**Auxin Efflux Carrier ZmPGP1 Mediates Root Growth Inhibition under Aluminum Stress**

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Auxin has been shown to enhance root growth inhibition under aluminum (Al) stress in Arabidopsis (Arabidopsis thaliana). However, in maize (Zea mays), auxin may play a negative role in the Al-induced inhibition of root growth. In this study, we identified mutants deficient in the maize auxin efflux carrier P-glycoprotein (ZmPGP1) after ethyl methane sulfonate mutagenesis and used them to elucidate the contribution of ZmPGP1 to Al-induced root growth inhibition. Root growth in the zmpgp1 mutant, which forms shortened roots and is hyposensitive to auxin, was less inhibited by Al stress than that in the inbred line B73. In the zmpgp1 mutants, the root tips displayed higher auxin accumulation and enhanced auxin signaling under Al stress, which was also consistent with the increased expression of auxin-responsive genes. Based on the behavior of the auxin-responsive marker transgene, DRSrec::RFP, we concluded that Al stress reduced the level of auxin in the root tip, which contrasts with the tendency of Al stress-induced Arabidopsis plants to accumulate more auxin in their root tips. In addition, Al stress induced the expression of ZmPGP1. Therefore, in maize, Al stress is associated with reduced auxin accumulation in root tips, a process that is regulated by ZmPGP1 and thus causes inhibition of root growth. This study provides further evidence about the role of auxin and auxin polar transport in Al-induced root growth regulation in maize.

Aluminum (Al) stress is a worldwide agricultural problem that limits crop growth and affects crop yields in acid soil (Kochian et al., 2004). Al, in the form of rather insoluble aluminosilicates or oxides, is a highly abundant soil element that becomes increasingly soluble when the soil pH falls below 5.5. The solubilized Al, mainly the Al³⁺ ion, inhibits root growth in most species even when present at modest levels (Ryan and Kochian, 1993; Von Uexküll and Mutert, 1995; Eswaran et al., 1997; Kochian et al., 2004; Ma, 2007). The consequences of poor root growth are a reduced efficiency of water and nutrient uptake and, thus, inhibition of overall plant growth (Kochian et al., 2004). Studies from wheat (Triticum aestivum), maize (Zea mays), sorghum (Sorghum bicolor), common bean (Phaseolus vulgaris), and the model plant Arabidopsis (Arabidopsis thaliana) all showed that the root apex transition zone (TZ), located between the apical meristem and the basal elongation region, is the main perception site of Al stress (Sivaguru and Horst, 1998; Rangel et al., 2007; Yang et al., 2014).

The root apex has been recognized as a unique site for perception and response to endogenous plant hormone signaling and exogenous environmental cues (Baluska et al., 2010). The root apex TZ is the most active zone for the action of distinct phytohormones such as auxin, cytokinin, ethylene, gibberellins, brassinosteroids, etc. (Baluska et al., 2010). Auxin acts antagonistically with cytokinin to control root meristem size through regulation of the cytokinin-responsive type B response regulators ARRI and ARR12 in the root apex TZ (Dello Ioio et al., 2007, 2008; Moubayidin et al., 2010). Cells of the root apex TZ also play a critical role in response to increased ethylene levels and thus mediate changes in root growth through the regulation of auxin biosynthesis and polar auxin transport (Růžička et al., 2007; Swarup et al., 2007).

The role of auxin in Al-regulated root growth inhibition is well known (Kollmeier et al., 2000; Doncheva et al., 2005; Teale et al., 2005; Zhou et al., 2010; Yang et al., 2013). In maize, external application of indole-3-acetic acid (IAA), an active form of auxin, to the elongation zone (EZ), but not to the meristematic zone, significantly alleviated Al-induced root growth inhibition.
In Arabidopsis, auxin treatment alters the expression of *Alsensitive1* (*ALS1*), thereby influencing the pattern of Al distribution in the cell (Zhu et al., 2013). Auxin also mediates Al-induced ethylene signaling to control root growth (Sun et al., 2007). In the soybean (*Glycine max*) root, auxin enhances the Al-induced exudation of citrate through the up-regulation of multidrug and toxic compound extrusion (MATE) and an increase in the phosphorylation of plasma membrane H^+~/ATPase (Wang et al., 2016a). In alfalfa (*Medicago sativa*), Al-induced root growth inhibition correlates with the inhibition of auxin synthesis in its apical buds and transport in the roots (Wang et al., 2016b).

