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Synthesis and mode of action studies of *N*-[(-)-jasmonyl]-*S*-tyrosin and ester seiridin jasmonate

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ABSTRACT

Recent analyses on fungal jasmonic acid (JA)-containing metabolites suggest a mode-of-action of these naturally occurring compounds as inactive storage pools of JA. Plants and/or fungi can catabolize JA into the bioactive jasmonyl-isoleucine (JA-Ile) that in turn activates the JA-Ile-pathway *in planta*. To extend our knowledge on JA-derivates related to natural occurring JA conjugates, *N*-[(-)-jasmonyl]-*S*-tyrosin (JA-Tyr) and the ester JA-Sei between JA and seiridin, a fungal disubstituted furanone, were synthesized. The classical procedures for ester synthesis were applied for compound JA-Sei, while *N*-[(-)-jasmonyl]-*S*-tyrosin was synthesized with an optimized procedure. JA-Tyr and JA-Sei were characterized by spectroscopic method (essentially 1D and 2D NMR spectroscopy and ESI-MS) and their stereochemical composition was determined by means of HPLC and circular dichroism analysis. Finally, the activity of these JA-derivates was analyzed *in planta*. JA-Tyr and JA-Sei trigger JA-regulated plant responses, such as protein degradation and growth inhibition. These effects require the conversion of JA into JA-Ile and its recognition by the plant JA-Ile perception complex COI1-JAZ. Overall, these data suggest a mode-of-action of JA-Tyr and JA-Sei as inactive pool of JA that can be transformed into the bioactive JA-Ile to induce the canonical JA-Ile-pathway.

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1. Introduction

Jasmonic acid (**1**, Fig. 1) is the basic compound of a naturally occurring family of substances named jasmonates which regulate many aspects of growth, development and defense responses in plants (Dewick, 2009; Osbourn and Lanzotti, 2009; Wasternack and Hause, 2013; Chini et al., 2016). In particular, JA is a signaling compound in plant defense, activating responses to wounding, herbivores and necrotrophic pathogens. JA and related compounds are also produced by fungi (Wasternack and Hause, 2013; Andolfi et al., 2014; Goossens et al., 2016). The JA-Ile biosynthetic pathway has been extensively studied in plants (Schaller and Stinzi, 2009; Wasternack and Hause, 2013; Chini et al., 2018; Wasternack and Song, 2017). The bioactive plant hormone JA-Ile [(+)−7-*iso*-JA-Ile] is generated by the conjugation of JA to Ile by the JA-amido synthetase JAR1 (Staswick and Tiryaki, 2004; Fonseca et al., 2009). JA-Ile is the ligand of the receptor complex and it acts as "molecular glue" to induce the formation of the COI1-JAZ co-receptor complex (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). JA-Ile-promoted COI1-JAZ interaction results in ubiquitination and proteasome degradation of the JAZ repressors, which in turn activates several transcription factors that regulate specific physiological responses (Chini et al., 2007, 2016; Thines et al., 2007).

Fungi are well-known producers of secondary metabolites belonging to different class of natural compounds (Turner and Aldridge, 1983; Cole et al., 2003; Lo Presti et al., 2015; Fonseca et al., 2017). Phytopathogenic fungi are one of the main causal agents of serious diseases of agrarian and forest plants with heavy consequential economic losses (Ballio and Graniti, 1991; Evidente and Motta, 2001; Evidente et al., 2010, 2011, 2013; Cimmino et al., 2014, 2015).

Lasiodiplodia strain, recently classified as *Lasiodiplodia*

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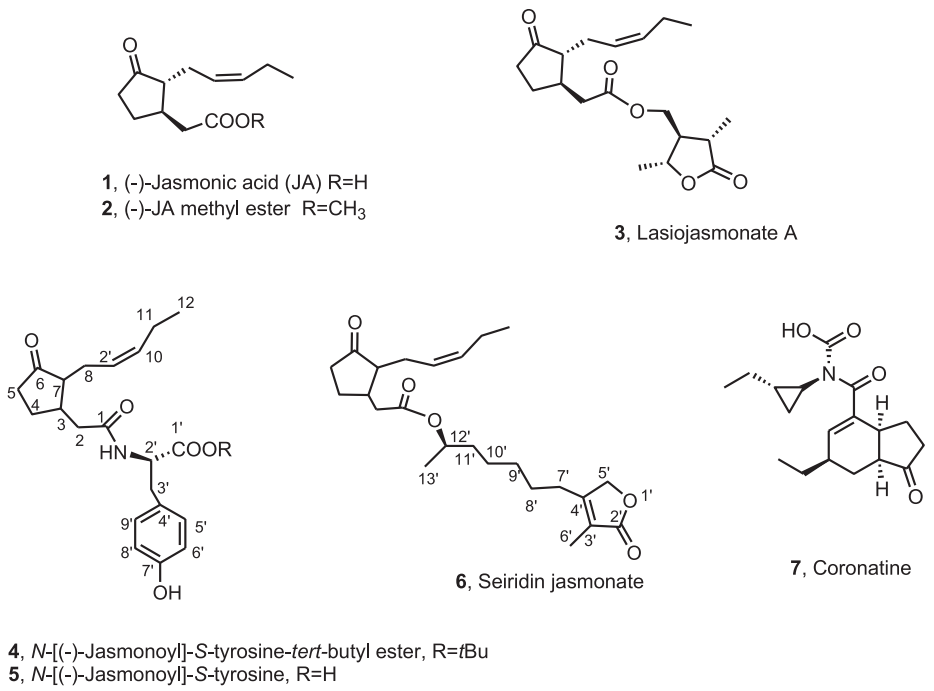


