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Phytoalexins and phytoanticipins from the wild crucifers *Thellungiella halophila* and *Arabidopsis thaliana*: Rapalexin A, wasalexins and camalexin

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Abstract

Investigation of phytoalexin production using abiotic elicitation showed that the phytoalexin rapalexin A was produced by both *Thellungiella halophila* and *Arabidopsis thaliana*, but while *A. thaliana* produced camalexin, *T. halophila* produced wasalexins A and B and methoxybrassenin B. Considering that the genome of *T. halophila* is being sequenced currently and that the wasalexin pathway present in *T. halophila* is expected to involve a number of genes also present in *Brassica* species, our discovery should facilitate the isolation of genes involved in biosynthetic pathways of phytoalexins of the most economically important crucifer species.

Keywords: Brassicaceae; *Brassica*; Camalexin; Crucifer; Phytoalexin; Rapalexin; Wasalexin

1. Introduction

The plant species *Thellungiella halophila* (salt cress) is highly tolerant to salt and drought stress (abiotic stresses), unlike its close crucifer relative, the well-known model plant *Arabidopsis thaliana* L. In contrast to *A. thaliana*, only a few genes were induced by salt stress in *T. halophila*, although the genes of both species have high sequence identity (90–95% at *c*DNA level, Taji et al., 2004). Recently, it was shown that the various genes induced by abiotic and biotic stresses in *A. thaliana* were overexpressed in *T. halophila* under unstressed conditions. For example, under normal growing conditions, *T. halophila* accumulated proline at much higher levels than *A. thaliana*, and was more tolerant to oxidative stress. For this reason, it was suggested that stress-inducible signaling pathways were constitutive and active in *T. halophila* even under normal growth conditions (Taji et al., 2004; Inan et al., 2004). Nonetheless, whether these signaling pathways induce changes on secondary metabolite profiles related with stress responses remains to be determined. In general, plant exposure to abiotic or biotic stresses initiates an elaborate cascade of metabolic reactions, some of which lead to the biosynthesis of secondary metabolites with active roles in self-defense. While phytoalexins are defense metabolites biosynthesized de novo, phytoanticipins are constitutive but their production can increase in response to various forms of stress (VanEtten et al., 1994). Plants are known to accumulate complex phytoalexin blends in response to stress, although the only phytoalexin reported from *A. thaliana* is camalexin (**9**) (Tsuji et al., 1992) and no phytoalexins from *T. halophila* are known (Pedras et al., 2007b). Considering the relevance of the species *A. thaliana* and *T. halophila* as model systems, we undertook to determine the phytoalexins and phytoanticipins produced by both crucifer species under abiotic stress conditions. Unexpectedly, it was established that the phytoalexin rapalexin A (**4**) was produced in both species; in addition, *T. halophila* produced wasalexins A (**1**) and B (**2**) and methoxybrassenin B (**3**), while only camalexin (**9**) was detected in *A. thaliana*. Although several genes from the camalexin pathway are known, no genes involved in the

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biosynthesis of many other crucifer phytoalexins have been cloned (Pedras et al., 2007a). Our discovery should facilitate such work since the wasalexin pathway present in *T. halophila* is expected to involve a number of genes also present in other crucifers, including the economically important crucifer genus *Brassica*.

2. Results and discussion

Time-course studies were carried out using 4- to 5-week-old plants sprayed with a $CuCl_2$ solution, as described in the Experimental. Sprayed plants were uprooted every 24 h for six consecutive days. The fresh aerial parts from both control and treated plants were frozen in liquid nitrogen, crushed, and then extracted. The extracts of both control and elicited plants were further partitioned into polar and non-polar fractions and then analyzed by the HPLC-DAD–MS.

