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Short communication

Folic acid induces salicylic acid-dependent immunity in Arabidopsis and enhances susceptibility to *Alternaria brassicicola*

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SUMMARY

Folates are essential for one-carbon transfer reactions in all organisms and contribute, for example, to *de novo* DNA synthesis. Here, we detected the folate precursors 7,8-dihydropteroate (DHP) and 4-amino-4-deoxychorismate (ADC) in extracts from *Arabidopsis thaliana* plants by Fourier transform ion cyclotron resonance-mass spectrometry. The accumulation of DHP, but not ADC, was induced after infection of plants with *Pseudomonas syringae* delivering the effector protein AvrRpm1. Application of folic acid or the DHP precursor 7,8-dihydroneopterin (DHN) enhanced resistance in Arabidopsis to *P. syringae* and elevated the transcript accumulation of the salicylic acid (SA) marker gene *PATHOGENESIS-RELATED1* in both the treated and systemic untreated leaves. DHN- and folic acid-induced systemic resistance was dependent on SA biosynthesis and signalling. Similar to SA, folic acid application locally enhanced Arabidopsis susceptibility to the necrotrophic fungus *Alternaria brassicicola*. Together, the data associate the folic acid pathway with innate immunity in Arabidopsis, simultaneously activating local and systemic SA-dependent resistance to *P. syringae* and suppressing local resistance to *A. brassicicola*.

Keywords: 7,8-dihydropteroate, *Arabidopsis thaliana*, cross-talk, folic acid, innate immunity, salicylic acid, systemic acquired resistance.

Plants recognize and respond to incoming signals to defend themselves against pathogen attack and the concomitant stress. Pattern recognition receptors (PRRs) located on the plant cell surface detect pathogen-associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI; Jones and Dangl, 2006; Spoel and Dong, 2012). A second layer of defence is activated by nucleotide binding/leucine-rich repeat (NLR) receptors directly or indirectly recognizing pathogen effectors, which leads to effector-triggered immunity (ETI; Jones and Dangl, 2006; Spoel and Dong, 2012). ETI is a relatively strong resistance that culminates in the hypersensitive response, a form of programmed cell death of the infected site and surrounding cells, restricting pathogen spread (Jones and Dangl, 2006; Mur *et al*., 2008; Spoel and Dong, 2012). Both PTI and ETI are associated with the enhanced accumulation of the phytohormone salicylic acid (SA) and the induction of systemic acquired resistance (SAR), a long-lasting, broad-spectrum disease resistance in systemic uninfected tissues (Fu and Dong, 2013; Spoel and Dong, 2012; Vlot *et al*., 2009).

Several putative long-distance signals have been associated with SAR, including methyl salicylate (Park *et al*., 2007), azelaic acid (AzA; Jung *et al*., 2009; Wittek *et al*., 2014), glycerol-3-phosphate (G3P; Chanda *et al*., 2011), dihydroabietinal (Chaturvedi *et al*., 2012), pipecolic acid (Navarova *et al*., 2012) and the predicted lipid transfer proteins DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) and DIR1-like (Champigny *et al*., 2013; Maldonado *et al*., 2002). In addition, SAR is associated with another lipid transfer protein, AZELAIC ACID INDUCED 1 (AZI1; Jung *et al*., 2009), two predicted apoplastic aspartyl proteases that repress and promote SAR, respectively (Breitenbach *et al*., 2014; Xia *et al*., 2004), and the putative carbohydrate-binding protein LEGUME LECTIN-LIKE PROTEIN1 (LLP1), which is essential for systemic, but not local, resistance to *Pseudomonas syringae* in Arabidopsis (Breitenbach *et al*., 2014). SAR is further influenced by abiotic factors, such as light (Griebel and Zeier, 2008; Liu *et al*., 2011; Zeier *et al*., 2004), and SAR signal perception requires an intact cuticle in the systemic uninfected tissue (Xia *et al*., 2009, 2010). An increasing body of evidence suggests that SAR signalling components interact with each other to promote SAR (Dempsey and Klessig, 2012; Gao *et al*., 2014; Kachroo and Robin, 2013; Shah *et al*., 2014; Shah and Zeier, 2013; Spoel and Dong,

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2012). For example, dihydroabietinal appears to cooperate with AzA and/or AZI1 in SAR (Chaturvedi *et al*., 2012), and DIR1 and AZI1 may act in a positive feedback loop with G3P promoting SAR downstream of AzA (Yu *et al*., 2013).

