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Rapid report

Direct and individual analysis of stress-related phytohormone dispersion in the vascular system of *Cucurbita maxima* after flagellin 22 treatment

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Summary

- The stress-related phytohormones, salicylic acid (SA) and abscisic acid (ABA), and the three jasmonates, jasmonic acid (JA), *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), were investigated in phloem and xylem exudates of *Cucurbita maxima*.
- Phloem and xylem exudates were separately collected and analysed via liquid chromatography–mass spectrometry.
- We show direct evidence for all three jasmonates, ABA, and SA in both phloem and xylem exudates of *C. maxima*. JA and JA-Ile concentrations are higher in xylem (JA: $c_{xylem} \approx 199.5$ nM, $c_{phloem} \approx 43.9$ nM; JA-Ile: $c_{xylem} \approx 7.9$ nM, $c_{phloem} \approx 1.6$ nM), whereas ABA and SA concentrations are higher in phloem exudates (ABA: $c_{xylem} \approx 37.1$ nM, $c_{phloem} \approx 142.6$ nM; SA: $c_{xylem} \approx 61.6$ nM, $c_{phloem} \approx 1319$ nM). During bacteria-derived flagellin 22 (flg22)-triggered remote root-to-shoot signalling, phytohormone concentration changed rapidly both in phloem and xylem.
- The unequal distribution of phytohormones suggests that phloem and xylem have distinct roles in defence responses. Our data shed light on systemic phytohormone signalling and help explain how plants cope with environmental challenges by lateral exchange between phloem and xylem. Our analysis is a starting point for further investigations of how phytohormones contribute to phloem- and xylem-based defence signalling.

Introduction

Various phytohormones have been identified as important components of signalling cascades in plant development and plant response to various (a) biotic challenges (Beckers & Spoel, 2005; Pozo *et al.*, 2005; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). Abscisic acid (ABA) is a well-known root-to-shoot signal produced in response to abiotic stress in the form of drought, heat, cold, salt or heavy metals (Christmann *et al.*, 2006; Zhang *et al.*, 2008), though it is also involved in the stress response to microbes (De Vleesschauwer *et al.*, 2010). Salicylic acid (SA) and its derivative, methyl salicylate, are key signals in systemic acquired resistance (SAR) and the hypersensitive response (HR); both are typically involved in the control of biotrophic pathogens (Métraux *et al.*, 1990; Vlot *et al.*, 2009; Dempsey *et al.*, 2011). The three jasmonates, *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), jasmonic acid (JA), and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), have been discussed as general intercellular and intracellular signalling compounds typically involved in multiple defence reactions to both necrotrophic microbial pathogens and herbivore attack (Verhage *et al.*, 2011; Pel & Pieterse, 2013; Wasternack & Hause, 2013).

However, JA-Ile has been identified as the proposed bioactive form of jasmonates that interacts with the corresponding receptor complex SCF^{COI1} (Fonseca *et al.*, 2009). The cross-talk between interacting hormone signalling cascades makes it necessary to consider stress-related phytohormones simultaneously (Engelberth *et al.*, 2001; Koornneef & Pieterse, 2008; Diezel *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011; Verhage *et al.*, 2011).

Although many studies have emphasized the role of jasmonates, SA derivates, and ABA in plant defences, the nature of the long-distance signal(s) is still controversial (Dempsey & Klessig, 2012; Matsuura *et al.*, 2012), at least in part because little is known about the distinct role of the translocation pathway – the vascular system.

The vascular system of higher plants pervades the plant from roots to leaf tips and ensures communication and nutrient supply among all plant organs and tissues. The balance between phloem and xylem guaranties the reliability of the short- and long-distance transport of various constituents, such as carbohydrates, proteins, inorganic ions, water, and secondary metabolites (van Bel *et al.*, 2011; Lucas *et al.*, 2013). Hence, the analysis of the composition of phloem and xylem sap offers crucial information about a plant's physiological status (Dinant *et al.*, 2010). Although most studies have observed amino acids, proteins, (in)organic ions and carbohydrates (e.g. Richardson *et al.*, 1982; Shelp, 1987) in the vascular sap, few have quantitatively analysed phytohormones (see Table 1). The importance of phloem and xylem as tubes for propagating phytohormones has often been suggested but is not well understood.

