Mechanostimulation of *Medicago truncatula* leads to enhanced levels of jasmonic acid

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Received 1 April 2008; Accepted 28 April 2008

Abstract

Wounding of plants leads to endogenous rise of jasmonic acid (JA) accompanied with the expression of a distinct set of genes. Among them are those coding for the allene oxide cyclase (AOC) that catalyses a regulatory step in JA biosynthesis, and for 1-deoxy-D-xylulose 5-phosphate synthase 2 (DXS2), an enzyme involved in isoprenoid biosynthesis. To address the question how roots and shoots of *Medicago truncatula* respond to mechanostimulation and wounding, *M. truncatula* plants were analysed in respect to JA levels as well as *MtAOC1* and *MtDXS2-1* transcript accumulation. Harvest-caused mechanostimulation resulted in a strong, but transient increase in JA level in roots and shoots followed by a transient increase in *MtAOC1* transcript accumulation. Additional wounding of either shoots or roots led to further increased JA and *MtAOC1* transcript levels in shoots, but not in roots. *In situ* hybridization revealed a cell-specific transcript accumulation of *MtAOC1* after mechanostimulation in companion cells of the vascular tissue of the stem. AOC protein, however, was found to occur constitutively in vascular bundles. Further, transcript accumulation of *MtDXS2-1* was similar to that of *MtAOC1* in shoots, but its transcript levels were not enhanced in roots. Repeated touching of shoots increased *MtAOC1* transcript levels and led to significantly shorter shoots and increased biomass. In conclusion, *M. truncatula* plants respond very sensitively to mechanostimulation with enhanced JA levels and altered transcript accumulation, which might contribute to the altered phenotype after repeated touching of plants.

Key words: Allene oxide cyclase, cell specific expression, 1-deoxy-D-xylulose 5-phosphate synthase 2, jasmonic acid, mechanostimulation, *Medicago truncatula*, wounding.

Introduction

Wounding by mechanical injuries or herbivore attack is a common event in the life of a plant and elicits a complex series of spatial and temporal responses. Different signalling pathways are activated, which involve various signalling molecules, such as jasmonic acid (JA), nitric oxide, ethylene, ABA, systemin, reactive oxygen species, and possibly electrical or hydraulic signals (Orozco-Cárdenas *et al.*, 2001; Stratmann, 2003; Chen *et al.*, 2004; Howe, 2004; Wasternack, 2006). For systemin and JA, signalling properties are well established, whereby tomato (*Solanum lycopersicon* = *Lycopersicon esculentum*) serves as the best-studied model in that respect (Schilmiller and Howe, 2005). A characteristic feature of the local wound response of plants is a transient increase of JA in the first hour followed by activation of defence genes such as those coding for proteinase inhibitors, enzymes of phytoalexin synthesis, amino acid metabolism or vegetative storage proteins, thionins, and defensins (Ryan, 2000; Howe, 2004). Local wounding, however, is followed by systemic activation of defence genes in leaves. Among others, systemin, JA, and/or related compounds were suggested to act as systemic signals, too. Grafting experiments using tomato mutants affected in JA biosynthesis and in JA signalling led to strong arguments for JA as an essential component of the systemic signal (Li *et al.*, 2000; Li *et al.*, 2002). The preferred generation of JA in vascular bundles (Stenzel *et al.*, 2003a) and the occurrence of the biosynthetic enzymes in companion cells and even in sieve elements of the vascular bundles (Hause...
et al., 2003) support this assumption. Similar data were found with Arabidopsis thaliana (Truman et al., 2007). Much less is known on signalling and responses in shoots and roots following wounding of either shoots or roots.

JA and its derivatives, commonly named jasmonates, are lipid-derived signals. They are synthesized via the octadecanoid pathway, where 12-oxophytodienoic acid (OPDA) is a central intermediate. The initial reaction is the 13-lipoxygenase-catalysed insertion of molecular oxygen into position 13 of α-linolenic acid. The resulting (13S)-hydroperoxy linolenic acid (13-HPOT) is the substrate for at least seven different pathways (Feusnser and Wasternack, 2002). Only the conversion of 13-HPOT by an allene oxide synthase (AOS) specifically acting with 13-HPOT leads to the formation of an unstable allene oxide that is further processed by an allene oxide cyclase (AOC), leading exclusively to the cis-(+) enantiomer (95,13S) of OPDA. It carries that enantiomeric structure which is present in the naturally occurring jasmonates. In the subsequent steps occurring in peroxisomes, OPDA is reduced by an OPDA reductase, followed by shortening the carboxylic acid side chain by three cycles of β-oxidation (Castillo et al., 2004; Athilhile et al., 2005; Delker et al., 2007).

