Propiconazole Is a Specific and Accessible Brassinosteroid (BR) Biosynthesis Inhibitor for *Arabidopsis* and Maize

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**Abstract**

Brassinosteroids (BRs) are steroidal hormones that play pivotal roles during plant development. In addition to the characterization of BR deficient mutants, specific BR biosynthesis inhibitors played an essential role in the elucidation of BR function in plants. However, high costs and limited availability of common BR biosynthetic inhibitors constrain their key advantage as a species-independent tool to investigate BR function. We studied propiconazole (Pcz) as an alternative to the BR inhibitor brassinazole (Brz). *Arabidopsis* seedlings treated with Pcz phenocopied BR biosynthetic mutants. The steady state miRNA levels of BR, but not gibberellic acid (GA), regulated genes increased proportional to the concentrations of Pcz. Moreover, root inhibition and Pcz-induced expression of BR biosynthetic genes were rescued by 24-epi-brassinolide, but not by GA$_4$, co-applications. Maize seedlings treated with Pcz showed impaired mesocotyl, coleoptile, and true leaf elongation. Interestingly, the genetic background strongly impacted the tissue specific sensitivity towards Pcz. Based on these findings we conclude that Pcz is a potent and specific inhibitor of BR biosynthesis and an alternative to Brz. The reduced cost and increased availability of Pcz, compared to Brz, opens new possibilities to study BR function in larger crop species.

**Introduction**

Brassinosteroids (BRs) are poly-hydroxylated steroidal hormones with profound effects on several physiological plant responses. They are involved in regulating cell elongation and division [1–2], vascular differentiation [3–4], photomorphogenesis [5–6], leaf angle inclination [7–8], seed germination [9–10], stomata development [11], as well as suppression of leaf senescence and abscission [12]. Radioactive tracer studies in cell cultures of *Catharanthus roseus* established the steps of the BR metabolic pathway [13]. This work was complemented by the characterization of several BR-deficient mutants in *Arabidopsis* [14–20], as well as crops like tomato, pea, and rice [21]. These studies showed that several steps of BR biosynthesis are mediated by cytochrome P450 monoxygenases (P450s) [13,21]. Although the importance of BRs for agricultural crops such as sorghum (*Sorghum bicolor*) and maize (*Zea mays* L.) has been recognized [22], only a few null-mutations have been reported in these species [23–24].

The field of chemical genomics greatly benefited from the use of chemical inhibitors/modifiers [25–26]. Potent and specific biosynthesis inhibitors are useful tools to evaluate the functions of endogenous substances, including phytohormones. Biosynthetic mutants and specific metabolic inhibitors displayed their effectiveness in mode of action studies of gibberellic acid (GA) and BRs [27–28].

Numerous triazole compounds have been shown to inhibit P450s, one of the largest and most ubiquitous group of plant enzymes that catalyze oxidative processes in life systems [29]. Paclobutrazol (Pac) and uniconazole (Ucz) are two triazole plant growth regulators (Fig. 1) that block sterol 14R-demethylation, phenocopy GA mutants, and reduce endogenous GA levels [30]. Both compounds inhibit P450 CYP701, which catalyzes an early step in GA biosynthesis [30]. Furthermore, Ucz also has been reported to slightly decrease the endogenous concentration of castasterone and inhibits BR-induced tracheary element differentiation [31–32]. These reports suggested that Ucz may also affect BR biosynthesis and later screens of structurally similar azoles led to the development of brassinazole (Brz) (Fig. 1), the first specific BR biosynthetic inhibitor [33–34].
Brassinolide Biosynthesis Inhibitors in Plants

Brz’s mode of action is to bind and block DWF4/CYP90B1 [35]. DWF4 is a P450 that mediates multiple rate-limiting C22-alpha-hydroxylations in the biosynthesis of BRs [35]. DWF4 expression is a target of regulation by both endogenous signals like auxin [36–37] and exogenous cues like temperature [38]. Brz and its even more specific derivative Brz2001 [39] became invaluable tools for BR research. Not only did they help to reveal the role of BRs in various plant species [40], they were also essential for the isolation and characterization of genes that function in BR signaling [41–43]. However, low accessibility and high costs associated with Brz/Brz2001 have limited their use in agricultural crops that often require large-scale applications. In this context, it would be beneficial to the research community to have access to potent, specific, and more cost efficientazole BR inhibitors in plants.