Local auxin biosynthesis mediates tissue or cellular auxin responses to control many plant growth and developmental responses (Stepanova et al., 2008; Tao et al., 2008). In Arabidopsis, Al stress induced a local auxin biosynthesis and an enhanced auxin signaling response in the root TZ, which eventually caused root growth inhibition (Yang et al., 2014; Liu et al., 2016). In response to Al stress, both the YUCCA (YUC) family of flavin-containing mono-oxygenases and the TRP AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of aminotransferases, two key enzymes in Trp-dependent auxin biosynthesis (Cheng et al., 2006; Stepanova et al., 2008; Tao et al., 2008; Zhao, 2010), were up-regulated to mediate Al-induced local auxin biosynthesis in the root TZ (Yang et al., 2014; Liu et al., 2016). These studies showed the critical role of local auxin biosynthesis in mediating Al-induced root growth inhibition.

Besides local auxin biosynthesis, the auxin gradient, which largely determines auxin activity, is also influenced by polar auxin transport (Friml and Palme, 2002; Swarup and Bennett, 2003; Tian et al., 2014). Polar auxin transport is governed by auxin influx carrier proteins, such as AUX1 (Marchant et al., 1999; Kramer, 2004; Yang et al., 2006), auxin efflux carrier proteins, such as PINs (Vieten et al., 2005; Petrásek et al., 2006), and by homologs of the human multiple drug resistance transporters ABC transporter (ABCB)/P-glycoprotein 1 (PGP1) and PGP19 (ABCB19) (Geisler and Murphy, 2006; Blakeslee et al., 2007; Henrichs et al., 2012).

PGP1 (ABCB1, Brachytic2-BR2, or multidrug resistance 1 [MDR1]) encodes a membrane-bound protein (Higgins, 1992) that belongs to the ATP-binding cassette (ABC) transporters family in Arabidopsis (Noh et al., 2001) and maize (Multani et al., 2003). In maize, BR2 has two transmembrane domains and two nucleotide binding domains (NBDs) and each half has one transmembrane domain and one NBD (Dhaliwal et al., 2014). The *br2* mutant plants express semidwarf stalks, and a similar phenotype was reported in sorghum (Multani et al., 2003) and Arabidopsis (Higgins, 1992). In the stem of *br2* mutants, auxin transport is decreased compared with the wild-type B73. Knöller et al. (2010) further found that BR2 functions in IAA export from the intercalary meristem through the analysis of IAA transport in *br2* mutants (Knöller et al., 2010). In *br2* mutant roots, IAA transport to the shoots is reduced at the root apex and gravitropic growth responses are also reduced (McLamore et al., 2010).

Although polar auxin transport is known to regulate root growth under Al stress in Arabidopsis (Sun et al., 2010; Wu et al., 2014), the underlying molecular mechanism(s) is not fully understood. Screening was conducted to characterize root-defective mutants in maize since the molecular regulation mechanism of root development in maize is much less understood compared to that in Arabidopsis. The three mutants used in this study display shorter roots and the absence of aboveground brace roots, but they also have strong, semidwarf stalks. After mapping, three allele variations were found at *ZmPGP1*, which encodes a well-known auxin transporter. Considering that auxin plays an important role in root growth regulation under Al stress in maize by an unknown mechanism, we analyzed the *zmpgp1* mutants and the role of *ZmPGP1* in Al stress-induced auxin accumulation in the root apex and root growth inhibition.

### RESULTS

#### The Characterization of *zmpgp1* Mutants

Three mutants, which have semidwarf stalks and the absence of aboveground brace roots, were identified from the ethyl methanesulfonate (EMS)-induced mutant population of the maize inbred line B73 and were named Mut001, Mut002, and Mut003 (Fig. 1, A and B). The F1 plants obtained by crossing each mutant to wild-type B73 all displayed the wild-type phenotype. The F2 generation of the Mut001 × B73 cross-segregated as 45 mutant and 152 wild-type phenotypes, and the ratios for the Mut002 × B73 and Mut003 × B73 crosses were 41 and 139, and 40 and 140, respectively (Fig. 1C). As these segregation ratios all approximated 1:3, we assumed that each mutant phenotype was under monogenic control, with the mutant allele acting as a recessive. To check for allelism between the three mutants, Mut001 was crossed with both Mut002 and Mut003; in both cases, the phenotypes of the F1 plants were identical with that of Mut001 (Fig. 1, A and B), suggesting that the defects of these three mutants were caused by a mutation of the same gene.

