemic acquired resistance) is briefly reviewed in Chapter 1.1 and the wound responsive pathway will be discussed in detail in the following Chapter 1.2 .

1.1 Systemic acquired resistance

The first step of the defence response to a pathogen is the recognition of pathogen elicitors. While many bacterial or fungal proteins or oligosaccharides are recognized non-specifically, other elicitors encoded by pathogen *avr* genes match with host receptors, encoded by the R gene [Nürnberger and Scheel, 2001].
Both interactions, incompatible and compatible, are manifested on macroscopic level as a hypersensitive response, which is characterised by necrotic lesions resulting from localised host cell death at the site of infection. The hypersensitive response lesions formation is connected to the accumulation of salicylic acid, jasmonic acid and also hydrogen peroxide [Kenton et al., 1999].
The host recognition of pathogen elicitors initiates early signalling events, such as protein phosphorylation/dephosphorylation, ion flux and accumulation of reactive oxygen species, like superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Subsequent transcriptional and posttranslational activation of transcription factors leads to induction of plant defence genes and biosynthesis of endogenous secondary signals [Yang et al., 1997]. There is a number of secondary signals found to have a role in plant pathogen defence (for review see [Bennett and Wallsgrove, 1994]). This includes cyanogenic glucosides, glucosinolates, alkaloids, plant phenolics, phytoalexins, plant terpenes and above all salicylic acid and jasmonic acid. The role of secondary metabolites involves anti-fedant activity, toxicity or signalling activity either for selfinduction of secondary metabolites themselves [Blechert et al., 1995] or for the expression of defence related genes. Salicylic acid is necessary, and in some plant species (tobacco, A*rabidopsis*) also sufficient, for the induction of pathogen-induced SAR. SA is synthesized via phenylalanine and the first step of the conversion is catalysed by the enzyme phenylalanine ammonia-lyase, which can be induced by SA (for review see [Raskin, 1995]). Methyl salicylate, a volatile compound synthesized from salicylic acid, can induce SAR in neighbouring and in healthy tissues by conversion back to salicylic acid [Shulaev et al., 1997]. Also a SA-binding protein SABP2 has been recently identified in tobacco leaves. However, it still remains to be elucidated how salicylic acid mediates the SAR induction.
Even though salicylic acid is required for resistance to many pathogens, SAR can develop independent of salicylic acid in some cases. For instance, infection of tobacco by the soft-rot pathogen *Erwinia carotovora* activates basic ß-1, 3-glucanase and basic chitinase, which are induced independently of salicylic acid and SA inhibits their expression [Vidal et al., 1997]. In another study, an A*rabidopsis* mutant, deficient in jasmonate synthesis, was extremely susceptible to the fungal root pathogen *Pythium mastophorum* and synthesis of defence antifungal proteins encoded by the defensin PDF1.2 gene and the thionin Thi2.1 gene was not induced by salicylic acid. Jasmonic acid was necessary for the induction of these genes [Vijayan et al., 1998]. Evidence is emerging that the plant growth regulators jasmonic acid and ethylene play key roles in these salicylic acid-independent pathways [Pieterse and Van Loon, 1999].

1.2 Wound response pathway

For defence against mechanical damage or damage caused by herbivore feeding, plants are endowed with pre-existing physical barriers that limit damage, such as cuticle or trichomes. However, the main potential of plant defence and healing lies in the competence of each cell to trigger defence gene expression. The defence proteins accumulated in both damaged and healthy tissue should fulfil certain functions. First, the defence proteins should guarantee the repair of damaged tissue. Second, they should inhibit growth of the predator insect by producing toxins or interfering with the digestion in the insect gut. Third, they should assure the activation of wound defence signalling pathway. Finally, plant metabolism has to be adjusted to new demands.
Different plant species accommodate common signalling molecules but different sets of genes are induced by these molecules in different plants.
The aspects of wound response pathway will be discussed in this chapter with focus on solanaceous species, particularly on tobacco, the model plant of this study. The corresponding wound signalling cascade for *Arabidopsis thaliana*, the model plant in other studies, is summarised for example in [León et al, 2001].

The early known events detected in wounded leaves are similar to that detected upon pathogen attack and include ion flux, changes in cytoplasmic calcium concentration, oxidative burst and changes in the protein phosphorylation pattern. These events are followed by the activation of transcription factors and synthesis of secondary messengers. A great score of studies confirmed jasmonic acid and its derivatives as a key signal molecule mediating wound response. The DNA-binding proteins and jasmonates, coupled with action of other phytohormones (ethylene, abscisic acid), mediate wound gene expression. Products of wound-induced genes can have direct defensive properties, like cell wall strengthening, proteinase inhibition, antifeeding function, anti-microbial function or toxification. Other wound-induced gene products can serve to mediate wound signal transmission and amplification. These proteins are involved in biosynthesis of wound-signalling molecules. Products of wound-defence genes can also attract predators or parasitoids of herbivores. This function is mainly accomplished by volatile compounds.

1.2.1 Early signalling events

Wounding, many pathogen-derived elicitors, glycans and systemin (see Chapter 1.2.5) all cause a rapid depolarisation of the electrical potential of the plasma membrane. This depolarisation is associated with an efflux of K^+ ions and influx of protons, leading to alkalisation of the extracellular space. Influx of another ion – Ca^{2+} – in cytoplasmic space is connected with the activation of calmodulin. Within a few minutes following damage appears the first phase of oxidative burst characterised by the production of reactive oxygen species – superoxide radical and hydrogen peroxide. The second activation of H_2O_2 appears over a period of hours and at least in tomato it is JA-dependent [De Bruxelles and Roberts, 2001]. The generation of H_2O_2 has been confirmed to occur both locally and systematically. The main source of ROS is the plasma-membrane-bound NADPH oxidase.

Ion fluxes as well as ROS are connected to the regulatory process of protein phosphorylation by specific protein kinases and of protein dephosphorylation by protein phosphatases. The reversible phosphorylation allows fast and specific signal transduction of extracellular stimuli to the cytosol and nucleus. One particular signal transduction mechanism, the mitogen-activated protein kinase cascade, has been found to be activated by a variety of stress stimuli, including pathogen infection, wounding, extreme temperature, drought, salinity, osmolarity, UV irradiation, ozone and ROS (for review see [Zhang and Klessig, 2001] or [Morris, 2001]). Active MAP kinase requires phosphorylation on tyrosine and threonine in a tripeptide motif. The activator of MAP kinase is MAP kinase kinase, which is activated by MAP kinase kinase kinase. Wounding has been reported to activate salicylic acid-induced MAPK, called SIPK, and to some extend also wound-induced MAPK, called WIPK. Prolonged activation of SIPK and delayed activation of WIPK seem to be required also for the hypersensitive response.
In animals and yeasts, most of the substrates for stress-activated MAPK are transcription factors. The activation of MAPK leads to the phosphorylation of transcription factors, which in turn, activate gene expression. However, so far, no substrates have been identified for any plant MAPKs. It has been only proposed that WIPK activates phospholipase A_2, releasing linolenic acid from the plasma membrane that acts as a substrate for biosynthesis of plant hormone – jasmonic acid [Naváez-Vásquez et al., 1999].

1.2.2 Biology of jasmonic acid

Already Charles Darwin postulated the existence of chemical messengers regulating plant development. Since that time many signalling molecules have been identified and some of them have analogues both in animals and plants.
Jasmonic acid belongs to naturally occurring growth regulators of higher plants resembling in structure animal postaglandins. Jasmonic acid, its methyl ester, its amino acid conjugates and biosynthetic precursor – 12-oxo-phytodienoic acid, collectively named as jasmonates, play a role in several developmental processes and in response to various biotic and abiotic stresses. The principle of jasmonates function is based on modulation of gene expression of a number of developmental and stress responsive genes. A detailed summary of physiological processes promoted or inhibited by jasmonates action is given in [Sembdner and Parthier, 1993] or [Creelman and Mullet, 1997a]. Most of the processes with house-keeping functions are down-regulated by jasmonates, such as inhibition of germination of nondormant seeds, inhibition of root growth or inhibition of photosynthetic apparatus, but others are promoted, like fruit ripening or pollen development. Stress stimuli, responses to which are mediated by jasmonates, are summarised in [Wasternack et al., 1998a] and illustrated in Fig. 1.2. Most of the stress genes responsive to these stimuli are up-regulated by jasmonates [Wasternack and Hause, 2000].

Native isomers of jasmonic acid and its naturally occurring derivatives are illustrated in Fig. 1.3. (3R,7S)-jasmonic acid (i.e. (+)-7-iso-jasmonic acid) is the product of the jasmonic acid biosynthetic pathway. However, the *cis* orientation of side chains is less stable and (3R,7S)-jasmonic acid is rapidly converted to the thermodynamically more

favourable *trans*-configuration, which is (3R,7R)-jasmonic acid (i.e.(–)-jasmonic acid). The epimerisation could, in part, regulate the life-time of the active signal within the cell [Farmer, 1994] because different physiological responses differ in its sensitivity to a given stereoisomer.

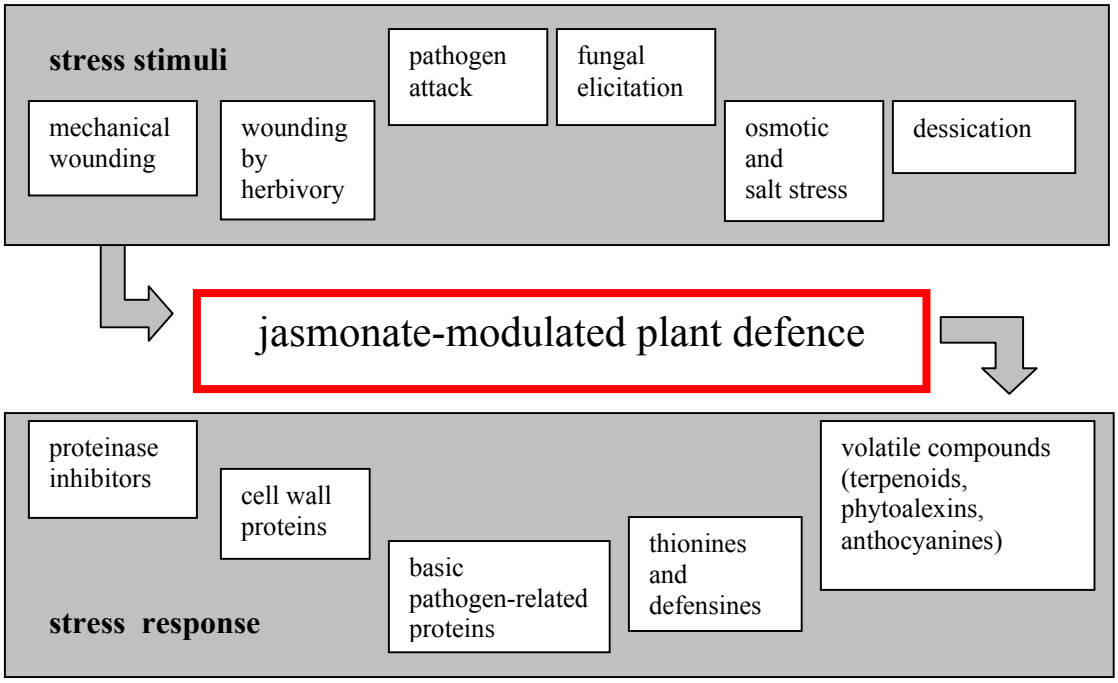


Fig.1.2 Stress factors, in which jasmonates mediate the induction of stress responsive genes resulting in the synthesis of various defence compounds.

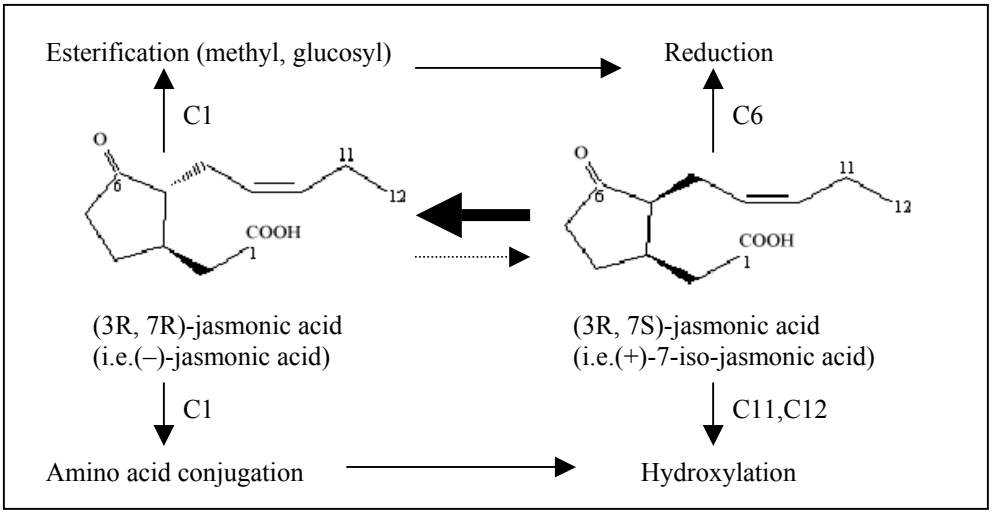


Fig. 1.3 Isomers and natural derivatives of jasmonic acid. Adopted from [Wasternack and Parthier, 1997].

The level of jasmonic acid in plants varies as a function of tissue, cell type, developmental stage and in response to several different environmental stimuli. Levels of JA, *in planta*, range from 0.01 – 3 μg/g FW [Schaller, 2001], which is less than 10 μM, with highest concentrations in growing tissues, like shoot apex, root tips, immature fruits and young leaves. Changes in plant gene expression are induced by nanomolar to micromolar concentrations of JA and/or JAME [Creelman and Mullet, 1995]. So far no receptor of jasmonates has been found, only G-box and as-1-like types of *cis* acting promoter sequences have been identified as jasmonate-inducible in promoters of some JA-inducible genes (pin 2, LOX 1 – see below).
Some evidence suggests that the induced jasmonate is, at least in part, synthesized *de novo* in response to environmental or developmental stimulus and is not simply released from some „storage“ conjugates or pools [Farmer, 1994].

1.2.3 Jasmonic acid biosynthetic pathway

Jasmonic acid and its derivatives biochemically belong to a group of cyclic oxylipines derived from C18 unsaturated fatty acids, called octadecanoids. The pathway for the octadecanoid biosynthesis is summarised in Fig. 1.4.
It is generally assumed that octadecanoids are produced from α-linolenic acid and because all enzymes of the octadecanoid biosynthesis are present in uninduced tissue, the substrate – α-linolenic acid – availability can limit metabolite flow in the pathway [Weiler et al., 1999]. Free α-linolenic acid is probably released from membrane-bound galactosyl lipids by action of phospholipase A_2 [Munnik, 1997], [Narváez-Vásquez et al., 1999]. It has been suggested that wounding or systemic signals like oligogalacturonides, chitosan or systemin (see Chapter 1.2.5) trigger the activation of phospholipase A_2 via calmodulin and MAPK and activated phospholipase A_2 then releases α-linolenic acid from membranes.
Free α-linolenic acid is subsequently converted to hydroperoxy polyunsaturated acid, called 13(S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid (13(S)HPOT). This oxygenation is catalysed at carbon 13 by 13-lipoxygenase. Generally lipoxygenases are nonheme iron containing enzymes catalysing dioxygenation of polyunsaturated fatty acids at carbon atom 9 (9-LOX) or at carbon atom 13 (13-LOX). Plant lipoxygenases can be grouped into two gene subfamilies, *type 1*-LOXs and *type 2*-LOXs. While the *type 1*-LOXs have no transit sequence, the *type 2*-LOXs carry a putative chloroplast transit peptide. So far known *type 2*-LOXs all belong to the subfamily 13-LOXs. An extensive review on lipoxygenases is given in [Feussner and Wasternack, 2002]. *Arabidopsis* LOX2, member of the *type-2* 13-lipoxygenases, is expressed upon wounding and involved in JA biosynthesis [Bell et al., 1995]. The expression of 13-LOX is induced by jasmonate [Feussner et al., 1995].
13(S)HPOT serves as a substrate for several enzymes, see Chapter 5.2, Fig. 5.13. Catalytic function of only one of these enzymes, allene oxide synthase, leads to JA biosynthesis. The product of AOS conversion is unstable epoxide, called (12,13(S)-epoxy-9Z,11E,15Z-octadecatrienoic acid (12,13-EOT). Cyclization of the 12,13-EOT by another enzyme, allene oxide cyclase, leads to the formation of the first cyclic and biologically active compound of the JA-pathway, called (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid (i.e. cis(+)-OPDA).

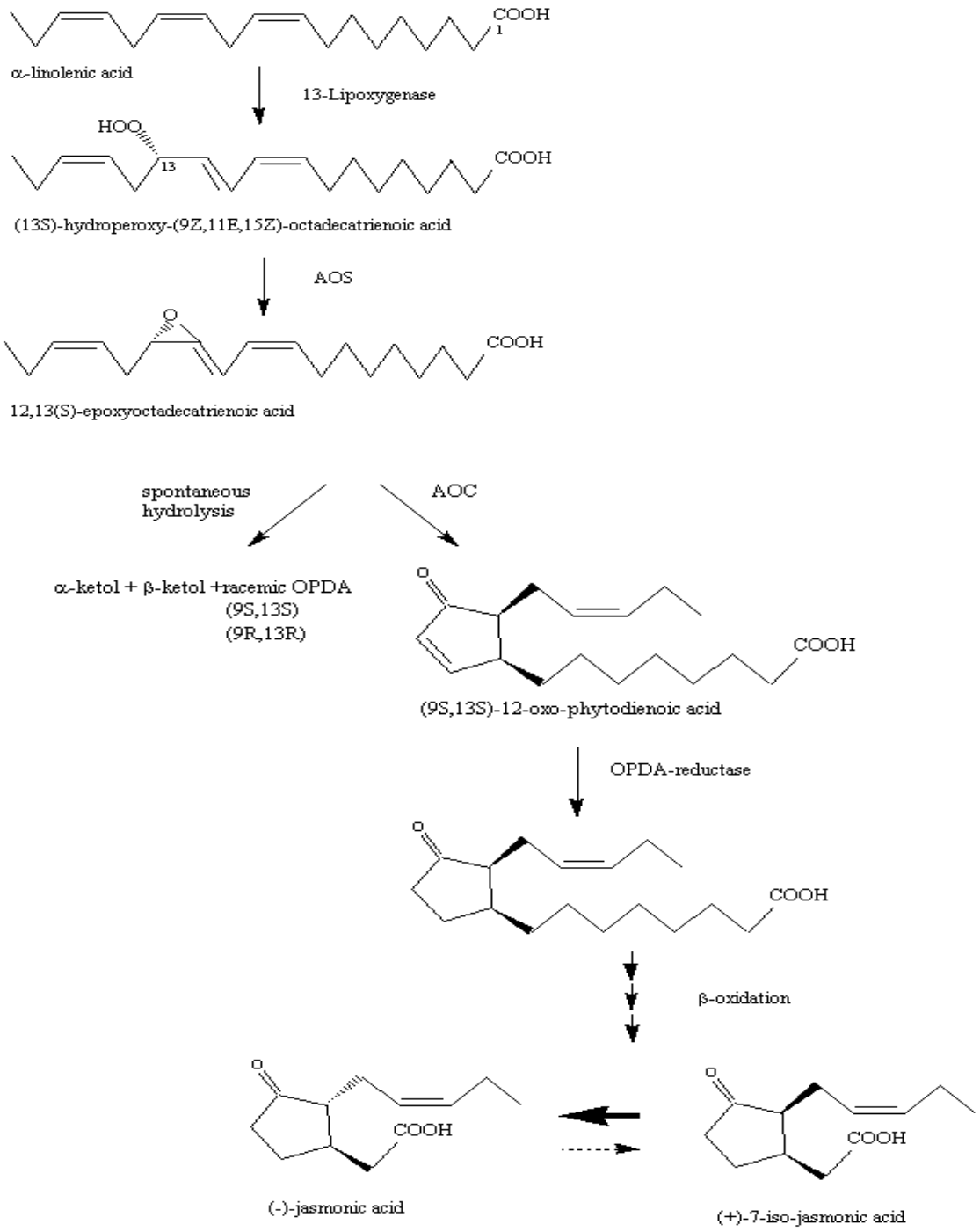


Fig. 1.4 Scheme of the octadecanoid biosynthetic pathway. Names of intermediates are written under schemes of their chemical structure. Names or abbreviations for enzymes are placed next to arrows. For details see text of Chapter 1.2.3.

Without the action of AOC the unstable 12,13-EOT would be spontaneously hydrolysed to α-ketols, γ-ketols and a racemic mixture of (9S,13S) and (9R,13R) isoforms of 12-

oxo-10,15(Z)-phytodienoic acid. It has been suggested that AOC is coupled to AOS to favour the formation of (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid over the ketols and racemic OPDA. Moreover it seems to be AOC, rather than LOX and AOS, that assures the specificity of the octadecanoid biosynthetic pathway for α-linolenic acid and not for linoleic acid [Ziegler et al., 1999].
While the coding sequences of AOS enzyme in some species (tomato, A*rabidopsis*, flax) contain chloroplastic targeting transit peptides, AOS of other plant species (barley) seems to be localized in the cytosol [Maucher et al., 2000]. Overexpression of flax AOS with a chloroplastic transit peptide in potato led to a constitutively higher level of JA [Harms et al., 1995]. However, overexpression of A*rabidopsis* AOS with a transit peptide in tobacco did not increase basal level of JA [Laudert et al., 2000]. The same unchanged basal level of JA has been reported upon overexpression of flax AOS without a transit peptide [Wang et al., 1999]. Only the wound stimulus led to the induction of defence mechanism manifested by elevated JA level or by higher defence gene expression in transgenic plants overexpressing AOS. This is an important result suggesting that it is not the absolute level of octadecanoids but the relative increase of the octadecanoids after stimulus, like wounding, that induces the defence mechanism [Schaller, 2001].
The (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid, formed with high probability in chloroplasts, is transported into the cytosol where OPDA-reductase reduces the 10,11-double bond of (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid and forms 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). Three OPDA-reductase isoforms have so far been identified in tomato and A*rabidopsis* and in both species OPR3 has been found to be the isoform that prefers the (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid – the natural precursor of JA biosynthesis [Schaller, 2001 and references therein]. The physiological process of tendril coiling in *Bryonia dioica* is connected to changed levels of 12-oxo-phytodienoic acid, but not to changed levels of jasmonic acid. The OPR3 enzyme seems to be the decisive component controlling the metabolite flow from C18-compound, like 12-oxo-phytodienoic acid, to the C12-metabolite, like jasmonic acid [Schaller et al., 2000].
The shortening of the carboxyl chain of OPC-8:0 by three cycles of β-oxidation is the terminal step of JA biosynthesis. This step is probably localized in peroxisomes and/or glyoxysomes, since these are the only sites where β-oxidation is known to occur in plants [Vick and Zimmerman, 1984].

AOS and LOX, but not AOC and OPR, have been proven to be JA-inducible, and AOS also OPDA-inducible. However, feedback mechanism in the jasmonic acid biosynthetic pathway is not probable at least in tomato [Miersch and Wasternack, 2000]. The whole octadecanoid pathway is under inductive control of many other factors, as summarised in Fig. 1.5 according the reviews by [Weiler et al., 1999], [Wasternack and Hause, 2000] and [Schaller, 2001].

A number of mutants have been established for the analysis of the wound response pathway and also for the revelation of the complex network of inductive relations in the octadecanoid biosynthetic pathway. The mutants were obtained by screening of a mutagenized population of tomato or A*rabidopsis* for plants that fail either to accumulate JA (JA biosynthetic mutants) or to express genes known to be JA-responsive

(JA responsive mutants). Both types of mutants show deficiency in expression of certain JA-induced genes. However, in case of biosynthetic mutants the wild type phenotype can be totally or partially restored by the exogenous application of jasmonate, while JA-responsive mutants stay insensitive to the jasmonate signal.

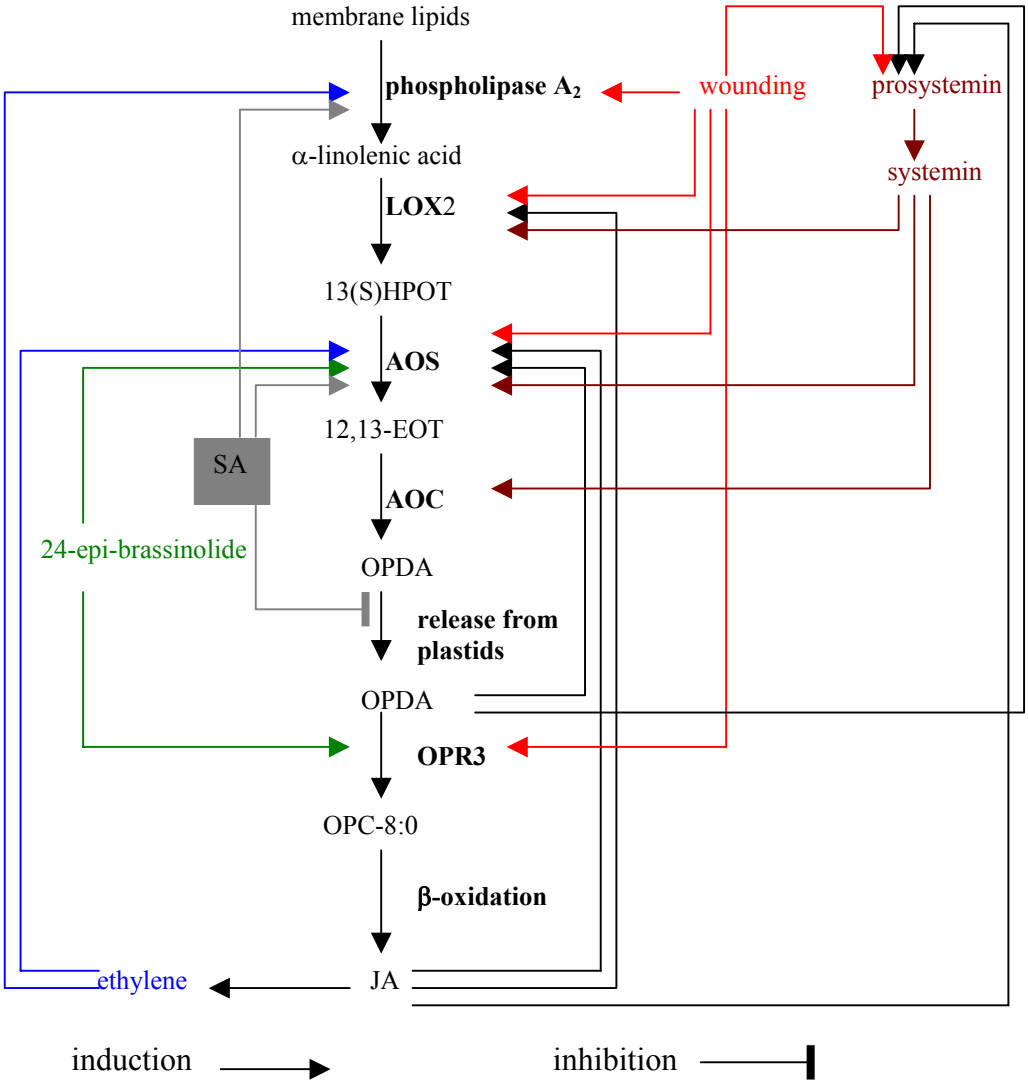


Fig. 1.5 Inductive control of the octadecanoid biosynthetic pathway. Abbreviations for enzymes are in bold.

A number of A*rabidopsis* mutants have been identified, like *fad3-2 fad7-2 fad8*, *aos*, *delayed dehiscence1* and *opr3* (biosynthetic mutants), and *jar1*, *jin1*, *jin4, and coi1* (response mutants). The *fad3-2 fad7-2 fad8* (fatty acid desaturase deficient) mutant has reduced levels of trienoic acids [McConn and Browse, 1996]. The *aos* mutant has a knock-out mutation in the AOS gene [Park et al., 2002]. The *delayed dehiscence1* [Sanders et al., 2000] and *opr3* [Stintzi et al., 2001] mutants are defective in the OPR3 gene. The *jin1*, *jin4* (jasmonate insensitive) mutants and *jar1* (jasmonate response) mutant are capable of growth on 10mM JA that inhibit wild type root growth. The *coi1*

(coronatine insensitive) mutant is resistant to coronatine, a chlorosis-inducing toxin with chemical structure and biological activity similar to those of jasmonic acid (for overview see [Creelman and Mullet, 1997b]). Other *Arabidopsis* mutants with constitutive expression of JA-responsive genes were recently isolated, such as *cev1, cex1, cet or cpr5* (for review see [Turner et al., 2002]).
Several tomato mutants have so far been reported, like *def1*, *spr-1* and *spr-2* (biosynthetic mutants), and *jai-1* (response mutant). The *def1* (defenseless) mutant is inhibited in the conversion of 13(S)HPOT to 12-oxo-phytodienoic acid. The *spr-1* and *spr-2* (suppressed in prosystemin-mediated response) mutants suppress prosystemin (see Chapter 1.2.5) signalling. The *jai-1* (jasmonic acid insensitive) mutant does not express two JA-induced genes upon treatment with JAME (for overview see [Li et al., 2001]).
Analysis of the mutants so far established clearly shows a crucial role for octadecanoids in wounding and herbivory defence. For instance, the *fad3-2 fad7-2 fad8*, *def1*and *coi1* mutants were all more susceptible to herbivore attack than wild type. Grafting experiments with mutants of tomato – *spr-2* and *jai-1* – suggest that activation of JA biosynthetic pathway in response to wounding is required for the production of a long-distance signal whose recognition in distal leaves depends on jasmonate signalling [Li et al., 2002].

1.2.4 Signalling molecules in wound response pathway

The former view that jasmonic acid is the sole biologically active octadecanoid had to be abandoned when it was shown that 12-oxo-phytodienoic acid is a powerful inducer of gene activation [Parchmann et al., 1997], [Stintzi et al., 2001]. Also the capacity of the chloroplast to store OPDA until the demand for it arises confirms the biological activity of OPDA [Weiler et al., 1999].
Recently, an alternative pathway of JA production via the hexadecanoid compound – dinor-oxo-phytodienoic acid (dinor-OPDA) originating from hexadeca-7(Z),10(Z),13(Z)-trienoic acid has been described. The level of dinor-OPDA can be also increased after wounding, as has been reported in potato and A*rabidopsis* [Weber et al., 1997].
Thus, jasmonic acid, its isomers, derivatives (Fig 1.3) OPDA and dinor-OPDA form a broad spectrum of octadecanoids and hexadecanoids, collectively called cyclic oxylipins. Each particular defensive or developmental process may require a certain profile of oxylipins [Wasternack et al., 1998b], [Kramell et al., 2000]. Also different plant species have different profiles of oxylipin molecules. These profiles are termed "oxylipin signature" [Weber et al., 1997].

The spectrum of elicitors of defence gene expression is not restricted only to certain oxylipin profiles. The complex network of inductive control of the octadecanoid biosynthetic pathway (Fig. 1.5) already indicates the involvement of other signalling molecules in the wound response pathway. These elicitors are ethylene, abscisic acid and glycans.
A significant effort has been made toward elucidating the ethylene signalling transduction pathway because ethylene is produced in response to many stresses and developmental processes [Chang and Shockey, 1999]. In Solanaceae ethylene acts in a concerted action with jasmonates. The expression of jasmonic acid-action marker gene –

proteinase inhibitor 2 gene – is ethylene dependent [O'Donnell et al., 1996]. Wound-induced increase in JA causes ethylene synthesis and its action in turn further amplifies the JA signal. The expression of basic PR genes is dependent on ethylene perception [Knoester et al., 1998] and the combination of ethylene and JAME causes synergistic induction of PR-1b gene and PR-5-osmotin gene [Xu et al., 1994].
The role of abscisic acid in the wound response pathway [Peña-Cortés et al., 1995] does not seem to be primary but is required for the plants to respond maximally, as shown by experiments with tomato plants deficient in ABA biosynthesis [Herde et al, 1999].
Cell wall glycans of fungi (chitosan) and plants (pectin) are oligogalacturonides that have also been found to elicit wound response in tomato by induction of early signalling events of the wound response pathway, by induction of JA and induction of ethylene. Moreover oligogalacturonides are the only elicitors able to mimic all aspects of signalling in the wounded leaf.

1.2.5 Wound signal transmission

Signalling molecules mentioned so far are considered to play a role locally, in damaged leaf. However, plants have the fascinating ability to transmit the wound signal to distant leaves and even to neighbouring plants. The systemic signal to distant undamaged parts of the plant is carried by the vasculature, while the SOS signal between plants is mediated by volatile compounds spread in the air.
There are several candidates for a transmitter of a systemic signal. Firstly, there are wound-induced electrical signals and hydraulic signals caused by the wound-induced release of water tension in the xylem. The role of these signals is not really understood. Moreover, these weak, transient signals alone cannot sustain the activation of defence genes over a period of several hours. Secondly, a peptide called systemin has been identified in tomato plants and proven to act as the systemic signal in this specie (for review see [Ryan, 2000]). The 18-amino-acid peptide systemin is processed from 200-amino-acid precursor prosystemin as a result of wounding. Prosystemin is expressed in cytosol [Bowles, 1993] in the vascular bundles of the aerial parts of the plant and cleaved to systemin by proteolytic enzymes released from vacuoles of damaged cells in wounded site. Also a systemin-binding protein has recently been identified. Systemin has an inductive role in jasmonate biosynthesis (Fig 1.5). Although related peptides have been identified in other solanaceous species, tomato systemin is inactive in inducing proteinase inhibitor synthesis in tobacco [Ryan, 2000] and this peptide-based systemic signal can be specific only for one plant family [De Bruxelles and Roberts, 2001].
Indirect defence mediated by volatile compounds serves as SOS signal for neighbouring plants and as attractant of predators and parasitoids of feeding herbivores. Volatile compounds, such as monoterpenes, sesquiterpenes or volatiles derived from the lipoxygenase pathway [Bate and Rothstein, 1998], are released from damaged storage organs and also synthesized newly. Herbivore-induced synthesis of volatiles is triggered both locally and systematically. The spectrum of herbivore-induced volatiles differs from volatiles emanated from mechanically wounded plants [De Bruxelles and Roberts, 2001]. Volatile compounds can also be induced by octadecanoids but different octadecanoids induce different classes of volatiles.

1.2.6 Cross-talk between defence signalling pathways

Two statements can be made based on results of research in recent years. First, it is impossible to view defence signalling as a small number of linear pathways. Second, one plant system as a model system is not sufficient because of significant differences in defence responses between species. For instance, both JA-dependent and JA-independent wound response pathways have been detected so far only in A*rabidopsis* [Titarenko et al., 1997]. The JA-independent pathway operates in the immediate vicinity of the wound site. Reversible protein phosphorylation regulates both JA-dependent and JA-independent wound response pathways [Rojo et al., 1998].
The wound response pathway should be seen as a broad network of signalling pathways, which acts in a synergistic and/or antagonistic way in dependence on elicitor, time course and cellular compartment. Signalling molecules involved in the wound response pathway influence synthesis of each other and relative levels of these compounds, rather than absolute values, are the decisive factor for "fine-tuning" the defence response. Some of the most striking evidence supporting the view of the wound response pathway as a network of signals is summarised here.