The triazole compound propiconazole (Pcz), 1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole, (Fig. 1) as a potent inhibitor of BR biosynthesis was first reported after examining its inhibitory effect on hypocotyl elongation of cress plants (Lepidium sativum) [27]. This inhibitory effect of Pcz was reversed by co-application with brassinolide. Based on the Pcz structure additional BR inhibitors, such as 2RS,4RS-1-[2-(4-trifluoromethylphenyl)-4-n-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole, were identified [27]. On the other hand, Pcz has been commercially used as fungistat (BannerMaxx, Syngenta) against a broad range of phytopathogenic fungi. Its fungistatic mode of action is the same as that of Ucz and Pac; blocking of lanosterol 14R-demethylase (CYP51A1) [44–45]. Pcz has also been studied extensively for its toxicity on plants, animals, humans, and the environment [46–47]. Here we present a molecular genetic analysis of Pcz’s effects on Arabidopsis and maize seedlings.

Our results indicate that Pcz is a potent and specific inhibitor of the BR metabolic pathway in plants.

Results

Arabidopsis seedlings treated with Pcz display dwarf phenotypes

To study Pcz’s impact on Arabidopsis, we treated wild-type Ws-2 plants with Pcz concentrations ranging from 0.1 to 5 μM for 5 days. Cotyledons showed a reduction in size and epinastic growth responses with treatments of Pcz (Fig. 2A–B). Subsequently, we evaluated the effect of Pcz on primary root length. The results showed a dose-dependent reduction of primary root length, where 0.1 μM Pcz decreased the elongation by 20% and 5 μM Pcz by 54% compared to mock conditions (Fig. 2A, 2C). No further significant decrease was observed at concentrations higher than 0.5 μM Pcz (Fig. 2C).

We then evaluated the overall efficacy and specificity of Pcz compared to equal concentrations of Brz and Ucz. Wild-type Ws-2 plants treated with 1 μM Pcz or Brz for 4 days showed comparable reductions in root length of 38% and 41%, respectively (Fig. 2D). In contrast, the impact of 1 μM Ucz on reduction of root length was significantly less with only a 25% decrease compared to mock treatments (Fig. 2D). We found a complementation of the phenotypes induced by 1 μM Pcz or Brz, but not 1 μM Ucz, with co-applications of 0.1 μM 24epi-brassinolide (BL), a bioactive epimer of brassinolide (Fig. 2D). Root length of plants co-treated with 1 μM Pcz plus 0.1 μM BL was not significantly different from mock treatments (Fig. 2D). On the contrary, the co-application of 1 μM Ucz and 0.1 μM BL showed no significant difference to treatments with 1 μM Ucz alone (Fig. 2D). These results suggest that Pcz and Brz have comparable efficacies in the inhibition of the BR biosynthetic pathway.

In some cases, triazole derivatives affect multiple targets; although usually to a varying extent [48]. To address whether Pcz inhibition is specific to the BR biosynthetic pathway or also affects P450 monooxygenases in GA biosynthesis, we compared co-applications of Pcz with BL or GA3. Again, Arabidopsis seedlings co-treated with Pcz and BL showed no difference in primary root length compared to mock conditions (Fig. 3A, 3C). In contrast, Pcz induced-inhibition of root length was not recovered by exogenous co-application of GA3 (Fig. 3A, 3C). We did find a slight increase (17%) in root length with the co-application of Pcz and GA3 relative to Pcz treatment, however, a similar increase (11%) was found for GA3 application compared to mock. Taken together, the results indicate that the inhibition of root growth caused by Pcz is complemented by the co-application of BL, but not GA3.

Arabidopsis brassinazole resistant1-1D (bzr1-1D) mutants carry a dominant gain of function mutation in the transcription factor and major component of BR signaling, BZR1, which results in a constitutive BR response even in the absence of BRs. Therefore, bzr1-1D mutants are sensitive indicators for BR inhibitor specificity [49]. Wild-type (Col-0) plants treated with 2 μM Pcz showed a reduction in hypocotyl length of 83%, whereas bzr1-1D mutants exhibited only 4% shorter hypocotyls, compared to mock (Fig. 3D–F). Although bzr1-1D plants were more resistant to Brz applications than wild type, 2 μM Brz did reduce the length of bzr1-1D hypocotyls by 37% compared to mock (Fig. 3E–F).

