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Research Article

Defence signalling marker gene responses to hormonal elicitation differ between roots and shoots

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Abstract. Phytohormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) play a key role in regulation of plant immune responses to different attackers. Extensive research over recent years has led to the identification of molecular markers for specific hormonal-regulated defence pathways. However, most of our current knowledge on the regulation of plant immunity derives from studies focused on above-ground organs, mainly on the model plant *Arabidopsis thaliana*. Therefore, it is unclear whether the paradigms based on experiments on above-ground organs are entirely transferable to roots. Here, we used the non-model plant *Brassica rapa* to study the regulation dynamics of hormonal-related marker genes in both roots and shoots. These markers were identified in *Arabidopsis* shoots after elicitation of the JA-, SA-, ET- or ABA-signalling pathways, and are commonly used to study induced responses. We assessed whether the regulation of those genes by hormonal elicitation differs between roots and shoots. To discern whether the differences in marker gene expression between roots and shoots are related to differences in hormone production or to differential responsiveness, we also measured actual hormone content in the treated tissue after elicitation. Our results show that some of the widely used markers did not show specific responsiveness to single hormone applications in *B. rapa*. We further found that hormonal elicitation led to different response patterns of the molecular markers in shoots and roots. Our results suggest that the regulation of some hormonal-related marker genes in *B. rapa* is organ specific and differs from the *Arabidopsis*-derived paradigms.

Keywords: *Brassica*; hormonal signalling; marker genes; phytohormones; plant defences.

Introduction

Phytohormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) act as signalling molecules that regulate plant responses to

insect herbivores, pathogens and beneficial microbes (reviewed by Erb and Glauser 2010; Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; Broekgaarden et al. 2015). Jasmonic acid is a key regulator of plant defences

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to necrotrophic pathogens, chewing insects and wound responses, whereas SA is mainly induced in response to biotrophic pathogens and phloem-sucking insects (Zarate et al. 2007; Pieterse et al. 2012). While the JA and SA signalling pathways (hereafter referred to as pathways) form the backbone of the plant's immune system, ABA and ET play a more modulatory role. In the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*), the JA pathway consists of two distinct and antagonistic branches, the MYC- and the ERF-branch, which are co-regulated by ABA and ET, respectively. The ET-regulated ERF-branch of the JA pathway is associated with plant defences against necrotrophic pathogens, while the ABA-regulated MYC-branch is associated with wounding and insect herbivory (Anderson et al. 2004; Lorenzo and Solano 2005; Pré et al. 2008; Verhage et al. 2011; Kazan and Manners 2013). Antagonistic and synergistic interactions between pathways are well known, with the antagonistic interactions between JA and SA pathways being the most intensively studied (Berrocal-Lobo et al. 2002; Leon-Reyes et al. 2009; Vos et al. 2015). These interconnections between hormonal pathways are known as 'crosstalk' and provide plants with a complex network that allows them to fine-tune their defences against different stimuli (Pieterse et al. 2012).

To link particular pathways with actual defence responses, molecular tools such as qPCR allow us to use the expression of several marker genes as indicators of the activation of specific pathways. For example, in *Arabidopsis* PATHOGENESIS-RELATED PROTEIN 1 (*PR1*) is used as a marker gene for the SA pathway (Gaffney et al. 1993; van Wees et al. 1999; van Loon et al. 2006); the basic helix-loop-helix leucine zipper transcription factor *MYC2* and the VEGETATIVE STORAGE PROTEIN 2 (*VSP2*) are used as markers for the ABA-modulated branch of JA pathway (Anderson et al. 2004; Lorenzo et al. 2004; Dombrecht et al. 2007), while the ETHYLENE RESPONSE FACTOR 1 (*ERF1*) and PLANT DEFENSIN 1.2 (*PDF1.2*) are commonly used as markers for the ET-modulated branch of the JA pathway (Penninckx et al. 1998; Lorenzo et al. 2003; Robert-Seilaniantz et al. 2011). In analogy, *ERF1* and the ETHYLENE RECEPTOR 1 (*ETR1*) genes are used as markers for the ET pathway (Lorenzo et al. 2003; Lorenzo et al. 2004) and LATE EMBRYOGENESIS ABUNDANT 4 (*LEA4*) as a marker for the ABA pathway (Hoth et al. 2002; Hundertmark and Hinch 2008).

The vast majority of studies on the identification and characterization of marker genes for specific pathways has been done on the shoot tissues of the model plant *Arabidopsis*. Marker genes have been validated in *Arabidopsis* shoots by analysing their expression patterns after exogenous application of single or combined phytohormone solutions (Hoth et al. 2002). The

information on marker gene responsiveness and the interactions between different pathways obtained in *Arabidopsis* shoots has been implemented in other plant species, including species of the closely related genus *Brassica* (Wang et al. 2011). In some *Brassica* spp., the responsiveness of several *Arabidopsis*-derived marker genes to exogenous hormonal application has been tested. For example, *VSP2* is up-regulated in response to JA application in *Brassica juncea* and *B. oleracea* (Mathur et al. 2013; Tytgat et al. 2013), *PR1* is responsive to SA application in *B. rapa* (Abe et al. 2011; Lee and Hong 2015) and *BnLEA4-1* is an ABA-responsive gene in *B. napus*, *B. juncea* and *B. carinata* (Dalal et al. 2009). Although *Arabidopsis* and *Brassica* spp. belong to the same family, marker gene responsiveness to activation of specific hormonal pathways might show important differences (Tytgat et al. 2013). Since an enormous amount of studies on plant immune responses to above-ground organisms is being conducted on *Brassica* spp., further validation of the *Arabidopsis*-derived marker genes in *Brassica* spp. is required (Soler et al. 2012; Maag et al. 2014; Kroes et al. 2016; Pineda et al. 2017).

Over the last 15 to 20 years, interest in the regulation of root-induced responses and how they affect above-ground defence responses has increased (van Dam et al. 2003; Papadopoulou and van Dam 2017). In natural environments, plant roots interact with a variety of organisms present in the rhizosphere (van der Putten 2003). It may therefore be expected that roots have an equally extensive signalling network as shoots for managing the diversity of below-ground interactions. Surprisingly, limited information on hormonal signalling and marker gene responsiveness is available for the root tissues of *Arabidopsis* or other plant species (Lawrence et al. 2012; Tytgat et al. 2013; Lu et al. 2015). A few available studies support the notion that transcriptional responses to hormonal elicitation in shoots and roots on the same plant may considerably differ (Lawrence et al. 2012; Tytgat et al. 2013). These findings raise the question whether the paradigms for hormonal signalling and the responses of the main marker genes as observed in shoots can be simply transferred to the roots of the same or different plant species. Considering the increasing research interest in plant immune responses to below-ground organisms (Barr et al. 2010; Martínez-Medina et al. 2017a, b; Tsunoda et al. 2017), it is imperative to investigate hormonal signalling pathways regulating defence responses in root organs. To do so, it is also important to identify marker genes appropriate for roots. At present, most marker genes have been developed for shoot organs and may thus be unreliable for root studies.