Here we individually analyse the presence of stress-related phytohormones in phloem and xylem exudates and in response to challenge with the bacterial flagellin 22 (flg22).

Materials and Methods

Plant material

The growth conditions for *Cucurbita maxima* Duch. plants and sampling of phloem and xylem exudates have been described (see Zimmermann *et al.*, 2013; also see Fig. 1). *Cucurbita maxima* plants (cv GeleReuzen; Enza Zaden, Enkhuizen, the Netherlands) were cultivated in pots in a glasshouse under standard conditions (21°C, 60–70% relative humidity, and a 14 h : 10 h, light : dark period). Plants were taken for experiments 21–24 d after germination. Cuts were placed at the stem 10–20 mm above soil.

Phytohormone determination

To concentrate samples, 3 ml of either exudate was first acidified by adding 15 µl formic acid (0.5% v/v final concentration) to a mix of internal phytohormone standards (Vadassery *et al.*, 2012). Subsequently, the sample was loaded onto a reversed-phase column (Oasis HLB 3cc; Waters, Eschborn, Germany) that was first pre-conditioned by the addition of 2 ml methanol (MeOH) followed by 2 ml water. After being washed with 2 ml 5% (v/v) MeOH/water solution containing 0.5% (v/v) formic acid, the column was eluted with 2 ml MeOH. The eluate was completely dried in a speed-vac, resolved in 0.1 ml MeOH and subsequently subjected to liquid chromatography–mass spectrometry analysis according to Vadassery *et al.* (2012).

Application of stimulus

Flagellin 22 (flg22; Davids Biotechnologie GmbH, Regensburg, Germany) was applied to the root system with a final concentration

Table 1 Reported concentrations of phytohormones in phloem and xylem exudates

	Phloem			Xylem		
Phytohormone	*c* (nM)	Species	Reference	*c* (nM)	Species	References
JA				170–500	*Brassica napus*	Ratzinger *et al.* (2009)
				70	*Solanum lycopersicum*	Matsuura *et al.* (2012)
JA–Ile				180	*S. lycopersicum*	Matsuura *et al.*(2012)
SA	0–10	*Cucurbita maxima*	Métraux *et al.* (1990)	100–180	*Brassica napus*	Ratzinger *et al.* (2009)
ABA	200–500	*Ricinus communis*	Zhong *et al.* (1996)	260	*R. communis*	Zhong *et al.* (1996)
	1800	*R. communis*	Jeschke *et al.* (1997)	180	*R. communis*	Jeschke *et al.* (1997)
	4000	*R. communis*	Else *et al.* (2001)	220	*R. communis*	Else *et al.* (2001)
	90	*R. communis*	Peuke *et al.* (2002)	1700	*R. communis*	Peuke *et al.* (2002)
	650–790[a]	*Oryza sativa*	Yokota *et al.* (1994)	60–100	*Cucurbita maxima*	Zhang *et al.* (2008)
				40–75	*Cucurbita ficifolia*	Zhang *et al.* (2008)
				60–140	*Cucumis sativus*	Zhang *et al.* (2008)
				50–95	*Cucumis melo*	Zhang *et al.* (2008)
				75–80	*Momordica charantia*	Zhang *et al.* (2008)
				75–100	*Benincasa hispida*	Zhang *et al.* (2008)
				2–42	*Brassica napus*	Ratzinger *et al.* (2009)
				and many more data available for *Helianthus annuus, Lupinus albus, Lycopersicon esculentum* and *Zea mays*		

Note: JA, jasmonic acid; JA-Ile, (+)-7-*iso*-jasmonoyl-L-isoleucine; SA, salicylic acid; ABA, abscisic acid.
Some values are converted to molar (*c* = nM) specification.
[a]Phloem sap was collected using stylectomy.