Fig. 1. Structures of jasmonic acid, its methyl ester and lasiojasmonate A (**1**-**3**), *N*-[(-)-jasmonoyl]-*S*-tyrosine and its tert-butyl ester (**4** and **5**), seiridin jasmonate ester (**6**) and coronatine (**7**).

mediterranea sp. nov. (Linaldeddu et al., 2015), produced *in vitro* (1*R*,2*R*)-(-)-jasmonic acid (**1**, Fig. 1), as the main phytotoxin, its methyl ester (JA-Me, **2**, Fig. 1), and three furanonenoyl esters, named lasiojasmonates A (LasA, **3**, Fig. 1) B and C, 16-*O*-acetylbotryosphaerilactones A and C, botryosphaerilactone A, (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone and (3*R*,4*S*)-botryodiplodin (Andolfi et al., 2014). A mode of action of LasA (**3**) was recently proposed (Chini et al., under review). The results show that LasA activates the plant JA-pathway and that its activity required the conjugation of JA to Ile by JAR1, the last biosynthetic step that generates the bioactive JA-Ile [(+)–7-*iso*-JA-Ile] (Staswick and Tiryaki, 2004; Fonseca et al., 2009). The activity of LasA also depends on the plant hormone receptor complex COI1-JAZ (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). Because this fungal metabolite contains JA as a moiety of its structure, it has been proposed that plants and/or fungi can process these compounds and release free JA. Therefore, LasA might serve as inactive conjugated JA that can be converted into JA and subsequently into the bioactive JA-Ile in specific conditions (Chini et al., under review).

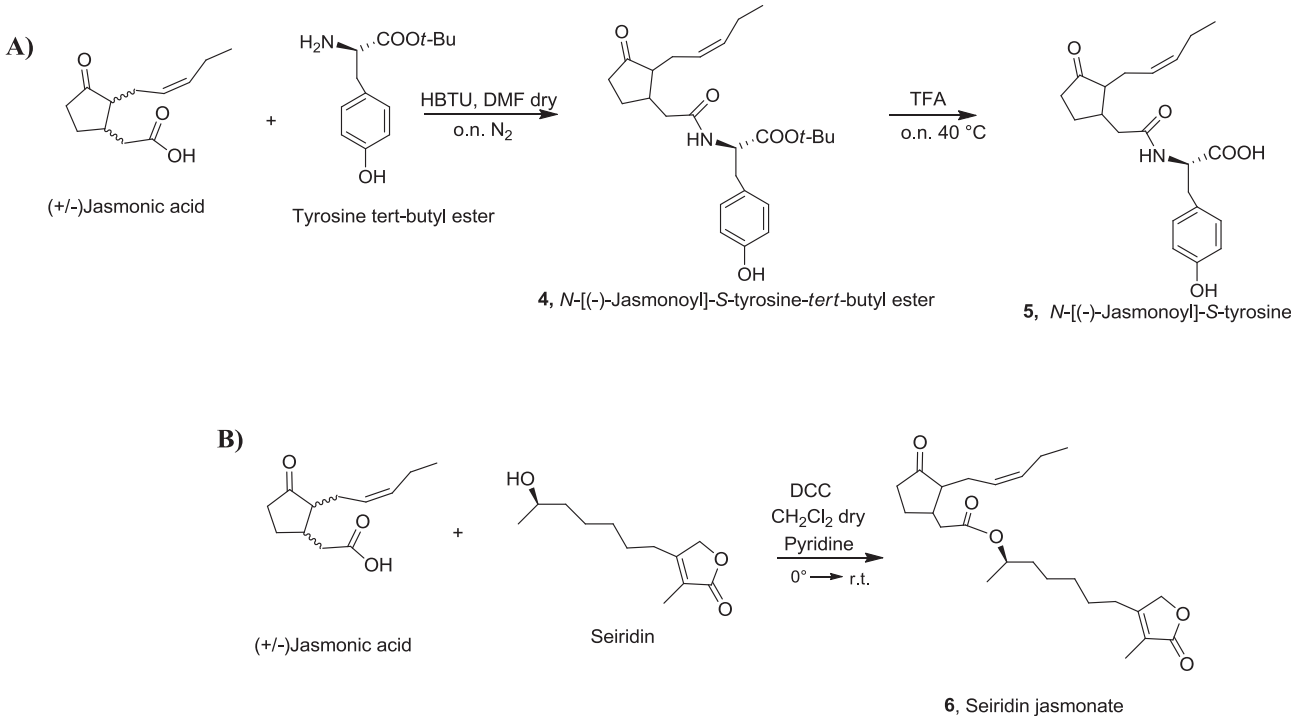
Considering that amino acid conjugates of JA occur naturally (Cross and Webster, 1970; Bohlmann et al., 1984; Brückner et al., 1988; Yan et al., 2016; Staswick et al., 2016; Wasternack and Song, 2017), and that the cylopentanone moiety has an important role in the direct binding of JA-Ile to its receptor COI1-JAZ, we proceeded with the synthesis of *N*-[(-)-jasmonoyl]-*S*-tyrosine-*tert*-butyl ester (**4**), the amide tyrosine-JA (JA-Tyr, **5**, Fig. 1) and the ester of JA with seiridin (JA-Sei, **6**, Fig. 1). Seiridin is produced as the main phytotoxin from *Seiridum cardinale*, the causal agent of cypress canker disease (Evidente et al., 1986), and it is a disubstituted furanone closely related to the trisubstituted didihydrofuranone **3**. The stereochemical composition of the prepared JA derivatives **5** and **6** could not be assigned by NMR, and was determined by means of HPLC separations and electronic circular dichroism (CD) measurements.

