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Ectopic expression of *ARGOS8* reveals a role for ethylene in root-lodging resistance in maize

Jinrui Shi*, Bruce J. Drummond, Jeffrey E. Habben, Norbert Brugire, Ben P. Weers, Salim M. Hakimi, H. Renee Lafitte, Jeffrey R. Schussler, Hua Mo, Mary Beatty, Gina Zastrow-Hayes and Dennis O'Neill

Corteva Agriscience, Agriculture Division of DowDuPont 7300 NW 62nd Avenue, Johnston, IA 50131, USA

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*For correspondence (e-mail jinrui.shi@pioneer.com).

SUMMARY

Ethylene plays a critical role in many diverse processes in plant development. Recent studies have demonstrated that overexpression of the maize *ARGOS8* gene reduces the plant's response to ethylene by decreasing ethylene signaling and enhances grain yield in transgenic maize plants. The objective of this study was to determine the effects of ethylene on the development of nodal roots, which are primarily responsible for root-lodging resistance in maize. Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was found to promote the emergence of nodal roots. Transcriptome analysis of nodal tissues revealed that the expression of genes involved in metabolic processes and cell wall biogenesis was upregulated in response to ACC treatment, supporting the notion that ethylene is a positive regulator for the outgrowth of young root primordia. In *BSV::ARGOS8* transgenic plants with reduced ethylene sensitivity due to constitutive overexpression of *ARGOS8*, nodal root emergence was delayed and the promotional effect of ACC on nodal root emergence decreased. Field tests showed that the *BSV::ARGOS8* plants had higher root lodging relative to non-transgenic controls. When *ARGOS8* expression was controlled by the developmentally regulated promoter FTM1, which conferred *ARGOS8* overexpression in adult plants but not in the nodal roots and nodes in juvenile plants, the *FTM1::ARGOS8* plants had no significant difference in root lodging compared with the wild type but produced a higher grain yield. These results suggest that ethylene has a role in promoting nodal root emergence and that a delay in nodal root development has a negative effect on root-lodging resistance in maize.

Keywords: ethylene, maize, nodal root, root lodging, *ARGOS8*, grain yield.

INTRODUCTION

Roots perform functions that are necessary for plant growth and development. Roots absorb water and nutrients and anchor plants in the soil. In maize, the root system consists of embryonic roots (i.e. the primary root and lateral seminal roots), stem-borne nodal roots and lateral roots that are formed from all roots (Abbe and Stein, 1954; Feldman, 1994). The stem-borne roots, including the crown roots at the basal nodes of the stem and the brace roots which emerge from the nodes above the soil surface and quickly enter the soil, form the main framework of the underground root system and are the basis for root-lodging resistance (Hochholdinger, 2009). Root lodging is the inability of plants to stand upright for optimum growth and harvestability (Ennos *et al.*, 1993; Landi *et al.*, 2007). The maize *rtcs* (*rootless concerning crown and seminal roots*) mutant deficient in the formation of nodal roots falls over at early developmental stages and cannot survive in typical agronomic environments without human intervention (Hetz *et al.*, 1996). Maize root lodging often occurs during the mid-growing season when heavy rainfall saturates soils and strong winds displace the root system from its normal vertical axis. In root-lodged plants, uptake of water and nutrients, as well as interception of light by the canopy, can be reduced and in some cases pollination is delayed, leading to reduced grain yield and poor grain quality (Carter and Hudelson, 1988; Pellerin *et al.*, 1990; Stamp and Kiel, 1992; Guingo and Hébert, 1997; Duvick, 2005; Liu *et al.*, 2012). Harvesting of root-lodged fields is often difficult and can result in additional yield losses.

Maize nodal roots develop sequentially at each of the seven or eight lowest nodes, beginning at the coleoptile node (Hoppe *et al.*, 1985). These roots originate from cells next to the peripheral cylinder of vascular bundles in the

stem, with young root primordia growing through the cortex, penetrating the nodal epidermis and emerging from the stem. Phytohormones play an important role in regulating the development of stem-borne roots (often referred to as adventitious roots, which include all roots formed from non-root tissues; for reviews see Orman-Ligeza *et al.*, 2013; Bellini *et al.*, 2014; Steffens and Rasmussen, 2016). Genetic studies in rice, tomato and Arabidopsis have shown that pin-formed (PIN) and AUX1 families of auxin efflux and influx carriers are required for the initiation and development of adventitious roots (Tyburski and Tretyn, 2004; Xu *et al.*, 2005; Morita and Kyozuka, 2007; Kitomi *et al.*, 2008; Liu *et al.*, 2009; Negi *et al.*, 2010; Vidoz *et al.*, 2010; Pacurar *et al.*, 2014). Auxin induces the expression of the maize *RTCS* gene and the rice ortholog *ADVENTITIOUS ROOTLESS1* (*ARL1*; also known as *CROWN ROOTLESS1* or *CRL1*), which are essential for the initiation of stem-borne roots (Liu *et al.*, 2005; Taramino *et al.*, 2007). The rice *ARL1*/*CRL1* and *CROWN ROOTLESS5* (*CRL5*; an AP2/ERF transcription factor) are targets of the auxin response factor gene family (Inukai *et al.*, 2005; Kitomi *et al.*, 2011). It has been reported that the rice *Oscand1* mutant is defective in crown root emergence and the gene responsible for the mutation is involved in auxin signaling to maintain the G_2/M cell cycle transition in the crown root meristem (Wang *et al.*, 2011). Cytokinins are antagonistic to auxin and suppress the formation of adventitious roots. In rice, treatment with cytokinin inhibits crown root initiation and loss-of-function mutation of WUSCHEL-related homeobox 11 (*WOX11*), a repressor of cytokinin signaling, reduces the number and the growth rate of crown roots (Zhao *et al.*, 2009). The rice CRL5 protein promotes crown root initiation through repression of cytokinin signaling by positively regulating type-A response regulators (Kitomi *et al.*, 2011).

Ethylene is another hormone in the hormonal regulatory network regulating adventitious root development (for review see Steffens and Rasmussen, 2016). In deepwater rice, tomato and *Rumex palustris*, submergence in flood water increases ethylene biosynthesis and ethylene induces adventitious root formation (Visser *et al.*, 1996; Lorbiecke and Sauter, 1999; Kim *et al.*, 2008; Vidoz *et al.*, 2010). In tomato, the *Nr* (*Never ripe*) mutant defective in ethylene signaling has fewer adventitious roots in young seedlings than the wild type (WT), while the *epinastic* mutant, which has elevated ethylene and constitutive ethylene signaling, has a greater number of adventitious roots (Negi *et al.*, 2010). Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) promotes adventitious rooting in WT plants. Treatment with ACC was found to negatively regulate transport of indole-3-acetic acid (IAA) in hypocotyls, whereas the *Nr* mutation increased IAA transport. Negi *et al.* (2010) proposed that ethylene regulation of adventitious root formation in tomato hypocotyls is auxin dependent. Clark *et al.* (1999) also reported that ACC promotes adventitious root formation in vegetative cuttings taken from older tomato plants, and showed that ethylene signaling is required for auxin-induced adventitious root formation in tomato and petunia (Clark *et al.*, 1999). These tomato studies, however, did not determine if ethylene promotes the initiation of root primordia or the outgrowth of young root primordia through the cortex (i.e. emergence). In deepwater rice, ethylene enhances adventitious root development during flooding by promoting the emergence of the root primordia which have been formed during normal development (Lorbiecke and Sauter, 1999). However, the role of ethylene in maize nodal root development is far less clear, and that is the focus of this study.