- There is a whole spectrum of cyclic oxylipins playing a role in plant defence.
- Except oxylipins there are also other elicitors of the wound response (ethylene, ABA, brassinosteroids, glycans, systemin) and most of them participate in inductive control of the octadecanoid biosynthesis.
- Other plant growth regulators, which are not directly involved in the wound response, regulate the response indirectly. These regulators are auxins and cytokinins. The endogenous level of indole-3-acetic acid decline upon wounding in tobacco and recovery of the initial levels of active IAA has been proposed as a mechanism to limit the duration of the wound response [Bowles, 1993]. Cytokinins have been proposed to control endogenous level of jasmonic acid and salicylic acid under wound stress conditions [Seo et al., 1997].
- Salicylic acid induces an accumulation of AOS (Fig. 1.5) but inhibits release of OPDA from chloroplasts [Weiler et al., 1999]. Similarly, JA inhibits both accumulation and action of SA. Although marker genes of systemic acquired resistance and wound response pathway may vary from species to species, at least in tobacco strong antagonistic effect of SA and JA on the expression of PR genes upon wounding has been reported [Niki et al., 1998]. JA and ethylene regulate the expression of some basic PR genes involved in SA-independent pathogen defence (Chapter 1.1). SA inhibits the expression of proteinase inhibitor genes but this effect can be overcome by exogenous application of JA and ethylene, suggesting the inhibition effect of SA not only in JA biosynthesis but also downstream of JA and ethylene signalling [Dong, 1998].
- Salicylic acid and jasmonic acid can act synergistically under some conditions. The PR-1b gene of tobacco is salicylic acid responsive but jasmonic acid methyl ester enhances accumulation of PR-1b protein. JAME and ethylene induce the PR-1b and the PR-5 – osmotin gene of tobacco [Xu, 1994]. A number of A*rabidopsis* defence genes overlap in their responsiveness to both jasmonic acid and salicylic acid [Reymond and Farmer, 1998].
- Both the wound response pathway and induced systemic resistance require JA and ethylene as secondary messengers. Both induced systemic resistance and systemic

recquired resistance require NPR1 protein [Maleck and Dietrich, 1999]. Both ROS and MAPK cascade are active signalling components in systemic acquired resistance and wound response pathway. These molecules mediate horizontal connections between signalling pathways.

1.2.7 Schematic model of the wound response pathway

A current simplified model of the wound response pathway, illustrated in Fig. 1.6, summarises so far known components of this signalling cascade in local and systemic leaf.
Elicitors of the wound response pathway – glycans, mechanical wounding or wounding by herbivory directly or via systemin – trigger early signalling events in damaged leaf (Chapter 1.2.1). The consequence of these signals is probably the activation of transcription factors and the release of polyunsaturated fatty acids from membranes. Subsequently genes responsible for healing processes and the biosynthesis of octadecanoids are activated (Chapter 1.2.3). The wound signal is amplified by the production of octadecanoids probably initially in vascular bundles, from where these secondary messengers are transported to palisade and mesophyll cells. In this tissue the defence proteins are synthesized [Ryan, 2000], [Hause et al., 2000]. The octadecanoids biosynthesis and whole wound response is modulated by action of a number of other plant regulators (Chapter 1.2.4), which influence the wound response as both inductors and inhibitors (Chapter 1.2.6). It has been proven that the wound signal is transmitted into distal undamaged parts of a plant (Chapter 1.2.5) but the origin of the signals responsible for the transmission and also the exact sequence of signalling events in systemic leaf are poorly understood. The gene expression profile in unwounded leaves differs from that in wounded leaf [De Bruxelles and Roberts, 2001].

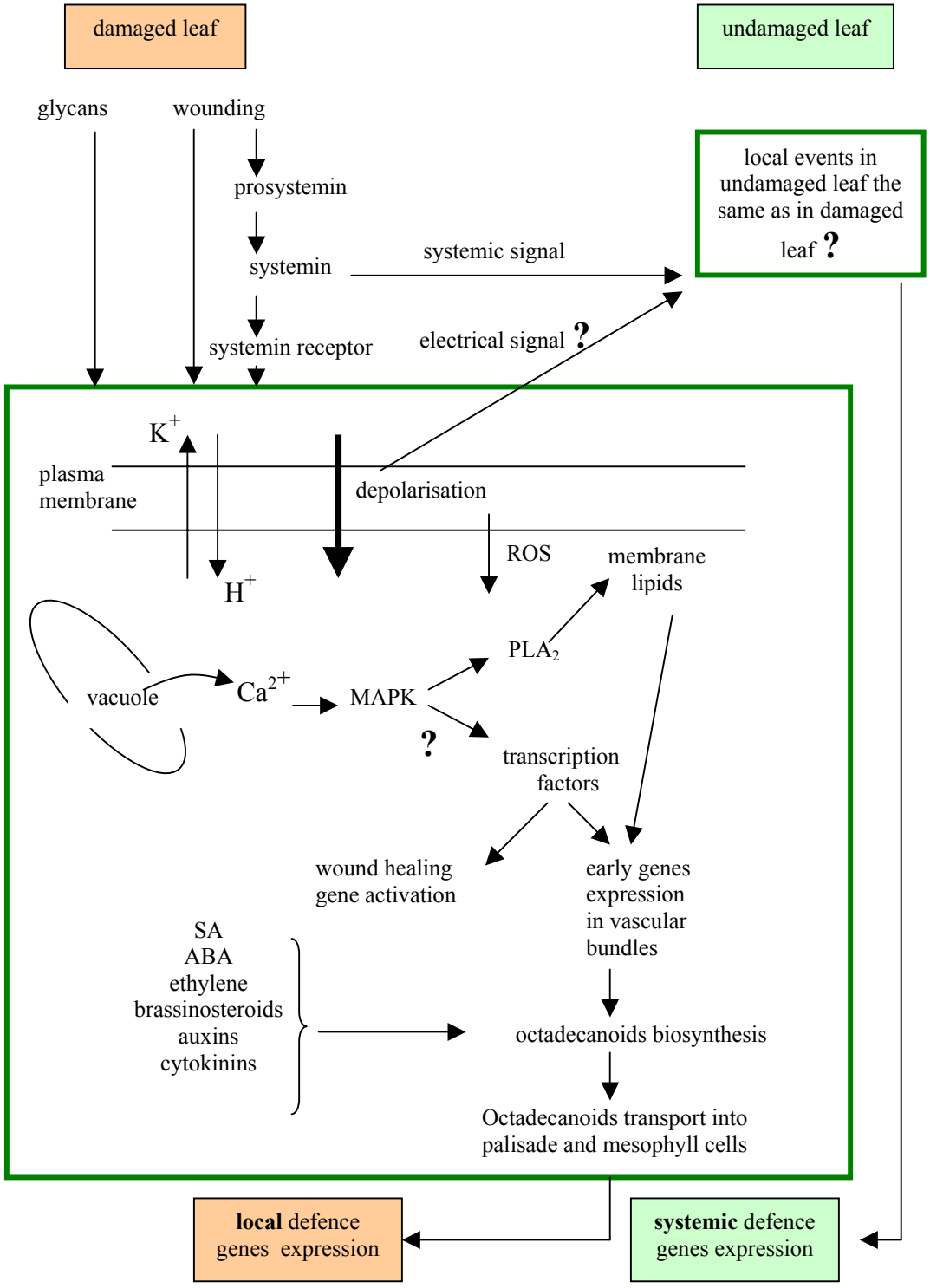


Fig. 1.6 Current model of wound response pathway. Summarised from reviews by [Weiler et al., 1999], [Ryan, 2000], [Wasternack and Hause, 2000] and [De Bruxelles and Roberts, 2001]. For details see text in Chapter 1.2.7.

Chapter 2

Recombinant antibodies in prokaryotic and eukaryotic cells

2.1 Recombinant antibodies in the prokaryotic cell.

2.1.1 Structure and function of antibody molecules

Unlike in plants, defence processes in animals are mediated by a spectrum of specialised mobile cells of the immune system. In the centre of the very complex immune response of animals stands an interaction between B-cell produced antibodies and an invader molecule – the antigen. Increasing knowledge and learning from the natural antibody system opens a great field of antibody engineering for research, diagnostic and therapeutic purposes.

A schema of a complete antibody molecule is illustrated in Fig. 2.1. The basic dimeric antibody structure comprises two heavy chains (H) of about 440 amino acids and two light chains (L) of about 220 amino acid residues. These chains fold into three domains. Two of the domains (Fab domains) are identical and form the arms of the Y. One light chain associates with the amino-terminal region of one heavy chain to form an antibody-binding site. The third domain (Fc) forming the base of the Y is fold together by the carboxy-terminal regions of the two heavy chains and is responsible for certain aspects of the immune response. The four polypeptide chains are held together by disulfide bridges (SS) and noncovalent bonds. The light chain can be divided into two regions, variable (V) and constant (C) region. The heavy chain contains one variable and three constant regions. Within variable regions four framework regions (F) and three hypervariable regions (CDR) can be discriminated. These CDRs form the majority of contact residues for the binding of the antibody to the antigen [Harlow and Lane, 1988].
The repertoire of antibodies is created during B-cell development by DNA rearrangement and combinatorial assembly of different genetic segments. V-, D- and J-segment form the heavy chain gene and V- and J-segment form the light chain gene (for overview see [Winter, 1998]). Mutagenesis is a second source of the antibody diversity (Chapter 2.1.4).

2.1.2 Recombinant antibody fragments

Current molecular techniques enable the construction of antibody fragment molecules without loss of their antigen-binding properties.
An overview of most frequently used antibody fragments is depicted in Fig 2.2. New antibody proteins can be designed as monovalent fragments by reducing in size (Fab)

[Plückthun and Skera, 1989]. The constant regions of Fab antibody fragments can be omitted and then Fv fragments are formed. It has been found that even a single VH chain can bind antigen (single VH domain). Alternatively one variable region of heavy-chain (VH) and one variable region of light chain (VL) can be bound together by a short hydrophilic linker to form a single-chain variable fragment (scFv) [Bird et al., 1988], which is, together with an Fv fragment, the smallest antibody fragment still containing the complete antigen binding site. The linker is usually a sequence of $(Gly_4Ser)_n$ with 10-25 amino acids in length [Plückthun et al., 1996]. If the linker is too short (5-10 residues), the interchain pairing of the variable domains dominates above the intrachain pairing and the single-chain molecule prefers to form bivalent diabodies (scFv-dimmer) [Holliger and Winter, 1997] or triabodies (scFv-trimmer). VH and VL regions can be also covalently linked by designing disulfide bonds between them (dsFv) [for review see Plückthun et al., 1996]. Another strategy of antibody engineering are miniantibodies (scZIP, sc HLX) [Pack and Plückthun, 1992] where the scFv is linked with the flexible hinge region from mouse IgG3 and an amphiphilic helix fused to the C-terminus of the antibody fragment [Dall'acqua and Carter, 1998].

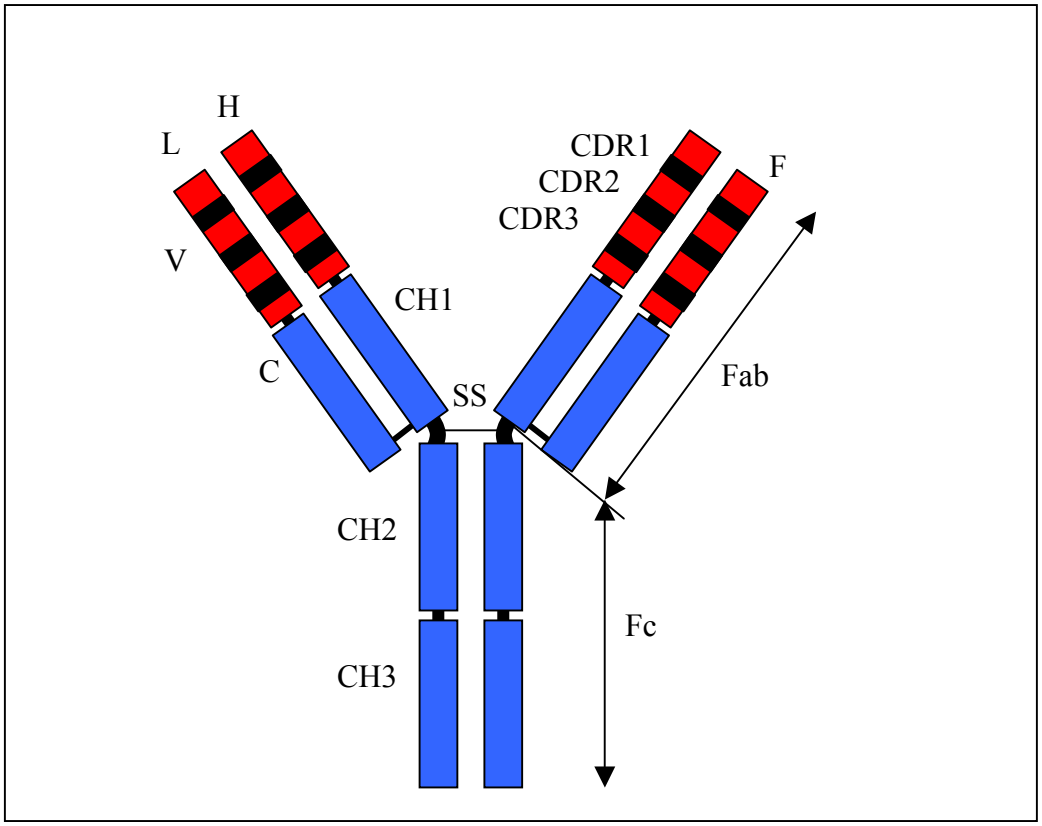


Fig. 2.1 Structure of complete dimeric antibody molecule. For the explanation of abbreviations see text in Chapter 2.1.1.

The use of such small antibody fragments offers some advantages. The linkage of VH and VL regions together and expression as one gene in equimolar concentration enables easier association of antigen-binding sites even in more reducing environments and

without molecular chaperones which are necessary for correct association of natural complete antibodies. Smaller antibody fragments also easier access intracellular compartments and are less immunogenic. Especially therapeutically important are the bispecific diabodies where two scFv molecules have specificity for two different epitopes [Fanger and Guyre, 1991], [Holliger et al., 1993] or bifunctional antibodies where the scFvs are fused to other molecules like enzymes and deliver these molecules into target tissue.

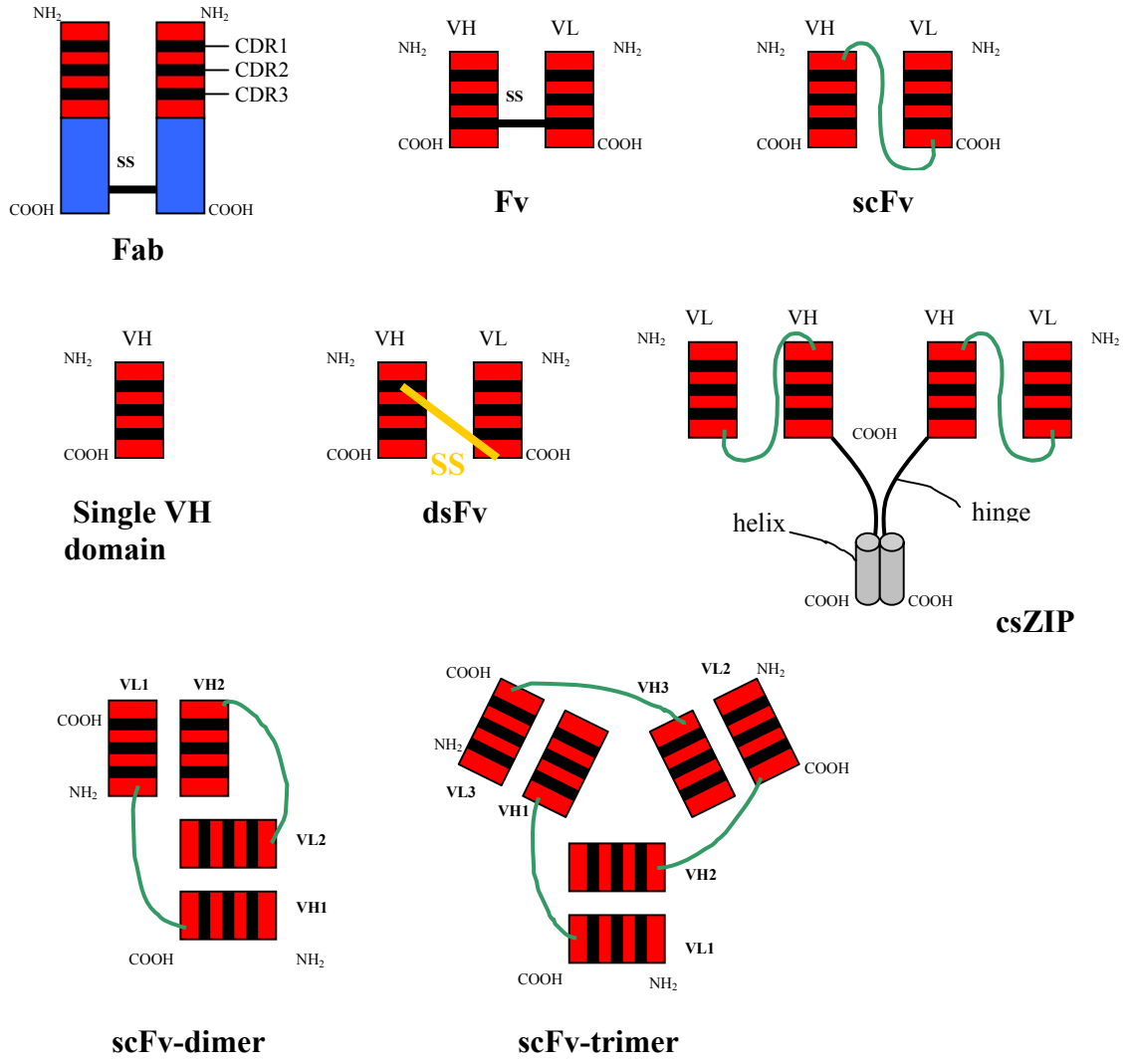


Fig. 2.2 Schematically represented overview of various antibody fragment molecules. For details see text in Chapter 2.1.2.

The choice of the right antibody format depends on biotechnological or therapeutical application. Generally Fab and scFv antibody fragments are mostly used. Fab fragments should be more stable but increasing folding problems may occur [Plückthun et al., 1996]. The scFv molecule can on the other hand be favoured kinetically during folding,

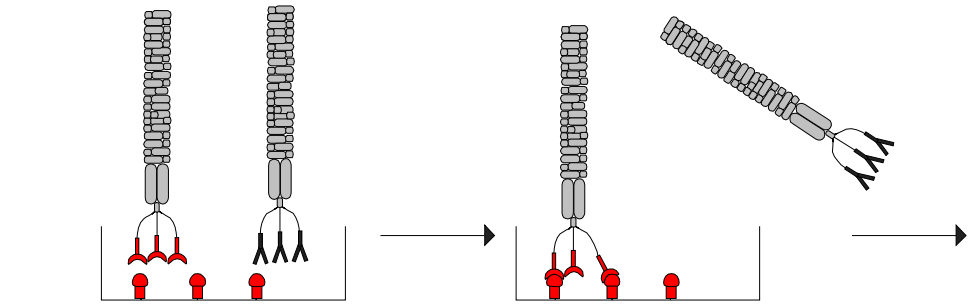
expressed to higher level then Fab in bacteria [Hudson, 1998] and penetrate more rapidly and deeper into the targeting tissue.

2.1.3 Antibody fragment libraries

Recent accomplishments in the protein engineering of antibodies offers alternatives to classical hybridoma technology, to the technique of the humanization of murine antibodies by grafting murine CDR regions onto a human antibody framework regions or to PCR cloning of rearranged VH and VL genes from hybridomas [Winter and Milstein, 1991].
A tremendous impact on the development of antibody engineering technology had the display of antibody fragments on the surface of filamentous phage fd or M13 that live on *Escherichia coli* [McCafferty et al., 1990], [Clackson et al., 1991], [Chiswell and McCafferty, 1992], [Winter et al., 1994]. The principle of antibody phage display is illustrated in Fig 2.3. (for review see [Rader and Barbas, 1997], [Hoogenboom, 1997]). The antibody phage display is accomplished by fusing the coding sequence of the antibody variable regions to the gene encoding one of the phage coat proteins (pIII, pVI, pVII, pVIII, or pIX) [Gao et al., 1999]. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle. The enrichment of specific phages is achieved by selection on immobilized target. Phages with the relevant antibody will be retained by binding to immobilized antigen, while non-adherent phages will be washed away. Bound phages can be eluted from the plate, reinfected into bacteria for further enrichment and used either for further round of selection or for analysis of binding.
Such analysis of binding should discriminate between selected variants according to their affinity of binding or their kinetics of binding. ELISA-based methods as well as screening using BIAcore have been described (for review see [Hoogenboom et al., 1998]).
A common strategy is the cloning of a scFv fragment in frame with the pIII minor coat protein into phagemid vector utilizing lacZ promoter for glucose-dependent IPTG-inducible fusion-protein expression. In this case a helper phage such as M13KO7 is needed which provides the protein machinery for packaging. By use of *Escherichia coli* antibody fragments can be secreted as soluble proteins to the periplasm or expressed into the cytoplasm. Alternatively, antibodies can be expressed as insoluble inclusion bodies in the periplasm or the cytoplasmic space and refolded subsequently *in vitro* (for review see [Plückthun et al., 1996]).
Attempts have already been made to select antibodies to difficult complex antigens, particulary to cell surface molecules [Hoogenboom et al., 1998] or prepare antibody arrays for screening of large number of antigens [De Wildt et al., 2000].

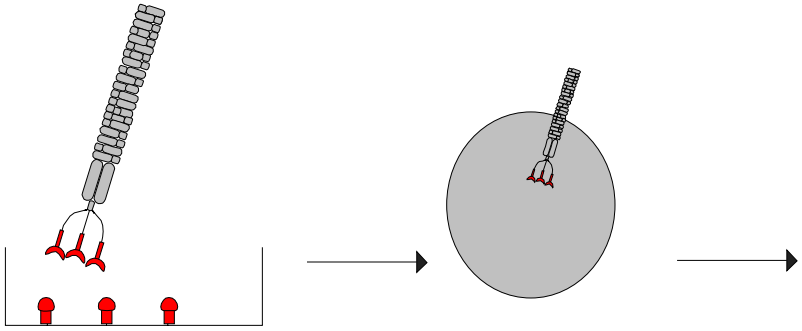
The affinity of phage display-selected antibodies depends to a large extend on the size of the displayed antibody repertoire. In principle three different types of libraries can be utilized [Little et al., 1995]: a) a library derived from an immunised donor; b) a naive V-gene library; c) a synthetic antibody library.
The antibody libraries from an immunised donor (for review see [Aujame et al., 1997]) are the least complex libraries obtained by PCR amplifying rearranged variable-region

gene segments of the immunised donor. The advantage of this library should be its particular richness in phage clones with higher affinity to the immunogen. The disadvantage is the necessity to prepare a new library for each new antigen.



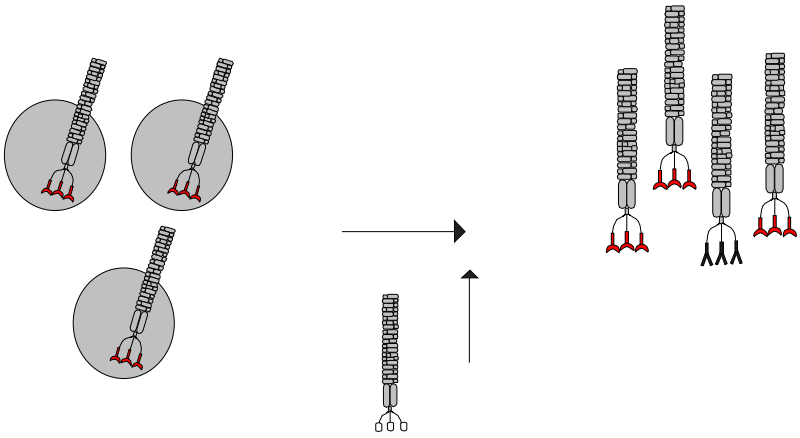
phages incubated with antigen

washing



elution of bound phages

infection of *E. coli*



amplification of *E.coli*

helper phage

phages preparation for next round of selection enriched by antigen-specific phage

Fig. 2.3 The principle of antibody phage display.

In naive V-gene libraries, in comparison to the first discussed type of library, the source of mRNA for PCR amplification are B-cells originating from a non-immunised donor. Such libraries can contain around 10^8 clones displaying 10^8 antibody fragments with different binding properties, what is about the estimated amount of different antibodies present in the immune system at any one time. Affinity of antibodies obtained from such libraries were comparable with antibody affinity from primary immune response, which is $10^6 – 10^8$ M^{-1}. This type of library can be used for selection of antibodies against any antigen, including toxic or non-immunogenic antigens. The disadvantage of such a library is the unequal expression of different V-gene families and unknown history of the B-cell donor.
Antibody libraries with the highest complexity can be achieved by in vitro assembly of V-gene segments and D/J segments via PCR. All the CDR regions, except of the CDR3 of the heavy chain, follow a certain canonical fold. The greatest structural diversity has been found in the CDR3 of the heavy chain. The antibody CDR regions can be randomised by using PCR amplification with an oligonucleotide containing a random sequence NNS_{16}, where N is an equimolar mixture of A, C, G and T nucleotides and S is an equimolar mixture of G and C nucleotides. Remarkable advantage of this type of library is the lack of negative selection to self-antigens as occurs *in vivo*. The disadvantage is the size of such library that has to be large enough to find higher-affinity antibodies.

2.1.4 Improvements and limitations of antibody-fragments production in *Escherichia coli*

Affinity maturation of antibodies is a classical property of the immune system. The antigen triggers hyper mutations in antigen-activated B-cells [Rajewsky, 1996]. The sense of the somatic hyper mutations in the late stage of immune response is to improve antibody's affinity. Affinity can be influenced by providing new contact residues or replacing low-affinity contact residues with the one with more favourable energetic. Point mutations are focussed at „hotspots" mainly at the periphery of the antigen-binding site. Replacement mutations are often over represented in CDRs and underrepresented in the framework regions.
Various techniques have also been developed for *in vitro* affinity maturation (for review see [Hoogenboom et al., 1998]). Random mutations can be introduced by growth of the phage in a bacterial mutator strain, by error-prone PCR, by chain shuffling, by oligonucleotide-directed mutagenesis or codon-based mutagenesis. Libraries constructed to improve the affinity of selected antibodies are called secondary libraries. Such libraries utilize loxP/Cre recombination system [Zahra et al., 1999], mutations in the centre of the CDR3 regions of VH and VL [Tomlinson et al., 1996], or (attP/attB) integration sites plus integrase [Aujame et al., 1997]. Affinity of such improved antibodies can reach a level below 10 nM, properties usually associated with antibodies from the second immune response [Vaughan et al., 1996].
The possibility of producing antibody fragments in *E.coli* has been a major break-through in the field of antibody engineering due to an easy accessibility of this method and especially in combination with phage display technology which also makes use of *E.coli* [Nilson, 1995]. However the Achilles heel of this technology is the dependence of

the expression titre on the antibody primary sequence and partially also on the expression vector and *E. coli* host strain (for review see [Dall'acqua and Carter, 1998]). Thus, alternative techniques emerged such as *in vitro* translation of proteins by using ribosome display [Hanes and Plückthun, 1997] or recloning of the antibody fragment coding sequence for expression in other hosts like yeast, insect cell or plant cell.

2.2 Recombinant antibodies in the eukaryotic cell

2.2.1 Antibodies in plants

Results of several studies of evolution of antibody production in the plant cell suggest that the antigen-binding properties of plant-derived antibodies are comparable to those of mouse-derived antibodies [Hein et al., 1991] or bacterial-derived antibody fragments [Bruyns et al., 1996]. Moreover, there are at least five characteristics of plant cells, which make them a valuable tool for the production of bioactive compounds like antibodies, enzymes, and vaccines. First, the processing and assembly of recombinant proteins in the eukaryotic plant cell is closer to the protein machinery of the mammalian cell than prokaryotic microbial expression system. Molecular chaperones responsible for correct folding of proteins have also been detected in plant cells. The difference in glycosylation pattern of plant antibodies, in comparison to mammalian, has no effect on the specificity or the affinity of antibodies [Ma and Hein, 1995]. Second, most plant transformation techniques result in the stable integration of the foreign DNA into plant genome, which enables sexual crossing of transgenic plants and introduction of new genes or multiple genes into the progeny. Third, plant cells can be propagated as clumps of cells on agar plates, as suspension culture or as protoplasts and later regenerated to whole plants. Fourth, the potential for protein production in large scale at an extremely competitive cost [Fiedler et al., 1999] is one of the main attractions. Energy and nutrient supply are of much lower costs than fermentation and the sterile conditions needed for microbial production. Fifth, storage of the recombinant protein of interest is possible simply in seeds [Fiedler and Conrad, 1995] almost indefinitely or in tubers [Artsaenko et al., 1998]. Difficulties with the utilisation of the plant expression system could be connected with the non-correct assembly of some proteins that may occur because of still poorly understood expression in the heterologous system. Also down stream processing of plant-produced antibody proteins for clinical use requires further development.

In the first report of the antibody production in plants [Hiatt et al., 1989] genes for light and heavy chain originating from hybridoma cells were cloned and induced separately into the genome of tobacco cells. Cross-pollination of regenerated transgenic plants expressing either light or heavy chain gives transgenics accumulating correctly assembled full-length antibody with binding affinity indistinguishable from that of the original hybridoma cells. This two-generation system has later been improved by double-transformation of genes for light and heavy chain into the same plant cell simultaneously [De Neve et al., 1993]. Another strategy is to introduce the light- and heavy-chain genes on single T-DNA [Düring et al., 1990]. This approach is very close to the strategy of expression of antibody scFv fragments in plant cells [Owen et al., 1992], as will be discussed in more detail in Chapter 3.

Generally recombinant proteins have been expressed in scores of plant species (summarised in [Larrick et al., 1998]), including Solanaceae (tomato, tobacco and potato), plants interesting for research like *Arabidopsis* and alfalfa and agriculturally interesting plants like pea, rice, soybean, wheat, banana. Antibody-derived molecules were produced in transgenic plants in format of scFv, Fab as well as in full length as IgG, IgM, hybrid IgG/A, and even as secretory IgA/G antibody [Ma et al., 1995]. Unlike in animals, a single cell of a transgenic plant was able to assemble the secretory IgA antibody, which demonstrates the overall flexibility of plant cells for recombinant protein production.

2.2.2 Immunomodulation

The technology of plant-derived antibodies has an increasing number of applications. One aspect is the utilisation of „plantibodies“ in immunotherapy for production of immunoconjugates, for production and storage of vaccines or for passive immunization with secretory IgA (for review see [Ma and Hein, 1995]. However the plantibody approach can go far more beyond the use of plants as bioreactors of therapeutically interesting molecules.
A very large area has been opened with the molecular technique of immunomodulation, which is a technique that allows the interference with cellular metabolism or pathogen infectivity by the ectopic expression of genes encoding antibodies or antibody fragments. Important prerequisites of the immunomodulation approach are: first, the availability of a specific antibody with high affinity for the target molecule, and second, an accumulation of the antibodies in high concentration in the right plant organ and plant cell compartment. These aspects will be discussed in more detail in Chapter 3.

The potential mechanisms of antibody-mediated *in vivo* modulation of proteins and signal molecules is reviewed in [De Jaeger et al., 2000] and illustrated in Fig 2.4.
Recombinant antibodies (RA) can compete with substrate, hormone or secondary metabolite (2.4(a)). RA can bind to a functional protein and affect the protein structure (2.4(b)). RA expression may mistarget low molecular weight compounds (2.4(c)). RA can bind the substrate, hormone or secondary metabolite (2.4(d)). RA interfere with DNA binding (2.4(e)) or with multiprotein-complex formation (2.4(f)).

Plants can be intra- or extra-cellulary immunized by production of pathogen-specific antibodies. In this way a resistance to artichoke mottled crinkle virus [Tavladoraki et al., 1993], tobacco mosaic virus [Voss et al., 1995] or necrotic yellow vein virus [Fecker and Koenig, 1999] were obtained. Plantibody immunization is also an elegant strategy to control mycoplasma infection, and challenging alternative to control resistance against multicellular pathogens, such as nematode, fungi or insect (for review see [De Jaeger, 2000]).
The expression of antibody fragments specific to regulatory proteins and hormones makes it possible to evaluate developmental and metabolic events that are controlled by this components. Generated antibody-antigen complexes can interfere with the function of enzymes, with receptor-like proteins or with DNA-binding proteins (for review see [Conrad and Manteuffel, 2001]).

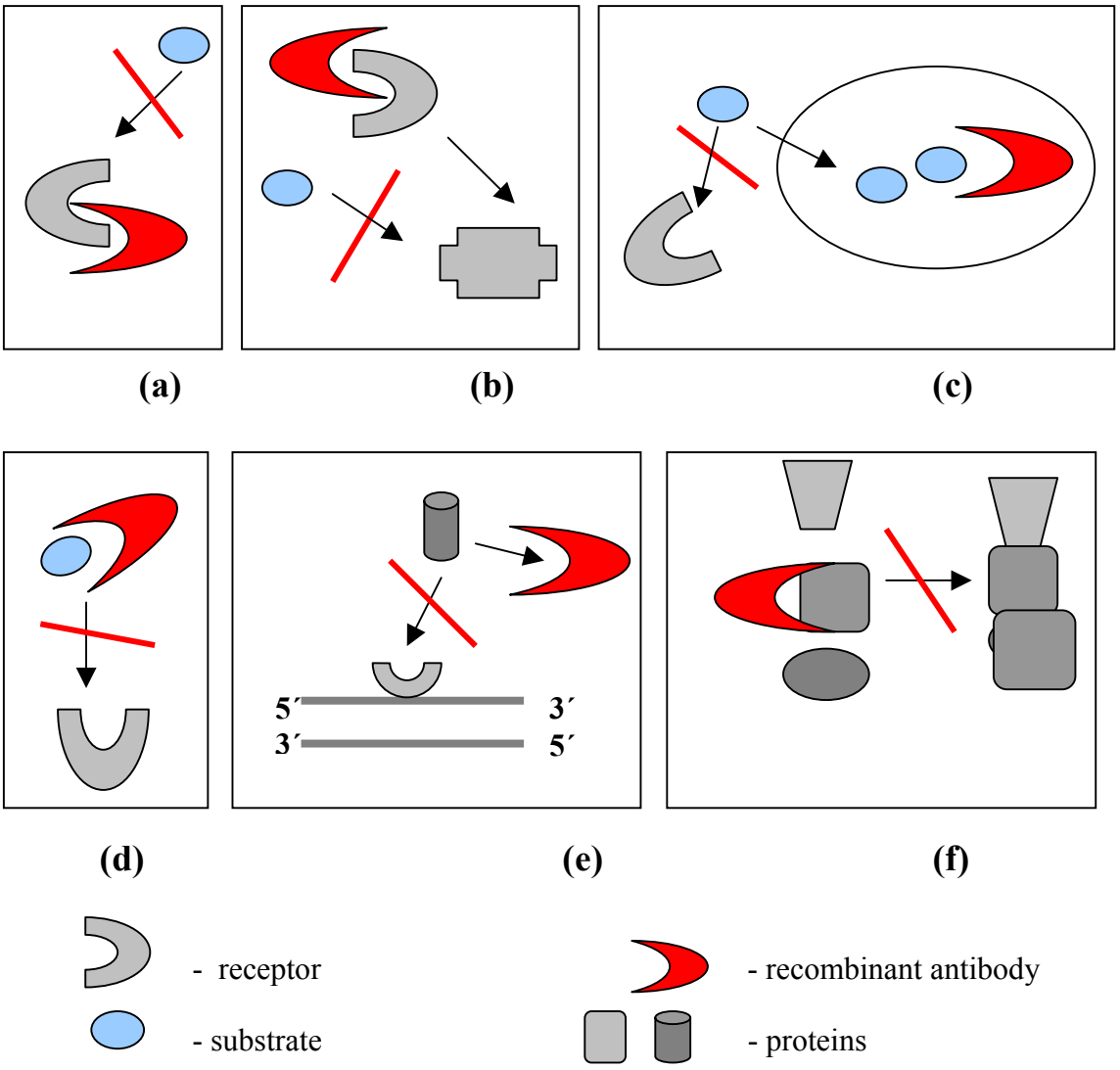


Fig 2.4 Potential mechanisms of *in vivo* immunomodulation of proteins and signal molecules. Modified from [Conrad and Manteuffel, 2001]. Recombinant antibodies (RA) compete with substrate, hormone or secondary metabolite (a). RA bind to a functional protein and affect the protein structure (b). RA expression mistarget low molecular weight compounds (c). RA bind the substrate, hormone or secondary metabolite (d). RA interfere with DNA binding (e) or with multiprotein-complex formation (f).