Finally, we studied the effect of JA-Tyr and JA-Sei on plant JA-regulated responses to understand their activity *in planta*. JA-Tyr and JA-Sei induce JA-regulated plant responses, including JAZ degradation and growth inhibition. These effects are dependent on JAR1 and COI1, suggesting that the activity of JA-Tyr and JA-Sei requires plant conversion of JA into JA-Ile and its consequent recognition by the COI1-JAZ receptor.

2. Results and discussion

2.1. Synthesis and characterization of N-[(-)-jasmonyl]-S-tyrosin and the ester seiridin jasmonate

JA is naturally conjugated with several amino acids and additional molecules (Wasternack and Song, 2017), and the cyclopentanone moiety has an important role in the binding of the ligand to its COI1-JAZ receptor complex (Sheard et al., 2010; Monte et al., 2014). Therefore, we synthesized the amide tyrosine-JA (JA-Tyr, **5**, Fig. 1) and the ester of JA with seridin (JA-Sei, **6,** Fig. 1), containing an alcoholic moiety related to 4-hydroxymethyl-3,5-dimethyldihydrofuran-2-one (LasA, **3**), reasoning that these JA-derivates might sterically affect the interaction with the COI1-JAZ complex (Monte et al., 2014). *N*-[(-)jasmonyl]-*S*-tyrosin (**5**) was synthesized starting from the commercially available racemic (±)-jasmonic acid and L-tyrosine *tert*-butyl ester according a previously reported procedure (Ulijn et al., 2015) as illustrated in Scheme 1A. The crude product obtained from reaction work-up was purified as reported in the Experimental Section yielding *N*-[(-)-jasmonoyl]-*S*-tyrosine-*tert*-butyl ester (**4**, Fig. 1**)** as colorless oil (67% yield). The ^{1}H NMR spectrum of **4** (Table 1) showed both the typical pattern system of L-tyrosine and JA in addition to the signal of the amidic proton (NH) observed as a doublet ($J = 7.7$ Hz) at δ 5.94 being coupled in the COSY spectrum (Berger and Braun, 2004) with the α-proton (HC-2′) resonating as a quartet ($J = 7.7$ Hz) at δ 4.75. This latter hydrogen, the X part of an ABX system, in turn coupled with the proton of the adjacent methylene group (H_2C-3′, the AB part) observed as two doublets of double



Scheme 1. Mechanisms of synthesis of (A) of *N*-[(-)-jasmonoyl]-*S*-tyrosin and its *tert*-butyl ester (**4** and **5**) and (B) seiridin jasmonate ester (**6**).

Table 1
^{1}H and ^{13}C NMR data of *N*-[(-)-jasmonoyl]-*S*-tyrosine-tert-butyl ester and *N*-[(-)-jasmonoyl]-*S*-tyrosine (**4** and **5**)[a,b].

4				**5** (CD_3OD)
Position	δ_C[c]	δ_H (*J* in Hz)	HMBC	δ_H (*J* in Hz)
1	170.8 s		H-7, H-2, H-3′,	
2	30.6 t	2.06 m (2H)[d]	H_2-8, H-7, H_2-5	2.07 m[g] (2H)
3	25.6 d	2.32 m[e]	H_2-8, H-7	2.32 m[h]
4	37.8 t	1.44 m (2H)[f]		1.86 m (2H)
5	54.0 t	2.32 m [d]	H_2-8, H-7, H-3	2.32 m[h]
		1.82 m		1.97 m
6	219.5 s		H_2-5, H-3	
7	41.1 d	2.58 ddd (18.7, 14.3, 4.6)	H_2-5	2.54 td (13.6, 4.2)
8	27.0 t	2.32 m [e]	H-10, H-9	2.32 m[h]
		2.06 m [d]		2.25 m
9	125.1 d	5.24 m	H_2-8	5.25 m
10	134.0 d	5.42 m	H_2-8, Me-12	5.42 m
11	20.9 t	2.06 m (2H)	Me-12, H-10, H-9, H_2-8	2.07 m[g] (2H)
12	14.1 q	0.95 d (7.5) (3H)	H_2-11, H-10	0.92 d (6.8) (3H)
1′	171.0 s		NH, H-2′	
2′	53.5 d	4.75 q (7.7)	H_2-3′, H-5′,9′	4.61 t (7.8)
3′	30.5 t	3.05 ddd (10.0, 7.7, 2.8)	H-5′,9′, H-2′	3.17 br d (12.0)
		2.96 ddd (10.0, 7.7, 2.8)		2.85 br d (12.0)
4′	127.7 s		H-2′	
5′,9′	130.5 d	6.99 dd (8.2, 2.8) (2H)	H_2-3′	7.06 d (7.8) (2H)
6′,8′	115.4 d	6.73 d (8.2) (2H)	H-5'.9′	6.71 d (7.8) (2H)
7′	155.1 s		H-6′,8′, H-5'.9′	
$(Me)_3\underline{C}$	82.6 s		$(\underline{Me})_3C$	
$(\underline{Me})_3C$	28.0 q	1.44 s (9H) [f]		
NH		5.94 d (7.7)		

