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Original Article

Accumulation of terpenoid phytoalexins in maize roots is associated with drought tolerance

Martha M. Vaughan[1], Shawn Christensen[2], Eric A. Schmelz[2], Alisa Huffaker[2], Heather J. McAuslane[3], Hans T. Alborn[2], Maritza Romero[2], Leon Hartwell Allen[2] & Peter E. A. Teal[2]

[1]Bacterial Foodborne Pathogens and Mycology, Agricultural Research Service, United States Department of Agriculture, Peoria, IL 61604, USA, [2]Chemistry Research Unit, Center of Medical, Agricultural, and Veterinary Entomology, Agricultural Research Service, United States Department of Agriculture, Gainesville, FL 32608, USA and [3]Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA

ABSTRACT

Maize (*Zea mays*) production, which is of global agro-economic importance, is largely limited by herbivore pests, pathogens and environmental conditions, such as drought. Zealexins and kauralexins belong to two recently identified families of acidic terpenoid phytoalexins in maize that mediate defence against both pathogen and insect attacks in aboveground tissues. However, little is known about their function in belowground organs and their potential to counter abiotic stress. In this study, we show that zealexins and kauralexins accumulate in roots in response to both biotic and abiotic stress including, *Diabrotica balteata* herbivory, *Fusarium verticillioides* infection, drought and high salinity. We find that the quantity of drought-induced phytoalexins is positively correlated with the root-to-shoot ratio of different maize varieties, and further demonstrate that mutant *an2* plants deficient in kauralexin production are more sensitive to drought. The induction of phytoalexins in response to drought is root specific and does not influence phytoalexin levels aboveground; however, the accumulation of phytoalexins in one tissue may influence the induction capacity of other tissues.

Key-words: abiotic and biotic stress; *ANTHER EAR2* (*AN2*); root-to shoot ratio; salt stress.

INTRODUCTION

To sustain increasing population demands, global food production will have to rise 50% within the next 40 years (Chakraborty & Newton 2011), and ongoing climate changes only accentuate this challenge. As a consequence of increased atmospheric CO_2, global warming is projected to intensify episodes of drought (Zhao & Running 2010), and may result in increased outbreaks of herbivore pests and pathogens (Chakraborty & Newton 2011; de Sassi & Tylianakis 2012).

Maize (*Zea mays*) is an essential part of the world's grain supply and food security. With the majority of the maize cropping system relying on natural precipitation, drought is a key limiting factor for productivity (Boyer & Westgate 2004; Barton & Clark 2014). Approximately 25% of the global maize-growing area is affected by drought on an annual basis (Bänziger & Araus 2007; Manavalan *et al*. 2011). The roots and shoots are integrated structural elements that enable the plant to draw from below and aboveground environmental resources necessary for growth, development and productivity. Root development in particular plays an important role in drought tolerance, and under dry soil conditions, root growth generally increases while shoot growth declines, resulting in an increased root-to-shoot ratio (Weerathaworn *et al*. 1992). In response to water deficit, maize seedlings with vigorous root development are more likely to sustain growth and be productive (Ruta *et al*. 2010). However, as with the phyllosphere, the rhizosphere harbours pests and pathogens that impose a constant threat, and therefore resistance to biotic stress should not be neglected in breeding efforts for drought tolerance.

In order to adapt to the multitude of biotic and abiotic challenges within their ecosystem, plants have evolved a complex defence mechanism, which relies on the rapid perception and activation of secondary metabolites. Among these defence metabolites, terpenoids constitute a large and structurally diverse group of chemicals. In addition to their physiological roles as phytohormones, such as the sesquiterpenoid abscisic acid (ABA) and the diterpenoid gibberellic acid (GA), terpenoids also function as phytoalexins, which are low-molecular-weight antimicrobial compounds that accumulate in response to biotic and abiotic factors (Kuc 1995; Pichersky & Gershenzon 2002; Tholl 2006; Vickers *et al*. 2009; Loreto & Schnitzler 2010; Schmelz *et al*. 2014). Cotton plants accumulate sesquiterpenoid phytoalexins, such as gossypol, hemigossypolone and heliocides, both above and belowground as defensive metabolites against pathogens and herbivores (Yang *et al*. 2013). Similarly, rice leaves and roots produce diterpenoid phytoalexins including momilactones and oryzalexins that contribute to resistance against fungal diseases such as rice blast, caused by *Magnaporthe grisea* (Peters 2006; Toyomasu *et al*. 2008). In addition, abiotic factors such as heavy metal toxicity and ultraviolet radiation induce the accumulation of rice phytoalexins (Kato-Noguchi

Correspondence: M. M. Vaughan. e-mail: Martha.vaughan@ars.usda.gov

& Kobayashi 2009). Recently, two novel families of acidic sesquiterpenoid and diterpenoid phytoalexins, termed zealexins and kauralexins, respectively, were identified in maize and found to exhibit both potent antifungal and insect antifeedant activity aboveground; however, little is known about their potential role belowground in response to biotic and abiotic stress.

Induced defence metabolites, including phytoalexins, are typically controlled by interconnected phytohormone signalling networks (Farmer & Ryan 1992; Howe & Schilmiller 2002; Turner *et al*. 2002; Howe 2004). The phytohormones jasmonic acid (JA) and salicylic acid (SA) are predominantly involved in the activation of defences related to biotic stress, whereas ABA has a predominant role in mediating responses to abiotic stress such as drought. While individual stress responses display measurable specificity (De Vos *et al*. 2005), plants are frequently simultaneously challenged by several stress factors resulting in the activation of diverse signals. Signalling crosstalk is viewed as a mechanism to integrate a multitude of signals resulting in appropriate responses. In particular, the 'optimal defence theory' predicts that defence-related biochemicals will be allocated to tissues of the greatest value or risk of attack (Zangerl & Rutledge 1996; Yates *et al*. 2005). However, the assignment of which tissue is of greatest value or risk is not necessarily fixed and can be adjusted based on the plant's current status, such as the quantity/duration of inflicted damage or environmental conditions. For example, under conditions of high light intensity, water deficit, and belowground herbivory, the roots might be considered of higher importance then the leaves.

Complex signalling mechanisms can have synergistic and antagonistic effects on each other at multiple levels including perception and biosynthesis (Adie *et al*. 2007; Fan *et al*. 2009; Ton *et al*. 2009; Hey *et al*. 2010; Cao *et al*. 2011; Robert-Seilaniantz *et al*. 2011). Abiotic stress often influences plant susceptibility to biotic stress by affecting secondary metabolite production (Gouinguene & Turlings 2002; Ramakrishna & Ravishankar 2011; Atkinson & Urwin 2012; Vaughan *et al*. 2014). Conversely, biotic stress, such as root herbivore damage, can intensify plant susceptibility to abiotic stress, and the systemic induction of belowground stress responses can influence aboveground defences and vice versa (Erb *et al*. 2009a).

The induced production of maize terpenoid phytoalexins is highly localized to the site of damage or infection where they are synthesized and accumulated in part through the regulation of JA signalling (Huffaker *et al*. 2011; Schmelz *et al*. 2011). *De novo* biosynthesis elicited by the combined action of the JA and ethylene signalling pathways is typical of maize terpenoid compounds (Schmelz *et al*. 2004, 2011; Huffaker *et al*. 2011; Kollner *et al*. 2013). Although the enzymatic biosynthetic pathways of maize phytoalexins have yet to be fully elucidated, the terpene synthase genes *TERPENE SYNTHASE*6/11 (*TPS*6/11) and *ANTHER EAR*2 (*AN2*) encoding two homologous β-macrocarpene synthases and the *ent*-copalyl diphosphate synthase, are presumably involved in the supply of precursors for zealexin and kauralexin biosynthesis, respectively (Huffaker *et al*. 2011; Schmelz *et al*. 2011). Although, *ent*-copalyl diphosphate is enzymatically converted to *ent*-kaurene, which is also the first tetracyclic intermediate in the production of the growth hormone GA, *AN2* is not likely a major contributor to GA biosynthesis as it has been shown that mutant *AN1* maize produces significantly less GA and displays a GA-responsive phenotype (Bensen *et al*. 1995; Harris *et al*. 2005; Schmelz *et al*. 2011).