BR biosynthesis mutants show reduced sensitivity towards Pcz

The azole ring of Brz binds to the heme prosthetic group of DWF4 (CYP90B1), the rate-limiting enzyme in BR biosynthesis, forming a coordination complex, which impairs DWF4 activity [35]. Mutants...
already deficient in BR biosynthesis should thus show a reduced sensitivity towards BR biosynthetic inhibitors such as Brz and Pcz. We tested wild-type and dwf7-1 seedlings with Pcz and compared their responses to Brz treatments (Fig. 4). DWF7 catalyzes the conversion of episterol to 5-hydroepisterol upstream of DWF4 [18]. Wild-type (Ws-2) plants treated with either 1 mM Pcz or Brz, or 10 mM Brz produced 46%, 37%, and 64% shorter roots compared to mock conditions, respectively (Fig. 4A, 4F). Although dwf7-1 showed a significant response to 1 mM Pcz and 10 mM Brz treatments, the relative reduction (25% and 30%, respectively) was still lower than Pcz/Brz treated Ws-2 (Fig. 4B, 4G).

bri1-5, a weak allele of the major BR receptor BRASSINOSTEROID RESISTENT1 (BRI1), and wild type respond similarly to Brz, but not to BL treatment [50–51]. This suggests that although bri1-5 is affected in BR signal transduction, it still responds to changes in BR homeostasis. We found that Pcz and Brz, at equal concentrations, had similar effect on bri1-5 and Ws-2 (Fig. 4A, 4C, 4F, 4H).

Since DWF4 has been shown to be a direct target of Brz [35], which is structurally similar to Pcz (Fig. 1), we also took a closer look at the effect of Pcz on dwf4 mutants. Ws-2 plants again showed a decrease in root length upon Pcz or Brz treatment (Fig. 5A, 5C), but dwf4-1 mutants did not exhibit a significant reduction (Fig. 5B, 5C).

Pcz specifically influences the expression of BR biosynthetic genes

Transcriptional feedback regulation of rate-limiting step enzymes is a critical mechanism to maintain hormone homeostasis [52–53]. Since triazole derivatives can have multiple targets, we evaluated the expression levels of both BR (DWF4, BR6ox2, CPD, BAS1 and BZR1) and GA (GA20ox1 and GA2ox1) related genes, to further assess the specificity of Pcz.

Using quantitative real-time PCR (qRT-PCR) we found a dose-dependent induction of BR biosynthetic gene expression (DWF4, BR6ox2, and CPD) with increasing Pcz concentrations (Fig. 6A). With the same treatment conditions the mRNA levels of BAS1, a gene involved in BR degradation [5], showed a downward trend (Fig. 6B). BZR1, a key regulator of BR signaling, did not show relevant changes in its expression upon Pcz treatments (Fig. 6B). The GA biosynthesis gene GA20ox1, which is feedback regulated by endogenous GA levels [30], also showed no increase in expression upon Pcz application (Fig. 6B).

The Pcz-induced increase in mRNA accumulation of DWF4 and BR6ox2 and concomitant decrease in BAS1 expression was offset by co-application of Pcz with BL, but not by Pcz with GA3 (Fig. 6C). Our results also showed an expected decrease in DWF4 and BR6ox2 expression upon BL and to a smaller extent GA20ox1 upon GA3 treatment (p = 0.06) (Fig. 6C–D). Together these findings suggest that Pcz specifically affects BR regulated genes and do not provide evidence for an inhibitory effect on GA biosynthesis.