[a,b]The chemical shifts are in δ values (ppm) from TMS. [b]2D ^{1}H, ^{1}H (COSY) and 2D ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectra. [d,e,f,g,h]These signals are overlapped.

doublets (*J* = 10.0, 7.7, 2.8 and *J* = 10.0, 7.7 and 2.8 Hz) at δ 3.05 and 2.96. These protons were allylic coupled with the two protons (H-5′,9′) of the *para*-disubstituted tyrosine benzene ring, resonating as a double doublet (*J* = 8.2 and 2.8 Hz) at δ 6.99 and these latter, in turn, were coupled with the adjacent protons (H-6′,8′) appearing as a doublet (*J* = 8.2 Hz) at δ 6.73. The two *cis*-coupled olefinic protons (H-10 and H-9) of the JA moiety resonated as two multiplets at δ 5.42 and 5.24 and were coupled with the protons of the adjacent methylene groups (H_2-11, H-8A and H-8B) appearing as multiplets at δ 2.06, 2.32 and 2.06, respectively. Protons H_2-11 coupled with the terminal methyl group (Me-12) observed as a doublet (*J* = 7.2 Hz) at δ 0.95. The two protons of C-8 also coupled between

themselves and with the adjacent methine proton (H-7) of the dihydrofuran-2-one ring. Proton H-7, resonating at δ 2.58 as a doublet of double doublets (J = 18.7, 14.3 and 4.6 Hz), was also coupled with the adjacent methine proton (H-3) bearing the carboxymethylene group, which resonated as a multiplet at δ 2.32, thus overlapping with the H-8A and H-5A signals. Proton H-8B overlapped with both multiplets of protons H_2C-2 and H_2C-11, resonating all at δ 2.06 (Pretsch et al., 2000). Finally, the singlet of the *tert*-butoxy group appeared at δ 1.44. The ^{13}C NMR spectrum (Table 1) also showed the signal patterns typical for both the JA and the dihydrofuranoyl residues. In addition, three carbonyls resonating at δ 219.5, 170.8 and 171.0, and signal of *tert*-butoxy at δ 28.0, signals for seven methines, four of which aromatic (C-6′,8′ and C-5′,9′), six methylenes and one methyl groups were observed. The couplings observed in the HSQC (Berger and Braun, 2004) spectrum allowed us to assign the signals at δ 134.0, 130.5, 125.1, 115.5, 54.0, 53.5, 41.1, 37.8, 30.6, 30.5, 27.0, 25.6, 20.9 and 14.1 to C-10, C-5′,9′, C-9, C-6′,8′, C-5, C-2′, C-7, C-4, C-2, C-3′, C-8, C-3, C-11 and C-12, respectively (Breitmaier and Voelter, 1987). The couplings observed in the HMBC spectrum allowed the assignment of the quaternary carbons. In particular, the couplings observed between C-6 with H_2-5 and H-3, C-1′ with NH and H-2′, C-1 with H-7, H-2 and H-3′, C-7′ with H-6′,8′ and H-5′,9′, C-4′ with H-2′ and the quaternary carbon of *tert*-butoxy with its germinal methyls, allowed us to assign the signals at δ 219.5, 171.0., 170.8, 155.1, 127.7 and 82.6 to C-6, C-1′, C-1, C-7′, C-4′ and $(Me)_3$C-O, respectively (Breitmaier and Voelter, 1987).

The structure of **4** was supported by the all other couplings observed in the HMBC spectrum (Berger and Braun, 2004) as reported in Table 1. The ESI MS spectrum of **4** showed the potassium cluster $[M+K]^+$ and the pseudomolecular ion $[M+H]^+$ at *m/z* 468 and 430 respectively.

Compound **4** was successively hydrolyzed with TFA (Scheme 1A) affording after TLC purification *N*-[(-)-jasmonyl]-*S*-tyrosin (**5**, Fig. 1) as another pure oil (98% yield). Its ^{1}H NMR spectrum (Table 1) differed from that of **4** essentially for the absence of the singlet of the trimethyl-*tert*-butoxy group and the upfield shift (Δδ 0.14) of H-2′ appearing a triplet (J = 7.8 Hz) at δ 4.61.

Seiridin jasmonate (**6**, Fig. 1) was obtained in satisfactory yield (50.4%) by reaction of the commercially available racemic JA and seridin isolated from the culture filtrates od *Seiridium cupressi* (Evidente et al., 1986) as detailed reported in the Experimental Section.

The ^{1}H NMR spectrum of **6** (Table 2) showed a signal pattern for the jasmonate moiety as well as that of seiridin residue which were very similar to that previously reported for this toxin (Evidente et al., 1986), except for the expected downfield shift (Δδ 1.18) of the signal of H-12' (H-2′ in seiridin) resonating as a sextet (J = 6.2 Hz) at δ 4.94. The ESI MS spectrum of **6** showed the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M+H]^+$at *m/z* 401 and 379, respectively.

Table 2
^{1}H NMR data of seiridin jasmonate (**6**)[a].