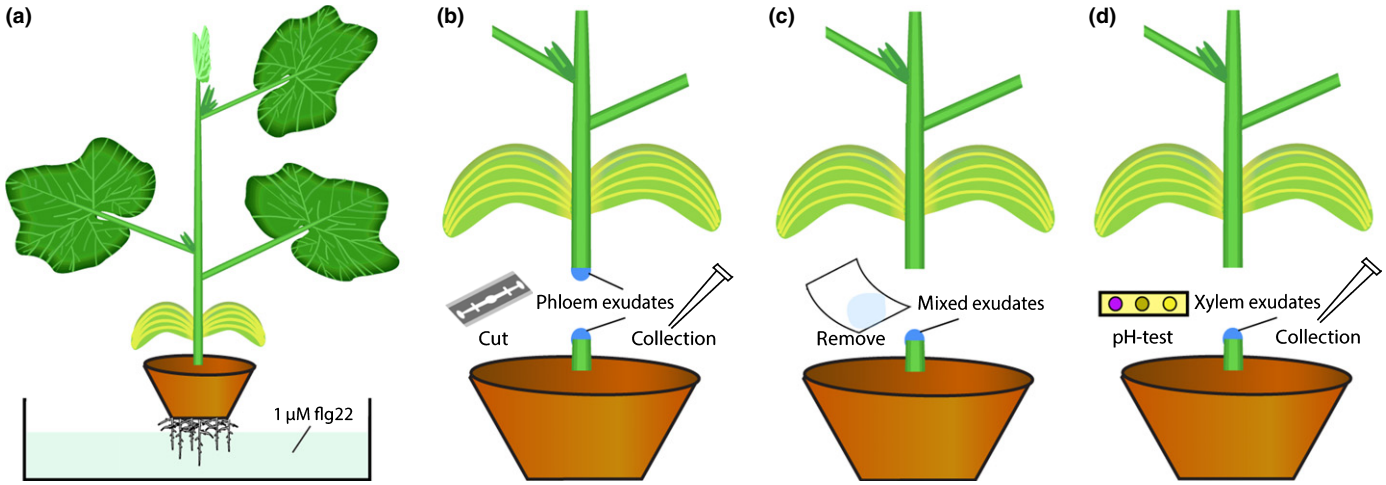

Fig. 1 Sampling procedure of phloem and xylem exudates in *Cucurbita maxima*. (a) Jutting roots are incubated in tap water containing 1.0 µM flagellin 22 (flg22) for 10, 30, or 180 min. (b) The stem is cut 10–20 mm above the soil level. The droplets exuded from both cut sides (apical and basal) within the first minute (< 2 min) are collected and represent diluted phloem sap. To achieve a high volume of phloem exudates, the apical side can be cut 1–3 times. (c) To harvest xylem exudate, the remaining gelled droplets of phloem exudate on the wound surface of the basal side are removed with a fluffless cloth after 2 min at the earliest (> 2 min). (d) The subsequent exudates represent xylem exudates; they are monitored for the characteristic pH-value (Zimmermann *et al.*, 2013). Volumes of phloem and xylem exudates ranged between 90 and 210 µl per plant. Plants were used 21–24 d after germination.

of 1 µM (Fig. 1). Afterwards, samples of phloem and xylem exudates were collected at 10, 30 and 180 min after flg22 treatment. The distance between the stimulus site at the root and the sampling location at the stem was *c.* 70–80 mm.

Statistical analyses

Statistical analyses were carried out using one-way analysis of variance (ANOVA) and *post hoc* test (Tukey; SigmaStat® 3.0; SPSS Inc., Chicago, IL, USA). The statistical significance level was set to 5% ($P < 0.05$).