Defining the mutation site in each mutant was achieved using the exome capture-based MutMap (EcMutMap) approach (Lu et al., 2018), an improved method of MutMap that is a way of rapid gene isolation used in rice (*Oryza sativa*) through crossing mutant to wild-type parent lines (Abe et al., 2012; Takagi et al., 2013). A set of 22,910 single nucleotide polymorphisms (SNPs) was obtained from a sample of 40 seedlings displaying mutant phenotypes from the Mut001 × B73 F2 population (Supplemental Table S1). Of these, four (SNP-192401755, -200076997, -200400381, and -201194537), located on chromosome 1, were linked to the mutation; the recombination frequencies were...
3.5, 1.7, 0.87, and 0.87%, respectively (Fig. 2). The region defined by these variants harbors \textit{ZmPGP1} (GRMZM2G315375, syn. \textit{ZmBR2} and \textit{ZmABCB1}). Mutations at this gene are known to cause semidwarf stalk phenotypes (Multani et al., 2003; Knöller et al., 2010), just as was observed in Mut001, 002, and 003 (Fig. 1).

Resequencing \textit{ZmPGP1} in the three mutants revealed that the Mut001 sequence contains a G-to-A transition at position 5332, which converted a Trp to a stop codon (Supplemental Fig. S1A). The Mut002 sequence harbored a G-to-A mutation at position 747, which locates at the border between the first intron and the second exon (Supplemental Fig. S1B, a). The RT-PCR analysis indicated that this point mutation might affect mRNA splicing since an additional transcript (444 bp) was detected in the \textit{zmpgp1-2} mutant (Supplemental Fig. S1B, b). The subsequent sequencing of the longer transcript (444 bp), which is only present in the \textit{zmpgp1-2} mutant, shows that it retains the first intron (Supplemental Fig. S1B, c), further confirming that the point mutation affects \textit{ZmPGP1} mRNA splicing. A 78-nucleotide segment had been inserted between positions 4465 and 4466 of the Mut003 sequence (Supplemental Fig. S1C), and the insertion site is just the D-loop of the NBD1, one of the NBDs of BR2, which might interfere with nucleotide binding (Higgins, 1992). Given that all three mutants had experienced an alteration in their \textit{PGP1} sequence and that the mutation had generated a semidwarf growth habit, we assumed that these mutations were responsible for the altered phenotype. The mutants were therefore redesignated as \textit{zmpgp1-1}, \textit{zmpgp1-2}, and \textit{zmpgp1-3}.

The \textit{zmpgp1} Mutant Has a Short-Root Phenotype and Displays Hyposensitivity to Auxin

\textit{ZmPGP1} mediates the export of IAA from the intercalary meristem (Multani et al., 2003; Knöller et al., 2010). The \textit{zmpgp1} mutant is associated with a semidwarf stature and lack of aboveground brace roots, and its
seedlings develop shortened primary roots (Fig. 3, A–D). When supplied with 40 nm 1-naphthaleneacetic acid (NAA), the elongation of the primary roots of wild-type B73 seedlings was more strongly inhibited (51% reduction) than that of either the zmpgp1-1 (42%) or zmpgp1-2 (39%) mutant seedlings (Fig. 3E). In addition, gravitropic analyses showed that roots of the three zmpgp1 mutant seedlings showed less gravitropic responses (Supplemental Fig. S2, A and B), which is also consistent with previous observations (McLamore et al., 2010).