Previously, we have generated transgenic maize plants with reduced ethylene sensitivity by overexpressing the maize *ARGOS8* gene (Shi *et al.*, 2015, 2016). *ARGOS8* is a member of the *ARGOS* gene family which contains a conserved TPT domain (Shi *et al.*, 2016). *ARGOS8* is expressed in developing kernels, but the transcript abundance in other tissues is very low (Shi *et al.*, 2017). The ARGOS8 protein is a component of the ethylene receptor signaling complex, interacting with the reversion-to-ethylene sensitivity1 like (RTL) proteins (Shi *et al.*, 2016). When overexpressed, *ARGOS8* reduces the ethylene response in transgenic maize and Arabidopsis plants by reducing ethylene signal transduction (Shi *et al.*, 2015, 2016). Maize hybrids with constitutive *ARGOS8* overexpression produce high grain yield under drought-stressed and well-watered conditions (Shi *et al.*, 2015, 2017). Because of the constitutive expression patterns of the transgene or the genome-edited allele, it is expected that ethylene signaling can be downregulated in tissues where reduced ethylene signaling is unnecessary and even harmful to achieving high grain yield. Given the promotional effect of ethylene on adventitious root development in rice and the predominant role of nodal roots in the resistance of maize plants to root lodging, we sought to determine whether ethylene is involved in maize nodal root development and whether reduced ethylene signaling affects root-lodging resistance.

In this report, ACC treatments and *ARGOS8* transgenic plants with reduced ethylene sensitivity were used to determine the effect of ethylene on nodal root emergence. We also examined transcriptional changes in nodal tissues upon ACC treatment and in plants with reduced ethylene sensitivity. Root lodging of transgenic plants with *ARGOS8* overexpression driven by constitutive promoters and a developmentally regulated promoter was evaluated in field conditions. The results show that ACC treatments promote nodal root emergence and plants with constitutive *ARGOS8* overexpression have delayed nodal root emergence as well as reduced root-lodging resistance. When the developmentally regulated FTM1 promoter was used

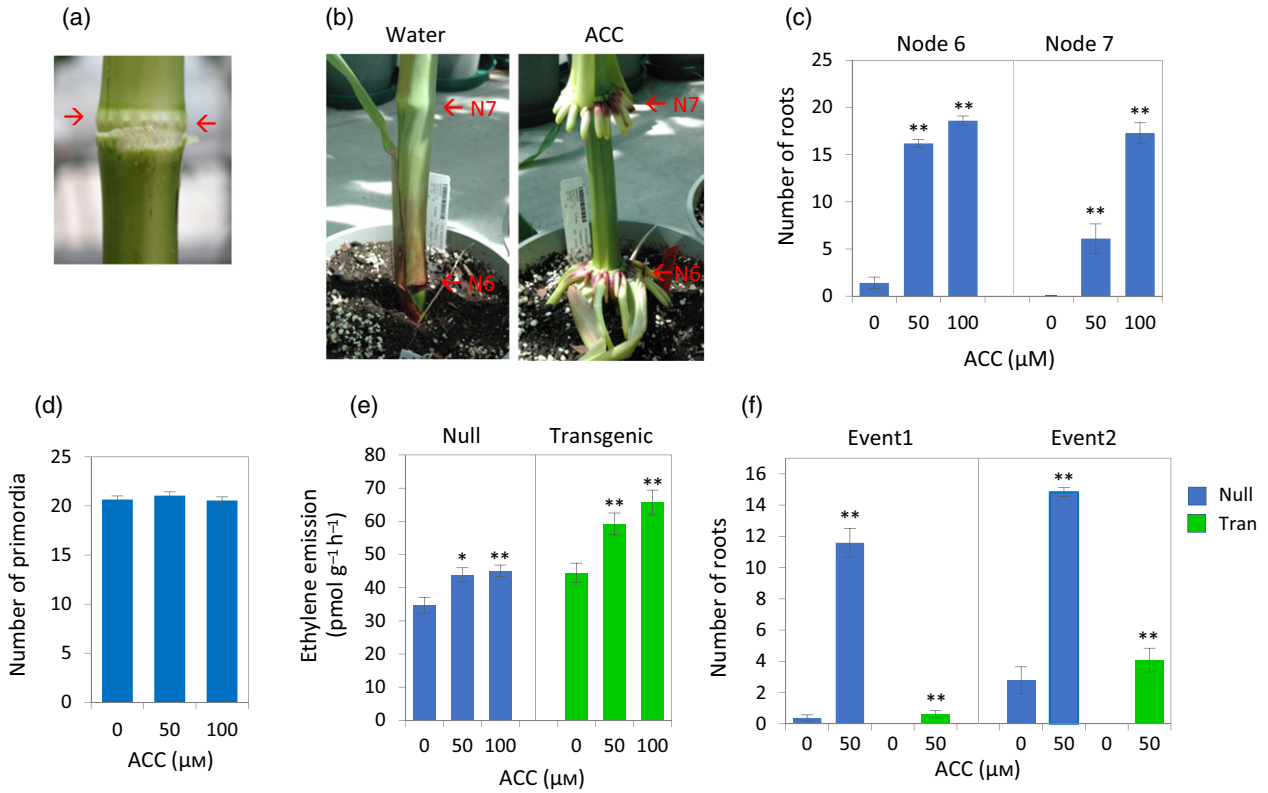

Figure 1. Effects of treatments with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) on maize brace root emergence and ethylene evolution

(a) Node 6 of a maize hybrid PHR03/PH12SG plant at V10 with the leaf sheath removed showing root primordia (arrows). (b) Images of node 6 (N6) and node 7 (N7) of the plants that were treated with water or 100 μM ACC for 7 days starting at V8. (c) Number of brace roots in the ACC-treated plants 6 days after initiation of the treatment (n = 10; error bar, SE). The ACC-treated plants were compared with water controls (Student's t-test, ** $P < 0.01$). (d) Number of primordia on node 7, including emerged and unemerged primordia. The plants were treated with ACC for 7 days starting at V9 and scored 8 days after the end of the ACC treatment (n = 20; error bars, SE). (e) Ethylene emission rates in the eighth leaves of ACC-treated plants and water controls. Samples were taken from the wild-type (i.e. non-transgenic segregants; null) and *BSV::ARGOS8* transgenic plants at V11 which had been treated with ACC for 5 days (n = 10; error bar, SE). The ACC-treated plants were compared with water controls (Student's t-test, *$P < 0.05$). (f) Number of brace roots on node 6 in the non-transgenic segregants (null) and *BSV::ARGOS8* transgenic plants (Tran) treated with 0 or 50 μM ACC for 7 days starting at V10 (n = 24; error bar, SE). The ACC-treated plants were compared with water controls (Student's t-test, **$P < 0.01$).

to restrict *ARGOS8* overexpression to adult plants, the transgenic plants withstood the effects of inclement weather like the non-transgenic controls but had a higher grain yield.