Pcz induces dwarfism in dark and light grown maize inbred W22 seedlings

Studies on crop species such as sorghum, rice, and maize often require large amounts of growth media which limits the use of cost
intensive inhibitors such as Brz. For that reason, we tested the effect of Pcz on both dark and light grown seedlings of the maize inbred line W22. A strong reduction of hypocotyl elongation is one of the most striking characteristics of the de-etiolation phenotype of dark grown Arabidopsis mutants deficient in BR biosynthesis [53]. Compared to mock, W22 seedlings grown in the dark for 8 d in the presence of 0.5 to 30 \( \mu \)M Pcz showed decreased mesocotyl elongation ranging from 27% to 64%, respectively (Fig. 7A, 7C). Ucz treatment of 0.5 to 30 \( \mu \)M reduced the length of the mesocotyl 25% to 73% (Fig. 7C). Similar to the mesocotyl, the lengths of true leaves were reduced from 21% to 51% by Pcz and from 20% to 56% by Ucz, respectively (Fig. 7D). Although both Pcz and Ucz treatment affected the coleoptile, the relative reduction in length was less pronounced than in mesocotyls and true leaves (Fig. 7E). Surprisingly, the primary root of W22 seedlings showed significant differences in sensitivity towards Pcz and Ucz. Pcz concentrations up to 30 \( \mu \)M had no significant effect on primary root length. In contrast, seedlings treated with equal or greater than 5 \( \mu \)M Ucz resulted in a more overall compact appearance (Fig. 7F). Interestingly, unlike in dark treatments, the primary root length of seedlings grown for 21 d in the light was reduced by 30% when treated with 5 \( \mu \)M Pcz relative to mock (Fig. 7H).

The genetic background of maize influences tissue specific sensitivity to Pcz

Given the great diversity between maize inbred lines [54], we assessed whether the genetic background influences the effects of Pcz and Ucz on dark grown maize seedlings. Thus, we repeated the dark assay using 3 additional maize inbred lines: Mo20W, A619, and B73. The length of the four evaluated tissues (mesocotyl, true leaves, coleoptile, and primary root) showed significant differences between Mo20W, A619, and B73 even in the absence of Pcz or Ucz (Fig. 8A–G). In addition, when treated with Pcz or Ucz we observed significant differences between the inbred lines in both their overall and tissue-specific sensitivity. In the presence of 1 or 10 \( \mu \)M Pcz the mesocotyl length of Mo20W was reduced by 57% and 56%, respectively, relative to mock treatment (Fig. 8A, 8D). Likewise, B73 treated with the same concentrations of Pcz exhibited 40% and 52% shorter mesocotyls, respectively (Fig. 8C–D). However, in the case of A619, only 10 \( \mu \)M Pcz had a significant but smaller impact (35%) on mesocotyl elongation, relative to mock (Fig. 8B, 8D). Comparable results were obtained for the response of true leaves to Pcz and Ucz treatments (Fig. 8A–C, 8E).

The primary roots of Mo20W, A619, and B73 showed a significant reduction in length when treated with 1 or 10 \( \mu \)M Ucz (Fig. 8A–C, 8G). Interestingly, Mo20W and B73 showed, in addition...
to the Ucz sensitivity, a decrease in primary root length when treated with 1 or 10 μM Pcz (Fig. 8A, 8C, 8G). The primary root length of A619 was reduced significantly only at 10 μM Pcz, but to a smaller extent than Mo20W and B73 (Fig. 8A–C, 8G). The coleoptile was the only tissue evaluated with comparable responses to both Pcz and Ucz in all three inbred lines (Fig. 8A–C, 8F).

To test whether the differences in Pcz efficacy are due to differences in BR response, we compared the effect of BL on root elongation between the maize inbreds. W22 and A619 plants treated with 20 μM BL exhibited the smallest relative reduction (37% and 44%, respectively) in root length (Fig. 9). On the other hand, Mo20W and B73 inbreds treated with the same amount of BL had 67% and 60% shorter roots than mock, respectively (Fig. 9). This result is consistent with our previous findings for Pcz sensitivity, and supports our hypothesis that genetic diversity influences BR responses in maize.

Figure 4. Sensitivity of Arabidopsis wild type, BR biosynthetic mutant dwf7-1, and signaling mutant bri1-5 to Pcz and Brz. (A–C) Seedlings of (A) Ws-2, (B) the BR biosynthetic mutant dwf7-1 (Ws-2), and (C) the BR signaling mutant bri1-5 (Ws-2) grown on ½ MS media with either 1 μM Pcz or Brz or 10 μM Brz. (D–E) Close-up of the shoot apices harboring cotyledons and the first pair of true leaves of (D) dwf7-1 and (E) bri1-5. (F–H) Average root lengths of (F) Ws-2, (G) dwf7-1 and (H) bri1-5 measured at the end of treatments (n>10). (F–H) Error bars represent standard deviation and lowercase letters indicate significant differences among treatments determined by “Post-hoc” test (p<0.05). Scale bar (A–E) 1 cm. doi:10.1371/journal.pone.0036625.g004

