Increased chloroplast area in the rice bundle sheath through cell-specific perturbation of brassinosteroid signalling

Authors

Lee Cackett[1], Leonie H. Luginbuehl[1], Ross-William Hendron[2], Andrew R. G. Plackett[3], Susan Stanley[1], Lei Hua[1], Na Wang[1], Steven Kelly[2], Julian M. Hibberd[1]

Addresses for authors

[1]Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, UK
[2]Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
[3]University of Birmingham, School of Biosciences, Edgbaston, B15 2TT, UK

Corresponding author:

Julian M. Hibberd (jmh65@cam.ac.uk)

Short title: BR signaling regulates chloroplast area and number

One-sentence summary:

Cell-specific manipulation of brassinosteroid signalling reveals a role in chloroplast development in rice, that can be used to increase chloroplast area in bundle sheath cells.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is Julian M. Hibberd.

Oryza sativa, bundle sheath, brassinosteroids, chloroplast development

Abstract

In the leaves of C_3 species such as rice (*Oryza sativa*), mesophyll cells contain the largest compartment of photosynthetically active chloroplasts. In contrast, plants that use the derived and more efficient C_4 photosynthetic pathway have a considerable chloroplast compartment in both bundle sheath and mesophyll cells. Accordingly, the evolution of C_4 photosynthesis from the ancestral C_3 state required an increased chloroplast compartment in the bundle sheath. Here, we investigated the potential to increase chloroplast compartment size in rice bundle sheath cells by manipulating brassinosteroid signalling. Treatment with brassinazole, a brassinosteroid biosynthesis inhibitor, raised leaf chlorophyll content and increased the number but decreased the area of chloroplasts in bundle sheath cells. Ubiquitous overexpression of the transcription factor-encoding *BRASSINAZOLE RESISTANT 1* (*OsBZR1*) increased bundle sheath chloroplast area by up to 45%, but these plants became chlorotic. However, when *OsBZR1* expression was driven by a bundle sheath-specific promoter, the negative effects on growth and viability were alleviated whilst chloroplast area still increased. In summary, we report a role for brassinosteroids in controlling chloroplast area and number in rice and conclude that cell-specific manipulation of brassinosteroid signalling can be used to manipulate the chloroplast compartment in rice bundle sheath cells.

Introduction

Increasing crop yield is considered imperative to feed a growing population and improving photosynthetic efficiency is recognised as one possible approach to achieve this (Smith et al., 2023). In land plants photosynthesis takes place in chloroplasts, the development of which is initiated by the perception of light and is modulated by various hormones (Cackett et al., 2022). This interplay allows the chloroplast content of each cell type to be tuned to the needs of the cell. For example, in C_3 species such as rice (*Oryza sativa*), carbon fixation occurs primarily in mesophyll cells that are densely packed with chloroplasts (Sage & Sage, 2009). Although bundle sheath cells in C_3 plants also contain chloroplasts, the proportion of cell volume they occupy is much lower than in mesophyll cells (Sage & Sage, 2009). In contrast, C_4 plants such as maize (*Zea mays*) contain a greatly enhanced chloroplast volume in bundle sheath cells (Lee et al., 2023). This allows photosynthetic reactions to be partitioned between the mesophyll and bundle sheath such that a biochemical pump concentrates CO_2 in bundle sheath cells where RuBisCO accumulates. This C_4 cycle reduces oxygenation of RuBisCO and the subsequent photorespiratory reactions, enabling photosynthetic efficiency to be increased by up to 50% (Sage et al., 2012). Understanding the differences in chloroplast biogenesis between cell types is therefore relevant to attempts to engineer C_3 leaves such that they operate a C_4-like photosynthesis.