The endogenous targets of immunomodulation can also be signal molecules, such as phytohormones.

The application of scFv against plant hormone abscisic acid led to disturbance of physiological processes controlled by this hormone, such as stomatal closure, seed development or dormancy, and revealed new insight into the role of this phytohormone [Artsaenko et al., 1999]. First the mistargeting of ABA has been shown by the expression of anti-ABA scFv under control of CaMV35S promoter in the endoplasmic reticulum of tobacco cells [Artsaenko et al., 1995]. Unlike the wild type plants, an

artificial sink of ABA in the ER of transgenic plants led to higher levels of ABA, and transgenics could not close their stomata in response to water stress, atmospheric CO_2 or light. Moreover morphological changes have been observed in guard cells and normal phenotype could be restored after long-time treatment with ABA [Wigger et al., 2002].
Similar effects of ABA deficiency, in spite of intracellular excess of ABA, have been observed in transgenic potato plants [Strauss et al., 2001].
The role of ABA in seed development was investigated by expression of anti-ABA scFv under control of seed specific USP promoter in the ER of tobacco seeds. Deficiency of functional ABA caused a switch from the seed maturation program to the germination program leading to seeds with lack of storage protein bodies, less oil, appearance of embryos with green cotyledons and accumulation of photosynthetic pigments [Philips et al., 1997].

The expression of anti-gibberellin scFv in the endoplasmic reticulum of tobacco cells leads to a dwarf phenotype as a result of trapping precursors of bioactive gibberellins [Shimada et al., 1999].

Previously the investigation of phytohormones action was carried out by application of an exogenous hormone. However, observed response of plant under such non-physiological levels of the hormone often does not reflect the function of the endogenous signalling molecule. Therefore genetical and molecular strategies are currently involved that remove the single regulatory molecule of interest from their site of action. This can be achieved by the selection of mutants by antisense or co-suppression techniques. Such approach allows time- and organ- specific blocking of certain gene activity. However compartment-specific interference is not possible. The cell-compartment-specific interference of phytohormone functions is one of the most important advantages of the immunomodulation. Moreover, specific antibodies trap only certain precursor or final product of a hormone biosynthetic pathway without affecting the function of other components of the pathway. In addition, plants with different levels of biologically active phytohormone can be investigated due to a different level of antibody expression. Another advantage is that the intracellular immunization or immunomodulation can be applied to any species that can be transformed. Finally, this technique does not require cloning of genes involved in the phytohormone biosynthesis.

Chapter 3

Introduction of scFv antibody fragments into the plant genome

3.1 Organ-specific and cell-compartment-specific accumulation of recombinant antibodies in plants

The choice of the right plant cell organ and cell compartment for the accumulation of recombinant antibodies depends on developmental and/or metabolic events that should be modulated by the antibody expression. The expression of antibodies in the developmental stage or in the cell compartment where the action of an antigen of interest does not take place leads to a failure of this molecular technique.
Many attempts have been described for expression and assembly of antibodies constitutively under control of the Cauliflower mosaic virus 35S promoter [Töpfer et al., 1993]. In such case expressed antibodies can be detected in all plant organs from flowers and leaves (for review see [Conrad and Fiedler, 1998]), to roots [van Engelen et al., 1994], hairy roots [Wongsamuth and Doran, 1997] or tubers of potato [Artsaenko et al., 1998].
However, the antibody accumulation exclusively in particular plant organs can be advantageous for control of a certain developmental stage of a plant. The accumulation of antibodies in early stage of seed maturation under control of legumin B4 promoter [Fiedler et al., 1997] or in later stage of seed development controlled by USP promoter [Phillips et al., 1997] has been reported. Antibodies can be potentially expressed in any other plant organ depending on the promoter region of the expression cassette.

Protein trafficking in eukaryotic cells is guided by signal sequences. Proteins without a signal peptide on its amino-terminal side are localized in the cytosol. The presence of a signal peptide specific for the endoplasmic reticulum, nucleus, peroxysomes, mitochondria or chloroplast determines the subcellular compartment where the nascent protein ends up (for review see [Alberts et al., 1994]).

3.2 Recombinant antibodies in the apoplast and in the endoplasmic reticulum of plant cells

The first successful attempts of full-size antibody expression were all ubiquitous and with a murine Ig signal peptide leading to the assembly of the complete antibodies in the endoplasmic reticulum of transgenic plants (for review see [Conrad and Fiedler, 1998]) followed by their secretion into apoplastic space. Antibody secretion across the plant cell wall into the apoplasm can be advantageous because this large aqueous space is a stable

environment with minimal hydrolytic processing [Ma and Hein, 1995]. The disadvantage is that apoplast is not the right compartment for modulation of antigens with an intracellular localization and action. Although the maximal level of the antibody accumulation depends on the plant species and on the intrinsic properties of the antibody itself, generally the accumulation level of secreted antibodies is highest for full-size antibodies and Fab fragments [De Jaeger et al., 2000].

If the plant expression cassette contains a signal peptide on an N-terminal side of the antibody-fragment coding sequence and a retention signal on a C-terminal side the antibody retains in the endoplasmic reticulum. The ER contains a number of molecular chaperones, which assist the folding and maturation of nascent protein (for review see [Galili et al., 1998]). Moreover this oxidizing environment facilitates the formation of intra- and inter-chain disulfide bonds necessary for the correct folding of proteins. The retention signal can be the tetrapeptide HDEL or KDEL. Soluble reticuloplasmins are thought to be retained in the ER by membrane receptors that recognize the H/KDEL sequence [Pagny et al., 1999]. These features make the ER a convenient cell compartment for stable antibody accumulation. Indeed many laboratories showed a correct cleavage of the ER signal sequence and an assembly of a functional antibody fragment in the ER (summarised in [Conrad and Fiedler, 1998]). For the immunomodulation in the ER, scFv fragments are the best choice of a format [De Jaeger et al., 2000]. The scFv proteins have been detected in the endoplasmic reticulum, ER-derived vesicles and in the nuclear envelope.

3.2.1 Cloning of anti-jasmonate scFv genes into the vector pRTRA 7/3 for retention in the endoplasmic reticulum of plant cells

Jasmonic acid-specific scFv antibodies and 12-oxo-phytodienoic acid-specific scFv antibodies, collectively named anti-jasmonate scFv antibodies, were selected by A. Hunger [Hunger, 2002] from a screening of Tomlinson's Human synthetic VH + VL scFv phagemid libraries A + B (for detailed protocol see web site http://www.mrc-cpe.cam.ac.uk/~phage/*g1p.html). Both libraries contain 10^9 clones with scFv coding sequences inserted into phagemid pIT1. Phagemids as vectors offers some advantages, such as monovalent display of scFv (or Fab) fragments for selection [Hoogenboom, 1997]. They favour the display of less then a single antibody fragment and thereby the avidity effect in the selection process is minimised [Winter, 1998]. Phagemids also enable production of soluble antibodies without another subcloning.
The pIT1 vector, illustrated in Fig. 3.1, contains an inducible lacZ promoter. The scFv gene is inserted between SFiI – NotI restriction sites and targeted into the periplasm by the pelB leader. The c-myc-tag sequence is recognized by the 9E10 antibody and used for the detection of secreted scFv antibodies. Between tag-sequence and a gene coding for gIII minor phage coat protein is introduced an amber codon. The scFv can be produced either for display on phage particle from infected amber suppressor strain (TG1 suppressor strain was used in this study) or secreted into the periplasm as a soluble fragment from infected non-suppressor strain (HB2151 strain has been used in this study).

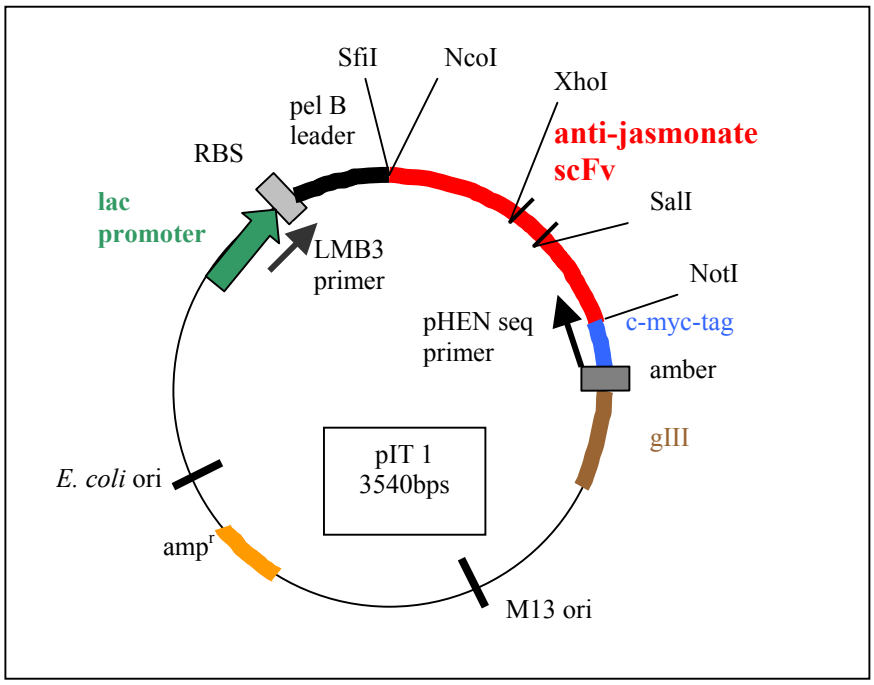


Fig. 3.1 Scheme of the pIT1 phagemid. For details to the expression cassette see text in Chapter 3.2.1. RBS – ribosome binding site.

Three obtained jasmonic acid-specific clones (namely, B11, C4 and F8) and three 12-oxo-phytodienoic acid-specific clones (namely D3, E10 and F2) were selected for further cloning. Single-stranded DNA of these clones has been used for radioactive sequencing with the primers LMB3 and pHEN seq. The sites of their matching to pIT 1 vector are shown in the Fig. 3.1. The nucleotide sequences of scFv genes have been compared with the nucleotide sequences of brassinosteroid-specific scFv [Fecker L.F., unpublished], with ABA-specific scFv [Artsaenko, 1996] and with anti-cytokinin – *meta* topolin – scFv [ten Hoopen P., unpublished]. The comparison done by ClustalW Multiple Sequence Alignment (http://dot.image.bcm.tmc.edu:9331) via the program Vector NTI Deluxe, v.4.0.1, allowed an identification of CDR's and framework regions and revealed differences between individual scFv's only in CDR1 and CDR3 of heavy chains and CDR1 of light chains. Table 3.2 summarises this differences on deduced amino acid level.

The anti-jasmonate scFv genes were inserted into the expression cassette for their retention in the ER of plant cells. The vector pRTRA 7/3, Fig. 3.3, containing anti-ABA scFv gene-c-myc fusion under control of the CaMV35S promoter, with the legumin B4 signal sequence and the KDEL retention sequence has been used [Artsaenko, 1996]. The vector was cleaved with BamHI, dephosphorylated and 3200bp fragment was ligated with anti-JA and/or anti-OPDA scFv gene. The BamHI restriction sites were incorporated into the scFv gene inserts by PCR with FORTOM and BACKLEGTOM primers. The PCR product, 800bp long, was cleaved with BamHI prior the ligation. XL1 Blue strain of *E.coli* was heat shock-transformed with the ligation product and ampicilin-resistant transformants were tested for the presence of scFv insert in the right

orientation by NcoI-NotI cleavage. The positive clones were sequenced with M13 Universal (MU) and M13 Reverse (MR) primers and also with LEG73 and TAG73 primers. The matching positions of all four primers to vector pRTRA 7/3 are depicted in the Fig. 3.3. The results of sequencing confirmed correct orientation and correct reading frame of the cloned scFv genes. Fig. 3.4, page 31, illustrates the schematic overview of the cloning procedure and the final expression cassette for retention of anti-jasmonate scFv antibody fragments in the endoplasmic reticulum of plant cells. The expression cassette was inserted between HindIII restriction sites.

Table 3.2 Differences on deduced amino acid level in CDR regions of chosen scFv antibodies selected against different plant hormones by phage display.

phytohormone specific clone		CDR region		
		CDR I - VH	CDR III - VH	CDR I - VL
JA	B11	RYPMR	NLRR	ILKSLL
JA	C4	KYIMG	KYRR	IRQLLM
JA	F8	RYPMR	GARK	IGAQLK
OPDA	E10	PYRMW	KSLL	IKNSLE
OPDA	F2	RYRMW	RHRR	ISRQLL
BRAS.	A1	NYMML	KARR	ILGPLS
mTR	A1	SYTMS	NRPF	INVNLA

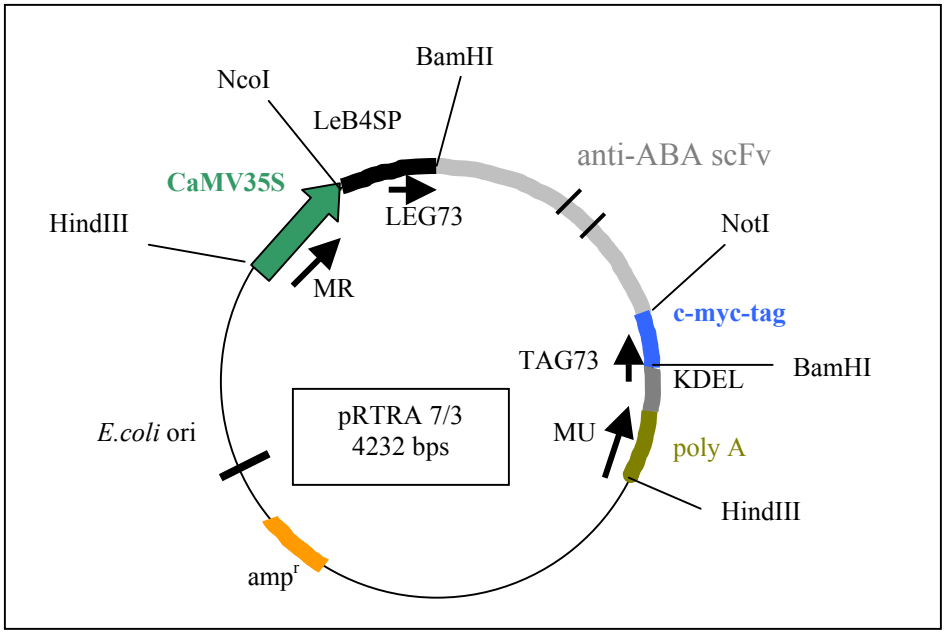


Fig. 3.3 Scheme of the vector pRTRA 7/3. For details to the expression cassette see text of Chapter 3.2.1.

3.3 The accumulation of functional antibodies in the cytosol of tobacco cells

The expression cassette without signal peptide allows the retention of recombinant antibodies in the cytoplasmic space. The accumulation of functional antibodies in high concentration in the cytoplasm is of highest interest because an action of scores of proteins and signalling molecules takes place in this cell compartment. For example most plant viruses are RNA viruses that replicate in the cytosol and an assembly of anti-viral antibodies in this compartment has highest impact on the reduction of the viral infection [Tavladoraki et al., 1993], [Zimmermann et al., 1998]. Unfortunately the cytosol is the compartment in which high antibody accumulation levels are the most difficult to obtain because of the lack of chaperones and the reducing environment of the cytosol, which prevents the formation of disulfide bridges [Biocca et al., 1995]. For this reason antibodies were in some studies expressed at low level and the visualization of the immunomodulation effect was prevented. Unlike full-size antibodies or Fab antibody fragments, the scFv antibody fragments have a higher chance of being correctly folded in the cytosol because flexible linker connects light and heavy chains and there is no need for the formation of inter-chain disulfide bonds. Indeed, several examples of sufficient accumulation of functional scFv antibodies in the cytosol have been reported [Owen et al., 1992], [Tavladoraki et al., 1993], [Zimmermann et al., 1998]. However, even scFv has et least one intra-chain disulfide bridge which is essential for correct folding. This is probably the reason why some scFv's can not be detected in the cytosol in spite of normal level of corresponding mRNA.
It was unexpectedly discovered that the addition of the ER-retention signal at the C-terminus of scFv fragment significantly improves expression level [Schouten et al., 1997]. The KDEL peptide has been suggested to protect sterically the scFv region susceptible to proteolysis. However even this tetrapeptide does not improve the cytosolic accumulation level of every scFv protein and the expression level of a particular scFv is also coding-sequence dependent.

3.3.1 Cloning of anti-jasmonate scFv genes into the vector pRTRA 7/3 for retention in the cytosol of plant cells

The cloning strategy for targeting of three anti-JA and three anti-OPDA scFv genes into the cytosol was the same as described in Chapter 3.2.1 with only one exception. The final expression cassette lacks the LeB4 signal peptide. Therefore, PCR amplification with FORTOM and BACKLEGTOM primers was omitted and the anti-JA and anti-OPDA scFv genes were cleaved from pIT1 phagemid by double restriction NcoI-NotI and directly ligated behind the promoter region of the pRTRA 7/3 vector.
Fig. 3.5 illustrates the schematic overview of the cloning procedure and the final expression cassette for retention of anti-jasmonate scFv antibody fragments in the cytosol of plant cells. The expression cassette was inserted between HindIII restriction sites.

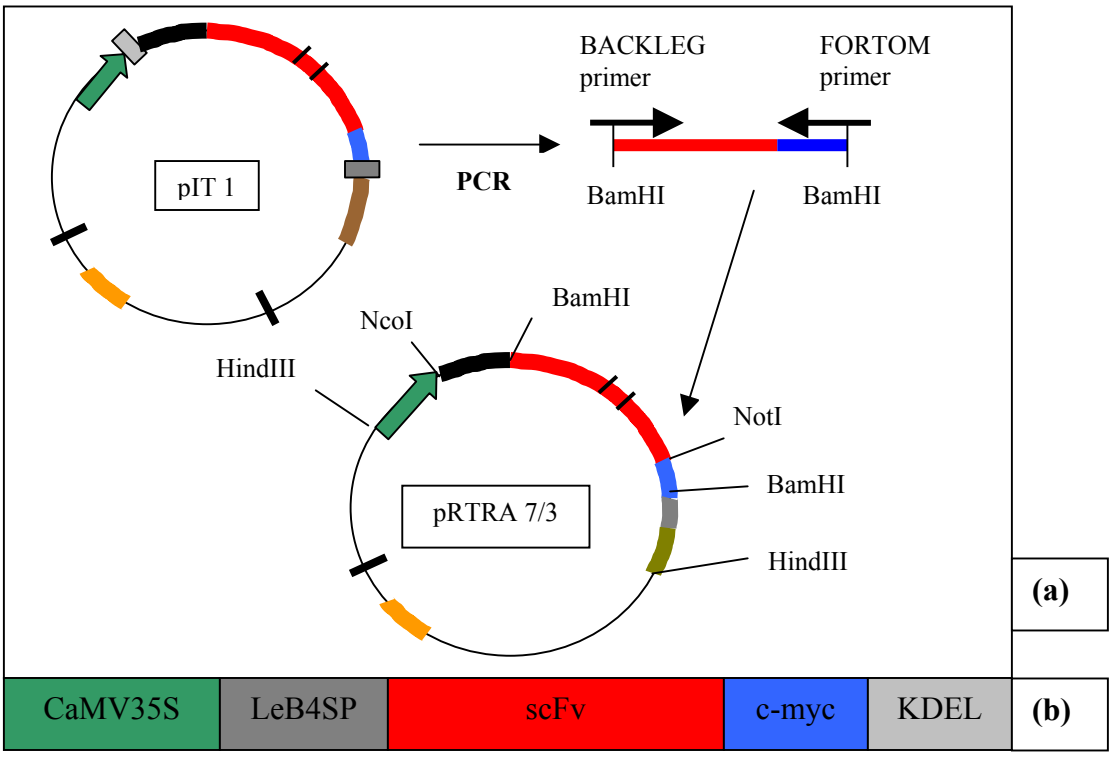


Fig. 3.4 Schematic overview of cloning of anti-jasmonate scFv genes into the pRTRA 7/3 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the endoplasmic reticulum of tobacco cells (b).

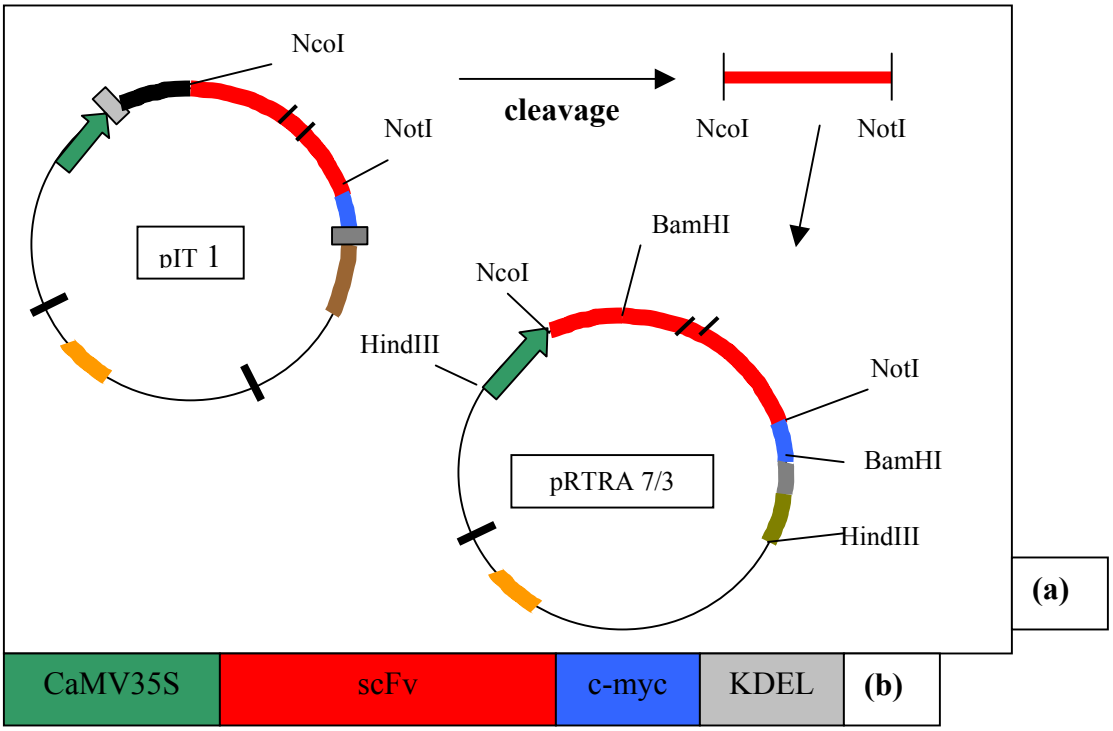


Fig. 3.5 Schematic overview of cloning of anti-jasmonate scFv genes into the pRTRA 7/3 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the cytosol of tobacco cells (b).

3.4 The accumulation of scFv antibody fragments in the stroma and on the outer envelope membrane of tobacco chloroplasts

Chloroplasts are semiautonomous organelles. The consequence of the evolution of the chloroplast from an original prokaryotic endosymbiont to a cellular organelle was the transfer of the vast majority of genes encoding chloroplast polypeptides to nuclear chromosomes (approximately 90%). The chloroplast genome of vascular plants contain only about 100 genes, most of which encode components of the photosynthetic electron transport machinery and elements of the transcriptional and translational apparatus [Stern et al., 1997]. Proteins destined for the internal compartments of the chloroplast (stroma, tylakoid and inner membranes) carry N-terminal transit sequences, which are necessary and sufficient to direct the import of a polypeptide into the chloroplast [Fuks and Schnell, 1997]. The import process of proteins with this chloroplast-targeting signal engages a recognition and translocation machinery at the chloroplast envelope called general import machinery. In contrast, chloroplast outer envelope membrane proteins appear to use an alternative mechanism of integration that does not involve the general import machinery and this alternative mechanism is up till now poorly understood.
The envelope translocation does not require a fully unfolded polypeptide chain even though polypeptide-unfolding activity associated with the outer membrane has been identified. Both, outer and inner chloroplast envelope membranes contain also molecular components and chaperones involved in the import process.
Reports concerning the import process into tylakoid membranes [Henry et al., 1997], into the stroma [Filho et al., 1996], or into the outer chloroplast envelope membrane [Li and Chen, 1996] recently emerged. Even though the chloroplast translocation process is far from being understood there have already been some attempts to utilize the chloroplast transport machinery for accumulation of foreign proteins. While the targeting of a foreign enzyme into the chloroplast has been successful [Creissen et al., 1995] the attempt to accumulate a single-domain antibody fragment into the chloroplast had less success. Camel single-domain antibodies (VHH) fused to tetanus toxoid have been targeted into chloroplasts of *Nicotiana tabacum*, however, no VHH proteins were found in the chloroplast extracts [Vû, 1999].

3.4.1 Cloning of anti-12-oxo-phytodienoid acid scFv genes into the pRT103 vector for retention in the stroma and on the outer envelope membrane of chloroplasts

In this study three different transit sequences have been used for the accumulation of anti-12-oxo-phytodienoci acid scFv antibodies in chloroplasts. First, the transit sequence of the small subunit of ribulose-biphosphate-carboxylase, targeting precursor of this enzyme into the stroma of chloroplasts, has been used and called ST3TP [Waegemann and Soll, 1996]. Second, the stromatal transit sequence of the protein ferredoxin has been utilized and called ST5TP [Van´t Hof, and De Kruijff, 1995]. For targeting of scFv into the outer chloroplastic envelope membrane the transit sequence of the outer chloroplast membrane protein OEP14 was used and called OCMTP [Li and Chen,1997].
There are several lines of evidence supporting the idea that the stroma of chloroplasts and the chloroplastic envelope membranes are the sites where the biosynthesis of 12-oxo-phytodienoic acid – precursor of jasmonic acid biosynthesis – takes place (see

Chapter 1.2.3). Therefore only the anti-OPDA scFv antibodies were candidates for cloning into the chloroplastic stroma and into the outer envelope membrane of chloroplasts. The F2 – anti-OPDA scFv gene has been utilized.

The coding sequences of all three above discussed transit peptides (ST3TP, ST5TP and OCMTP) were available in plasmid pRT103 and scFv expression was detected in regenerated transgenic plants [Rosso M., unpublished]. The pRT103 vector, depicted in Fig. 3.6, contains an expression cassette with anti-ABA scFv and a transit sequence for stromatal targeting (ST3TP or ST5TP) or a transit sequence for outer envelope membrane targeting (OCMTP) under the CaMV35S ubiquitous promoter.

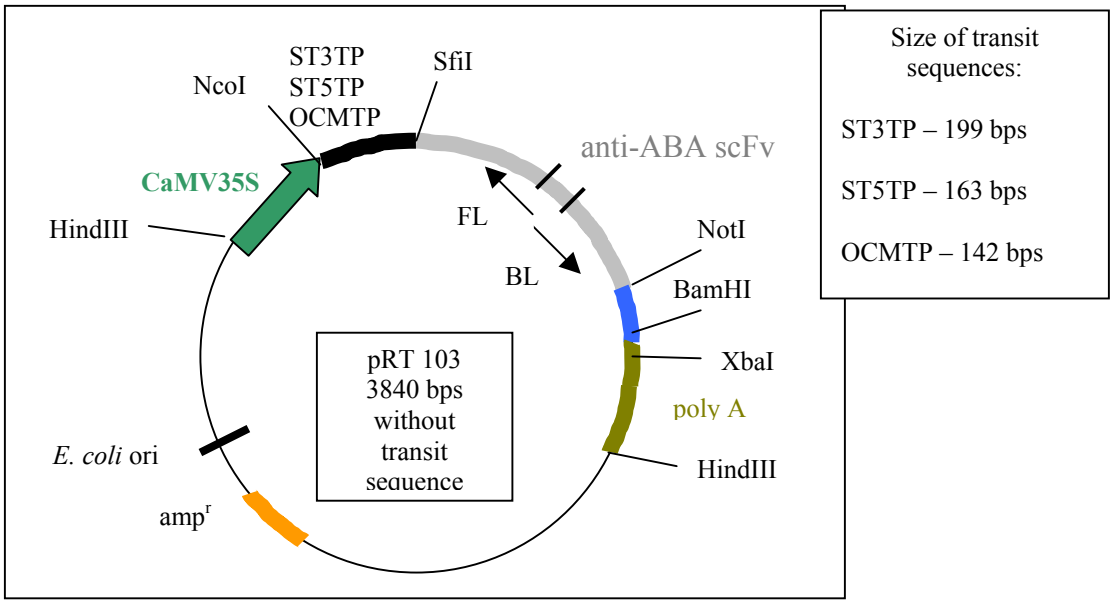


Fig. 3.6 Scheme of the vector pRT103. For details see text of Chapter 3.4.1.

Cloning of the anti-OPDA scFv gene for the retention in the stroma of tobacco chloroplasts consisted of only one cloning step. The anti-OPDA scFv-F2 gene was double digested with SfiI-NotI enzymes and cloned into the SfiI-NotI site of the pRT103 vector with ST3TP or ST5TP transit sequence. The presence of anti-OPDA scFv gene in the ampicilin resistant clones was confirmed by control restriction and by sequencing with primers BACKLINK (BL) and FORLINK (FL). Matching sites of these primers are included in the Fig. 3.6. Fig. 3.8, page 34, shows the schematic overview of the cloning procedure and the final expression cassette for retention of anti-OPDA scFv antibody fragments in the stroma of tobacco chloroplasts. The expression cassette was inserted between HindIII restriction sites.

The expression cassette for the retention of anti-OPDA scFv antibody F2 gene in the outer envelope membrane of tobacco chloroplasts was designed with utilization of two vectors. The pRT103 vector with the OCMTP transit sequence, Fig 3.6, and vector pRTHook which contains the 237bp long insert of the c-myc-tag sequence fused to the transmembrane domain sequence (Hook domain). This domain should anchor the scFv antibody in the outer membrane of the chloroplast. Fig. 3.7 shows a scheme of the

pRTHook vector. First, the anti-OPDA scFv gene has been inserted into SfiI-NotI site of the pRT103 vector with the OCMTP transit sequence. Afterwards the c-myc-Hook domain fusion has been ligated into the NotI-restricted and dephosphorylated vector pRT103 with the anti-OPDA scFv gene.

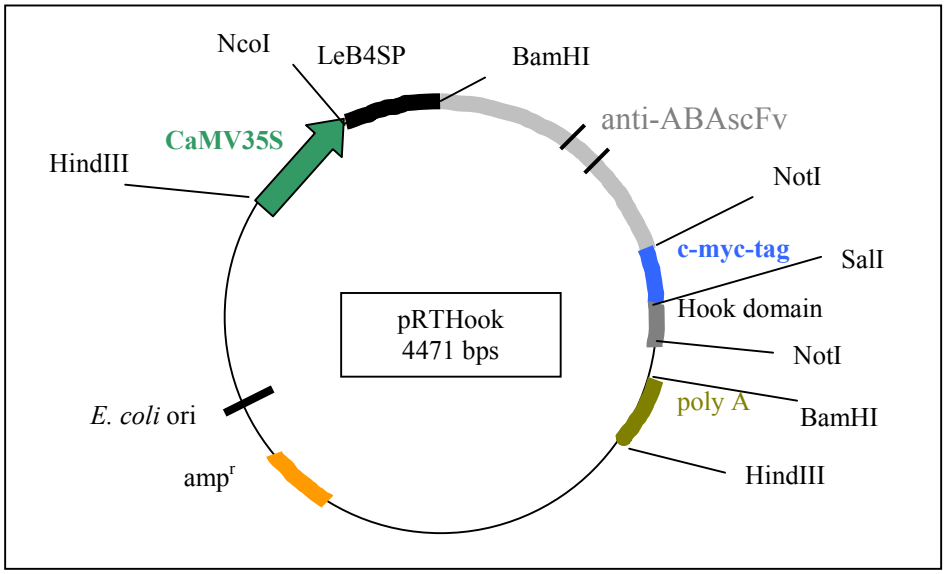


Fig. 3.7 Scheme of the pRTHook vector. For details see text of Chapter 3.4.1.

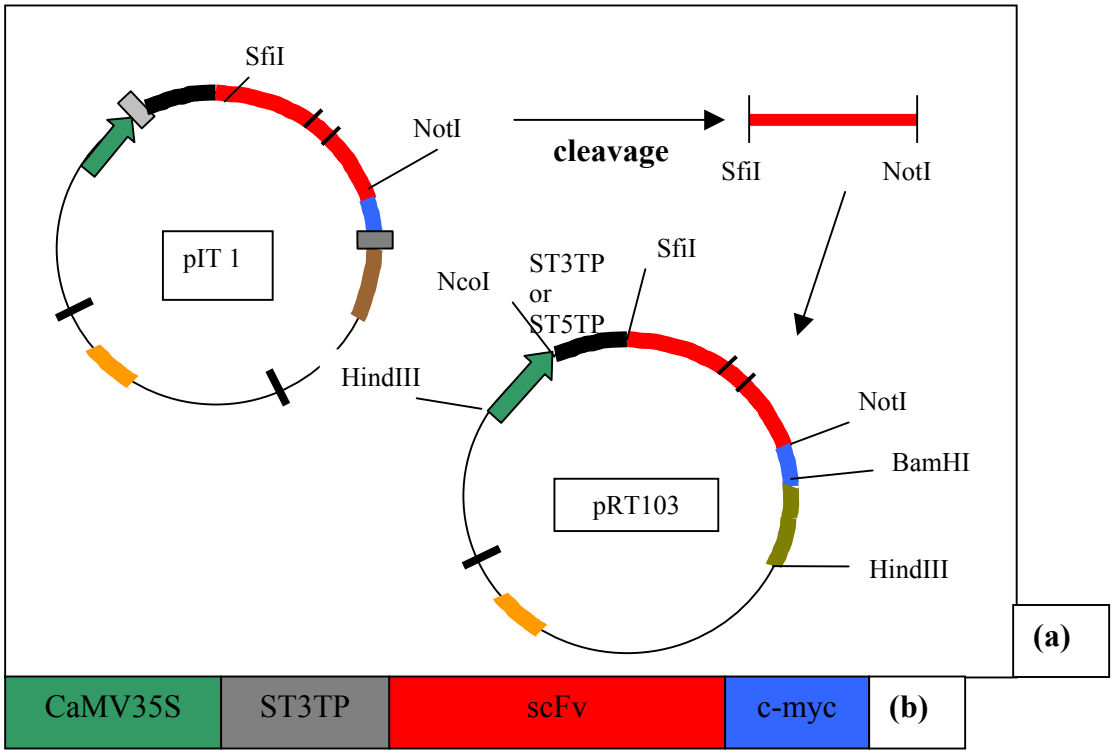


Fig. 3.8 Schematic overview of cloning of the anti-OPDA scFv gene into the pRT103 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the stroma of tobacco chloroplasts (b).