Among the biosynthetic enzymes, the AOC is regarded to be crucial for JA biosynthesis due to the establishment of the naturally occurring enantiomeric structure of JA (Wasternack and Hause, 2002). AOC has been cloned and is encoded by a single-copy gene in tomato (Ziegler et al., 2000) and Hordeum vulgare (Maucher et al., 2004), and by small gene families in Arabidopsis thaliana (Stenzel et al., 2003b) and Medicago truncatula (Isayenkov et al., 2005) and many other plant species (see www.ncbi.nlm.nih.gov). All AOC-cDNAs carry a transit peptide for plastid targeting and localization in chloroplasts was confirmed immunohistochemically (Ziegler et al., 2000; Stenzel et al., 2003b; Isayenkov et al., 2005).

Mechanical wounding is qualitatively similar but not identical to wounding by herbivores in terms of local and systemic responses (Korth and Dixon, 1997; Kessler and Baldwin, 2002; Mithöfer et al., 2005). Herbivore attack, in contrast to mechanical wounding, is accompanied by oral secretions. These contain compounds such as volicitin which are able to induce plant defence genes (Halitschke et al., 2003; Engelberth et al., 2004). Both stresses, however, result in the production of secondary compounds such as isoprenoids (Ament et al., 2004; Arimura et al., 2005; Leitner et al., 2005). In many organisms, including bacteria and plants, the rate-limiting step for isoprenoid biosynthesis is catalysed by 1-deoxy-d-xylulose 5-phosphate synthase (DXS) (Eisenreich et al., 1998; Carretero-Paulet et al., 2002; Estevez et al., 2001). In the first step of the plastid-located methyl-d-erythritol 4-phosphate (MEP) pathway, DXS converts D-glyceraldehyde 3-phosphate and pyruvate to 1-deoxy-d-xylulose 5-phosphate. The corresponding gene has been shown to be inducible by jasmonates (Van der Fits and Memelink, 2000; Sanchez-Hernandez et al., 2006; Arimura et al., 2007).

Usually, the early and transient elevation of JA in response to wounding is followed by the activation of genes coding for JA biosynthetic enzymes (Wasternack and Hause, 2002). In tomato, Arabidopsis, and M. truncatula, genes encoding AOC are also JA-inducible (Strassner et al., 2002; Stenzel et al., 2003a; Isayenkov et al., 2005) suggesting a feed forward regulatory loop. Most of the stress responses including wounding have been shown to be organ-specific in terms of transcript accumulation (Swindell, 2006). As far as is known, however, there are few data on the response of roots to their wounding. Compared with that following nematode infection, an accumulation of transcripts in roots after mechanical injury was monitored (Veronico et al., 2006). By contrast, a JA-induced de novo nicotine synthesis in roots of Nicotiana species was detected after leaf wounding and points to a systemic response of roots to wounding of shoots (Zhang and Baldwin, 1997; Baldwin, 1998; Shi et al., 2006).

In this paper, the question how roots and shoots of M. truncatula respond to mechanostimulation and wounding is addressed. The levels of JA and transcripts of MtAOC1 as well as of DXS2-I were monitored. The data suggest a high sensitivity of M. truncatula to mechanostimulation performed by harvesting the plants that leads to the accumulation of JA and the transcripts under analysis. Moreover, effects of repeated touching of plants on their phenotype were analysed.

Materials and methods

Plant material and treatments

Plants of Medicago truncatula Gaertn. var. Jemalong (obtained from Austra Hort Pty, Australia), were grown in a phytochamber (Percival, CLF, Emersacker, Germany) at 23 °C, with a 16 h light period and in pots filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay Deutschland, Pinneberg). Every pot was fertilized once per week with 10 ml Long Ashton fertilizer (Hewitt, 1966). Plants were harvested after 5 weeks by careful removal of the expanded clay. Wounding of roots and leaves was performed by squeezing the complete root system and all leaflets with tweezers. Subsequently, roots of all plants were covered with wet filter paper to avoid drought stress. After the indicated time periods, roots and shoots were frozen separately in liquid nitrogen. Material from three to five different plants was pooled to minimize biological differences. All experiments were done at least in triplicate. To monitor the effect of touching, plants were grown as described above. One-half of the plants remained untouched for the whole growth period of 5 weeks. Complete shoots of the plants of the second half were touched by hand three times per week for 10 s each.