Figure 5. Resistance of dwf4-1 to Pcz and Brz. (A–B) Seedlings of (A) Ws-2 and (B) dwf4-1 grown on ½ MS media for 7 days, and then transferred to media containing 1 μM Pcz or Brz for 3 more days of growth. (C) Average root lengths of Ws-2 and dwf4-1 measured at the end of treatments (n>10). Error bars represent standard deviation and lowercase letters indicate significant differences among treatments determined by “Post-hoc” test (p<0.05). Scale bar (A–B) 1 cm. doi:10.1371/journal.pone.0036625.g005
Discussion

Phytohormone biosynthesis inhibitors allow the species-independent study of hormonal function during plant development. Inhibitor studies can also support the isolation and characterization of hormone deficient mutants without prior knowledge of the mutant phenotype. Pcz has previously been reported to impair the hypocotyl growth of cress seedlings and that this inhibition is reversible by the co-application of BL [27]. Chemical modification of Pcz also revealed structural elements essential for its inhibitory properties [27]. Pcz’s high accessibility and economical aspects prompted us to conduct a comparative analysis with the established BR inhibitor Brz.

Pcz treatment of Arabidopsis seedlings produced typical BR-deficient phenotypes such as: epinastically growing and dark-green cotyledons, reduced hypocotyl length, and a significantly shorter primary root (Fig. 2). Using root length as a reference we found that even relatively low Pcz levels of 0.5 μM resulted in strong inhibition (Fig. 2). As shown in independent experiments, the impairment of root growth in Arabidopsis through Pcz treatment can essentially be restored to length of mock-treated seedlings by BL, but not GA3 (Fig. 3). The slight effect of GA3 on root elongation was independent of Pcz treatment and may not indicate a recovery of Pcz inhibition. In contrast, BL treatment had a dramatic effect on root elongation in Pcz treated seedlings (Fig. 3).

In the absence of BR, the transcription factor BZR1 and its homolog BZR2 (BES1) are phosphorylated by the GSK3/SHAGGY-like protein kinase BIN2 [49,55–56]. Phosphorylation negates BZR1’s DNA-binding capacity and increases its cytoplasmic retention by phosphopeptide-binding 14-3-3 proteins [57–58]. The dominant bzr1-1D mutation increases BZR1’s dephosphorylation by the phosphatase PP2A [59]. BZR1 therefore remains nuclear localized and stabilized even in the absence of BRs causing bzr1-1D plants to show a constitutive BR response [49,59]. In contrast to wild type, bzr1-1D mutants showed only a minor inhibition of hypocotyl growth in the presence of Pcz (Fig. 3). Current evidence in rice and cress suggests that Brz inhibits BR biosynthesis but also affects GA responses [39]. We found that bzr1-1D plants were more sensitive to Brz than Pcz (Fig. 3) which suggests that Pcz is more specific. This hypothesis is supported by our finding that roots co-treated with Brz and BL, but not with Pcz and BL, were significantly shorter than mock (Fig. 2).

Ucz has been extensively studied as an inhibitor of GA biosynthesis [30]. Circumstantial evidence reported by Yokota et al. [31] and Iwasaki and Shibaoa [32] indicates that Ucz might also act as a demethylase inhibitor in BR biosynthesis. We observed shorter roots and hypocotyls of Ucz-treated Arabidopsis seedlings. Although Pcz- and Ucz-induced phenotypes were similar, co-application of Ucz and BL was not significantly different to Ucz alone (Fig. 2). Based on these analyses, Pcz-

Figure 6. Pcz impact on the expression of BR- and GA-related genes. (A–D) Quantitative real-time PCR analysis of the transcript levels after treatment with 0.2, 1, 5 or 10 μM Pcz of (A) BR biosynthetic genes DWF4, BR6ox2, and CPD or (B) BAS1, BZR1, and GA20ox1, involved in BR degradation, BR signaling and GA biosynthesis, respectively. (C, D) Expression pattern of (C) BR biosynthesis and signaling or (D) BR degradation, GA biosynthesis and GA degradation (GA2ox1) genes measured after treatments with 1.5 μM Pcz, 0.01 or 1 μM BL or 1 μM GA3 or co-applications of Pcz (1.5 μM) with 0.01 or 1 μM BL or 1 μM GA3. (A–D) Data points represent the average of three independent biological replicates with three technical replicates each. Error bars represent standard deviation. Asterisks indicate significant differences to the respective mock determined by Student’s t-test (p<0.05). Ubiquitin conjugating enzyme 21 (UBC21) was used as internal control.

