Oxylipin dynamics in *Medicago truncatula* in response to salt and wounding stresses

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Multiple stresses are becoming common challenges in modern agriculture due to environmental changes. A large set of phytochemicals collectively known as oxylipins play a key role in responses to several stresses. Understanding the fine-tuned plant responses to multiple and simultaneous stresses could open new perspectives for developing more tolerant varieties. We carried out the molecular and biochemical profiling of genes, proteins and active compounds involved in oxylipin metabolism in response to single/combined salt and wounding stresses on *Medicago truncatula*. Two new members belonging to the CYP74 gene family were identified. Gene expression profiling of each of the six CYP74 members indicated a tissue- and time-specific expression pattern for each member in response to single/combined salt and wounding stresses. Notably, hormonal profiling pointed to an attenuated systemic response upon combined salt and leaf wounding stresses. Combined, these results confirm the important role of jasmonates in legume adaptation to abiotic stresses and point to the existence of a complex molecular cross-talk among signals generated by multiple stresses.

Abbreviations − ABA, abscisic acid; AOS, allene oxide synthase; DES, divinyl ether synthase ; ET, ethylene ; HPL, hydroperoxide lyase ; JA, jasmonates ; JA-Ile, jasmonoyl-isoleucine ; LW, leaves wounding ; LW/S, combined leaves wounding and salt stress; OPDA, oxo-phytodienoic acid; PUFA, polyunsaturated fatty acids; RW, roots wounding; RW/S, combined root wounding and salt stress; S, salt stress; SA, salicylic acid; UPLC, ultra-high performance liquid chromatography.

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Introduction

Plants are commonly exposed to multiple stresses, which can specifically activate different signalling pathways. Several studies have shown that the idea to extrapolate the response to single stress from combined stresses is too simplistic due to the large variety of synergistic or antagonistic signals generated by each single stress in different tissues/organs (aerial or above-ground organs) and stress timing (concomitant or delayed stresses) (De Vleesschauwer et al. 2013, Matschi et al. 2015, Nguyen et al. 2016, Dworak et al. 2016, Verma et al. 2016).

Distinct metabolic pathways that regulate plant responses to diverse environmental signals have been described extensively (Yamaguchi 2008, Zaho 2010, De Domenico et al. 2012, Kmiecik et al. 2016, Taurino et al. 2014, Thatcher et al. 2016, Zhang et al. 2016). However, recent studies point out the existence of a complex coordination in plant responses to pathogens and abiotic stresses, including the expression of overlapping sets of genes (AbuQamar et al. 2006, Fujita et al. 2006, Deb et al. 2016) likely involved in plant reactions to different adverse conditions. Furthermore, hormones such as ethylene (ET), salicylate (SA), jasmonates (JA) and abscisic acid (ABA), acting either synergistically or antagonistically, depending on different stressors, can orchestrate a complex set of early and late responses either locally or distally (Angulo et al. 2015, Balmer et al. 2013, Caarls et al. 2017).

It is widely accepted that one of the most important phyto-hormone mediating the plant cell response to (a)biotic stresses is represented by jasmonoyl-isoleucine (JA-Ile), the primarily active form of jasmonic acid, able to translocate through membranes and to move through xylem from roots to leaves and backward in the model species *Arabidopsis thaliana* (Koo et al. 2009, Chini et al. 2016, Poudel et al. 2016, Li et al., 2017). Recently, an independent role as signalling molecule has also been proposed for OPDA-Ile-like related compounds (Arnold et al. 2016, Wasternack and Hause 2016).

Jasmonates are derived from the oxidation of polyunsaturated fatty acids (PUFA), together with a large set of other phytochemicals collectively known as oxylipins, which comprise aldehydes, ketols, epoxides and divinyl ethers. All these compounds participate either in direct or indirect defence mechanisms (Hughes et al. 2009). After PUFA hydroperoxide biosynthesis, mediated by 9- or 13-lipoxygenases (Santino et al. 2005, Santino et al. 2013), other enzymes, including allene oxide synthase (AOS), hydroperoxide lyase (HPL) and divinyl ether synthase (DES), convert these reactive compounds into more stable oxylipins; AOS, HPL and DES form a closely related group of cytochromes P450, named CYP74, specialised in the metabolism of hydroperoxides (Hughes et al. 2009, Wasternack 2007, Wasternack and Hause 2013).