Al-Induced Root Growth Inhibition Is Alleviated in the zmpgp1 Mutant

The basipetal auxin transport controls Al signaling between the TZ and the EZ in the root apex of maize (Kollmeier et al., 2000). However, no genetic evidence has been provided for this. Since ZmPGP1 acts as an auxin efflux carrier and regulates auxin transport in the roots of maize (Knöller et al., 2010; McLamore et al., 2010), the Al-induced inhibition of root growth in the zmpgp1 mutants was examined in more detail. When exposed to 0, 5, and 10 µm AlCl₃ for 48 h, the roots of all three zmpgp1 mutant seedlings expressed a relatively higher elongation rate than that of wild-type B73 seedlings (Fig. 4), indicating a resistant phenotype under Al stress. However, when treated with 25 µm AlCl₃ (Fig. 4A), the epidermis of all roots was damaged; therefore, 10 µm AlCl₃ was chosen for treatment with Al. The 24-h treatment with 10 µm AlCl₃ expressed the same tendency as the 48-h treatment (Fig. 4B). Consistent with this

Figure 2. Positional cloning of the pgp1 mutation. A, Crossing-overs occurring in (a) neither, (b) one, and (c) both homologs. B, The positions and frequency of crossing-over (%) of the four informative nucleotide variants show that the candidate gene region includes PGP1. C, Structure of GRMZM2G315375 (ZmPGP1) and a snapshot of the mutation positions.
observation, the roots of both \textit{zmppg1-1} and \textit{zmppg1-2} mutants absorbed less hematoxylin stain (Fig. 5), an indication that they experienced a lower degree of Al toxicity. The root growth inhibition-resistant phenotype in the \textit{zmppg1} mutants is also supported by the reduced Al levels in two of the \textit{zmppg1} mutants (Fig. 5B), which further confirms the results of hematoxylin staining (Fig. 5A).

Root citrate exudation plays a critical role in improving Al tolerance (Kochian et al., 2004). \textit{ZmMATE1} encodes a citrate efflux carrier, which is up-regulated by Al stress, and the expression of \textit{ZmMATE1}, mostly concentrated in root tissues, significantly increases Al tolerance (Maron et al., 2010). To further explore if the decrease of Al content was caused by \textit{ZmMATE1}-induced citrate exudation, we detected the expression of \textit{ZmMATE1} in the roots under Al stress. Consistent with the decreased Al content, the expression level of \textit{ZmMATE1} was significantly up-regulated in the \textit{zmppg1-1} and -2 mutants (Fig. 5C). \textit{ZmMATE1} was also up-regulated by Al stress in B73 (Fig. 5C).

\textbf{Figure 3.} Maize plants harboring the pgp1 mutation form shortened roots and are hyposensitive to NAA. A and C, Fourteen-day-old (A) and 4-d-old (C) B73 and \textit{zmppg1} seedlings grown hydroponically in 0.5× Hoagland solution. The primary root is marked by red arrowheads in A. Bar = 10 cm in A and 1 cm in C. B and D, Length of the 14-d-old (A) and 4-d-old (C) seedling primary root of B73 and the three \textit{zmppg1} mutants. E, Four-day-old B73 and \textit{zmppg1} seedlings (the PRs were about 6–8 cm) were treated for 2 d with 40 nM NAA.*. Significant difference according to a Student’s \textit{t} test at \( P < 0.05 \). Error bars indicate the se \((n = 20)\).
Figure 4. Primary root growth in the zmppp1 mutant is more tolerant of Al stress than the wild type. A, Root elongation after treatment with 0, 5, 10, and 25 µM Al\(^{3+}\). * and **, All three mutants differ significantly from B73 according to a Student’s t test at \(P < 0.05\) and 0.01, respectively. Error bars indicate the se (\(n = 20\)). B, Relative elongation rate of primary root after exposure to 10 µM Al\(^{3+}\) for 24 and 48 h. * and **, Significant difference according to a Student’s t test at \(P < 0.05\) and 0.01, respectively. Error bars indicate the se (\(n = 20\)). C, The root tips of 4-d-old seedlings imaged before (upper) and after (lower) 48 h exposure to 10 µM Al\(^{3+}\). For the convenience of measurement, the points, 2 cm to the root tip, are marked by black. White arrows indicate the marked positions. Bar = 1 cm.
Auxin Alleviates Al-Induced Inhibition of Root Growth