Quantitative analysis of JA

About 1 g fresh weight of plant material frozen in liquid nitrogen was homogenized in a mortar and extracted with 5 ml 80% (v/v) methanol. To quantify JA, [3H6]JA was added in an appropriate amount before extraction. Ion exchange chromatography on DEAE Sephadex A-25 cartridges, reversed phase HPLC, and gas
chromatography–mass spectrometry/selected ion monitoring analyses were performed as described (Hause et al., 2002).

Quantitative reverse-transcription (RT)-PCR

Total RNA was isolated from 100 mg of complete root systems as well as of whole shoots using the Plant RNAeasy Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA synthesis was performed with the first-strand cDNA synthesis kit for RT-PCR (Promega, Madison, WI, USA) using 1 µg of total RNA in a volume of 10 µl with 0.5 µg oligo(dt)15 primers (MWG BIOTEC, Ebersbach, Germany). After cooling on ice, 9 µl of a mixture containing 4 µl M-MLV RT 5× reaction buffer, 4 µl dNTP mix, and 1 µl M-MLV reverse transcriptase RNase minus, point mutant was added and incubated at 40 °C for 10 min and then at 42 °C for 50 min.

All PCR reactions were performed in three technical replicates. TaqMan™ probes and primers, both for MEF1a (elongation factor 1-α as constitutive expression control) and MaOAC1, were purchased from Applied Biosystems (Assays-by-Design service, Foster City, CA, USA) as described (Isayenkov et al., 2004, 2005). Real-time PCR was carried out using the ABI Prism 7000 sequence detection system with optical tubes and caps from Applied Biosystems (Assays-by Design service, Foster City, CA, USA) using 1 µl of total RNA in a volume of 10 µl with 0.5 µg oligo(dt)15 primers (MWG BIOTEC, Ebersbach, Germany). After cooling on ice, 9 µl of a mixture containing 4 µl M-MLV RT 5× reaction buffer, 4 µl dNTP mix, and 1 µl M-MLV reverse transcriptase RNase minus, point mutant was added and incubated at 40 °C for 10 min and then at 42 °C for 50 min.

For quantitative determination of transcripts of DXS2-1 (Walter et al., 2002), 3 µl of 1:1 diluted cDNA (15 ng of reverse transcribed total RNA) or 3 µl of diluted control reaction were mixed with SYBR Green PCR Mastermix (Applied Biosystems, Warrington, UK), 1 pmol of forward primer, and 1 pmol of reverse primer in a final volume of 10 µl in three independent replicates. The following primers and annealing temperature were used: forward primer, 5′-CAC CTT GTA TCT ATA AAT CAT TAA GTC TCT TCT-3′; reverse primer, 5′-CGG AAT CAC TCT TCT CAA CCA AGA-3′; 60 °C. To normalize DXS2-1 expression for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed MEF1a were measured using the following primers and temperature: forward primer, 5′-AGA AGG AAG CTG CTG AGA TGA AC-3′; reverse primer, 5′-TGA CTG TGC AGT AGT ACT TGG TG-3′; 60 °C. The efficiency of each primer pair was in the range of 0.95–1.0. Real-time PCR was performed using the Mx3005P QPCR system (Stratagene, La Jolla, CA, USA) with the following protocol: denaturation (95 °C for 10 min), amplification (40 cycles of 95 °C for 30 s, primer-specific annealing temperature for 1 min, and 72 °C for 30 s), and melting curve (95 °C for 1 min, 60 °C for 30 s, heating up to 95 °C with a heating rate of 0.1 °C s⁻¹). Data were evaluated with the MxPro software (Stratagene). To correct for well-to-well fluorescent fluctuations, normalization of the SYBR Green–dsDNA complex signal to the constitutively expressed gene and to a control sample.