In the case of *T. halophila* ecotype Shandong, HPLC-DAD–MS analysis of the non-polar fraction led to the identification of several elicited metabolites by comparison of their UV spectra and retention times with those of our current phytoalexin library (Pedras et al., 2006). The elicited metabolites included the phytoalexins wasalexins A (**1**) and B (**2**), methoxybrassenin B (**3**), and rapalexin A (**4**) (Table 1) and the phytoanticipins indolyl-3-acetonitrile (5), 4-methoxyindolyl-3-acetonitrile (**6**, i.e. arvelexin), 1-methoxyindolyl-3-acetonitrile (**7**), and indole-3-carboxaldehyde (**8**) (Table 2). These phytoanticipins were present in extracts of elicited plants in substantially larger amounts than in control plants (Table 2). Interestingly, two very diverse plant species (*Wasabi japonica* syn. *Eutrema wasabi* and *Thlaspi arvense*) were reported to produce wasalexins A (**1**) and B (**2**) (Pedras et al., 1999; Pedras et al., 2003b), whereas methoxybrassenin B (**3**) was previously isolated from *Brassica oleracea* (Monde et al., 1991) and rapalexin A (**4**) from *Brassica rapa* (Pedras et al., 2007a). Isolation of metabolites produced by *T. halophila* ecotype Yukon after 24 h of elicitation showed a metabolite profile similar to that of ecotype Shandong.

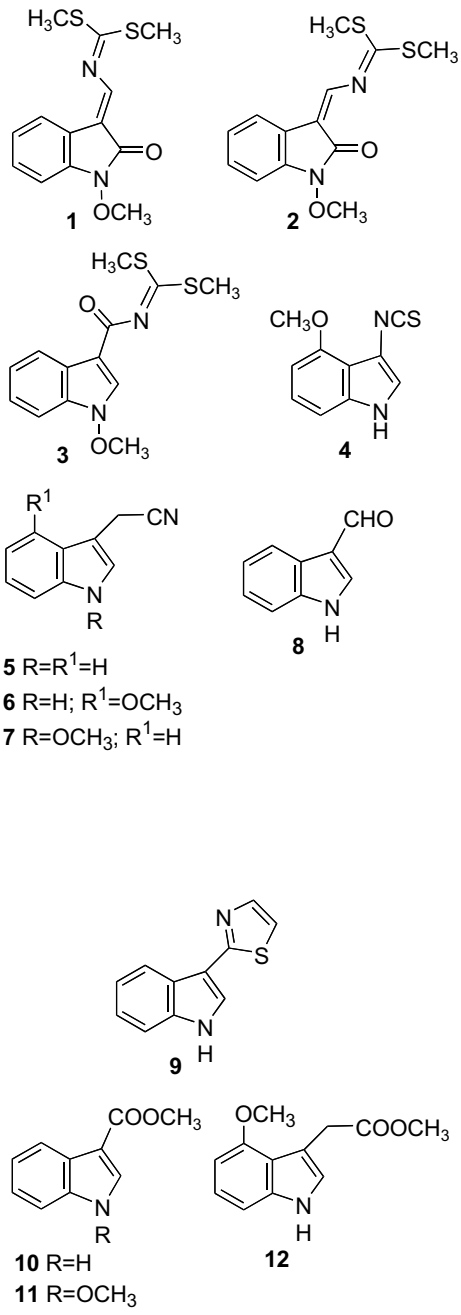

Table 1
Production of phytoalexins (μmol/100 g fresh weight) **1**–**4** by *Thellungiella halophila* ecotype Shandong and phytoalexins **4** and **9** by *Arabidopsis thaliana* ecotype Columbia upon elicitation with $CuCl_2$

Phytoalexin[a]	24 h	48 h	72 h	96 h	120 h	144 h
Wasalexin A (**1**)	4.5 ± 0.7	7.1 ± 2.8	5.4 ± 1.5	4.5 ± 0.2	3.5 ± 0.9	3.0 ± 1.1
Wasalexin B (**2**)	0.90 ± 0.14	1.4 ± 0.56	1.1 ± 0.3	0.90 ± 0.04	0.70 ± 0.27	0.60 ± 0.22
Methoxybrassenin B (**3**)	1.54 ± 0.21	2.43 ± 0.95	1.84 ± 0.48	1.57 ± 0.04	1.18 ± 0.29	1.02 ± 0.33
Rapalexin A (**4**)[b]	N.D.[c]	N.D.	N.D.	N.D.	N.D.	N.D.
Camalexin (**9**)	0.020 ± 0.004	0.013 ± 0.007	0.014 ± 0.005	0.017 ± 0.018	0.010 ± 0.002	N.D.