Position	δ_H (J in Hz)		
2	2.10 m (2H)[b]	12	0.98 t (7.7) (3H)
3	2.39 m[c]	5′	4.67 q (1.8) (2H)
4	1.40 m (2H)[d]	6′	1.85 t (1.8) (3H)
5	2.39 m[c]	7′	2.10 m (2H) [b]
	1.90 m		
7	2.70 m	8′	1.40 m (2H)[d]
8	2.39 m[c]	9′	1.40 m (2H)[d]
	2.31 m		
9	5.29 m	10′	1.40 m (2H)[d]
10	5.46 m	11′	1.40 m (2H)[d]
11	2.10 m (2H) [b]		
		12′	4.94 sex (6.2)
		13′	1.24 d (6.2)

[a]The chemical shifts are in δ values (ppm) from TMS. [b,c,d]Signals are overlapped.

2.2. Analysis of the stereoisomeric composition of N-[(±)-jasmonyl]-S-tyrosin-tert-butyl ester (4) and seiridin (±)-jasmonate (6)

Although the synthesis of products **4**–**6** was started from racemic (±)-jasmonic acid, their ^{1}H and ^{13}C NMR spectra revealed in each case a single set of signals, rather than two sets of signals, or a set of split signals, as expected for a mixture of diastereomers. The preferential formation of a single diastereomer of **4** and **6** might in principle occur, however a complete diastereoselectivity looked extremely unlikely. Therefore, the nature of products **4** and **6** was further investigated by electronic circular dichroism (CD) and HPLC.

Enantioselective HPLC analysis of **4** and **6** with two different chiral columns, based on cellulose and amylose (see Experimental Section) (Kramell et al., 1997), revealed that **4** and **6**, and consequently also **5** prepared from **4**, were in fact a mixture of diastereomers. A baseline separation was achieved for **4** and a sufficient separation for **6**. Peak integrals were in accord with a 50:50 ratio within the experimental uncertainty (Supporting Information).

CD spectra were measured on **4** and **6** dissolved in methanol and compared with literature data for various esters of JA acid, including a series of aminoacid conjugates (Kramell et al., 1988, 1997). In all reported cases, the esters of (+)-JA display a positive CD band around 300 nm with an intensity of $\Delta\varepsilon \approx +1\ M^{-1}cm^{-1}$; vice-versa, the esters of (−)-JA display a negative CD band around 300 nm with an intensity of $\Delta\varepsilon \approx -1\ M^{-1}cm^{-1}$ (Kramell et al., 1988, 1997). This band is allied with the n-π* transition of the carbonyl chromophore (Berova et al., 2007), therefore it senses the chirality of the JA moiety. Our samples of **4** and **6** showed very weak CD spectra in the same region (see Supporting Information): **4**, $\Delta\varepsilon$ (298 nm) = + 0.030 $M^{-1}cm^{-1}$; **6**, $\Delta\varepsilon$ (297 nm) = + 0.016 $M^{-1}cm^{-1}$. In both cases, the existence of a couple of diastereomers may be envisaged, that is, the coupling products of (+)-JA and (−)-JA with *S*-tyrosine-*tert*-butyl ester and seiridin, respectively. Our products **5** and **6** are therefore confirmed to be mixtures of diastereomers, that is, [(±)-jasmonyl]-(*S*)-tyrosine and (*R*)-seiridin (±)-jasmonate, respectively.

2.3. Effect of JA synthetic derivates 5 and 6 on JAZ stability

We tested the effect of JA-Tyr (**5**) and JA-Sei (**6**) on JA-mediated responses in *Arabidopsis thaliana* plants. JA and its natural analogue coronatine (COR, **7**, Fig. 1) promote a quick degradation of the JAZ repressors via the canonical JA-Ile pathway (Chini et al., 2007; Thines et al., 2007). The stability of JAZ1 was therefore evaluated after treatment with JA-Tyr (**5**) and JA-Sei (**6**). Both JA-derivates induce JAZ1-GUS degradation, similarly, but to a lesser extent, to JA (Fig. 2). To assess the requirement of the canonical JA-Ile pathway for the activity of JA-Tyr (**5**) and JA-Sei (**6**), we analyzed the stability of JAZ1-GUS in the *jar1-1* and *coi1-1* mutants, impaired in JA to JA-Ile conjugation and JA-Ile perception, respectively. As shown in Fig. 2, the induction of JAZ1 degradation by JA-Tyr (**5**) and JA-Sei (**6**) was dependent on COI1 and JAR1 because no JAZ1-GUS destabilization was observed in the mutant background, similarly to JA. As anticipated, COR (**7**) induces JAZ degradation in a JAR1-independent and COI1-dependent manner (Fig. 2).

In summary, JA-Tyr (**5**) and JA-Sei (**6**) can promote the degradation of the JAZ1 protein, and their activity required the canonical JA-Ile pathway.