The correct orientation of the c-myc-Hook domain fusion has been checked by XbaI digestion followed by SalI restriction and has also been confirmed by sequencing with primers BACKLINK and FORLINK, whose matching sites are shown in the Fig. 3.6. The correct orientation of the c-myc-Hook domain fusion is illustrated in Fig. 3.9 together with the schematic overview of the cloning procedure and the final expression cassette for the retention of anti-OPDA scFv antibody fragments in the outer envelope chloroplastic membrane. The expression cassette was inserted between HindIII restriction sites.

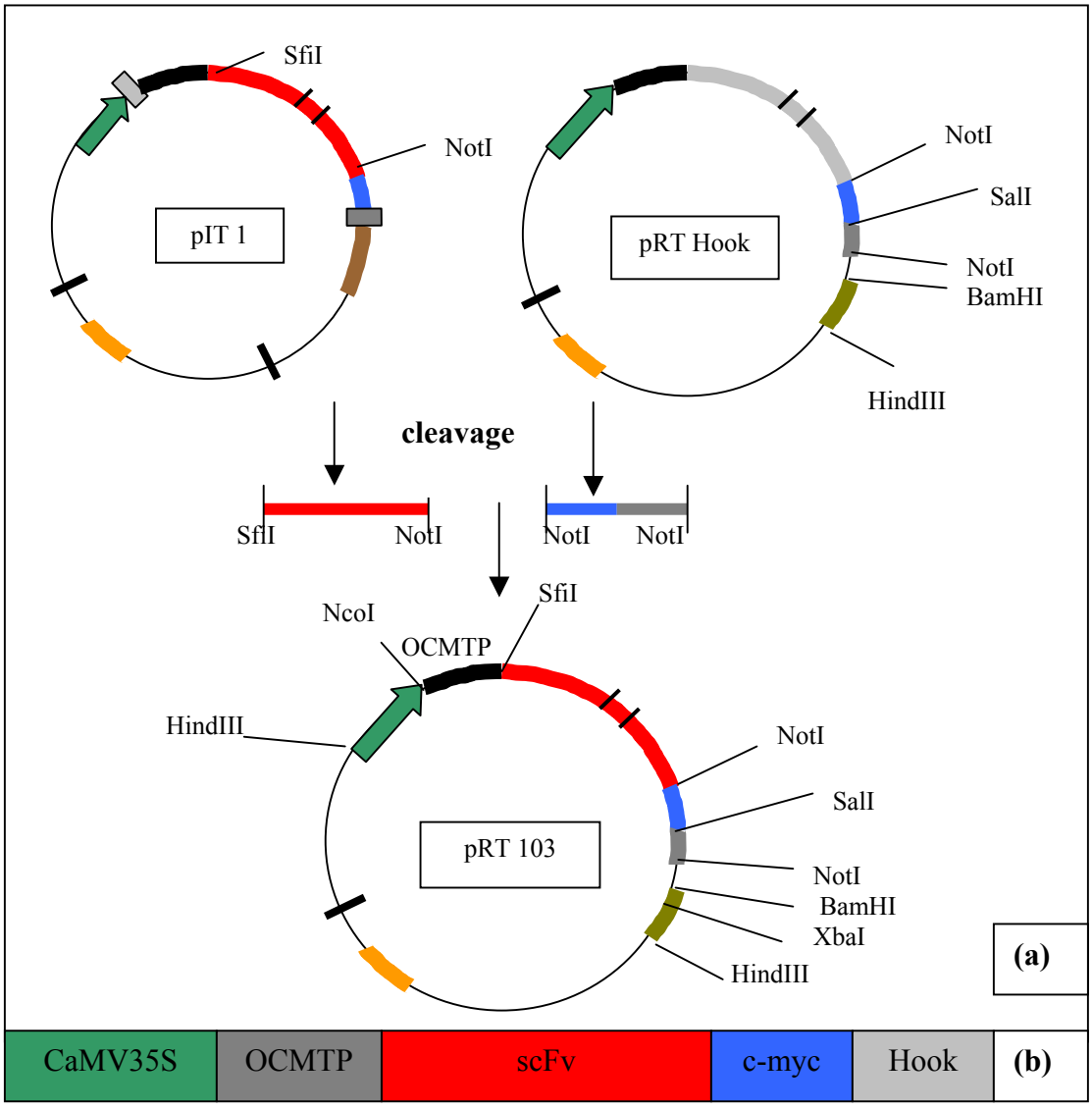


Fig. 3.9 Schematic overview of cloning of the scFv gene into the pRT103 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the outer envelope chloroplastic membrane (b).

3.5 *Agrobacterium tumefaciens*-mediated gene transfer

Various techniques have been used in the last decades to introduce foreign genes into plants to achieve an alteration of the properties of the recipient. These transformation techniques include transformation of protoplasts by electroporation [Lindsey and Jones, 1987], particle bombardment [Klein et al., 1987], microinjection [Reich et al., 1986] and above all transformation mediated by *Agrobacterium tumefaciens* [Zambryski et al., 1983], reviewed extensively for example in [White, 1993]. The principle of A*grobacterium*-mediated gene transfer is based on two regions essential for the transfer, the *vir* region and the T-DNA region. The *vir* region is not transferred itself; genes of the *vir* region are induced by phenolic compounds produced after wounding of a plant. The T-DNA region is defined by T-DNA border repeat sequences, which are highly conserved and made up of 25bp DNA sequences. Any DNA placed between the borders will be transferred into the plant. The right border (RB) seems to play a more essential role than the left border (LB) because the T-DNA transfer starts always from RB and only around RB has always been found „overdrive" DNA sequence that functions as T-DNA transfer enhancer. V*ir* region gene products recognize the border sequences, cleave T-DNA from the plasmid and direct the T-DNA, which is mostly linear single-stranded DNA, to the nucleus of the plant cell. The T-DNA covalently integrates into plant nuclear DNA and transcription is done by RNA polymerase II. The majority of T-DNA insertions by A*grobacterium*-mediated transfer are stably inherited in normal Mendelian fashion.

3.5.1 Cloning of the expression cassettes into binary vector pBIN19

Fig. 3.10 shows an overview of four expression cassettes for targeting of anti-jasmonate scFv antibody fragments into the ER (a), into the cytosol (b), into the chloroplastic stroma (c) and on the outer envelope chloroplastic membrane (d) of tobacco cells.

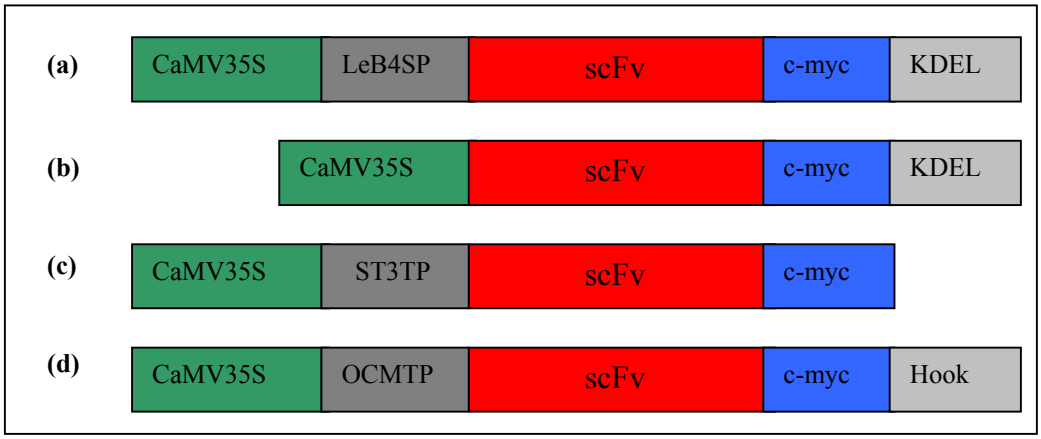


Fig 3.10. Overview of expression cassettes for accumulation of anti-jasmonate scFv antibodies into different subcellular compartments of tobacco cells.

HindIII fragments of size 1.5 kbp containing the expression cassettes were isolated from plasmids pRTRA 7/3 and pRT103 and cloned into the binary vector pBIN19 restricted

with HindIII enzyme and dephosphorylated. The scheme of the pBIN19 vector is shown in Fig. 3.11.

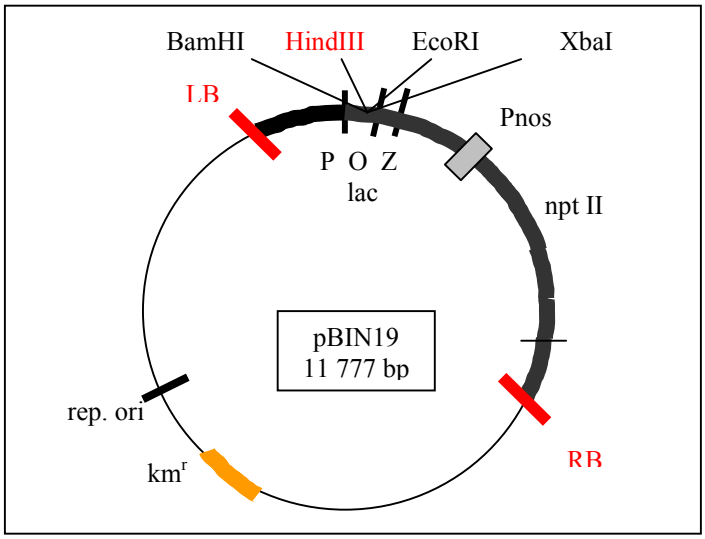


Fig. 3.11 Scheme of the binary vector pBIN19.
RB, LB – right and left T-DNA borders; P,O,Z – lac operons; Pnos –nopaline synthase promotor; nptII – neomycine phosphotransferase gene, km^r – kanamycin resistance.

The plasmid contains a kanamycin bacterial selection marker and replicons for replication in both *E.coli* and *A. tumefaciens*. Between left and right border of T-DNA is a multiple restriction site including a HindIII site and the neomycin phosphotransferase II gene enabling selection of transgenic plants on kanamycin. The binary vector pBIN19 with the expression cassette was electroporated into the *Agrobacterium tumefaciens* that provides vir gene products in *trans*. *A. tumefaciens* strain 2260 has been used for the transformation of tobacco cells, Chapter 7.2.11. The confirmation of stable incorporation of the expression cassettes in *Agrobacterium* was done by the extraction of the vector, HindIII restriction and separation of 1% agarose gel followed by southern blot analysis. The HindIII fragment was hybridised with the 800bp long scFv gene-c-myc fusion restricted BamHI from the pRTRA 7/3 vector. As an example, the result of the southern blot analysis with some of the analysed agrobacterium-clones containing different scFv's in different expression cassettes is summarised in Fig. 3.12.
The selection and characterisation of transgenic plants is discussed in details in the following chapters.

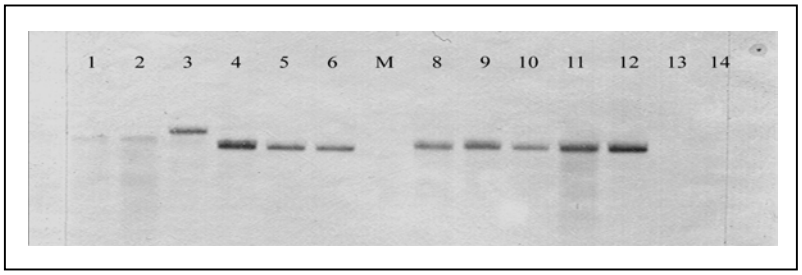


Fig. 3.12
Southern blot analysis of *Agrobacterium* clones.

1 and 2 – clones with ST3TP, 3 – clone with OCMTP, 4-6 – clones without signal peptide, M – marker, 8-12 – clones with LeB4SP, 13 – agrobacterium clone with the pBIN19 vector only, 14 – "empty" *Agrobacterium* clone.

Chapter 4

Selection and characterization of transgenic plants

4.1 Selection of transgenic plants according to scFv expression level

Transgenic plants, regenerated from the leaf-discs transformed with *Agrobacterium*-clones containing anti-jasmonate scFv, have been screened for transformants with the highest level of scFv accumulation in different plant cell compartments. The selection has been done by western blot analysis.

Table 4.1 summarises regenerated, kanamycin-resistant (Km-resistant) transgenic plants with three different anti-JA scFvs (B11, C4 and F8) in the ER of tobacco cells. Plants were designed as JAI, JAII and JAIII, respectively. Transgenic plants with two different anti-OPDA scFvs (E10 and F2) in the ER of tobacco cells were designed as OPDAIV and OPDAV, respectively. Table 4.1 shows the total number of plants analysed and the number of plants reaching different levels of scFv accumulation in the ER, ranging from 1 ng – 25 ng of scFv / 20 μg of total soluble protein. The expression level of scFv in % of TSP is also given therein. The corresponding expression cassette for these "ER plants" is illustrated in Fig. 3.10 (a).
Similarly, table 4.2 gives an overview of total number of regenerated, Km-resistant, transgenic plants expressing the three different anti-JA scFv antibodies in the cytosol of tobacco cells. Plants were designed as JAVI, JAVII and JAVIII, respectively. The table 4.2 shows a number of these plants reaching expression levels of scFv between 1 ng – 25 ng of scFv / 20 μg of TSP. The same data are given in the table 4.2 for Km-resistant transgenic plants with three different anti-OPDA scFvs (D3, E10 and F2) in the cytosol of tobacco cells. Plants were designed as OPDAIX, OPDAX and OPDAXI, respectively. The corresponding expression cassette for these "cytosolic plants" is illustrated in Fig. 3.10 (b).
As expected, the anti-jasmonate scFv is accumulated in the cytosol to a significantly lower level than in the ER due to the reducing environment of the cytosol, as discussed in Chapter 3.3. Moreover, differences in the expression level between different scFvs are obvious. The dependence of antibody intracellular stability on primary antibody sequence is known but not an in advance predictable phenomenon. For this reason more than one anti-JA and anti-OPDA scFv have been used for cloning into expression cassettes and for transgenic plants regeneration.
Finally, table 4.3 gives an overview of Km-resistant transgenic plants accumulating anti-OPDA-F2 scFv in the stroma of tobacco chloroplasts, plants designed as ST3 and ST5, and Km-resistant plants expressing the anti-OPDA-F2 scFv in the outer chloroplastic membrane of tobacco cells, plants designed as OCM. The corresponding expression cassettes of these "chloroplastic plants" are illustrated in Fig. 3.10 (c) and Fig. 3.10 (d).

Table 4.1 Summary of regenerated transgenic plants expressing anti-JA and anti-OPDA scFv in the endoplasmic reticulum of tobacco cells.

expression level in		designation of plants (scFv sequence)				
ng scFv/ 20µg of TSP	% TSP	JAI (B11)	JAII (C4)	JAIII (F8)	OPDAIV (E10)	OPDAV (F2)
25ng	0,125%	0	0	0	0	0
10ng	0,050%	6	7	0	2	2
5ng	0,025%	9	14	7	17	7
1ng	0,005%	12	16	17	31	21
total number of analyzed plants		65	74	79	93	92

Table 4.2 Summary of regenerated transgenic plants expressing anti-JA and anti-OPDA scFv in the cytosol of tobacco cells.

expression level in		designation of plants (scFv sequence)					
ng scFv/ 20µg of TSP	% TSP	JAVI (B11)	JAVII (C4)	JAVIII (F8)	OPDAIX (D3)	OPDAX (E10)	OPDAX (F2)
25ng	0,125%	0	0	0	0	0	0
10ng	0,050%	0	0	0	0	0	0
5ng	0,025%	0	1	8	0	0	3
1ng	0,005%	7	12	13	2	4	14
total number of analyzed plants		84	53	135	70	61	65

Table 4.3 Summary of regenerated transgenic plants expressing anti-OPDA-F2 scFv in the stroma and in the outer membrane of tobacco chloroplasts.

expression level in		designation of plants (scFv sequence)		
ng scFv/ 20µg of TSP	% TSP	OCM (F2)	ST3 (F2)	ST5 (F2)
25ng	0,125%	0	5	0
10ng	0,050%	0	26	2
5ng	0,025%	8	10	15
1ng	0,005%	23	11	11
total number of analyzed plants		86	95	110

Transgenic plants with the highest expression level of scFv in the particular plant cell compartment have been chosen for further investigation. The selected transgenic plants are summarised in table 4.4.

Table 4.4 Designation of selected transgenic plants expressing anti-jasmonate scFvs in different subcellular compartments.

ScFv-targeting plant cell compartment	designation of transgenic plants	scFv sequence
endoplasmic reticulum	JAII	C4
cytosol	JAVII	C4
stroma of chloroplast	ST3	F2
outer chloroplastic membrane	OCM	F2

4.2 Immunolocalization of anti-OPDA-F2 scFv in the chloroplastic subcompartments of tobacco cells

To verify the expected targeting of the anti-OPDA scFv into chloroplastic subcompartments, three independent lines of ST3 plants and three independent lines of OCM plants have been used for immunolocalization of the scFv in the stroma and in the outer chloroplast membrane of tobacco cells. High expression level of scFv in all chosen transgenic plants has been confirmed, prior to this experiment, by western blot analysis. Wild-type plants, *Nicotiana tabacum*, cv. Samsun NN, of the same developmental stage and grown under the same conditions as the transgenic plants, were taken as a negative control. Histochemical analysis of the transgenic and wild-type plants has been done by Dr. Bettina Hause, IPB-Halle, Halle, Germany. Single-chain Fv antibody proteins were detected by anti-c-myc-tag antibodies and visualised by FITC conjugated secondary antibodies. Results obtained by light microscopy after immunofluorescence staining are shown in Fig. 4.5.

There was no signal in wild-type plants, as shown in Fig. 4.5 (a). Chloroplasts show only autofluorescence. A green fluorescence given by detected scFv in chloroplasts of ST3 transgenic plants confirmed the presence of scFv antibodies in the stromatal subcompartment of chloroplasts of the transgenic plants (Fig. 4.5 (b)). ScFv has been localized in chloroplasts also in all three OCM lines, Fig. 4.5 (c), but unlike in the ST3 plants, rather in a spot-like pattern, which could mean an incorporation of the scFv protein in the outer membrane in between other functional proteins of the outer chloroplastic membrane.

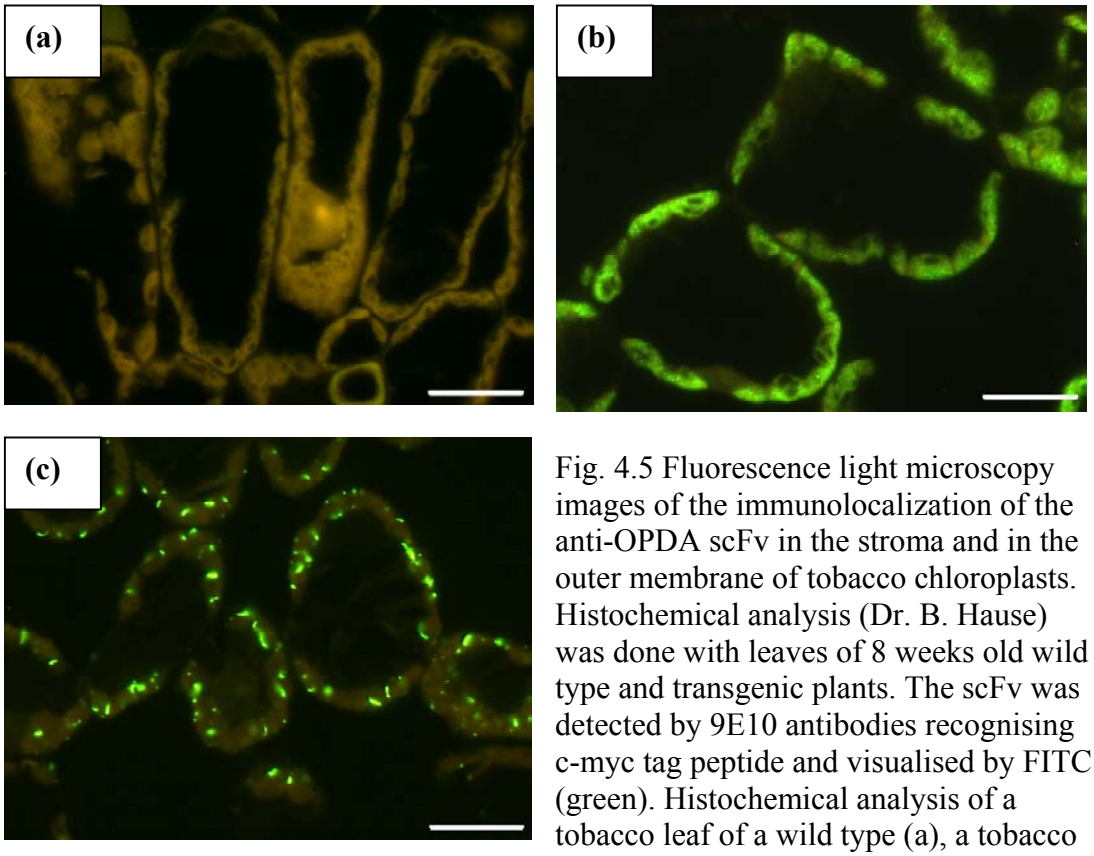


Fig. 4.5 Fluorescence light microscopy images of the immunolocalization of the anti-OPDA scFv in the stroma and in the outer membrane of tobacco chloroplasts. Histochemical analysis (Dr. B. Hause) was done with leaves of 8 weeks old wild type and transgenic plants. The scFv was detected by 9E10 antibodies recognising c-myc tag peptide and visualised by FITC (green). Histochemical analysis of a tobacco leaf of a wild type (a), a tobacco leaf of a transgenic plant with anti-OPDA scFv in the stroma of chloroplasts (b) and a tobacco leaf of a transgenic plant with anti-OPDA scFv in the outer chloroplastic membrane (c).

4.3 Developmental changes caused by the expression of anti-jasmonate scFv antibodies

No significant changes have been observed in F_0 generation of regenerated transgenic plants.

However, the expected developmental disturbance caused by the ectopic expression of anti-jasmonate antibodies in subcellular compartments could be the disturbance in gametophyte development.

The experiments with JA-insensitive mutant of tomato *jai1* [Li et al., 2001] revealed a role of jasmonic acid in female reproductive development. This result, however, stands in contrast to studies in A*rabidopsis* where JA biosynthesis and perception are essential for male, but not female, gametophyte development. *Arabidopsis* triple mutant *fad3-2 fad7-2 fad8* defective in trienoic acids was male sterile but only when the level of linolenic acid in flower organs decreased under 1% of the wild-type level of linolenic acid. The observation that *fad3-2 fad7-2 fad8* mutants were fully fertile despite having less than 5% of linolenic acid in their tissues suggests that the threshold requirement for linolenic acid must be very low [McConn and Browse, 1996]. Considering that linolenic acid, substrate of JA biosynthesis, might be the limiting factor of JA biosynthesis

(Chapter 1.2.3) and that the endogenous level of JA in plant tissues is normally in range of nanomol to micromol, the threshold of JA required for the disturbance of gametophyte development might be even lower.
Taking together, these results indicate species-specific differences in the role of jasmonates in gametophyte development and a very low level of jasmonates required for the disturbance of male or female fertility. If the ectopic expression of anti-jasmonate antibodies does not lead to a decrease of jasmonates availability under this threshold, the immunomodulated tobacco plants will not manifest any disturbance in gametophyte development.

The inhibition of nondormant seed germination is a well documented aspect of jasmonates function (Chapter 1.2.2). Up to 50 μM of jasmonic acid methyl ester had no effect on seed germination in *Arabidopsis* but as little as 5 μM JAME inhibited germination when 2 μM abscisic acid was also present. However, as little as 1 μM JAME inhibited germination of both *Brassica napus* and *Linum usitatissimum* [Staswick et al., 1992 and references therein].

Similar seed germination experiment has been performed with heterozygotic transgenic plants expressing anti-jasmonate scFv in the endoplasmic reticulum and in the cytosol of tobacco cells.
Seeds of wild-type plants were incubated on Murashige-Skoog medium containing either 10 μM JAME or only water, as a control. While germination of water treated seeds was normal, the germination of wild-type nondormant tobacco seeds on 10 μM JAME was significantly reduced (Fig. 4.6 (a)).
In contrast, transgenic tobacco plants accumulating anti-JA scFv in the ER or in the cytosol showed germination of nondormant seeds on 10 μM JAME very similar to that of uninfluenced seed germination on medium with water (Fig. 4.6 (b) and Fig. 4.6 (c)).
The differences between the transgenic plants and wild type in seed germination after application of 1 μM JAME were observed as well (data not shown), only slightly less pronounced.

Nondormant seeds of transgenic plants accumulating scFv against 2-phenyl-oxazol-5one (oxazolone) [Fiedler and Conrad, 1995], a compound not naturally present in plants, have been investigated for their germination in the presence of JAME to check that the ectopic expression of recombinant scFv does not influence the germination process. As well as wild-type plants, the transgenic tobacco plants with anti-oxazolone scFv in the ER manifested normal germination on medium with water but germination was highly inhibited by the presence of 10 μM JAME (Fig. 4.7 (a)). Transgenic tobacco plants with anti-jasmonate scFv could again overcome the JAME-mediated inhibition of nondormant seeds germination (Fig. 4.7 (b) and 4.7 (c)).

These results indicate that the specific ability of transgenic tobacco plants, expressing ectopically anti-JA scFv antibodies in the ER or in the cytosol, to overcome the JA-mediated inhibition of nondormant seed germination is a consequence of intracellular accumulation of exclusively anti-jasmonic acid recombinant antibodies and confirms that the anti-JA scFv antibodies are active in the transgenic plants.

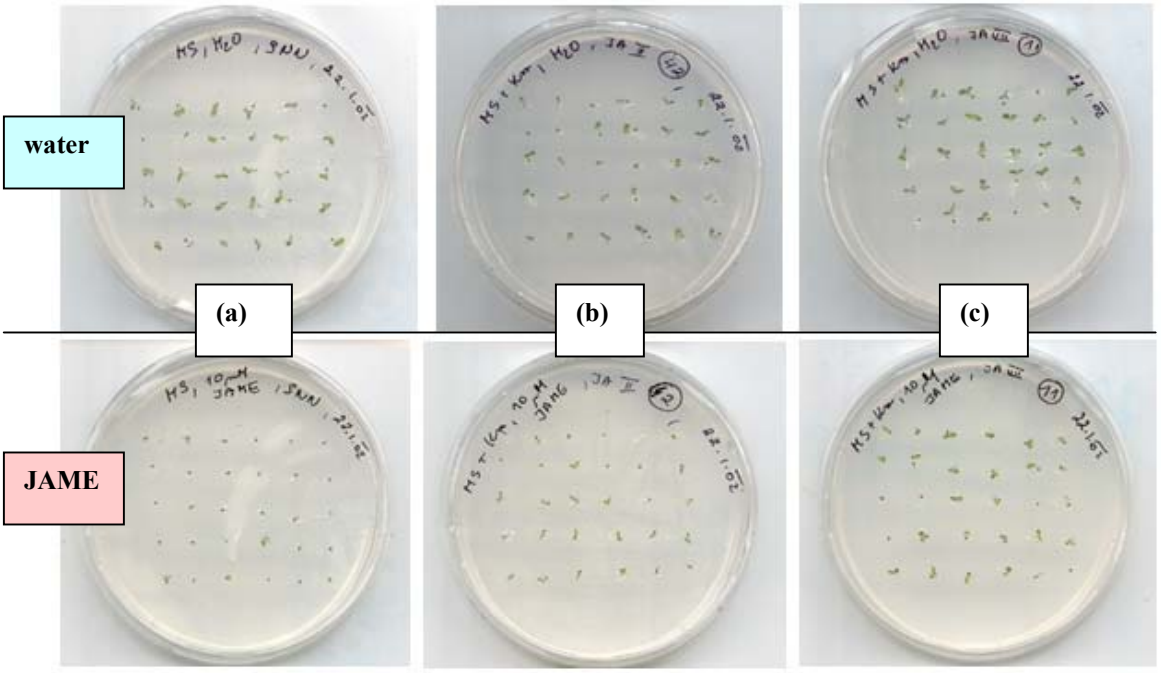


Fig. 4.6 Seed germination of tobacco seeds on Murashige-Skoog medium (specified in Chapter 7.1.17) in the absence or presence of 10 µM jasmonic acid methyl ester. Seeds of a wild type (a), seeds of a transgenic plant with anti-JA scFv in the endoplasmic reticulum (b) and seeds of a transgenic plant with anti-JA scFv in the cytosol (c) are shown 7 days after imbibition.

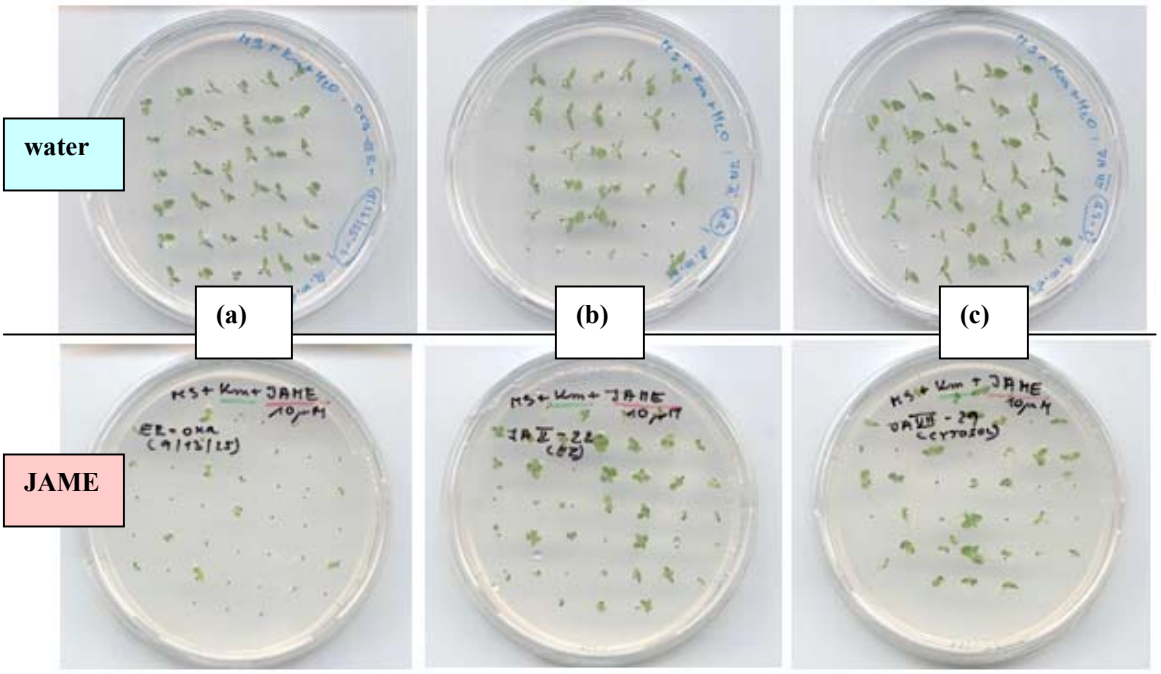


Fig. 4.7 Seed germination of tobacco seeds in the absence and presence of 10 µM JAME. Seeds of a transgenic plant with anti-oxazolone scFv in the ER (a), seeds of a transgenic plant with ant-JA scFv in the ER (b) and seeds of a transgenic plant with anti-JA scFv in the cytosol (c) are shown 12 days after imbibition.

4.4 Analysis of F1 generation of transgenic tobacco plants

To verify stable inheritance of foreign recombinant scFv in the next generation, seeds of four selected types of transgenic plants (JAII, JAVII, ST3 and OCM) were placed on Murashige-Skoog medium (Chapter 7.1.17) containing kanamycin. Seeds of wild-type plants placed on the Murashige-Skoog medium without antibiotic have been used as a negative control. Western blot analysis of transgenic seedling revealed that the scFv antibody expression in F_1 generation reaches the same level as was detected in F_0 generation.
Fig. 4.8 shows, as an example, western blot analysis of 5 independent lines of JAII plants and 5 independent lines of JAVII plants with different levels of scFv accumulated in the ER or in the cytosol, respectively.
Similarly, Fig. 4.9 depicts an example of western blot analysis of 5 independent lines of ST3 plants and 5 independent lines of OCM plants accumulating different levels of scFv in chloroplastic subcompartments.

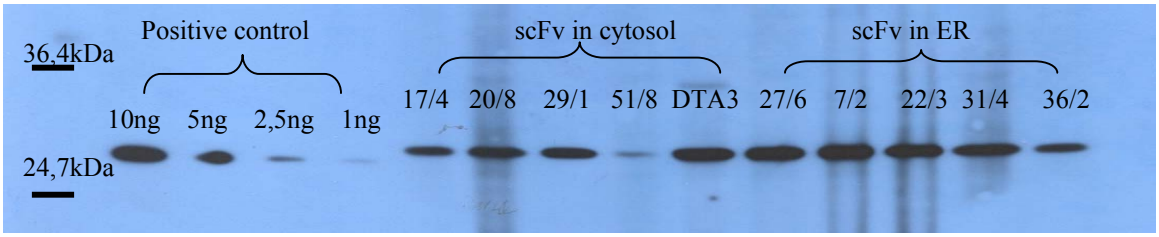


Fig. 4.8 Western blot analysis of F_1 generation of transgenic plants with anti-jasmonate scFvs in the ER or in the cytosol of tobacco cell.

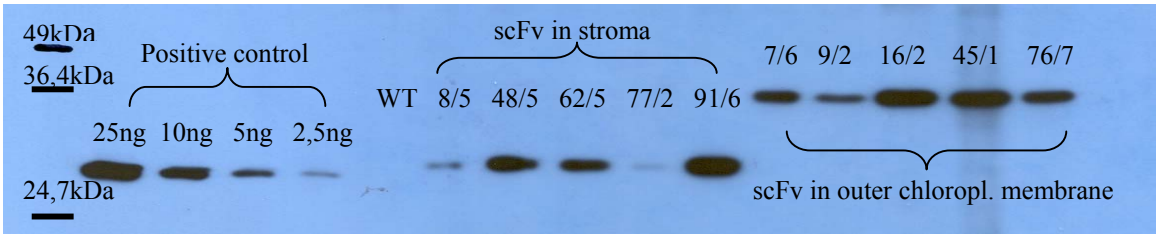


Fig. 4.9 Western blot analysis of F_1 generation of transgenic tobacco plants with anti-jasmonate scFvs in the stroma or in the outer membrane of chloroplasts.

In order to investigate the antigen-binding activity of anti-jasmonic acid scFv and anti-12-oxo-phytodienoic acid scFv antibodies produced in plants, the crude leaf protein extract of F_1 generation of JAII and ST3 transgenic plants has been purified via affinity matrix column (Chapter 7).

Anti-abscisic acid, anti-24-epi-brassinolide and anti-dihydro-zeatin riboside antibodies were tested with abscisic acid-BSA (ABA-BSA), 24-epi-brassinolide-BSA (Bras-BSA), dihydro-zeatin riboside-BSA (diHZR-BSA) conjugates and with BSA alone by enzyme-linked immunosorbent assay [Harlow and Lane, 1988] to test the conjugates (Fig. 4.10 (a), (b) and (c)). This ELISA confirmed all three antibodies could recognise exclusively their counterpart phytohormone coupled to BSA.

Rabbit polyclonal anti-JA serum and non-immunised rabbit serum were tested by ELISA for their binding to (3R,7R)-jasmonic acid-BSA (JA-BSA) conjugate and to BSA alone. Unlike the non-immunised serum, the anti-JA antibodies in the polyclonal serum could recognise exclusively the (3R,7R)-jasmonic acid coupled to BSA (Fig. 4.10 (d)).