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mediated suppression of root and hypocotyl elongation is likely the result of a specific inhibition of the BR biosynthetic pathway.

DWF7/STE1 is a Δ7 sterol C-5 desaturase that converts avenasterol to dehydroavenasterol or episterol to 5-dehydroepisterol early in BR biosynthesis [18]. Significant reduction of root elongation with Pcz treatment was found in wild type and to lesser extent also for duf7-1 seedlings, whereas roots of duf7-1 mutants did not exhibit a significant decrease (Fig. 4, Fig. 5). The BR metabolic pathway is likely non-linear, as downstream BR intermediates can be found in most monogenic BR biosynthetic mutants, including duf7-1 [18,20,60]. Therefore, Pcz treatment may further reduce endogenous BR pools in duf7-1 mutants. Loss of function mutations in DWF4 result in a more severe phenotype which could be the reason why no further reduction in root length was observed in duf4-1 upon Pcz treatment. Alternatively, since DWF4 could be the target of Pcz – like for Brz [35] – an additional inhibition of growth can not be expected in an already genetically disrupted duf4-1 mutant. From our findings we conclude that BR biosynthesis mutants show a reduced sensitivity towards Pcz.

Another line of evidence that Pcz is a specific and potent BR biosynthesis inhibitor comes from transcriptional analyses of BR and GA regulated genes in Arabidopsis. BR homeostasis relies on the feedback regulation of DWF4 transcription [52]. Thus, differences in DWF4 expression reflect even minor changes in BR biosynthesis. As expected, we found that BL treatment reduced the expression of DWF4 and other BR-biosynthetic genes in wild type, whereas Pcz application resulted in a dose-dependent increase of DWF4, CPD and BR6ox2 transcripts (Fig. 6). The induction of CPD expression relative to DWF4 and BR6ox2 was lower upon Pcz application, however, CPD is primarily post-transcriptionally regulated [51]. The Pcz dependent induction of BR biosynthetic gene expression was offset by the co-application of GA, this Pcz dependent induction was not reverted (Fig. 6).

PHYB ACTIVATION TAGGED SUPPRESSOR1 (BAS1/CYP72B1) catalyzes the conversion and inactivation of BL to 26-Hydro-BL. Similar to DWF4, BAS1 is feedback regulated by endogenous BR levels [5]. We observed that BAS1 expression was induced by BL application and repressed by Pcz treatment (Fig. 6). The co-application of Pcz with BL, but not with GA3, countered the effect of Pcz on BAS1 repression.
GA20-oxidases catalyze the sequential conversions of GA3 to GA20, late in the GA biosynthetic pathway [61]. Similar to an expected induction upon GA 3 application, but did not display regulation of GA20-oxidases by both GA and Pac, but not Pcz.

Interestingly, the GA-dependent increase in expression was impaired by simultaneous treatment with Pcz (Fig. 6). This indicates the presence or absences of 1.25, 5, or 20 μM BL, respectively. Average length of the primary root measured at the end of treatment (n=15). Error bars represent standard deviation. Statistical analysis determined by “Post-hoc” test (p<0.05) is shown in Table S3.

With respect to our recent study, we presented independent lines of evidence which indicate that Pcz resistance of W22 roots raised the question if this is a feature specific to W22 inbreds. We therefore tested the effect of genetic diversity in maize inbreds [54] towards Pcz response using the lines Mo20W, A619, and B73. Significant differences between these inbred lines in the length of four analyzed tissues were observed even under mock conditions. Furthermore, we found significant differences in tissue specific sensitivity towards Pcz and Ucz (Fig. 8). In general, Mo20W showed the highest sensitivity and A619 the highest resistance towards both inhibitors. Concerning tissue-specific responses, the coleoptile was the only organ which showed an even response to both Pcz and Ucz treatment in Mo20W, A619, and B73. In contrast, Pcz sensitivity in the roots and true leaves ranged from resistant (A619) to highly susceptible (B73 and Mo20W). The degree of Pcz response in maize roots seems therefore dependent on the genetic background of the maize line. The data also indicates differential hormonal regulation of tissue growth in aerial organs of maize inbreds. In rice and wheat tissue culture, accumulation of Pcz against a concentration gradient has been reported [69]. This indicates active uptake systems in these grass species. In *Monilinia fructicola*, the ABC transporter MfABC1 is induced upon Pcz treatment, which suggests a possible role for transporters of the ABC family [70] in Pcz uptake in plants and fungi [71]. Differences in either root uptake, *in planta* transport, and/or Pcz catabolism may be responsible for the observed variances between maize inbreds.