Chloroplast biogenesis is primarily controlled by transcriptional regulators belonging to the *GOLDEN2-LIKE* (*GLK*), *GATA NITRATE-INDUCIBLE CARBONMETABOLISM-INVOLVED* (*GNC*) and *CYTOKININ RESPONSIVE GATA FACTOR 1* (*CGA1*) gene families (reviewed by Cackett et al., 2022). Recently, an additional regulator from the *RR-TYPE MYOBLASTOMA RELATED* (*RR-MYB*) gene family has been identified (Frangedakis et al., 2024) but it is not yet known how it responds to signals inducing chloroplast biogenesis, nor whether other transcription factors are involved. Overexpression of *GLK*s in multiple species increases chlorophyll and chloroplast production and can stimulate this in tissues that normally contain a very limited chloroplast compartment (Kobayashi et al., 2012, 2013; Nakamura et al., 2009; Wang et al., 2017). Constitutive overexpression of *ZmG2* in rice grown in the field increased photosynthesis, vegetative biomass, and grain yield (Li et al., 2020). Overexpression of *GNC* and *CGA1* increased chloroplast planar area in Arabidopsis (*Arabidopsis thaliana*) (Hudson et al., 2011) and rice bundle sheath cells (Hudson et al., 2013; Lee et al., 2021; Lim et al., 2024). However, neither overexpression of *GLK* or *CGA1* stimulated chloroplast biogenesis in the rice bundle sheath to the extent that their chloroplast content matched that of C_4 sorghum (*Sorghum bicolor*) or maize. Whilst unknown regulators may control the enhanced biogenesis of bundle sheath chloroplasts in C_4 species, it is also plausible that known components initiating this process are responsible, but the complete network of control has not yet been elucidated.

The brassinosteroid (BR) signalling pathway acts to repress chlorophyll accumulation and chloroplast biogenesis in the dark and is inhibited after light is perceived and de-etiolation is induced. In the dark, brassinosteroids act with PHYTOCRHOME INTERACTING FACTORS (PIFs) to negatively regulate photosynthesis gene expression (Oh et al., 2012). Upon exposure to light, PIFs are degraded in response to phytochrome signalling and the induction of *GNC* expression represses BR signalling, allowing activation of chloroplast biogenesis (reviewed by Cackett et al., 2022). In the dark, Arabidopsis BR-related mutants such as *deetiolated2 (det2), dwarf4 (dwf4)*, *constitutive photomorphogenic dwarf (cpd), brassinosteroid-insensitive1 (bri1)* and *brassinosteroid-insensitive 2* (*bin2)* show characteristics of de-etiolation including differentiated chloroplasts, short hypocotyls, development of true leaves and expression of light-regulated genes (Azpiroz et al., 1998; Chory et al., 1991; Clouse et al., 1996; Li et al., 2001; Szekeres & Né, 1996; Tachibana et al., 2022). The transcription factor BRASSINAZOLE RESISTANT 1 (BZR1) mediates the brassinosteroid-modulated negative control of photomorphogenesis by repressing genes involved in light-signalling and chloroplast development including photoreceptors such as phytochrome B and phototropin1, transcription factors such as *GATA2, GATA4*, *GLK1&2* and photosynthesis genes associated with chlorophyll biosynthesis (Luo et al., 2010; Sun et al., 2010; Wang et al., 2020; Yu et al., 2011). It is thought that the BZR1 mediated repression of chlorophyll biosynthesis avoids overaccumulation of protochlorophyllide in the dark so that when light is perceived photooxidative damage is minimized and greening promoted (Wang et al., 2020).

The role of brassinosteroids and BZR1 during de-etiolation, organ development, cell elongation and chlorophyll accumulation is well documented in Arabidopsis and, to a lesser extent, in rice. However, to our knowledge there are no analyses in rice demonstrating if or how brassinosteroids control chloroplast biogenesis in terms of size and number per cell. We therefore assessed how pharmacological and genetic perturbations to brassinosteroid signalling affect the planar area and number of chloroplasts in the bundle sheath of rice. Our analysis indicated that brassinosteroids alter the number and area of chloroplasts in the rice bundle sheath during de-etiolation and at later stages of development. Ubiquitous overexpression of *OsBZR1* resulted in larger chloroplasts in the bundle sheath but had adverse effects on plant health and yield. However, when overexpression of *OsBZR1* was driven by a bundle sheath specific promoter, increased bundle sheath cell chloroplast area was maintained whilst the adverse effects on growth mitigated. Overall, these data are consistent with an approach in which cell-specific manipulation of brassinosteroid signalling could be used to manipulate chloroplast number and size in rice.