In this study, we first tested whether genes known as markers for the main defence-related hormonal

pathways (i.e. JA, SA, ABA and ET) in the *Arabidopsis*-shoot model are regulated similarly by hormonal elicitation in *B. rapa* shoots. The complete genome sequence of *B. rapa* is available (<http://brassicadb.org>). It shares 93 % of its gene families with *Arabidopsis* (Wang et al. 2011), which makes it a good model plant among *Brassica* spp. for molecular and genomic studies. Then, we investigated whether the regulation of the molecular markers after hormonal elicitation is similar in shoot and in root organs of *B. rapa*. Based on the great homology between *Brassica* and *Arabidopsis*, we expect that the regulation of the tested marker genes by hormonal elicitation in *B. rapa* shoots will be similar to that of *Arabidopsis* shoots. However, we expect differential regulation in root and shoot tissues of *B. rapa* (Tytgat et al. 2013). To test these assumptions, we analysed the expression patterns of *VSP2*, *PR1* and *ERF1* over time (from 4 to 48 h) in *B. rapa* shoots and roots after local elicitation with either methyl jasmonate (MeJA), ABA, SA or ethephon, an ET-releasing compound. Furthermore, we analysed the regulation of the predicted *B. rapa* 18 kDa seed maturation protein-like (hereafter referred to as *BrLEA4*) as a novel putative marker gene for ABA pathway in *B. rapa* shoots and roots. *BrLEA4* was selected as a homolog of the *Arabidopsis* *LEA4-5* gene, previously shown to be highly ABA responsive in vegetative tissues (Hoth et al. 2002). Moreover *LEA* genes are also responsive to ABA in soybean, tomato and maize (Martínez-Medina et al. 2013; Fernández et al. 2014; Zamora-Briseño and de Jiménez 2016). Furthermore, to link changes in marker gene expression with that in the accumulation of the phytohormones themselves, we also measured phytohormone levels in the same tissues. In this way, we could discern whether discrepancies in marker gene expression between roots and shoots were attributed to differences in actual hormonal levels in the treated tissues or to different marker gene responsiveness. We found that marker gene responsiveness to specific hormonal pathways in *B. rapa* deviated to some extent from those of the *Arabidopsis*-shoot model. In addition, we found that the same marker genes show differential regulation depending on the plant organ. Overall, our findings indicate that plant species and the specific plant organ should be considered in marker gene selection when studying regulation of plant defence responses.

Methods

Plant growth

Brassica rapa seeds, originating from a wild population (Maarsen, The Netherlands), were germinated on glass beads in plastic containers closed with a transparent

plastic lid. The containers were kept for 1 week in a climate chamber (Percival Scientific, Perry, IA, USA) at 20 °C, with a 16-h light and 8-h dark cycle, 60 % relative humidity and 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation. Seedlings were then transplanted in 11 × 11 × 12 cm pots, filled with 1:1 mixture of potting soil (Floradur B Pot Clay Medium, Floragard Vertriebs GmbH, Oldenburg, Germany) and sand (Gerhard Rösl GmbH, Jesewitz OT Liemehna, Germany). During the transplantation plants were fertilized with the Osmocote®Pro 3-4M slow-release fertilizer (Everris International B.V., The Netherlands). The plants were grown in a greenhouse with a 16-h light (27 °C) and 8-h dark (21 °C) cycle at 50 % relative humidity, and watered as needed. Four weeks after seed germination, the plants that had five fully expanded leaves were used for the experiments.

Hormonal application

The roots or shoots of *B. rapa* plants were either treated with 100 μM MeJA (Sigma-Aldrich, Munich, Germany), 1 mM SA (Carl Roth, Karlsruhe, Germany), 10 μM ABA (Sigma-Aldrich, Munich, Germany) or 7 mM ethephon (2-chloroethylphosphonic acid, Sigma-Aldrich, Munich, Germany). Hormone solutions were amended with 0.015 % (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). Control plants were treated with a water solution containing 0.015 % Silwet L77.

Different groups of plants were used for root and shoot treatments. Shoot treatment was performed by applying 1 mL of MeJA, ABA, SA, ethephon or control solution to the upper adaxial side of the fourth fully expanded leaf (counted from the soil). In case of MeJA, ABA, SA and the respective control plants, 1 mL was also applied to the lower, adaxial surface of the same leaf. Root treatment was performed by applying 50 mL of MeJA, ABA, SA, ethephon or control solutions to the saucers from which roots quickly absorbed the solution. To avoid ethephon evaporating into the surrounding air, ethephon-treated plants were covered with transparent foil. As control plants for the ethephon treatment, a separated set of plants treated with control solution was used, which was also covered with a transparent foil. The plants were harvested at 4, 8, 24 or 48 h after hormonal application and five biological replicates (single plants) per time point were used. Roots were carefully washed to remove the adherent soil. The roots of root-treated plants and the local leaf of the shoot-treated plants were harvested, immediately frozen in liquid nitrogen and stored at -80 °C.

Quantitative RT-PCR analysis

Total RNA was extracted from ~100 mg of ground plant tissue using innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany) and treated with DNase I (Biozym Scientific,

Hessisch Oldendorf, Germany) following the manufacturers' instructions. For each sample, 1 µg of purified total RNA was subjected to reverse transcription using oligo(dT)₂₀ primer and RevertAid HMinus enzyme (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Undiluted cDNA was used for real-time quantitative RT-PCR (qPCR) analyses of *PR1* and *VSP2* in root samples. For the rest of the qPCR analyses 10-fold diluted cDNA was used. qPCRs were performed by using SYBR Green qPCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. All the qPCRs were run in 96-well plates with a PikoReal 96 instrument (Thermo Scientific, Waltham, MA, USA) under the following conditions: incubation at 50 °C for 2 min and 95 °C for 5 min, followed by 40 cycles of incubation at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Relative quantification of mRNA levels was performed using the comparative 2^{-ΔCT} method (Livak and Schmittgen 2001). Expression values were normalized by using the housekeeping gene *TIP41* (Chen et al. 2010; Chandna et al. 2012). Gene-specific primers listed in Table 1 were used for qPCR analysis. For *BrLEA4* primer design, a nucleotide sequence of *Arabidopsis LEA4-5* (AT5G06760) was subjected to NCBI nBLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *B. rapa* sequence (LOC103855696) predicted to encode for 18 kDa seed maturation protein-like (referred here as *BrLEA4*) showed 100 % similarity with the *Arabidopsis LEA4-5* sequence and was selected for a primer design. Primers were designed using the Primer3 tool (v. 0.4.0, <http://bioinfo.ut.ee/primer3-0.4.0/>). The specificity and efficiency of all primers used in this study were tested. Primer specificity was tested with agarose gel electrophoresis and melting curve analysis following qPCRs.

Primers resulting in a single product were selected. PCRs were performed by using GoTaq® DNA Polymerase (Promega, Madison, WI, USA), according to manufacturer's instructions on a Techne® Prime Elite thermal cycler (Bibby Scientific Ltd, Stone, UK). PCRs were run for 95 °C for 2 min, 35 cycles of incubation for 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 5 min. In order to determine the gene-specific PCR efficiency, a 10-fold serial dilutions of cDNA were used to generate a standard curve. The correlation coefficient (R^2) and the PCR efficiency were calculated by using the slopes of the standard curves [see Supporting Information—Fig. S1]. The linear R^2 for all the primers ranged from 0.938 to 0.999 over 100-fold of cDNA dilution.

Phytohormone measurements

Phytohormone extraction and purification were performed as described by Machado et al. (2013) with some modifications. Briefly, shoot and root tissue (50–100 mg per sample) was extracted with 1 mL ethyl acetate containing 40 ng of each of the following internal phytohormone standards: D₆-ABA, D₆-SA (OIChemIm Ltd, Olomouc, Czech Republic) and D₆-JA (HPC Standards GmbH, Borsdorf, Germany). Samples were vortexed for 10 min, centrifuged at 14 000 rpm for 2 min at 4 °C and the supernatants were evaporated until dryness in a Speed-Vac (Labconco, USA) at room temperature. Pellets were then suspended in 200 µL methanol:water (70:30) and dissolved using a Fisherbrand FB 15061 ultrasonic bath (Fisher Scientific, UK) for 15 min. Phytohormones were analysed using liquid chromatography (Bruker Advance UHPLC, Bremen, Germany) coupled to mass spectrometer (Bruker Elite EvoQ Triple quad, Bremen, Germany) (LC-MS), as described by Schäfer et al. (2016).

Table 1. Gene information and primers sequences used for gene expression analysis. n.a.: not available.