Several studies have reported a key role of jasmonates in stress signalling from above- to below-ground tissues and vice versa in response to wounding and herbivore attack, drought or salt stresses (Nahar et al. 2011, De Domenico et al. 2012, Santino et al. 2013, Wasternack and Hause 2013, Savchenko et al. 2014, Taurino et al. 2014, Goossens et al. 2016). In this context, our long-term goal is to understand the fine tuning of the hormonal signalling that orchestrates the response of legumes to

combined (a)biotic stresses. Here, we report about the local and systemic oxylipin signalling from *M. truncatula* root to shoot and backward in the early response to single or combined salt/wounding stresses. In particular, we investigated the contribution of each member of the CYP74 gene family in the response to single and combined stresses in *M. truncatula* at both above- and below-ground levels. Furthermore, hormonal profiling confirmed the participation of jasmonates, ABA and SA in early response signalling to these stressors.

Material and methods

Plant growth, maintenance and stress treatment

Seeds of *M. truncatula* (Jemalong A17) were sterilised and germinated as described by de Lorenzo and co-workers (2007). Germinated seedlings were grown in a greenhouse in pots containing a mixture of sand and perlite (3/1, w/w; 5 plants/25 × 20 cm). The seedlings were maintained at 25 ± 2°C, 50 ± 5% relative humidity under a 16-h photoperiod for 4 weeks. The pots were irrigated daily with 100 ml of water.

Salt (S), wounding (W) and combined wounding/salt (W/S) stresses were imposed on different groups of 4-week-old plants. A first group was watered with 200 mM NaCl solution to induce salt stress. Leaf wounding stress (LW) was imposed on a second group of plants by means of cuttings all leaves by a tweezer. To impose root wounding stress (RW), plants of a third group were gently removed from the soil trying to avoid any root damage; the roots were then wounded with dented forceps and the plants were replaced in new soil. Finally, a combination of the two former stresses (RW/S) was imposed on a fourth plant group by first inducing the mechanical root wounding stress and, after 15 min, inducing the salt stress. The same timing of stress induction was used to obtain the combination of leaf wounding and salinity (LW/S) on a last group of plants. Plants were harvested at 0 (unstressed), 1, 6, 24 and 48 hours after the onset of the stress. At least three replicates for each treatment were used. Plants were removed from the pots and roots and leaves were collected separately and frozen in liquid nitrogen; the roots were thoroughly rinsed with water, shortly dry-blotted on filter paper and immediately frozen. Samples were then stored at -80°C.

Nucleic acid extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen tissues pulverised in liquid nitrogen, using the SV Total RNA isolation kit (Promega, Madison, USA) according to the manufacturer's protocol; RNA aliquots were preserved at −80°C. First-strand cDNA was synthesised using 1 μg of total RNA, oligo(dT)18 and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol. The transcription profiles of *Mthpl1* (GenBank acc.no. AJ316562), *Mthpl2* (AJ316563), *Mthpl3* (DQ011231), *Mthpl4* (CM001217), *Mtaos1* (AJ316561) and *Mtaos2* (CM17001219) genes in *M. truncatula* roots and leaves at different times after stress imposition were analysed by using real-time quantitative reverse transcription-PCR (qRT-PCR), which was performed in a 7500 Real-Time

PCR System (Applied Biosystem, Warrington, UK). For the amplification reaction, a SYBR Green assay with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) was performed in a reaction volume of 25 µl. The constitutively expressed actin (XM_003621971) gene served as an internal reference to normalise gene expression. Primers and respective concentrations used for qRT-PCR are listed in Appendix S1 in Supporting information. The following amplification conditions were used: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Specificity of the PCR amplification was confirmed by dissociation curve analyses. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method. The PCR efficiency of each oligonucleotide pair was calculated from each linear regression of standard curves. Real-time PCR derived data were quantified relatively by using the Relative Expression Software Tool (REST; Pfaffl et al. 2002), taking into account the divergent efficiencies. Three replicates were performed for each experiment in addition to a no-template control included for each primer pair.

Gene identification, sequence alignment and comparative analyses of CYP74 proteins