Al stress-induced root growth inhibition was postulated to result from the low auxin accumulation in maize root tips (Kollmeier et al., 2000). In this study, we conducted a dosage-dependent assay to test the effects of auxin on Al-induced inhibition of root growth in maize. The results showed that auxin cotreatments (10, 20, and 50 nm NAA) alleviated Al-induced root growth inhibition, displaying a higher relative elongation rate than that of the untreated control with auxin (Fig. 6A). Consistently, Al and auxin cotreated maize roots showed weaker hematoxylin staining than those of the untreated control, indicating less Al toxicity under the
same Al stress condition (Fig. 6B). To further study the mechanism of auxin-alleviated Al toxicity, we assessed the transcription of ZmMATE1, which encodes a citrate efflux carrier and is induced by Al stress in maize (Maron et al., 2010), using reverse transcription quantitative PCR (RT-qPCR) in the root tips treated with 0 (−NAA) or 100 (+NAA) nM NAA combined with Al stress. The results showed that auxin up-regulated the expression of ZmMATE1 in the presence or absence of Al stress (Fig. 6D). Furthermore, we also observed that, though the zmpp1 mutants showed more resistance to Al stress in the absence of auxin, the cotreatment with 20 nM NAA eliminated the difference between B73 and the zmpp1 mutants, both displaying a similar relative root elongation rate (Fig. 6C).

Al-Stressed Roots of the zmpp1 Mutant Accumulated a Higher Level of Auxin Than Wild-Type Roots

Al stress depletes auxin from the root TZ and EZ, and thus causes inhibition of root growth in maize (Kollmeier et al., 2000). In this study, using a maize DR5rev:RFP marker (auxin response reporter) line, we examined the local auxin accumulation under Al stress using a fluorescence microscope. Under the treatment with 10 μM AlCl_3 for either 2 or 12 h, we observed a
strong reduction of DR5rev:RFP signal in maize root tips (Fig. 7A), indicating a decrease of endogenous auxin, which was confirmed by a measurement of the free IAA content of the root tip (Fig. 7B). When exposed to 50 µM AlCl₃ for 6 h, the root tips (1–5 mm) of Al-stressed seedlings displayed significantly reduced free IAA levels compared to the untreated control (Fig. 7B). However, in the zmppg1 mutant, which has increased free IAA levels and DR5rev:RFP signal in root tips, Al-stressed seedling root tips had higher free IAA levels and DR5rev:RFP signal than that of the wild-type B73 control (Fig. 7, A and B).

Auxin Signaling Is Enhanced in the zmppg1 Mutant Root Tips

To further confirm the increased free IAA levels and auxin signaling shown by DR5rev:RFP in the zmppg1 mutant root tips (Fig. 7), the transcription of a set of auxin-responsive genes (ZmIAA2, ZmIAA10, ZmIAA21, and ZmGH3) was assessed using RT-qPCR in both wild-type and zmppg1 mutant roots. Exposure of 50 µM AlCl₃ for 6 h substantially down-regulated the expression of ZmIAA2, ZmIAA10, ZmIAA10, and ZmGH3 in all maize roots (Fig. 8), which is consistent with the reduced free IAA levels in Al-treated maize roots (Fig. 7). However, in the zmppg1 mutant, with or without Al stress treatment, the expression of ZmIAA2, ZmIAA10, ZmIAA10, and ZmGH3 in roots was significantly higher than that in B73 (Fig. 8).

ZmPGP1 Transcription Is Up-Regulated by Al Stress

In Arabidopsis, TAA1 and YUCs, the classical auxin synthesis genes, are induced by Al stress, which causes auxin accumulation in the root TZ and eventually leads to the inhibition of root growth (Yang et al., 2014; Liu et al., 2016). To address if ZmPGP1 responds to Al stress, we examined the expression of ZmPGP1 in root tips under Al stress using RT-qPCR with two independent pairs of primers. The results showed that, in response to exposure to 50 µM AlCl₃ for 6 h, the expression of ZmPGP1 was significantly up-regulated (Fig. 9), indicating that Al stress could affect auxin transport through the regulation of ZmPGP1 expression.