RESULTS

ACC promotes nodal root emergence in maize

The greenhouse-grown maize hybrid PHR03/PH12SG developed the first tier of brace roots on node 6, which also bears the fifth leaf (the coleoptile node designated node 1; Hoppe *et al.*, 1985). The brace roots grew into the soil and developed lateral roots. In plants at vegetative stage 8 (V8), the young root primordia had formed on nodes 6 and 7 but were arrested, appearing as a circle of deep green dots and surrounded by a pale green halo underlying the stem epidermis (Figure 1a). The V8 plants were treated with the ethylene precursor ACC by watering them with an aqueous solution of 1 L per plant per day of 0, 50 or 100 μM ACC. Brace roots began to emerge on node 6 3 days after initiation of the treatment, and subsequently on node 7 2 days later (Figure 1b). At day 7, the 50 μM ACC-treated plants had on average 16.2 and 6.1 roots on nodes 6 and 7, respectively, but only 1.4 roots were found on node 6 in water controls (Figure 1c). In the plants treated with 100 μM ACC, almost all primordia on both node 6 and node 7 had broken through the stem surface by day 7. The total number of primordia on node 7, including emerged and unemerged, was not affected by the ACC treatment (Figure 1d). The application of ACC increased ethylene biosynthesis in the plants, as revealed by increased ethylene emission in leaves (Figure 1e), indicating that ethylene is most likely responsible for the promotion of nodal root emergence.

To confirm the promotional effect of ACC on nodal root emergence, we tested *ARGOS8* transgenic plants, which

have reduced ethylene sensitivity (Shi *et al.*, 2015, 2016). In the transgenic plants, maize *ARGOS8* was overexpressed under control of the banana streak virus (BSV) promoter which confers strong constitutive expression (Schenk *et al.*, 2001). To determine the brace root response, the *BSV::ARGOS8* hybrid PHR03/PH12SG plants were treated at V10 with 50 μM ACC. The ACC treatment did not affect the transcript abundance of the *ARGOS8* transgene (Figure S1 in the online Supporting Information), as expected. Like WT plants, the ACC-treated transgenic plants had increased ethylene emission relative to water controls (Figure 1e). Although the ACC treatment also promoted the emergence of brace roots on node 6 in the transgenic plants, the magnitude of the effect was considerably smaller than that in non-transgenic segregants (Figure 1f). On node 7, brace roots were observed in some of the non-transgenic plants 7 days after the initiation of the treatment, but not in any transgenic plants.

Maize seedlings grown in the greenhouse were used to study underground nodal root response. When 17-day-old seedlings were treated with ACC, nodal roots began to emerge on node 5 in the treated non-transgenic plants 6 days after the initiation of treatment. In contrast, there were no roots visible in water (0 μM ACC) controls until 4 days later (Figure 2a). By this time, most of the root primordia on node 5 in 100 μM ACC-treated plants had penetrated through the node epidermis. Like the brace roots, the underground nodal roots in the *BSV::ARGOS8* transgenic plants were not as responsive to ACC as those in the non-transgenic plants (Figure 2a,b). At 3 days after the end of the 16-day ACC treatment, the 50 μM ACC-treated non-transgenic plants had on average 11.3 roots on node 5 with a mean root length of 152.1 mm, while the transgenic plants had 8.3 nodal roots on the same node and the root length averaged 57.7 mm (Figure 2c,d). For the plants treated with 100 μM ACC, nearly all the root primordia on node 5 had emerged in both transgenic and non-transgenic plants and the number of nodal roots was comparable, but the non-transgenic segregants had longer roots (Figure 2c, d). The total number of primordia on node 5, including emerged and unemerged, was not significantly different between the transgenic and non-transgenic plants or between ACC-treated and water controls (Figure S2), suggesting that both ACC treatment and *ARGOS8* overexpression affected nodal root emergence rather than initiation of root primordia.

Under normal growth conditions without exogenously applied ACC (i.e. water controls), nodal root emergence was delayed in the *BSV::ARGOS8* transgenic plants compared with the non-transgenic segregants (Figure 2a). In the 35-day-old plants with overlying leaf sheaths removed, nodal roots were found on node 5 in all non-transgenic plants examined (Figure 2b). Of the 24 transgenic plants from two events, only 17 had nodal roots. The number of node 5 roots per plant was higher and the root length longer in the non-transgenic plants (Figure 2c,d). In the V14 plants, brace roots were present on node 6 in 20 out of 28 non-transgenic plants examined, averaging 8.7 roots per plant, while only one plant with two roots was found from the same number of transgenic plants (Figure S3). The delay of nodal root emergence in the transgenic plants overexpressing *ARGOS8* further supports the hypothesis that ethylene is involved in the regulation of nodal root emergence in maize.

Greater root lodging in *BSV::ARGOS8* transgenic plants

To determine if a delay in nodal root emergence affects resistance to root lodging, *BSV::ARGOS8* transgenic and non-transgenic hybrids were grown in fields in nine locations across the USA in 2013. Root lodging was evaluated in typical agronomic environments. Out of the nine locations, seven did not have lodging pressure. Only two locations experienced a combination of strong winds and heavy rainfall before flowering, allowing discrimination of root lodging. Compared with non-transgenic controls, the transgenic plants had a significantly higher frequency of root lodging (Table 1).

A reduction in ethylene sensitivity in the transgenic plants overexpressing *ARGOS8* driven by the maize UBIQUITIN1 promoter (UBI1) had been shown to improve grain yield under drought stress conditions and in well-watered environments (Shi *et al.*, 2015). Relative to the UBI1 promoter, the BSV promoter conferred a considerably higher level of constitutive *ARGOS8* overexpression (Figure 3a). In the 2013 field test, the transgenic PHR03/PH12SG hybrid plants gave a yield similar to non-transgenic controls (Tables 1 and S1). The reason for the lack of increase in yield in the *BSV::ARGOS8* plants is that the highly expressed *ARGOS8* may have reduced ethylene sensitivity too much (Shi *et al.*, 2015), while high grain yield requires an optimal level of ethylene signaling in transgenic plants. In the same genetic background, moderate expression of *ARGOS8* under control of the maize constitutive GOS2 promoter (Shi *et al.*, 2017), which confers weaker expression than UBI1 (Figure 3), was found to increase grain yield by 4.8 bushels per acre (bu ac^{-1}) (Tables 1 and S1). Although the percentage of root-lodged *GOS2::ARGOS8* plants was slightly higher than that in non-transgenic controls, the difference was not statistically significant (Table 1). However, further testing of the *GOS2::ARGOS8* construct in different hybrids revealed that the effects of *ARGOS8* overexpression on root lodging are influenced by genetic background. Hybrid plants derived from the *GOS2::ARGOS8* transgenic PH184C inbred line developed brace roots later than WT controls in the field (Figure 4a,b). Greenhouse-grown transgenic PH184C/PH1V69 plants also showed delayed nodal root development and reduced promotional effects of ACC on nodal