Because of their only recent identification, it has not been determined whether zealexins and kauralexins are produced in maize root tissues or whether they can be systemically induced. With growing awareness of the role belowground, root stress plays in inducing physical and physiological changes in plants, which can impact multiple trophic levels and affect the entire ecosystem (Kaplan *et al*. 2008; van Dam 2009; Chakraborty & Newton 2011; de Sassi & Tylianakis 2012), the potential functions of maize root phytoalexins are of considerable ecological relevance (Erb *et al*. 2009a)

In this study, we characterized the role of terpenoid phytoalexins in maize root defence responses to biotic and abiotic stress. We demonstrate that the accumulation of drought-induced phytoalexins in maize roots is positively correlated with the root-to-shoot ratio of different maize lines and show that kauralexin-deficient mutant *an2* is significantly more susceptible to drought. Furthermore, we show that the accumulation of phytoalexins in one tissue may influence the production of phytoalexins in another tissue. Finally, we discuss the above and belowground defensive strategy of maize under water deficit and its potential ecological influence.

MATERIALS AND METHODS

Plant material and growth conditions

All experiments were conducted in environmental Conviron E15 (Pembina, ND, USA) growth chambers controlled at 28 °C day/25 °C night, 500 μmol m^{-2} s^{-1} photosynthetic photo flux density 12 h photoperiod, and between 50 and 60% relative humidity. Unless otherwise specified, experiments were conducted using hybrid *Z. mays* var. *Golden Queen* (Southern States Cooperative, Inc., Richmond, VA, USA). Four seeds were germinated in each 10.5 × 10.5 × 12 cm high plastic pot filled with MetroMix 200 (Sun Gro Horticulture Distribution, Inc., Bellevue, WA, USA) supplemented with 14-14-14 Osmocote (Scotts Miracle-Gro, Marysville, OH, USA).

In order to overcome experimental difficulties associated with the accessibility of root tissue and the interference of soil particles with metabolite analysis, the previously described aeroponic culture system (Vaughan *et al*. 2011) was adapted for maize. Briefly, seeds were germinated in potting mix (as described earlier) and then each 1-week-old seedling was transferred individually to a 20 oz (~0.6 L) plastic cup (prepared by drilling five 5 mm diameter holes in the bottom) filled with Seramis® (ACE Gardening Products Inc., Ontario, Canada) clay pellets. Unless otherwise stated, plants were watered daily with Hoagland's solution. This aeroponic root culture not only provided convenient access to root tissue following belowground root–organism interaction experiments, but also provided a simple method, which

allowed the removal of an intact plant from a substrate under one set of conditions and placement into a substrate of different conditions at any point during growth with minimal damage. Root tissue was separated from granules by submerging the belowground plant portion in water. While holding the plant stem and moving it back and forth in water, the heavier clay pellets fell to the bottom while the root mass remained intact (Vaughan *et al*. 2011). The seed and scutellum, which has previously been reported to contain high levels of phytoalexins (Schmelz *et al*. 2011), was excluded from the root mass of all samples analysed.

For experiments conducted with 2-week-old seedlings (V2 stage), maize seeds were germinated directly in Seramis clay granules. With the exception of a 1–2 d delay in seed germination, plant growth appeared comparable with that in potting mix.

Quantification of maize phytohormones and terpene phytoalexins

The quantification of maize phytohormones, kauralexins and zealexins, was performed using a previously developed method (Huffaker *et al*. 2011; Schmelz *et al*. 2011). In summary, metabolites were extracted from pulverized tissue with 1-propanol and methylene chloride, and collected by vapour-phase extraction following carboxylic acid methylation. The analysis was then performed using gas chromatography/chemical ionization – mass spectrometry. Quantity estimates of zealexins and kauralexins were based on the internal standard $^{13}C_{18}$-linolenic acid. The concentration of zealexins (related metabolites 1–6 as numbered and described in Huffaker *et al*. 2011) and kauralexins (A1–A3 and B1–B3 as described by Schmelz *et al*. 2011) are reported as cumulative total amounts. Total terpene phytoalexins represent combined total amounts of both zealexin and kauralexin metabolites. A more detailed quantitative analysis of individual constitutive and induced zealexin and kauralexin metabolites are displayed in Supporting Information Fig. S1. Quantity estimates of ABA, JA and SA were based on corresponding deuterated internal standards.

Biotic stress treatments of maize roots

To determine if the induction of phytoalexins was specific to aboveground tissues, maize roots were subjected to biotic stress treatments. Root fungal inoculations were conducted by dipping the rinsed roots of 1-week-old plants in a 1×10^6 spores per mL *Fusarium verticillioides* (Northern Regional Research Laboratory stock no. 7415) suspension or a control (0.1% Tween 20) suspension for approximately 30 s. The suspensions were prepared as previously described (Vaughan *et al*. 2014). Following the root treatment, seedlings were placed in aeroponic culture for 3 weeks of growth after which the youngest roots (at the bottom 10 cm of the root mass) were collected, weighed, frozen in liquid N_2, pulverized and stored at –80 °C until metabolite analysis. The percentage of water content of each root mass was determined from the fresh and dry weight of a tissue aliquot. Using this percentage, the dry weight was then estimated from the fresh weight of each sample analysed, so that the concentration of metabolites could be reported on a dry weight basis. Thus, the differences in water content would not interfere with the metabolite concentration calculations and comparisons. The average concentration of each family of phytoalexins was estimated from four *F. verticillioides*-treated biological replicates and compared with the average from four control treated root samples using a Student's *t*-test.

Diabrotica balteata LeConte larvae were utilized as a belowground generalist root herbivore of maize. Larvae were reared according the methods of (Huang *et al*. 2002), with minor modifications. Briefly, adult beetles were maintained in an incubator at 25 ± 1 °C, 70% relative humidity, and a photoperiod of 14:10 (L: D), and fed a mixture of lima bean leaves (Fordhook 242, Johnny's Selected Seeds, Winslow, ME, USA) and slices of United States Department of Agriculture-certified organic sweet potato. Beetles oviposited into containers provisioned with moistened layers of cheesecloth and eggs were collected weekly and sterilized in sodium hypochlorite solution. Eggs were placed on sprouted corn seeds (open-pollinated field corn population, University of Florida's Institute of Food and Agricultural Sciences Everglades Research and Education Center) and allowed to hatch. Larvae fed for 2 weeks on corn roots and then were transferred to containers of sharp builders' sand to pupate. Newly emerged beetles were collected and placed into the incubator to begin the next generation. Ten second and third instar larvae (approximately 10 d post hatching) were released into the Seramis clay granules of V2 maize seedlings in aeroponic culture. The larvae were allowed to feed on the roots for 24 h prior to root recovery and metabolite analysis. The average concentration of phytoalexins from four herbivore damaged roots were compared with roots from undamaged plants cultured similarly using a Student's *t*-test.

Abiotic stress treatments of maize roots

To simulate precise drought stress treatments, 2-week-old maize seedlings (V2 stage) germinated and grown in aeroponic culture were removed from the clay substrate and placed in new clay substrate of known volumetric water content (VWC). The percentage of water content was formulated such that the volume of Hoagland's solution added was either 60, 50, 40, 30 or 20% (300, 250, 200, 150, 100 mL) of the 500 mL clay substrate in each cup containing the transferred seedling. Furthermore, salt stress, which can result in a similar biochemical response as drought-induced osmotic stress (Zhu *et al*. 1997), was imposed by watering a second set of 2-week-old maize seedlings grown in aeroponic culture with Hoagland's solution containing 0, 50, 100, 500 or 1000 mM sodium chloride (NaCl) (Fisher BioReagents, Fairlawn, NJ, USA). Sets of five biological replicate plants for each specified VWC or NaCl concentration were treated for 24 h prior to tissue collection and metabolite analysis. Following an analysis of variance (ANOVA), each mean was compared with its corresponding control (60% VWC or 0 mM NaCl) using Fisher's least significant difference (LSD) test.