Nonetheless, our results also indicated a correlation of Pcz- and BL-sensitivity between the inbred lines. Compared to Mo20W and B73, W22 and A619 plants exhibited a smaller inhibition of root elongation in the presence of either Pcz or higher concentrations of BL (Fig. 8, Fig. 9). We therefore conclude that the genetic diversity between these maize lines influences their response to BRs.

Conclusion

We presented independent lines of evidence which indicate that Pcz inhibits BR metabolism and induces BR deficiencies in both *Arabidopsis* and maize seedlings. *Arabidopsis* seedlings treated with Pcz phenocopied BR deficient mutants such as *dwf7* and *dwa4*. Similarly, Pcz-induced dwarf phenotypes were discovered in both light and dark grown maize seedlings. Growth responses towards Pcz and Ucz were not equally expressed in all measured tissues of
maize. Tissue specific sensitivity of Pcz in the coleoptile, mesocotyl, true leaves, and primary roots alludes to differential BR biosynthesis and/or signal transduction for the different maize tissues. Genetic variation of maize inbred lines implies that genetic enhancers and suppressors play a key role in Pcz-induced physiological responses. We presented that Pcz is a potent alternative to the commonly used Brz with a comparable specificity and efficacy. In contrast to Brz/Brz2001, Pcz is easily accessible and the associated costs are much lower, allowing its use for large-scale chemical genomics and field testing.

Materials and Methods

Plant material and growth conditions

Seeds of Arabidopsis thaliana were surface-sterilized before being sprinkled on 0.8% agar-solidified media containing 0.5 × Murashige and Skoog salts and 1% sucrose. After one day of stratification at 4°C, plates were transferred to a growth room and grown under a 16 h light period. For the kzy1-1D experiments, seedlings were stratified for 48 h at 4°C, irradiated for 6 hours to promote germination and then transferred to a growth chamber and grown in the dark at 22°C.

Maize plants were grown under greenhouse conditions at 27°C (day) and 21°C (night). Unless indicated otherwise, plants were grown in coarse Vermiculite (SunGro Horticulture, Bellevue, WA) and Perlite Vermiculite Packaging Industries, Inc., North Bloomfield, OH). Plants were fertilized with 200 ppm Miracle-Gro Excel (Scotts, Marysville, OH) adjusted to pH 6 following manufacturer recommendations.

Chemical treatments and morphometric analysis

Seedlings of 3-day old Ws-2 wild type were transferred to agar-solidified media supplemented with Pcz (Banner Maxx, Syngenta, Greensboro, NC), Brz (gift from Shozo Fujioka, Riken, Japan) and Ucz (Consise, Fine Americas Inc., Walnut Creek, CA) alone or in combination with BL (Sigma Aldrich, St Louis, MO) or GA3 (Gold Biotechnology, St. Louis, MO). Media plates were placed vertically to ease morphometric analysis of the root and each plate contained more than 10 seedlings. After 3 days of treatment, images were taken and the growth parameters were analyzed using ImageJ software [72]. For treatments with Pcz and Brz of BR images were taken and the growth parameters were analyzed using ImageJ [72]. Plant height was measured from the root-shoot transition zone to the highest leaf collar, whereas the length of the main root was used to determine root length.

For light grown Pcz experiments, W22 seeds were planted 5 cm deep in 24.5 cm wide pots with coarse Vermiculite and watered every fifth day. Pcz was added at indicated concentrations to the water solution. After 21 days plants were harvested, photographed, and analyzed using ImageJ [72]. Plant height was measured from the root-shoot transition zone to the highest leaf collar, whereas the length of the main root was used to determine root length.

RNA extraction and qRT-PCR detection of gene transcripts

Arabidopsis thaliana Ws-2 seeds were surface sterilized and stratified for 48 h at 4°C, followed by growth for 4 days at 100 μmol/m²/sec, 16:8 h light/dark cycle at 25°C. The seedlings were then transferred into Erlenmeyer flasks prefilled with 50 ml ½ MS with 1% sucrose liquid media (pH 5.7) and were allowed to grow for 2 d at 100 rpm under the conditions described above. For the treatments, 500 × stock solutions were made in 50% DMSO (0.1% final concentration) and 100 μl of each stock solution, or 50% DMSO for the mock treatment, were applied at the beginning of the light cycle on day 7. After 10 h at 100 rpm the seedlings were harvested and immediately frozen in liquid nitrogen.