Results and Discussion

A main reason for the lack of integrated knowledge about phytohormones in the vasculature is the difficulty in obtaining unimpeded access to vascular sap. Cucurbits spontaneously exude vascular sap from the wound surface. This characteristic allows both phloem and xylem exudates to be sampled separately from the same site of an individual plant, which is a prerequisite for a comparative quantitative analysis (Zimmermann *et al.*, 2013). Thus, we used *Cucurbita maxima* as a model to study the distribution of phytohormones in xylem and phloem and, in addition, defence-related alterations in the vascular sap in more detail.

All considered metabolites – the three jasmonates, *cis*-OPDA, JA, JA-Ile, as well as SA and ABA – were detected in the exudates of both phloem and xylem (Fig. 2), and significant (ANOVA, $P < 0.05$) differences among the composition of phloem and xylem exudates were assessed. However, it has recently been found that the process of exudation forces out the content of phloem by an osmotically driven lateral water influx from the xylem/apoplast into the sieve elements (SEs; Zimmermann *et al.*, 2013). As a consequence, the collected phloem sap is diluted. Therefore, the measured values underestimate the *in vivo* situation

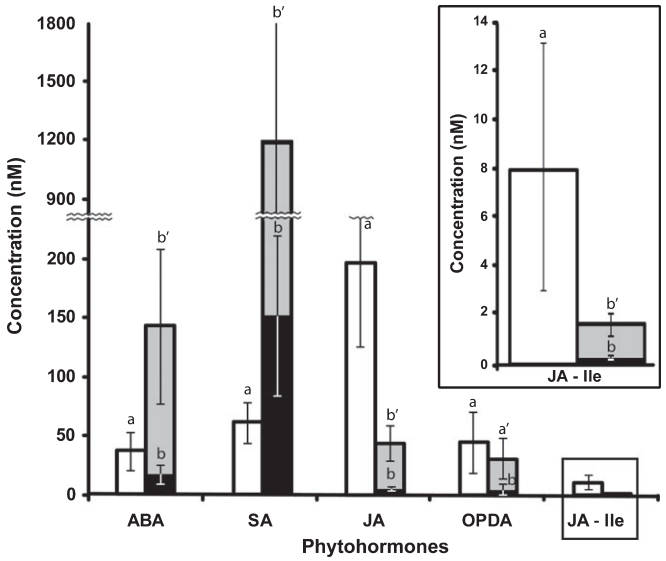

Fig. 2 Analysis of phytohormones in phloem and xylem exudates of unstressed *Cucurbita maxima* plants. The concentrations (in nM) of the phytohormones *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), jasmonic acid (JA), (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), salicylic acid (SA), and abscisic acid (ABA) are individually determined in phloem (black bars) and xylem (white bars) exudates from the same site of a stem-cut plant ($n_{plant} = 8$). The dilution of phloem exudates with xylem water is considered with a factor of eight (grey bars) to respect the *in vivo* situation (Zimmermann *et al.*, 2013). The statistical significance is tested with one-way ANOVA by pairwise comparison of xylem and phloem exudates (also phloem ×8) with Student's *t*-test ($P < 0.05$) and marked with different letters (superscript apostrophe for phloem ×8). Vertical lines represent standard deviations of eight replicates.

by *c.* 7–8-fold. This factor was determined by carbohydrate measurements in exudates (Zimmermann *et al.*, 2013). After the application of the dilution factor, a significant difference between phloem and xylem exudates remains for ABA, SA, JA and JA-Ile,

whereas almost equal concentrations of *cis*-OPDA were calculated in phloem and xylem exudates (Fig. 2).