Alignment and comparative analyses were performed on HPLs and AOSs proteins from *Medicago truncatula* (*Mt*HPL1, CAC86898; *Mt*HPL2, CAC86899; *Mt*HPL3, AAY30368; *Mt*HPL4, KEH40767; *Mt*AOS1, CAC86897; *Mt*AOS2, KEH36125) and from other plant species: *Lotus japonicum* (*Lj*HPL, BAJ78218), *Glycine max* (*Gm*HPL, AGH32771; *Gm*AOS1, ABB91776; *Gm*AOS2, ACA79943), *Cucumis melo* (*Cm*HPL, AF081955), *Cucumis sativus* (*Cs*HPL, AGZ95025), *Arabidopsis thaliana* (*At*HPL, OAP00833; *At*AOS, AAM91133), *Solanum tuberosum* (*St*HPL, NP_001275329; *St*AOS1, CAD29735; *St*AOS2, AAN37417), *Solanum lycopersicum* (*Sl*HPL, NP_001234420; *Sl*AOS, NP_001274707), *Medicago sativa* (*Ms*HPL, CAB54849); *Prunus dulcis* (*Pd*AOS, CAE18065). Nucleotide and amino acid sequences were retrieved from the NCBI databank (http://www.ncbi.nlm.nih.gov/pubmed). Sequence alignments and visualisation were done using the Blast program (http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted by busing the PhylML program (Felsenstein 1989) at the web service Phylogeny.fr (http://www.phylogeny.fr/; Dereeper et al. 2008, Dereeper et al. 2010). The dendrogram was inferred using the neighbour-joining method (Saitou and Nei 1987). The percentages of the replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Protein extraction, SDS/PAGE and Western blot analysis

Of each plant tissue stored at -80°C, 100 mg were ground in liquid nitrogen, re-suspended in denaturing buffer and boiled. Extracted proteins (40 ng) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). Western blot analyses were performed according to the ECL protocol (Amersham) and using antibody directed against *St*AOS and *St*HPL of

S. tuberosum, kindly provided by prof. José Juan Sánchez-Serrano (Centro Nacional de Biotecnología-CSIC, Cantoblanco, E-28049 Madrid, Spain). An actin monoclonal antibody (mAbGEa, Thermo Fisher, Waltham, MA) was used to verify equal loading of total protein samples.

Determination of hormone levels in *M. truncatula*

Fresh tissue was frozen in liquid nitrogen and lyophilised. Determination of levels of ABA, SA, oxo-phytodienoic acid (OPDA), JA, JA-Ile in the plant under single and combined stresses was performed as described by Flors et al. (2008). Briefly, dry tissue (0.05 g) was immediately homogenised in 2.5 ml of ultrapure water and a mixture of internal standards. After centrifugation (5000 *g*, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid and subsequently partitioned twice against an equal volume of diethyl ether. The aqueous phase was discarded, and the organic fraction was evaporated in a Speed Vacuum Concentrator (Eppendorf, Hamburg, Germany) at room temperature; the solid residue was re-suspended in 1 ml of a water/methanol (90/10) solution and filtered through a 0.22-µm cellulose acetate filter. A 20-µl aliquot of this solution was then directly injected into the UPLC system. The UPLC was interfaced with a triple quadrupole tandem mass spectrometer (Waters, Saint-Quentin en Yvelines, France); LC separation was performed using an Acquity UPLC BEH C18 analytical column (Waters) at a flow rate of 300 µl min^{-1}. Quantifications were performed using MassLynx 4.1 software (Waters), using internal standards (Sigma-Aldrich, St Louis, MO) as a reference for extraction recovery and standard curves as quantifiers. Values represent means and standard deviations from three biologically independent experiments. Data were statistically analysed using two-way ANOVA with a significance level of $P < 0.05$.

Statistical analysis

Statistical analysis was performed by using the software package GraphPad PRISM 5.03. Data were subjected to one-way ANOVA, followed by Tukey's test to determine the statistical differences between control and stressed plants. Significance was defined as $P < 0.05$.

Results

Phylogenetic relationships among CYP74 members of *M. truncatula*

To understand the role of the CYP74 gene family in the *Medicago truncatula* adaptation to multiple stresses, a phylogenetic approach was preliminary carried out to verify the existence of other, not yet described, members within this family. This study allowed us to identify two new genes belonging to the CYP74 family. The new members were named *Mt*HPL2 and *Mt*HPL4, thus bringing the number of CYP74-encoding genes so far detected in the *Medicago truncatula* genome up to six. Of them, four genes encode putative HPLs, whereas the other two genes share a high sequence identity with AOSs. According to the comparative analyses of sequences, three HPLs (*Mt*HPL1, *Mt*HPL2 and *Mt*HPL4) can be ascribed to the 9/13-HPL subfamily (Hughes et al. 2009). The remaining *Mt*HPL3 is a 13-HPL

and, similarly to other 13-HPL from other plant species, shows a plastidial localisation (De Domenico et al. 2007).

The dendrogram in Fig. 1 highlights the phylogenetic relationship among CYP74 members from different plant species according to their substrate specificity. Notably, *Mt*HPL4 appears to be more strictly related to *Mt*HPL2 (about 81% identity) than *Mt*HPL1 (about 63%). A lower sequence identity (about 38%) was detected between 13-HPL (*Mt*HPL3) and 9/13 HPLs of *M. truncatula*. Concerning the two putative AOS proteins (*Mt*AOS1 and *Mt*AOS2), they shared about 71% of identity.