Results

Brassinosteroids modulate chloroplast size and number in the rice bundle sheath

To initiate an understanding of the role of brassinosteroids in modulating chloroplast biogenesis in rice we applied the active brassinosteroid, brassinolide (BL), or the biosynthesis inhibitor, brassinazole (Brz) to seedlings during de-etiolation. For this, seeds were germinated for two days in the dark, transferred to BL- or Brz-containing media for 4 days in the dark and then exposed to light for 0, 4 and 12 hours. In control plants, greening and expansion of the first leaf were evident after exposure to light as expected (**Fig. 1A**). Consistent with previous analyses (Hong et al., 2002; Mori et al., 2002), BL treatment inhibited de-etiolation such that rice seedlings showed reduced leaf expansion and greening (**Fig. 1A, Supp fig. 1**). Quantification of whole seedling chlorophyll levels confirmed that its accumulation was reduced compared with controls (**Fig. 1B**). In contrast to the BL treatment, seedlings treated with Brz showed an increase in accumulation of chlorophyll after exposure to light compared with controls (**Fig. 1B**). Chloroplast content in the bundle sheath cells of the first leaf of controls and treated seedlings was imaged using confocal laser scanning microscopy at 0, 4 and 12 hours after light exposure (**Fig. 1C**). Brz treatment resulted in more chloroplasts per bundle sheath cell compared with controls after 12 hours of light, whereas BL treatment produced no significant difference (**Fig. 1D & E**). Both BL and Brz treatments resulted in smaller bundle sheath chloroplasts compared to control plants, in terms of mean planar area (**Fig. 1F & G**). Bundle sheath cell size decreased compared to the control as a result of the BL treatment and was unchanged in the Brz treatment compared with the control (**Supp. fig. S2**). Overall, these data are consistent with previous studies from other species reporting that brassinosteroids modulate chlorophyll accumulation during de-etiolation but also indicate that brassinosteroids can control chloroplast number and planar area.

To determine whether changes to chloroplast number and size were maintained later in development, plants were grown in BL or Brz and leaf 4 harvested once it was fully expanded. Both treatments impacted overall plant growth and development. For example, plants treated with BL developed the same number of leaves as controls, but leaf length was reduced (**Fig. 2A**). Brz caused faster development such that more leaves were evident (**Fig. 2A**), and they contained more veins than controls (**Supp. fig. S3**). Consistent with the initial de-etiolation experiments, chlorophyll content of leaf 4 was reduced by addition of BL (**Fig. 2B**) whilst it had no significant impact on chloroplast area and number in the bundle sheath at this developmental stage (**Fig. 2C, D & E**). Conversely, Brz treatment increased bundle sheath chloroplast number and decreased chloroplast area compared with controls (**Fig. 2C, D & E**) but with no change in chlorophyll content (**Fig. 2B**). Overall, these results indicate that brassinazole, an inhibitor of brassinosteroid biosynthesis, modulates the area and number of chloroplasts in rice bundle sheath cells both during de-etiolation and at later stages of leaf development.