Immunocytochemistry and in situ hybridization

Immunocytochemical analysis of stems was performed as described (Isayenkov et al., 2005). Small pieces of stems were fixed with 4% (v/v) paraformaldehyde/0.1% (v/v) Triton X-100 in phosphate-buffered saline (135 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄) for 2 h at room temperature. After dehydration in a graded series of ethanol, the specimens were infiltrated with polyethylene glycol 1500 (Merck KgaA, Darmstadt, Germany) at 50 °C. Cross-sections 10 µm thick were used for immunolabelling. The rabbit polyclonal antibody raised against recombinant LeAOC (Ziegler et al., 2000) was used at a dilution of 1:1000. The use of pre-immune serum at the same dilutions served as a control and revealed no signals. As secondary antibody, goat anti-rabbit IgG conjugated with AlexaFluor488 (Molecular Probes, Leiden, The Netherlands) was used according to the manufacturer’s instructions. Counterstaining was performed with DAPI (4,6-diamidino-2-phenylindol; Sigma-Aldrich, Steinheim, Germany). Sections were analysed by epi-fluorescence microscopy using a Zeiss ‘Axiolmager’ (Zeiss, Jena, Germany) equipped with a CCD camera or by confocal laser scanning microscopy using a LSM510 META (Zeiss).

For in situ hybridization, cross-sections (16 µm thick) of the same embedding were collected in sieves, rinsed in 0.1 M TRIS–HCl, pH 8.0, and incubated with 10 µg ml⁻¹ proteinase K (Sigma-Aldrich) in 0.05 M TRIS–HCl, pH 7.5, and 5 mM EDTA for 30 min at 37 °C. After incubation with 1% (w/v) bovine serum albumin and 2 mg ml⁻¹ glycine in 0.1 M TRIS–HCl, pH 8.0, for 30 min, sections were equilibrated in 0.1 M triethanolamine, pH 8.0, and then acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. After acetylation, sections were dehydrated in a graded series of ethanol and air-dried. For hybridization, a solution consisting of 50% (v/v) formamide, 4× SSC, 350 µg ml⁻¹ RNA, 0.5% (w/v) blocking reagent (Roche Diagnostics, Mannheim, Germany) and 40 U ml⁻¹ RNase inhibitor (Merck, Darmstadt, Germany), and containing denaturated digoxigenin (DIG)-labelled sense or antisense RNA (DIG RNA labelling kit; Roche Diagnostics) were applied and sections were incubated at 45 °C overnight. After subsequent washing steps with 50% formamid/4× SSC, 4× SSC, and 0.2× SSC at 45 °C for 10 min each, sections were incubated with 20 µg ml⁻¹ RNase A at 37 °C for 30 min, followed by washing with 0.2× SSC at 45 °C for 5 min. After a short equilibration in TBS (0.1 M TRIS–HCl, pH 7.5, 0.15 M NaCl) and blocking of sections with 1% blocking reagent in TBS for 30 min at room temperature, immunological detection of DIG-labelled RNA hybrids was performed using a 1:2000 diluted anti-DIG-fab fragment conjugated with alkaline phosphatase (Roche Diagnostics) according to the supplier’s protocol. The colorimetric reaction was performed for 3 h at 37 °C with detection buffer (0.1 M TRIS–HCl, pH 9.5, 0.1 M NaCl, and 50 mM MgCl₂) containing 0.4 M nitroblue tetrazolium, 0.5 M 5-bromo-4-chloro-3-indolyl-phosphate, and 10 mM levamisol (Sigma-Aldrich). The reaction was stopped by washing the sections in TE (10 mM TRIS–HCl, pH 8.0, and 1 mM EDTA). After transfer of the sections to slides, micrographs were taken using a Zeiss ‘Afioelmager’ microscope. All micrographs were processed through the Photoshop 8.0.1 program (Adobe).

Determination of chlorophyll content and phenotypic analysis

For chlorophyll determination, leaf material was homogenized in liquid nitrogen. Fifty milligrams of each sample were extracted twice with 1.5 ml absolute methanol. The supernatant (in total 3 ml) was collected and diluted 1:1 with methanol. The total chlorophyll content in 1 ml diluted extract was measured spectrophotometrically (DU 640 Beckman Spectrophotometer; Beckmann Instruments, Munich, Germany) against methanol at 664.5 nm wavelength, showing adsorption maxima of the extract. Determination was carried out in three independent replicates. As phenotypic markers of growth, the weight of shoots and roots and length of the longest shoot were determined for each plant.