Drought time course experiment

To further evaluate the induction of root phytoalexins under more natural conditions, maize grown in potting mix was subjected to 7 d of consecutive drought throughout which a subset of biological replicates were sacrificed for biochemical analyses. Drought treatment was imposed by withholding water from 1-month-old plants (V8 stage). Each day at noon throughout the time course the soil VWC was measured using an EC-5 soil moisture sensor attached to the ProCheck sensor read-out system (Decagon Devices, Pullman, WA, USA), and the root mass of five biological replicates was collected. Each biological replicate represents the root mass from two plants grown in the same pot. An ANOVA followed by Fisher's LSD was used to compare each mean throughout the time course to the mean at day 1. Comparable results were obtained from two independent experiments, as well as a separate experiment conducted with maize grown in aeroponic culture.

Treatment of maize roots with phytohormones

To determine the potential involvement of individual signalling pathways in stimulating the induction of root phytoalexins with drought, well-watered roots were separately treated with the individual phytohormones and then analysed for phytoalexin accumulation. Treatments were applied to 2-week-old plants (V2 stage) in aeroponic culture by saturating the clay substrate with a control Hoagland's solution or solution containing 150 μM ABA, 50 μM JA or 1 mM SA (Sigma-Aldrich, St. Louis, MO, USA) for 24 h prior to collection for analysis. The average concentration ($n = 5$) of total phytoalexins in the roots of different treatments was then compared by performing an ANOVA followed by a Tukey–Kramer honestly significant difference (HSD) test.

Drought-induced phytoalexins and root-to-shoot ratio of different maize varieties

To determine if there was any association between the accumulation of phytoalexins and the root-to-shoot ratio, eight different maize varieties were drought stressed and evaluated. *Golden Queen* (*GQ*), *Silver Queen* (*SQ*), *Silver King* (*SK*), *B73*, *W22*, *Ms71*, *CML322* and *MP708* seeds were grown under well-watered conditions (60% VWC) for 2 weeks. Plants were subsequently transplanted to a substrate of 40% VWC where they were allowed to acclimate and grow for two additional days prior to removal for measurements and metabolite analysis. As a control for transplant manipulations, a set of plants were similarly removed from 60% VWC and replanted in substrate of 60% VWC. Following the treatment, five plants of each variety were dried in a 50 °C oven for 4 d, and the root-to-shoot ratio was then calculated by dividing the dry root weight by the dry shoot weight. The concentration of induced phytoalexins was calculated by subtracting the average quantity of phytoalexins in well-watered roots (60% VWC) from the average quantity in drought stressed roots (40% VWC). In order to determine the relationship between the two variables, the average root-to-shoot ratio for each variety was then plotted against the concentration of induced phytoalexins and the coefficient of determination (R^2) was estimated by the linear regression in Excel (Microsoft, Redmond, WA, USA).

Evaluation of *an2* mutant maize susceptibility to drought

Using the Activator (Ac) and Dissociation (Ds) system (Ahern *et al.*, 2009; Vollbrecht *et al.*, 2010), a reverse-genetic screen was performed using a donor Ds element (B.W06 .0419) located within the *An2* gene (GRMZM2G044481) in a near-isogenic *W22* inbred line. Seed was received from the Ac/Ds Tagging Facility at the Boyce Thompson Institute for Plant Research as a 1:2:1 segregating population for a stable Ds insertion in the fourth exon (http://acdstagging.org/). Detection of homozygous mutant seed was carried out by both genotyping with a Ds-flanking *AN2* gene-specific primer (5′-GATCGCCTGGAGCGTCTCGG-3′) and a Ds-end primer (5′-ACCCGACCGGATCGTATCGG-3′) and by chemotyping for the presence/absence of maize kauralexins.

Furthermore, the mutant plants were analysed for detectable levels of *AN2* (AY562491) transcript in well-watered and drought-stressed plants using quantitative RT-PCR with primers 5′-TGTTCTTGTGAAGGCAGTTC-3′ and 5′-TC ATTCGAGCTAAAAGCAGA-3′ as previously described (Schmelz *et al*. 2011; Vaughan *et al*. 2014). As it was at least plausible that the *an2* mutants were also deficient in gibberellin (GA), the transcript levels of GA marker genes, *anther ear1* (*AN1*; NM_001111859; 5′-ACGGCTCCTTCTT GTTCTCTC-3′ and 5′-AGTCCATGCACTGCTCAATCT-3′), *GA 2-oxidase* (*GA2ox;* NM_001154780; 5′-GCATCCA CCGTGAAGTTACA-3′ and 5′-GACTAGCCGATTGCAA AAGC-3′), and *GA 3-oxidase* (*GA3ox;* JX307642.1; 5′- GA CTAGCCGATTGCAAAAGC-3′ and 5′-TAGCTGCGGA ACGGAATTAG-3′), in *W22* plants were compared with the levels in *an2* plants via quantitative RT-PCR. Threshold cycle (Ct) values of each gene was normalized to the endogenous control ribosomal protein L17 (RPL17; AF034948; 5′-CAA AGTCTCGCCACTCCA-3′ and 5′-CGTCCGTGAGCACG GTA-3′). The amount of each gene transcript was calculated relative to its corresponding average in *W22* controls. Transcript fold-changes were calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008) using CFX Manager software, version 3.0 (Bio-Rad, Hercules, CA, USA).

W22 and *an2* plants (four seeds per pot) were grown in potting mix till the V8 stage and then drought was imposed by withholding water for 5 d throughout which the VWC was recorded with the EC-5 soil moisture sensor. Every day at noon, the plants were examined for curling of the youngest leaves, which was designated as symptoms of severe drought. At which point the VWC was recorded. At the end of the 5 d drought treatment, the root tissues were collected and analysed for phytoalexin content. The quantity of zealexins and kauralexins for *W22* and *an2* roots exposed ($-H_2O$) and not exposed to the drought ($+H_2O$) treatment were compared in

separate ANOVAs with Tukey–Kramer HSD tests ($n = 5$). The average VWC at which *W22* and *an2* plants exhibited severe drought symptoms was compared using a Student's *t*-test ($n = 5$).

In order to maintain a specific VWC for a longer period of time so that differences in plant growth under variable limited water conditions could be measured, a method was developed utilizing a mixture of the porous clay Seramis pellets and non-absorbent 6 mm glass beads (Walter Stern, Inc., Port Washington, NY, USA). By combining these two components at different proportions, a substrate with a fairly stable and uniform VWC could be obtained by watering daily and allowing excess water to flow freely from the drainage holes.

Initially, the clay pellets were used to develop a VWC calibration curve using the raw data obtained from EC-5 soil moisture sensors and then the maximum VWC was measured for Seramis clay pellets alone, 1:1 Seramis: glass beads, 1:3 Seramis: glass beads, and only glass beads. The average VWC of each mixture was confirmed by both gravimetric calculations (from wet and dry weight measurements) and EC-5 soil moisture sensors measurements.

Seed of the *W22* parent line and the *an2* maize mutant were germinated in the substrate mixes and watered daily with Hoagland's solution at noon. Three weeks after germination, the plants were removed from the substrate, dried in a 50 °C oven, and the above and belowground tissue weights were determined. In order to control for differences in biomass under control well-watered conditions (100% Seramis/60% VWC), the percentage of weight loss with drought treatments was calculated for *W22* and *an2* plants and then compared with an ANOVA followed by a Tukey–Kramer HSD test for individual mean difference.

Tissue specificity of drought-induced phytoalexins and the influence of aboveground stress

To determine if the drought-induced accumulation of phytoalexins was specific to the root tissues, the aboveground tissues were examined for a systemic induction of phytoalexins. As previously described, Golden Queen maize was germinated in aeroponic culture, and at the V2 stage was transplanted to substrate of 60% VWC ($+H_2O$, well-watered) or 30% VWC ($-H_2O$, water deficit) for 24 h. The leaf, stem and root tissues were then separated and independently analysed. The average concentration of phytoalexins in each tissue ($n = 4$) under well-watered or water-deficit conditions were compared using a simple Student's *t*-test.