With regard to other plant species, *M. truncatula* CYP74 enzymes appear to be strictly related to similar enzymes from other legumes, i.e. *M. sativa* and soybean, with which a sequence identity higher than 68% was recorded.

Gene expression profiling of *M. truncatula* CYP74-encoding genes in response to combined salt/wounding stresses

The expression pattern of *CYP74* genes of *M. truncatula* was monitored at different time points in roots and leaves in response to single/combined salt and wounding stresses.

The results of the qRT-PCR, regarding the expression profiles of all the CYP74-encoding genes of *M. truncatula*, are summarised as a heatmap in Fig. 2. The results of the statistical analysis on expression levels are also shown in Appendix S2.

Concerning the *AOS* genes, our results indicated that they reacted rapidly (1 h) to wounding stress either in aerial and below-ground tissues, with a lower induction in the latter (Fig. 2A). Another interesting point refers to the response to wounding in distal tissues. Indeed, root wounding was able to trigger an up-regulation of AOS genes in distal leaves, which was not observed upon leaf wounding. Salt stress induced an early induction (tenfold at 1 h) of *MtAOS1* in the roots (Table S2) and a later activation of *MtAOS2* in leaves, with about a 35-fold up-regulation at 48 h (Fig. 2C).

Regarding combined stresses, the concomitant application of salt and leaf wounding delayed and reduced the expression of both *AOS*s (Fig. 2D) in aerial tissues if compared to the maximum recorded after a single stress (Fig. 2A).

Conversely, when salt and root wounding were applied, they acted synergistically, inducing an up-regulation of both the *AOS* genes either locally or distally, with a similar or even stronger induction to that recorded after single stresses (Fig. 2E and Appendix S2).

In conclusion, *M. truncatula AOSs* are more responsive to shoot wounding, and a concomitant NaCl application impacted the *AOS* expression levels differently, depending on whether wound stress was imposed on aerial or below-ground tissues.

With regard to *HPL*-encoding genes, the qRT-PCR results clearly indicated distinctive expression profiles for these genes. In control healthy plants, *MtHPL1*, *MtHPL2*, and *MtHPL4* genes showed a constitutive higher expression in aerial in comparison with below-ground tissues. Conversely, stress application generally resulted in a higher induction of *MtHPL1* (LW/S at 48h), *MtHPL2* (RW up to 24

h; S at 24 h; LW/S up to 6 h and RW/S at 1 h) and *MtHPL4* (S at 48 h and WR/S at 48 h) genes in below-ground tissues in comparison with aerial ones. A specific shoot expression was observed for *MtHPL3*, which mainly reacted distally to root wounding.

A closer look to each gene indicated that *MtHPL1* responded to most of the stresses here considered in leaves, even though the highest expression levels were recorded in the roots (46-fold; see Appendix S2) after combined LW/S stress (Fig. 2D).

A different expression pattern was observed for *MtHPL2*. According to our results, this gene better responded locally to root wounding (13-fold up-regulation at 48 h) and salt stress (17-fold at 24 h; Appendix S2) (Fig. 2 B and C). Under combined RW/S stresses, this gene reacted at early time points (about 8-fold; see Appendix S2), whereas LW/S treatment resulted in its sustained activation starting from 6 h up to 48 h (Fig. 2D and E).

Gene *MtHPL3* generally showed a weak response to the stresses here considered, with the highest induction in distal leaves upon root wounding (Fig. 2B).

Finally, gene *MtHPL4* showed the highest induction upon combined stresses in roots (about 40-fold, after RW/S combined stresses; Fig. 2E and Appendix S2).

These results highlight the finely tuned regulation of *HPLs* in a stress-dependent and tissue-specific manner.

Biochemical profiling of the CYP74 in *M. truncatula* leaves and roots subjected to single/double stressors

With the aim to parallel gene expression profiling with CYP74 protein levels upon single/combined wounding/salt stresses, we carried out Western blot analysis using specific antibodies raised against potato AOS and HPL. Our preliminary data indicate that these antibodies showed a good specificity towards *M. truncatula* orthologs (data not shown). Fig. 3 shows the trend of AOS and HPL accumulation in leaves and roots from control and stressed plants.

In leaves, AOS enzymes early (at 1 h) reacted better to distal than to local wounding. The concomitant application of salt stress delayed AOS accumulation in leaves, which showed the highest levels between 6 and 24 h upon combined LW/S and RW/S, respectively (Fig. 3D and 3E). In roots, AOSs reacted to local wounding and salt stress, with an earlier induction upon RW (from 1 h) and a delayed accumulation upon salt stress (from 6 h; Fig. 3B and C). In contrast to leaves, the accumulation trend of AOSs in roots was not significantly delayed upon combined stresses in comparison to single stresses (Fig. 3E).