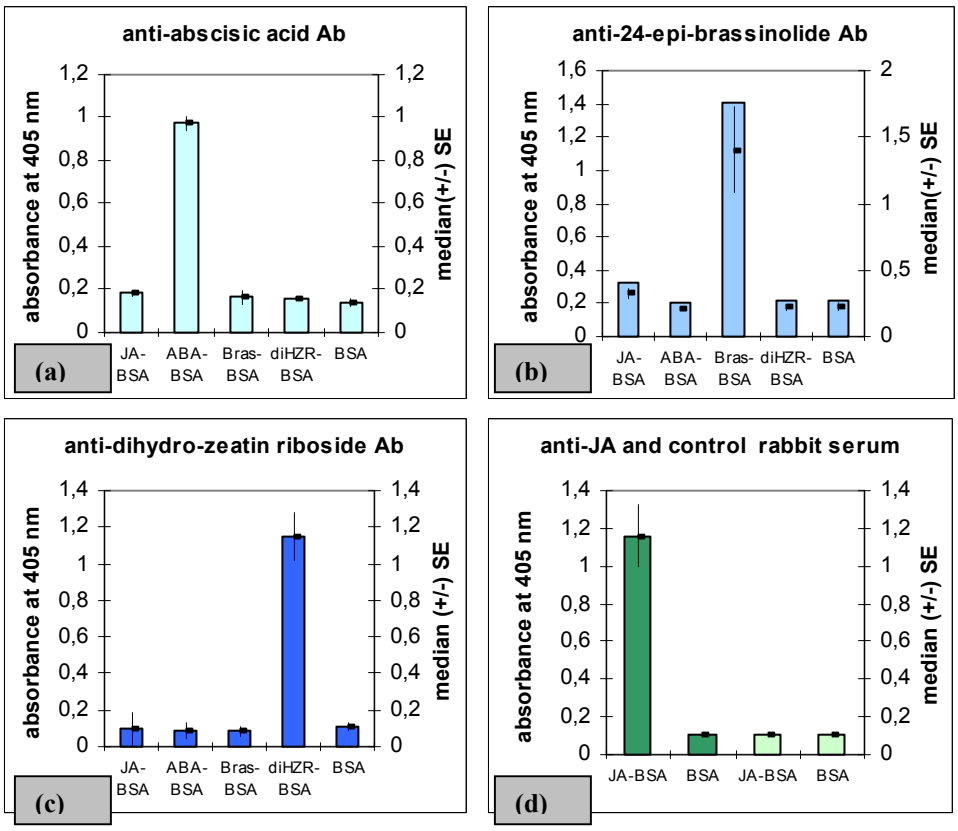


Fig. 4.10 ELISA of anti-phytohormone antibodies (Ab) with phytohormone-BSA conjugates. The mouse anti-ABA antibodies were detected by anti-mouse IgG-alkaline phosphatase conjugate (a), the anti-24-epi-brassinolide scFvs were detected by c-myc-tag antibodies followed by anti-mouse IgG-ALP conjugate (b), and the rabbit anti-dihydro-zeatin riboside antibodies were detected by anti-rabbit IgG-ALP conjugate (c). The rabbit anti-JA polyclonal (dark green) and unimmunised (light green) serum were detected by anti-rabbit IgG-ALP conjugate (d). Each bar is an median value of five replications.

Thus, the conjugates (3R,7R)-jasmonic acid-BSA, abscisic acid-BSA, 24-epi-brassinolide-BSA and dihydro-zeatin riboside-BSA were found to be functional and could be used in a test of the specificity of the plant produced anti-jasmonate single-chain Fv antibodies. (9S,13S)-12-oxo-phytodienoic acid-BSA conjugate was also included in the specificity assay.

The plant-purified anti-(3R,7R)-jasmonic acid scFv or anti-(9S,13S)-12-oxo-phytodienoic acid scFv was applied on microtitre plate coated with these five conjugates and BSA. The bound scFv antibodies were detected by 9E10 antibodies, recognising the c-myc-tag peptide, followed by rabbit anti-mouse IgG-alkaline phosphatase conjugate.

The ELISA revealed that plant-produced anti-(3R,7R)-jasmonic acid scFv antibody binds exclusively to (3R,7R)-jasmonic acid-BSA conjugate and confirmed the specificity of the anti-JA scFv antibodies for naturally occurring (3R,7R)-jasmonic acid (Fig. 4.11(a)).

The anti-(9S,13S)-12-oxo-phytodienoic acid scFv antibodies do not bind other plant growth regulators but show high binding activity for (3R,7R)-jasmonic acid and no binding for (9S,13S)-12-oxo-phytodienoic acid (Fig. 4.11(b)). The results of sequencing (Chapter 3.4.1) confirmed that the anti-OPDA scFv is not identical with the anti-JA scFv. Therefore, a possible explanation of the ELISA result is that the original anti-OPDA scFv available from phage display library has no specificity for (9S,13S)-12-oxo-phytodienoic acid and the anti-OPDA antibodies can specifically recognise only (3R,7R)-jasmonic acid and together with it the common structure of all jasmonates. This structure consists of a planar pentanone ring, the pentenyl chain inserted at C7 and the keto group at C6 (Fig. 1.3). However, the (9S,13S)-12-oxo-phytodienoic acid-BSA conjugate was the only one that could not be tested for its ability to bind anti-OPDA antibodies because no control anti-OPDA serum was available Thus, the (9S,13S)-12-oxo-phytodienoic acid-BSA conjugate might be non-functional.
In conclusion, the plant produced anti-(9S,13S)-12-oxo-phytodienoic acid scFv can specifically recognize the common structure of all jasmonates but these antibodies can not discriminate between the length of the side chain at C3, the double bond in the pentanone ring and the three-dimensional position of the pentenyl chain, which are the structural differences between (3R,7R)-jasmonic acid and (9S,13S)-12-oxo-phytodienoic acid.

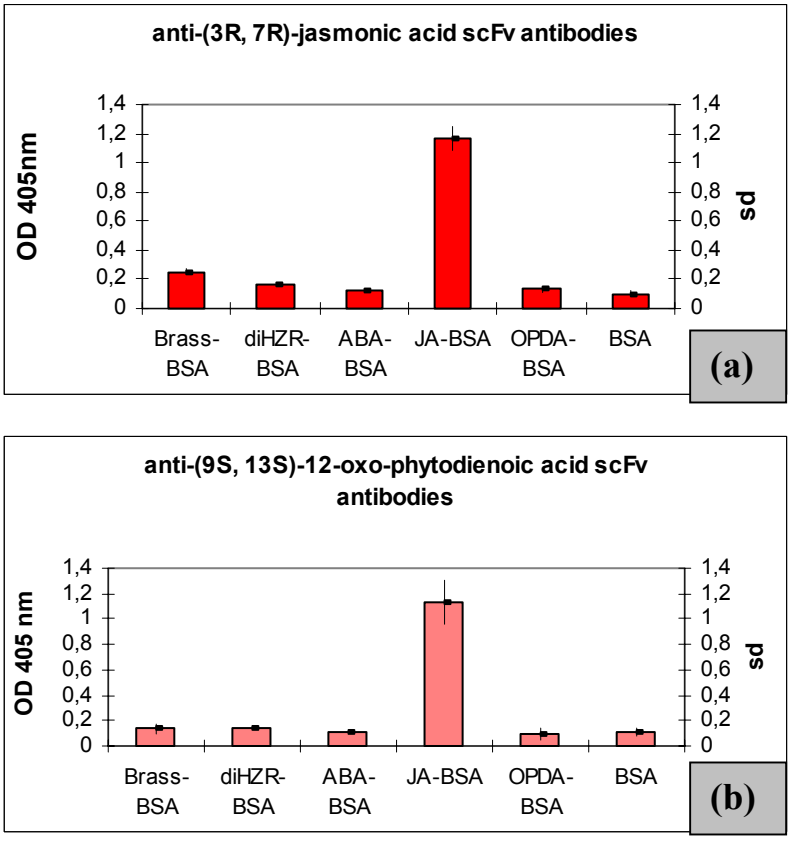


Fig. 4.11 ELISA detecting binging of anti-JA scFv (a) and anti-OPDA scFv (b) to JA-BSA and OPDA-BSA conjugate and to other phytohormone-BSA conjugates. Each bar is a median value of five replications.

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 („*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].

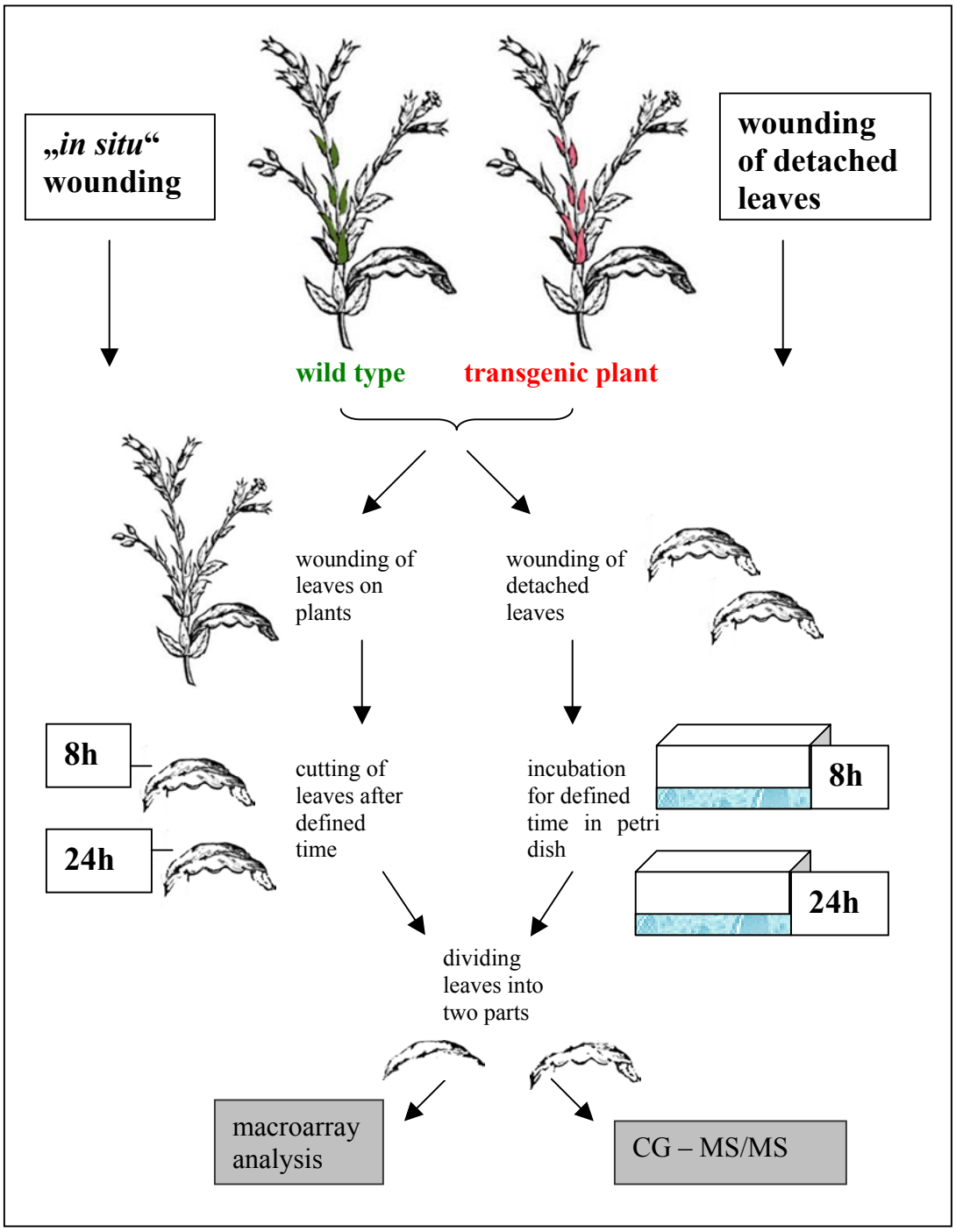


Fig. 5.1 Schematic representation of mechanical wounding of attached and detached leaves.

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 [α-^{33}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 filtr 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.

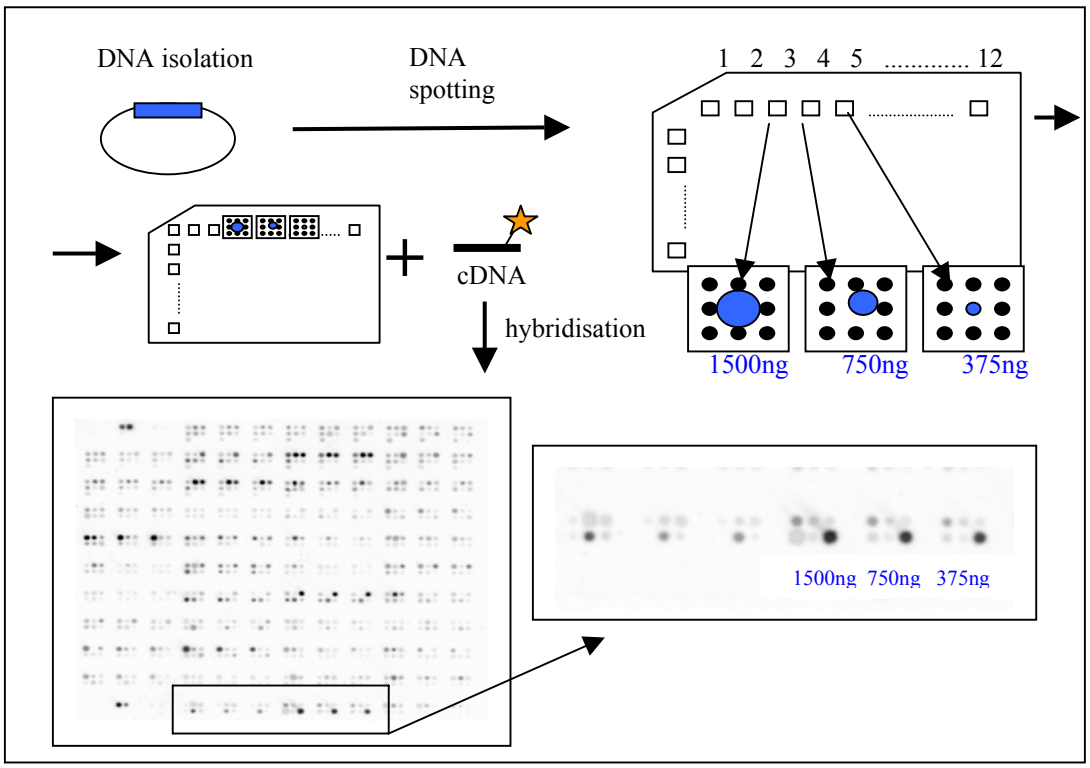


Fig. 5.2 The schematic representation of macroarray used in this study. Purified DNA was spotted on a nylon membrane in a pattern 3x3. DNA, each corresponding to a particular gene, was applied in concentrations 1500 ng, 750 ng and 375 ng in neighbouring patterns. Messenger RNA purified from total RNA via dynabeads was reverse transcribed and cDNA labelled with [α-^{33}P]dCTP. 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.

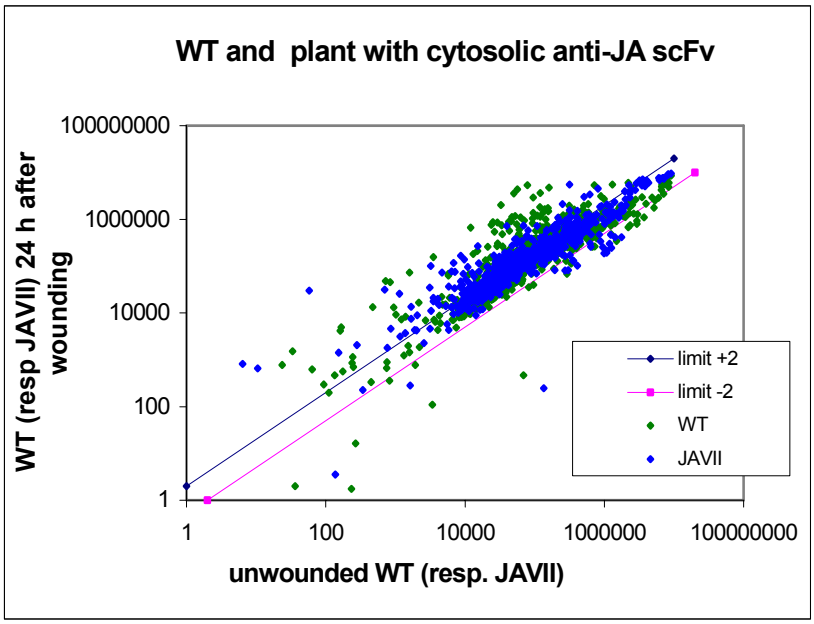


Fig. 5.3 Scattered plot of gene expression of unwounded and 24 hours wounded detached wild-type leaf (green) and JAVII transgenic leaf (blue). Lines show twofold gene induction (line +2) or repression (line -2).

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, JAII, 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.

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

Results, summarised in Fig. 5.5 and Fig. 5.6, are median of four independent experiments with each, wild type, JAII 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 JAII 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.

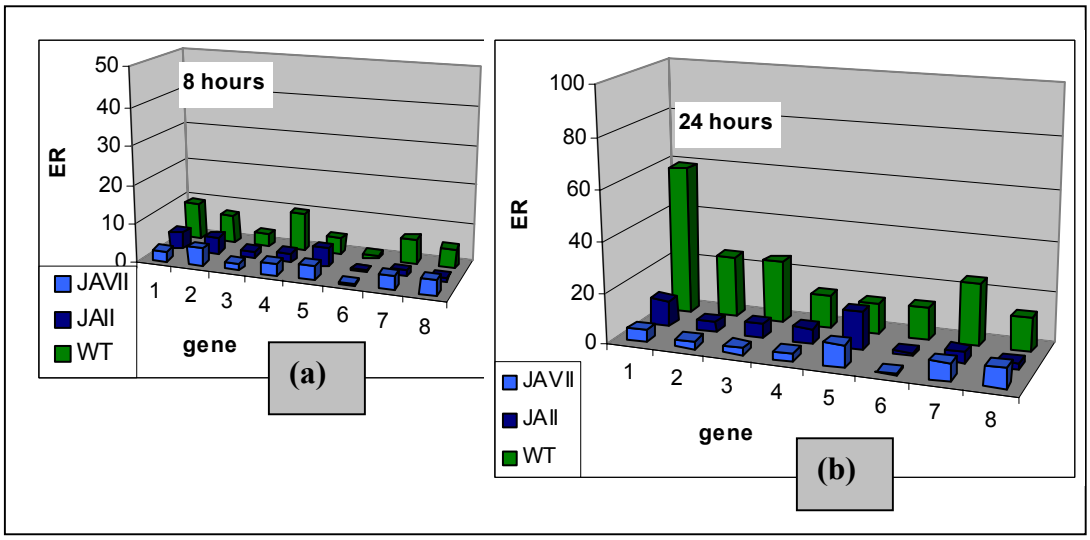


Fig. 5.5 cDNA macroarray analysis of gene expression at 8 hours (a) and 24 hours (b) after mechanical wounding. Expression ratio (ER) represents the wound-induction of above listed genes in wild type (green), JAVII transgenic (light blue) and JAII transgenic (dark blue) plants. Description of the genes 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 JAII) 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 [α-^{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, JAII, ST3 and OCM transgenic plants.

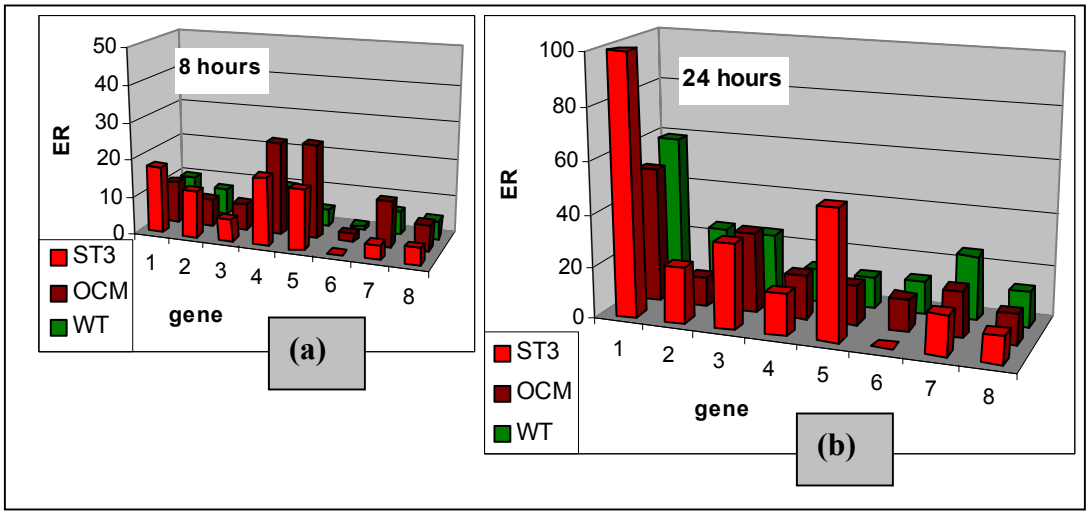


Fig. 5.6 cDNA macroarray analysis of gene expression at 8 hours (a) and 24 hours (b) after mechanical wounding. The expression ratio (ER) represents the wound-induction of the selected genes in wild type (green), ST3 transgenic (light red) and OCM transgenic (dark red) plants. Description of the genes 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 JAII 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.

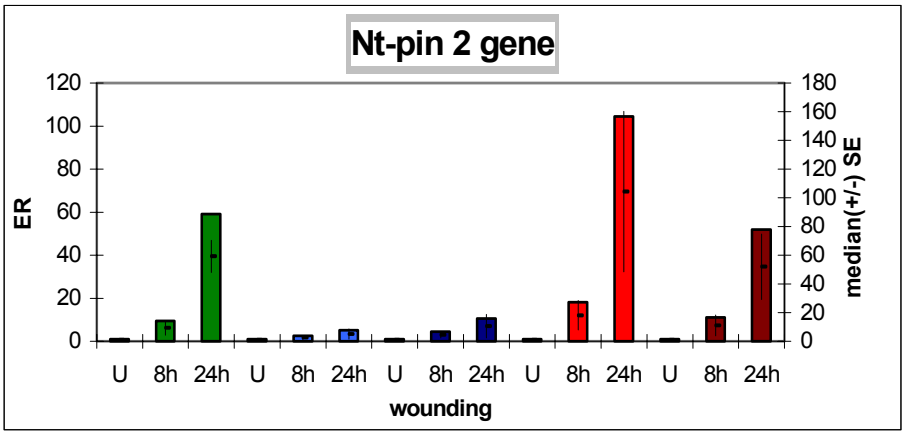


Fig. 5.7 (a)

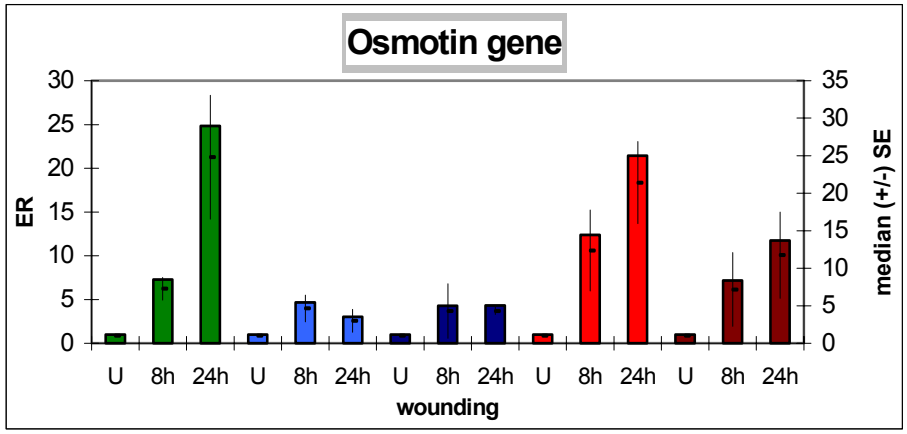


Fig. 5.7 (b)

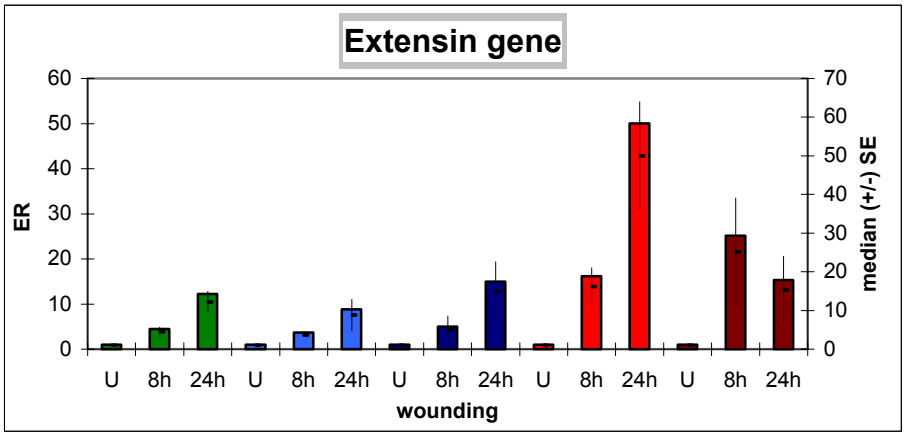


Fig. 5.7 (c)

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.

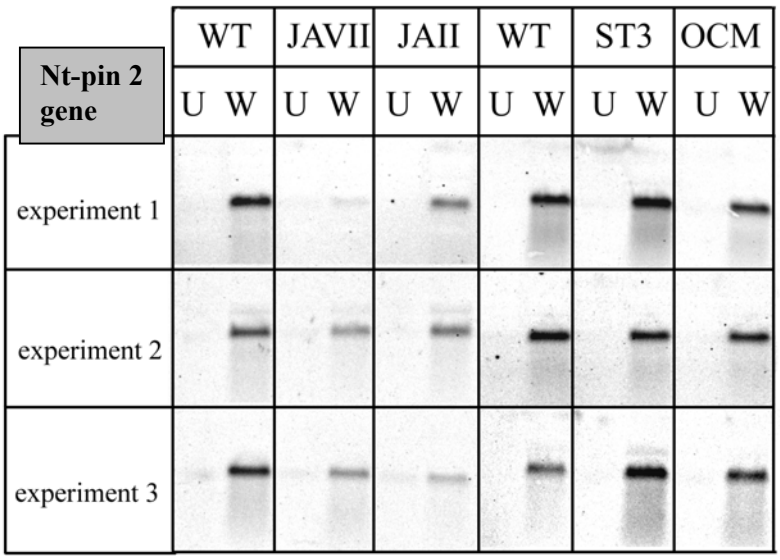


Fig. 5.8(a)
The expression profile of the Nt-pin 2 gene in wild type and transgenic plants after mechanical wounding. Result of northern blot analysis. Total RNA was isolated from unwounded leaf (U) or 24 hours-wounded leaf (W) of wild type (WT), transgenic plants with anti-JA scFv in the cytosol (JAVII)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

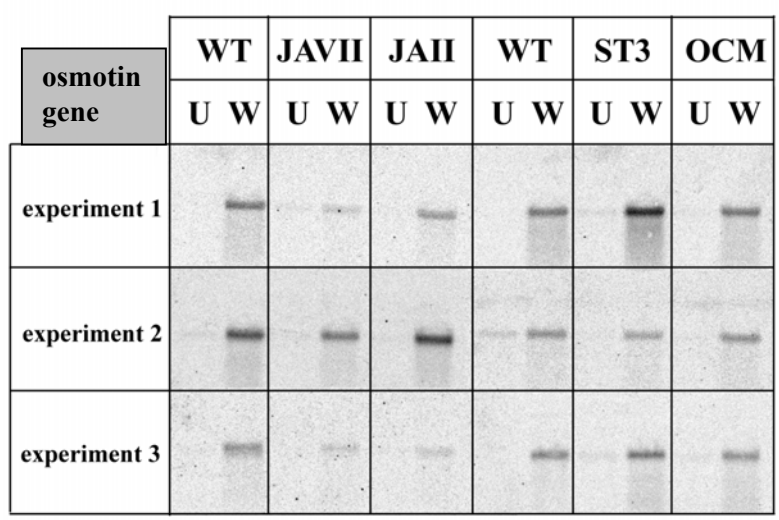


Fig. 5.8(b) The expression profile of the osmotin gene in wild type and transgenic tobacco plants after wound induction.
Result of northern blot analysis. For explanation of abbreviations see Fig. 5.8 (a).

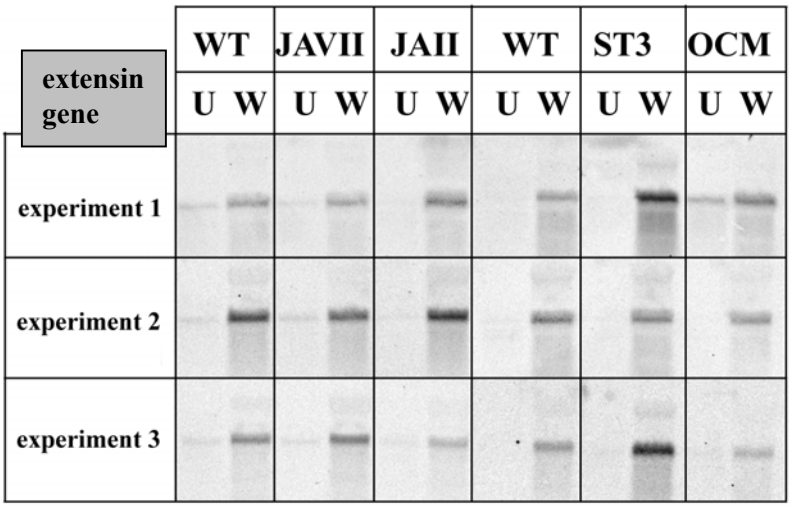


Fig. 5.8(c)
The expression profile of the extensin gene in wild type and transgenic plants after mechanical wounding.
Result of northern blot analysis. For explanation of abbreviations see Fig. 5.8 (a).

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.

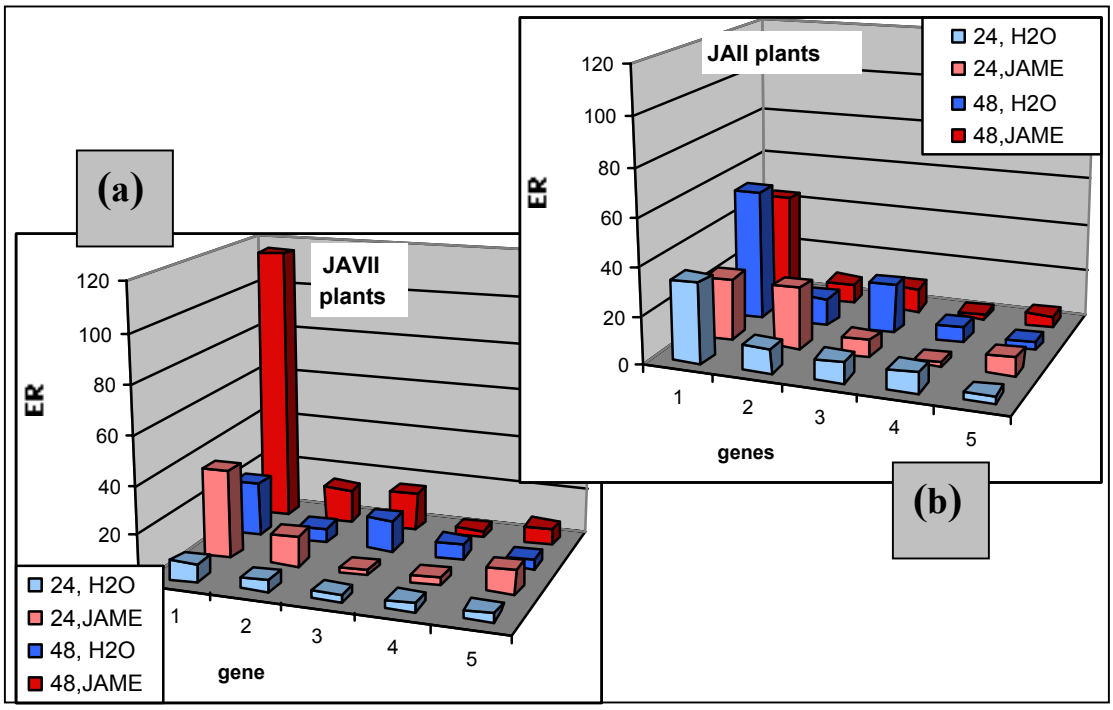


Fig. 5.9 cDNA macroarray analysis of JAME-treated and water-treated leaves of transgenic plants. Expression ratio (ER) represents the median of two experiments with JAVII plants (a) and two experiments with JAII plants (b). Description to the numbers of genes is given in the Table. 5.4.

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.

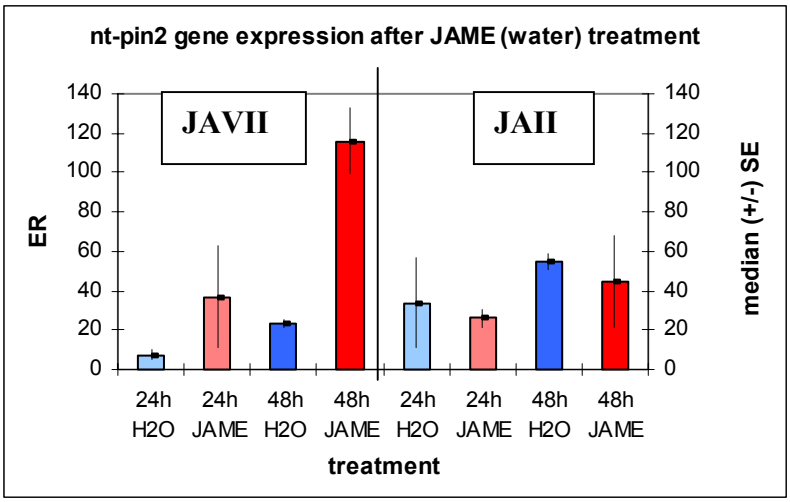


Fig. 5.10 The expression profile of the Nt-pin 2 gene in transgenic plants after JAME (water) treatment. Result of cDNA macroarray analysis. The y-axis on the left corresponds to the median value and the y-axis on the right to the median ± SE. ER: expression ratio.

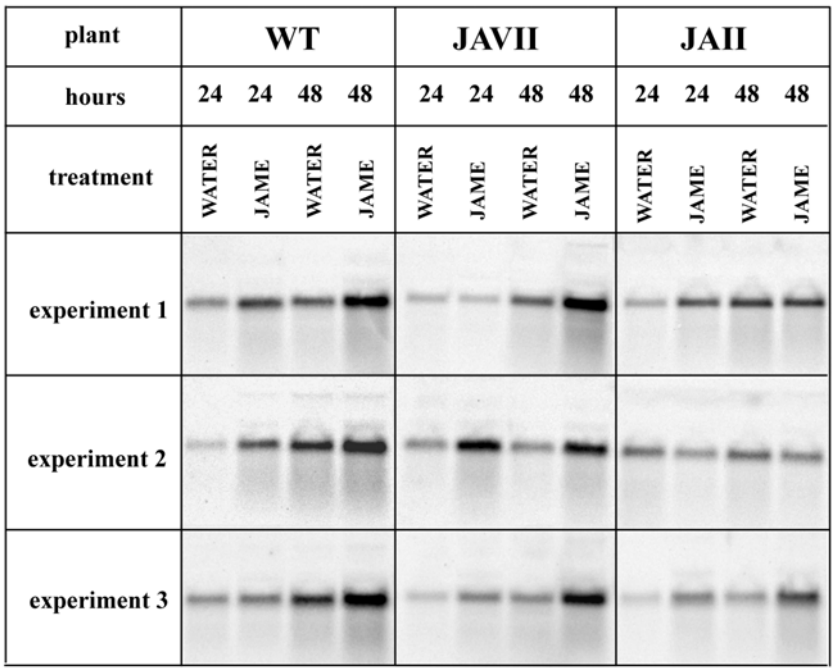


Fig. 5.11
The expression profile of the Nt-pin 2 gene in wild type and transgenic plants after JAME (or water) treatment. Results of northern blot analysis. Analysis of three wild-type plants (WT) and three transgenic plants accumulating anti-JA scFv either in the cytosol (JAVII) or in the ER (JAII) 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 JAII 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.