Results

JA content and transcript accumulation of MaOAC in shoots and roots of M. truncatula

For plants grown in solid medium such as soil or expanded clay, harvest of roots may represent a mechanostimulation.
Therefore, the effect of harvest was monitored in comparison with additional wounding of roots and shoots on the corresponding JA content. Five-week-old plants were harvested carefully to exclude wounding of roots during removal of the growth substrate. Surprisingly, both untreated shoots and roots exhibited a significant and transient increase in JA content (Fig. 1). Whereas roots showed a 4-fold increase with a maximum at 30 min, JA levels of shoots were raised nearly 8-fold between 30 min and 1 h after harvest. Additional wounding of roots performed by squeezing the whole roots system with tweezers, did not result in an additional increase of JA levels, either in roots wounded on intact plants or in roots wounded after separation from the shoot (Fig. 1, upper part). This is slightly different in shoots. Here, harvest of plants and wounding of roots cause a similar increase in JA content, whereas wounding of all leaves of the separated shoots tended to result in slightly higher JA levels (Fig. 1, lower part). For all treatments, however, the JA levels dropped down to basal levels of untreated tissues after 4 h.

To inspect whether the increase in JA levels by harvest and wounding was accompanied by altered expression of JA biosynthesis genes, quantitative determination of transcript accumulation of \( \text{MtAOC1} \) was recorded by qRT-PCR. \( \text{MtAOC1} \) mRNA levels were altered with similar kinetics for roots and shoots by all three types of treatments (Fig. 2). In unwounded roots, harvest already caused a significant accumulation of \( \text{MtAOC1} \) transcripts with a transient maximum between 1 h and 2 h after harvest. There was no significant increase in transcript accumulation after additional wounding, either in roots of whole plants or in roots separated from shoots at the time of harvest. In shoots, harvest and wounding caused a similar increase in JA content, whereas wounding of all leaves of the separated shoots tended to result in slightly higher JA levels (Fig. 1, lower part).

**Fig. 1.** Harvest- and wound-induced accumulation of JA in roots and shoots of 5-week-old \( M. \) truncatula plants. Plants were carefully removed from expanded clay and stayed untreated (white columns) or were wounded on roots (cross-hatched columns). From a third batch of plants shoots and roots were separated from each other and both were wounded (dark grey columns). At each time point, up to five plants were pooled, and total RNA was extracted from roots and shoots separately. Real-time RT-PCR was carried out in triplicate for each sample. The mean JA levels of plants directly after harvest (time point 0) was set to 1. Data are presented as mean values ±SD of at least three biological replicates. Different letters designate statistically different values separately for each treatment (ANOVA with Tukey HSD test, \( P < 0.05 \)).

**Fig. 2.** Harvest- and wound-induced transcript accumulation of \( \text{MtAOC1} \) in roots and shoots of 5-week-old \( M. \) truncatula plants. Relative \( \text{MtAOC1} \) transcript levels were determined by real-time RT-PCR analysis using TaqMan probes. Plants were carefully removed from expanded clay and stayed untreated (white columns) or were wounded on roots (cross-hatched columns). From a third batch of plants, shoots and roots were separated from each other and both were wounded (dark grey columns). At each time point, up to five plants were pooled, and total RNA was extracted from roots and shoots separately. Real-time RT-PCR was carried out in triplicate for each sample. The mean \( \text{MtAOC1} \) transcript level of plants directly after harvest (time point 0) was set to 1. Data are presented as mean values ±SD of at least three biological replicates. Different letters designate statistically different values separately for each treatment (ANOVA with Tukey HSD test, \( P < 0.05 \)).
of wounding. This was slightly different in shoots, where transcripts accumulated to a much higher level and additional wounding of all leaves caused a minor enhancement of transcript levels in comparison with non-wounded plants. Similar to JA levels, MtAOC1 transcripts dropped down to basic levels of untreated plants 4 h after treatment.

Cell-specific occurrence of MtAOC1 transcripts and protein

To examine where MtAOC1 transcripts and protein accumulate after harvest, MtAOC1 mRNA and protein were localized by in situ hybridizations and immunocytochemistry, respectively, both of them in stem tissues at the maximum of transcript accumulation (Fig. 3). Two hours after harvest, MtAOC1 transcripts could be visualized in the phloem (Fig. 3A, B). The close-up revealed that transcripts occurred preferentially in companion cells, which are characterized by their typical pair-wise position next to sieve elements. Hybridization with a sense probe (Fig. 3C) as well as hybridization of plant material embedded directly after harvest (data not shown) did not exhibit specific labelling.