Concerning HPLs, our results indicate that these proteins better responded to local stress. For example, wounding and salt stresses resulted in an early (1-6 h) and late (24-48 h) accumulation of HPLs (Fig. 3A, B and C).

Combined stresses mainly antagonised on the levels of HPLs, with a reduction of the recorded signal in aerial tissues and a delayed accumulation in below-ground tissues (Fig. 3D and E).

Taken together, gene and protein profiling showed that both AOS and HPL are differentially regulated in a stress- and tissue-dependent manner in response to combined salt/wounding stresses.

Hormonal profiling in *M. truncatula* roots subjected to single/double stressors

Since *AOS* and *HPL* gene families are directly linked to the biosynthesis of oxylipins, we firstly targeted our analysis within this pathway, although subsequently, it was extended to ABA and SA to further understand the hormonal balance upon combined stresses. Abscisic acid was chosen since it is one of the main hormones involved in abiotic stress signalling, while SA was selected based on its participation in plant responses to biotic stresses. The jasmonic acid precursor OPDA, JA and the active JA-Ile levels were monitored in roots and leaves from control and stressed plants. Fig. 4 shows a heatmap representation of quantitative levels of these phyto-hormones. The results of the relative statistical analysis on their absolute quantification from a targeted MS/MS analysis (expressed as ng g^{-1} dry tissue) are reported in Appendix S3.

Analytical quantification indicated that the levels of these hormones in the absence of stress were higher in the shoot compared to the root, with the only exception of OPDA, which showed consistently higher levels (fivefold) in roots than leaves of non-stressed plants. Additionally, SA levels were comparable in both organs.

As expected, leaf wounding resulted in a local early burst of jasmonates, with a 3- and 6-fold increase of JA and JA-Ile, respectively, at 1 h after stress onset (Fig. 4A and Appendix S3). The distal organs (roots) also responded, although to a lesser extent than the shoots (Appendix S3). An increase of the local pool of ABA (about 2-fold compared to the control) was also observed at later time points, whereas a distal increase of SA was recorded early in roots.

Intriguingly, when wounding was applied to roots, local jasmonate levels were lower than those triggered by leaf wounding stress (about 8- and 2-fold lower levels for JA and JA-Ile, respectively; see Appendix S3). Similarly, the response of leaves to root wounding was also attenuated (about 3- and 2-fold JA-Ile lower levels for JA and JA-Ile, respectively). In particular, OPDA locally increased up to 2-fold in comparison with control levels recorded in roots. Together with jasmonates, root wounding stress triggered an increase of SA (about 3-fold from 1 h) and ABA (about 30% at later time points) in aerial tissues (Fig. 4B and Appendix S3).

Salt application did not result in significant changes in jasmonates and OPDA levels, even though this stress induced a 2- to 3-fold increase in ABA and SA in distal leaves (Fig. 4C).

Under combined LW and S stresses, the levels of jasmonates in leaves were similar to those recorded after single LW, but were greatly reduced in distal roots (Fig. 4D). A similar antagonistic effect was also observed for SA (Fig. 4D).

As shown in Fig. 4E, the combination of RW/S stresses had a positive impact on the local pool of JA-Ile and OPDA, which about doubled the levels recorded after single RW stress. Of note is the significant reduction of SA at early time points recorded after RW/S stresses, in comparison with

levels recorded after single salt stress. Finally, the same stress combination negatively impacted the levels of OPDA and SA (about 3- and 2-fold lower than those recorded after single RW stress) in distal leaves.

Discussion

Understanding the fine-tuned responses of plants to multiple stresses is crucial to identify synergic or antagonistic signals originated by different stressors at local and systemic levels and could open new perspectives to improve the tolerance of main crops to the increasing sets of (a)biotic stresses which the future agriculture will have to face with in the next years. Among them, wounding and soil salinity are becoming common challenges as a consequence of massive changes in agricultural practices and/or environmental conditions. Wounding can often be a consequence of abiotic (wind, heavy rain, hail) or biotic (chewing insects, nematodes) stresses in aerial or below-ground tissues. Salinity is becoming a serious concern in most Mediterranean countries as well as in arable, irrigated areas due to the low quality of irrigation water and the massive use of fertilisers.

Oxylipins are a class of secondary metabolites deriving from polyunsaturated fatty acid metabolism. Most of them act as important regulators of plant defence response to (a)biotic stresses. The present study was aimed at investigating, at molecular and biochemical levels, the participation of this pathway to the *M. truncatula* response to single or combined salt/wounding stresses.