Gene name	Accession number	<i>Arabidopsis thaliana</i> locus	Sequence (5'-3')	Marker for	Reference
<i>VSP2</i>	Bra020470	AT5G24770	F: TCTACGCCAAAGGACTTGCT R: CCCGTATCCATATTGAGCGTA	JA pathway	T. O. Tytgat (unpubl. data)
<i>PR1</i>	n.a.	AT2G14610	F: CTACGCCGACCGACTAAGAG R: CTACTCCCGCCAAGTTCTC	SA pathway	Mathur et al. (2013)
<i>ERF1</i>	Bra023744 Bra023746	AT3G23240	F: CGGCGGAGAGAGTTAAAGAG R: AACACCCATCCTCGTAGCTG	ET pathway	Mathur et al. (2013)
<i>BrLEA4</i>	Bra005911	AT5G06760	F: TCAGCCACTCACTCAACCAC R: GTCCGACCAGTTCCAGTGTT	ABA pathway	Present study
<i>TIP41</i>	Bra011516	AT4G34270	F: TGCGAAAGGGTATCCAGTTG R: ATCACCGGAAGCCTCTGAC	Housekeeping gene	T. O. Tytgat (unpubl. data)

Separation was achieved on a Zorbax Eclipse XDB-C 18 column (50 × 4.6 mm, 1.8 mm; Agilent Technologies, Boeblingen, Germany) with 0.05 % formic acid in water and 0.05 % formic acid in acetonitrile as mobile phases A and B, respectively. Samples were analysed in a randomized sequence including acetonitrile samples in between as controls. Data acquisition and processing were performed using the ‘MS data Review’ software (Bruker MS Workstation, version 8.2). Phytohormone levels were calculated over the amount of fresh mass of plant material (ng⁻¹ mg⁻¹ fresh mass).

Statistical analysis

Gene expression and phytohormone level data were log transformed to meet the assumptions of normality and homogeneity of variances and then subjected to two-way ANOVA (R software, version 3.1.2). The data were analysed per treatment using a model containing treatment (control, hormonal application), time (4, 8, 24, 48 h) and their interaction term as factors. Following two-way ANOVA, one-way ANOVA was performed for each time point to analyse the effect of hormonal application when time point had a significant effect. Tukey test was performed on the interaction effect (treatment × time), when the interaction was significant. Phytohormone level data were corrected for instrument carry over by subtracting the average value found in acetonitrile control samples from the experimental-samples data.

Following subtraction, negative values were replaced with 0.00001 for data analysis and visualization. For the visualization of data, fold changes (FC) in gene expression and phytohormone levels were calculated by dividing the normalized expression or phytohormone levels [see Supporting Information—Tables S1–S3] of each treated plant by the average expression or phytohormone levels of the respective control group. The data obtained from the MeJA, ABA and SA experiments were also analysed for the overall treatment effect with a two-way ANOVA model containing control, MeJA, SA and ABA as treatment term [see Supporting Information—Tables S4 and S5].

Results

Effect of hormonal application on marker gene expression

Gene expression analysis in *B. rapa* shoots showed that irrespective of the time point after treatment, *VSP2* expression was significantly up-regulated by MeJA and ABA application compared to control plants (treatment effect, $F_{1,24} = 18.36$, $P < 0.001$ and $F_{1,24} = 5.68$, $P = 0.025$, respectively, Table 2, Fig. 1A). Salicylic acid and ethephon application did not have a significant effect on *VSP2* expression, though *VSP2* tended to be down-regulated by SA treatment (treatment effect, $F_{1,24} = 3.00$, $P = 0.096$,

Table 2. Statistical analyses (*F*- and *P*-values) of the effects of local hormonal application on gene expression levels in *Brassica rapa* shoots. The expression levels of *VSP2*, *PR1*, *ERF1* and *BrLEA4* were measured in *B. rapa* shoots after MeJA, ABA, SA or ethephon application to the shoots ($n = 3–4$ per treatment and harvest time). The data were analysed per hormonal treatment group using a two-way ANOVA model containing treatment (control, hormonal application), time (4, 8, 24, 48 h) and their interaction term as factors. Statistically significant effects ($P \leq 0.05$) are indicated in bold.

Treatment	Factor	Gene			
		<i>VSP2</i>	<i>PR1</i>	<i>ERF1</i>	<i>BrLEA4</i>
MeJA	Treatment (1)	$F_{1,24} = \mathbf{18.36}$, $P < \mathbf{0.001}$	$F_{1,22} = 0.24$, $P = 0.63$	$F_{1,24} = 0.95$, $P = 0.34$	$F_{1,22} = \mathbf{5.02}$, $P = \mathbf{0.035}$
	Time (2)	$F_{3,24} = 2.18$, $P = 0.117$	$F_{3,22} = 0.51$, $P = 0.679$	$F_{3,24} = 0.97$, $P = 0.425$	$F_{3,22} = \mathbf{6.17}$, $P = \mathbf{0.003}$
	Interaction (1.2)	$F_{3,24} = 0.88$, $P = 0.465$	$F_{3,22} = 1.94$, $P = 0.152$	$F_{3,24} = 1.1$, $P = 0.37$	$F_{3,22} = 1.09$, $P = 0.373$
ABA	Treatment (1)	$F_{1,24} = \mathbf{5.68}$, $P = \mathbf{0.025}$	$F_{1,22} = 0.16$, $P = 0.692$	$F_{1,22} = 0.12$, $P = 0.73$	$F_{1,20} = 3.83$, $P = 0.064$
	Time (2)	$F_{3,24} = 1.33$, $P = 0.287$	$F_{3,22} = 1.11$, $P = 0.367$	$F_{3,22} = 0.98$, $P = 0.42$	$F_{3,20} = \mathbf{11.8}$, $P < \mathbf{0.001}$
	Interaction (1.2)	$F_{3,24} = 0.25$, $P = 0.863$	$F_{3,22} = 1.23$, $P = 0.323$	$F_{3,22} = 0.05$, $P = 0.986$	$F_{3,20} = \mathbf{3.31}$, $P = \mathbf{0.041}$
SA	Treatment (1)	$F_{1,24} = 3.00$, $P = 0.096$	$F_{1,23} = \mathbf{5.82}$, $P = \mathbf{0.024}$	$F_{1,24} = 2.43$, $P = 0.133$	$F_{1,21} = \mathbf{4.31}$, $P = \mathbf{0.05}$
	Time (2)	$F_{3,24} = 0.79$, $P = 0.512$	$F_{3,23} = \mathbf{3.58}$, $P = \mathbf{0.03}$	$F_{3,24} = 1.88$, $P = 0.16$	$F_{3,21} = \mathbf{5.31}$, $P = \mathbf{0.007}$
	Interaction (1.2)	$F_{3,24} = 0.41$, $P = 0.745$	$F_{3,23} = 0.43$, $P = 0.736$	$F_{3,24} = 0.83$, $P = 0.492$	$F_{3,21} = 1.09$, $P = 0.376$
Ethephon	Treatment (1)	$F_{1,24} = 1.46$, $P = 0.239$	$F_{1,24} = 0.92$, $P = 0.346$	$F_{1,22} = \mathbf{29.16}$, $P < \mathbf{0.001}$	$F_{1,24} = 1.89$, $P = 0.183$
	Time (2)	$F_{3,24} = 2.43$, $P = 0.090$	$F_{3,24} = 0.38$, $P = 0.766$	$F_{3,22} = 1.94$, $P = 0.153$	$F_{3,24} = 2.27$, $P = 0.106$
	Interaction (1.2)	$F_{3,24} = 2.48$, $P = 0.085$	$F_{3,24} = 0.06$, $P = 0.979$	$F_{3,22} = 1.42$, $P = 0.262$	$F_{3,24} = 2.24$, $P = 0.11$