To analyse cell-specific occurrence of MtAOC protein in stems, cross-sections and immunohistological staining by an antibody specifically recognizing MtAOC were used (Isayenkov et al., 2005) (Fig. 4). MtAOC was clearly detectable in parenchymatic cells of the vascular bundles, the pith, and of the cortex. Analyses of immunolabelled sections by confocal laser scanning microscopy revealed the localization of MtAOC in plastids of the corresponding cells (Fig. 4C, D). There were no differences in cell-specific occurrence and labelling intensity detectable after the different treatments as harvest and additional wounding (data not shown). No signal was detectable in any of the cells when the tissues were probed with a pre-immune serum (Isayenkov et al., 2005, and data not shown).

Transcript accumulation of MtDXS2-1 in roots and shoots of M. truncatula

To analyse whether the harvest- and wound-induced increase in JA-levels lead to expression of a JA-responsive gene, quantitative RT-PCR was performed to determine transcript levels of MtDXS2-1. In contrast to MtAOC1, transcript levels of MtDXS2-1 remained nearly unaffected in roots, independent of the treatment. A slight increase was detectable at 4 h after treatment (Fig. 5, upper part). By contrast, a strong, but transient increase in transcript levels occurred in shoots 1 h after harvest of plants (Fig. 5, lower part). There was no additional increase in MtDXS2-1 mRNA levels upon wounding of roots or shoots. Transcript levels in shoots dropped down to basal levels of untreated plants 4 h after onset of treatment.

Repeated mechanostimulation causes alteration of the phenotype of M. truncatula

The harvest-induced rise in JA levels as well as in MtAOC1 and MtDXS2-1 transcript levels of roots and preferentially of shoots suggests that M. truncatula is highly sensitive in respect to JA formation and JA responses upon mechanostimulation. Therefore, the effects of repeated touching of shoots were analysed. Plants were grown for 5 weeks either without any touching (Fig. 6A) or shoots were slightly touched three times per week for 10 s (Fig. 6B). Touched plants exhibited a stunted

Fig. 3. Localization of MtAOC1 transcripts in stems of M. truncatula 2 h after wounding of roots. In situ hybridization was performed on cross-sections 16 μm thick. (A) Hybridization with DIG-labelled antisense RNA for MtAOC1 shows staining in the phloem of the vascular bundles. (B) The close-up of one vascular bundle visualizes the occurrence of the signals in companion cells (arrows). (C) Control performed using DIG-labelled sense RNA shows no staining in the complete section. Scale bars represent 100 μm in all micrographs.
phenotype and elevated chlorophyll content. This is reflected in various growth parameters listed in Table 1. The shoot length of touched plants decreased significantly, whereas shoot weight and chlorophyll content increased significantly. Although the root weight was not changed, the increased shoot weight led to an increase in the total biomass of touched plants in comparison to non-touched plants.

To check whether touching results in similar responses as harvest, JA levels and \textit{MtAOC1} transcript levels were monitored 60 min after touching the shoots for 10 s. The control set of plants remained untouched. The JA content of shoots and roots did not differ significantly between touched and untouched plants due to high variations in biological replicates (data not shown). However, relative \textit{MtAOC1} transcript levels of shoots of touched plants exhibited a 4.4-fold increase compared with the untouched plants (0.104±0.031 versus 0.024±0.002; \(P < 0.01\)). Even in the roots of touched plants there was a 2.4-fold increase of \textit{MtAOC1} transcript level detectable in comparison with untouched plants (0.048±0.011 versus 0.020±0.002; \(P < 0.01\)).

**Discussion**

The activation of herbivore- and wound-induced defence responses involves a complex network of plant signalling cascades (Rojo \textit{et al.}, 2003; Stratmann, 2003; Schaller \textit{et al.}, 2004). Thereby, jasmonates represent the best characterized class of signals mediating the elicitation of these responses (Schilmiller and Howe, 2005; Delker \textit{et al.}, 2007; Farmer, 2007). Upon wounding, JA levels increase locally, but jasmonates are also involved in the regulation of systemic response (Howe, 2004) as shown by grafting experiments with a JA-insensitive tomato and a mutant, which is defective in JA biosynthesis (Li \textit{et al.}, 2002; Ryan and Moura, 2002). Much less is known, however, on how roots respond to wounding in terms of jasmonate biosynthesis. Regarding root wounding, only transcript accumulations in wounded roots after nematode
infection were reported (Veronico et al., 2006). To get insights into the root response to wounding and especially the possible role of jasmonates in that process, the accumulation of JA in shoots and roots of M. truncatula upon mechanostimulation (harvest) and additional wounding was analysed.