The CYP74 gene family harbours six members in the *M. truncatula* genome

With the aim to provide wide overview about the role of the *M. truncatula* CYP74 gene family to multiple stresses, we searched in the *M. truncatula* genome and reported for the first time the existence of two new CYP74 members. Indeed, out of the six genes here considered, four (*MtAOS*1, *MtAOS*2, *MtHPL1* and *MtHPL*3), have been previously characterised (De Domenico et al. 2007, Hughes et al. 2009), whereas two (Mt*HPL2* and Mt*HPL4*) are reported for the first time in the present study. Amino acid alignment and phylogenetic analyses clearly indicated a strict relationship between these genes and *MtHPL1*; thus, they likely encode 9/13-HPLs. Remarkably, out of the four putative *HPL*-encoding genes, only one is a 13-HPL (*MtHPL3*). Therefore, this HPL might be the only potential competitor of the two plastidial AOSs (*MtAOS1* and *MtAOS2*) involved in the first step of jasmonate biosynthesis, as both these enzymes use the same substrates and share the same plastidial localisation (Farmaki et al. 2007).

The six members of the CYP74 family of *M. truncatula* show specific activation patterns upon salt and wounding stress

The expression profiles of the six members of the CYP74 family of *M. truncatula* upon single stress or the combination of wounding and salt stresses clearly indicated a tissue- and stress-specific regulation for each single gene.

For example, both AOS genes early reacted in aerial tissues in response to local or distal wounding, although *MtAOS2* was more responsive than *MtAOS1* to both the stresses considered here. The same was true for the HPL genes, which showed specific activation patterns in response to local or distal stresses. For example, *MtHPL2* and *MtHPL4* locally reacted to salt and root wounding stress, whereas *MtHPL1* better responded to salt stress in distal organs.

Regarding the tissue-specific expression of AOS/HPL genes, *MtHPL3* should be mentioned, whose levels were undetectable in below-ground tissues.

Signals from salt and wounding stresses are different

The results reported here indicate that wounding was able to trigger an early and strong up-regulation of AOS/HPL genes in aerial tissues, together with a stronger jasmonate biosynthesis in aerial tissues in comparison with below-ground tissues, even upon root wounding.

It should be noted that AOSs did not respond to distal wounding of below-ground tissues, and only the *MtHPL2* gene showed a modest up-regulation in response to this stress. However, gene expression profiling did not parallel with chemical quantification, since jasmonate levels in roots were higher in response to distal wounding than to local wounding. These results indicate a translocation of these stress-signalling molecules from shoots to roots or the presence of a stable pool of enzymes committed to jasmonate biosynthesis.

In contrast to wounding, salt stress triggered a late response, inducing specific genes either locally (i.e. up-regulating *MtHPL1*, *HPL2* and *HPL4* at 24-48 h) or distally (*MtAOS2*). The same was clearly observed at the protein level, with both AOS and HPL enzymes showing the highest levels at 48 h.

The local and systemic jasmonate burst upon leaf wounding has already been reported for *A. thaliana* and *M. truncatula* (Glauser et al. 2008, Landgraf et al. 2012). Landgraf et al. (2012) showed that single or repeated wounding was able to enhance *MtAOC1* transcripts and JA levels either locally or distally, thus suggesting a shoot-to-root signalling and a systemic priming effect of leaf wounding. Here, we showed that similarly to leaf, root wounding was also able to trigger a root-to-shoot signalling with a consequent induction of OPDA, JA-Ile and SA in aerial tissues.

It should be noted that OPDA levels, in contrast to jasmonates, were similar at all the time points both in aerial and below-ground tissues. These results point to an autonomous signalling behaviour of this phyto-hormone in *M. truncatula*. Hasegawa et al. (2011) have proposed that OPDA might orchestrate the expression of genes involved in the root-to-shoot communication during the late responses of *Arabidopsis* to stress. Our results confirmed that OPDA could play a similar role in *M. truncatula*. Indeed, starting from 24 h after stress onset, OPDA levels were higher than those recorded for JA and JA-Ile in belowground tissues. Conversely, this hormone was repressed in aerial tissues upon local wounding. Savchenko et al. (2014) showed that OPDA uncouples the conversion to JA upon drought stress and acts as a stomatal closure regulator in different plant species. Interestingly, our results

indicate that under wounding and salt stress, OPDA was over-accumulated as an additive phenomenon (Fig. 4E). Therefore, OPDA can exert a signal role independently from jasmonates in *M. truncatula*.