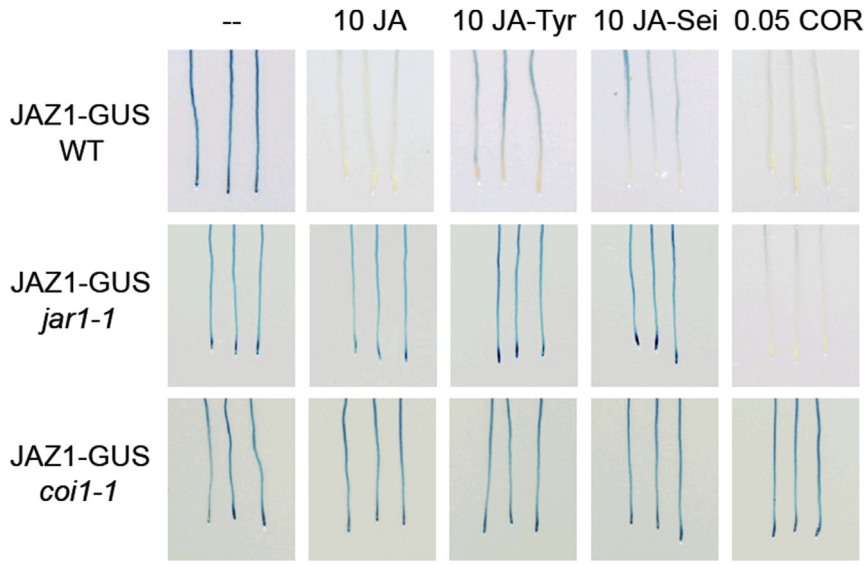


Fig. 2. JA-derivates (**5** and **6**) induce degradation of JAZ1 protein. GUS-staining for visualization of JAZ1 stability in roots of transgenic *Arabidopsis* 35S:JAZ1-GUS line in wild-type (WT) and mutant backgrounds. 6-day-old seedlings were treated for 1 h with mock solution (-), 10 μM JA, JA-Tyr, JA-Sei or 50 nM COR. JA-derivates (**5** and **6**) induce the degradation of JAZ1-GUS in a JAR1-and COI1-dependent manner.

2.4. Effect of JA synthetic derivates *5* and *6* on plants

The effect of JA-derivates **5** and **6** was also tested in JA-dependent growth inhibition, a typical physiological response regulated by JA. Wild-type (WT) *Arabidopsis* plants were grown in the presence of JA-Tyr (**5**) and JA-Sei (**6**). Both JA-derivates induced growth inhibition, similarly to JA (Fig. 3). To test if the activity of these JA-derivates required the canonical plant JA-Ile pathway, *jar1-1* and *coi1-1* mutants were also grown in presence of JA-Tyr (**5**) and JA-Sei (**6**). Growth of *jar1-1* plants (impaired in JA-Ile biosynthesis) is partially inhibited by JA-Tyr (**5**) and JA-Sei (**6**), similarly to JA, whereas *coi1-1* mutants are completely insensitive to the JA-derivates (Fig. 3). As expected, growth inhibition induced by COR (**7**) requires JAR1 but is COI1-dependent (Fig. 3).

Overall, these results show that JA-Tyr (**5**) and JA-Sei (**6**) trigger JA-regulated JAZ-degradation and growth inhibition and that their activity requires the canonical JA-pathway.

In conclusion, two JA derivatives of, JA-Tyr (**5**) and JA-Sei (**6**), were synthetized and HPLC and CD analysis confirmed that the two products were both obtained as diastereomeric mixtures from the coupling of *rac*-JA with enantiopure *S*-tyrosine-*tert*-butyl ester and seiridin. The results on bioassay *in planta* show that JA-Tyr and JA-Sei trigger the canonical plant JA-Ile-pathway, requiring JAR1 and COI1, key plant components of the JA conjugation to JA-Ile or JA-Ile perception respectively (Figs. 2 and 3). These data suggest that JA-Tyr and JA-Sei can be catabolized to release JA, which is consequently converted into JA-Ile activating plant JA responses. Supporting this hypothesis, the activity of JA-Tyr and JA-Sei requires an intact JA-Ile biosynthetic pathway and perception complex. Recently, a similar activity has been described for the natural fungal JA furanonyl ester LasA (Chini et al., under review).

Plants evolved a cleaving amidohydrolase activity on JA-conjugates; for example, the *Arabidopsis* enzyme IAR3/ILL6 deconjugates JA-Ile into JA (Widemann et al., 2013). The compound specificity might be quite loose because IAR3/ILL6 can also deconjugate 12-OH-JA-Ile into 12-OH-JA (Widemann et al., 2013). Therefore, JA-Tyr and JA-Sei may undergo a plant-mediated JA-deconjugation to liberate JA, the immediate precursor of the bioactive JA-Ile, consequently activating plant JA responses. A similar mode-of-action has been recently proposed for the natural fungal JA-derivate LasA (Chini et al., under review). Although the JA-derivates analyzed here are synthetic, our results reinforce the hypothesis that LasA and additional natural JA-conjugates act as pool of inactive conjugated JA converted into active JA-Ile and activating plant JA-responses in specific conditions. As a future development, we plan to resolve *rac*-JA into its enantiomers and prepare JA-Tyr, JA-Sei and possibly other derivatives as pure isomers instead of diastereomeric mixtures. At that point, we will be able to test and verify the impact of the stereochemistry of the JA moiety on the activity of its derivatives (Miersch et al., 1999; Fonseca et al., 2009).

Production of jasmonates or mimics to unbalance the plant immune system is a usual strategy of several pathogens (Gimenez-Ibanez et al., 2016; Fonseca et al., 2017). The JA-Ile mimic coronatine, produced by some strains of *Pseudomonas syringae*, is probably the best-studied example. COR is a functional analog of the bioactive JA-Ile hormone acting by direct binding to the JA-Ile receptor COI1-JAZ (Brooks et al., 2005; Sheard et al., 2010). Due to the antagonist relationship between JA- and SA-regulated defenses, the activation of the JA-Ile-pathway in turn restricts the SA-dependent immune responses, facilitating the bacterial infection. In contrast, fungal production of JA-conjugates such as LasA and the consequent induction of JA-Ile-promoted plant cell dead would occur at late fungal infection stages to facilitate fungal propagation (Chini et al., under review). Similarly, JA-Tyr and JA-Sei, although not natural JA-derivates, act as activators of the canonical JA-Ile-pathway, supporting the idea that LasA and additional natural JA-conjugates act as storage pool of inactive JA-conjugates quickly convertible into active JA-Ile to trigger plant JA-responses in specific conditions.