In the work presented here, wounding was performed on roots of complete plants or on shoots and roots separated from each other. Unexpectedly, JA accumulated already after mechanostimulation caused by harvest in both parts of the plant, independently on whether they had been separated from each other or not. Also in aeroponically grown M. truncatula plants, mechanical disturbances (performed by shaking the plants) lead to increased JA levels (K Pawlowski, personal communication). Subsequent wounding did not lead to an additional increase in JA levels. This is surprising, because up to now elevated jasmonate levels were only detected after wounding, herbivory, pathogen attack, or osmotic stress (Wasternack, 2006).

Usually, the wound-induced elevation of jasmonate levels occurs transiently followed by activation of genes coding for JA biosynthetic enzymes (Wasternack and Hause, 2002). In tomato, Arabidopsis and M. truncatula, most genes encoding enzymes of JA biosynthesis are JA-inducible (Strassner et al., 2002; Stenzel et al., 2003a; Isayenkov et al., 2005). As shown here, upon mechanostimulation by harvest of M. truncatula, the rise in JA precedes also the accumulation of MtAOC1 transcripts. Similar to the increase in JA levels, additional wounding did not lead to significantly enhanced levels of MtAOC1 transcripts either in shoots or in roots. This prompted the analysis of tissue-specific occurrence of MtAOC1 transcripts and AOC protein in stems upon mechanostimulation. MtAOC1 transcripts have been detected in vascular tissues only. The detailed analysis revealed the preferential occurrence of MtAOC1 transcripts in companion cells where AOC protein is also located. This is similar to tomato, where AOC is located exclusively in vascular tissues, in sieve elements, and in companion cells (Hause et al., 2003). In tomato, the biosynthesis of jasmonates upon wounding is restricted to the veins of a leaf and has been shown to be reminiscent of the location of

Fig. 5. Harvest- and wound-induced transcript accumulation of MdDXS2-1 in roots and shoots of 5-week-old M. truncatula plants. Relative MtDXS2-1 transcript levels were determined using real-time RT-PCR analysis. Plants were carefully removed from expanded clay and stayed untreated (white columns) or were wounded on roots (cross-hatched columns). From a third batch of plants shoots and roots were separated from each other and both were wounded (dark grey columns). At each time point, up to five plants were pooled, and total RNA was extracted from roots and shoots separately. Real-time RT-PCR was carried out in triplicate for each sample. The mean MdDXS2-1 transcript level of plants directly after harvest (time point 0) was set to 1. Data are presented as mean values ±SD from at least four biological replicates. Different letters designate statistically different values for each treatment (ANOVA with Tukey HSD test, P < 0.01).

Fig. 6. Effect of repeated touching of shoots on plant growth of M. truncatula. Plants were grown without touching (A) or with repeated touch stimulation performed by touching shoots for 10 s three times per week (B).
Table 1. Effect of repeated touching of shoots on plant growth of *M. truncatula*

Plants were grown without movement of shoots (control) or shoots were slightly touched for 10 s three times per week. After 5 weeks, the fresh weight of shoots and roots, shoot length, as well as chlorophyll content of leaves were determined. Data are given as mean ±SD (n=20). *P*-values were calculated using Student's *t*-test.

| Shoot length (cm) | Control | Touch treatment | P-value |
|-------------------|---------|-----------------|---------|
| Shoot weight (g)  | 1.85 ±0.54 | 2.35 ±0.45 | 0.0025 |
| Root weight (g)   | 2.04 ±0.49  | 2.23 ±0.32 | 0.1533 |
| Total biomass (g) | 3.88 ±0.86  | 4.58 ±0.67 | 0.0059 |
| Chlorophyll (E<sub>664.5nm</sub> g<sup>-1</sup>) | 301±58  | 418±53 | 0.00018 |

*JA-inducible proteins specifically in the vascular bundle (Stenzel *et al.*, 2003a). The immunological detection of AOC protein in stems shown here for *M. truncatula* revealed, however, the occurrence of AOC in parenchymatic cells of the vascular bundles, of the pith, and of the cortex. This was independent of the treatment, but differed from leaves and roots, which showed AOC protein exclusively restricted to vascular bundles (Isayenkov *et al.*, 2005). The protein constitutively present seems to be active in biosynthesis of JA after mechanostimulation. This could be realized by activation of the enzyme or by increased substrate availability as proposed for tomato (Hause *et al.*, 2000; Stenzel *et al.*, 2003a). Obviously, vascular tissues of *M. truncatula* stems carry the capacity to form jasmonates and—regarding MtAOC1 as a gene induced upon endogenous rise of JA—produce jasmonates after mechanostimulation.