Combined salt and wounding stresses generated complex signals, acting synergistically or colliding

The application of combined stresses resulted in unpredicted results, such as the over-expression of some genes whose inductions were not observed under single stresses (i.e. the over-expression of *MtAOS2* and *MtHPL2* in roots at 1 h after RW/S or *MtHPL4* at 48 h after LW/S). In other cases, salt and wounding stress signals antagonised the down-regulation of some genes in comparison with the maximum recorded values after a single stress (i.e. *MtAOS1*, *MtAOS2*, *MtHPL2*, *MtHPL3* and *MtHPL4*, which were all down-regulated in leaves under RW/S stresses). Finally, in few cases, stress signals acted synergistically, and some genes reached their maximum levels under combined stresses (i.e. *MtHPL4* at 48 h under combined RW/S stresses or *MtHPL1* and *MtHPL2* at 48 h under combined LW/S stresses).

This complex expression pattern observed under combined stresses paralleled with a general shift in the protein level maximum towards later time points, with a trend similar to that observed under single salt stress.

Phytohormone levels under combined stresses revealed some interesting peculiarities, i.e. as in the case of combined RW/S, which did not significantly impact the local levels of jasmonates, but repressed ABA locally and OPDA and SA distally. It is also noteworthy that under LW/S stress, the shoot-to-root signalling mediated by jasmonates was also drastically reduced, together with a significant reduction of the local/distal pool of ABA in comparison with the maximum levels recorded after single salt stress. Therefore, the jasmonate/ABA-mediated defence signalling might be attenuated under these circumstances, and the defence response might be consequently affected. In this context, Landgraf et al. (2012) have reported that repeated leaf wounding altered the colonisation of either beneficial (the arbuscular mycorrhizal fungus *Glomus intraradices*) or pathogenic (the oomycete *Aphanomyces euteiches*) microorganisms. Guo et al. (2016) have recently reported that the drought-induced up-regulation of ABA-signalling decreased the SA-dependent, but increased the JA-dependent response in *M. truncatula* A17 plants, thus potentially affecting the phloem defences and, consequently, aphid penetration under drought stress.

Whether the reduced systemic signalling observed upon concomitant salt and leaf wounding stresses could result in a higher susceptibility of *M. truncatula* to biotic stresses still needs to be confirmed. Our previous results (Santino et al.; unpublished) point to a specific down-regulation of a root-specific protease inhibitor gene under combined salt and leaf wounding stresses, thus confirming the antagonistic effect of salt on the jasmonate-mediated stress signalling pathway.

Finally, in a previous work on chickpea responses to drought stress, we reported increased levels of OPDA and jasmonates in the tolerant ICC4958, but not in the susceptible ICCC1882 variety (De

Domenico et al. 2012). Taken together, these results confirm the important role of jasmonates in legume adaptation to abiotic stresses and point to the existence of a complex molecular cross-talk among signals generated by multiple stresses plants commonly face. The fine-tuned interplay among jasmonates, OPDA, ABA, SA and other hormones has the fundamental role of organising early and late defence responses and reprogramming plant growth, reproduction and senescence for an optimal adaptation of the plant to adverse environmental conditions.

Author contributions

S.D.D., M.T. and A.S. designed experiments and performed the experiments. A.S. obtained funding and supervised the study. S.D.D. and A.G. performed phylogenetic and gene expression analysis. M.T., V.P. and V.F. carried out the biochemical analyses. M.T., S.D.D. V.P., V.F. A.S., analysed the data. A.S. wrote the paper. All authors gave their contribution in the writing, read and approved the final manuscript.

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Supporting information

Additional supporting information may be found in the online version of this manuscript:

Appendix S1. Primers and respective concentrations used for qRT-PCR analysis.

Appendix S2. Genes expression level and statistical analysis.

Appendix S3. Hormonal amounts in tissues and statistical analysis.

Figure legends

Fig. 1. Phylogenetic tree from a set of CYP74 proteins. The dendrogram was generated using the PhylML program. The following groups are indicated: 13-HPL [MtHPL3 (AAY30368), MsHPL (CAB54849), LjHPL (BAJ78218), StHPL (NP_001275329), AtHPL (OAP00833), PdHPL (CAE18065)]; 9/13-HPL [MtHPL2 (CAC86899), MtHPL4 (KEH40767), MtHPL1 (CAC86898), GmHPL (AGH32771), CmHPL (AF081955), CsHPL (AGZ95025)]; 13-AOS [MtAOS2 (KEH36125), MtAOS1 (CAC86897), GmAOS2 (ACA79943), GmAOS1 (ABB91776), StAOS1 (CAD29735), StAOS2 (AAN37417), AtAOS (NP_199079), SlAOS (NP_001274707)].