DISCUSSION

As an auxin efflux carrier, PGP1/ABCB1/MDR1 has been identified in Arabidopsis (Titapiwatanakun and Murphy, 2009), maize, and sorghum (Multani et al., 2003; Knöller et al., 2010). Though disruption of PGP1 causes relatively subtle plant growth defects, which might result from the redundant roles with other PGPs in Arabidopsis (Noh et al., 2001; Geisler et al., 2005; Yang and Murphy, 2009), the pgp1 mutants in both maize and sorghum have a dwarf phenotype with compact lower stalk internodes (Multani et al., 2003). Similar to the Arabidopsis PGP1 ortholog, which controls auxin efflux out of meristematic regions in the shoot and root, ZmPGP1 was also found to function in IAA export from intercalary meristems (Knöller et al., 2010). Here, three EMS-induced zmppg1 mutants were used to investigate the contribution of PGP1 on root growth in the presence of Al stress. According to our study, ZmPGP1 controls local auxin accumulation in...
root tips and regulates root growth under Al stress in maize. This result provides further evidence about the role of auxin polar transport in Al-induced root growth regulation in maize.

### Auxin Decreases Al Accumulation through Up-Regulation of ZmMATE1 Expression in Maize

The MATE1 family, involved in Al-activated citrate efflux, was first described in sorghum (SbMATE; Magalhaes et al., 2007) and barley (Hordeum vulgare; HvAACT1; Furukawa et al., 2007) and the similar mechanism was identified in Arabidopsis (Liu et al., 2009), wheat (Ryan et al., 2009), and rice (Yokosho et al., 2011). In maize, ZmMATE1 is induced by Al stress and subsequently activates citrate exudation in root apices (Maron et al., 2010) and the constitutively higher expression of ZmMATE1 in some Al-tolerant lines is associated with Al tolerance due to the increased citrate exudation (Maron et al., 2010; Guimaraes et al., 2014). Here, our results showed that ZmMATE1 had constitutively higher expression in the root apices of zmpgp1 mutants (Fig. 5C), which have higher free IAA levels and increased auxin responses (Figs. 7 and 8). ZmMATE1 was also up-regulated by auxin in the absence of Al stress (Fig. 6D), which was consistent with a recent report in soybean (Wang et al., 2016). Considering the decreased Al contents caused by auxin treatment (Fig. 6, A and B) and the previous conclusion mentioned above, we assumed that the mutation of PGP1 causes higher auxin accumulation and then induces the expression of ZmMATE1, which promotes Al efflux in the root apices.
ZmPGP1 Regulates Auxin Accumulation in Maize Root Tips and Regulates Root Growth under Al Stress

In Arabidopsis, the role of auxin polar transport has been highlighted by the analysis of aux1-7 and pin2 mutants, which have defects in auxin influx and efflux, respectively, and both display greater tolerance to Al stress compared to the wild-type control (Sun et al., 2010). Although it has been suggested that auxin in polar transport is also involved in the regulation of Al-induced root growth in maize and alfalfa (Kollmeier et al., 2000; Wang et al., 2016), there is no supportive further evidence in either species. However, here, we showed that the zmpgp1 mutant was indeed more tolerant of Al stress than the wild type, as mutant seedlings challenged with Al stress were able to elongate their primary root more successfully than could the B73 control (Fig. 4). The zmpgp1 mutant root tips accumulated free IAA and maintained a more effective auxin signaling response (Fig. 7). The interpretation of these observations is that ZmPGP1 acts to drain auxin from the root TZ and EZ as a response to Al stress, resulting in the auxin level falling below a threshold sufficient to support normal root growth in maize.

AI Stress Depletes Auxin from the Root TZ and EZ to Cause Root Growth Inhibition in Maize