In light of the dilution, the higher concentration of ABA in the phloem exudates ($c = 142.5 \pm 65.3$ nM) compared to the xylem exudates ($c = 37.1 \pm 16.1$ nM) was unexpected. ABA is a root-borne signal that is released to the above-ground plant part (Christmann *et al.*, 2006; Zhang *et al.*, 2008). Recently it was shown that ABA is exported from the producing cells in the roots into the xylem by the ABC-transporter AtABCG25 (Kuromori *et al.*, 2010). Hence, the xylem should be the most likely translocation pathway. Consistent with our results, however, previous comparative studies have reported higher ABA concentrations in the phloem exudates of *Ricinus communis* (Table 1; Jeschke *et al.*, 1997; Else *et al.*, 2001). ABA in the phloem was suggested to be loaded within leaves and earlier was proposed to act as a reservoir (Wilkinson & Davies, 2002). Despite abundant surveys of ABA in the vascular sap, fundamental knowledge is lacking about how ABA is distributed between the phloem and xylem in unstressed plants (Table 1; Zhong *et al.*, 1996; Peuke *et al.*, 2002).

Substantial differences between phloem and xylem exudates were found for SA and JA independent of the effect of dilution, suggesting that each has a preferred translocation pathway (Fig. 2). A higher concentration of SA was found in phloem exudates (1.5-fold) than in xylem exudates ($c_{xylem} = 61.6 \pm 17.2$ nM, $c_{phloem} = 165 \pm 60.6$ nM); a higher concentration of SA has also been reported for *Ricinus communis* (Rocher *et al.*, 2006). The content of SA *in vivo* is even higher ($c_{phloem} = 1319 \pm 484$ nM) when the dilution factor is taken into account. For unknown reasons, Métraux *et al.* (1990) observed low SA concentrations (*c.* 0–10 nM) in the phloem exudates of unstressed *C. maxima* plants, while concentrations in *R. communis* were at least 20 times higher. Unlike SA, the highest amounts of JA by far (36-fold) were detected in xylem exudates ($c_{xylem} = 199.5 \pm 71.5$ nM, $c_{phloem} = 5.5 \pm 1.9$ nM), which confirms other data available (Ratzinger *et al.*, 2009). Further details about the distribution of JA within phloem and xylem are not available yet.

At first glance, the different distribution patterns of JA, SA and ABA in phloem and xylem exudates are surprising: JA (pKa = 4.5), SA (pKa = 3.02) and ABA (pKa = 4.87) are weak organic acids and thus share similar physico-chemical properties. Any enrichment of these compounds within the SEs/phloem would have been expected due to the ion trap mechanism (Delrot *et al.*, 1981; Kramer, 2006; Rocher *et al.*, 2006). The protonated forms of JA, SA and ABA can permeate membranes and are supported more by the weak acidic environment of the xylem (pH = 4.8–5.5; Crafts, 1936; Richardson *et al.*, 1982; Zimmermann *et al.*, 2013) than by the alkaline/neutral environment of the SEs/phloem (pH = 7.5–8.0; Crafts, 1936; Richardson *et al.*, 1982; Zimmermann *et al.*, 2013). However, it is worth to mention that at pH 5.0 in particular JA and also *cis*-OPDA are predicted to be mainly in their poorly permeant dissociated form. Within the xylem/apoplast, the balance between protonated and de-protonated forms is shifted to the protonated one. Hence, there may be a net influx from the xylem/apoplast into the SEs/phloem. Inside the SEs/phloem, JA, SA and ABA are immediately de-protonated, and the nonmembrane-permeable anion is trapped by the SEs/phloem; the consequence is an increase of JA, SA and ABA. Indeed, the high level of SA in the phloem exudate of *Ricinus* seedlings was previously explained by the combination of the ion trap mechanism and a postulated specific carrier system (Rocher *et al.*, 2006, 2009). The higher concentration of ABA in the phloem may also be assisted by a trans-membrane translocation system as described for xylem loading in *Arabidopsis* (Kuromori *et al.*, 2010; Kang *et al.*, 2011). However, such importer/exporter that might be involved in phloem loading still needs to be identified.