Ubiquitous overexpression of OsBZR1 increases chloroplast area in bundle sheath cells but has adverse effects on plant growth

Given that perturbation of BR signalling through exogenous treatments gave rise to changes in chloroplast area and number in rice bundle sheath cells, we sought to determine whether analogous changes could be achieved through genetic activation of BR-responsive gene expression. As BZR1 is the primary transcription factor that mediates BR-responsive gene expression in Arabidopsis (Luo et al., 2010; Sun et al., 2010; Wang et al., 2020; Yu et al., 2011), we chose to investigate whether manipulation of the expression of the orthologous regulatory gene in rice could achieve the desired changes in chloroplast development. Phylogenetic interrogation of the BZR1 gene family revealed that there is a single gene in rice (LOC_Os07g39220) that is putatively orthologous (i.e. equally related) to BZR1, BES1 (BZR2), BEH1 and BEH2 in Arabidopsis (**Supp. fig. S4**), with no other rice gene homologs in this same clade. Accordingly, we hypothesised that this single gene is likely the primary transcription factor that mediates BR-responsive gene expression in rice. Moreover, previous investigations had implicated this gene in brassinosteroid signalling in rice leading to the naming of the gene *OsBZR1* (Bai et al., 2007). Thus, both overexpression and RNAi lines were generated to alter the expression of Os*BZR1* in the rice leaf.

No reduction in transcript abundance was detected in T_2 *OsBZR1* RNAi plants (**Supp. fig. S5B**), and so phenotyping was not undertaken. However, ubiquitous overexpression using the maize UBIQUITIN promoter was successful. Here, three independent homozygous single copy transgenic lines along with their respective null segregants (lines which had been through the transformation process but do not contain the genetic modification themselves) were identified (**Supp. fig. S6A & B**). RT-qPCR and bulk RNA sequencing on T_2 plants was conducted to confirm the level of endogenous *OsBZR1* and transgene expression (**Supp. fig. S6C, D and E**). Hereafter these lines are referred to as UBQ Null 1, UBQ OE 1, UBQ Null 2, UBQ OE 2, UBQ Null 3 and UBQ OE 3. Brightfield microscopy was used to image isolated bundle sheath and mesophyll cells from fully expanded leaf 8 (**Fig. 3A**). The individual chloroplast planar area was significantly larger for all three overexpression lines compared with the respective nulls in bundle sheath cells (**Fig. 3B**). UBQ OE 1 showed the largest effect with individual chloroplast area being increased by 45% (**Fig. 3B**). The extent to which chloroplast area increased corresponded with the degree of *OsBZR1* overexpression within each line (**Supp. fig. S6C**). There were no statistically significant differences in chloroplast area in mesophyll cells in any UBQ OE line (**Fig. 3C**). To confirm these findings with a higher throughput approach (Billakurthi & Hibberd, 2023) allowing a larger number of chloroplasts to be assessed, we next used confocal laser scanning microscopy (**Fig. 3D**). Consistent with the analysis of single cells after brightfield microscopy, this showed that overexpression of *OsBZR1* increased individual chloroplast area in bundle sheath cells in leaf 4 and leaf 8 compared with corresponding null lines (**Fig. 3E and F**). The number of chloroplasts per bundle sheath cell in leaf 4 or leaf 8 was calculated. Although there was a statistically significant difference in chloroplast number between null and UBQ overexpressor lines when leaf 4 was assessed, the absolute values were 10 and 8 chloroplasts per cell respectively, and so this difference was small. Furthermore, there were no statistically significant differences when leaf 8 was assessed (**Supp. fig. S7A and B**). Scanning electron microscopy detected no evident changes in bundle sheath chloroplast ultrastructure between null and overexpression lines (**Fig. 3G**). There were no consistent differences in whole leaf chlorophyll content (**Supp. fig. S8**) and only one of the three UBQ OE lines showed a significant difference in bundle sheath cell size compared with the corresponding null line (**Supp. fig. S9A and B**). The rate of net photosynthesis in young fully expanded leaves was not affected by ubiquitous overexpression of *OsBZR1* (**Fig. 3H**). Notably, the UBQ OE leaves senesced rapidly soon after maturation (**Fig. 3I**) and the number of seeds produced by all UBQ OE plants was significantly lower than corresponding nulls (**Fig. 3J**). We therefore sought to test whether a more targeted mis-expression of *OsBZR1* in the rice bundle sheath could maintain chloroplast development without inducing premature senescence and decreased yield.