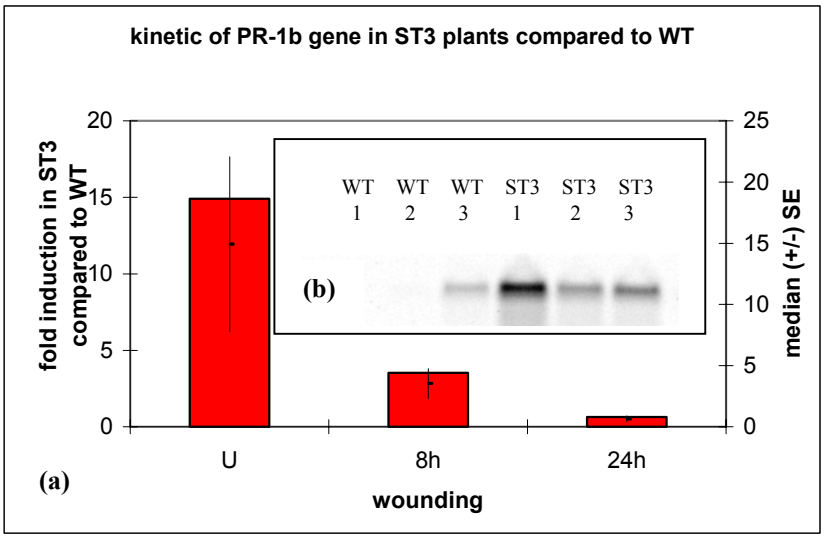


Fig. 5.12 Kinetics of the induction of the PR-1b gene in unwounded (U) and wounded detached leaves of transgenic tobacco plants with anti-12-oxo-phytodienoic acid antibodies in the stroma of chloroplasts (ST3) compared to wild-type plants (WT). Results of macroarray analysis (a). The northern blot (b)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

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 lipoxygenase 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.

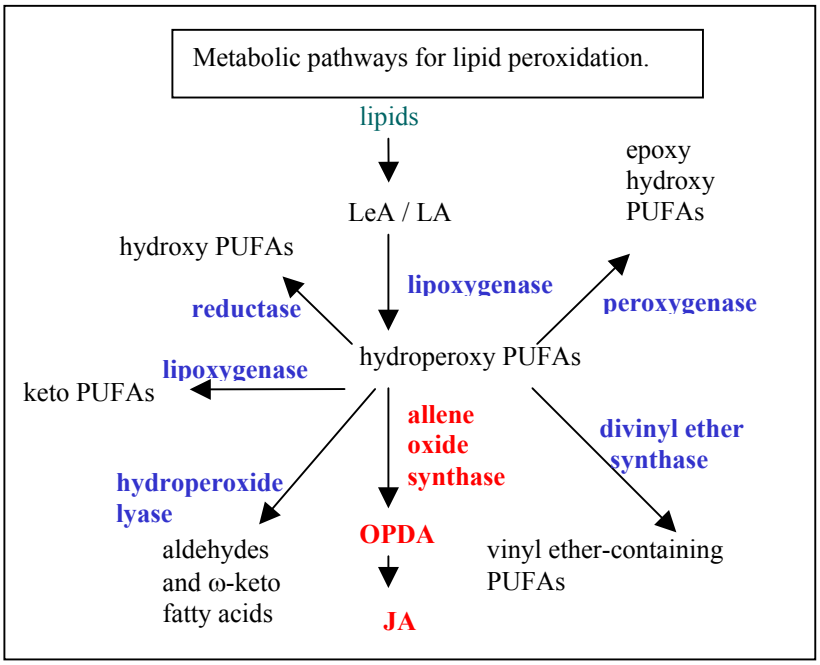


Fig. 5.13 LOX-dependent catabolism of polyunsaturated fatty acids. α-LeA – α-linolenic acid, LA – linoleic acid, PUFAs – polyunsaturated fatty acids. Enzymes are written in bold, the octadecanoid pathway is in red.

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, hexadecaenoic 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.

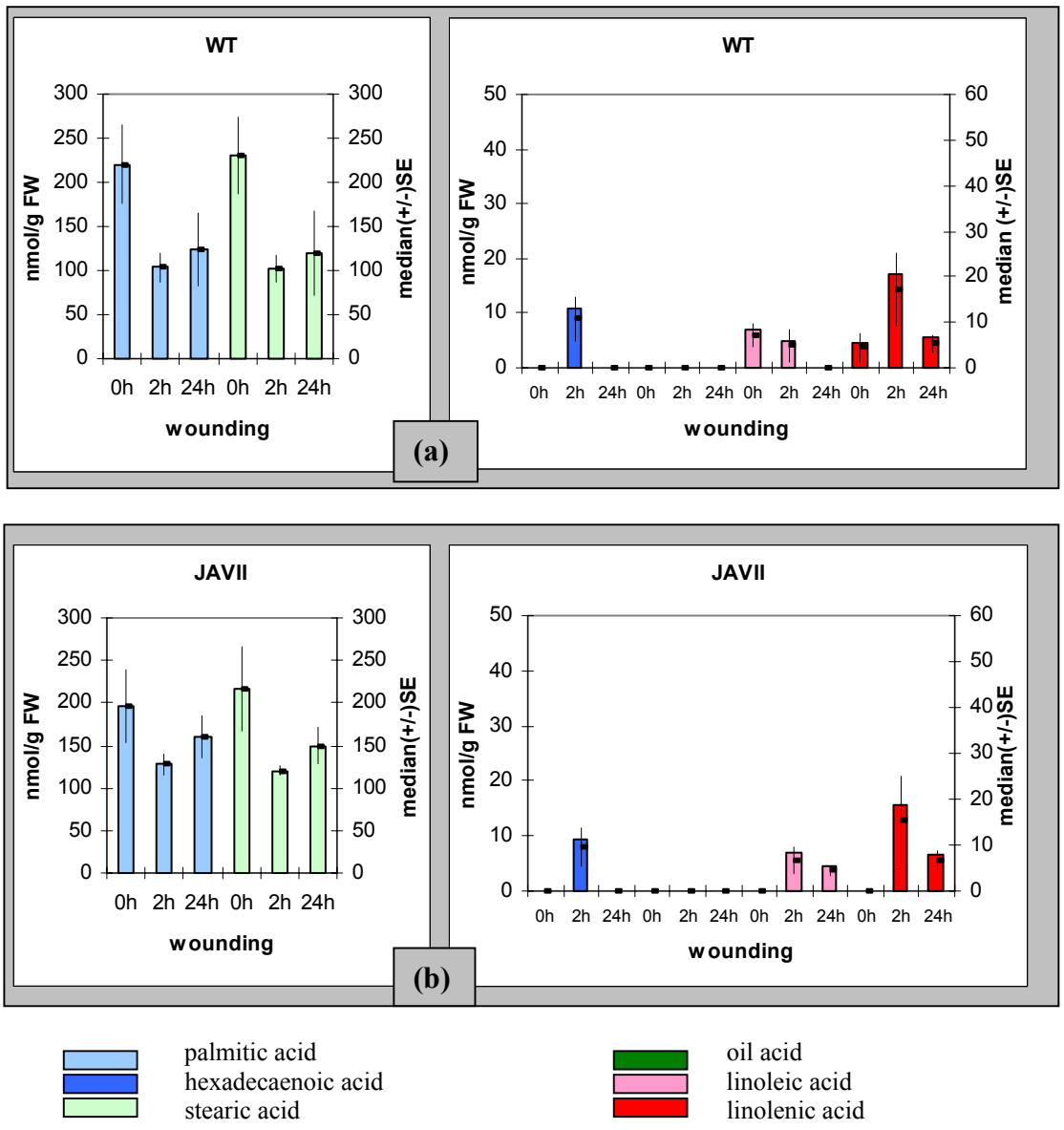


Fig. 5.14 Levels of free fatty acids (palmitic acid, hexadecaenoic acid, stearic acid, oil acid, linoleic acid and linolenic acid) in wild type (a) and in JAVII transgenic plants (b) measured by gas chromatography. The y-axis on the left corresponds to the levels of free fatty acids and the y-axis on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

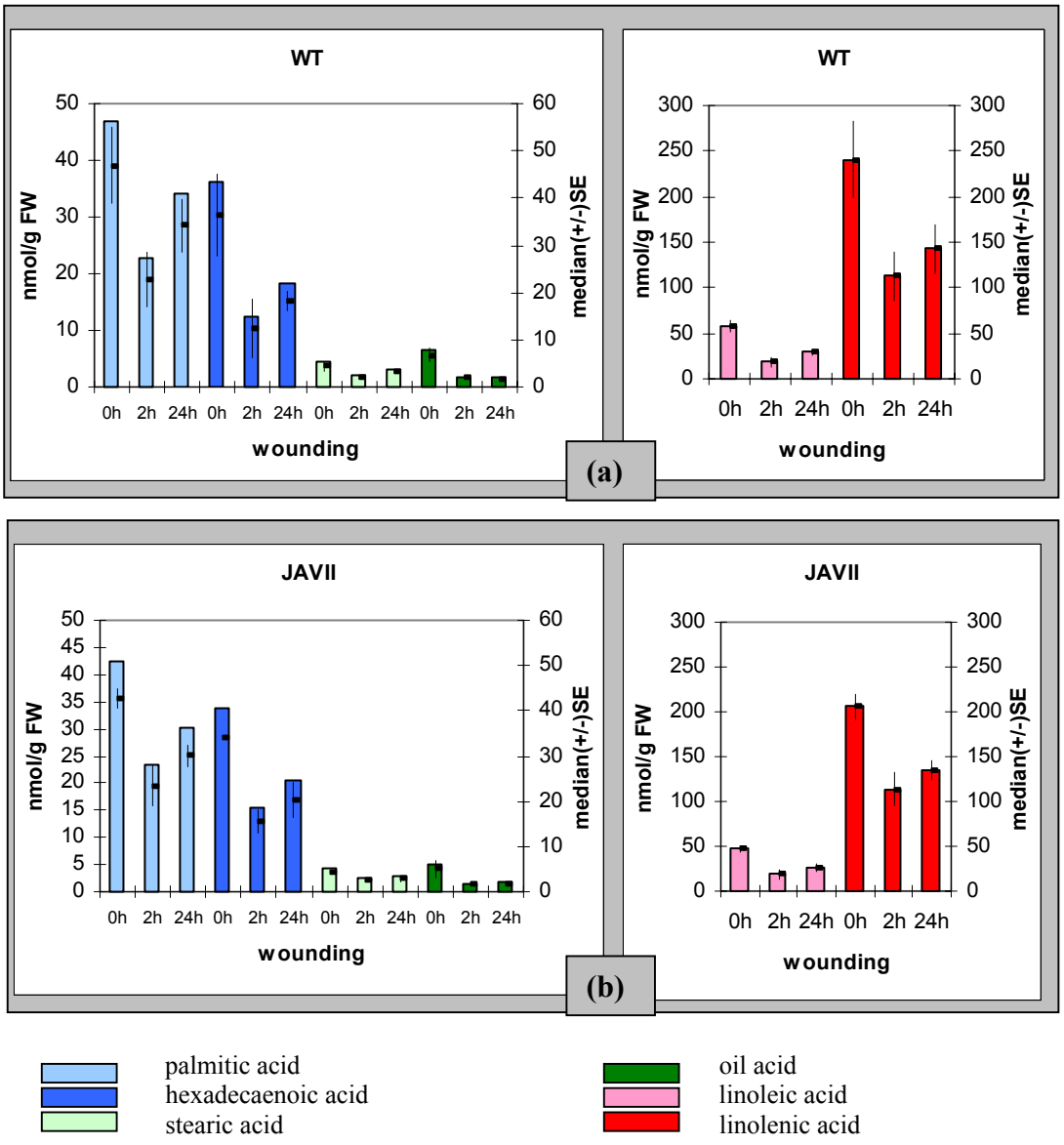


Fig. 5.15 Levels of membrane-bound fatty acids in wild type (a) and JAVII transgenic plants (b) measured by gas chromatography. The y-axis on the left corresponds to the levels of membrane-bound fatty acids and the y-axis on the right to the median ± standard deviation of 3 independent experiments.

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 JAII, 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 etheral 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*-isoforms 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, JAII 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.

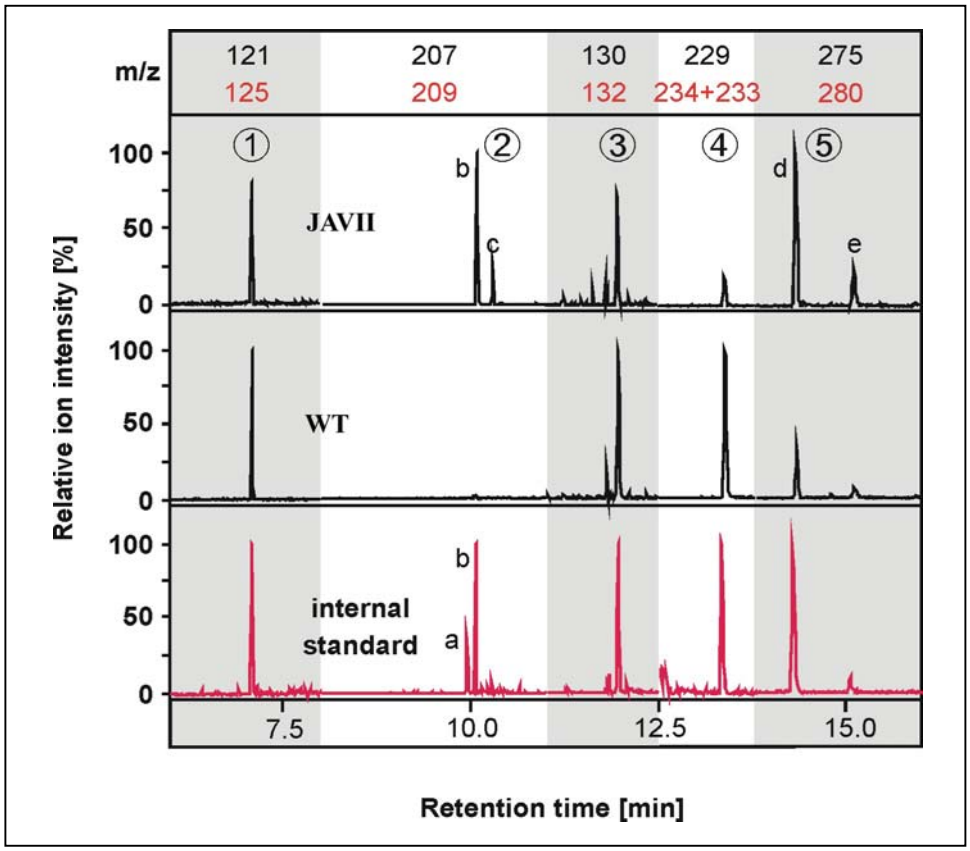


Fig. 5.16 Chromatograms obtained from GC-MS/MS phytohormone analysis of wild type and transgenic tobacco plant. Relative signal intensities of characteristic daughter ions of five endogenous compounds detected in 2h-wounded leaf of wild-type plant (a) and JAVII transgenic plant (b) are plotted. Relative 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, JAII, 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.

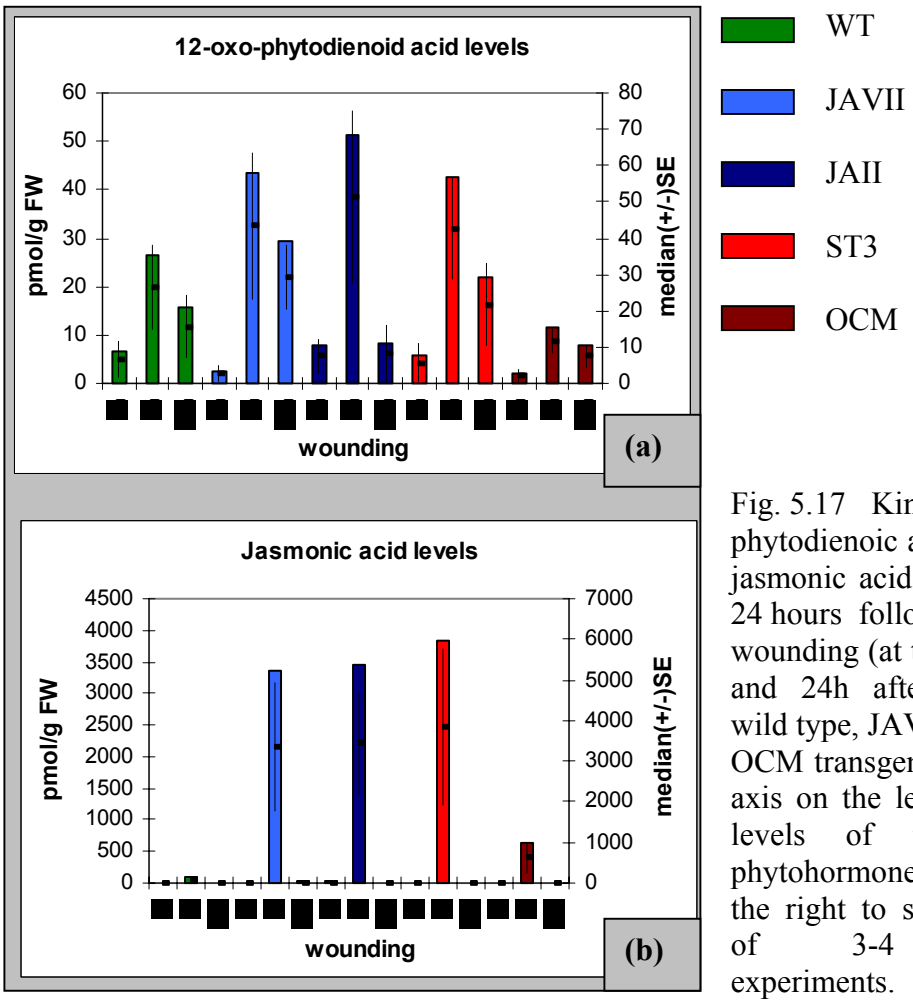


Fig. 5.17 Kinetic of 12-oxo-phytodienoic acid levels (a) and jasmonic acid levels (b) during 24 hours following mechanical wounding (at time points 0h, 2h and 24h after wounding) of wild type, JAVII, JAII, ST3 and OCM transgenic plants.. The y-axis on the left corresponds to levels of the endogenous phytohormones and y-axis on the right to standard deviation of 3-4 independent experiments.

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, JAII 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, JAII and ST3 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.

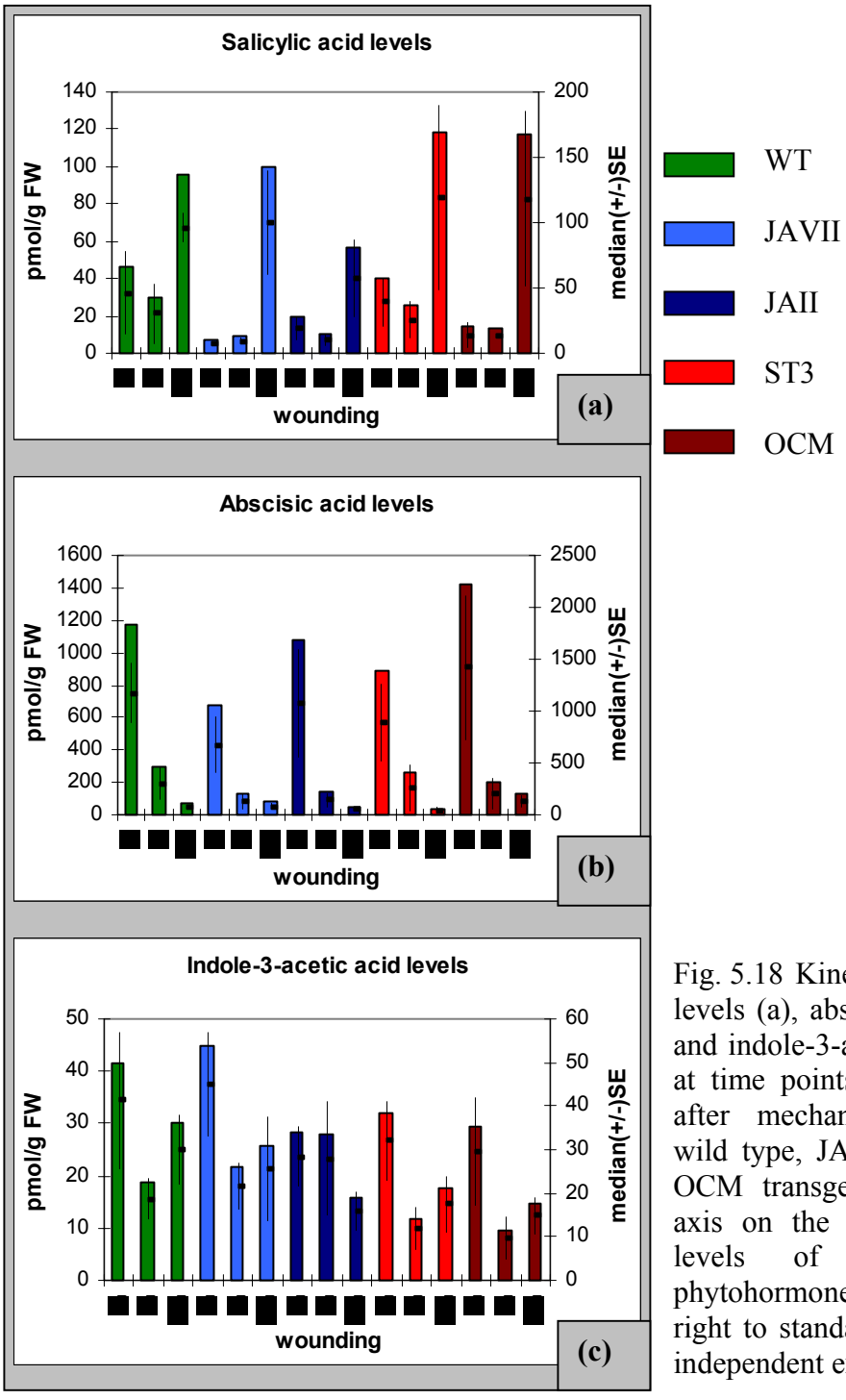


Fig. 5.18 Kinetic of salicylic acid levels (a), abscisic acid levels (b) and indole-3-acetic acid levels (c) at time points 0 h, 2 h and 24 h after mechanical wounding of wild type, JAVII, JAII, ST3 and OCM transgenic plants. The y-axis on the left corresponds to levels of the endogenous phytohormones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

Chapter 6

Discussion

Jasmonates are oxylipin compounds acting as secondary messengers in signalling cascades regulating gene expression of a number of genes in response to various environmental stimuli as well as in distinct stages of plant development.
A molecular technique of immunomodulation has been adopted to create transgenic plants showing symptoms of jasmonate deficiency and thus to study the role of jasmonates in physiological processes induced by these oxylipins. The availability of jasmonates has been disturbed by the intracellular expression of single-chain Fv antibodies selected either against the physiological active (3R, 7R)-jasmonic acid or against its biosynthetic precursor – 12-oxo-phytodienoic acid, having also biological activity *per se* (Chapter 1.2). The analysis of transgenic tobacco plants was focused mainly on the plant response to mechanical wounding, the abiotic stimuli where jasmonates were found to be a key signalling molecule.
A number of questions were set up with this experimental approach or emerged during the investigation:

- Will the anti-jasmonate single-chain Fv antibodies produced in plant cells be a functional and reliable tool for immunomodulation?
- Would the immunomodulation of jasmonate functions influence the development or morphology of transgenic plants accumulating anti-jasmonate single-chain Fv?
- Will the intracellular accumulation of anti-jasmonate scFv antibodies alter the gene expression profile of house-keeping or stress-inducible genes?
- Could the possible symptoms of jasmonate deficiency be restored by the application of exogenous jasmonates?
- Will the immunomodulation of jasmonic acid biosynthetic pathway influence the overall content of polyunsaturated fatty acids – shared substrates with other lipoxygenase-dependent metabolic routes?
- Would the expression of anti-jasmonate scFv antibodies influence endogenous jasmonate levels?
- Would the potential modulation of jasmonate levels have any impact on endogenous levels of other phytohormones, known to cross-talk with the jasmonic acid biosynthetic pathway?
- Are these effects, if any, cell-compartment specific?

The attempts to answer these questions will be given in this Chapter.

- **Will the anti-jasmonate single-chain Fv antibodies produced in plant cells be a functional and reliable tool for immunomodulation?**

Anti-jasmonic acid single-chain Fv antibodies and anti-12-oxo-phytodienoic acid single-chain Fv antibodies, collectively called anti-jasmonate scFv antibodies, were selected from synthetic VH+VL scFv phagemid libraries [Hunger, 2002]. The jasmonate-specific scFv genes were cloned into plant expression vectors and integrated via *Agrobacterium tumefaciens* stable into the genome of *Nicotiana tabacum*, cv. Samsun NN, plants. The recombinant anti-jasmonate antibodies were targeted into different plant cell compartments because the intracellular localisation of functional jasmonates is not known.

The anti-jasmonic acid scFv antibodies were directed by an amino-terminal signal sequence into the lumen of the endoplasmic reticulum (Fig. 3.4) or retained in the cytosol (Fig. 3.5) of tobacco cells. The accumulation level of the antibody protein was in both compartments increased by the KDEL retention sequence placed on the carboxy-terminal side of the scFv genes behind the c-myc-tag sequence. Western blot analysis confirmed stable accumulation of anti-jasmonic acid antibodies in both F_0 and F_1 generations of transgenic plants (Fig. 4.8). The maximal expression level of anti-jasmonic acid antibodies in the ER reached 0.05 % of total soluble protein. This accumulation level is lower than the yield of expression reported in other studies (reviewed in [Conrad and Fiedler, 1998]) and could be explained by dependence of the scFv expression level on the antibody coding sequence. The expression level of anti-jasmonic acid antibodies in the cytosol was, as expected, lower than in the ER, maximally 0.025 % of total soluble protein. Anti-jasmonic acid scFv was purified from leaves of transgenic plants and the binding activity of the antibodies was determined by ELISA. The plant produced anti-jasmonic acid scFv antibodies were confirmed to bind specifically the natural (3R,7R)-jasmonic acid conjugated to BSA (Fig. 4.11a). No binding to BSA alone or to other phytohormones tested (abscisic acid, 24-epi-brassinolide and dihydro-zeatin riboside) was observed.
Significant differences between transgenic and wild-type plants were observed during nondormant seed germination in the presence of 10 μM jasmonic acid methyl ester. The jasmonate-induced inhibition of nondormant seed germination is a well documented aspect of jasmonates function (Chapter 1.2.2). While no differences in germination of water-treated seeds of wild type and transgenic plants with anti-JA scFv were observed, the germination of nondormant seeds treated with 10 μM jasmonic acid methyl ester was significantly reduced only in wild-type plants (Fig. 4.6) The methyl jasmonate-treated transgenic seeds germinated like the water-treated seeds, indicating trapping of the exogenous methyl jasmonate by the endogenously expressed anti-jasmonic acid antibodies. Wild-type-like inhibition of seed germination of control transgenic plants with anti-oxazolone scFv antibodies in the ER (Fig. 4.7) confirmed that the differences in the seed germination are not caused non-specifically by the ectopic expression of single-chain Fv.
The specific ability of transgenic tobacco plants, containing anti-jasmonic acid scFv in the cell cytosol or in the ER, to overcome the jasmonic acid-mediated inhibition of seed germination confirms that the anti-jasmonic acid antibodies are active in the transgenic

plants and can be used as a reliable tool for immunomodulation of (3R,7R)-jasmonic acid.

There are several lines of evidence supporting the idea that chloroplasts are the site of biosynthesis of 12-oxo-phytodienoic acid. Therefore the anti-12-oxo-phytodienoic acid scFv antibodies were directed not only to the cell cytosol and into the ER, but also by a stroma-specific amino-terminal transit sequence into the stroma of tobacco chloroplasts (Fig. 3.8) or by an outer chloroplast membrane-specific amino-terminal transit sequence into the outer chloroplast membrane and anchored by a transmembrane domain (Fig. 3.9). Histochemical analysis (Dr. B. Hause) confirmed the localisation of the anti-OPDA scFv in the stroma and on the outer membrane of chloroplasts, as shown in the fluorescence light microscope-images in Fig. 4.5. Western blot analysis revealed stable accumulation of the anti-OPDA scFv antibodies in F_0 as well as in F_1 generation of transgenic plants (Fig. 4.9) and allowed the selection of the plants with the highest scFv expression. Maximal expression levels of anti-OPDA scFv in the cytosol and in the outer chloroplastic membrane were 0.025 % of total soluble protein. Accumulation levels of these antibodies in the ER were maximally 0.05 % of total soluble protein and reached 0.125 % of total soluble protein in the stroma of chloroplasts. The anti-12-oxo-phytodienoic acid scFv antibodies were purified from leaves of transgenic plants and the binding activity of the antibodies determined by ELISA. The plant-produced anti-12-oxo-phytodienoic acid scFv antibodies specifically bind (3R,7R)-jasmonic acid but the ability of the anti-OPDA scFv to bind (9S,13S)-12-oxo-phytodienoic acid coupled to BSA could not be confirmed (Fig. 4.11b).
The plant produced anti-(9S,13S)-12-oxo-phytodienoic acid scFv can specifically recognize the common structure of all jasmonates consisting of a planar pentanone ring, the pentenyl chain inserted at C7 and the keto group at C6 (Fig. 1.3). However, these antibodies can not discriminate between the length of the side chain at C3, the double bond in the pentanone ring and the three-dimensional position of the pentenyl chain, which are the structural differences between (3R,7R)-jasmonic acid and (9S,13S)-12-oxo-phytodienoic acid. The anti-12-oxo-phytodienoic acid scFv antibodies recognise specifically cyclic metabolites of the JA-biosynthetic pathway and can be used for the immunomodulation of these compounds. However, the antibodies can not be used for modulation of availability of exclusively (9S,13S)-12-oxo-phytodienoic acid.

- **Would the immunomodulation of jasmonate functions influence the development or morphology of transgenic plants accumulating anti-jasmonate single-chain Fv?**

The ectopic expression of functional anti-jasmonate scFv antibodies in subcellular compartments of tobacco cells does not change the overall morphology and plant development under natural conditions. While changes in gene expression profile are induced by nanomolar to micromolar concentrations of jasmonic acid [Creelman and Mullet, 1995], the concentration threshold for the regulation of developmental processes seems to be lower, as demonstrated in case of gametophyte development (Chapter 4.3). The ectopic expression of the anti-jasmonate recombinant antibodies probably does not reach this threshold.

- **Will the intracellular accumulation of anti-jasmonate scFv antibodies alter the gene expression profile of house-keeping or stress-inducible genes?**

A great number of studies confirmed jasmonic acid as a secondary messenger playing a primary role in the plant wound response (Chapter 1.2).
Leaves of wild-type plants and transgenic plants, reaching the highest expression levels of anti-jasmonate antibodies in different subcellular compartments, were mechanically wounded. The gene expression profile of untreated leaves and leaves 8 hours and 24 hours after wounding were analysed by macroarray and by northern blot analysis.
While "*in situ*" wounding lead to a wound induction neither in wild-type plants nor in transgenic plants, mechanical wounding of detached leaves caused strong induction of wound inducible genes in wild-type plants and in transgenic plants accumulating anti-12-oxo-phytodienoic acid scFv antibodies in the stroma or in the outer membrane of chloroplasts (Fig. 5.6). In contrast, the accumulation of anti-jasmonic acid recombinant antibodies in the cytosol or in the ER of tobacco cells caused significant deficiency in the expression of wound inducible genes. This effect was more pronounced in plants with the cytosolic localisation of anti-jasmonic acid single-chain Fv than in plants with the ER-localised anti-JA scFv and more evident in leaves 24 hours after wounding than in leaves 8 hours after wounding(Fig. 5.5).
From eight genes identified by macroarray, whose mRNA levels changed repeatedly more than threefold following wounding of wild type or transgenic plants (table. 5.4), three genes were selected as probes for northern blot 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 *Nicotiana tabacum* proteinase inhibitor 2 gene expression in wounded JAVII (or JAII) plants was dramatically different from that in wounded wild type, the osmotin gene transcript levels differed only moderately and in the extensin gene expression no dissimilarities were found (Fig. 5.5(b)). Total RNA was isolated from unwounded and 24 hours-wounded leaves of transgenic plants and wild-type plants and hybridised with *Nicotiana tabacum* proteinase inhibitor 2 gene, Fig. 5.7(a) and Fig. 5.8(a), with the gene coding for PR5-osmotin-like protein, Fig. 5.7(b) and Fig. 5.8(b), and with the gene coding for extensin-like cell wall protein, Fig. 5.7(c) and Fig. 5.8(c). Northern blot analysis confirmed the results of the macroarray.
The question remains whether the selected wound-induced genes are activated through the octadecanoid pathway.

From 247 genes tested, 8 genes were identified in wild type or transgenic plants whose transcript abundance was repeatedly changed more than threefold as a consequence of mechanical wounding. However, not all wound-inducible genes are jasmonate-induced and *vice versa*. A closer survey of the selected genes can provide the answer whether the octadecanoid pathway is involved in their induction or the increase in transcript levels of these genes is connected to other processes induced by wounding, independent of jasmonates signalling. In the following, the description of particular traits of the single genes selected by macroarray, table. 5.4, will be given in more detail.

- ***Nicotiana tabacum* proteinase inhibitor 2 gene (Nt pin 2).**

Proteinase inhibitors are plant defence proteins with specificity against serine, cysteine or aspartate proteinases. Inhibitors of proteinases reduce the nutritional quality of herbivore feeding by inhibiting protein digestion [Ryan, 2000]. Serine proteinase inhibitor 1 (pin 1) and serine proteinase inhibitor 2 (pin 2) are two nonhomologous inhibitors well characterised in Solanaceae. They were found to be induced by a number of stimuli, such as wounding, chewing by insects, systemin, oligosaccharides, chitosan and also by phytohormones – jasmonic acid and abscisic acid [Peña-Cortés et al., 1995, and references therein]. The rate of synthesis of pin 1 and pin 2 in response to methyl jasmonate was shown to be similar to the rates of synthesis induced by wounding [Farmer et al., 1992]. Many laboratories reported wound- and jasmonate-inducibility of pin 1 and pin 2 genes in Solanaceae [Herde et al., 1999], [Van Dam et al., 2001] or other species like *Capsicum annuum* [Moura et al., 2001], and used pin 1 and pin 2 gene as a marker gene of wound induction and jasmonate action [Hildmann et al., 1992], [Harms et al., 1995], [Wasternack et al., 1998b], [Miersch and Wasternack., 2000].

- **PR5-osmotin-like protein**

Osmotin and osmotin-like proteins have been classified as members of group 5 of plant pathogen related proteins (Chapter 1). Tobacco osmotin gene expression was found to be activated by ethylene, abscisic acid, wounding, NaCl, viral and fungal infection [Zhu et al., 1995]. The osmotin promoter region contains two GCC *cis*-acting elements, which are required for the ethylene responsiveness of several basic PR protein genes [Sato et al., 1996], [Xu et al., 1998] and recognised by EREBP family of transcription factors (Chapter 1). The observed wound induction of the tobacco osmotin gene [Neale et al., 1990], [Zhu et al., 1995] varied considerably depending on the degree of wounding and was demonstrated to be mediated through ethylene [Nelson et al., 1992]. Moreover ethylene and methyl jasmonate were found to induce synergistically PR5-osmotin protein in tobacco seedlings [Xu et al., 1994].