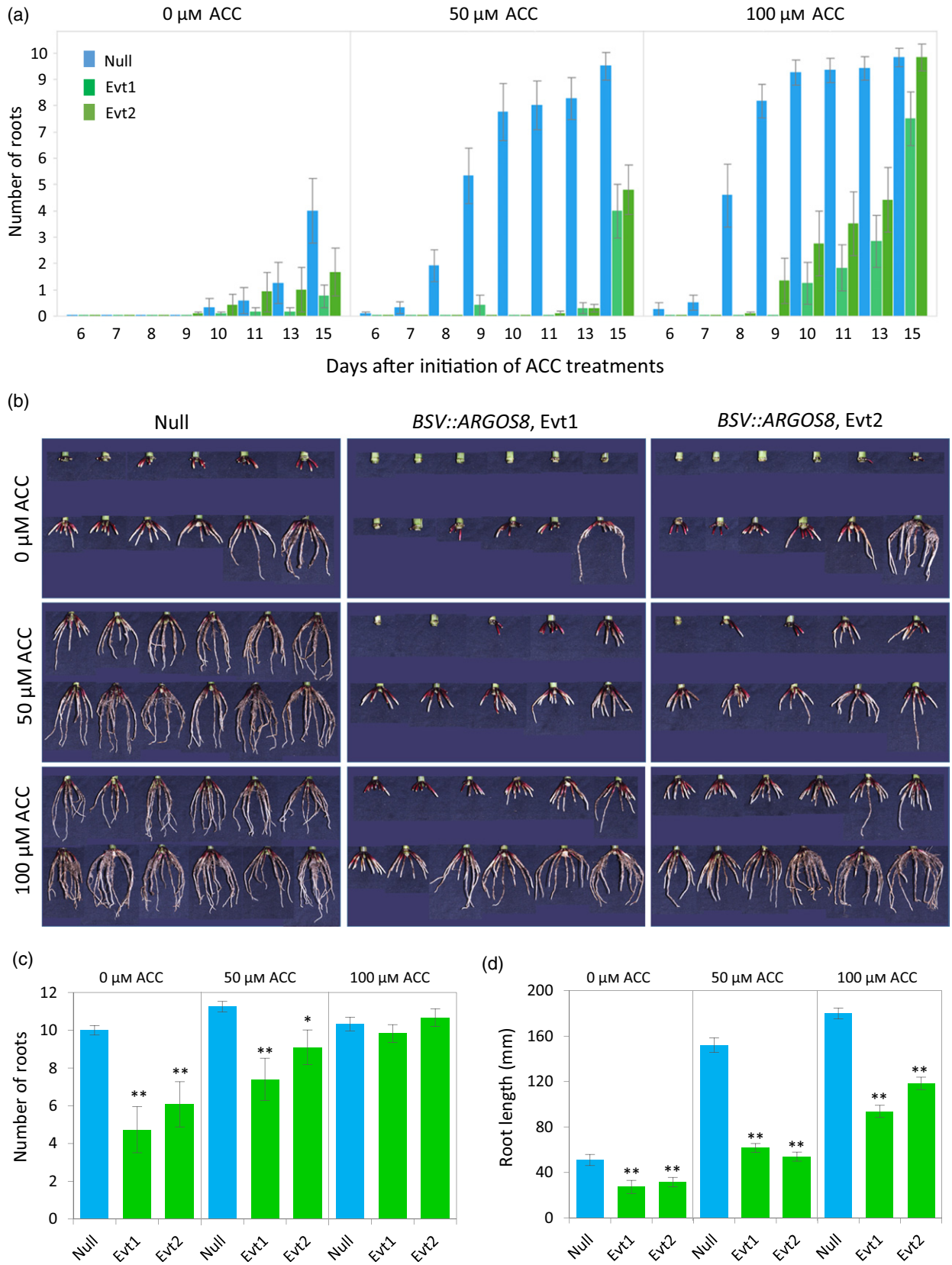

Figure 2. Emergence and growth of underground nodal roots on node 5 in maize plants.
(a) Time courses of nodal root emergence in response to 1-aminocyclopropane-1-carboxylic acid (ACC) treatment in the *BSV::ARGOS8* transgenic plants and non-transgenic controls. Seventeen-day-old seedlings of two events (Evt1 and Evt2) and bulked non-transgenic segregants (Null) were treated with 0, 50 or 100 μM ACC for 16 days (n = 12; error bar, SE).
(b) Images of isolated node 5 with nodal roots from 10 or 12 individual plants per genotype and treatment 3 days after the end of the ACC treatment.
(c) Number of roots on node 5 (n = 12; error bar, SE). Transgenic plants (Evt1 and Evt2) were compared with Null (Student's t-test, $*P < 0.05$, $**P < 0.01$).
(d) Length of roots on node 5. The data represent means of the length of individual roots (n = 61–135; error bar, SE).

Table 1 Root lodging and grain yield in maize PHR03/PH12SG hybrid overexpressing *ARGOS8*. Transgenic plants (Trans) of three *ARGOS8* constructs (nine to ten events per construct) and non-transgenic controls (Null) were grown in nine locations across the USA in 2013. Two locations had significant root-lodging pressures allowing us to evaluate root-lodging resistance. All analyses were implemented using ASReml with output of the model presented as best linear unbiased predictions

Trait	Location	Construct	Trans	Null	Difference (95% CI)	n	P-value
			Per cent	Per cent			
Root-lodged plants	Location 1	*BSV::ARGOS8*	48.9	13.0	35.7 (24.9–46.5)	27	0.00
		GOS2::ARGOS8	18.2	13.0	5.2 (−5.4 to 15.7)	30	0.32
		FTM1::ARGOS8	16.8	13.0	3.6 (−6.9 to 14.0)	30	0.50
	Location 2	*BSV::ARGOS8*	6.3	0.6	5.7 (2.4–9.1)	26	0.00
		GOS2::ARGOS8	1.0	0.6	0.5 (−2.9 to 3.8)	29	0.78
		FTM1::ARGOS8	0.9	0.6	0.4 (−3.0 to 3.7)	29	0.83
			bu ac^{-1}	bu ac^{-1}			
Grain yield	All locations	*BSV::ARGOS8*	144.2	143.0	1.2 (−0.5 to 2.9)	733	0.17
		GOS2::ARGOS8	147.8	143.0	4.8 (3.1–6.4)	826	0.00
		FTM1::ARGOS8	145.8	143.0	2.8 (1.1–4.4)	830	0.00

n = total comparisons: number of locations × number of events × replicates.

root emergence relative to WT controls (Figure S4). In a multiple-location field test in 2016, the *GOS2::ARGOS8* transgenic hybrids had a significantly higher percentage of root-lodged plants than WT controls (Figure 4c). However, these hybrids had a greater grain yield than controls (Figure S5). These results confirmed that constitutively overexpressing *ARGOS8* delays nodal root development and negatively affects root-lodging resistance.

To determine if increased grain yield and reduced root-lodging resistance can be decoupled, we tested the transgenic plants which overexpressed *ARGOS8* under control of the non-constitutive FTM1 promoter. The FTM1 promoter was derived from the maize *FLORAL TRANSITION MADS1* gene (also known as *ZMM4*; Danilevskaya *et al.*, 2008). This promoter confers developmentally regulated gene expression, primarily after the transition from vegetative to reproductive growth in developing apical and lateral inflorescences and to a lesser extent in several other tissues in adult plants, but not in embryonic and juvenile tissues (Danilevskaya *et al.*, 2008). For 24-day-old seedlings, there were very few transcripts of *ARGOS8* in nodal roots and nodes in the *FTM1::ARGOS8* transgenic plants, similar to that found in non-transgenic plants (Figure 3a). Among the four constructs, the highest level of *ARGOS8* expression in those tissues was found in the *BSV::ARGOS8* plants (Figure 3a). The absence of *ARGOS8* overexpression in tissues of the juvenile plants implies unaffected ethylene sensitivity in the *FTM1::ARGOS8* transgenic plants at early developmental stages. In adult *FTM1::ARGOS8* plants, *ARGOS8* was overexpressed in leaves, stalks, tassels, silks, cobs and developing seeds, and the abundance of the transcripts were nearly as high as in the *GOS2::ARGOS8* plants (Figure 3b). Field tests showed that the *FTM1::ARGOS8* hybrid PHR03/PH12SG yielded 2.8 bu ac^{-1} more than non-transgenic plants ($P < 0.05$; Tables 1 and S1). The percentage of root-lodged plants was not significantly different between the transgenic plant and controls in the two locations where root-lodging pressure existed (Table 1). These results indicate that restricting *ARGOS8* overexpression to adult plants can minimize the risk of root lodging and increase grain yield in maize.