A key SAR signalling component acting upstream of SA in innate immunity is ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Falk *et al*., 1999). EDS1 is essential for PTI and ETI mediated by a subset of NLR receptors (Aarts *et al*., 1998; Zhu *et al*., 2011), but not for ETI mediated by the NLR receptors RPM1 and RPS2 responding to the *Pseudomonas syringae* effector proteins AvrRpm1 and AvrRpt2, respectively (Aarts *et al*., 1998; Bent *et al*., 1994; Dangl *et al*., 1992). Nevertheless, EDS1 is essential for SAR induced by *P. syringae* delivering AvrRpm1 and AvrRpt2 (Rietz *et al*., 2011; Truman *et al*., 2007). We have recently associated the SAR-deficient phenotype of the *eds1* mutant in response to AvrRpm1 with a reduced accumulation of AzA and two of its lipid peroxidation precursors (Wittek *et al*., 2014). In addition, we observed an apparently *EDS1*-independent accumulation of the folate precursors 7,8-dihydropteroate (DHP) and 4-amino-4-deoxychorismate (ADC; Fig. 1) after Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) analysis of extracts from *AvrRpm1*-expressing plants (data not shown). These compounds caught our attention because folates may promote plant growth and/or yield (Burguieres *et al*., 2007; Song *et al*., 2013), whereas the chloroplastic folate precursor *para*-aminobenzoic acid (PABA; Fig. 1) enhances resistance in pepper to *Xanthomonas axonopodis* and *Cucumber mosaic virus* (Song *et al*., 2013).

Here, we analysed the accumulation of folates after infection of 4–5-week-old *Arabidopsis thaliana* ecotype Col-0 wild-type (wt) and *eds1-2* mutant plants (Bartsch *et al*., 2006) with *Pseudomonas syringae* pathovar *tomato* (*Pst*) delivering AvrRpm1 (*Pst/AvrRpm1*; Aarts *et al*., 1998). The plants were maintained in short day (10-h) conditions as described by Breitenbach *et al*. (2014) and fully expanded leaves were syringe infiltrated with 10 mM $MgCl_2$ (mock treatment) or 10^5 colony-forming units (cfu)/mL of *Pst/AvrRpm1*. Two days later, metabolites were extracted from 50 mg of tissue per sample in 1.5 mL of methanol (MeOH)–H_2O (1:1, v/v) in an ultrasound bath for 10 min. After centrifugation of the samples at the highest speed (depending on the rotor), the supernatant was diluted 1:20 with MeOH–H_2O (1:1, v/v). Subsequently, samples from untreated, mock-treated and infected plants were analysed by FTICR-MS focusing on negatively