To assess the influence of aboveground biotic stress on the drought-induced accumulation of root phytoalexins, maize at the V8 stage of development was subjected to 5 d of drought, and subsequently stalk inoculated with *F. verticillioides* as previously described (Vaughan *et al*. 2014). Briefly, 0.1 mL of 1×10^6 mL^{-1} *F. verticillioides* spore solution was injected into a 5 cm vertical incision through the apical meristem. Mock-inoculated (control) stems were identically likewise wounded and treated with sterile 0.1 mL of 0.1% Tween 20 solution. Both stem and root samples were collected 48 h after inoculations. Designated watering regimes or drought treatments were sustained throughout this 48 h period. Samples were collected from the inoculation site by shaving both sides of the incision area, which directly received the treatment. The entire root mass was removed from the clay substrate and samples were collected from the youngest root tissues, which included the root tips (bottom 10 cm of root mass). Tissue samples from two independently inoculated or control plants were combined for each of four biological replicates. The mean concentration of phytoalexins in the control- and pathogen-inoculated stem tissues under well-watered or water-deficit conditions were compared with an ANOVA followed by a Tukey–Kramer HSD test. The concentrations in root tissues under the same treatments were compared in a separate ANOVA.

RESULTS

Belowground biotic stress induces phytoalexins in maize roots

To confirm the belowground occurrence of induced phytoalexins, metabolites of maize roots exposed to pathogen infection and herbivore damage were analysed. Three weeks after treatment with *F. verticillioides*, root tissues appeared diseased and displayed brown discolorations compared with control roots grown under the same aeroponic conditions. Metabolite analysis revealed that *F. verticillioides* treated roots contained approximately 20- and fivefold more zealexins and kauralexins, respectively, in comparison with control roots (Fig. 1a,b; $n = 4$, $P < 0.01$). Similarly, roots exposed to 24 h of *D. balteata* larval feeding contained significantly higher concentrations of zealexin and kauralexin phytoalexins than controls (Fig. 1c,d; $n = 4$, $P < 0.05$). Although both families of phytoalexins were induced by biotic stress, the zealexin metabolites were more abundant in response to pathogen infection while the kauralexins predominated following root herbivore damage. Nevertheless, together, these results with previous publications (Huffaker *et al*. 2011; Schmelz *et al*. 2011) verify that phytoalexins are induced by biotic stress factors both above and belowground.

Abiotic stress induces phytoalexins in maize roots

Given that defence metabolites are often influenced by abiotic stress (Ramakrishna & Ravishankar 2011), the amount of phytoalexins in roots experiencing reduced subterranean VWC and salt stress was analysed. In general, roots in substrates of lower water content contained higher quantities of zealexins and kauralexins. Root tissues placed in substrate at 30% VWC had fourfold more zealexins in comparison with plants placed in control substrate at 60% VWC (Fig. 2a; $n = 5$, $P < 0.01$). The concentration of kauralexins significantly increased in root tissues by 2.2-fold (Fig. 2b; $n = 5$, $P < 0.05$) under more modest drought conditions

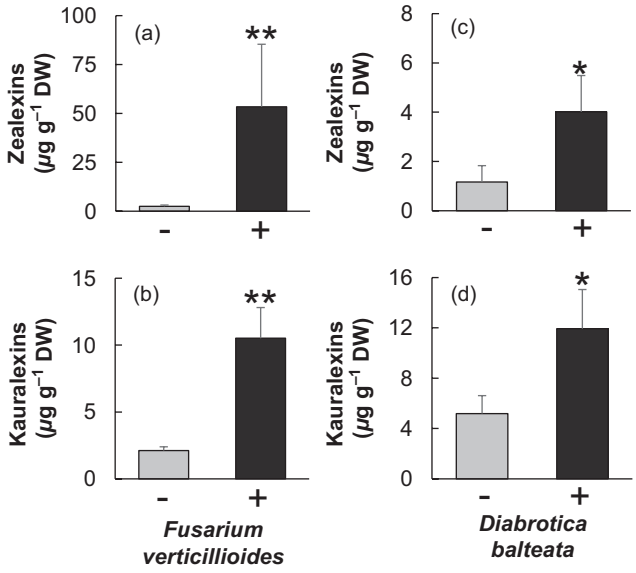

Figure 1. Maize roots accumulate terpenoid phytoalexins in response to biotic stress. Concentration of zealexin (a) and kauralexin (b) metabolites in maize roots dipped in a control 0.1% tween solution (−) or a 1×10^6 spores per mL^{-1} *Fusarium verticillioides* (+) solution and allowed to grow in aeroponic culture for 3 weeks. The concentration of zealexin (c) and kauralexin (d) phytoalexins in non-infested roots (−) and roots infested with 10 *Diabrotica balteata* larvae for 24 h. Values represent averages ± SEM. Asterisks above bars indicate significant differences (Student's *t*-test, $n = 4$, $*P < 0.05$, $**P < 0.01$). DW, dry weight.

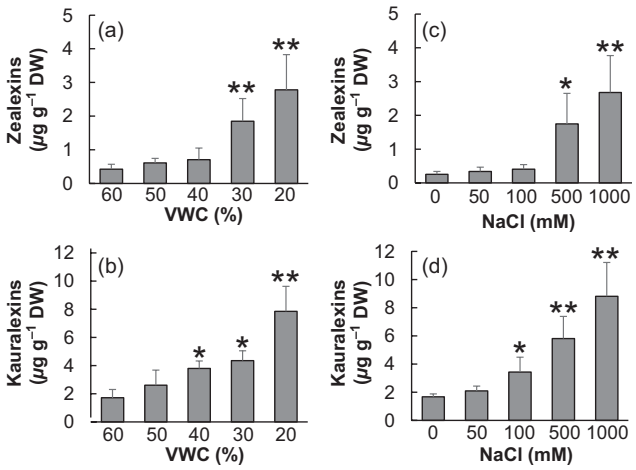

Figure 2. Drought and salt stress induces root terpenoid phytoalexins. Maize seedlings at the V2 stage were transferred from the saturated Seramis clay pellets in which they were germinated to new substrate of reduced volumetric water content (VWC) and allowed to acclimate for 24 h at which point the root mass was extracted and analysed. The concentration of zealexin (a) and kauralexin (b) phytoalexins were measured of roots in substrate of declining VWCs. Similarly, maize seedlings in aeroponic culture were watered with Hoagland's solutions containing various concentrations of NaCl and allowed to acclimate for 24 h. Average quantities of zealexins (c) and kauralexins (d) in maize roots treated with 0 mM to 1 M NaCl were estimated. Values represent averages (±SEM) and were compared by an analysis of variance (ANOVA) followed by a Fisher's least significant difference (LSD) test for individual means ($n = 5$). Asterisks above bars indicate VWCs and NaCl concentrations at which zealexin and kauralexin metabolite levels were significantly greater than the levels for the 60% VWC and 0 mM NaCl concentrations, respectively ($*P < 0.05$, $**P < 0.01$). DW, dry weight.

(i.e. 40% VWC). The concentrations of phytoalexins also increased in root tissues exposed to salt stress. Treatment with 500 mM NaCl significantly induced the concentration of zealexins by approximately fivefold (Fig. 2c; $n = 5$, $P < 0.05$), while the lower concentration of 100 mM NaCl was sufficient to significantly induce the quantity of kauralexins by twofold in comparison with control root tissues mock-treated with 0 mM NaCl (Fig. 2d; $n = 5$, $P < 0.05$).