Transcript profiles of nodal tissues in ACC-treated plants

Ethylene-induced transcriptional changes in nodal tissues were investigated with RNA sequencing. Wild-type plants (21 days old) were treated with 100 μM ACC. The *BSV::ARGOS8* transgenic plants which have reduced ethylene sensitivity were included for comparison with untreated WT plants. To identify differentially expressed (DE) genes prior to nodal root emergence, a cross section (about 2 mm thick) of node 5, containing root primordia, was excised 48 and 72 h after the initiation of the ACC treatment (Figure 5a). The differential expression at the gene level was analyzed in pairwise comparisons. The DE genes are defined as those with false discovery-corrected $P < 0.05$ and an absolute value of $\log_2$(fold change) ≥ 1.

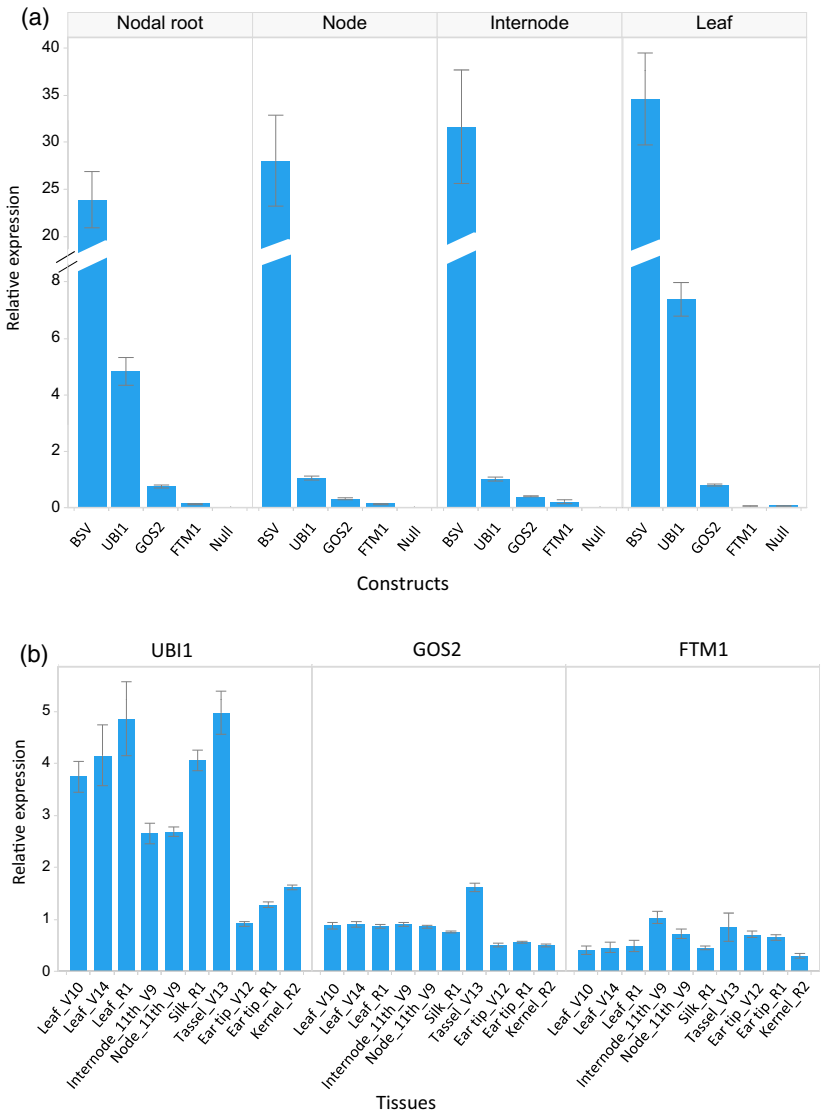

Figure 3. Transcript expression in transgenic *ARGOS8* and control plants
(a) Relative expression levels of maize *ARGOS8* transcripts in greenhouse-grown 24-day-old transgenic and non-transgenic plants (Null). Quantitative RT-PCR was conducted using *ARGOS8*-specific primers. Two events per construct (promoter BSV, UBI1, GOS2 or FTM1) and eight replicates per event (error bars, SE).
(b) Relative expression levels of the *ARGOS8* transgene in field-grown transgenic plants. Quantitative RT-PCR was conducted using primers derived from the OS-UBIQUITIN terminator used in the constructs. Three events per construct and three replicates per event (error bars, SE).

Comparisons of the ACC-treated WT plants with water controls revealed that 963 and 1303 genes were DE 48 and 72 h after the initiation of the treatment, respectively. There were 504 overlapping DE genes between the two sets (Figure 5b, Table S2). Among those, 370 were upregulated and 134 downregulated by ACC and the direction of change of expression of these genes was consistent between the two time points. The function of the ACC upregulated genes was explored using the Gene Ontology (GO) classification system. The significantly enriched GO categories include metabolic process (e.g. organic acid, amine, L-phenylalanine, hemicellulose and cell wall macromolecule metabolism; methyltransferase, acyl transferase and hydrolase activity), nucleosome assembly, protein dimerization, transition metal ion binding and cell wall biogenesis (Figure 5c, Table S2). These categories are related to cell growth. Increased expression of these genes is consistent with the faster outgrowth of the young root primordia and emergence of nodal roots in the ACC-treated plants. Consistent with the well-known role of ethylene in stress responses and defense to infection, the GO terms response to biotic stimulus, oxidative stress, osmotic stress, water deprivation, organic substance and hormone stimulus were significantly enriched in this set of ACC-induced genes. In addition, the GO terms oxidoreductase activity (GO 16491) and peroxidase activity (GO 4601) were overrepresented in the ACC-treated plants (Figure 5c, Table S2). The ACC-upregulated genes include 17 putative peroxidases. It has been reported that water deficit suppresses the expression of several peroxidase genes in the crown region and significantly reduces the number of nodal roots in the model C4