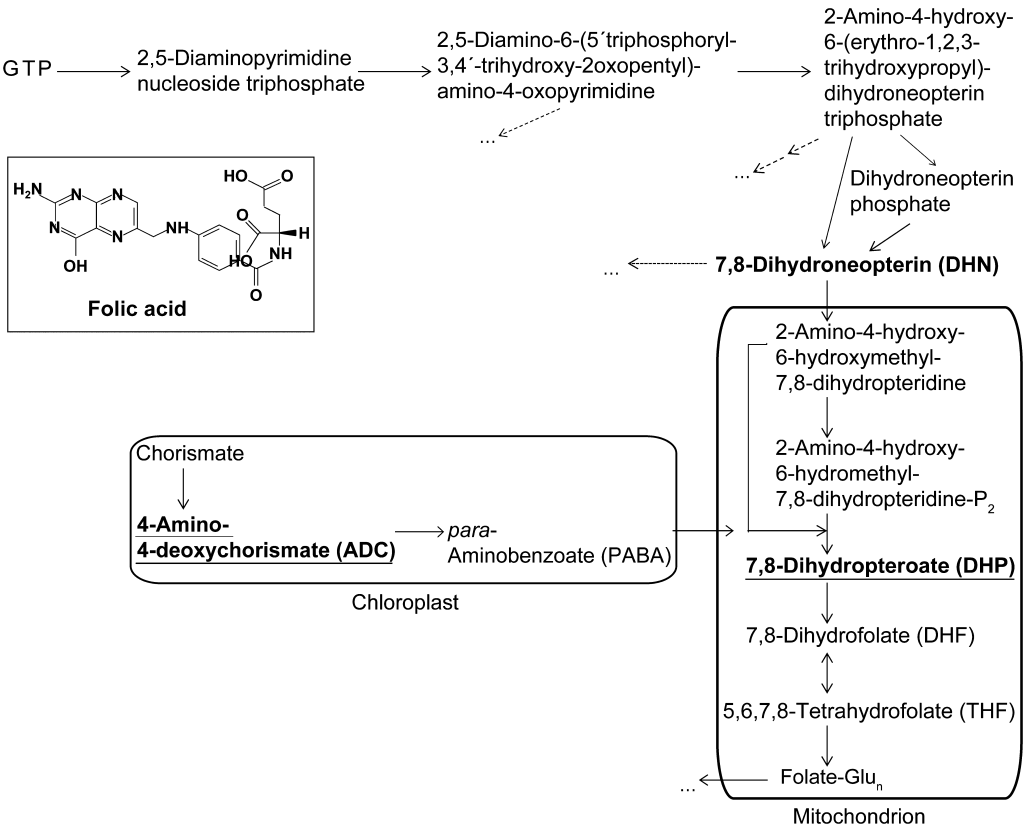

Fig. 1 Folate biosynthesis pathway. The pathway was adapted from http://www.genome.jp/kegg/pathway and Hanson and Gregory (2011). The compounds that were annotated by Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) are highlighted in bold and underlined, and the compounds that were applied to plants are highlighted in bold. The part of the pathway that is not encircled in organelles is located to the cytosol. Inset: folic acid structure from www.chemspider.com.

Table 1 Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS)-assisted annotation of 7,8-dihydropteroate (DHP) and 4-amino-4-deoxychorismate (ADC) in Arabidopsis extracts, including the theoretical mass (from KEGG), the FTICR-MS-determined experimental mass and the difference between the theoretical and experimental mass in ppm. $[M–H]^–$, negatively charged ions.

Annotated metabolite	Theoretical mass $[M–H]^–$	Experimental mass $[M–H]^–$	Error (ppm)
DHP	313.105462	313.1059906	−1.688
ADC	224.056446	224.0564606	−0.065

charged ions ($[M-H]^–$) as described by Wittek *et al*. (2014). We detected masses that were annotated as DHP and ADC when queried against the KEGG, Knapsack and Human Metabolome DataBase (Table 1; Afendi *et al*., 2012; Kanehisa *et al*., 2014; Wishart *et al*., 2007). DHP was not detected in the samples from untreated plants and was just above the detection limit in two of three biologically independent samples from mock-treated plants (Fig. 2a). *Pst/AvrRpm1* infection enhanced DHP accumulation in wt plants (Fig. 2a), suggesting that DHP is associated with Arabidopsis responses to infection or the effector AvrRpm1. Because the DHP levels were similar in *Pst/AvrRpm1*-infected wt and *eds1-2* mutants, which are defective for SAR but not for ETI (Aarts *et al*., 1998; Breitenbach *et al*., 2014; Truman *et al*., 2007), the data suggest that a putative role of DHP in plant immunity is not limited to SAR. DHP is synthesized in mitochondria from two precursors, 2-amino-4-hydroxy-6-hydromethyl-7,8-dihydroneopterin-P_2, derived from cytosolic pterin precursors, and PABA (Fig. 1). Here, we detected the immediate PABA precursor ADC, which seemed to accumulate to similar levels in the extracts from untreated, mock-treated and *Pst/AvrRpm1*-infected wt and *eds1-2* mutant plants (Fig. 2b), suggesting that ADC accumulation was not affected by infection. In conclusion, the accumulation of the folate precursor DHP, but not ADC, was induced in Arabidopsis by *Pst/AvrRpm1*, putatively associating DHP with plant responses to infection or the effector AvrRpm1.