Such a system is very likely due to the high permeability of ABA in SEs (Hartung *et al.*, 2002). However, the findings for JA are unexpected, since, in addition to the ion trap mechanism, diverse enzymes (lipoxygenase, ACC-oxidase/synthase) involved in jasmonate biosynthesis were detected directly in phloem exudates (Avdiushko *et al.*, 1994; Walz *et al.*, 2004) and within SEs and the companion cells of tomato plants (Hause *et al.*, 2000, 2003; Stenzel *et al.*, 2003). Thus, it is tempting to speculate that JA, like SA, accumulates in the phloem and is actively exported from the phloem into the xylem. Whereas a study using exogenously applied carbon-14 (^{14}C)-labelled JA suggested a transport from leaves to roots in *Nicotiana sylvestris* (Zhang & Baldwin, 1997), grafting experiments with jasmonate insensitive and jasmonate biosynthesis mutants, respectively, showed that a root to shoot direction for jasmonates is likely (Howe, 2004). Consistent with these later results, more recent studies reported a synthesis of JA in the root from where it is translocated to the shoot *via* the xylem (López-Ráez *et al.*, 2010). However, the mechanism by which the protonated JA has to be prevented from refluxing is still unknown.

The absence of literature that refers to direct JA measurements in the vascular sap motivated us to investigate the related metabolites *cis*-OPDA and JA-Ile. *cis*-OPDA, the biosynthetic precursor of JA, has diverse signalling functions. Recently, inhibition of seed germination and interaction with ABA was reported (Dave & Graham, 2012). Unlike JA and JA-Ile, *cis*-OPDA did not accumulate preferentially in the xylem exudates when the dilution factor is taken into account ($c_{xylem} = 48.6 \pm 25.5$ nM, $c_{phloem} = 29 \pm 16.3$ nM), raising the possibility for the presence of an independent distribution. The conjugate JA-Ile (Fig. 2, inset) is a highly active wound-response signal (Fonseca *et al.*, 2009; Matsuura *et al.*, 2012). Notably, the highest relative differences (40-fold and five-fold, respectively, when dilution is included) between phloem and xylem exudates were observed for JA-Ile ($c_{xylem} = 7.9 \pm 5$ nM, $c_{phloem} = 0.2 \pm 0.06$ nM). This finding supports as well the hypothesis of a preferred translocation pathway for certain phytohormones. The observed low concentrations of JA-Ile in comparison to JA and *cis*-OPDA may be indicative of a highly efficient signal. A previous study of *S. lycopersicum* demonstrated much higher amounts of JA-Ile in the xylem (18-fold) in comparison to our results, and the concentration of JA-Ile was found to be twice as high as that of JA (Table 1; Matsuura *et al.*, 2012). The detection of methyl-jasmonate and methyl-salicylate in vascular exudates would strongly amplify our knowledge; however, an advancement of the sampling procedure is a precondition due to the volatile characteristic and will be a challenge of future work.

The different lateral distribution patterns of the jasmonates, SA and ABA, raise questions about the role of phloem and xylem during signalling. No previous studies have proposed an adequate explanation of the imbalanced distributions. Now that it is possible to determine the concentrations of various phytohormones in parallel in xylem and phloem independently, studies addressing the role and physiology of these signalling compounds under different stress situations can be performed. Because phloem and xylem are known to interact in order to ensure the translocation of photoassimilates, they should be seen as a functional unit