Correspondingly, with each consecutive day of drought as the soil VWC declined, the concentration of phytoalexins gradually increased (Fig. 3). Although plant vigour appeared to decline throughout the time course, severe water stress symptoms, such as leaf curing and chlorosis, were not visible until day 7 of withholding water. On day 5, at which point the VWC was approximately one-third of the starting amount (Fig. 3a), both the concentration of zealexin and kauralexin metabolites were significantly induced three- and fourfold, respectively (Fig. 3b,c; $n = 5$, $P < 0.05$) and continued to rise as drought persisted. Interestingly, the drought-induced accumulation of phytoalexins was preceded by a gradual increase in JA and coincided with a spike in ABA concentrations at days 4, 5 and 6. ABA reached a maximum eightfold induction at day 5 while JA peaked on day 6 with levels of 4.6-fold above controls (Fig. 3d,e; $n = 5$, $P < 0.05$). The quantity of SA was also evaluated; however, no significant changes were detected (Fig. 3f; $P > 0.05$).

Treatment of roots with ABA is sufficient to stimulate the accumulation of phytoalexins

In order to determine the effect of individual phytohormone signalling pathways, phytoalexin concentrations were measured in roots treated with ABA, JA or SA. Treatment with 150 μM ABA increased total root phytoalexins by approximately fivefold compared with the untreated control (Fig. 4; $P < 0.01$). In contrast, treatment with JA or SA had no effect on phytoalexin production.

Drought-induced root phytoalexin concentrations display positive relationships with root-to-shoot ratios across inbred maize lines

The variation in root-to-shoot ratios among inbred maize lines grown in aeroponic culture positively correlated with the quantity of drought-induced root phytoalexins ($R^2 = 0.9066$; Fig. 5). All of the eight lines examined accumulated higher quantities of phytoalexins with drought. The lines *B73*, *Ms71* and *W22* displayed the largest increase in

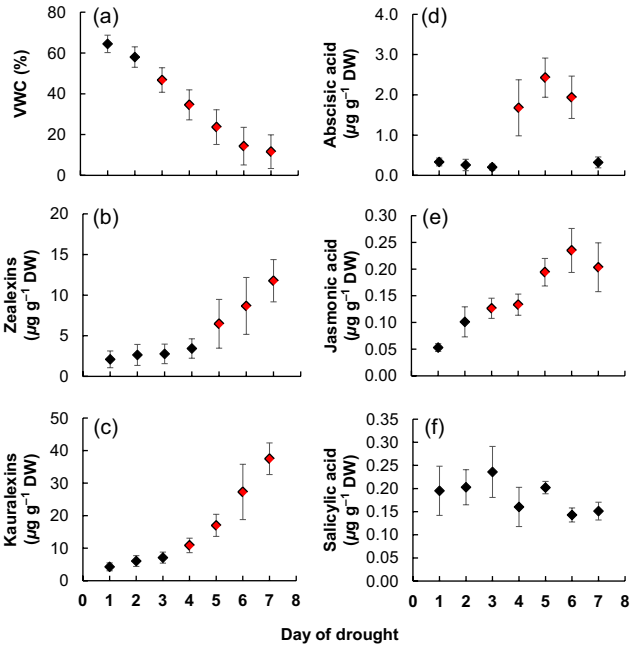

Figure 3. The increase in terpenoid phytoalexins with drought is preceded by a gradual increase in jasmonic acid (JA) and coincides with a spike in abscisic acid (ABA). Water was withheld from V8 stage plants for 7 consecutive days throughout which the percentage of volumetric water content (VWC) was monitored using an EC-5 soil moisture sensor (a). Each day, a subset of roots was collected for extraction and quantification of zealexins (b), kauralexins (c), ABA (d), JA (e) and salicylic acid (f). Values represent the mean (±SEM) of five samples at each corresponding day of drought. Each mean was compared with the initial mean of the metabolite at day 1 by analysis of variance (ANOVA), Fisher's least significant difference (LSD). Points that represent metabolite levels significantly different from day 1 are represented in red ($P < 0.05$). DW, dry weight.

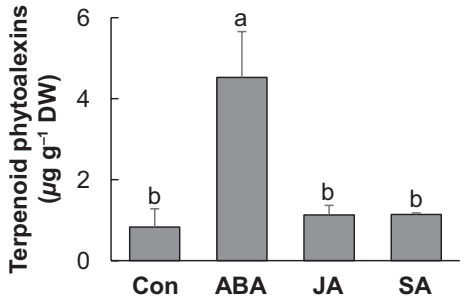

Figure 4. Treatment with abscisic acid (ABA) increases the concentration of root terpenoid phytoalexins. Average (±SEM) concentration of total terpenoid phytoalexins (sum of both zealexin and kauralexin compounds) in seedling roots treated for 24 h with different phytohormones by saturating the clay substrate with a control nutrient solution (Con) or a solution containing 150 μM ABA, 50 μM jasmonic acid or 1 mM SA. Different letters denote significant differences between treatments [analysis of variance (ANOVA) followed by Tukey–Kramer honestly significant difference (HSD), $n = 5$, $P < 0.01$]. DW, dry weight.

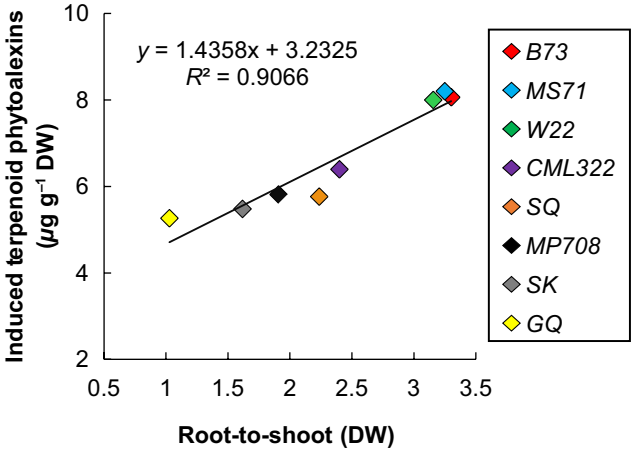

Figure 5. Relationship between drought-induced phytoalexins and root-to-shoot ratio among different maize lines. Two-week-old *Golden Queen (GQ), Silver Queen (SQ), Silver King (SK), B73, W22, Ms71, CML322* and *MP708* maize seedlings were placed in clay substrate of 40% volumetric water content (VWC) for 2 d following which the dry weight (DW) root-to-shoot ratio was estimated and correlated with the average quantity of induced root terpenoid phytoalexins. The linear regression and estimated coefficient of determination (R^2) was performed in Microsoft Excel 2013.

phytoalexins and the highest root-to-shoot ratio. Curiously, *Golden Queen* (*GQ*) sweet corn, our primary model in the current study, displayed the lowest root-to shoot ratio and accumulated the least phytoalexins in response to drought (Fig. 5). These results demonstrate a positive relationship between the root-to-shoot ratio and drought-induced phytoalexins, suggesting a potential function for phytoalexins in drought stress tolerance.

Kauralexin-deficient *an2* mutants are susceptible to drought

Consistent with the notion that the copalyl diphosphate synthase, *AN2*, is involved in kauralexin biosynthesis, mutant plants homozygous for the Ds insertion in *AN2* had reduced *AN2* transcript levels and produced minimal amounts of kauralexins (Fig. 6a, Supporting Information Fig. S2a). The transcript abundance of *AN2* was approximately 95% less in well-watered *an2* mutant roots in comparison with *W22* wild-type roots, and with drought, where the transcript abundance of *AN2* increased 16-fold, in *W22* roots, the amount of transcript in *an2* roots was 98% less. Even under conditions of drought, the concentration of kauralexins in *an2* roots was significantly less than well-watered *W22* wild-type roots. There was no significant difference in zealexin production between *an2* mutant maize and *W22* under both conditions of sufficient (+H_2O) or limited water (–H_2O) (Fig. 6b).