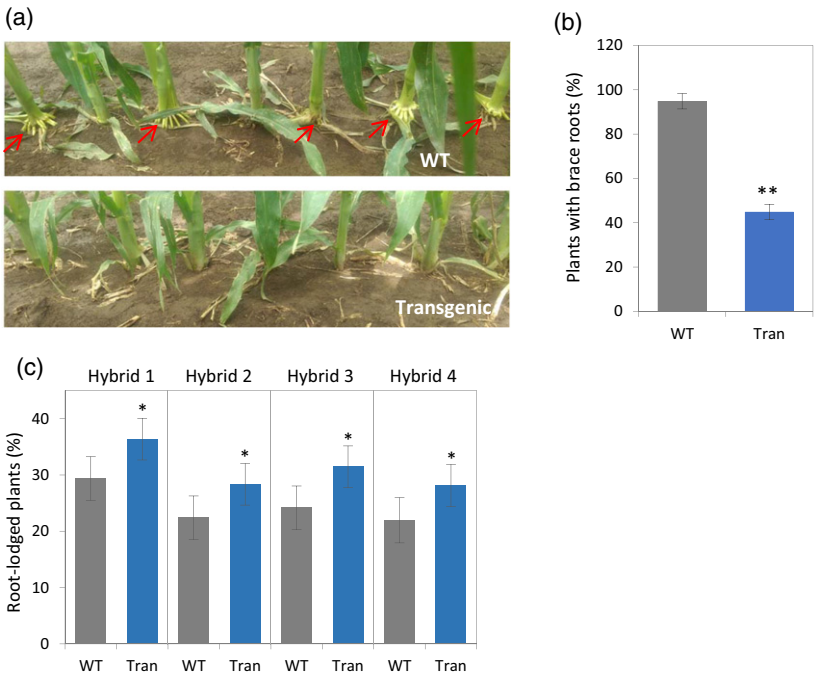

Figure 4. Nodal roots and root lodging of the *GOS2::ARGOS8* transgenic plants grown in the field.
(a) Images of the transgenic and wild type (WT) hybrid PH184C/PH1V69 plants 10 days before silking grown in the field in 2017 showing delayed nodal root development in the transgenic plants. Red arrows indicate brace roots on the lowest aboveground node in WT plants.
(b) Percentage of plants with brace roots in the *GOS2::ARGOS8* transgenic (Tran) and WT PH184C/PH1V69 plants. Two-row plots with 57–58 plants per plot ($n = 2$; error bars, SE; Student's t test, $^{**}P < 0.01$).
(c) Percentage of root-lodged plants in the *GOS2::ARGOS8* transgenic (Tran) hybrids and WT controls. Four hybrids were produced by crossing the transgenic PH184C inbred with four inbred testers. Transgenic plants and WT controls were planted in seven locations across the USA in 2016 (hybrid 1, PH184C/PH1V69). Transgenic plants were compared with WT. Data analyses were implemented using ASReml with output of the model presented as best linear unbiased predictions (error bars, SE; $^{*}P < 0.05$).

grass *Setaria viridis* (Sebastian *et al.*, 2016). In rice, NADPH oxidase was the major enzyme responsible for production of reactive oxygen species (ROS) which mediate the ethylene-induced growth of the young root primordia (Steffens *et al.*, 2012). For the 134 ACC-suppressed genes, only two GO terms, transcription factor activity (GO 3700) and *O*-acyltransferase activity (GO 8374), were significantly enriched (Table S2).

When the RNA sequencing data from *BSV::ARGOS8* plants were compared with those from the WT, 12 genes were found to be upregulated and 22 downregulated at 48 h (i.e. 23-day-old plants) in the transgenic plants (Figure 5d, Table S2). A larger number of DE genes were detected at 72 h, 548 being upregulated and 110 downregulated. A comparison of these DE genes with those identified in the ACC treated plants revealed 63 genes that were upregulated by ACC and downregulated in the transgenic plants, or vice versa (Figure 5e, Table S2). One of these genes is the AP2 EREB transcription factor 114 (EREBP114; GRMZM2G008234) which is the maize ortholog of the rice *CROWN ROOTLESS5* (Kitomi *et al.*, 2011; Schnable *et al.*, 2012). The others include a receptor protein serine/threonine kinase (GRMZM2G075438), an ethylene signaling component (*RTL3*, GRMZM2G121208), 10 transcriptional factors [AP2/ERF (1), NAC (1), *WUSCHEL*-related homeobox (1), basic helix–loop–helix (2), MYB-related (3) and C2H2-type zinc finger (2)], 20 enzymes (e.g. trehalose-6-phosphate synthase, GRMZM5G890599; phytosulfokines2, GRMZM2G146222; anionic peroxidase1, GRMZM2G108207; heme peroxidase, AC197758.3_FG004; respiratory burst NADPH oxidase, GRMZM2G300965) and three cortical cell-delineating proteins (Table S2).

DISCUSSION

The promotion of stem-borne adventitious root formation by ethylene under stressed conditions is well known in rice and tomato plants (Clark *et al.*, 1999; Lorbiecke and Sauter, 1999; Negi *et al.*, 2010; Vidoz *et al.*, 2010). In maize, nodal roots originate at the stem nodes during the normal developmental process and form the framework of the root system. In this study, *BSV::ARGOS8* transgenic plants with reduced ethylene signaling and ACC treatments were used to determine if ethylene plays a role in nodal root development in maize. We found that nodal root emergence was accelerated in maize plants treated with ACC. The transgenic plants with reduced ethylene sensitivity due to *ARGOS8* overexpression showed delayed emergence of nodal roots. As expected, the promotional effect of ethylene on nodal roots was less pronounced in the ACC-treated transgenic plants than in the WT. It has been reported that the ethylene-producing growth regulator ethephon promoted the development of brace roots in field-grown maize hybrids at growth stage V10 (Langan and Oplinger, 1987). In another study, maize seedlings growing in liquid media through which ethylene was bubbled showed increased adventitious root formation (Jackson *et al.*, 1981). Taken together, these results demonstrate that ethylene is a positive regulator of nodal root development in maize.

Although nodal roots emerged earlier in the ACC-treated maize plants, the sequence of nodal root development