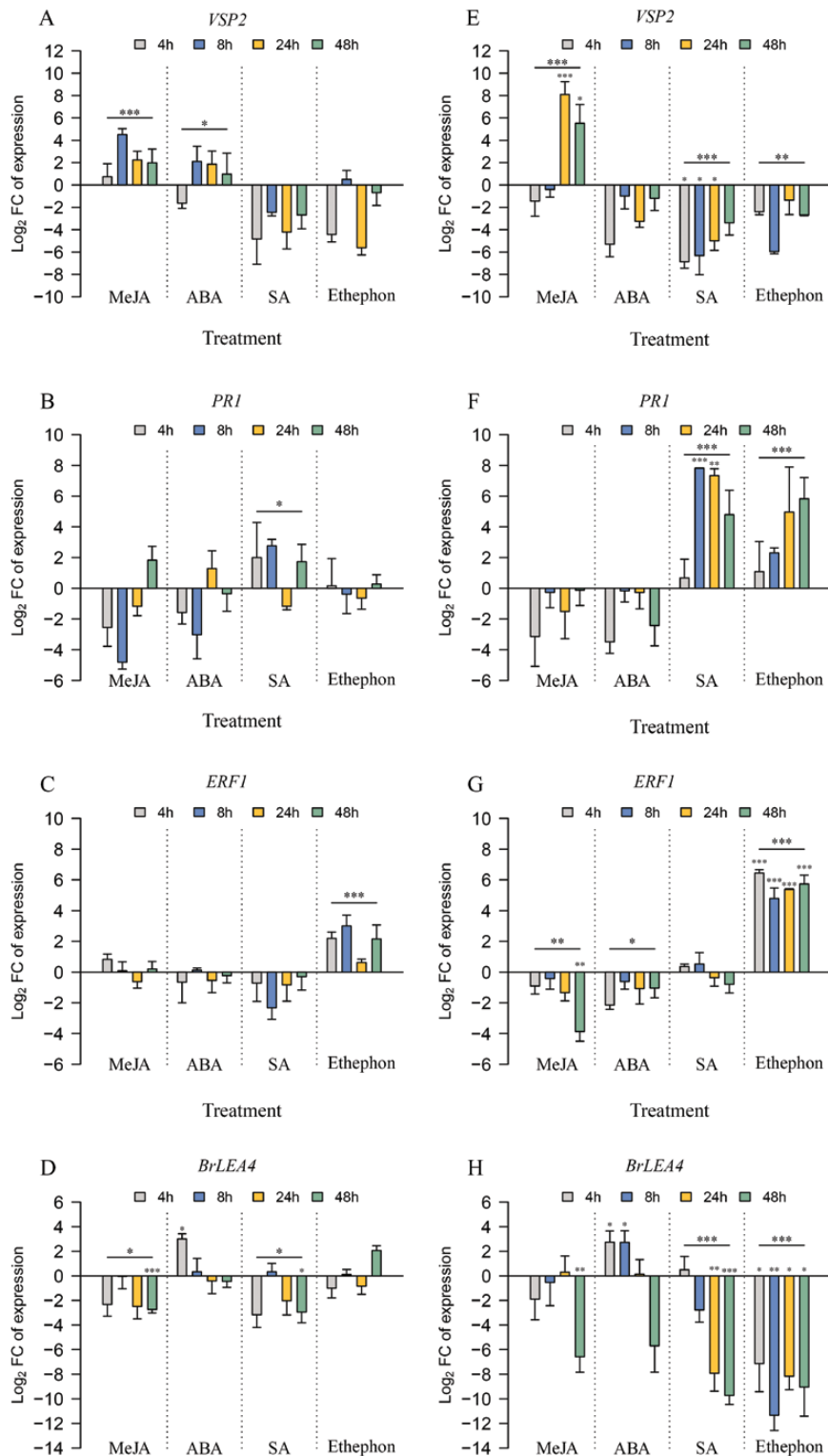


Figure 1. Relative expression of the hormonal-related marker genes in *Brassica rapa* shoots (left column, panels A–D) and roots (right column, panels E–H) in response to hormonal application. Expression levels of (A, E) *VSP2*, (B, F) *PR1*, (C, G) *ERF1* and (D, H) *BrLEA4* were measured at 4, 8, 24 and 48 h after local MeJA, ABA, SA or ethephon application. Data were normalized over the housekeeping gene *TIP41*, and are represented as mean log₂ fold changes (log₂ FC + SE) in relation to the respective control. In each hormonal treatment, asterisks over the horizontal line represent the overall significant treatment main effect and those over individual bars indicate significant differences between the treatment group and their respective control plants, according to two-way ANOVA ($n = 3\text{--}4$ per treatment and harvest time) * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 2, Fig. 1A), as well as by ethephon 4 and 24 h after application (interaction effect, $F_{3,24} = 2.48$, $P = 0.085$, Table 2, Fig. 1A). In *B. rapa* roots, *VSP2* expression was significantly up-regulated specifically in response to MeJA application. The effect of MeJA application on *VSP2* expression was time-dependent (interaction effect, $F_{3,22} = 6.07$, $P = 0.004$, Table 3), with statistically significant up-regulation at 24 and 48 h after treatment (Tukey *post hoc* test, $P = 0.001$ and $P = 0.029$, respectively, Fig. 1E). By contrast, SA and ethephon application significantly down-regulated *VSP2* expression (two-way ANOVA, Table 3, Fig. 1E). Although ABA application had no significant effect on *VSP2* expression, there was a trend for down-regulation of this gene in ABA-treated roots (treatment effect, $F_{1,19} = 3.46$, $P = 0.078$, Table 3).

PR1 expression was significantly up-regulated in SA-treated shoots compared to control (treatment effect, $F_{1,23} = 5.82$, $P = 0.024$, Table 2, Fig. 1B). Methyl jasmonate, ABA or ethephon application had no significant effect on *PR1* expression (treatment effect, $F_{1,22} = 0.24$, $P = 0.63$; $F_{1,22} = 0.16$, $P = 0.692$ and $F_{1,24} = 0.92$, $P = 0.346$, respectively, Table 2, Fig. 1B). In the roots, expression of *PR1* was strongly up-regulated in response to SA application (treatment effect, $F_{1,20} = 45.69$, $P < 0.001$, Table 3, Fig. 1F). The effect of SA treatment on *PR1* expression in the roots was time-dependent (interaction effect, $F_{3,20} = 3.56$, $P = 0.033$, Table 3), with a significant up-regulation at 8 and 24 h after application compared to

control plants (Tukey *post hoc* test, 8 h: $P = 0.001$; 24 h: $P = 0.002$). Interestingly, ethephon application also up-regulated the expression of *PR1* in roots, irrespective of the time after treatment (treatment effect, $F_{1,22} = 14.97$, $P = 0.001$; interaction effect, $F_{3,22} = 1.15$, $P = 0.35$, Table 3, Fig. 1F). Methyl jasmonate nor ABA application significantly affected *PR1* expression in roots (treatment effect, $F_{1,22} = 0.00$, $P = 0.964$ and $F_{1,22} = 0.13$, $P = 0.722$, respectively, Table 3, Fig. 1F).

In the shoots, expression of *ERF1* was significantly up-regulated by ethephon treatment (treatment effect, $F_{1,22} = 29.16$, $P < 0.001$, Table 2, Fig. 1C). Application of MeJA, ABA or SA had no significant effect on *ERF1* shoot expression (treatment effect, $F_{1,24} = 0.95$, $P = 0.34$; $F_{1,22} = 0.12$, $P = 0.73$ and $F_{1,24} = 2.43$, $P = 0.133$, respectively, Table 2, Fig. 1C). Also in the roots, *ERF1* was significantly up-regulated by ethephon application (treatment effect, $F_{1,20} = 234.81$, $P < 0.001$, Table 3, Fig. 1G). Interestingly, local *ERF1* expression was significantly down-regulated by MeJA and ABA application to the roots (treatment effect, $F_{1,19} = 12.84$, $P = 0.002$ and $F_{1,20} = 6.72$, $P = 0.017$, respectively, Table 3, Fig. 1G). The down-regulation of *ERF1* by MeJA was time-dependent (interaction effect, $F_{3,19} = 4.34$, $P = 0.017$, Table 3, Fig. 1G) and was significant 48 h after the treatment (Tukey *post hoc* test, 48 h: $P = 0.002$). Application of SA did not significantly affect *ERF1* expression in roots (treatment effect, $F_{1,18} = 0.02$, $P = 0.888$, Table 3).

Table 3. Statistical analyses (*F*- and *P*-values) of the effects of local hormonal application on gene expression levels in *Brassica rapa* roots. The expression levels of *VSP2*, *PR1*, *ERF1* and *BrLEA4* were measured in *B. rapa* roots after MeJA, ABA, SA or ethephon application to the roots ($n = 3-4$ per treatment and harvest time). The data were analysed per hormonal treatment group using a two-way ANOVA model containing treatment (control, hormonal application), time (4, 8, 24, 48 h) and their interaction term as factors. Statistically significant effects ($P \leq 0.05$) are indicated in bold.