Although *AN2* is homologous and functionally equivalent to *AN1*, which is involved in GA biosynthesis (Bensen *et al.* 1995; Harris *et al.* 2005), *an2* mutants did not display morphological consequences characteristic of GA deficiency. Under

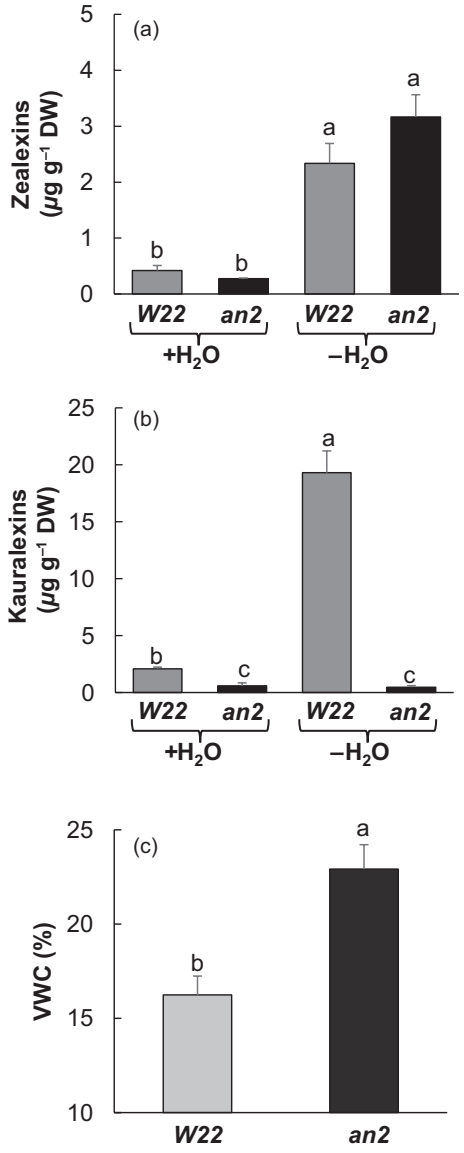

Figure 6. Mutant maize plants homozygous for a Ds insertion in *AN2* produce less kauralexins and are more susceptible to drought. The average (±SEM) concentration of zealexin (a) and kauralexin (b) metabolites in *W22* and *an2* maize roots following 5 d of water (+H_2O) or water-deficit treatment (–H_2O) was estimated and compared. Letters above bars indicate significant differences [analysis of variance (ANOVA), Tukey–Kramer honestly significant difference (HSD), $n = 5$, $P < 0.01$]. The volumetric water content of soil at which point 2-week-old *W22* and *an2* plants exposed to drought by withholding irrigation exhibited first signs of leaf curling the volumetric water content was recorded and compared (c) (Student's *t*-test, $n = 5$, $P < 0.01$). DW, dry weight.

non-stress conditions, *an2* mutant plants were not significantly different in height nor did they exhibit any aberrant floral development. Additionally, transcript levels of the GA marker genes *AN1*, *GA2ox* and *GA3ox* did not suggest any reduction in GA metabolism, which could have resulted in a reduction in root growth and development (Supporting Information Fig. S2). In fact, *AN1* transcription was induced twofold and both *GA2ox* and *GA3ox* transcript levels were not significantly different from *W22* plants.

During the 5 d drought treatment, *an2* mutant plants exhibited signs of severe drought stress a day sooner than *W22* plants. This did not appear to be due to an increase in water usage, but rather because of increased sensitivity to limited VWC (Fig. 6c).

With equivalent daily watering regimes, the method of using mixtures of clay pellets and glass beads at different proportions enabled long-term plant growth in substrates of defined VWC. The VWC was directly proportional to the percentage of clay pellets in the mixture ($R^2 = 0.9967$). The VWC of clay pellets alone averaged 55% throughout the 24 h interval between watering; a proportion of 1:1 (clay pellets: glass beads) had an average of 27% VWC, and a proportion of 1:3 (clay pellets: glass beads) had approximatly13% VWC. Glass beads alone averaged at 4% VWC. *W22* and *an2* seed germinated and grew in all substrate mixtures with exception of glass beads alone, which did not retain enough water to sustain the plants.

The shoot growth in particular was most affected by reduced substrate VWC (Fig. 7). Both *W22* and *an2* shoot weights significantly declined in substrates of declining VWC ($n = 5$; $P < 0.05$). Aboveground *an2* shoot growth was not significantly different from *W22*; however, the root-to-shoot ratio of *W22* was approximately 1.6-fold greater than *an2* in control substrate (100% clay pellets) at 55% VWC. The average root mass of *W22* plants did not significantly decline at 27 or 13% VWC; however, the root mass of an2 at 13% VWC was approximately half the weight of the root mass at 27% VWC ($n = 5$; $P < 0.05$). Nevertheless, limited VWC reduced *an2* growth much more severely than *W22* growth (Fig. 7c). In comparison with corresponding control plants in substrate at 55% VWC, the percentage of weight lost by *an2* plants was more than double the average weight lost by *W22* plants in substrate at 27% VWC ($n = 5$; $P < 0.05$).

Induction of terpenoid phytoalexins in response to drought is root specific; however, stress in root and shoot tissues may influence reciprocal production potentials

Since belowground osmotic stress caused by drought or root herbivore damage can systemically impact the production of defence metabolites in aboveground tissues (Erb *et al.* 2009b), the concentration of phytoalexins was evaluated in above and belowground tissues under well-watered and drought stressed conditions. Drought-induced accumulation of phytoalexins was specific to belowground tissues and did not influence constitutive phytoalexin levels within the stem or leaves (Fig. 8). However, the accumulation of phytoalexins in belowground tissues may have influenced the aboveground induction potential and vice versa (Fig. 9). *F. verticillioides* infected stalk tissues of watered plants accumulated five times more total phytoalexins than infected drought-stressed plants, which also accumulated phytoalexins in the roots ($n = 5$, $P < 0.01$). Additionally, the

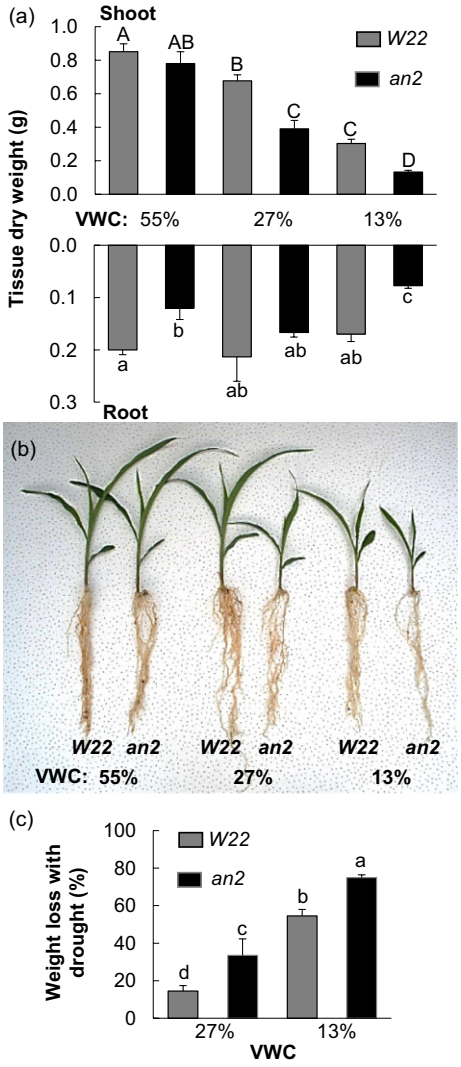

Figure 7. Mutant *an2* maize deficient in kauralexin production grow significantly less than *W22* wild-type controls at reduced substrate volumetric water contents (VWC). *W22* and *an2* seed were planted in mixtures of different proportions of clay pellets and glass beads, which were able to precisely maintain average VWCs of 55, 27 and 13% with daily watering throughout a growing period of three weeks. The average (±SEM) shoot and root dry tissue weights were compared separately (a) [analysis of variance (ANOVA), Tukey–Kramer honestly significant difference (HSD), $n = 5$, $P < 0.05$]. Capital letters indicate differences among stalk means and lower case letters indicate significant differences among root means. Representative image of *W22* and *an2* plants grown is substrates at VWCs of 55, 27 and 13% (b). The percentage of weight lost with different VWC treatments as compared with control at 55% VWC was calculated for *W22* and *an2* maize and then compared with an ANOVA followed by a Tukey–Kramer HSD test for individual mean difference (c) ($n = 5$, $P < 0.05$).