[a] Results are presented as means ± standard deviation.
[b] Rapalexin A (**4**) was detected only after column fractionation and was isolated from *T. halophila* (ca. 0.56 nmol/100 g fresh weight) and *A. thaliana* (0.91 nmol/100 g fresh weight) 24 h post-elicitation.
[c] Not detected.

Table 2
Production of phytoanticipins (μmol/100 g fresh weight)[a] by *Thellungiella halophila* ecotype Shandong and *Arabidopsis thaliana* ecotype Columbia

Phytoanticipin/plant species	Elicitor	24 h	48 h	72 h	96 h	120 h	144 h
Indolyl-3-acetonitrile (**5**)							
T. halophila	Control	N.D.[b]	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A. thaliana	Control	10.7 ± 0.1	1.25 ± 0.00	1.35 ± 0.00	2.13 ± 0.00	1.38 ± 0.00	N.D.
	$CuCl_2$	6.47 ± 0.18	3.30 ± 0.03	4.72 ± 0.12	6.67 ± 0.17	3.19 ± 0.01	N.D.
4-Methoxyindolyl-3-acetonitrile (**6**, arvelexin)							
T. halophila	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A. thaliana	Control	0.43 ± 0.09	0.15 ± 0.08	0.41 ± 0.04	0.43 ± 0.03	0.25 ± 0.16	N.D.
	$CuCl_2$	0.69 ± 0.36	0.58 ± 0.17	0.89 ± 0.26	1.33 ± 0.30	0.66 ± 0.14	N.D.
1-Methoxyindolyl-3-acetonitrile (**7**)							
T. halophila	Control	0.30 ± 0.02	0.60 ± 0.02	0.70 ± 0.03	0.70 ± 0.05	1.20 ± 0.03	N.D.
	$CuCl_2$	0.60 ± 0.05	0.70 ± 0.05	0.30 ± 0.02	1.60 ± 0.17	0.80 ± 0.03	N.D.
A. thaliana	Control	91.8 ± 0.6	32.6 ± 0.2	42.4 ± 0.4	58.7 ± 0.3	57.0 ± 0.2	N.D.
	$CuCl_2$	70.8 ± 29.1	55.2 ± 16.8	79.6 ± 20.7	122 ± 12	49.3 ± 11.9	N.D.
Methyl indole-3-carboxylate (**10**)[c]							
T. halophila	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A. thaliana	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Methyl 1-methoxyindole-3-carboxylate (**11**)[d]							
T. halophila	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A. thaliana	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Methyl 4-methoxyindolyl-3-acetate (**12**)[e]							
T. halophila	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
A. thaliana	Control	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	$CuCl_2$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Results are presented as means ± standard deviation.
[b] Not detected.
[c] Methyl indole-3-carboxylate (**10**) was detected only after column fractionation and was isolated only from *A. thaliana* (0.45 nmol/100 g fresh weight).
[d] Methyl 1-methoxyindole-3-carboxylate (**11**) was detected only after column fractionation and was isolated only from *A. thaliana* (0.77 nmol/100 g fresh weight).
[e] Methyl 4-methoxyindolyl-3-acetate (**12**) was detected only after column fractionation and was isolated only from *T. halophila* (0.36 nmol/100 g fresh weight).

Additional experiments carried out with *A. thaliana* ecotype Columbia showed the phytoalexins camalexin (**9**) and rapalexin A (**4**) (Table 1), and phytoanticipins indolyl-3-acetonitrile (**5**), arvelexin (**6**), 1-methoxyindolyl-3-acetonitrile (**7**), indole-3-carboxaldehyde (**8**), methyl indole-3-carboxylate (**10**), methyl 1-methoxyindole-3-carboxylate (**11**), and methyl 4-methoxyindolyl-3-acetate (**12**), a new metabolite (Table 2). Compound **12** was reported earlier as an intermediate in the synthesis of psilocin (Sakagami and Ogasawara, 1999) but not as a natural product, whereas methyl 1-methoxyindole-3-carboxylate (**11**) was reported as a phytoalexin from wasabi plants (Pedras et al., 1999). The ^{1}H NMR spectral data of compound **12** was identical to those of the synthetic compound (Sakagami and Ogasawara, 1999). A substantial increase in the amounts of indolyl-3-acetonitrile (**5**) and arvelexin (**6**) was observed after spraying plants with $CuCl_2$. As well, in the polar fraction of the aerial parts of *A. thaliana*, tryptophan could be identified in larger amounts than in control plants (Table 2). This is not surprising since tryptophan is a precursor of many of the indolyl metabolites reported above (Pedras et al., 2007b).