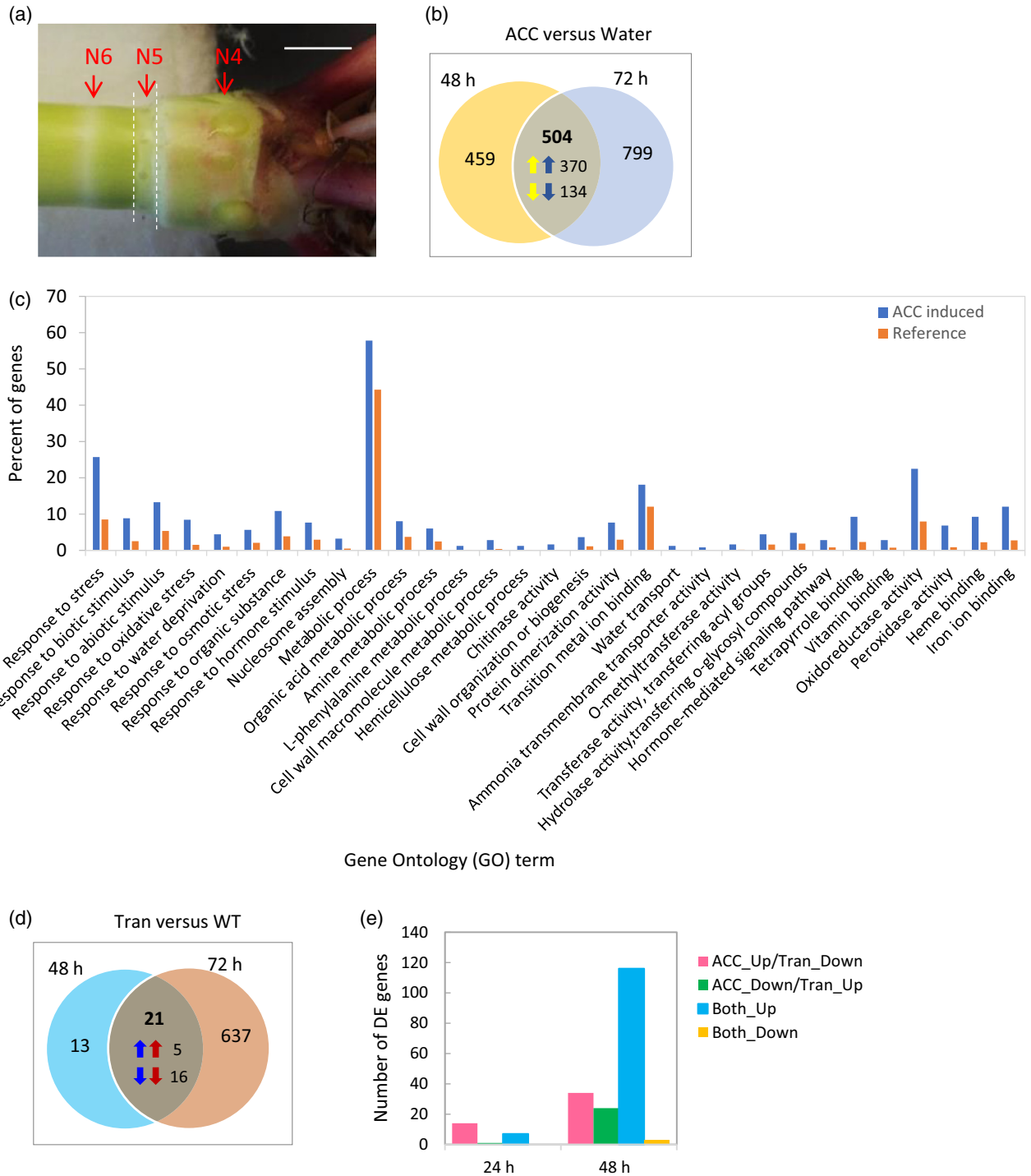

Figure 5. Ethylene-induced transcriptomic changes in nodes of maize plants
(a) Image of basal nodes of a 21-day-old seedling showing nodes 4, 5 and 6 (N4, N5 and N6). Dashed lines indicate the sampling regions. Bar = 5 mm.
(b) Venn diagram showing differentially expressed (DE) genes in nodal tissues treated with 0 or 100 μM ACC at 48 and 72 h. Arrows indicate up- or down-regulation by 1-aminocyclopropane-1-carboxylic acid (ACC).
(c) Overrepresentation of Gene Ontology terms (false discovery-corrected $P < 0.05$) among the ACC upregulated DE genes.
(d) Venn diagram showing DE genes between the *BSV::ARGOS8* transgenic plants and WT plants.
(e) Number of DE genes in four groups classified based on response to ACC treatments (ACC) in WT and response to overexpression of the *BSV::ARGOS8* transgene (Tran) in untreated plants (Up, upregulated; Down, downregulated).

along the stem remained unchanged. As in the untreated plants, the roots on a node in the ACC-treated plants did not penetrate the stem epidermis until the roots on the lower node had emerged, indicating that there are other regulators which control the growth of root primordia and certain conditions must be met in the node before ethylene can exert its promotional effect. In deepwater rice, the epidermal cells overlying the tips of the root primordia are a physical barrier restricting the emergence of the stem-borne roots, and programmed cell death in the epidermal cells is required for the root primordia to emerge from the stem surface (Lorbiecke and Sauter, 1999; Steffens *et al.*, 2012). However, the role of the epidermal cells in restricting root primordia in maize is not clear. For the nodes examined in this study, root primordia had formed within the nodal tissues prior to ACC treatments (Figure 1a). The accelerated emergence of nodal roots was a result of enhanced growth of the young root primordia, which was reflected in the transcriptomic changes in the nodal tissues. A GO analysis of the DE genes revealed that the ACC-upregulated genes are associated with GO categories nucleosome assembly, protein dimerization, metabolic process, cell wall biogenesis and transporter activities. Increased expression of the genes in these GO categories can support active cell division and expansion, leading to fast tissue growth. It has been shown that accelerated growth of the young root primordia in rice generates a mechanical force which is one of the two signals (the other one being ethylene) that regulate programmed cell death of the epidermal cells overlying the root primordia, facilitating root emergence in rice (Steffens *et al.*, 2012).

Root lodging is one of the major problems in high-yield maize production. Early establishment of nodal roots and adequate nodal root development can provide plants resistance to root lodging (Pellerin *et al.*, 1990; Stamp and Kiel, 1992; Ennos *et al.*, 1993). Extensive breeding efforts have taken place to develop lodging-resistant hybrids, but as complex traits, selection for root-lodging resistance and simultaneous selection for high grain yield is quite challenging. Targeted genetic engineering can be an alternative approach. Two maize mutants with defects in nodal root development have been isolated. The *rtcs* mutant is devoid of nodal roots (Hetz *et al.*, 1996). The *RTCS* gene encodes an auxin-inducible LOB domain protein acting downstream of the auxin response factor ARF34 (Taramino *et al.*, 2007; Majer *et al.*, 2012; Xu *et al.*, 2015). The *rootless1* mutant produces fewer crown roots at the first two nodes, and lacks brace roots (Jenkins, 1930). These genes can be overexpressed in transgenic plants to test if they can enhance nodal root formation. In this study, we found that modulation of ethylene signaling also can alter nodal root development, uncovering new targets (i.e. ethylene pathway genes) for regulating root-lodging resistance. Because reduced ethylene signaling delays nodal root emergence and increases root lodging in the *BSV::ARGOS8* transgenic plants and ACC treatments promote nodal root formation, increasing ethylene signaling by altering the expression of ethylene pathway genes in a tissue-specific fashion could enhance resistance to root lodging. In addition, transcript profiling of the nodal tissues revealed other candidate genes for improving root-lodging resistance, such as *EREBP114*, the maize ortholog of the rice *CROWN ROOTLESS5* (Kitomi *et al.*, 2011), and *WUSCHEL-RELATED HOMEOBOX 4*, which belongs to the same gene family as the rice *WOX11* (Zhao *et al.*, 2009), respiratory burst NADPH oxidase and ROS pathway genes.

Ethylene promotes nodal root emergence, which could lead to improved resistance of the plant to root lodging. However, ethylene is involved in many aspects of plant growth and development as well as the response of plants to both biotic and abiotic stresses. Maize can show enhanced grain yield when the sensitivity of the plant to ethylene is reduced or expression of an ethylene biosynthesis gene is silenced (Habben *et al.*, 2014; Shi *et al.*, 2015, 2017). When constitutively overexpressed, *ARGOS8* increased kernel number per ear (Shi *et al.*, 2015), but delayed nodal root emergence and reduced plant resistance to root lodging. Using the developmentally regulated FTM1 promoter for *ARGOS8* overexpression in adult plants, we obtained transgenic plants which have a greater yield and have minimal root-lodging problems. The *FTM1::ARGOS8* transgene is expressed at a low level in the basal nodes of the stem and nodal roots in young plants; consequently the effect on ethylene signaling in these tissues was negligible. This study shows that selective modification of ethylene signaling at different developmental stages can decouple improved grain yield from root lodging.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The seeds of maize hybrid PHR03/PH12SG and transgenic plants were sown in Ellepot® plugs (Ellepot A/S, https://www.ellepot.com/) in 4- by 8-cell flats and 2-week-old seedlings were transferred to 2-L pots and grown in the greenhouse. The soil mixture consisted of peat (48.7%), Turface (44.6%; https://www.turface.com/), perlite (2.3%), vermiculite (4.4%), lime [3 pounds per cubic yard (lbs yd^{-3})], Peters' Professional 11-5-11 UniMix Starter (1 lb yd^{-3}; ICL Specialty Fertilizers, https://icl-sf.com/), Suffusion wetting agent (0.5 lbs yd^{-3}; OHP Inc., http://www.ohp.com/) and Osmocote Plus 15-9-12 controlled-release fertilizer (3 lbs yd^{-3}; ICL Specialty Fertilizers). Nutrient was supplemented once a week with water-soluble fertilizer dissolved in tap water (95 p.p.m.; Peters Excel 15-5-15 Cal-Mag Special; ICL Specialty Fertilizers). To determine the effects of ethylene on nodal root formation, plants at various vegetative growth stages were treated with the ethylene precursor ACC (Accela ChemBio Inc., http://www.accelachem.com/) by watering them daily with an aqueous solution of ACC at three concentrations (0, 50 or 100 μM). Plant vegetative growth stages were determined using the leaf collar method. Stem nodes were numbered according to their order of appearance, the coleoptile node being designated node 1 (Hoppe *et al.*, 1985).