Treatment	Factor	Gene			
		<i>VSP2</i>	<i>PR1</i>	<i>ERF1</i>	<i>BrLEA4</i>
MeJA	Treatment (1)	$F_{1,22} = 22.57$, $P < 0.001$	$F_{1,22} = 0.00$, $P = 0.964$	$F_{1,19} = 12.84$, $P = 0.002$	$F_{1,23} = 0.88$, $P = 0.358$
	Time (2)	$F_{3,22} = 2.19$, $P = 0.117$	$F_{3,22} = 0.57$, $P = 0.639$	$F_{3,19} = 12.16$, $P < 0.001$	$F_{3,23} = 4.61$, $P = 0.011$
	Interaction (1.2)	$F_{3,22} = 6.07$, $P = 0.004$	$F_{3,22} = 0.36$, $P = 0.786$	$F_{3,19} = 4.34$, $P = 0.017$	$F_{3,23} = 3.24$, $P = 0.04$
ABA	Treatment (1)	$F_{1,19} = 3.46$, $P = 0.078$	$F_{1,22} = 0.13$, $P = 0.722$	$F_{1,20} = 6.72$, $P = 0.017$	$F_{1,22} = 2.5$, $P = 0.128$
	Time (2)	$F_{3,19} = 8.2$, $P = 0.001$	$F_{3,22} = 0.50$, $P = 0.684$	$F_{3,20} = 3$, $P = 0.06$	$F_{3,22} = 4.03$, $P = 0.02$
	Interaction (1.2)	$F_{3,19} = 0.59$, $P = 0.626$	$F_{3,22} = 1.23$, $P = 0.322$	$F_{3,20} = 0.33$, $P = 0.805$	$F_{3,22} = 6.02$, $P = 0.004$
SA	Treatment (1)	$F_{1,20} = 25.4$, $P < 0.001$	$F_{1,20} = 45.69$, $P < 0.001$	$F_{1,18} = 0.02$, $P = 0.888$	$F_{1,23} = 21.68$, $P < 0.001$
	Time (2)	$F_{3,20} = 5.51$, $P = 0.006$	$F_{3,20} = 1.31$, $P = 0.299$	$F_{3,18} = 8.25$, $P = 0.001$	$F_{3,23} = 8.75$, $P < 0.001$
	Interaction (1.2)	$F_{3,20} = 0.75$, $P = 0.538$	$F_{3,20} = 3.56$, $P = 0.033$	$F_{3,18} = 0.94$, $P = 0.444$	$F_{3,23} = 9.5$, $P < 0.001$
Ethephon	Treatment (1)	$F_{1,18} = 9.62$, $P = 0.006$	$F_{1,22} = 14.97$, $P = 0.001$	$F_{1,20} = 234.81$, $P < 0.001$	$F_{1,24} = 43.49$, $P < 0.001$
	Time (2)	$F_{3,18} = 0.97$, $P = 0.43$	$F_{3,22} = 0.63$, $P = 0.606$	$F_{3,20} = 5.54$, $P = 0.006$	$F_{3,24} = 4.45$, $P = 0.013$
	Interaction (1.2)	$F_{3,18} = 1.65$, $P = 0.213$	$F_{3,22} = 1.15$, $P = 0.35$	$F_{3,20} = 0.72$, $P = 0.549$	$F_{3,24} = 0.81$, $P = 0.503$

In shoots, the overall expression of *BrLEA4* was not significantly affected by ABA treatment, though there was a trend for up-regulation (treatment effect, $F_{1,20} = 3.83$, $P = 0.064$, Table 2, Fig. 1D). Although *BrLEA4* was specifically up-regulated at 4 h after ABA application (one-way ANOVA, $F_{1,6} = 7.92$, $P = 0.031$), at later time points, *BrLEA4* expression returned to basal levels. Shoot treatment with ethephon did not have any significant effect on the *BrLEA4* expression (treatment effect, $F_{1,24} = 1.89$, $P = 0.183$, Table 2, Fig. 1D). However, MeJA and SA treatments significantly down-regulated the expression of this gene in *B. rapa* shoots (two-way ANOVA, Table 2, Fig. 1D). In *B. rapa* roots, expression of *BrLEA4* was significantly up-regulated by ABA treatment at 4 and 8 h after application (one-way ANOVA, 4 h: $F_{1,6} = 7.5$, $P = 0.034$; 8 h: $F_{1,6} = 6.19$, $P = 0.047$), while at 48 h there was a trend for down-regulation of this gene (one-way ANOVA, $F_{1,4} = 6.65$, $P = 0.061$, Fig. 1H). By contrast, SA and ethephon treatments overall significantly down-regulated *BrLEA4* expression compared to their respective controls (treatment effect, $F_{1,23} = 21.68$, $P < 0.001$ and $F_{1,24} = 43.49$, $P < 0.001$, respectively, Table 3, Fig. 1H). The effect of SA treatment on *BrLEA4* expression was time-dependent (interaction effect, $F_{3,23} = 9.5$, $P < 0.001$, Table 3, Fig. 1H), showing a significant down-regulation at 24 and 48 h after the treatment (Tukey *post hoc* test, 24 h: $P = 0.006$; 48 h: $P < 0.001$, Fig. 1H). The effect of MeJA application on *BrLEA4* expression was time-dependent as well (interaction effect, $F_{3,23} = 3.24$,

$P = 0.04$, Table 3, Fig. 1H). *BrLEA4* expression was significantly down-regulated by MeJA application at 48 h (one-way ANOVA, $F_{1,5} = 18.15$, $P = 0.008$).

Effect of hormonal application on phytohormone levels in *B. rapa* shoots and roots

In *B. rapa* shoots, JA levels were significantly increased by MeJA application compared to control plants (treatment effect, $F_{1,19} = 485.13$, $P < 0.001$, Table 4, Fig. 2A). Salicylic acid application, on the other hand, significantly reduced JA levels (treatment effect, $F_{1,22} = 5.81$, $P = 0.025$), while neither ABA nor ethephon application had significant effects on JA levels in shoots (treatment effect, $F_{1,22} = 0.57$, $P = 0.458$ and $F_{1,24} = 2.23$, $P = 0.148$, respectively, Table 4, Fig. 2A). In *B. rapa* roots, a similar pattern emerged: MeJA application significantly increased JA levels, while ABA, SA or ethephon application did not have any effect on root JA levels when compared to the respective control plants (treatment effect, $F_{1,22} = 1240.19$, $P < 0.001$; $F_{1,21} = 2.34$, $P = 0.141$; $F_{1,23} = 0.89$, $P = 0.355$ and $F_{1,23} = 2.08$, $P = 0.163$, respectively, Table 5, Fig. 2D). Shoot SA levels were significantly increased by SA treatment (treatment effect, $F_{1,19} = 912.11$, $P < 0.001$, Table 4, Fig. 2B). Methyl jasmonate, ABA or ethephon application had no significant effect on SA shoot levels (treatment effect, $F_{1,22} = 1.96$, $P = 0.175$; $F_{1,22} = 1.93$, $P = 0.179$ and $F_{1,24} = 0.03$, $P = 0.873$, respectively, Table 4, Fig. 2B). In roots, SA levels were also significantly increased in response to SA application, while

Table 4. Effects of local hormonal application on phytohormone levels in *Brassica rapa* shoots. The levels of JA, SA and ABA were measured at 4, 8, 24 and 48 h after MeJA, ABA, SA and ethephon application. For each measured phytohormone, the data were analysed per treatment using a two-way ANOVA model containing treatment (control, hormonal application), time (4, 8, 24, 48 h) and their interaction term as factors ($n = 3-4$ per treatment and harvest time). Statistically significant effects ($P \leq 0.05$) are indicated in bold.