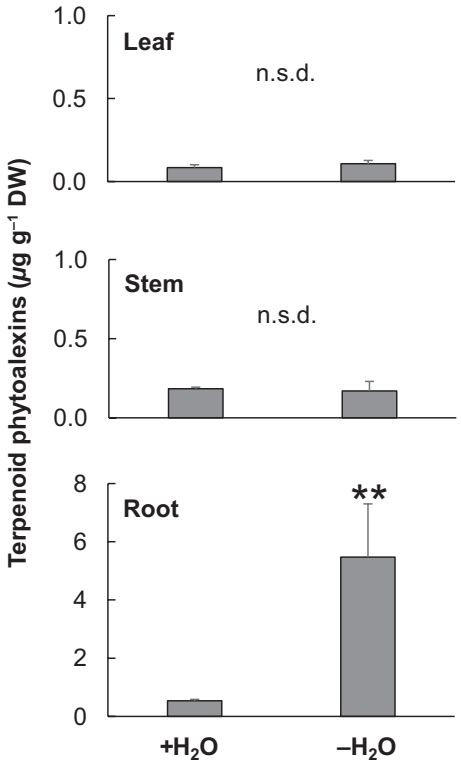

Figure 8. Drought-induced accumulation of terpenoid phytoalexins is root specific. Maize seedlings were placed in substrate of reduced volumetric water contents (VWC) (30%) for 24 h. The plant the leaf, stem and root tissues were then divided and independently analysed for their terpenoid phytoalexin content. The average (±SEM) quantity of phytoalexins in tissues from well watered seedlings ($+H_2O$) were compared with corresponding quantities in tissues of seedlings experiencing reduced VWC ($-H_2O$) using a simple Student's *t*-test, $n = 4$, n.s.d. denotes no significant difference, ** indicates significant differences $P < 0.01$.

concentration of phytoalexins in the roots of drought stressed plants with aboveground *F. verticillioides* stalk infection was sixfold less than plants with only drought stress ($n = 5$, $P < 0.05$).

DISCUSSION

Our results on maize zealexins and kauralexins are consistent with a role in belowground defences that are regulated by both biotic and abiotic stress. While to the best of our knowledge this represents the first report demonstrating the presence of terpenoid phytoalexins in maize roots, localized products of *TPS6/11*and *AN2* in the roots were previously predicted based on transcriptional analyses (Kollner *et al.* 2008; Allardyce *et al.* 2013). In response to root herbivore feeding and pathogen infection, these phytoalexins are induced (Fig. 1), and likely serve similar defensive functions against belowground biotic stressors as those previously demonstrated for aboveground tissues (Huffaker *et al.* 2011; Schmelz *et al.* 2011). Furthermore, we demonstrated that zealexins and kauralexins accumulate in roots under conditions of drought and high salinity, which suggests that these terpenoids also play a role in osmotic stress tolerance (Figs 2 and 3). The induced accumulation of root phytoalexins is positively correlated with the root-to-shoot ratio of inbred maize lines (Fig. 5), and *an2* mutant plants deficient in

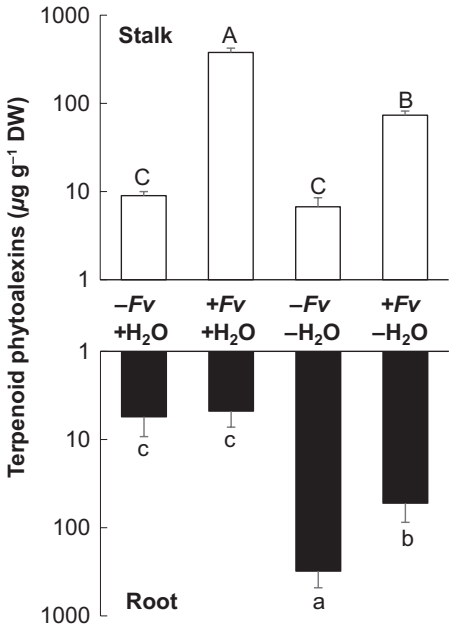

Figure 9. The accumulation of terpenoid phytoalexins in belowground dampens the induction potential aboveground and vice versa. Following 5 d of water ($+H_2O$) or water-deficit treatment ($-H_2O$) aeroponically grown maize at the V8 stage was inoculated aboveground in the stalk with either control solution (*−Fv*) or a 0.1 mL of 1×10^6 mL^{-1} *Fusarium verticillioides* spore solution (*+Fv*). Forty-eight hours after stalk infection, both the stem and root tissues were collected and analysed for terpenoid phytoalexins. The average (±SEM) concentration of terpenoid phytoalexins in the control- and pathogen-inoculated stem tissues under well-watered or water-deficit conditions were compared (top) with an analysis of variance (ANOVA) followed by a Tukey–Kramer honestly significant difference (HSD) test. The phytoalexin concentrations in root tissues under the same treatments were compared using a separate ANOVA. Capital letters indicate differences among stalk means and lower case letters indicate significant differences among root means ($n = 5$; $P < 0.05$).

kauralexin production are more sensitive to water deficit (Figs 6c and 7). Together, these results additionally suggest a role of root phytoalexins in drought stress tolerance.

Secondary defence metabolites frequently display dual functionality against biotic and abiotic stress factors. In addition to their role in defence against biotic stress above and belowground, terpenoid compounds also function in thermotolerance and quenching oxidative stress (Delfine *et al*. 2000; Loreto *et al*. 2004). Benzoxazinoids (BXs), which prior to the discovery of the maize terpenoid phytoalexins were thought to be the primary metabolites of maize direct defences, are also stimulated by osmotic stress and accumulate in the shoot partly through the systemic signalling of ABA (Richardson & Bacon 1993; Erb *et al*. 2009a).

It is worth noting that while stress induces phytoalexin accumulation in both stem and root tissues, the source of the induction appears to influence which of the two families of terpenoid phytoalexins predominates. Zealexin compounds are most abundant with pathogen infection both above and belowground (Huffaker *et al*. 2011; Vaughan *et al*. 2014), while the kauralexins are more abundant with drought and *Diabrotic balteata* feeding damage (Fig. 1). Additionally, osmotic stress appears to favour the induction of the mono-unsaturated B series of kauralexins (Supporting Information Fig. S1) (Schmelz *et al*. 2011). These results suggest specificity and selectivity of phytoalexin production in response to the different stressors.

The stimulated accumulation of secondary metabolites by water stress has been widely demonstrated as a means of stress tolerance (Pedranzani *et al*. 2003; Porcel & Ruiz-Lozano 2004; Zhu *et al*. 2009; Ramakrishna & Ravishankar 2011). For example, anthocyanins accumulate in the roots of maize under conditions of drought or high salinity, and seedlings with greater quantities of anthocyanins are considerably more drought resistant (Chalker-Scott 1999). Similarly, under limited water conditions, the enhanced formation of diterpenoid antioxidants, such as α-tocopherol and isorosmanol, protects rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) from oxidative damage, which in turn increases their tolerance to drought (Munné-Bosch *et al*. 1999, 2001). The accumulation of maize root phytoalexins could correspondingly serve an adaptive function under conditions of limited water availability; however, the antioxidant potential of maize phytoalexins has yet to be evaluated and the mechanisms involved requires further investigation.