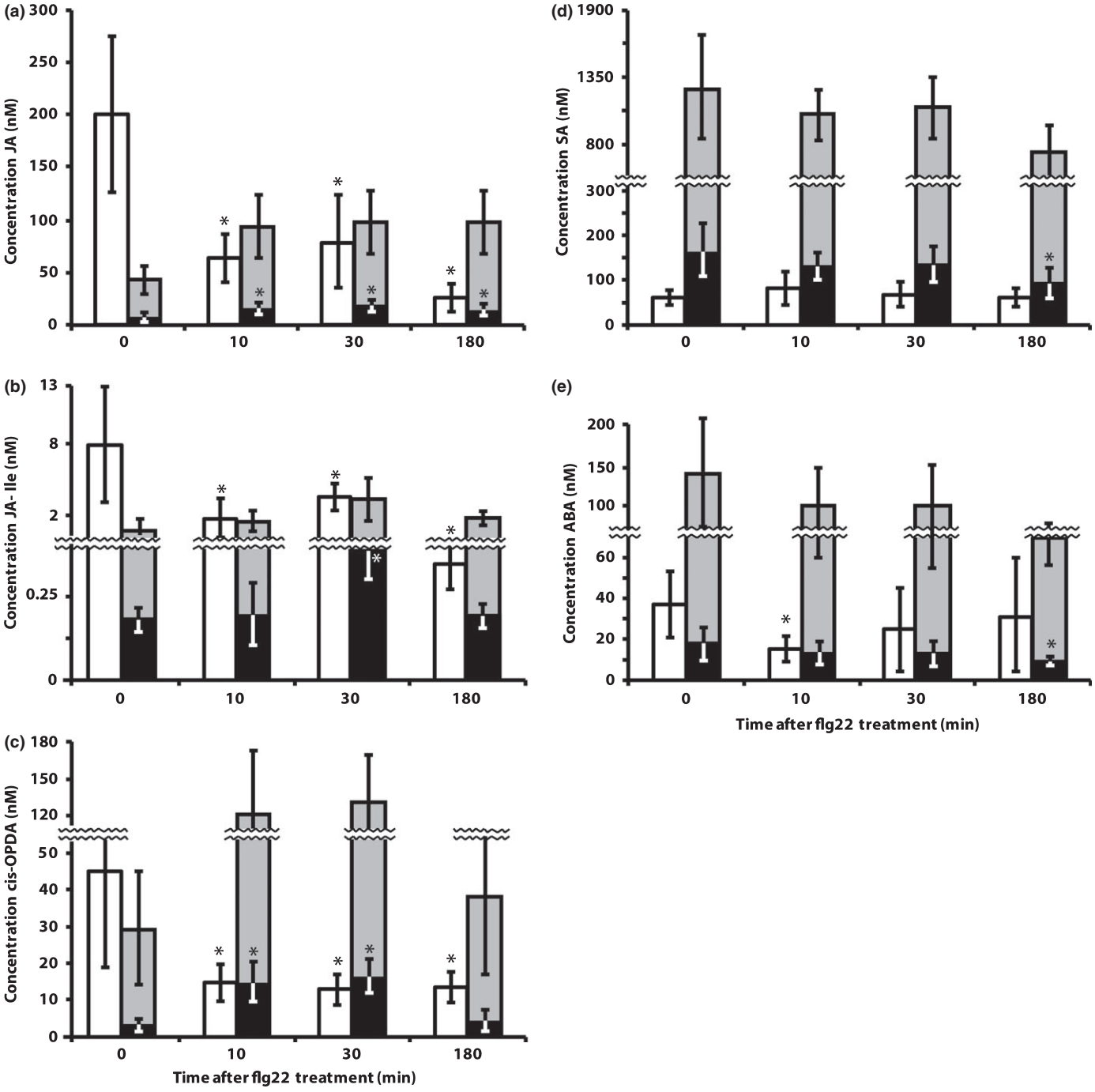

Fig. 3 Analysis of phytohormones in phloem and xylem exudates of flagellin 22 (flg22)-treated *Cucurbita maxima* plants. Distinct xylem (white bars) and phloem (black bars) exudates are separately collected at the stem cut 10, 30 or 180 min after incubation of *Cucurbita maxima* roots in 1 μM flg22. The dilution of phloem exudates is considered with a factor of eight (grey bars) to represent the *in vivo* situation (Zimmermann *et al.*, 2013). Time point 0 min represents untreated plants. The concentrations (c = nM) of jasmonic acid (JA), (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), salicylic acid (SA), and abscisic acid (ABA) are directly determined in phloem and xylem exudates (a–e). Each bar represents the mean of 7–15 replicates (plants). Vertical lines, ± standard deviation (SD). The statistical significance is tested with one-way ANOVA (*post hoc* test: Tukey; $P < 0.05$) and marked with asterisks. Note that the illustrated significance compares untreated plants (= 0 min) with the different flg22 time points (10, 30 and 180 min) separate for xylem and phloem exudates.

collaborating during short- and long-distance phytohormone signalling.