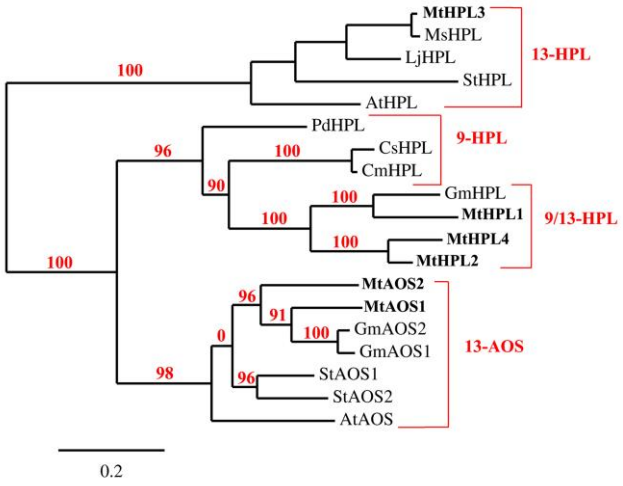

Fig. 2. Gene expression profile in control and stressed *M. truncatula* plants. Heatmaps relative to the gene expression profiles of *MtAOS1*, *MtAOS2*, *MtHPL1*, *MtHPL2*, *MtHPL3* and *MtHPL4* at different time stress points (1, 6, 24 and 48 h). For each gene, a colour scale bar (from blue, minimum expression to red, highest level was used after comparison with control unstressed leaves, whose value was scored as 1.0). (A) Leaf wounding (LW); (B) root wounding (RW); (C) salt stress (S); (D) LW/S combined stress; (E) RW/S combined stress.

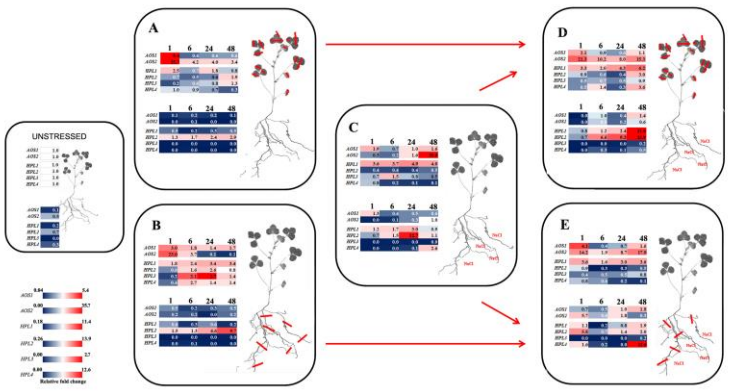

Fig. 3. Levels of CYP74 enzymes in control and stressed *M. truncatula* plants. Western blot analysis on total protein samples from control and stressed tissues using specific anti-HPL or anti-AOS antibodies. (A) Protein levels upon leaf wounding, LW. (B) Protein levels upon root wounding, RW. (C) Protein levels upon salt stress, S. (D) LW/S protein levels upon combined stress. (E) RW/S protein levels upon combined stress. An actin monoclonal antibody was used as loading control.

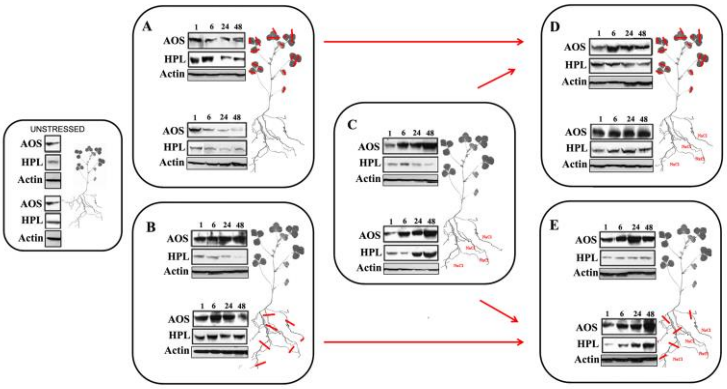

Fig. 4. Hormonal profiles in control and stressed *M. truncatula* plants. Heatmap representation of hormonal levels in *M. truncatula* roots and leaves collected at 1, 6, 24 and 48 hours after stress induction. For each hormone, a colour scale bar (from blue = 0 to red = highest level) was used. (A) Leaf wounding, LW; (B) root wounding, RW; (C) salt stress, S; (D) LW/S combined stress and (E) RW/S combined stress. OPDA, oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; ABA, abscisic acid; SA, salicylic acid.