Treatment	Factor	Measured phytohormone		
		JA	SA	ABA
MeJA	Treatment (1)	$F_{1,19} = 485.13$, $P < 0.001$	$F_{1,22} = 1.96$, $P = 0.175$	$F_{1,21} = 22.82$, $P < 0.001$
	Time (2)	$F_{3,19} = 68.46$, $P < 0.001$	$F_{3,22} = 17.41$, $P < 0.001$	$F_{3,21} = 7.67$, $P = 0.001$
	Interaction (1.2)	$F_{3,19} = 35.80$, $P < 0.001$	$F_{3,22} = 4.00$, $P = 0.02$	$F_{3,21} = 1.22$, $P = 0.327$
ABA	Treatment (1)	$F_{1,22} = 0.57$, $P = 0.458$	$F_{1,22} = 1.93$, $P = 0.179$	$F_{1,20} = 619.21$, $P < 0.001$
	Time (2)	$F_{3,22} = 2.69$, $P = 0.071$	$F_{3,22} = 7.87$, $P = 0.001$	$F_{3,20} = 80.47$, $P < 0.001$
	Interaction (1.2)	$F_{3,22} = 0.19$, $P = 0.902$	$F_{3,22} = 2.58$, $P = 0.079$	$F_{3,20} = 75.15$, $P < 0.001$
SA	Treatment (1)	$F_{1,22} = 5.81$, $P = 0.025$	$F_{1,19} = 912.11$, $P < 0.001$	$F_{1,21} = 15.09$, $P = 0.001$
	Time (2)	$F_{3,22} = 2.22$, $P = 0.114$	$F_{3,19} = 39.42$, $P < 0.001$	$F_{3,21} = 3.7$, $P = 0.028$
	Interaction (1.2)	$F_{3,22} = 0.29$, $P = 0.834$	$F_{3,19} = 13.89$, $P < 0.001$	$F_{3,21} = 0.6$, $P = 0.62$
Ethephon	Treatment (1)	$F_{1,24} = 2.23$, $P = 0.148$	$F_{1,24} = 0.03$, $P = 0.873$	$F_{1,24} = 10.93$, $P = 0.003$
	Time (2)	$F_{3,24} = 1.22$, $P = 0.323$	$F_{3,24} = 1.14$, $P = 0.353$	$F_{3,24} = 2.18$, $P = 0.117$
	Interaction (1.2)	$F_{3,24} = 0.34$, $P = 0.794$	$F_{3,24} = 0.34$, $P = 0.798$	$F_{3,24} = 1.94$, $P = 0.149$

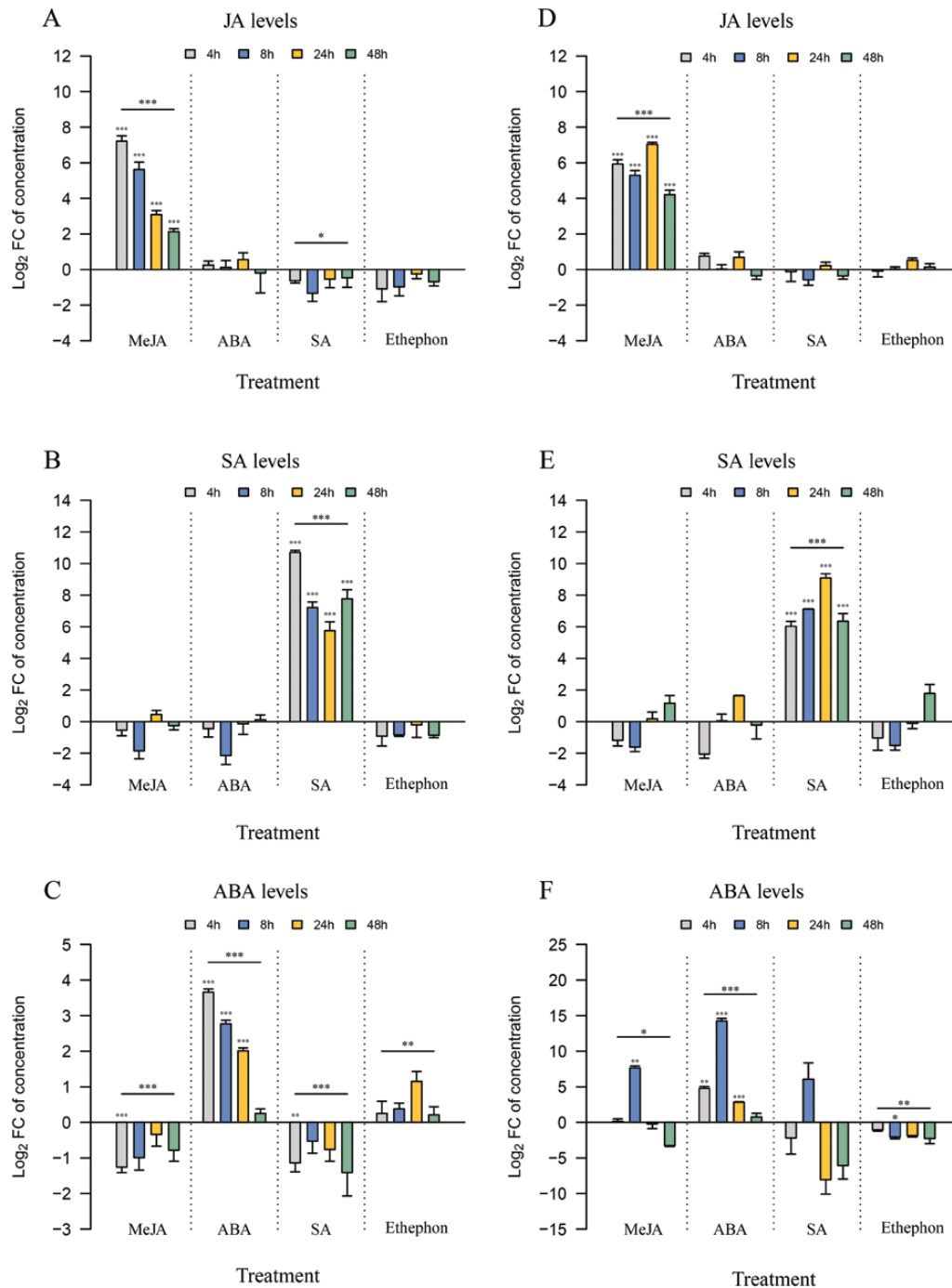


Figure 2. Phytohormone levels in *Brassica rapa* shoots (left column, panels A–C) and roots (right column, panels D–F) in response to hormonal application. The levels of (A, D) JA, (B, E) SA and (C, F) ABA were measured at 4, 8, 24 and 48 h after local MeJA, ABA, SA or ethephon application. Bars represent \log_2 fold changes (\log_2 FC + SE) of concentrations in relation to the respective control. In each hormonal treatment, asterisks over the horizontal line represent the overall significant treatment main effect and those over individual bars indicate significant differences between the treatment group and their respective control plants, according to two-way ANOVA ($n = 3\text{--}4$ per treatment and harvest time, except for ABA-treated roots at 24 h where $n = 2$) * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

MeJA, ABA or ethephon application had no significant effect on root SA levels (treatment effect, $F_{1,23} = 677.76$, $P < 0.001$; $F_{1,22} = 0.33$, $P = 0.573$; $F_{1,21} = 0.18$, $P = 0.679$ and $F_{1,23} = 0.07$, $P = 0.793$, respectively, Table 5, Fig. 2E).

Shoot ABA levels were significantly increased in response to both ABA or ethephon application (treatment effect, $F_{1,20} = 619.21$, $P < 0.001$ and $F_{1,24} = 10.93$, $P = 0.003$, respectively, Table 4, Fig. 2C). The effect of

Table 5. Effects of local hormonal application on phytohormone levels in *Brassica rapa* roots. The levels of JA, SA and ABA were measured at 4, 8, 24 and 48 h after MeJA, ABA, SA and ethephon application. For each measured phytohormone, the data were analysed per treatment using a two-way ANOVA model containing treatment (control, hormonal application), time (4, 8, 24, 48 h) and their interaction term as factors ($n = 3-4$ per treatment and harvest time, except for ABA treatment at 24 h where $n = 2$). Statistically significant effects ($P \leq 0.05$) are indicated in bold.