- **PR-1b and SAR 8.2 protein.**

Pathogenesis-related protein PR-1b and salicylic acid responsive protein SAR 8.2 are pathogen related proteins activated normally during the development of systemic acquired resistance (Chapter 1.1) and used as marker genes of the resistance [Heo et al., 1999]. The PR-1b gene, believed to be induced by salicylic acid, was found to be synergistically induced by ethylene and methyl jasmonate [Xu et al., 1994]. The author also reported several fold higher induction of the PR-1b gene after the combination of salicylic acid and methyl jasmonate treatment than after the application of salicylic acid alone. Transcripts of the PR-1b gene are absent in healthy tissue [Sano et al., 1996]. However, wounding of tobacco wild-type plants led to significant increase of the PR-1b gene expression and this induction was even stronger in transgenic tobacco plants overexpressing a flax AOS in the cell cytosol [Wang et al., 1999]. Rather weak wound induction of the PR-1b gene was observed in this study in wild-type and transgenic tobacco plants (Chapter 5.1.5).

- **Pathogen related protein 1 (prp1).**

Prp1 is a basic pathogen defence protein of potato with a high similarity to glutathione S-transferase from tobacco [Hahn and Strittmatter, 1994]. Glutathione S-transferase catalyses the nucleophilic addition of glutathione to a variety of hydrophobic molecules,

which is one of several detoxifying mechanisms in plant and animal cells. In potato the prp1 protein accumulates only in response to fungal infection [Martini et al., 1993]. The activity of glutathione S-transferase is in tobacco modulated by indole-3-acetic acid [Hahn and Strittmatter, 1994] and the expression of the genes for glutathione synthesis is in *Arabidopsis* strongly stimulated by jasmonic acid [Xiang and Oliver, 1998]. Glutathione synthesis is also promoted by oxidative stress [Smith et al, 1985].
One of the widespread defence mechanisms of higher plants is called cross-tolerance, the induced tolerance to additional abiotic and biotic stresses after exposure to a specific oxidative stress [Xiang and Oliver, 1998]. Wound- and JA-induced cross-tolerance to ozone stress has also been demonstrated in tobacco [Örvar et al., 1997]. These findings indicate that the prp1 potato gene, used in the macroarray in this study, hybridised to the glutathione S-transferase gene of tobacco. The tobacco glutathione S-transferase transcript abundance was observed as a result of wound- and jasmonic acid-induced cross-tolerance to oxidative stress.

- **3-hydroxy-3-methylglutaryl coenzym A reductase (HMGR) and 5-epi-aristolochene synthase (EAS).**

HMGR catalyses conversion of 3-hydroxy-3-methylglutaryl coenzym A to mevalonic acid, precursor to all isoprenoid compounds. In plants, these include terpenoid phytoalexins, glycoalkaloids, sterols, abscisic acid, gibberellins and carotenoids. Wounding and pathogen challenge result in the activation of two different isogenes of HMGR and lead to the increase in HMGR activity associated with sesquiterpene phytoalexins accumulation [Yang et al., 1991]. EAS converts farnesyl diphosphate to 5-epi-aristolochene, a precursor of capsidol, which is the major tobacco sesquiterpene phytoalexin [Starks et al., 1997]. Wounding induces the release of volatile compounds from damaged tissue (Chapter 1.2.5) and pathogen elicitor-induced accumulation of capsidol was proven to correlate with the induction of EAS [Mandujano-Chávez et al., 2000]. Choi and coauthors reported that jasmonates differentially regulate HMGR in potato [Choi et al., 1994]. Results of another report indicate that methyl jasmonate induces sesquiterpene metabolism in tobacco cell culture but via different mechanisms than fungal elicitors do [Mandujano-Chávez et al., 2000]. The induction of isoprenoid compounds by methyl jasmonate as well as by 12-oxo-phytodienoic acid has also been observed [Blechert et al., 1995].

- **Extensin-like cell wall protein.**

Extensin is a hydroxyproline-rich protein, a member of one class of structural cell wall proteins. Extensin genes of tobacco were found to be induced by wounding, pathogen infection, ethylene, cytokinin stress [Memelink et al., 1993], salicylic acid [Tire et al., 1994], sucrose in combination with wounding [Ahn et al., 1996] and in *Arabidopsis* also by methyl jasmonate and abscisic acid [Merkouropoulos et al., 1999]. Different members of the extensin family are expressed in different plant organs [Elliott and Shirsat, 1998] or may contain promoter elements induced by different stimuli [Hirsinger et al., 1997].

Seven defence genes, from eight genes selected in this study by macroarray analysis, were confirmed by results of the above referred studies as wound inducible. The octadecanoid pathway mediates, at least partially, the transcription activation of six of

the genes selected upon mechanical wounding of wild-type and transgenic tobacco plants. The accumulation of anti-jasmonic acid single-chain Fv antibodies in the cytosol and in the ER of tobacco cells leads to the deficiency in defence response induced by wounding, mediated by jasmonates and manifested by the reduced levels of expression of following defence genes: *Nicotiana tabacum* proteinase inhibitor 2 gene, PR-5 osmotin-like gene, glutathione S-transferase gene, 5-epi-aristolochene synthase gene, 3-hydroxy-3-methylglutaryl coenzym A reductase gene and pathogenesis-related protein PR-1b gene. The extensin gene is wound-induced in wild type and the transgenic plants to the same extend and seems to be independent on the modulation of functional jasmonates. Repeatedly observed increase in the transcript level of SAR 8.2 gene requires further investigation.
Nothing is known about putative jasmonate receptors or the localisation of functional jasmonates (Chapter 1.2). However, the most likely mechanism to explain the observed reduced defence response is the binding of endogenous jasmonic acid by the anti-jasmonic acid scFv antibodies accumulated in the cell cytosol or in the ER of transgenic tobacco plants. The anti-JA scFv antibodies accumulated in the ER may trap jasmonic acid in this compartment and thereby form an artificial sink of jasmonic acid in the ER. The cytosol-localised anti-JA antibodies may capture the jasmonic acid directly in the cytosol, resulting in a more pronounced deficiency in jasmonate-induced gene expression. This indicate that the place of putative jasmonic acid function is the cytosol of the cell.

- **Could the possible symptoms of jasmonate deficiency be restored by the application of exogenous jasmonates?**

Symptoms of jasmonate deficiency were manifested by reduced expression levels of several wound-induced defence genes in transgenic plants with the accumulation of anti-jasmonic acid recombinant antibodies in the cell cytosol and in the ER. The application of 200 μM jasmonic acid methyl ester on wounded leaves of the transgenic plants could restore wild-type-like expression level of *Nicotiana tabacum* proteinase inhibitor 2 gene (Nt pin 2) after 24 hours or 48 hours of JAME treatment (Fig. 5.9, 5.10 and 5.11). The normalisation changed the transcription abundance of the other defence genes only slightly or not at all (Fig. 5.9). A possible explanation could be the wound-induced substantial changes of Nt pin 2 transcript abundance and rather moderated induction of other selected defence genes, observed even in wild-type plants, making the normalisation visible mainly in case of Nt pin 2 gene expression level. The defence gene expression should also be considered as a result of complex signalling network of defence pathways (Chapter 1.2.6). Thus, another possible interpretation is a more direct involvement of jasmonic acid in the Nt pin 2 gene expression and the wound-induction of the other selected defence genes through the interaction of jasmonates with other signalling molecules, like ethylene (in case of PR-5 osmotin) or salicylic acid (in case of PR-1b) or through other stimuli (in case of prp1, EAS and HMGR).
Summarizing, wild-type-like gene expression could be reproducibly restored by the application of exogenous methyl jasmonate only in case of *Nicotiana tabacum* proteinase inhibitor 2 gene. Both significantly lower gene induction upon mechanical wounding and more pronounced involvement of other signalling stimuli seem to be the

cause of the non-complete restoration of wild-type-like transcript abundance of the other defence genes selected in this study.

- **Will the immunomodulation of jasmonic acid biosynthetic pathway influence the overall content of polyunsaturated fatty acids – shared substrates with other lipoxygenase-dependent metabolic routes?**

Polyunsaturated fatty acids (linoleic and α-linolenic acids) undergo in higher plants dioxygenation by lipoxygenase and afterwards further conversion in different metabolic pathways. The jasmonic acid-biosynthetic pathway is one of the metabolic routes (Fig. 5.13).
The analysis of free and membrane-bound fatty acid derivatives in wild-type and transgenic tobacco plants accumulating anti-jasmonate scFv antibodies in the cytosol, in the ER, in the chloroplast stroma and in the outer chloroplastic membrane of tobacco cells was performed by gas chromatography (Chapter 5.2).
The content of membrane-bound or free polyunsaturated fatty acids is not changed upon the immunomodulation of jasmonic acid or its biosynthetic precursor. This result indicates no effect of the immunomodulation on the distribution of polyunsaturated fatty acids, substrates of different lipoxygenase-dependent metabolic routes.

- **Would the expression of anti-jasmonate scFv antibodies influence endogenous jasmonate levels?**

The state of knowledge of biology of jasmonates (Chapter 1.2) including the inductive control of jasmonate biosynthetic pathway (Fig. 1.5), the principle of immunomodulation of hormonal functions (Chapter 2.2) and also the results of macroarrray analysis (Chapter 5.1) all raise the questions whether the endogenous jasmonate can induce its own biosynthetic pathway, which mechanisms regulate the temporary character of jasmonate induction and what is the contribution of other phytohormones to these processes.
Analysis of acidic phytohormones – jasmonic acid, 12-oxo-phytodienoic acid, salicylic acid, abscisic acid and indole-3-acetic acid – in wild type and transgenic tobacco plants with the intracellular accumulation of anti-jasmonate single-chain Fv antibodies was performed by gas chromatography-tandem mass spectrometry (Chapter 5.3).
Each analysed phytohormone showed different behaviour during 24 hours following mechanical wounding.
The most dramatic changes were observed in the case of jasmonic acid, whose content was calculated as a sum of *cis*- and *trans*-isoforms. A dramatic increase in jasmonic acid level was measured 2 hours after mechanical wounding in leaves of transgenic tobacco plants with the intracellular localisation of anti-jasmonate antibodies in the cytosol, in the endoplasmic reticulum and in the stroma of tobacco chloroplasts. The level of jasmonic acid in plants with the outer chloroplastic membrane-localised anti-jasmonate antibodies increased moderately and in wild type was negligible in comparison to the jasmonic acid level in the transgenic plants (Fig. 5.17 (b)).

Transient increase in 12-oxo-phytodienoic acid 2 hours after wounding, calculated as a sum of *cis*- and *trans*-isoforms, was also observed but two orders of magnitude lower than the jasmonic acid level (Fig. 5.17 (a)).
As suggested above, formation of antibody-jasmonic acid complexes in the cytosol and in the ER may prevent jasmonic acid from downstream processing of a wound signal, leading to the deficiency in defence response manifested by the reduced defence genes induction in the transgenic plants. The dramatic increase in endogenous levels of jasmonic acid two hours after mechanical wounding observed in transgenic plants with anti-jasmonate scFv antibodies in the cytosol, in the ER and interestingly also in the chloroplastic stroma of tobacco cells seems to be as well caused by the accumulation of anti-jasmonate antibodies in these cell compartments. The induction of JA exceeded by more than two orders of magnitude the endogenous level of jasmonic acid in wounded wild type. This enormous accumulation was transient, indicating a disturbed, so far unknown, control mechanism of induction of endogenous jasmonic acid upon mechanical wounding.

- **Would the potential modulation of jasmonate levels have any impact on endogenous levels of other phytohormones, known to cross-talk with the jasmonic acid biosynthetic pathway?**

Wound-induced changes in the concentrationt of salicylic acid and indole-3-acetic acid were complementary to the levels of jasmonic acid and 12-oxo-phytodienoic acid (Fig. 5.18 (a) and (c)). This observation supports the idea of an inhibitory effect of jasmonic acid on levels of salicylic acid and *vice versa*, and also the idea of a recovery of initial levels of indole-3-acetic acid as a mechanism limiting the duration of the wound response (Chapter 1.2.6).
The kinetics of abscisic acid was surprising. While wounding causes an increase in the abscisic acid level in tomato [Herde et al., 1999] and in potato [Bowles, 1993] the level of abscisic acid in wild type as well as in transgenic tobacco plants with anti-jasmonate recombinant antibodies were very high in unwounded leaves and decreased upon wounding by one order of magnitude (Fig. 5.18 (b)). It has been reported that abscisic acid is not a primary component of the wound response pathway [Ryan, 2000] but minimal threshold value of abscisic acid is essential for the mediation of proteinase inhibitor 2 gene expression upon wounding in tomato [Herde et al., 1999]. It is likely that abscisic acid reacts upstream of jasmonic acid in the wound-signalling cascade [Hildmann et al., 1992]. On the ground of these investigations an elevated abiscisic acid level upon wounding was expected. The set up conditions in the phytochamber, where all transgenic and wild-type plants were grown, were standard (see Chapter 7) and controlled, and can not explain the measured unusual profile of abscisic acid upon wounding.
The intracellular accumulation of functional anti-jasmonate recombinant antibodies does not have an impact on the endogenous levels of salicylic acid, abscisic acid and indole-3-acetic acid. Relative and transient wound-induced changes of concentrations of endogenous levels of salicylic acid, abscisic acid and indole-3-acetic acid are expected to be involved in the temporal character of the wound-induced elevated jasmonates level. However, the mentioned control mechanism regulating the increase of the

endogenous jasmonate level upon wounding is independent of the relative changes of concentrations of these three acidic phytohormones and seems to be disturbed by the ectopic expression of specific recombinant anti-jasmonate antibodies.

- **Are these effects, if any, cell-compartment specific?**

The immunomodulation of jasmonate functions is cell-compartment specific. Several lines of evidence support this assumption. First, transgenic plants accumulating anti-jasmonate single-chain Fv antibodies in different cell compartments show significantly different expression profiles of defence genes upon mechanical wounding. While the localisation of anti-jasmonate scFv in the cytosol and in the ER cause deficiency in the wound-induced defence genes expression, the chloroplastic localisation of anti-jasmonate single-chain Fv antibodies lead to wild-type-like expression profile of the defence genes. Second, differences between transgenic plants with anti-jasmonate antibodies in different cell compartments were observed during the normalisation of wild-type-like gene expression levels. While the expression level of *Nicotiana tabacum* proteinase inhibitor 2 gene in transgenic plants with the cytosol-localised anti-jasmonic acid recombinant antibodies could be restored to the wild-type level, only slight changes in Nt pin 2 gene expression upon the application of exogenous jasmonic acid were repeatedly observed in plants with the ER-localised anti-jasmonic acid recombinant antibodies.

Model of intracellular events upon immunomodulation of jasmonate functions.

The following model describes intracellular events taking place upon mechanical wounding of transgenic plants with subcellular expression of functional anti-jasmonate single-chain Fv antibodies.

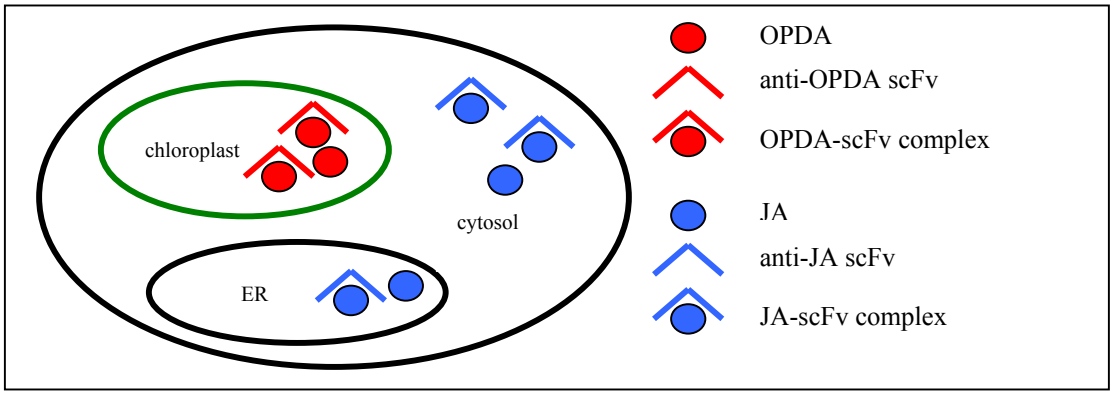


Fig. 6.1 Localisation and putative functions of anti-jasmonate scFv antibodies in the subcellular compartments of tobacco cells.

Putative events in tobacco cells with the cytosol-localised anti-jasmonic acid scFv:
Basal levels of jasmonic acid and 12-oxo-phytodienoic acid as well as gene expression in unwounded leaves of the transgenic plants are in accordance with that of wild type.
Induced jasmonic acid 2 hours after wounding is bound by the recombinant antibodies. Cells do not recognise an excess of JA and a so far unknown intracellular regulation mechanism promotes production of more JA. Increased level of OPDA could suggest preferential conversion of OPDA to JA. However a more detailed study of the kinetic of JA induction and enzymatic activity of JA biosynthetic enzymes during the first two hours following wounding are required to decide from which sources the induced jasmonic acid originates.
24 hours after wounding levels of JA and OPDA decrease. Mechanisms responsible for the decrease of JA concentration to the basal level, possibly mediated by other phytohormones, are not disturbed by the accumulation of endogenous anti-JA antibodies. Formed antibody-jasmonic acid complexes prevented jasmonic acid to react with its putative downstream signalling components leading to the deficiency in defence response manifested by the reduced defence genes induction.

Putative events in tobacco cells with the ER-localised anti-jasmonic acid scFv:
Basal levels of jasmonic acid and 12-oxo-phytodienoic acid as well as gene expression in unwounded leaves of the transgenic plants are in accordance with that of wild type.
Antibodies accumulated in the ER bind jasmonic acid passing into this cell compartment and form an artificial sink of JA in the ER. Established antibody-jasmonic acid complexes lead to the inactivation of a fraction of the endogenously wound-induced JA or the exogenously applied JA. An unknown intracellular mechanism again forces cells to overcome the assumed deficit of functional JA, which results in the dramatic increase of JA concentration.
Catabolic mechanisms decrease endogenous JA concentration to a basal level measured 24 hours after wounding. Like in the case of the cytosolic localisation of anti-JA scFv, the trapping of functional JA causes a non-sufficient defence response.

Putative events in tobacco cells with the chloroplastic stroma-localised anti-12-oxo-phytodienoic acid scFv:
Basal levels of jasmonic acid and 12-oxo-phytodienoic acid in unwounded leaves of the transgenic plants are in accordance with that of wild type. PR-1b gene expression is interestingly increased.
Antibodies in the chloroplast bind to the first cyclic metabolite of the JA biosynthetic pathway – 12-oxo-phytodienoic acid. Alternative sources of OPDA are activated, such as putative JA-biosynthesis in the cytosol [Wang et al., 1999] or membrane bound OPDA [Stelmach et al., 2001], followed by the release of OPDA and its promoted conversion to JA. Cells are again forced to produce the excess of JA.
An attempt was made to create transgenic plants with anti-jasmonate scFv antibodies localised in both cytosol and chloroplast, and thereby to provide more information about a putative cytosolic JA-biosynthetic pathway. Unfortunately, no plants could be regenerated from the leaf discs infected with *Agrobacterium tumefaciens* containing expression cassettes for antibody targeting into the cytosol as well as into the stroma of chloroplasts (data not shown).
Functional catabolic mechanisms lead to a decrease of OPDA and JA concentration at 24 hours after wounding. Absence of antibodies in the cytosol and absence of an

artificial sink in the chloroplast or in the ER enable wild-type-like defence gene expression.

Events in cells with the outer chloroplastic membrane-localised anti-12-oxo-phytodienoic acid scFv:
Wild-type-like levels of phytohormones and defence gene expression indicate the non-visible immunomodulation effect in this cell subcompartment. Possible explanation could be masking of antibody binding sites by immobilisation of the antibodies in the chloroplastic membrane. Alternatively, other compounds of chloroplastic membrane, possibly membrane bound OPDA [Stelmach et al., 2001], may compete for the antibody binding sites.

Conclusions from the proposed model:

The ectopic expression of anti-jasmonate scFv antibodies does not change morphology and development of tobacco plants but disturbs a so far unknown regulatory mechanism of jasmonate induction upon mechanical wounding. This regulatory mechanism is first, operating already during the first two hours following wounding, second, independent of the relative changes of concentrations of acidic phytohormones (salicylic acid, abscisic acid and indole-3-acetic acid) and third, influenced by the accumulation of anti-jasmonate scFv antibodies in the cytosol as well as in the chloroplast, both putative cell compartments of the octadecanoid biosynthetic pathway. The immunomodulation of jasmonate functions: (a) has no effect on the intracellular content of the substrate of the jasmonic acid biosynthetic pathway, (b) has no effect on the temporal character of the wound-induced jasmonate signal, (c) is cell compartment specific and (d) indicates that the cytosol of the cell is the subcellular compartment of jasmonic acid function.

Perspectives for further investigation.

The proposed model of the immunomodulation of jasmonate functions raises new questions for further investigation. The most acute questions are:

- Which regulating mechanism of JA and OPDA induction upon wounding is disturbed by the immunomodulation of jasmonate functions causing the dramatic increase in jasmonic acid level?
- From which sources originates the excess of jasmonic acid? Is the phytohormone released from some storage compartment [Herde et al., 1999] or is the activity of the octadecanoid biosynthetic enzymes increased and jasmonic acid originates from *de novo* synthesis?
- Is the wounded tobacco leaf tissue more resistant to pathogens, whose defence is mediated by jasmonates? Is the increased expression level of acidic PR-1b protein in unwounded transgenic plants with anti-OPDA recombinant antibodies in the chloroplastic stroma connected to this resistance?
- What would be the effect of organ-specific immunomodulation of jasmonate function in vascular bundles and flower organs, both characterised by preferential expression of AOS and AOC – enzymes of the octadecanoid biosynthetic pathway [Hause et al., 2000], [Maucher et al., 2000], [Ziegler et al., 2000]?

Chapter 7

Material and methods

7.1 Material

7.1.1 Plant material

Nicotiana tabacum, cv.Samsun NN plants grown from seeds in sterile conditions were used for the leaf disk transformation.
Nicotiana tabacum, cv.Samsun NN plants of F1 generation grown from seeds for one month in sterile conditions and another two months in the phytochamber with controlled conditions were used for the wound stress experiments.
Conditions in the phytochamber: 16 hours light (7h – 23h), 8 hours dark
humidity: 70 %
temperature: 23 ºC
fresh air: 40 %
Soil mixture: compost 40 %, sand (0 – 2 mm) 20 %, peat 20 %, substrate II (Klasmann-Dielmann, D) 20 %.

7.1.2 *Agrobacterium tumefaciens* strain

C58C1Rf^r (pGV 2260 in C58C1) [Deblaere et al., 1985].

7.1.3 Escherichia coli strains

1. TG1: *K12, Δ(lac-pro), supE, thi, hsdD5/FtraD36, pro A$^+$B$^+$, lac1^q, lacZΔM15.*
2. HB2151: *K12, ara, Δ(lac-pro), thi/F' pro A$^+$B$^+$, lac1^{q}ZΔM15.*
3. XL1 Blue: *recA1, endA1 gyrA96 thi-1, hsdR17, supE44 relA1 lac[F'proAB lac1^{q}ZΔM15 Tn10 (Tet^r)]*

7.1.4 Vectors

phagemid pIT	amp^r **site** http://www.mrc-cpe.cam.ac.uk/~phage/*g1p.html
pRTRA7/3	amp^r [Artsaenko, 1996]
pRT103	amp^r [Töpfer et al., 1993]
pRTHook	amp^r [Artsaenko, 1996]
pBIN19	km^r [Frisch et al., 1995].

7.1.5 ScFv genes in pIT vector

anti-JA scFv genes [Hunger, 2002].
anti-OPDA scFv genes [Hunger, 2002].

7.1.6 Primers

- PCR primers:

FORTOM	5'-GTC ACG GGA TCC ATT CAG ATC CTC TTC TGA GAT GAG TT -3' [Amersham Pharmacia Biotech, D]
BACKLEGTOM	5'-ACA CGT GGA TCC GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG G -3' [Amersham Pharmacia Biotech, D]

- Sequencing primers:

LMB3	5'-CAG GAA ACA GCT ATG AC -3' http://www.mrc-cpe.cam.ac.uk/~phage/*g1p.html)
PHENseq	5'-CTA TGC GGC CCC ATT CA -3' http://www.mrc-cpe.cam.ac.uk/~phage/*g1p.html)
M13 universal	5'-GTA AAA CGA CGG CCA GT-3' [Boehringer/Mannheim, D]
M13 reverse	5'-AAC AGC TAT GAC CAT G-3' [Boehringer/Mannheim, D]
LEG 73	5'-GCT TTC ACT TTC CTT GCT TC-3' [Boehringer/Mannheim]
TAG 73	5'-GAG AGA CTG GTG ATT TTT GCG-3' [Boehringer/Mannheim, D]
FORLINK	5'-GCC ACC TCC GCC TGA ACC -3' [Metabion GmbH, D]
BACKLINK	5'-GGT GGA GGC GGT TCA GG -3' [Metabion GmbH, D]

7.1.7 Protein markers

SmartLadder [Eurogentec, D], western blot bench marker [GibcoBRL, D], western blot positive control – plant scFv purified via affinity matrix [G. Mönke, unpublished].

7.1.8 Enzymes

Alkaline phosphatase [Amersham, D], Klenow enzyme [USB, D], restriction enzymes [GibcoBRL, D], RNase A [GibcoBRL, D], Superscript ™ II Rnase H$^-$ Reverse Transcriptase [GibcoBRL, D], Taq polymerase [GibcoBRL, D], T4-DNA ligase [GibcoBRL, D].

7.1.9 Antibodies , antibody conjugates and BSA-conjugates

Anti-c-myc antibodies 9E10 ([Munroe and Pelham, 1986], were produced in our laboratory), anti-jasmonic acid polyclonal serum [kindly provided by Prof. Wasternack, IPB, Halle, Germany], anti-abscisic acid I5-I-C5 antibodies [kindly provided by Prof. Weiler, Ruhr-Uni, Bochum, Germany], anti-24-epi-brassinolide scFv [L. Fecker, unpublished; isolated in our laboratory], anti-dihydro-zeatine riboside antibodies [kindly provided by Prof. Strnad, UP, Olomouc, Czech Republic], anti-mouse IgG alkaline phosphatase conjugate [Sigma, D], anti-rabbit IgG alkaline phosphatase conjugate [Sigma, D], anti-mouse Ig horseradish peroxidase-linked whole molecule (IgG-HRP) [Amersham Pharmacia Biotech, D], JA-BSA and OPDA-BSA conjugates [kindly provided by Dr. Kramell, IPB, Halle, Germany], 24-epi-brassinolide-BSA conjugate [L. Fecker, unpublished], diHZR-BSA conjugate [P. ten Hoopen, unpublished], ABA-BSA conjugate [Artsaenko, 1996].

7.1.10 Antibiotics

Ampicilin sodium (Amp)[Duchefa, NL], carbenicillin disodium (Cb) [Duchefa, NL], cefotaxime sodium (Cla) [Duchefa, NL], kanamycin monosulfate (Km) [Duchefa, NL].

7.1.11 Plant hormones, fatty acid and phytohormone standards

α-naphthalene acetic acid (NAA) [Sigma, UK], 6-benzylamino-purine (BAP) [Sigma, UK], (3R, 7R)-jasmonic acid and (3R, 7R)-jasmonic acid methyl ester [kindly provided by Dr. Kramell, IPB, Halle, Germany], heptadecanoic acid [kindly provided by Dr. Feussner, IPK, Gatersleben, Germany], $[^2H]_4$-salicylic acid, $[^{13}C]_2$-jasmonic acid, $[^2H]_2$-indole-3-acetic acid, $[^2H]_6$-abscisic acid, $[^2H]_5$-12-oxo-phytodienoic acid [kindly provided by Dr. Müller, Ruhr-Uni, Bochum, Germany].

7.1.12 KITs

α-^{33}P-dCTP [NAN, USA], DIG DNA labelling and detection kit [Boehringer / Mannheim, D], Dynabeads® mRNA purification kit [Dynal, Norway], ECL Western blotting analysis system [Amersham Pharmacia Biotech, D], Megaprime DNA labelling kit [Amersham Pharmacia Biotech, D], RNeasy® kit [QIAGEN, D], QIAEX II gel extraction kit [QIAGEN, D], QIAGEN plasmid kit [QIAGEN, D].

7.1.13 Special laboratory reagents

Bacto-agar [Difco, USA], bacto-peptone [Difco, USA], DEPC [Sigma, D], Bio-Rad protein assay [Bio-Rad, D], bovine serum albumin [Sigma, D], ethidium bromide [Roth, D], 37% formaldehyde [Roth, D], formamide [Roth, D], glycine [Roth, D], hybridisation solution Roti-Hybri-Quick [Roth, D], IPTG [Boehringer/Mannheim, D], Marvel dried skimmed milk fat free [Marvel, GB], MES [AppliChem, D], Murashige-Skoog medium basal salt mixture [Duchefa, NL], NBT/BCIP stable mix [GibcoBRL, D], p-nitrophenyl phosphate (p-NPP) [Sigma, D], phenol/chloroform [Roth, D], polyethylenglycol 6000 (PEG6000) [Merck, D], Rotiphorese® Gel30 [Roth, D], Sucrose [Duchefa, NL], TEMED [Gibco BRL, D], Tris [Roth, D], Triton X-100 [Serva, D], Tween20 [Serva, D], yeast extract [Difco, USA].

7.1.14 Special laboratory tools

Filter paper Whatman no.4 [Whatman®, USA], fuji imaging plate BAS-III [Fuji foto film, Japan], microtitre plates Falcon 3915 [Becton Dickinson and company, USA], nylon membranes Hybond N+ [Amersham Pharmacia Biotech, D], Parafilm® – laboratory film [American National Can™, USA], ProbeQuant G-50 Micro columns [Amersham Pharmacia Biotech, D], Protran® nitrocellulose transfer membrane [Schleicher & Schuell, D], rProteinL™coupled to CNBr-activated sepharose [ACTIgen, D], silica-based aminopropyl matrix [Varian, D], syringe filters 0.2µm Nalgene® [Schütt Labortechnik, D], VectaSpin Micro™centrifuge tube filters anopore – 0,2µm [Whatman®, USA], X-ray film [Retina, D].

7.1.15 Special laboratory equipment

Biofuge fresco [Heraeus, D], ELISA reader Dynatech MR 7000, GeneAmp® PCR system 9700 [PE Applied Biosystems, USA], Gene-Pulser™ [Bio-Rad, D], hybridisation oven [Bachofer, D], phosphoimager Storm 860 [Molecular Dynamics, USA], spectrophotometr Spectronic Genesys 5 [Milton Roy, USA], spotting robot BG600 [BioGrid, GB], transfer electrophoresis unit [Schütt Labortechnik, D], transilluminator 2020E UV/white [Stratagene, D], ultrasonic device Sonorex RK510S [Bandelin, D], vacuum centrifuge concentrator 5301[Eppendorf, D].

7.1.16 Buffers

- A-PBS – 100 mM $Na_2HPO_4.12H_2O$, 100 mM $NaH_2PO_4.H_2O$, 0.15 M NaCl, pH = 7.2.
- Carbonate buffer – 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH = 9.6.
- denaturation solution – 1 M NaCl, 0.4 M NaOH.
- EB buffer – 1 M Tris-HCl pH = 9.0, 1% SDS (used DEPC-treated ddH_2O).
- EDAC solution – 1 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide / 10 µl methanol.
- HA solution – 30 mM Potassium acetate, 10 mM $CaCl_2$, 100 mM KCl, 50 mM $MnCl_2$, 15 % glycerol (sterile filtrated).
- HB solution – 10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15 % glycerol (autoclaved).
- HIP solution – iso-hexan/isopropanol (3:2) with 0.0025% 2-butyl-6-hydroxytoluen.
- Lysis buffer – 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH = 8.0.
- Lysosym buffer – Lysis buffer with 4 mg/ml lysozyme.
- 4x Marvel buffer – 80 mM Tris-HCl pH = 7.8, 720 mM NaCl.
- 10x MEM solution – 0.2 M MOPS, 0.08 M Sodium acetate pH = 7.0, 5 mM EDTA.
- neutralisation solution – 1 M NaCl, 0.5 M Tris-HCl pH = 7.5.
- P2 buffer – 0.2 M NaOH, 1% SDS.
- P3 buffer – 3 M Sodium acetate pH = 4.8.
- PBS – 0.03 M $Na_2HPO_4.2H_2O$, 0.017 M $NaH_2PO_4.H_2O$, 0.1 M NaCl, pH = 7.2.
- SDS-sample buffer – 10 ml contain: 1 ml glycerol, 1.4 ml Stacking buffer, 2 ml 10 % SDS, 0.5 ml β-mercaptoethanol, 5.1 ml ddH_2O, 0.001% bromphenol blue.
- Stacking buffer – 0.5 M Tris-HCl pH = 6.8, 0.4% SDS.
- 20x SSC – 0.3 M Sodium citrate, 3 M NaCl.
- TBE buffer for agarose gel – 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA.
- TE – 10 mM Tris-HCl pH = 8.0, 1 mM EDTA.

7.1.17 Media

All media are calculated for a volume 1 L and were sterilized by autoclaving for 20 min at 120 °C. Antibiotics, hormones or other components, specified in Chapter 7.2, were added after autoclaving the media. For a solidified medium 15 g bacto-agar was added per 1 L liquid medium.

- CPY medium – 1 g yeast extract, 5g bacto-peptone, 5 g Sucrose, 0,5 g $MgSO_4.7H_2O$.
- LB medium – 5 g yeast extract, 10 g bacto-peptone, 10 g NaCl.
- KB medium – 5 g yeast extract, 20 g tryptone, 0.7 g KCl, 4 g $MgSO_4.7H_2O$, pH = 7.6.
- Murashige-Skoog medium – 4.4g Murashige-Skoog medium basal salt mixture, 0.5 g MES, 30 g Sucrose, pH = 5.7.

- SOC medium – 5 g yeast extract, 20 g tryptone, 0.58 g NaCl, 0.18 g KCl. 2 M glucose was added after autoclaving.
- 2xTY medium – 10 g yeast extract, 16 g tryptone, 5 g NaCl.
- TBY medium - 5 g yeast extract, 8 g tryptone, 5 g NaCl, 5 g $MgSO_4.7H_2O$.

7.2 Methods

7.2.1 *E.coli* transformation (Electroporation, heat shock transformation)

Electroporation

A pulse (2.5 V, 25 µF, 200 Ω) was applied from the Bio-Rad Gene-Pulser™ on the cuvette containing 50 µl of electroporation-competent *E.coli* cells with 1 µl of plasmid DNA. Cells were resuspended in 1ml of SOC medium, incubated for 45 min at 37 °C and plated on TBY medium with 100mg/l Amp. In case of *A. tumefaciens* transformation, the cells were incubated at 28 °C and plated on CPY medium with 50mg/l Km and 100mg/l Cb.

Heat shock competent cells

5 ml of KB medium was inoculated with a single colony of XL1 Blue cells and incubated overnight at 37 °C. 1 ml of the overnight culture was used for the inoculation of 100 ml KB medium. The culture was incubated at 37 °C by shaking until the optical density at 560 nm reached 0.3. Cells were centrifuged for 10 min at 4 °C (4500 rpm), the pellet was resuspended in 25 ml ice cold solution HA and let on ice for 1 hour. Cells were centrifuged for 10 min at 4 °C (4500 rpm) and the pellet was resuspended in 4 ml ice cold solution HB. 100 µl aliquots of heat shock-competent cells were frozen in liquid nitrogen and stored at -80 °C.