Overexpression of OsBZR1 in the bundle sheath increases chloroplast area without inducing premature senescence

The promoter of the rice *SULPHITE REDUCTASE* (*SIR*) gene generates strong expression in bundle sheath cells (Hua et al., 2024) and so we used it to drive expression of rco*OsBZR1* (**Supp. fig. S10A**). Three independent homozygous and single copy overexpression lines (**Supp. fig. S10B**) along with corresponding null segregants were identified. RT-qPCR and bulk RNA sequencing on T_2 plants was performed to confirm the level of endogenous *OsBZR1* and transgene expression (**Supp. fig. S10C, D and E**). Hereafter these are referred to as BS Null 1, BS OE 1, BS Null 2, BS OE 2, BS Null 3 and BS OE 3. As with the ubiquitous overexpressor, individual mesophyll and bundle sheath cells from the BS Null and OE lines were isolated and brightfield microscopy used to quantify chloroplast planar area (**Fig. 4A, B and C**). All three overexpression lines contained larger bundle sheath cell chloroplasts when compared with corresponding null lines (**Fig. 4B**). Interestingly, chloroplast areas in mesophyll cells were also increased compared with the corresponding nulls in two out of the three lines (**Fig. 4C**). This may be due to very low levels of basal expression from the *SiR* promoter in mesophyll cells, or a non-cell autonomous response whereby changes in one cell-type impact other cells. Analysis of bundle sheath cells from leaf 4 and 8 by confocal laser scanning microscopy also indicated that bundle sheath cell chloroplasts were larger in BS OE plants compared with nulls (**Fig. 4D, E & F**). There were no significant changes in the number of chloroplasts per bundle sheath cell in leaf 4 or leaf 8 in the BS OE lines compared with corresponding nulls (**Supp. fig. S11 A and B**). No clear differences in ultrastructure between BS null and BS OE lines were discernible from scanning electron microscopy (**Fig. 4G**), nor could we detect differences in bundle sheath cell size (**Supp. fig. S12 A and B**). To initiate an understanding of whether increased chloroplast area was due to *OsBZR1* driving BR-related changes rather than perturbing other signalling pathways, the BS null and BS OE lines were grown in brassinazole which inhibits BR biosynthesis. For two of the three BS OE lines, the positive effect of bundle sheath cell-specific overexpression of *OsBZR1* was suppressed by Brz application (**Supp.**

fig. S13). These data imply, but do not unequivocally prove, that BZR1 is acting via the BR signalling pathway to modify chloroplast area, and so further work will be needed to fully understand the mode of action of BZR1 in rice in the future.

Despite the statistically significant increase in bundle sheath chloroplast area, neither chlorophyll content nor rate of photosynthesis were consistently increased by cell-specific overexpression of *OsBZR1* (**Fig. 4H & I**). However, unlike the ubiquitous overexpressors, the leaves of the BS OE plants did not show premature senescence (**Fig. 4J**), and the number of seeds produced per plant did not differ from controls (**Fig. 4K**). Overall, these data confirm that increasing *OsBZR1* expression can increase chloroplast area in rice bundle sheath cells, but also that cell-specific perturbation avoided deleterious effects on growth.

Ubiquitous overexpression of OsBZR1 perturbs stress-, brassinosteroid- and hormone-related pathways whilst bundle sheath cell-specific overexpression does not