Treatment	Factor	Measured phytohormone		
		JA	SA	ABA
MeJA	Treatment (1)	$F_{1,22} = 1240.19, P < 0.001$	$F_{1,22} = 0.33, P = 0.573$	$F_{1,18} = 7.07, P = 0.016$
	Time (2)	$F_{3,22} = 8.59, P < 0.001$	$F_{3,22} = 3.07, P = 0.049$	$F_{3,18} = 16.12, P < 0.001$
	Interaction (1.2)	$F_{3,22} = 14.17, P < 0.001$	$F_{3,22} = 4.05, P = 0.02$	$F_{3,18} = 9.83, P < 0.001$
ABA	Treatment (1)	$F_{1,21} = 2.34, P = 0.14$	$F_{1,21} = 0.18, P = 0.679$	$F_{1,19} = 98.24, P < 0.001$
	Time (2)	$F_{3,21} = 1.69, P = 0.199$	$F_{3,21} = 2.98, P = 0.055$	$F_{3,19} = 7.69, P = 0.001$
	Interaction (1.2)	$F_{3,21} = 2.77, P = 0.067$	$F_{3,21} = 3.11, P = 0.048$	$F_{3,19} = 20.21, P < 0.001$
SA	Treatment (1)	$F_{1,23} = 0.89, P = 0.355$	$F_{1,23} = 677.76, P < 0.001$	$F_{1,21} = 2.22, P = 0.151$
	Time (2)	$F_{3,23} = 3.48, P = 0.032$	$F_{3,23} = 9.88, P < 0.001$	$F_{3,21} = 2.76, P = 0.068$
	Interaction (1.2)	$F_{3,23} = 0.67, P = 0.581$	$F_{3,23} = 4.90, P = 0.009$	$F_{3,21} = 4.44, P = 0.015$
Ethephon	Treatment (1)	$F_{1,23} = 2.08, P = 0.163$	$F_{1,23} = 0.07, P = 0.793$	$F_{1,21} = 10.21, P = 0.004$
	Time (2)	$F_{3,23} = 1.77, P = 0.18$	$F_{3,23} = 3.08, P = 0.048$	$F_{3,21} = 3.02, P = 0.053$
	Interaction (1.2)	$F_{3,23} = 0.47, P = 0.71$	$F_{3,23} = 2.64, P = 0.074$	$F_{3,21} = 0.14, P = 0.935$

ABA application on ABA levels was time-dependent (interaction effect, $F_{3,20} = 75.15, P < 0.001$, Table 4), with statistically significant increases at 4, 8 and 24 h after treatment compared to the respective control plants (Tukey *post hoc* test, $P < 0.001$ for each time point, Fig. 2C). Methyl jasmonate and SA shoot application significantly reduced ABA levels (treatment effect, $F_{1,21} = 22.82, P < 0.001$ and $F_{1,21} = 15.09, P = 0.001$, respectively, Table 4, Fig. 2C). In roots, as expected, ABA levels were significantly increased in response to ABA application, but in contrast to shoots also after MeJA application (treatment effect, $F_{1,19} = 98.24, P < 0.001$ and $F_{1,18} = 7.07, P = 0.016$, respectively, Table 5,

Fig. 2F). The effect of both ABA and MeJA application on ABA levels was time-dependent (interaction effect, $F_{3,19} = 20.21, P < 0.001$ and $F_{3,18} = 9.83, P < 0.001$, respectively, Table 5, Fig. 2F). Abscisic acid-treated roots showed significant increases in ABA levels at 4, 8 and 24 h after the treatment (Tukey *post hoc* test, $P = 0.013, P < 0.001$ and $P = 0.034$, respectively, Fig. 2F), whereas MeJA application increased ABA levels at 8 h after the treatment compared to control plants (Tukey *post hoc* test, $P = 0.003$, Fig. 2F). Salicylic acid root application had no significant effect on ABA levels, whereas ethephon application significantly reduced ABA levels compared to the respective control plants (treatment

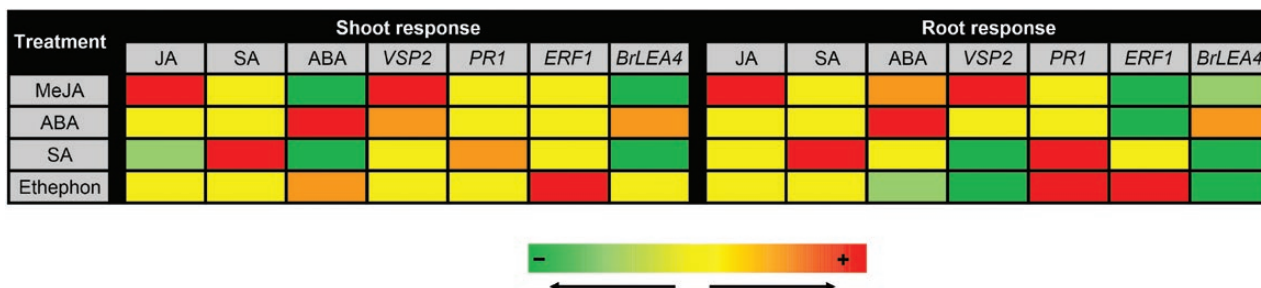


Figure 3. Summarizing scheme of the changes in the phytohormone levels and hormonal-related marker genes in *Brassica rapa* shoots and roots after local hormonal elicitation. Light green indicates reduction/down-regulation and dark green indicates a strong reduction/down-regulation of phytohormone/gene expression levels measured in the same treated organ. Orange indicates increase/up-regulation of phytohormone/gene expression levels and a strong increase/up-regulation is indicated in red. Yellow indicates no changes compared to the respective control group.

effect, $F_{1,21} = 2.22$, $P = 0.151$ and $F_{1,21} = 10.21$, $P = 0.004$, respectively, [Table 5](#), [Fig. 2F](#)).

Discussion

Over recent years, many experimental studies have greatly improved our understanding of hormonal-mediated regulation of plant immunity. These studies also identified marker genes for the main defence-related hormonal pathways. However, most of the knowledge on plant immunity and molecular markers is restricted to shoot organs, and in a great extent to the *Arabidopsis* model. In this study, we found that while the majority of the widely used marker genes are regulated by hormonal elicitation in *B. rapa* similarly to *Arabidopsis*, some of the markers are not unique to a single pathway ([Fig. 3](#)). Moreover, our data demonstrate that the response of the marker genes to certain hormonal pathways further differs between roots and shoots.

In line with previous studies in *Arabidopsis* we found that *VSP2* was up-regulated in shoots by local MeJA elicitation. This is showing that *VSP2* is a marker gene of the ABA-regulated MYC-branch of the JA pathway ([Anderson et al. 2004](#); [Lorenzo et al. 2004](#); [Verhage et al. 2011](#); [Vos et al. 2013](#)). Further studies using *Brassica* species revealed that *VSP2* is also up-regulated by elicitation with JA or MeJA in the shoots ([Abe et al. 2011](#); [Mathur et al. 2013](#); [Tytgat et al. 2013](#); [Lee and Hong 2015](#)), indicating that *VSP2* responsiveness to JA is conserved among different plant species of the Brassicaceae. Interestingly, we found that ABA shoot application similarly induced the up-regulation of *VSP2* without affecting JA accumulation, indicating that in *B. rapa*, *VSP2* expression can be elicited independently of JA accumulation in the shoot. Consequently, studies investigating the mechanisms underlying herbivore- or pathogen-induced responses in *Brassica* shoots should consider that *VSP2* up-regulation can be associated with the activation of not only JA-dependent but also of ABA-dependent defences, whereby the latter may be independent of JA accumulation.

In contrast to shoot analyses, those of roots showed that MeJA application resulted in both JA and ABA accumulation and lead to *VSP2* up-regulation. On the other hand, elicitation with ABA and accumulation of ABA alone (without increasing JA levels) did not affect *VSP2* regulation. Taken together, these results indicate that *VSP2* is a more suitable and exclusive marker gene for the JA pathway in *B. rapa* roots than in shoots. Our findings were unexpected as a previous study showed that *VSP2* expression was not affected in *B. oleracea* roots at 6, 18 or 30 h after JA application ([Tytgat et al. 2013](#)). The differences between our findings and those

of [Tytgat et al. \(2013\)](#) are probably due to differences in phytohormone concentrations and mode of application. Interestingly, we found a temporal delay in the induction of *VSP2* in roots compared to that in shoots, even though JA levels increased similarly within 4 h in both organs. This raises the question whether roots and shoots process increases in JA levels differently, e.g. by making different variants of the many possible conjugates ([Erb and Glauser 2010](#)).