In conclusion, these results demonstrate that, despite the close genetic relationship between *A. thaliana* and *T. halophila*, the two species differ in the phytoalexin and phytoanticipin qualitative contents (Table 3), though both produce rapalexin A (**4**). Although several genes from the camalexin pathway are known, including a recent report of another P450 (Nafisi et al., 2007), the genes involved in the biosynthesis of other crucifer phytoalexins, including the most economically important genus *Brassica*, have not been cloned (Pedras et al., 2007b). Considering that the genome of *T. halophila* is being sequenced (http://www.jgi.doe.gov/sequencing/why/CSP2007/thellungiella.html), and that the wasalexin pathway present in *T. halophila* is expected to involve a number of genes also present in *Brassica* species, our discovery should facilitate the isolation of genes involved in brassica phytoalexin pathways.

Table 3
Metabolites from *Thellungiella halophila* ecotypes Shandong and Yukon and *Arabidopsis thaliana* ecotype Columbia

Compounds (structure number, HPLC t_R, method[a])	*T. halophila*		*A. thaliana* Columbia
	Shandong	Yukon	
Wasalexin A (**1**, $t_R = 25.4$ min, A; $t_R = 23.2$ min, B)	X	X	N.D.[b]
Wasalexin B (**2**, $t_R = 25.0$ min, A; $t_R = 20.9$ min, B)	X	X	N.D.[b]
Methoxybrassenin B (**3**, $t_R = 25.3$ min, A; $t_R = 23.7$ min, B)	X	X	N.D.[b]
Rapalexin A (**4**, $t_R = 24.9$ min, A)	X	X	X
Indolyl-3-acetonitrile (**5**, $t_R = 12.2$ min, A)	X	X	X
Arvelexin (**6**, $t_R = 14.0$ min, A)	X	X	X
1-Methoxyindolyl-3-acetonitrile (**7**, $t_R = 17.6$ min, A)	X	X	X
Indole-3-carboxaldehyde (**8**, $t_R = 7.5$ min, A)	X	N.D.[b]	X
Camalexin (**9**, $t_R = 16$ min, very broad peak A)	N.D.[b]	N.D.[b]	X
Methyl indole-3-carboxylate (**10**, $t_R = 12.9$ min, A)	N.D.[b]	N.D.	X
Methyl 1-methoxyindole-3-carboxylate (**11**, $t_R = 19.1$ min, A)	N.D.[b]	N.D.	X
Methyl 4-methoxyindolyl-3-acetate (**12**, $t_R = 14.5$ min, A)	N.D.[b]	N.D.	X
Tryptophan ($t_R = 2.4$ min, A)	X	X	X

[a] Method A: Hypersil ODS column (5 μm particle size silica, 4.6 mm i.d. × 200 mm), H_2O–CH_3CN, 75:25 to 100% CH_3CN, in 35 min, linear gradient, 1.0 ml/min; method B: Eclipse XSB C-18 column (5 μm particle size silica, 4.6 mm i.d., 150 mm), 0.2% formic acid in H_2O and 0.2% formic acid in CH_3CN, 75% H_2O to 75% CH_3CN in 35 min, to 100% CH_3CN in 5 min), 1.0 ml/min.
[b] Not detected.

3. Experimental

3.1. General

All solvents were of HPLC grade and used as such. All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON. Analytical HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode array detector (wavelength range 190–600 nm), degasser, and a column equipped with an in-line filter. Mobile phase: method A, Hypersil ODS column (5 μm particle size silica, 4.6 mm i.d. × 200 mm), H_2O–CH_3CN, 75:25 to 100% CH_3CN, in 35 min, linear gradient, and 1.0 ml/min flow rate; method B, Eclipse XSB C-18 column (5 μm particle size silica, 4.6 mm i.d., 150 mm), 0.2% formic acid in H_2O and 0.2% formic acid in CH_3CN (75% H_2O to 75% CH_3CN in 35 min, to 100% CH_3CN in 5 min) and a flow rate of 1.0 ml/min. Other conditions are as previously reported (Pedras et al., 2006).