We assessed collaboration between phloem and xylem in stress-related root-to-shoot signalling after the application of 1 μM flg22 to roots (see Fig. 1 for methods). It has been reported that binding of bacteria-derived flg22 to the plant's transmembrane receptor kinase FLAGELLIN SENSITIVE2 (FLS2) induces innate immune responses that affect the defence-related phytohormone homeostasis (Nürnberger & Brunner, 2002; Chinchilla *et al.*, 2006, 2007; Boller & Felix, 2009).

As shown in Figure 3, significant (ANOVA, $P < 0.05$) flg22-triggered remote effects were detected for all examined phytohormones in both phloem and xylem after flg22-application to roots; this is consistent with a role of phytohormones in immune signalling (Engelberth *et al.*, 2001; Flors *et al.*, 2007; Koornneef & Pieterse, 2008; Diezel *et al.*, 2009; Dempsey & Klessig, 2012; Pieterse *et al.*, 2012). We observed significant short-term responses, especially for jasmonates and SA, after 10–180 min, which complemented previous long-term studies that reported such responses for time ranges between 0.5 and 3 d following an application of *Pseudomonas syringae*, tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium* (Métraux *et al.*, 1990; Rasmussen *et al.*, 1991; Smith-Becker *et al.*, 1998). Also, short-term effects of phloem-located JA and JA-Ile have been recently observed after mechanical wounding of *Cucurbita maxima* leaf edges (Gaupels *et al.*, 2012).

The present findings of simultaneous phytohormone alterations suggest that xylem and phloem interact. Inside the phloem, the reaction pattern of SA and ABA in response to flg22 treatment was unlike the reaction pattern of jasmonates (Fig. 3a–e). Initially, levels of SA (−43.6%) and ABA (−46.5%) significantly decreased until 180 min, whereas levels of jasmonates significantly increased until 180 min. The findings of anticyclical pattern of SA and jasmonates may confirm previous results and may indicate that an innate immunity response has been initiated (Flors *et al.*, 2007; Koornneef & Pieterse, 2008; Diezel *et al.*, 2009; Vlot *et al.*, 2009). The apparent suppression of JA in the xylem may also be explained by a lateral exchange. The significant increase of JA in the phloem (+89.7%) and the simultaneous decrease of JA in the xylem (−89%) indicates that a lateral exchange has occurred. This conclusion also is supported by the significant decline of *cis*-OPDA in the xylem (−74.5%) and the coincidental rise of *cis*-OPDA in the phloem (+31%; Fig. 3c). The xylem-originated *cis*-OPDA might serve as substrate for the essential enzymes of JA biosynthesis detected in vascular parenchyma cells, companion cells and SEs (Hause *et al.*, 2000, 2003; Stenzel *et al.*, 2003). Presumably *de novo* synthesized JA is translocated from cell to cell via plasmodesmata into SEs. It is very interesting that the lateral distribution of jasmonates between phloem and xylem in untreated plants (Fig. 2) significantly changed after 30 min of flg22 application (Fig. 3a) or, rather, completely reversed (*cis*-OPDA), when the dilution of phloem exudates was considered, and indicated additionally a lateral exchange. However, besides lateral exchanges as part of allocation processes (Schultz *et al.*, 2013), it is also tempting to speculate that the particular stress factor, flg22, has a negative effect on xylem loading of jasmonates. Other biotic challenges such as herbivory or different pathogens should be employed in future studies to address this question.

The lateral exchange of phytohormones between xylem and phloem during plant defence signalling suggests that the process of long-distance signalling is more inclusive than the longitudinal delivery of a signalling constituent. Future studies should focus on the molecular mechanisms by which phloem and xylem collaborate during short- and long-distance phytohormone signalling.

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