Transgene constructs and plant transformation

To generate constructs overexpressing maize *ARGOS8* in maize plants, the coding sequence was PCR-amplified from the inbred B73, verified by DNA sequencing and integrated between a promoter and the rice UBIQUITIN terminator in a Gateway-modified derivative of pSB11 (Ishida *et al.*, 1996) using the Invitrogen Gateway system (Thermo Fisher Scientific, https://www.thermofisher.com/). The promoters used in this study include the BSV promoter and the maize UBIQUITIN1 (UBI1), GOS2 and FLORAL TRANSITION MADS1 (FTM1) promoter. The transfer DNA (T-DNA) region contains the bialaphos resistance gene (phosphinothricin-*N*-acetyl-transferase) as a selectable marker for transgenic plant selection. A visible marker (red fluorescent protein) was added in some of the constructs to facilitate seed sorting. These plasmids were then co-integrated into the super binary pSB1 vector in *Agrobacterium* tumefaciens strain LBA4404 (Komari *et al.*, 1996) by electroporation. Maize transformants were produced in the proprietary inbred lines PHR03 or PH184C by using *Agrobacterium*-mediated transformation as described (Cho *et al.*, 2014). Single-copy T-DNA integration events that expressed the transgene were selected and advanced for crosses to WT plants and further characterization.

Transgene expression analysis

To determine mRNA expression of the transgene in maize with reverse transcription PCR, cDNA was synthesized with a mixture of oligo(dT) and random hexamer primers using Applied Biosystems HCAP Reverse Transcription Kit (Life Technologies, cat. no. 4368813, https://www.thermofisher.com/). Quantitative RT-PCR experiments were performed using TaqMan Universal Master Mix (Life Technologies) and a TaqMan probe. Relative quantification values were determined using the difference in Ct from the target gene and internal controls. Maize *eIF4-γ* was used as an internal control.

Ethylene emission analysis

Ethylene measurements were conducted on leaf disks taken from plants grown in the greenhouse. Thirty leaf disks (1 cm in diameter) were allowed to release wound-generated ethylene for 2 h, placed in amber glass vials (volume 9.77 ml) containing a disk of filter paper wetted with 50 μl of distilled water and then sealed with aluminum crimp seals. After an incubation period of 24 h, 1-ml samples were taken from the headspace of each sealed vial. The ethylene content was quantified by gas chromatography as previously described (Habben *et al.*, 2014). The ethylene production rate was expressed as pmol per hour per gram of dry weight.

Gene expression analysis by RNA sequencing

Nodal tissues were sampled from WT plants treated with ACC for 48 and 72 h and water controls as well as *BSV::ARGOS8* transgenic plants without ACC treatments. Four biological replicates consisting of four individual plants each were collected for each genotype, treatment and time point. Total RNAs were isolated from the nodal tissues with the Qiagen RNeasy kit for total RNA isolation (Qiagen, http://www.qiagen.com/). Sequencing libraries from the resulting total RNAs were prepared using the TruSeq mRNA-Seq kit according to the manufacturer's instructions (Illumina, https://www.illumina.com/) and sequenced on the Illumina HiSeq 2500 system with Illumina TruSeq SBS version 3 reagents. Sequences were trimmed based on quality scores, mapped to the maize B73 reference genome sequence v.4 and analyzed via the RSEM pipeline (Li *et al.*, 2010; Li and Dewey, 2011). DESeq2 was used to identify DE genes (Love *et al.*, 2014). The DE genes are defined as those with false discovery-corrected $P < 0.05$ and an absolute value of $\log_2$(fold change) ≥ 1. The DE genes were analyzed for overrepresentation of GO terms using BiNGO (Maere *et al.*, 2005). Those GO terms with a false discovery-corrected $P < 0.05$ were deemed overrepresented.

Maize hybrid field study

To evaluate root-lodging resistance in hybrids, field trials were conducted in multiple locations across the USA in small plots (approximately 8.4 m^2) with two to four replications at each location. Hybrid seed for these trials was generated by crossing heterozygous transgenic inbred lines with inbred testers. Hybrid seed segregated 1:1 for the transgene, and visible selection markers linked to the transgene were used to identify and separate the transgene-positive F_1 seed from the transgene-negative F_1 seed. The non-transgenic segregants (null) served as controls. For those constructs without visible markers, homozygous transgenic inbred plants and WT were crossed with inbred testers in the same nursery to produce experimental and control hybrid seeds. The field study in 2013 was carried out at research centers in Woodland CA, York NE, Garden City KS, Plainview TX, Miami MO, Marion IA, Johnston IA and Princeton IN. Fertilizer at each location was applied to achieve maximum yields. Weeds and pests were controlled according to local practices. Root-lodged plants (i.e. plants tilting at more than 30°; Landi *et al.*, 2007) were recorded at the locations where natural lodging due to severe weather conditions occurred before flowering. Grain mass and grain moisture data for all locations were collected using a small plot combine. Grain yield was adjusted to a constant 15% moisture. The root-lodging study of the *GOS2::ARGOS8* transgenic hybrids was performed in 2016 at research centers in Ivesdale IL, San Jose IL, Buda IL, Garden City KS and Marion IA. All locations experienced natural root-lodging events during the growth season.

The field experimental design was set up as split plot configuration where hybrid background was the whole plot and events were the subplot. Data analysis was performed using ASReml (VSN International Ltd, https://www.vsni.co.uk/), and the values reported are best linear unbiased predictions (BLUPs; Gilmour *et al.*, 2009). A mixed model framework was used to perform the multi-location analysis. In the analysis, the main effect of constructs and events was considered as a fixed effect whereas hybrids and interactions between event and hybrid were treated as random. The main effect of locations and location interactions with event and hybrid were considered as random effects. The blocking factors such as replicates and spatial variation in the field within each location were considered as random. For grain yield, three components of spatial effects including row, column and autoregressive correlation as AR1 × AR1 were included to reduce noise caused by spatial variation in the field. However, the spatial adjustments were not applied to analysis of lodging data to avoid overfitting. The significance test between event and nulls (or WT) within and across hybrids was performed using a *P*-value of 0.05 in a two-tailed test.

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CONFLICT OF INTEREST

The authors are employees of Corteva Agriscience, Agriculture Division of DowDuPont.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Transcript abundance of the *ARGOS8* transgene in 1-aminocyclopropane-1-carboxylic acid-treated *BSV::ARGOS8* transgenic plants and non-transgenic segregants.

Figure S2. Number of primordia on node 5 in *BSV::ARGOS8* transgenic and non-transgenic plants.

Figure S3. Percentage of plants with nodal roots on node 6 at the vegetative stage V14.

Figure S4. Effects of 1-aminocyclopropane-1-carboxylic acid on nodal root emergence in maize PH184C/PH1V69 hybrid plants.

Figure S5. Grain yield of maize *GOS2::ARGOS8* transgenic events.

Table S1. Grain yield of maize transgenic events from three *ARGOS8* constructs.

Table S2. RNA sequencing analysis of nodal tissues.

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