3.2. Plant material and growth conditions

Seeds of *A. thaliana* (Columbia ecotype CS 6673) were purchased from The Arabidopsis Information Resource (TAIR). Seeds were sown in Petri dishes and placed in the dark at 4 °C for 4 days. The Petri dishes were transferred to a growth cabinet under white fluorescent lamps (75–100 μmol/m^2 s) at 22 °C/16 h light and 16 °C/8 h dark cycles. Seedlings were transplanted into larger pots at ca. 7 days and were watered regularly up to 8 weeks.

Seeds of *T. halophila* (Yukon CS22664 and Shandong CS22504 ecotypes) were purchased from TAIR. Seeds were sown in Petri dishes and placed in the dark at 4 °C for 10–14 days. The Petri dishes were transferred to a growth cabinet under white fluorescent lamps (75–100 μmol/m^2 s) at 25 °C/16 h-light and 16 °C/8 h dark cycles. Seedlings were transplanted into larger pots at ca. 7 days and were watered regularly. When plants were about 2.5 cm in diameter they were returned to cold room at 4 °C for 7 days in the dark with little watering up to 8 weeks (only for the purpose of obtaining seeds).

3.3. Plant extracts and chromatographic fractionation for identification of metabolites

Initially, time-course studies for the detection of phytoalexins were carried out with 4-week-old *T. halophila*. Plants were sprayed with $CuCl_2$ (10^{-3} M) and were uprooted every 24 h for six consecutive days. The fresh aerial part samples from both control and treated plants were weighed, frozen in liquid nitrogen, crushed, and then soaked in MeOH for 3 h. The MeOH was decanted, dried and evaporated to dryness under reduced pressure. The residues from both control and elicited plants were weighed and then further partitioned into polar and non-polar fractions and analyzed by the HPLC-DAD–MS. Similar experiments were carried out with *A. thaliana*. These analyses allowed a preliminary identification of induced metabolites that were further confirmed after larger amounts of plant extracts were obtained for isolation and spectroscopic characterization of each compound, as follows.

Extraction of *T. halophila* (Shandong ecotype): 5-week-old plants were sprayed with $CuCl_2$ (10^{-2} M), the aerial parts were collected after 24 h, weighed (140 g), frozen in liquid nitrogen and soaked in MeOH under continuous stirring for 3 h. The MeOH was decanted, dried and evaporated to dryness under reduced pressure to afford a residue (ca. 3 g). Column chromatography (silica gel) of the plant extracts first using a gradient of CH_2Cl_2–MeOH followed by preparative TLC led to identification of metabolites shown in Tables 1 and 2. Identification of each

metabolite was carried out using NMR and HRMS spectroscopy, and HPLC-DAD–MS data, as well as direct comparison with authentic samples available in our metabolite library (Pedras et al., 2006).

Extraction of *A. thaliana* (Columbia ecotype): Similar experiments carried out as reported above for *T. halophila* (Shandong ecotype) led to identification of all metabolites reported in Tables 1 and 2. The spectroscopic data obtained for characterization of the metabolite **12** isolated for the first time from *A. thaliana* is reported below.

Methyl 4-methoxyindolyl-3-acetate (**12**). HPLC $t_R = 14.5$ min, method A; ^{1}H NMR (500 MHz, CD_3CN) δ: 3.66 (*s*, 3H), 3.83 (*s*, 3H), 3.85 (*s*, 2H), 6.49 (*d*, $J = 7.5$ Hz, 1H), 6.99–7.07 (*m*, 3H) 9.11 (*brs*, NH); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.73 (*s*, 3H), 3.89 (*s*, 3H), 3.96 (*s*, 2H), 6.50 (*d*, $J = 8$ Hz, 1H), 6.97 (*d*, 1H, $J = 8$ Hz), 7.04 (*brs*, 1H), 7.10 (*dd*, $J = 8$ Hz, 1H) 8.00 (*brs*, NH). MS-ESI *m*/*z* (relat. int.) 242 $[M+Na]^+$ (2), 220 $[M+H]^+$ (9), $[M+H–HCOOMe]^+$ 160 (100). HREIMS *m*/*z* measured: 219.0896 (219.0896 calcd. for $C_{12}H_{13}NO_3$).

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