Subsequently, we characterized the resistance-inducing capacity of the DHP precursor 7,8-dihydroneopterin (DHN) and the artificial folate folic acid (Fig. 1), which can be intracellularly metabolized to tetrahydrofolate (Hanson and Gregory, 2011). We focused first on SAR, which was induced as described by Wittek *et al*. (2014) by a primary inoculation of the first two true leaves of 4–5-week-old Col-0 wt plants by syringe infiltration of different concentrations of DHN or folic acid, or 10^6 cfu/mL of *Pst/AvrRpm1* as a positive control, or 10 mM $MgCl_2$ or 0.1% MeOH as a negative control. Three days later, the next two upper leaves were inoculated with 10^5 cfu/mL of virulent *Pst* and the resulting *Pst* titres were determined at 4 days post-inoculation (dpi) as described by Breitenbach *et al*. (2014). As expected, the primary *Pst/AvrRpm1* inoculation induced SAR, reducing the systemic *Pst* titres compared with those in the negative control plants (Fig. 3a). In addition, application of 250 μM, but not 1 mM, 100 μM, 50 μM or 25 μM, DHN reduced the growth of the systemic *Pst* inoculum compared with that in the negative control plants, indicating that the application of 250 μM DHN induced systemic resistance (Fig. 3a). Similarly, folic acid induced systemic resistance to *Pst* when applied at 50 or 25 μM, but not when applied at 1 mM, 250 μM or 100 μM (Fig. 3a). Thus, both DHN and folic acid induced systemic immunity in a concentration-dependent manner. It is currently unclear why the concentration window for SAR induction by DHN or folic acid application was so narrow, but this was not unprecedented. A similarly narrow concentration window was observed for SAR induction by 9-oxo nonanoic acid application, a precursor of the putative SAR signal AzA, to Arabidopsis (Wittek *et al*., 2014), suggesting that SAR is sensitive to the concentration of its associated signalling compounds. Notably, this might (partially) explain the inverse relationship between the concentration of a SAR-inducing pathogen inoculum and the extent of SAR (Mishina and Zeier, 2007).

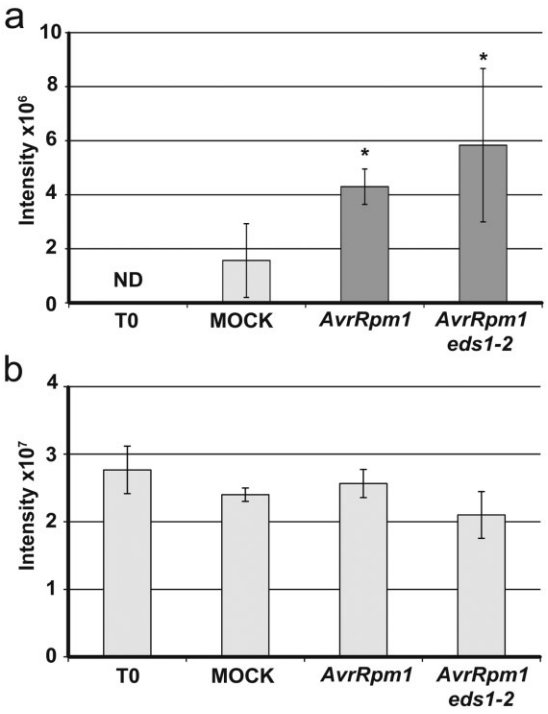

Fig. 2 7,8-Dihydropteroate (DHP), but not 4-amino-4-deoxychorismate (ADC), is induced in Arabidopsis by *Pseudomonas syringae* pathovar *tomato* (*Pst*) delivering AvrRpm1 (*Pst/AvrRpm1*). Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) signal intensities of the mass peaks that were annotated as DHP (a) and ADC (b) in untreated wild-type (wt) leaves (T0), in wt leaves at 2 days post-inoculation (dpi) of 10 mM $MgCl_2$ (MOCK), and in wt and *eds1-2* mutant leaves (the mutant is indicated) at 2 dpi of *Pst/AvrRpm1*. Plotted values are the average ± standard deviation of three biologically independent replicates (except for two replicates for DHP in mock-treated leaves, which was undetectable in the third replicate). ND, not detectable.