In Arabidopsis, using the auxin-responsive marker DR5rev:GFP, Al stress induced a local up-regulation of auxin response in the root TZ as a result of a localized increase in auxin synthesis (Yang et al., 2014; Liu et al., 2016). Consistently, both the behavior of mutants and the effect of exogenously supplying auxin have demonstrated that the Al-induced accumulation of auxin was responsible for the inhibition of root growth (Yang et al., 2014; Liu et al., 2016). Providing auxin magnifies the toxicity of Al through an alteration in the ALS1-mediated distribution of Al ions within the plant cell (Larsen et al., 2007; Zhu et al., 2013). However, the aggravating role of auxin in Al-induced root growth inhibition in Arabidopsis was not observed in maize (Kollmeier et al., 2000). The exogenous application of auxin to the root tips strongly alleviated the inhibition of root growth in maize, which leads to the hypothesis that Al stress depletes auxin from the root TZ and EZ to cause root growth inhibition (Kollmeier et al., 2000). Here, varying the concentration of auxin provided to Al-stressed maize seedlings confirmed that, if provided in sufficient amounts, auxin was able to reverse much of the restraint on root growth imposed by Al stress (Fig. 6A). As the zmpgp1 mutants accumulated higher amounts of free IAA, the primary roots of the three mutants displayed higher elongation rates than the wild type when the seedlings were faced with AI stress (Figs. 4 and 7). The auxin-responsive marker DR5rev:RFP allowed for a more detailed topological view of the effect of AI stress on the auxin response in maize. In the wild-type root tip, Al stress reduced the DR5rev:RFP signal (Fig. 7A), responding to the drop in the localized auxin level (Fig. 7B). Similar observations were also detected in wheat, displaying highly down-regulated expression of auxin response genes including GH3, IAA2, IAA10, and IAA21 (Liu et al., 2017). We hypothesize that Al stress drains auxin from the root tip. In the zmpgp1 mutant root tips, free IAA levels were higher, as was the enhanced DR5rev:RFP signal compared to the wild-type B73 control (Fig. 7), indicating that ZmPGP1 is responsible for depleting auxin from the root TZ and EZ in response to Al stress and results in low auxin levels and root growth inhibition.

In summary, this study provides direct evidence to highlight the critical role of auxin polar transport in AI-induced root growth regulation in maize and experimentally confirms the previous hypothesis that auxin alleviates root growth inhibition under Al stress in maize through mutants analysis. It appears that the alleviating role of auxin in Al-inhibited maize root elongation contrasts the aggravating role in Al-induced Arabidopsis root growth inhibition. The contrasting response of these two species is particularly intriguing. Such a fundamental difference is consistent with high auxin accumulations in Arabidopsis root TZs while a reduced auxin level in maize root TZs under Al stress. Therefore, investigations about how AI stress increases auxin accumulations in Arabidopsis root TZs while it depletes auxin from maize root TZs will highlight the opposite roles in different plant species.

MATERIALS AND METHODS

Mutant Screening and Genetic Analysis

A population of 12,000 EMS mutagenesis maize (Zea mays) derivatives was grown in the field and phenotyped. Selection was based on the combination of a semidwarf stature and the absence of aboveground brace roots. Each of the three selections was crossed with the inbred line B73, the same cultivar used for mutagenesis. The resulting F1 plants were self-pollinated and the F2 progeny were grown in the field, where 80-d-old plants were scanned for the expression of the mutant phenotype. Intercrosses of the three mutants was carried out to check for allelism, based on the phenotype of the F1 plants.

Positional Cloning Using the EcMutMap Method

EcMutMap (exome capture-based MutMap; Lu et al., 2018) is an improved method of MutMap that is a way of rapid gene isolation in rice through crossing mutants to wild-type parent lines (Abe et al., 2012; Takagi et al., 2013). The EcMutMap method was applied to identify the mutant gene in the selected mutants. DNA was isolated using the CTAB method from 40 F2 seedlings showing the mutant phenotype and was mixed in an equimolar fashion. The resulting DNA was subjected to exome capture-based sequencing using a GAIIx device (Illumina). The resulting filtered reads were mapped onto the B73 reference sequence using MAQ software (Li et al., 2008). A set of transition variants was selected (Supplemental Table S1), and the polymorphisms validated by sequencing the amplicons derived from each of the 40 templates. Where the variant site was associated with a pair of peaks, either G/A (Fig. 2A, b) or C/T, a crossing-over in one of the two homologs was inferred: a single peak corresponding to G (Fig. 2A, a) or C was interpreted as the occurrence of crossing-over in both homologs, while a single A (Fig. 2A, c) or T peak implied that no crossing-over had occurred. On this basis, the crossing-over rate (G/G+T or C/C+T) of each variant was calculated. Variants unlinked to the phenotype or located on different chromosomes were expected to segregate 1:1; those fully linked to the causative locus were not expected to segregate with respect to the phenotype. Variants at a position on the same chromosome

Plant Physiol. Vol. 177, 2018  829
DNA was extracted using the CTAB method (Dellaporta et al., 1983). The ZmPGP1 gene was checked in each mutant through sequencing PCR-amplified fragments and the primers are shown in Supplemental Table S2.