Rises in levels of JA as well as MtAOC1 transcripts in response to mechanostimulation occurred to a much higher extent in shoots than in roots. The lower response level of roots in term of JA-induced transcript accumulation is also reflected in the transcript accumulation of *MtDXS2-1*. In this case, enhanced transcript levels could be monitored in shoots only. The present data point to a regulation of *MtDXS2-1* transcript accumulation by wound-induced jasmonates in shoots, because the rise in JA precedes the transient maximum of *MtDXS2-1* transcript levels. The lower level of JA in roots, however, might be insufficient to induce the *MtDXS2-1* expression. This is supported by the fact that the promoter of *MtDXS2-1* is not activated in roots by wounding but by application of at least 100 μM JA for 24 h (D Floss and B Hause, unpublished results). The recorded member of the DXS gene family, DXS2, codes for the key enzyme involved in the MEP pathway leading to isoprenoids (Estevez *et al.*, 2001), and has been shown to be up-regulated in response to wounding of leaves and JA treatment (Bede *et al.*, 2006; Sanchez-Hernandez *et al.*, 2006). The resulting release of wound-induced terpene volatiles is therefore controlled through a JA-regulated MEP pathway and contributes to the well-described indirect defence mechanisms of plants against herbivores (Leitner *et al.*, 2005; Arimura *et al.*, 2007; Gao *et al.*, 2007).

The data shown here suggest that even the harvest represents a mechanostimulation of plants leading to increased JA levels in roots and shoots. This raises the question how *M. truncatula* plants react to repeated mechanostimulation of shoots such as repeated touching over several weeks. This ‘touch response’ is well described for *Arabidopsis* in terms of phenotypic alterations such as delay in flowering and an inhibition of inflorescence elongation (Braam, 2005). In *Arabidopsis*, over 2.5% of total genes were touch-inducible including genes related to calcium and kinase signalling, cell wall modifications, and disease resistance responses (Lee *et al.*, 2005). Among these touch-induced genes are also those coding for AOS and AOC3. It is tempting to speculate that touch-responsive genes might be regulated at least partially by increased jasmonate levels. Due to the fact that *M. truncatula* is regarded to be a model system for plant functional genomics and is frequently used for studying biological processes that are unique and pertinent to legumes (Suzuki *et al.*, 2005), effects of mechanostimulation occurring upon harvest of plants should be considered in experiments regarding global transcript accumulations.

Touch-treated *M. truncatula* plants showed an altered phenotype in comparison with non-touched plants. They exhibited a stunted phenotype reflected by decreased shoot length and increased biomass, and an elevated chlorophyll content. These features represent morphological traits that are characteristic of plants treated with JA or plants exhibiting a constitutive high level of JA (Wasternack and Hause, 2002). The *Arabidopsis* mutant cev1, which has constitutively elevated JA levels, produces plants that have stunted roots and shoots and accumulate anthocyanins (Ellis *et al.*, 2002). Furthermore, growth inhibition preferentially of roots is mediated by JA and has been used for numerous screens to identify mutants affected in JA signalling (Berger, 2002; Yan *et al.*, 2007). For touch-treated *M. truncatula* plants, an increase in JA levels has not been detected, but MtAOC1 transcript levels were significantly increased both in shoots and in roots. Due to the accumulation of MtAOC1 transcripts upon endogenous rise of JA, this increased transcript level may visualize a subtle JA increase, which, after repeated touching, might lead to the phenotype described.

In conclusion, the data presented here revealed that *M. truncatula* plants respond very sensitively to mechanostimulation. Mechanostimulation by harvest leads to a rapid increase in JA levels followed by increased transcript accumulation of JA-responsive genes. Even repeated mechanostimulation performed by touching...
obviously changed the phenotype of plants. It will be interesting to analyse whether repeated touching of plants will influence other features of the plants such as biotic interactions with pathogens or symbionts. It could be reasonable that enhanced JA levels over time due to repeated mechanostimulation influence such interactions.

Acknowledgements

The authors thank Otto Miersch for the GC-MS measurements and the synthesis of [2H6]JA, Martin Wolfram for determination of chlorophyll contents, and Daniela Floss for providing primers for MtDXS2-1. Claus Wasternack and Dieter Strack are acknowledged for critically reading the manuscript.

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