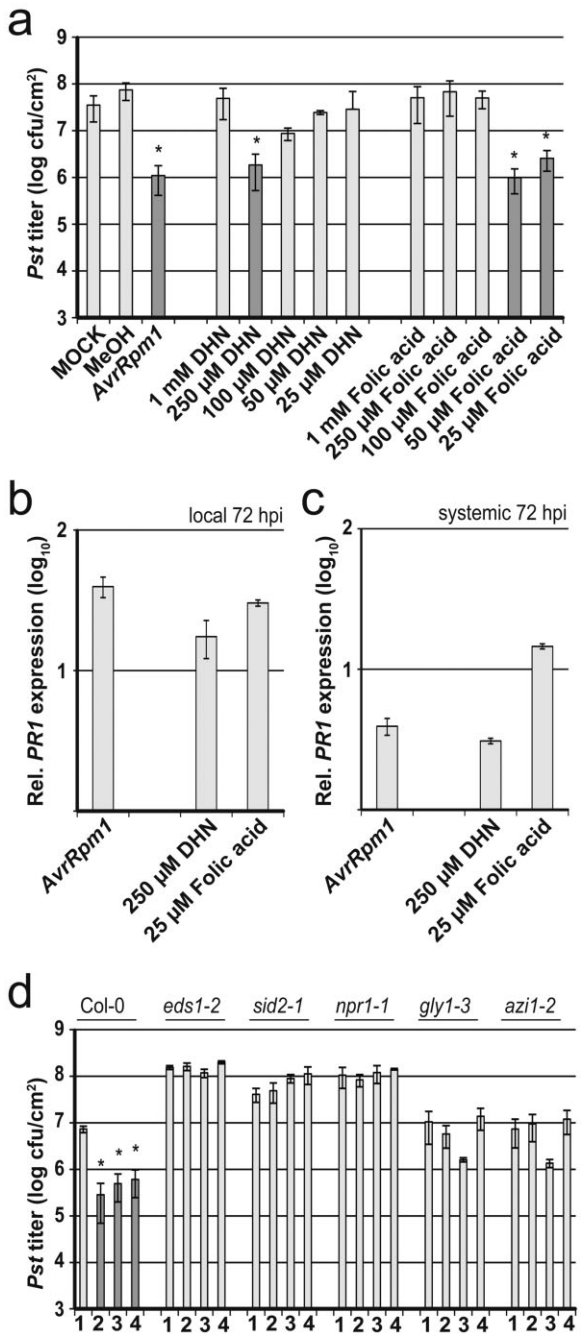

Fig. 3 7,8-Dihydroneopterin (DHN) and folic acid induce salicylic acid (SA)-dependent systemic immunity. (a) Systemic resistance assays. Col-0 plants were locally treated with 10 mM $MgCl_2$ (MOCK), 0.1% methanol (MeOH), *Pseudomonas syringae* pathovar *tomato* (*Pst*) delivering AvrRpm1 (*Pst/AvrRpm1*) (*AvrRpm1*) or with different concentrations of DHN or folic acid as indicated below the panel. Three days later, systemic leaves were infected with *Pst* and the resulting *Pst* titres are shown at 4 days post-inoculation (dpi). Plotted values are the average ± standard deviation of at least three replicates each. (b,c) *PATHOGENESIS-RELATED1* (*PR1*) transcript accumulation. Col-0 plants were locally treated with *Pst/AvrRpm1* (*AvrRpm1*), 250 µM DHN or 25 µM folic acid as indicated below the panel. Three days later, *PR1* transcript accumulation was analysed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), as described in Wittek *et al*. (2014), in treated (b) and systemic untreated (c) leaves. *PR1* transcript accumulation was normalized to that of *TUBULIN* and is shown relative to the normalized *PR1* transcript accumulation in corresponding mock-treated samples (10 mM $MgCl_2$ for *Pst/AvrRpm1* and 0.1% MeOH for DHN and folic acid). (d) Col-0 plants and the mutants indicated above the panel were locally treated with 0.1% MeOH (1), 250 µM DHN (2), 50 µM folic acid (3) or 25 µM folic acid (4) as indicated below the panel. Systemic immunity was analysed as in (a). (a,d) Asterisks above the bars indicate statistically significant differences from the MOCK or MeOH controls (*$P < 0.05$, Student's *t*-test). These experiments were repeated two (b,c) to three (a,d) times with similar results. Rel., relative; hpi, hours post-inoculation.