3. Experimental

3.1. General

Optical rotations were measured in a MeOH solution on a Jasco P-1010 digital polarimeter; ^{1}H and ^{13}C NMR spectra were recorded at 400/100 and/or 600/125 MHz in $CDCl_3$, unless otherwise noted, on Bruker spectrometers. Carbon multiplicities were determined by DEPT spectra (Berger and Braun, 2004). DEPT, COSY-45, HSQC,

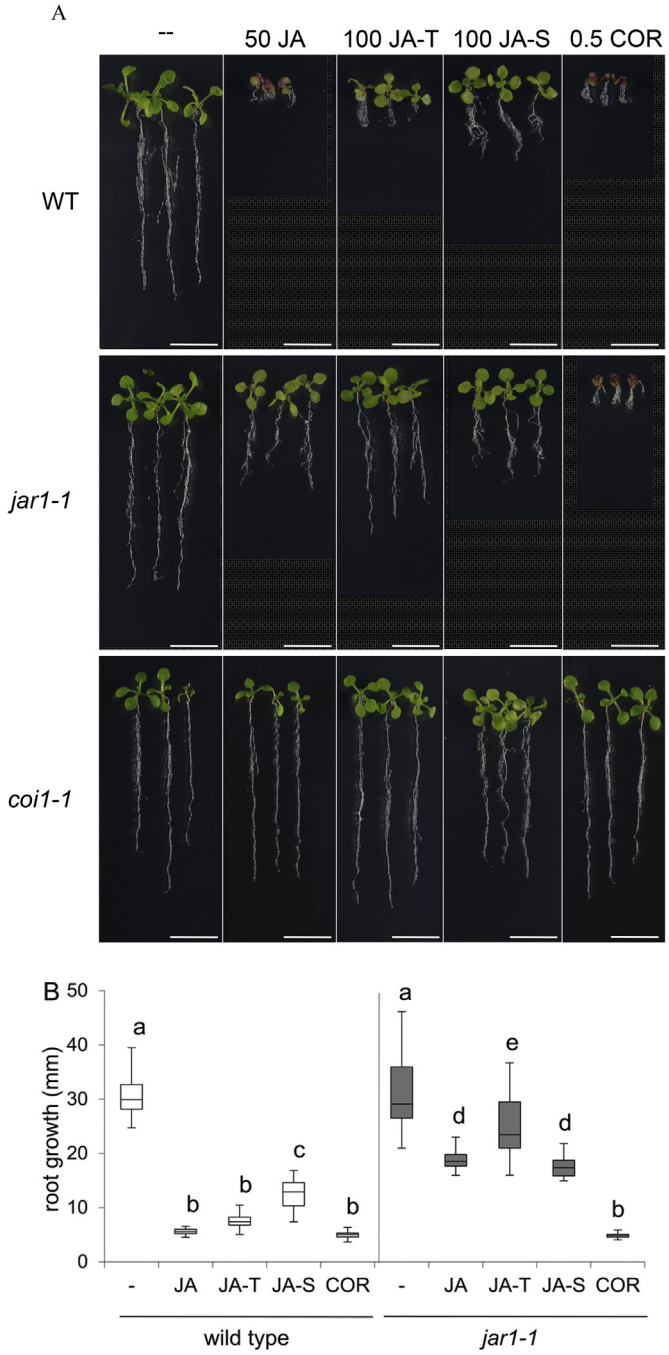


Fig. 3. Effect of JA-derivates on *Arabidopsis* plants. (A) Wild-type Col-0 (WT) and mutant *Arabidopsis* seedlings grown for 10 days on control medium (-) or medium supplemented with 100 μM JA-Tyr, JA-Sei, 50 μM JA or 0.5 μM COR. Bars, 1 cm. (B) Ten days after germination, root growth (mm) was measured in wild-type Col-0 (WT, white bars) and *jar1-1* (grey boxes) mutant seedlings (N = 12–25). Data presented as box-plots; horizontal lines are medians, boxes show the upper and lower quartile range and error bars show the full data range. The experiments were repeated at least 2 times with similar results. Letters stand for statistical differences (One-way ANOVA with post-hoc Tukey HSD, $p < .01$). JA-derivates (**5** and **6**) induce the root growth inhibition in a JAR1-and COI1-dependent manner.

HMBC and NOESY experiments (Berger and Braun, 2004) were performed using Bruker microprograms. CD spectra were measured with a Jasco J-715 spectropolarimeter. The spectra were recorded on solutions of **4** and **6** in CH_3OH (concentrations 6.53 mM and 4.45 mM, respectively), and quartz cells with various pathlengths from 0.02 to 1 cm. ESI MS and LC/MS analysis were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity). The HPLC separation were performed using a Phenomenex LUNA (C18 (2) 5u 150 × 4.6 mm). Enantioselective HPLC analyses were carried out by using Phenomenex Lux Cellulose-1 (5 u, 250 × 4.6 mm) or Lux Amylose-2 (3 u, 250 × 4.6 mm) columns on a Jasco PU-1580 instrument with UV-1575 detector (λ = 220 nm). Analytical and preparative TLCs were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm) plates. The spots were visualized by exposure to UV light (254 nm) and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min and/or by exposure to iodine vapors. (±) Jasmonic acid, HBTU, DCC, DIPEA solution for peptide synthesis (~2 M in 1-methyl-2-pyrrolidinone) and L-tyrosine *tert*-butyl ester were purchased from Sigma-Aldrich (San Louis, MO, USA). Seiridin was purified from the culture filtrates of *S. cardinale* (Evidente et al., 1986).