Plant Growth and Al Stress Treatment

Grains were surface-sterilized by immersion in 5% (w/v) NaClO for 30 min, imbibed in distilled water, and germinated for 4 d on moistened paper. The seedlings were grown hydroponically in a 28°C/25°C (day/night) chamber at ~60% relative humidity under a 16-h-light/8-h-dark photoperiod (~100 μmol m⁻² s⁻¹). The culture solution, 0.5× Hoagland liquid solution [0.51 g/L KNO₃, 0.82 g/L Ca(NO₃)₂, 0.49 g/L MgSO₄·7H₂O, 0.136 g/L KCl, 0.6 ml/L FeSO₄·7H₂O, 0.286 mg/L H₃BO₃, 1.81 mg/L MnCl₂·4H₂O, 0.08 mg/L CuSO₄·5H₂O, 0.22 mg/L ZnSO₄·7H₂O, and 0.09 mg/L H₂MoO₄·4H₂O], was renewed every 2 d. The seedlings with primary root lengths of approximately 6 to 10 cm were chosen for experimental stress treatment. Before Al treatment, the seedlings were precultured for 24 h in 0.5 mm CaCl₂ solution (pH 4.5) for acclimation and then the seedlings were exposed for a variable time to an AlCl₃ concentration of between 0 and 50 µm with or without additional NAA (Baluska et al., 2010) root apex transition zone: a signalling-response nexus in the root. Trends Plant Sci 15: 174–178.

Total RNA was extracted from root tips using TRIzol reagent (Invitrogen). The cDNA first strand was synthesized from 1 µg total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer’s protocol. A set of RT-qPCRs were performed using a MyiQ real-time PCR detection system (Bio-Rad) based on the FastStart Universal SYBR Green Master’s protocol. A set of RT-qPCRs were performed using a MyiQ real-time PCR detection system (Bio-Rad) based on the FastStart Universal SYBR Green Master’s protocol. A set of RT-qPCRs were performed using a MyiQ real-time PCR detection system (Bio-Rad) based on the FastStart Universal SYBR Green Master’s protocol.

Transcription Profiling by RT-qPCR

For the gravitropic analyses, 3-d-old seedlings with 3- to 5-cm-long roots were chosen for the treatment. The roots were kept vertical for 1 h then horizontal for 3 h on 0.5× Murashige and Skoog plates according to Bjorkman’s method (Bjorkman and Cleland, 1991). Photographs were taken at the indicated times and the curvatures were measured from digital images using Image J software. Twenty roots were measured per treatment for each of the three replicates.

Supplemental Data

The following supplemental materials are available.

Determination of IAA Concentration

Four-day-old seedlings of B73 and zmppg1 mutants were treated with either 0 or 50 µm AlCl₃, for 6 h. Then, 4-mm-long root tips (1–5 mm behind the tip) were excised and immediately frozen in liquid nitrogen. The samples were ground to powder in liquid nitrogen. Subsequently, 1 ml of 80% (v/v) methanol was added to each sample and vortexed. The subsequent extraction and quantification of IAA followed the methods described by Zhou et al. (2010).

Dilution, the concentration of Al was determined by graphite furnace atomic absorption spectrometer (GF-AAS, SHIMADZU, Japan).

Confocal Microscopy

To visualize the auxin-signaling response in the root, a maize transgene marker line carrying DR5rev:RFP (Gallavotti et al., 2008) was crossed with the zmppg1 mutant, and the homozygous zmppg1/DR5rev:RFP line carrying the mutant pgp1 allele and DR5rev:RFP was screened for this study. Four-day-old seedlings of zmppg1/DR5rev:RFP and the DR5rev:RFP line were exposed to 50 µm AlCl₃, for 0, 2, or 12 h and their root tips were subsequently scanned for the strength of RFP signal using an LSM-700 confocal laser scanning microscope (Zeiss).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ZmIAA2 (Zm00001d033976), ONM09067, ZmIAA10 (Zm00001d041416, ONM32956), ZmIAA21 (Zm00001d013302, AKQ63038), ZmGH3 (Zm00001d011377, AKQ96711) and ZmMATE1 (Zm00001d035115, AKQ78379).

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