SAR is typically associated with the local and systemic induction of the SA and SAR marker gene *PATHOGENESIS-RELATED1* (*PR1*; Van Loon *et al*., 2006). Here, *Pst/AvrRpm1* infection enhanced *PR1* transcript accumulation compared with that in $MgCl_2$-treated plants in treated (Fig. 3b) and systemic untreated (Fig. 3c) tissues at 72 h post-inoculation (hpi). Similarly, local applications of 250 µM DHN and 25 µM folic acid enhanced *PR1* transcript accumulation compared with that in MeOH-treated plants at 72 hpi, both locally and systemically (Fig. 3b,c), suggesting that DHN and folic acid induced SAR-like systemic resistance. We have shown previously that *eds1-2* mutant plants do not respond to SAR signals in petiole (phloem) exudates from *Pst/AvrRpm1*-infected wt leaves (Breitenbach *et al*., 2014). Likewise, local applications of 250 µM DHN and 25 or 50 µM folic acid to the first two true leaves of 4–5-week-old *eds1-2* plants did not trigger systemic resistance to *Pst* (Fig. 3d). Similarly to *eds1-2*, the SA biosynthesis mutant *salicylic acid induction deficient2* (*sid2*) and the SA signalling mutant *nonexpressor of PR genes1* (*npr1*) display enhanced susceptibility to *Pst* and compromised SAR (Cao *et al*., 1997; Wildermuth *et al*., 2001). Here, *sid2-1* (Wildermuth *et al*., 2001) and *npr1-1* (Cao *et al*., 1997) plants did not respond to local applications of DHN or folic acid with reduced growth of a systemic *Pst* challenge inoculum compared with that in negative control plants that were locally treated with 0.1% MeOH (Fig. 3d). These data suggest that systemic resistance induced by DHN and folic acid depends on SA biosynthesis and signalling. In Arabidopsis, SAR, but not local resistance to *Pst*, depends on G3P, the accumulation of which is compromised in the *gly1* mutant (Chanda *et al*., 2011; Kachroo *et al*., 2004; Yu *et al*., 2013), and AZI1 (Jung *et al*., 2009; Yu *et al*., 2013). Notably, *gly1-3* and *azi1-2* mutant plants did not respond with systemic resistance to local applications of DHN or 25 µM folic acid (Fig. 3d). A moderate reduction in *Pst* growth in systemic tissue was detected on challenge infection of *gly1-3* and *azi1-2* plants that had received a local treatment with 50 µM folic acid compared with that in negative control plants (Fig. 3d). Because systemic immunity induced by folic acid was compromised in the *gly1-3* and *azi1-2* mutants, the data suggest that G3P and AZI1 were at least partially required for this response. Together, folic acid and the natural folate pre-