Heat shock transformation

100 µl of heat shock-competent XL1 Blue cells were incubated for 15 min on ice with 1 µl of plasmid DNA and afterwards heated for 90 seconds at 42 °C. Cells were resuspended in 100 µl of LB medium, incubated for 10 min at 37 °C and plated on a solidified TBY medium with 100mg/l Amp.

7.2.2 Production of soluble scFv from *E.coli*

Non-suppressor strain HB2151 of *E. coli* was transformed with pIT vector containing the scFv gene. The culture, inoculated with an individual colony, was incubated overnight in 2xTY medium with 100 mg/l Amp and 1% glucose. A small inoculum (5µl) of this culture was transferred into medium 2xTY with 100mg/l Amp and 0.1% glucose and incubated in 37 °C by shaking until the optical density at 600 nm reached approximately 0.9. IPTG was added to a final concentration of 1 mM IPTG and the culture was shaken overnight at 30 °C. After spinning down the bacterial pellet, the supernatant was used in ELISA.

7.2.3 Purification of scFv from plant extract

1ml of rProteinL™coupled to CNBr-activated sepharose was transferred into a small column and washed with 20 ml of A-PBS buffer. Approximately 0.1 mg of a frozen tobacco leaf was extracted in SDS-sample buffer and used as a control of the scFv expression – probe A. 5-10 g of the same tobacco leaf was grinded in liquid nitrogen and 3 volumes of PBS containing 0.1 % of Triton X100 was added. The sample was centrifuged at 4 °C for 5 min (4500 rpm) and the supernatant centrifuged again in 2ml-tubes at 4 °C for 20 min (14000 rpm). The obtained supernatant was applied on the washed protein L column. The application of the supernatant was repeated two times. 0.5 ml of the crude plant extract was used as a control of the extraction – probe B. 0.5 ml of the solution after purifying via the column was used as a control of the binding effectivity – probe C. The protein L column was washed with 100 ml of A-PBS buffer. ScFv antibodies were eluted with 45 ml of 0.1 M glycine pH = 2.5, into 2ml-tubes, each containing 60 µl of 1 M Tris. The column was washed with 100ml of A-PBS buffer followed by the application of 10 ml of 22% ethanol in A-PBS buffer. A western blot analysis was performed to detect scFv in the fractions and in probes A, B and C. Fractions containing scFv antibodies were concentrated in PEG 6000 for 3 hours in a dialysing tube. The protein content was determined by Bio-Rad Protein Assay.

7.2.4 ELISA with soluble anti-jasmonate scFv in bacterial supernatant or anti-jasmonate scFv purified from plant extract

A falcon microtitre plate was coated overnight at RT with 80 µl of 10 µg/ml BSA or with JA-BSA conjugate at a concentration 50 µg/ml (or 80 µl JA-BSA conjugate in glycerol diluted 1:10000) in carbonate buffer. The ELISA plate was flipped over to discard excess liquid and incubated for 2 hours with 90 µl of 2% BSA in PBS to block unspecific binding sites. 80 µl of bacterial supernatant diluted 1:2 in 2 % BSA in PBS (or 80 µl of plant-purified anti-jasmonate scFv at a concentration 1 µg/ml in PBS) was added and incubated for 1 hour at 25 °C. The solution was discarded and the plate washed five times with PBS containing 0.05 % Tween 20 (PBST). 80 µl of anti-c-myc antibodies diluted 1:100 in 2 % BSA in PBS was applied for 45 min at RT followed by another three times washing with PBST. The plate was incubated with 80 µl of anti-mouse IgG-ALP conjugate diluted 1:2000 in 2 % BSA in PBS for 45 min, washed three times with PBST and developed with substrate solution – 1 mg/ml pNPP. The intensity of yellow colour developed after 1 hour was measured with the ELISA reader at 405 nm. ELISA with protein L-purified anti-24-epi-brassinolide scFv diluted 1:20 in PBS was performed the same way as described above, except 24-epi-brassinolide-BSA conjugate diluted 1:200 was used in stead of JA-BSA conjugate.

7.2.5 ELISA with anti-JA, anti-ABA or anti-diHZR serum

A falcon microtitre plate was coated overnight at RT with 80 µl of 10 µg/ml of BSA or with corresponding conjugate (JA-BSA conjugate in glycerol diluted 1:10000, ABA-BSA conjugate diluted 1:2000 or diHZR-BSA conjugate diluted 1:200) in carbonate buffer. The ELISA plate was flipped over to discard excess liquid and incubated for 2 hours with 90 µl of 2 % BSA in PBS. 80 µl of the antibody serum (anti-JA serum diluted 1:10000, anti-ABA serum diluted 1:500 or anti-diHZR serum diluted 1:5000) in PBS was added and incubated for 1 hour at 25 °C. Solution was discarded and the plate washed five times with PBS containing 0.05% Tween 20 (PBST). 80 µl of anti-mouse

IgG-ALP conjugate (or anti-rabbit IgG-ALP conjugate in case of anti-diHZR serum) diluted 1:2000 in 2 % BSA in PBS was applied for 45 min at RT. The plate was washed three times with PBST and developed with substrate solution – 1 mg/ml pNPP. Intensity of yellow colour developed after 1 hour was measured with the ELISA reader at 405 nm.

7.2.6 PCR amplification of scFv gene

Vector pIT containing anti-jasmonate scFv gene diluted 1:100 in sterile water was used as a template for PCR reaction. The reaction was performed with 10 μM FORTOM primer, 10 μM BACKLEG primer, 25 mM dNTP mix and Tag polymerase.
PCR conditions were the following:
95 °C / 4 min, (65 °C / 0.45 min, 72 °C / 0.45 min, 93 °C / 1 min) 30 time, 65 °C / 0.45 min, 72 °C / 10 min.

7.2.7 Plasmid minipreparation from *E.coli* and from *A. tumefaciens.*

Plasmid minipreparation from *E.coli* was done as described in [Sambrook et al., 1989].

Plasmid preparation for sequencing and spotting
Plasmid DNA extraction for sequencing and DNA spotting was performed with the QIAGEN plasmid kit. The sequencing was done by S. König and carried out with an ALF DNA sequencer [Pharmacia, D].

Plasmid minipreparation from *A. tumefaciens*
A.tumefaciens cells were grown overnight in CPY medium containing 50 mg/l Km and 100 mg/l Cb. The bacterial pellet was resuspended in 100 μl of ice-cold lysosyme buffer and incubated for 10 min at RT. 200 μl of P2 buffer was added and incubated for 10 min at RT. 50 μl P2 buffer containing 10 % of phenol / chlorophorm was added and gently vortexed. 150 μl P3 buffer was added, the sample incubated for 15 min in -20 °C and centrifuged at RT for 3 min (14000 rpm). DNA was precipitated with 1 ml of 96 % ethanol for 15 min in -80 °C, centrifuged at RT for 3 min (14000 rpm). The pellet was washed with 70 % ethanol, dried and resuspended in 30 μl of 50 % TE buffer containing 0.01 % RNAse A.

7.2.8 Southern blot analysis

Vector pBIN19, isolated from *Agrobacterium tumefaciens*, containing the expression cassette with scFv was cleaved with HindIII restriction enzyme and separated on 1% agarose gel. The DNA was transferred to Hybond N+; positively charged nylon membrane under alkaline conditions. The Dig DNA labelling and detection kit was used for labelling of 800bp scFv DNA isolated from pRTRA 7/3 vector and for the detection. Hybridisation was performed in the Roti-Hybri-Quick hybridisation solution at temperature 65 °C overnight. Posthybridisation washing was done two times for 5 min in (2x SSC + 0.1 % SDS) at RT and two times for 15 min (0.1x SSC + 0.1 % SDS) at 65 °C.

7.2.9 Western blot analysis

Leaf discs were homogenised in 80 μl of SDS-sample buffer, boiled for 10min, cooled for 10 min on ice and centrifuged at RT for 10 min (14000 rpm). The protein content

was determined by the Bio-Rad Protein Assay. 20 µg of total soluble protein per slot was loaded on 12.5 % SDS-polyacrylamide gel next to the bench marker. The SDS-polyacrylamide gel electrophoresis and the protein transfer to Protran® nitrocellulose transfer membrane was done as described in [Sambrook et al., 1989]. For detection, the nitrocellulose filter was blocked for 2 hours with 5 % Marvel dried skimmed fat free milk in 1x Marvel buffer (5 % MM), incubated for 2 hours in anti-c-myc primary antibody diluted 1:50 in 5 % MM and afterwards washed in 0.5 % MM. Anti-mouse IgG-HRP diluted 1:2000 in 5 % MM has been used as secondary antibody. After 1 hour incubation the filter was thoroughly washed twice in 0.5 % MM, twice in 1x Marvel buffer and twice in PBS buffer before the visualisation with the ECL Western blotting analysis system. Expression level was estimated by comparing the signal intensity of the band of a sample on X ray film with the signal intensity of the purified scFv used as a positive control.

7.2.10 Northern blot analysis

Isolation of total RNA

0.2 – 0.5 g of frozen leaf material was grinded in liquid nitrogen, transferred into 2ml-tubes and extracted with 0.7 ml phenol/chloroform and 0.7 ml EB buffer. The sample was vortexed for 10 seconds and centrifuged at 4 °C for 2 min (14000 rpm). The upper phase was transferred into a new tube, extracted again with 0.7 ml phenol/chloroform, vortexed for 10 seconds and centrifuged at 4 °C for 5 min (14000 rpm). The upper phase was transferred into a new tube and centrifuged again at 4 °C for 5 min (14000 rpm). Precipitation has been done with 1/10 volume of 3M sodium acetate buffer, pH = 5.1, and 3 volumes of ice-cold 96 % ethanol. The sample was incubated at -80 °C for 1 hour and then centrifuged at 4 °C for 5 min (14000 rpm). The precipitate was dissolved in 0.2 ml DEPC-treated sterile ddH_2O and centrifuged at 4 °C for 10 min (14000 rpm). An equal volume of 4 M LiCl was added to the supernatant and let to precipitate overnight on ice. The sample was centrifuged at 4 °C for 15 min (14000 rpm), the pellet washed in 70 % ethanol and stored in 0.2 ml 70 % ethanol at -80 °C. Directly before use the pellet was centrifuged at 4 °C for 2 min, dried briefly and dissolved in 50 µl DEPC-treated sterile ddH_2O.

Hybridisation conditions

9 µl of 37 % formaldehyde, 30 µl formamide, 7.5 µl 10x MEM solution, 7 µl 5x RNA loading buffer (RNeasy kit) and 0.7 µl ethidium bromide was added to 10 µg of total RNA in 30µl of DEPC-treated ddH_2O. RNA was incubated at 65 °C for 15 min and separated on 1 % agarose gel containing 1x MEM solution and 1/6 volume of 37 % formaldehyde. RNA transfer to Hybond N+; positively charged nylon membrane was done in 20x SSC solution. RNA was fixed on the membrane by auto-cross-link with UV light. DNA of the extensin gene, the Nt-pin 2 gene and the osmotin gene was restricted from plasmid and purified via the QIAEXII gel extraction kit. The Megaprime DNA labelling kit was used for the labelling with [α-^{33}P]dCTP. The unincorporated radio-nucleotides were separated via the ProbeQuant G-50 Micro column. Prehybridisation was performed in the Roti-Hybri-Quick hybridisation solution for 2 hours at temperature 65 °C. Filters were hybridised with the labelled DNA in the same hybridisation solution

at 65 °C overnight. Posthybridisation washing was done two times for 5 min in (2x SSC + 0.1 % SDS) at 65 °C, one time for 5 min in (1x SSC + 0.1 % SDS) at 65 °C and two times for 15 min in (0.2x SSC + 0.1 % SDS) at 65 °C. Filters were put on Whatman paper slightly wetted with 0.2x SSC, wrapped into Saran wrap and exposed for 1 – 2 days with an fuji imaging plate. The plate was scanned with the phosphoimager Storm 860.
Membranes were striped with 0.5 % SDS solution heated to 100 °C and poured on the membrane.

7.2.11 *Agrobacterium*-mediated leaf disk transformation

Agrobacterum tumefaciens 2260 strain transformed with pBIN19 binary vector was grown in CPY medium containing 50 mg/l Km and 100 mg/l Cb. The presence of scFv gene in pBIN19 binary vector was confirmed by Southern blot analysis prior the transformation. Leaf discs of wild type *Nicotiana tabacum* plant, grown from seeds in sterile conditions, were incubated for 1 hour with the *Agrobacterium* culture and then incubated on Murashige-Skoog medium for another 2 days at 25 °C. Infected leaf segments were transferred on Murashige-Skoog medium containing 100 mg/l Km, 400 mg/l Cla, 0.2 mg/l NAA and 1 mg/l BAP. Leaf discs were every 14 days replaced on new medium containing these phytohormones and antibiotics until callus and shoots began to form (4-6 weeks). Developed shoots were transferred on Murashige-Skoog medium with 100 mg/l Km and 400 mg/l Cla. Plantlets with regenerated roots were used for a Western blot analysis for determination of the scFv expression level and transferred to soil.

7.2.12 Seed germination experiment

Seeds of wild type *Nicotiana tabacum* plants, cv. Samsun NN, and transgenic plants were incubated on Murashige-Skoog medium containing 50 mg/l Km and either 0.2mM jasmonic acid methyl ester or equal volume of sterile dH_2O. Petri dishes were covered with parafilm and placed in cultivating room with conditions 16 hours light/ 8 hours dark, temperature 25 °C. Differences in seed germination between wild type and transgenic plants were obvious from the 6. day. From the stage of seedlings there were no developmental differences observed between transgenic plants and wild type.

7.2.13 DNA spotting

E. coli clones with DNA of interest were grown in 2xTY medium with selection antibiotics, plasmid DNA was purified via the QIAGEN plasmid kit and electrophoresed on 1% agarose gel to confirm quality of the DNA. The DNA concentration was determined by the comparison of the intensity of the purified-DNA dot with the intensity of standard-DNA dots, both spotted on 1% agarose plate containing 0.005% of ethidium bromide. DNA was diluted in 50% TE buffer for final concentrations 1500 ng, 750 ng and 375 ng, and spotted with the BioGrid spottin robot BG600 on Hybond N+; positively charged nylon membranes. Spotting was done from 384 well plates with the three concentrations of DNA in neighbouring wells. 0.4mm PIN was used for the spotting of 160nl of DNA per spot, with pattern 3x3 on the membrane. Membranes with spotted DNA were denaturated for 3 min in the denaturation solution and neutralised two times for 3 min in the neutralisation solution. Filters were dried for approx. 10 min and DNA fixed on the membranes by UV cross-link.

7.2.14 Macroarray analysis

Synthesis of labelled cDNA

Dynabeads® mRNA purification kit was used for the purification of mRNA from total RNA isolated from tobacco leaf tissue. Messenger RNA bound to the Dynabeads Oligo $(dT)_{25}$ was reverse transcribed by use of Superscript ™ II Rnase H^- Reverse Transcriptase and eluted from the Dynabeads. The 2^{nd} strand cDNA, labelled with [α-^{33}P]dCTP, was synthesized by using the Megaprime DNA labelling kit. The labelled cDNA was eluted from the Dynabeads and applied on the VectaSpin Micro™centrifuge tube filters anopore – 0.2µm for the separation of remaining beads.

Hybridization conditions for macroarray

A nylon filter with spotted DNA was prehybridised for 2 hours in the Roti-Hybri-Quick hybridisation solution prewarmed to temperature 65 °C. Dynabeads-free labelled cDNA was added and hybridisation was performed overnight at 65 °C. Posthybridisation washing was done two times for 5 min in (2x SSC + 0.1 % SDS) at 65 °C, one time for 5 min in (1x SSC + 0.1 % SDS) at 65 °C and two times for 15 min in (0.2x SSC + 0.1 % SDS) at 65 °C. Filters were put on Whatman paper slightly wetted with 0.2x SSC, wrapped into Saran wrap and exposed for two days with an fuji imaging plate. The plate was scanned with the phosphoimager Storm 860.

Stripping of the membranes was done by the incubation of the membrane one time for 5 min in 2x SSC at 90 °C, one time for 15 min in (0.4 M NaOH + 0.1 % SDS) at 65 °C and twice in (2x SSC + 0.1 M Tris pH = 7.5 + 0.1 % SDS) at RT.

7.2.15 Determination of fatty acids

HIP extraction

About 0.5 g of leaf material was added to 10 ml HIP solution containing 20 µl heptadecanoic acid as an internal standard. Samples were homogenised for 45 seconds with an Ultra Turrax under a stream of argon. The extracts were shaken in horizontal position for 10 min at RT, centrifuged for 10 min at 4 °C (3800 rpm) and upper phase transferred into a new tube. 6.7% potassium sulphate was added to a volume 32.5 ml and samples shaken again for 10 min at RT and centrifuged for 10 min at 4 °C (3800 rpm). The upper organic phase containing the oxylipin fatty acid derivatives was dried under nitrogen, resuspended three times in 400 µl chloroform and dried again under nitrogen. The extract was dissolved in 200 µl methanol.

Methylation

To 50 µl of the HIP extract was added 350 µl methanol and 10 µl of freshly prepared EDAC solution. The sample was shaken for 2 hours at RT and then twice extracted with 1 ml of hexan. In the first extraction 200 µl of 0.1 M Tris has been added. The upper hexan-phase was dried under nitrogen and resuspended in 10 µl acetonitril. 1 µl of the methylated extract of fatty acid derivatives was used for the GC analysis.

Measurement
Analysis of fatty acid derivatives was carried out by gas chromatography. Agilent GC 6890 system coupled with FID detector and with capillary HP INNOWAX column (0.32 mm x 30 m, 0.5 µm coating tickness, Agilent, Germany) was used. Helium was used as carrier gas. Samples were measured with splitter opening 1:60, injector temperature 220˚C and temperature program: 150˚C for 1min, 150-200˚C at 15˚C/min, 200-250˚C at 2˚C/min and 250˚C for 10min.

7.2.16 Determination of endogenous levels of phytohormones

Extraction
About 0.5 g of tobacco leaf was extracted in 2 ml of methanol prewarmed to 60 °C and containing 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 dried in the vacuum centrifuge concentrator 5301. The extracts were dissolved in 50 µl methanol and 200 µl diethyl ether with 5 min of ultrasonic treatment. Samples were centrifuged for 2 min at RT (14000 rpm) and the supernatant applied on the column with silica-based aminopropyl matrix. The column was washed with 250 µl isopropanol / chloroform (1:2) and eluted twice with 200 µl diethyl ether containing 2% acetic acid. Extracts were dried in the vacuum centrifuge, dissolved in 30 µl methanol and methylated with 100 µl ethereal diazomethane (safety precautions!). Remaining solutions were dried under a stream of nitrogen and samples were dissolved in 7 µl chloroform. 1 µl was used for the GC-MS/MS analysis.

Measurement
Samples were applied on Varian Saturn 2000 ion-trap mass spectrometer connected to a Varian CP-3800 gas chromatograph. Gas chromatograph with ZB-50 fused silica capillary column (0.25 mm x 30 m, 0.25 µm coating tickness, Phenomenex, Germany) and helium as carrier gas was used. Samples were measured with splittless injection, splitter opening 1:100 after one minute, injector temperature 260 °C and temperature program: 50 °C for 1min, 50 – 250 °C at 20 °C/min and 250 °C for 10min. Mass spectrometer working in mode of chemical ionization – multi reaction monitoring (CI-MRM) with methanol as reactant gas and positive ion detection had following settings of endogenous compounds and internal standards:
a/ endogenous compounds – SA m/z = 153 $[M+H]^+$, 0.60 V; IAA m/z = 190 $[M+H]^+$, 0.50 V; JA m/z = 225 $[M+H]^+$, 0.50 V; ABA m/z = 261 $[M-H_2O]^+$, 0.50 V; OPDA m/z = 307 $[M+H]^+$, 0.60 V.
b/ internal standards – $[^2H]_4$-SA m/z = 157 $[M+H]^+$; $[^2H]_2$-IAA m/z = 192 $[M+H]^+$; $[^{13}C]_2$-JA m/z = 227 $[M+H]^+$; $[^2H]_6$-ABA m/z = 267 $[M-H_2O]^+$; $[^2H]_5$OPDA m/z = 312 $[M+H]^+$.
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.

Summary

Plant cells, as well as animal cells, possess sophisticated intracellular signalling mechanisms that enable them to coordinate their behaviour for the benefit of the whole organism.
Jasmonates are a group of signalling molecules which plant cells use to mediate responses to adverse environmental conditions and to regulate distinct stages of plant development. Jasmonic acid and its derivatives are cyclic oxylipins derived from unsaturated fatty acids. Jasmonates modulate the gene expression of a number of stress-responsive and house-keeping genes involved in the defence and developmental processes, respectively.
In this study a molecular technique of immunomodulation has been adopted to investigate the mechanisms by which jasmonates operate.
Genes coding for recombinant single-chain Fv antibodies, selected against the naturally occurring (3R, 7R)-jasmonic acid and against its biosynthetic precursor – (9S,13S)-12-oxo-10,15(Z)-phytodienoic acid, were cloned into plant expression vectors for production of the antibodies in all plant organs but different cell compartments.

Transgenic *Nicotiana tabacum* plants, that were generated by *Agrobacterium*-mediated gene transfer, accumulated stable and heritable anti-jasmonate recombinant antibodies in their cytosol, in the endoplasmic reticulum, in the stroma and outer membrane of chloroplasts. The antibodies bind specifically the common structure of all jasmonates that is essential for the physiological activity.

Mechanical wounding is an abiotic stimulus, where jasmonates are assumed to be a key signalling molecule mediating response to this damage. Therefore, wounded leaves of transgenic and wild-type tobacco plants were investigated in special regard to their intracellular content of polyunsaturated fatty acids, endogenous levels of acidic phytohormones and gene expression profile.

The most important results of this study are:

1. The ectopic expression of functional anti-jasmonate scFv antibodies in subcellular compartments of tobacco cells does not change the overall morphology and plant development under natural conditions.
2. The accumulation of anti-jasmonic acid single-chain Fv antibodies in the cytosol and in the ER of tobacco cells lead to deficient wound response, mediated by jasmonates and manifested by the reduced levels of defence gene expression. The most likely mechanism to explain the observed reduced defence response is the binding of endogenous jasmonic acid by the anti-jasmonic acid scFv antibodies accumulated in the cell cytosol and in the ER of transgenic tobacco plants. Results of the analysis of transgenic plants indicate that the anti-JA scFv antibodies form an artificial sink of jasmonic acid in the ER. However, the place of putative jasmonic acid function seems to be the cytosol of the cell.
3. The immunomodulation of jasmonate functions is cell-compartment specific.
4. The application of exogenous methyl jasmonate reproducibly restore the wild-type-like gene expression only in case of one selected defence gene – *Nicotiana*

tabacum proteinase inhibitor 2. Both significantly lower gene induction upon mechanical wounding and more pronounced involvement of other signalling stimuli seem to be the cause of the non-complete restoration of wild-type-like transcript abundance of the other defence genes selected in this study.

5. The content of membrane-bound or free polyunsaturated fatty acids is not changed upon the immunomodulation of jasmonic acid or its biosynthetic precursor. This result indicates no effect of the immunomodulation on the distribution of substrate of the jasmonic acid biosynthesis.
6. The immunomodulation of functional jasmonate in the cytosol as well as in the chloroplast results in a dramatic increase in endogenous levels of jasmonic acid upon mechanical wounding. The induction was observed two hours after mechanical wounding of leaves of transgenic plants with anti-jasmonate scFv antibodies in the cytosol, in the ER and in the chloroplastic stroma of tobacco cells. The enormous and transient accumulation of jasmonic acid exceeded by more than two orders of magnitude the endogenous level of jasmonic acid observed in the wounded wild type.
7. The intracellular expression of functional anti-jasmonate recombinant antibodies does not have an impact on the endogenous levels of three acidic phytohormones – salicylic acid, abscisic acid and indole-3-acetic acid.
8. A so far unknown control mechanism regulating the increase in endogenous jasmonate levels upon wounding seems to be disturbed by the ectopic expression of the anti-jasmonate single-chain Fv antibodies. The suggested regulation mechanism: first, operates already during the first two hours following wounding, second, is independent of the relative changes of concentrations of the three acidic phytohormones and third, is influenced by the accumulation of anti-jasmonate scFv antibodies in the cytosol as well as in the chloroplast, both putative cell compartments of the octadecanoid biosynthetic pathway.

Zusammenfassung

Pflanzenzellen verfügen ebenso wie tierische Zellen über ausgeklügelte Mechanismen des intrazellulären Signaltransfers, die es ihnen ermöglichen, ihr Verhalten zum besten des ganzen Organismus zu regulieren.
Jasmonate sind eine Gruppe von Signalmolekülen die Pflanzenzellen nutzen, um Antworten auf feindliche Umweltbedingungen zu vermitteln und bestimmte Entwicklungsprozesse zu steuern. Jasmonsäure und ihre Derivate sind zyclische Oxylipine, die sich von ungesättigten Fettsäuren ableiten. Sie modulieren die Expression einer Anzahl stressabhängiger Gene sowie einiger Gene für Grundfunktionen, die an Abwehrreaktionen und verschiedenen Entwicklungsprozessen beteiligt sind.
In der vorliegenden Arbeit wurde die molekulare Technik der Immunmodulation genutzt, um die Wirkmechanismen von Jasmonaten zu untersuchen. Rekombinante single-chain Fv-Antikörper wurden durch Bindung an natürlich vorkommende (3R, 7R)-Jasmonsäure und an deren biosynthetische Vorstufe (9S, 13S)-12-oxo-10, 15(Z)-Phytodiensäure, selektiert. Gene, die diese rekombinanten Antikörper kodieren, wurden

in pflanzliche Expressionsvektoren zur ubiquitären Produktion der Antikörper in verschiedenen Kompartimenten der Zellen kloniert.

Transgene *Nicotiana tabacum*-Pflanzen, die durch *Agrobacterium*-vermittelten Gentransfer erzeugt wurden, akkumulierten stabile und vererbbare rekombinante anti-Jasmonat Antikörper im Cytosol, im ER, im Stroma und der äußeren Membran der Chloroplasten. Die Antikörper binden spezifisch die gemeinsame Struktur aller Jasmonate, die essentiell für ihre physiologische Aktivität ist.

Mechanische Verletzung ist ein abiotischer Reiz, bei dessen Vermittlung Jasmonate als Siganlmolekül eine Schlüsselrolle spielen sollen. Daher wurden verletzte Blätter von transgenen und Wildtyp-Tabakpflanzen unter besonderer Berücksichtigung ihres intrazellulären Gehaltes an mehrfach ungesättigten Fettsäuren, der endogenen Level saurer Phytohormone und ihrer Genexpressionsprofile untersucht.

Die wichtigsten Ergebnisse diese Untersuchung sind:

1. Die ektopische Expression funktionaler anti-Jasmonat scFv-Antikörper in subzellularen Kompartimenten der Tabakzellen führt zu keiner Veränderung des Erscheinungsbildes und der Pflanzenentwicklung unter natürlichen Bedingungen.
2. Die Akkumulation von anti-Jasmonat scFv Fv Antikörpern im Zytosol und im ER der Tabakzellen führt zu verminderter Jasmonat –vermittelter Verwundungsreaktion und zeigt sich auch bei der verminderten Expression der Gene, die an Verteidigungsmechanismen beteiligt sind. Die wahrscheinlichste Ursache für die beobachtete verminderte Verwundungsantwort ist eine Bindung der endogenen Jasmonsäure durch die in Zytosol und ER der transgenen Tabakpflanzen akkumulierten anti-Jasmonat scFv-Antikörper. Befunde aus der Analyse der transgenen Pflanzen zeigen, daß die anti-Jasmonat scFv-Antikörper einen künstlichen „sink“ für Jasmonäure im ER bilden. Allerdings scheint der vermutliche Wirkort der Jasmonsäure im Zytosol der Zelle zu liegen.
3. Die Immunmodulation der Jasmonatfunktionen ist spezifisch für Zellkompartimente.
4. Durch exogen appliziertes Methyljasmonat konnte reproduzierbar eine Wildtyp-artige Genexpression wiederhergestellt werden, dies allerdings nur im Fall eines ausgewählten Genes, nämlich *Nicotiana tabacum* Proteinase Inhibitor 2. Signifikant herabgesetzte Induktion nach mechanischer Verwundung und die stärkere Beteiligung anderer Reizauslöser sind von entscheidender Bedeutung bei der Wiederherstellung Wildtyp-artiger Transkriptmengen weiterer in dieser Studie erfasster Gene.
5. Der Gehalt membrangebundener oder freier mehrfach ungesättigter Fettsäuren ändert sich nach der Immunmodulation der Jasmonsäure oder ihrer Biosynthesevorstufe nicht. Die Immunmodulation hat keinen Einfluss auf die Verteilung der Substrate des Jasmonsäurebiosyntheseweges.
6. Die Immunmodulation funktioneller Jasmonate sowohl im Zytosol als auch in den Chloroplasten verursacht einen dramatischen Anstieg der endogenen Jasmonatkonzentration nach Verwundung. Diese Konzentrationserhöhung wurde 2 Stunden nach Verwundung von Blättern transgener Tabakpflanzen mit anti-Jasmonat scFv-Antikörpern im Zytosol, im ER und im Stroma von Chloroplasten

gemessen. Die Jasmonatakkumulation war transient und um 2 Größenordnungen höher als die Jasmonatakkumulation nach Verwundung in Wildtypblättern.

7. Die intrazelluläre Akkumulation funktionaler rekombinanter anti-Jasmonat Antikörper hat keinen Einfluss auf die endogenen Gehalte von Salicylsäure, Abscisinsäure und Indol-3-Essigsäure.
8. Ein bisher unbekannter Kontrollmechanismus, der die Zunahme endogener Jasmonate nach mechanischer Verwundung von Pflanzen steuert, könnte durch die ektopische Expression von anti-Jasmonat scFv-Antikörpern gestört werden. Dieser hypothetische Regulationsmechanismus wirkt bereits während der ersten 2 Stunden nach Verwundung, ist unabhängig von relativen Konzentrationsveränderungen von Salicylsäure, Abscisinsäure und Indol-3-Essigsäure. Dieser Kontrollmechanismus wird sowohl durch die Akkumulation von anti-Jasmonat-Antikörpern im Zytosol als auch in den Chloroplasten beeinflusst. Wichtige Schritte des Oktadekanoidstoffwechselweges finden in beiden Kompartimenten statt.

Shrnutí

Rostlinná buňka, stejně jako živočišná buňka, vlastní vysoce komplexní mechanismy, které jí umožňují koordinovat své chování ve prospěch celého organismu.
Jasmonáty jsou skupinou signálních molekul, pomocí kterých rostlinné buňky zprostředkovávají odpověď na nepříznivé okolní podmínky a regulují některá stádia rostlinného vývoje. Kyselina jasmonová a její deriváty jsou cyklické oxylipiny odvozené od nenasycených mastných kyselin. Jasmonáty regulují genovou expresi některých genů zapojených do reakce na stress a genů ovlivňujících některé vývojové procesy.
Ke studiu funkčních mechanismů jasmonátů byla v této studii použita molekulární technika immunomodulace.
Geny kódující rekombinantní single-chain Fv protilátky, vyselektované proti přirodnímu isomeru – (3R,7R)-jasmonové kyselině – a proti jejímu biosyntetickému předchůdci – (9S,13S)-12-oxo-10,15(Z)-phytodienoové kyselině, byly vklonovány do rostlinného expresního vektoru k produkci protilátek ve všech rostlinných orgánech ale v různých buněčných kompártmentech.

Transgenní *Nicotiana tabacum* rostliny, které byly získány genovým přenosem zprostředkovaným bakteriemi druhu *Agrobacterium tumefaciens*, produkovaly stabilně a dědičně rekombinantní protilátky proti jasmonátu v buněčné cytoplasmě, v endoplasmatickém retikulu, ve stromatu a ve vnější membráně chloroplastů. Protilátky vážou specificky obecnou strukturu všech jasmonátů, která je nezbytná pro fyziologickou funkci.

Mechanické zranění je neživý stress, kde jsou jasmonáty pokládány za klíčovou signální molekulu zprostředkovávající odpověď na toto poškození. Z tohoto důvodu byly analyzovány mechanicky zraněné listy transgenních a nekultivovaných (wild-type) tabáků s ohledem na jejich vnitrobuněčný obsah nenasycených mastných kyselin, endogenní hladiny fytohormonů kyselého charakteru a genový expresní profil.

Nejdůležitější výsledky této studie jsou:

1. Ektopická exprese funkčních anti-jasmonátových protilátek v buněčných kompártmentech buněk tabáku nemění za přírozených podmínek celkovou morfologii a vývoj transgenních rostlin.
2. Akumulace protilátek proti jasmonátu v cytosolu a v ER buněk tabáku způsobuje nedostatečnou odpověď na zranění, zprostředkovanou jasmonáty a projevující se sníženou hladinou exprese obranných genů. Nejpravděpodobnějším mechanismem vysvětlujícím pozorované snížení obranné odezvy je vazba endogenní jasmonové kyseliny anti-jasmonatovými protilátkami akumulovanými v cytoplasmě a v ER transgenních rostlin tabáku. Výsledky analýzy těchto transgenních rostlich indikují tvorbu umělého nedostatku kyseliny jasmonové v ER. Avšak místem předpokládané funkce kyseliny jasmonové je pravděpodobně buněčný cytosol.
3. Immunomodulace funkce jasmonátů je závislá na buněčném kompartmentu.
4. Aplikace exogenního methyl esteru kyseliny jasmonové vede k obnovení genové exprese, pozorované jinak u nekultivovaných tabáků, pouze u jednoho vyselektovaného genu – inhibitoru proteáz druhu *Nicotiana tabacum*. Příčinou nekompletního obnovení přirozené transkripční hladiny dalších genů, vyselektovaných v této studii, hrají pravděpodobně výrazně nižší genová indukce po mechanickém zranění i zřetelnější vliv dalších signálních stimulů.
5. Obsah volných a membránami-vázaných mastných kyselin se immunomodulací kyseliny jasmonové nebo jejího prekurzoru nemění. Z tohoto výsledku vyplývá, že immunomodulace nemá vliv na distribuci substrátu biosyntézy kyseliny jasmonové.
6. Immunomodulace funkčních jasmonátů v cytosolu i v chloroplastu způsobuje po mechanickém zranění dramatický nárůst endogenní hladiny kyseliny jasmonové. Tato indukce byl pozorována dvě hodiny po mechanickém zranění listů transgenních rostlin s anti-jasmonátovými protilátkami v cytosolu, v ER a ve stromatu chloroplastů. Enormní, ale dočasná, akumulace kyseliny jasmonové přesáhla více než dvěma řády endogenní hladinu kyseliny jasmonové pozorovanou u nekultivovaných, mechanickým zraněním poškozených, tabáků.
7. Exprese funkčních rekombinantních protilátek proti jasmonátům v buněčných kompártmentech nemění endogenní hladinu tří fytohormonů kyselého charakteru – kyseliny salicylové, kyseliny abscisové ani kyseliny indol-3-acetové.
8. Doposud neznámý, kontrolní mechanismus regulující endogenní hladinu jasmonátů po mechanickém zranění je pravděpodobně narušen expresí specifických rekombinantních protilátek proti jasmonátům. Tento navrhovaný regulační mechanismus: za prvé, operuje již dvě hodiny po zranění, za druhé, je nezávislý na relativních změnách koncentrací zmíněných tří fytohormonů kyselého charakteru a za třetí, je ovlivněn akumulací protilátek proti jasmonátu v cytosolu i v chloroplastu, obou kompartmentech předpokládané biosyntézy kyseliny jasmonové.

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Declaration

I hereby declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aid cited in the manuscript.

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