As further evidence for the involvement of particularly the more abundant kauralexins in drought stress tolerance, *an2* mutant plants exhibited severe symptoms of leaf curling at a higher VWC than control plants. Moreover, under conditions of limited moisture availability, *an2* growth was significantly less than the W22 parent line (Fig. 7). In agreement with this premise, there is a correlation between the root-to-shoot ratio and induced phytoalexin levels in maize seedlings under drought stress (Fig. 5). Root size, morphology and architecture are important factors for determining plant fitness, measured as yield, under drought conditions (Malamy 2005). Quantitative trait loci (QTL) for total ear number and grain yield are co-located on chromosome 1 in the bin 1.03 region with QTLs for root traits, including root-to-shoot ratio (Ruta *et al*. 2010; Rahman *et al*. 2011). Interestingly, *AN2*, which is involved with kauralexin biosynthesis is located in the adjacent bin 1.04 on chromosome 1 only about 20–25 Mbp away from the estimated region of QTLs involved in determining the root-to shoot ratio (MaizeGDB). The relatively close proximity of these genes would suggest a potential linkage. While elucidation of the mechanism by which kauralexins and potentially also zealexins enhance drought tolerance is beyond the scope of this paper, these findings have identified a phenotypic trait that connects both the potential for enhanced drought tolerance and biotic stress resistance, which can be targeted by breeders.

Although it has been suggested that the ent-copalyl diphosphate synthase *AN1* predominantly provides the precursor for GA synthesis and *AN2* is more involved in defence-related secondary metabolism (Harris *et al*. 2005; Schmelz *et al*. 2011), it is possible that the *an2* mutants are deficient in both kauralexin production and GA metabolism, which could potentially explain some of the observed results.

The *an1* deletion mutants were able to still produce ent-kaurene to approximately 20% of wild-type levels presumably through substrate provided by the enzymatic activity of *AN2* (Bensen *et al*. 1995). However, *AN1* transcription was induced in the *an2* mutants, which would supposedly compensate for the reduced activity of AN2 and furthermore, the *an2* mutants do not display any constitutive morphological characteristics of GA deficiency.

Our results, suggesting that ABA and JA are both involved in drought-induced production of phytoalexins in roots, are consistent with other reports. Upon exposure to drought or high salinity, JA levels have been shown to increase in rice (Moons *et al*. 1997; Kiribuchi *et al*. 2005), soybeans (Creelman & Mullet 1995) and tomatoes (Pedranzani *et al*. 2003). Experiments in rice have suggested that the production of JA during drought stress promotes the synthesis of ABA (Kim *et al*. 2009). This would also appear to be the case in maize, considering that increased JA levels preceeded a spike in ABA concentrations (Fig. 3). Treatment with JA alone, however, did not induce phytoalexin production (Fig. 4) presumably because of the necessity of a concurrent release of ethylene (Huffaker *et al*. 2011; Schmelz *et al*. 2011). Nevertheless, root treatment with ABA was sufficient to induce the accumulation of root phytoalexins, affirming previous reports showing the indelibility of phytoalexins by ABA (Jiang & Zhang 2001; Mansouri & Asrar 2011; Yang *et al*. 2012).

Our findings would argue that a more holistic approach is essential to understand the regulated outcome of the defence response. As integrated elements of the whole, the status of both the above and belowground organs most likely influences the regulatory interactions between biotic and abiotic stress. While multiple studies have focused on the physiology and biochemistry of the shoot in response to drought, relatively few have simultaneously considered the roots, although they constitute the primary site of drought perception, resistance and recovery (Porcel & Ruiz-Lozano 2004). Consistent with the optimal defence theory, under conditions of limited water supply terpenoid defence metabolites are allocated to maize roots (Fig. 9). This allocation of resources belowground is logically an optimal investment to protect the vital organs necessary in acquiring the limiting factor (water) for growth, development and reproduction from the existing osmotic stress and potentially from pest or pathogen attack. At least one putative explanation for the difference in phytoalexin accumulation above and belowground with and without drought stress, would be that the energy appropriation to the stem or root tissues may place constraints on the capacity of those organs to induce phytoalexins (Fig. 9). *F. verticillioides* stalk infection, which increased the production of zealexins and kauralexins at the site of attack aboveground, also appeared to dampened the accumulation of phytoalexins in the roots with drought.

The characterization of above and belowground phytoalexin defence responses to biotic and abiotic stress, has expanded our understanding of maize defensive strategies, particularly under conditions of drought. Plants possess a variety of chemical defences, and to collectively optimize resources these different compounds are distributed between and within tissues based on status quo in order to efficiently maximize protection of the whole (Stamp 2003). Under conditions of osmotic stress, maize appears to distribute its two main classes of direct defence metabolites differently. While maize roots accumulate greater levels of phytoalexins in response to water stress, root herbivore damage, or exogenous application of ABA (Figs 1–4), the concentration of belowground BXs remain unaltered (Erb *et al*. 2011). However, these same stressors boost the production of BXs aboveground in the shoot tissue (Richardson & Bacon 1993; Erb *et al*. 2011). Furthermore, treatment of maize roots with ABA (which can mimic drought stress signalling) leads to the augmented production of chlorogenic acid in response to aboveground herbivory (Erb *et al*. 2009b), while drought stress dampens the induced accumulation of phytoalexins pathogen-infected stems (Fig. 9).

Therefore, during drought stress, maize appears to invest in root protection with phytoalexins, but also enhances aboveground defences by increasing the concentration of BXs. This defensive strategy is holistic and displays the equal importance of shoots and roots in plant survival. However, by modulating defence compounds by tissue type and relying more heavily on particular types of defences versus others, plant susceptibility or resistance will mainly depend on the sensitivity of the pest or pathogen to the composite of defences being employed. For example, water stress caused by root-herbivore damage enhances maize resistance to the necrotrophic fungus *Setosphaeria turcica*, which is sensitive to BX concentrations (Erb *et al*. 2009a; Ahmad *et al*. 2011). On the other hand, the mycotoxigenic fungus *F. verticillioides*, which is capable of detoxifying BXs (Glenn *et al*. 2001, 2002), but displays reduced growth in the presence of terpenoid phytoalexins (Vaughan *et al*. 2014), thrives on drought-stressed maize (Miller 2001).

Changes in above and belowground plant defences because of abiotic stress and/or interacting abiotic and biotic stress responses are of significant ecological relevance (Bezemer & van Dam 2005; Chakraborty & Newton 2011; Gonzalez-Megias & Menendez 2012; de Sassi & Tylianakis 2012). It is becoming more and more evident that the physical and physiological responses of plants to above and belowground stressors can ultimately impact multiple trophic levels and the entire ecosystem (Van Der Putten 2003; De Deyn & Van der Putten 2005; Kaplan *et al*. 2008; van Dam 2009; Erb *et al*. 2009a). Considering that global climate changes are projected to increase the frequency, duration and severity of drought throughout many agriculturally relevant regions (Barton & Clark 2014), our results should provide additional insight into the ecological implications of these changes and hopefully aid in the adaptation of maize agriculture with the least amount of agro-economic loss.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Constitutive and induced quantities of individual zealexin and kauralexin metabolites in maize roots. Detailed quantities of individual zealexin metabolites (Z-1-Z-6 as numbered and described in Huffaker *et al*. 2011) and kauralexin metabolites (K-A1-K-A3 and K-B1-K-B3 as described by Schmelz *et al*. 2011) in (a) control and *Fusarium verticillioides* infected roots, (b) control and *Diabrotica balteata* damaged roots, and (c) roots exposed to 1 and 7 d of drought were estimated by gas chromatography/chemical ionization – mass spectrometry. Corresponding cumulative totals and statistical analyses are presented in Figs 1 and 3b,c.
Figure S2. Gibberellin (GA) marker genes were not correspondingly reduced as *AN2* in mutant plants. Transcript abundance of *anther ear2* (*AN2*), and the GA marker genes *anther ear1* (*AN1*), *GA 2-oxidase* (*GA2ox*) and *GA 3-oxidase* (*GA3ox*), in *an2* and *W22* roots were analysed via qRT-PCR. Data points have been normalized to the endogenous control *ribosomal protein L17* (*RPL17*) AF034948), and are relative to transcript abundance in *W22* maize roots. Comparisons were made using a Student *t*-test, $n = 4$, individual P values are represented within the graphs.