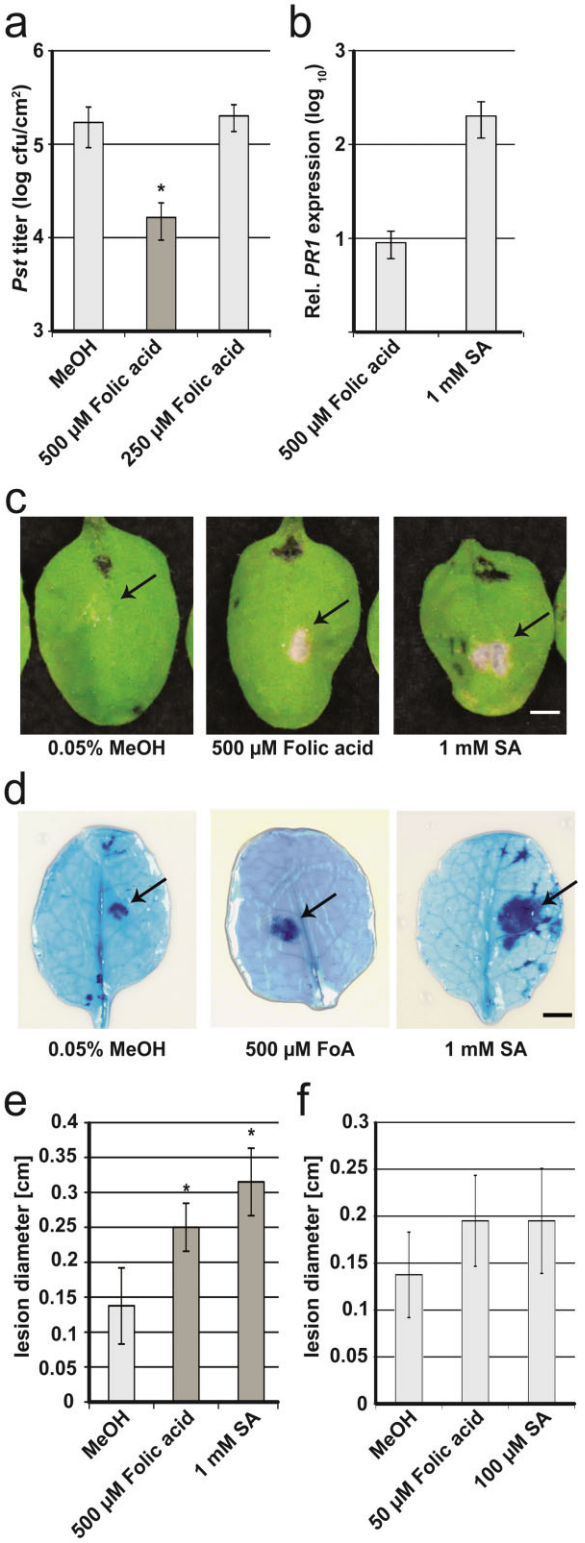

Fig. 4 Folic acid locally induces susceptibility in Arabidopsis to *Alternaria brassicicola*. (a–d) Col-0 plants were sprayed with 0.05% methanol (MeOH) or with 250 or 500 µM folic acid or 1 mM salicylic acid (SA) as indicated below the panels. (a) Twenty-four hours after the spray treatment, the treated leaves were syringe infiltrated with *Pseudomonas syringae* pathovar *tomato* (*Pst*) and the resulting *Pst* titres are shown at 4 days post-inoculation (dpi). Values represent the average ± standard deviation of at least three replicates each. (b) Twenty-four hours after the spray treatment, *PATHOGENESIS-RELATED1* (*PR1*) transcript accumulation was analysed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). *PR1* transcript accumulation was normalized to that of *TUBULIN* and is shown relative to the normalized *PR1* transcript accumulation in mock-treated samples (0.05% MeOH). (c–e) Twenty-four hours after the spray treatment, ~600 *A. brassicicola* spores were spotted onto the treated leaves. At 6 dpi, the resulting lesions were photographed (representative photographs in c), stained with trypan blue (d) or measured (e). Arrows in (c) and (d) indicate lesions caused by *A. brassicicola*; the black spots in (c) are pen marks indicating the inoculated leaves. (f) Col-0 plants were locally treated (by syringe infiltration) with 0.025% MeOH, 50 µM folic acid or 100 µM SA. Three days later, systemic untreated leaves were infected with *A. brassicicola* spores as in (c–e). The resulting lesions were measured at 6 dpi. Plotted values in (e) and (f) represent the average ± standard deviation of 20 replicates each. (a,e) Asterisks above the bars indicate a statistically significant difference from the MOCK or MeOH controls (*$P < 0.05$, Student's *t*-test). (a–f) These experiments were repeated at least three times with similar results. Rel., relative.

cursor DHN appear to induce a true SAR response that is associated with the systemic induction of *PR1* and is dependent on SA, G3P and AZI1.

Because DHN and folic acid applications locally induced *PR1* transcript accumulation (Fig. 3b), we investigated the local response to *Pst* after spray treatment of Arabidopsis plants with folic acid. To this end, 4–5-week-old plants were sprayed with 250 or 500 µM folic acid in 0.01% Tween-20 or with 0.05% MeOH in 0.01% Tween-20 as a negative control. One day later, the treated leaves of the plants were syringe infiltrated with 10^5 cfu/mL of *Pst* and the resulting *Pst* titres were monitored at 4 dpi. Application of 500 µM, but not 250 µM, folic acid reduced *Pst* growth (Fig. 4a), indicating that folic acid induced local resistance to *Pst* in a concentration-dependent manner. Local folic acid-induced resistance was accompanied by enhanced *PR1* transcript accumulation in the treated leaves at 24 hpi that was ~20-fold less than the *PR1* induction observed 24 h after spray treatment of plants with 1 mM SA in 0.01% Tween-20 (Fig. 4b). Because SA signalling acts antagonistically with jasmonic acid (JA) signalling (Pieterse *et al.*, 2012; Robert-Seilaniantz *et al.*, 2011), which is involved in resistance to necrotrophic pathogens (Glazebrook, 2005), we investigated the local and systemic folic acid-induced response in Arabidopsis to the necrotrophic fungus *Alternaria brassicicola*. Three- to four-week-old plants grown in 14-h days (other conditions as in Breitenbach *et al.*, 2014) were sprayed with 500 µM folic acid in 0.01% Tween-20 or with 0.05% MeOH in 0.01% Tween-20 as a negative control, or 1 mM SA in 0.01% Tween-20 as