Expression analysis of *PR1* in *B. rapa* shoots confirmed that this gene is a unique marker gene for the SA pathway. It was induced specifically after activation of the SA pathway and not by the JA, ABA or ET pathways. Our results are in line with studies in *Arabidopsis* and *Brassica* spp. shoots showing that *PR1* is a suitable marker gene for the SA pathway ([Zhang et al. 2003](#); [Nobuta et al. 2007](#); [Abe et al. 2011](#); [Lee and Hong 2015](#)). Moreover, *PR1* is also a widely used marker for the SA pathway in tomato, maize or soybean, among other species ([Martínez-Medina et al. 2013](#); [Fernández et al. 2014](#)). Surprisingly, and in contrast to shoot behaviour, our results showed that in *B. rapa* roots *PR1* was induced not only in response to SA treatment, but also after ethephon application even though the latter did not affect SA levels. These findings indicate that *PR1* induction in the roots does not necessarily require SA accumulation, supporting the notion that shoots and roots respond differently to specific hormonal pathways. Studies on the interactions between plants and soil biota often assess changes in the *PR1* expression as a marker for the activation of the SA pathway, following the paradigms based on shoot-derived data ([Paparú et al. 2007](#); [Chen et al. 2016](#); [Martínez-Medina et al. 2017a, b](#)). Although ethephon could have an effect that is independent of ET ([Lawton et al. 1994](#)), our results suggest that specifically in *B. rapa*, *PR1* may also respond to ET elicitation.

Our data also demonstrated that *ERF1* is a suitable marker gene for the ET pathway in *B. rapa* shoots as well as in roots. We found *ERF1* up-regulation specifically in ethephon-treated shoots, where surprisingly, ABA levels were also increased. However, ABA accumulation alone was not sufficient to induce *ERF1* expression, as the expression of this gene was not affected in ABA-treated shoots. *ERF1* is a widely used marker gene for ET pathway in different plant species, such as tomato, legumes and *Arabidopsis* ([Anderson et al. 2010](#); [Tian et al. 2014](#); [Broekgaarden et al. 2015](#); [Huang et al. 2015](#)). Studies on *Arabidopsis* shoots have shown that *ERF1* can be activated by the ET or the JA pathway, or synergistically by both phytohormones. Jasmonic acid-induced *ERF1* expression is associated with activation of the ERF-branch of the JA pathway, which is co-regulated by ET ([Penninckx et al. 1998](#); [Solano et al. 1998](#); [Lorenzo](#)

et al. 2003; Robert-Seilaniantz et al. 2011; Pieterse et al. 2012). In *Arabidopsis*, ABA is known to act antagonistically on the ERF-branch of the JA pathway (Lorenzo et al. 2004; Verhage et al. 2011; Vos et al. 2013). However, the fact that we found *ERF1* up-regulation together with increased ABA levels in ethephon-treated shoots suggests that ABA might not have an antagonistic effect on the ERF-branch of the JA pathway in *B. rapa* shoots, as it does in *Arabidopsis* shoots. In contrast, ethephon application to roots decreased ABA levels and led to *ERF1* up-regulation, indicating that ABA and ET indeed may act antagonistically on *ERF1* expression in *B. rapa* roots.

We further tested *BrLEA4* as a novel marker gene for the ABA pathway in *B. rapa*. We showed that *BrLEA4* is an early ABA-inducible gene in *B. rapa* shoots and roots. *LEA* genes, encoding for late-embryogenesis abundant proteins, are used as molecular markers for the ABA pathway in different plant species, such as soybean, tomato, maize and other (Martínez-Medina et al. 2013; Fernández et al. 2014; Zamora-Briseño and de Jiménez 2016). Among different *LEA* genes, *LEA4* was shown to be highly ABA inducible in vegetative tissues of *Arabidopsis* and *Brassica* spp. (Hoth et al. 2002; Dalal et al. 2009). Remarkably, we found that in both shoots and roots, JA or SA accumulation down-regulated *BrLEA4* expression. In the shoots this coincided with suppressed ABA levels, further suggesting that ABA accumulation is required for *BrLEA4* induction in shoots. Interestingly, ethephon application to the roots, which suppressed local ABA levels, also resulted in *BrLEA4* down-regulation. Taken together, our findings suggest that *BrLEA4* is a suitable marker gene for ABA pathway in *B. rapa* shoots and roots. In addition, our results showed that elicitation of the JA or the SA pathway in shoots and the JA, SA or ET pathways in roots suppresses the induction of this gene in *B. rapa*, further supporting the notion that *BrLEA4* is a unique marker gene for the ABA pathway. Based on our results we therefore suggest *VSP2* as a marker gene for the JA or ABA pathways, *PR1* as a marker gene for the SA pathway, *ERF1* for the ET pathway and *BrLEA4* for the ABA pathway in *B. rapa* shoots. For *B. rapa* roots, we propose *VSP2* as a marker gene for the JA pathway, *PR1* for the SA or ET pathways, *ERF1* for the ET pathway and *BrLEA4* for the ABA pathway.

In addition to the effects of single hormonal applications on specific marker gene responses, we also found evidence for hormonal crosstalk. For example, SA application reduced JA levels, especially in shoots. This may have been the reason that *VSP2* was down-regulated in shoots and roots of SA-treated *B. rapa* (Koornneef et al. 2008; Pieterse et al. 2009). Methyl jasmonate application, on the other hand, only mildly repressed SA or *PR1* expression. Jasmonic acid–salicylic acid negative crosstalk is one of the best-described hormonal interaction

processes (Pieterse et al. 2009). However, the nature of the interaction is highly dynamic and may also involve ET (Koornneef et al. 2008; Leon-Reyes et al. 2010). The negative crosstalk between ABA and ET is also well described, especially in the context of regulating abiotic stress responses. Increases in ABA levels lead to stomatal closure, which is a functional adaptation to drought stress (Nguyen et al. 2016). Ethylene, on the other hand, is involved in responses to flooding and waterlogging. Its production may result in the formation of adventitious root aerenchyma to overcome anoxia or quiescence responses that help the plant to survive while (partly) under water. In response to complete flooding, ET may also stimulate shoot elongation to ensure shoot contact with the air before asphyxiating (Pierik et al. 2006). We found evidence for negative ET–ABA crosstalk in the roots of *B. rapa* since ethephon treatment reduced ABA levels and increased *ERF1* expression. Interestingly, in the shoots we observed that ethephon treatment enhances ABA levels. Despite this increase in shoot ABA, *ERF1* expression was increased by ethephon application as well, but not as strongly as in the roots. Possibly these differences between root and shoot hormonal responses are related to differences in the interactions they experience in their natural environment. In general, hormonal crosstalk is very dynamic and complex. Future studies, for example with combined applications of multiple phytohormones (Koornneef et al. 2008), could shed more light on the nature of hormonal crosstalk processes in *B. rapa*.

Conclusions

Although most of the *Arabidopsis*-derived marker genes tested in this study are also suitable markers in *B. rapa*, some e.g., *VSP2* and *PR1* fail to show specificity for one pathway. Furthermore, we demonstrated that the responsiveness of some marker genes to specific phytohormones is organ specific since roots behaved differently to shoots. Consequently, plant organ should be taken into consideration in marker gene selection. Overall, our findings suggest that the link between marker gene expression profiles and the activation of specific hormone-inducible pathways should be interpreted with caution. It is therefore advisable to combine analyses of multiple marker genes with those of phytohormone levels to ascertain more certainly which hormonally regulated defence pathways are activated.

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Contributions by the Authors

G.V.P., A.M.-M. and N.M.V.D. designed the research; G.V.P. performed the experiments; G.V.P. and A.M. performed qRT-PCR analysis; G.V.P. and K.G. analysed phytohormone levels; G.V.P. performed the statistical analyses; G.V.P., A.M.-M. and N.M.V.D. executed data interpretation and wrote the manuscript with input from all the authors.

Conflict of Interest

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Figure S1. Amplification efficiencies of the primers used in this study.

Table S1. Relative expression (SE) of marker genes in *Brassica rapa* shoots in response to hormonal application to the shoots.

Table S2. Relative expression (SE) of marker genes in *Brassica rapa* roots in response to hormonal application to the roots.

Table S3. Levels of phytohormones (SE) in *Brassica rapa* shoots and roots in response to hormonal application.

Table S4. *F*- and *P*-values of a two-way ANOVA model on gene expression levels in *Brassica rapa* shoots and roots.

Table S5. *F*- and *P*-values of a two-way ANOVA model on the phytohormone levels in *Brassica rapa* shoots and roots.

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