To gain insight into how ubiquitous and cell-specific overexpression of *OsBZR1* re-programmed gene expression we conducted bulk RNA sequencing on mature leaf 4 from UBQ Null, UBQ OE, BS Null and BS OE plants. Principal Component Analysis (PCA) showed that ubiquitous overexpression of *OsBZR1* caused the most variance and impacted on both the first and second components (**Fig. 5A**). In contrast, when *OsBZR1* was expressed in the bundle sheath there were few overall transcriptional changes, with samples from BS Null and BS OE clustering together (**Fig. 5A**). The trends in changes to transcript abundance were also evident from a heatmap derived from Pearson's correlation analysis (**Fig. 5B**). Notably, correlation in mRNA abundance was weakest between UBQ OE and corresponding null lines whereas BS OE lines had high correlation with the corresponding BS null lines (**Fig. 5B**). Transcripts whose abundance was significantly different were identified by comparing each overexpression line with the corresponding null. Only genes with statistically significant changes (i.e. adjusted *p*-value < 0.05) were retained for subsequent analyses. This identified 6975, 1762 and 522 significantly up-regulated genes, and 5871, 295 and 90 genes down-regulated genes in the three ubiquitous overexpressors (**Fig. 5C, Supp. dataset S1 & S2**). Notably, the extent to which transcript abundance was perturbed corresponded to the degree of *OsBZR1* overexpression in these lines (**Fig. 5C,**

Supp. fig. S6C). As would be expected from the PCA and Pearson's correlation analysis, overexpression of *OsBZR1* from the bundle sheath promoter generated limited alterations to transcript abundance with only 14, 35 and 358 significantly up-regulated genes, and 8, 119 and 564 significantly down-regulated genes compared with null lines (**Fig. 5C, Supp. dataset S1 & S2**). As with the ubiquitous overexpressor, the extent to which transcript abundance was perturbed corresponded to the degree of *OsBZR1* overexpression in these lines (**Fig. 5C, Supp. fig. S10C**).

Gene Ontology (GO) enrichment analyses on transcripts that were up- and down-regulated identified several terms of interest, with the majority of these terms enriched in the ubiquitous overexpressing lines and not in the bundle sheath overexpressing lines (**Fig. 5D, Supp. dataset S3**). For example, GO terms for "protein amino acid phosphorylation", "protein ubiquitination", and "regulation of transcription" indicate a global change in transcription and protein degradation after ubiquitous *OsBZR1* overexpression. We note that several genes contributing to the GO terms involved in protein phosphorylation or transcription including four *Wall Associated Kinase* (*WAK*), six *Receptor-like Cytoplasmic Kinase* (*RLCK*) and nine *WRKY* transcription factors were significantly up-regulated in all three ubiquitous overexpressors, but this was not the case when *OsBZR1* expression was driven in the bundle sheath (**Fig. 5E, Supp. dataset S4**). Moreover, only the ubiquitous overexpressing line had enriched GO terms "defense response", "chitin catabolic process" and "apoptosis" that are indicative of biotic and/or abiotic stress responses and are consistent with the increased senescence observed in the ubiquitous overexpressors. Specific genes associated with biotic and abiotic stress responses and senescence were also significantly up-regulated in ubiquitous overexpressors (**Fig. 5E**). Consistent with the lack of GO term enrichment in bundle sheath specific *OsBZR1* lines, transcript abundance of the genes involved in biotic and abiotic stress responses and senescence was not altered (**Fig. 5E, Supp. dataset S4**). It was notable that transcripts derived from the *OsACS2, OsCYP74A2* and *OsDOG* genes involved in ethylene, jasmonic acid and gibberellic acid synthesis were more abundant in all three ubiquitous *OsBZR1* overexpression lines compared to corresponding nulls, indicating possible hormonal crosstalk had been induced (**Fig. 5E, Supp. dataset S4**). Again, these genes were not differentially expressed in the lines when *OsBZR1* was driven from the bundle sheath promoter. Interestingly, the only enriched GO term specific to the bundle sheath overexpressor was "cellulose biosynthetic process" (**Fig. 5D**).