3.2. Synthesis of N-[(-)-jasmonoyl]-S-tyrosin (*5*)

(±) Jasmonic acid (10.00 mg, 0.048 mmol), L-tyrosine *tert*-butyl ester (11.36 mg, 0.048 mmol) and HBTU (18.32 mg, 0.050 mmol) were dissolved in 1.5 mL of dry DMF under nitrogen atmosphere. 100 μL of DIPEA solution (~2 M in 1-methyl-2-pyrrolidinone) was then added to this solution and the resulting mixture was stirred overnight. Then, water was added (4 mL), and the mixture was extracted with EtOAc (3 × 5 mL). The organic phase was then washed with 5 ml of 1 N $NaHCO_3$ and 5 ml of 1N HCl, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the residue was chromatographed on preparative TLC using $CHCl_3$-*i*-Pro-OH (97:3, v/v) yielding 18.7 mg of a compound which was further purified on reverse phase column, eluted with $CHCN_3$-H_2O (6:4, v/v), yielding *N*-[(-)-jasmonoyl]-*S*-tyrosine- *tert*-butyl ester (**1**) a colorless oil (13.6 mg, 67%). **4** had: ^1H and ^{13}C NMR spectra see Table 1; ESIMS (+) *m/z* 468 $[M+K]^+$, 430 $[M+H]^+$. **4** dissolved in dry CH_2Cl_2 (3 mL) was deprotected by reaction with TFA (80 μL), the solution was heated at 45 °C for 15 min, then left at room temperature for 24 h. The solvent and the excess acid was removed under reduced pressure yielding *N*-[(-)-jasmonoyl]-*S*-tyrosine (**5**) as pure compound (13.30 mg. 98%). **5** had: ^1H NMR spectrum see Table 1; ESIMS (+) *m/z* 769 $[2M+Na]^+$, 412 $[M+K]^+$, 374 $[M+H]^+$.

3.3. Synthesis of seiridin jasmonate (*3*)

(±) Jasmonic acid (10.00 mg, 0.048 mmol), seiridin (10.01 mg, 0.048 mmol) and DCC (11.70 mg, 0.056 mmol) were dissolved in dry CH_2Cl_2 at 0 °C. Then, 100 μL of pyridine was added and the mixture was stirred at 0 °C for 1 h. The reaction was stirred for other 2 h at room temperature, then the solvent was removed under reduced pressure. The residue (25 mg) was purified by preparative TLC using $CHCl_3$-*i*-Pro-OH (97:3, v/v) yielding compound **6** (4.90 mg, 50.4%) and unreacted jasmonic acid (3 mg). **6** had: ^1H NMR see Table 2; ESIMS (+) *m/z* 401 $[M+Na]^+$, 379 $[M+H]^+$.

3.4. Stereoselective HPLC analysis of optical purity of N-[(-)-jasmonyl]-S-tyrosin-tert-butyl ester (*4*) and the ester seiridin jasmonate (*6*)

Baseline separation of the diastereoisomers of **4** was achieved using Lux Cellulose-1 chiral stationary phase using 1.0 mL min^{-1} of *n*-hexane-*i*-Pro-OH (90:10, v/v) as the eluant. Retention times of the two main components were $t_R = 28.9$ min and $t_R = 35.6$ min. For **6**, partial resolution of the diastereomeric mixture ($t_R = 51.8$ min and $t_R = 53.8$ min) was attained by using a Lux Amilose-2 column with 1.0 mL min^{-1} of *n*-hexane-*i*-Pro-OH (80:20 v/v) as the eluant.

3.5. Plant material and growth conditions

Arabidopsis thaliana Col-0 is the genetic background of wild-type and *jar1-1* loss-of-function mutant used in this study (Staswick and Tiryaki, 2004). For root-growth inhibition assays, 12 to 30 seeds of each line were germinated for 10 days in absence or in the presence of 50 μM jasmonic acid, 0.5 μM coronatine, 100 μM JA-Tyr or JA-Sei. JA and COR are commercially available (Sigma). Pictures were taken with a Nikon D1x digital camera and root length calculated with ImageJ as described in Chini et al. (2018). Statistical analyses were performed using One-way ANOVA with post-hoc Tukey HSD Test for comparing multiple treatments.

3.6. JAZ1-GUS degradation assays

The 35S:JAZ1-GUS in wild-type, *coi1-30* and *jar1-1* background were described by Thines et al. (2007), Monte et al. (2014) and Chini et al., under review. 35S:JAZ1-GUS seedlings were grown vertically on MS plates and 6-day-old seedlings were treated for 1 h with 5 μM JA, 100 μM JA-Tyr or JA-Sei solution as described in Chini (2014). Samples were then placed in staining solution (X-Gluc, Glycosynth) and incubated overnight at 37 °C to visualize GUS activity. The analysis was performed using 10–20 plants per sample and images taken with a Nikon D1x camera. This experiment was repeated three times with similar results.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2017.12.017.

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