Total RNA was isolated from seedlings, as described by Eggermont et al. [73]. For qRT-PCR analysis, total RNA was pre-treated with DNaseI (Invitrogen) and cDNA was synthesized using Reverse Transcriptase (Invitrogen). Ubiquitin conjugating enzyme 21 used as internal control was amplified with UBC21_Rev1 (300 nM) and UBC21_For1 (500 nM); GA2ox1-Rev2 (500 nM) as well as GA20ox1-For1 (1100 nM) and BAS1_REV1 (1100 nM); BZR1_REV1 (300 nM) and BZR1_FOR1 (500 nM); and BR6ox2_REV1 (300 nM) and BR6ox2_For1 (500 nM); CPD_REV1 (300 nM) and CPD_For1 (500 nM); BAS1_REV1 (300 nM) and BAS1_For1 (500 nM); GA2ox1-REV1 (500 nM) and GA2ox1_For1 (500 nM); and GA2ox1_REV1 (300 nM). Primer sequences are listed in Table S4. All primers showed >90% efficiency at their indicated concentrations. qRT-PCRs were performed as described previously [23,74] using the StepOnePlus instrument (Invitrogen). Each data point represents the average of three independent biological replicates (approximately 30 samples per replicate), with three technical replicates.

Statistical analysis

The Microsoft Excel®, add-in XL Toolbox (ver. 3.02, http://xltoolbox.sourceforge.net) was used to obtain all descriptive and comparative statistics. Analyses of variance (ANOVA) for sets of data groups were performed with “Multiple comparisons/Post-hoc” testing. Once a significant difference (p<0.05) was detected, “Post-hoc” tests, using the Holm-Sidak algorithm, were performed to test which of the possible multiple comparisons between the data groups were significant [75].

Supporting Information

Table S1 Statistical analysis of Figure 7 C–F. Statistical analysis was performed using ANOVA with “Post Hoc” test using the Holm-Sidak algorithm. Adjusted α and adjusted p-values are shown and significance of p-values was indicated with bold text. (DOC)

Table S2 Statistical analysis of Figure 8 D–G. Statistical analysis was performed using ANOVA with “Post Hoc” test using the Holm-Sidak algorithm. Adjusted α and adjusted p-values are shown and significance of p-values was indicated with bold text.
**Table S3** Statistical analysis of Figure 9. Statistical analysis was performed using ANOVA with “Post Hoc” test using the Holm-Sidak algorithm. Adjusted \( \alpha \) and adjusted \( p \)-values are shown and significance of \( p \)-values was indicated with bold text.

| Reference | Test Statistics |
|-----------|----------------|
| Neff MM, Nguyen SM, Malancharuvil EJ, Fujioka S, Noguchi T, et al. (1999) | \( F = 4.23, \alpha = 0.05 \) |
| Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) | \( F = 4.23, \alpha = 0.05 \) |
| Kauschmann A, Jessop A, Koncz C, Szekeres M, Willmitzer L, et al. (1996) | \( F = 4.23, \alpha = 0.05 \) |

**Table S4** Oligo sequences. Primer sequences used for qRT-PCR as described in Material and Methods. Sequences are listed from 5’ to 3’.

| Oligo Sequence | Description |
|---------------|-------------|
| Table S4 Oligo sequences. | Primer sequences used for qRT-PCR as described in Material and Methods. Sequences are listed from 5’ to 3’ |

**Table S5** Number of visible leaves and leaf collars of Pce treated W22. W22 maize seedlings grown in the light for 3 weeks in the presence of 0, 0.2, 1, or 5 \( \mu M \) Pcz. All visible, including immature leaves of treated plants (\( n > 13 \)) were counted. The leaf collar was recorded if a ligule was developed. Student’s \( t \)-test was used to obtain the indicated \( p \)-values for the comparison with mock.

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**Author Contributions**

Conceived and designed the experiments: TH CC SC BS. Performed the experiments: TH CC NBB JSB JYZ. Analyzed the data: TH CC NBB JSB JYZ SC BS. Wrote the paper: TH CC NBB JSB SC BS.
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