We next examined the expression of genes involved in brassinosteroid signalling, genes acting downstream of BZR1 or genes involved in BR biosynthesis and catabolism to better understand how overexpression of *OsBZR1* perturbed these processes (**Fig. 5F, Supp. dataset S4**). In ubiquitous overexpressors, the brassinosteroid receptor *OsBRI1* was significantly down-regulated whilst the BR-signalling kinase *OsBSK2* and the endogenous *OsBZR1* gene were significantly up-regulated (**Fig. 5F, Supp. dataset S4**). This was not evident when *OsBZR1* was overexpressed in the bundle sheath. Putative rice orthologs (*OsXTH9* and *OsSAUR39*) of Arabidopsis genes *AtTCH4* and *AtSAUR-AC1* that have been used as BR-induced marker genes (Iliev et al., 2002; Nakamura et al., 2003; Xu et al., 1995) were significantly up-regulated in the constitutive overexpressors. Additionally, *OsRAmy3D* and *OsLIC* which have been previously reported to be induced by BZR1 in rice (Qiao et al., 2017, Xiong et al., 2022) were significantly up-regulated in the constitutive overexpressor (**Fig. 5F, Supp dataset S4**). Transcripts from two brassinosteroid biosynthesis genes *OsDWF4* and *OsDWF2* were less abundant when *OsBZR1* was expressed constitutively and when expressed in the bundle sheath (**Fig. 5F, Supp. dataset S4**). Additionally, two genes associated with brassinosteroid catabolism *OsCYP734A2* and *OsCYP734A4* were significantly up-regulated but only in the ubiquitous overexpressors (**Fig. 5F, Supp. dataset S4**).

Finally, we interrogated the data to better understand how *OsBZR1* overexpression impacted genes involved in chloroplast function and biogenesis. We detected relatively few changes here, possibly due to the fact that the transcriptome of mature leaves was analysed at a stage during which chloroplast development may be complete. Transcripts derived from the chlorophyll biosynthesis gene *OsPORA* were more abundant in ubiquitous overexpressors, but this was not the case when *OsBZR1* was expressed in the bundle sheath (**Fig. 5F, Supp. dataset S4**). The *OsGLK2* transcription factor was significantly down-regulated in one of the ubiquitous overexpressing lines but slightly up-regulated when *OsBZR1* was overexpressed in the bundle sheath (**Fig. 5F, Supp. dataset S4**). Two genes involved in chloroplast division *PDV1a* and *PDV1c* were decreased when *OsBZR1* was constitutively overexpressed which could indicate the mechanism behind BZR1-modulated changes in chloroplast area (**Fig. 5F, Supp. dataset S4**). The expression of other master regulators of chloroplast development (*OsCGA1*, *OsGNC, OsMYBS1* and *MYB-related family protein*) were unchanged when *OsBZR1* was overexpressed (**Supp fig. S14**).

Discussion

A role for brassinosteroids in controlling chloroplast size and number

As key phytohormones, brassinosteroids have been studied for decades with much of the work performed in *Arabidopsis thaliana*. One particularly well-documented role is the control of photomorphogenesis, including the induction of hypocotyl elongation combined with the inhibition of cotyledon expansion, chlorophyll biosynthesis and chloroplast differentiation in the dark (Asami et al., 2000; Chory et al., 1991; Komatsu et al., 2010; Tachibana et al., 2022; Yu et al., 2011; Zhang et al., 2021). Brassinosteroids are important for chloroplast development in Arabidopsis by modulating the activity of GOLDEN2-LIKE transcription factors (GLK1&2), master regulators of chloroplast development which control the expression of chloroplast targeted proteins encoded by photosynthesis-associated nuclear genes (*PhANGs*) important for the correct development of chloroplast ultrastructure and chlorophyll accumulation (Tachibana et al., 2024, Zhang et al., 2021). However, to our knowledge there are no reports demonstrating that brassinosteroids control chloroplast size or number in Arabidopsis or rice. The results reported here show that exogenous treatment with an active brassinosteroid and a brassinosteroid biosynthesis inhibitor altered chloroplast planar area and number in rice bundle sheath cells and both ubiquitous and cell-specific overexpression of *OsBZR1* resulted in increased chloroplast area. The transcript abundance of *OsPDV1a* and *OsPDV1c*, genes involved in chloroplast division, was significantly decreased upon overexpression of *OsBZR1*. However, the overexpression lines showed no biologically meaningful changes in bundle sheath cell chloroplast number. These results imply that overexpression of BZR1 does not have a material effect on chloroplast division (despite the fact that the expression of PDVs was down-regulated in the UBQ OE lines). Together these results support a previously unknown role for brassinosteroids in modulating chloroplast size throughout plant development.