a positive control. One day later, the treated leaves were infected with ~600 *A. brassicicola* spores that were spotted onto the middle of the leaves, essentially as described by Spoel *et al*. (2007). The resulting lesions were measured with a ruler at 6 dpi. Similarly to SA (Spoel *et al*., 2007), folic acid application promoted the *A. brassicicola*-induced lesion size, indicating that both compounds enhanced the susceptibility of the plants to *A. brassicicola* (Fig. 4c–e). In addition to SA, a local infection of one Arabidopsis leaf half with virulent *Pst* enhanced susceptibility in the other half of the same leaf to *A. brassicicola* (Spoel *et al*., 2007). Notably, Spoel *et al*. (2007) associated these SA- and *Pst*-induced responses with the attenuated induction of the JA-responsive *PLANT DEFENSIN1.2* (*PDF1.2*) gene by *A. brassicicola*. Enhanced susceptibility to *A. brassicicola* was not observed in systemic untreated leaves of locally *Pst*-infected plants (Spoel *et al*., 2007). Similarly, the effect of folic acid and SA on susceptibility to *A. brassicicola* was limited to the local treated tissue and was not significant in the systemic leaves of plants that were locally treated with 50 μM folic acid or 100 μM SA by syringe infiltration (Fig. 4f). Taken together, the data suggest that folic acid induces local and systemic SA-dependent immune responses in Arabidopsis, possibly including local antagonistic effects on JA-mediated resistance to *A. brassicicola*.

Folates contribute to one-carbon transfer reactions that are essential in all organisms, for example for the biosynthesis of purines and some amino acids (Hanson and Gregory, 2011; Hanson and Roje, 2001). Mammals depend on their diet for folates, which are targets for biofortification of crop plants to alleviate folate insufficiency in humans (Bekaert *et al*., 2008). This and other studies associate folates with plant innate immunity. For example, elevated folate content in rice seeds is associated with the induction of defence-related genes, including a number of NLR receptors, such as RPM1 (Blancquaert *et al*., 2013b). In addition, PABA-induced resistance in pepper to *X. axonopodis* and *Cucumber mosaic virus* is associated with the induction of SA-related *PR* genes (Song *et al*., 2013). We show here that the folate precursor DHN and folic acid induce local and systemic SA-mediated defence in Arabidopsis, and suggest that folic acid suppresses JA-mediated responses to *A. brassicicola*. Folic acid induced SAR when applied at a five-fold lower concentration than DHP, which might be associated with the fact that DHP must be transported into the chloroplast for conversion into dihydrofolate (Hanson and Gregory, 2011; Fig. 1). In addition, over-expression of the enzyme that converts GTP to DHN-triphosphate does not enhance folate accumulation in Arabidopsis (Blancquaert *et al*., 2013a) and it is possible that exogenous DHN is converted to DHP in the cytosol (Storozhenko *et al*., 2007). The cytosolic enzymes that are responsible for this process are induced by abiotic stress and enhance Arabidopsis resistance to oxidative stress, but not the accumulation of folates, suggesting a role for pterins in abiotic stress resistance (Navarrete *et al*., 2012; Storozhenko *et al*., 2007). Taken together, it is possible that the DHP induction that was recorded after infection of Arabidopsis (Fig. 2) was cytosolic and independent of folate. In addition, DHN might induce SA-mediated responses in Arabidopsis via cytosolic DHP by a different, folate-independent mechanism compared with folic acid.

Summarizing, the application of DHN and folic acid triggers local and systemic SA-dependent immunity in Arabidopsis. DHN- and folic acid-induced systemic immunity is dependent on SA biosynthesis and signalling and on the SAR signalling components G3P and AZI1. Because folic acid application suppresses Arabidopsis immunity to the necrotrophic fungus *A. brassicicola*, the protection of folate-fortified plants from disease might require special attention.

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