Using OsBZR1 to engineer chloroplast volume specifically in rice bundle sheath cells

The role of BRs in increasing chloroplast area provided a candidate to manipulate the chloroplast compartment of rice bundle sheath cells, an important characteristic if C_4 photosynthesis is to be engineered into this species (Hibberd et al., 2008). Although ubiquitous overexpression of *OsBZR1* resulted in a significant increase in the area of individual chloroplasts in the bundle sheath, it did not impact mesophyll chloroplast area. This is possibly because the chloroplast compartment of mesophyll cells is already large, and so there is limited capacity to increase this further. However, this is not consistent with the bundle sheath cell-specific overexpressor where two of the three lines showed significant increases in the area of individual mesophyll chloroplasts. Therefore, it is possible that other factors limit chloroplast area in the mesophyll cells of the ubiquitous overexpressor and further analysis, for example use of single cell transcriptomics, would be valuable to elucidate these mechanisms.

Although the increased bundle sheath cell chloroplast area in the UBQ OE plants was extremely promising in terms of engineering this cell type, it had severe effects on plant health and yield. This is consistent with previous reports of misexpression of components of brassinosteroid biosynthesis or signalling leading to defects in plant growth (Clouse et al., 1996; Kim et al., 2020; Manghwar et al., 2022; Nolan et al., 2020). Exogenous application of brassinosteroids has been shown to have growth-promoting effects when a low dose is applied, whilst higher doses show growth retardation (Chaiwanon & Wang, 2015; González-García et al., 2011; Nolan et al., 2020). The growth defects reported here for rice demonstrate conservation in the brassinosteroid signalling pathway between rice and Arabidopsis. Transcriptome analyses of the *OsBZR1* ubiquitous overexpressors showed differentially expressed genes with enriched GO terms including “biotic stress response”, “abiotic stress response” and “senescence”, consistent with the negative effects on plant development.

Endogenous tissue-specific control of brassinosteroid signalling ensures proper growth and avoids deleterious effects of ubiquitous brassinosteroid action (Nolan et al., 2020). Indeed, studies using tissue-specific promoters to complement brassinosteroid mutant phenotypes have revealed that cell type-specific confinement of brassinosteroid signalling is essential for proper shoot and root development (Chaiwanon & Wang, 2015; Hacham et al., 2011; Kang et al., 2017; Nolan et al., 2020, 2023; Savaldi-Goldstein et al., 2007; Vragović et al., 2015). This cell type-specific signalling can be harnessed for the development of plants with improved stress resistance and yield. For example, overexpression of BRL3, a vascular-enriched brassinosteroid receptor in Arabidopsis, conferred drought stress tolerance without a growth penalty whereas altering the ubiquitously expressed BRI1 receptor conferred drought tolerance but at the expense of growth (Fàbregas et al., 2018). And, overexpression of C-22 hydroxylases, which increase BR levels, using a promoter that limits expression mainly to vegetative organs resulted in significant increases in grain yield

(Wu. et al, 2008). The results reported here therefore confirm that endogenous tissue-specific control of brassinosteroid signalling is important in rice.

As cell specific gene expression in Arabidopsis and rice had proved useful (Fàbregas et al., 2018), we used a bundle sheath cell-specific promoter for rice (Hua et al., 2024) to express *OsBZR1* only in bundle sheath cells. This resulted in an increase in bundle sheath cell chloroplast area of up to 34% and had no detectable negative impacts on plant growth. Moreover, the transcriptome of these plants showed no enrichment in stress- or senescence-related GO terms, indicating that the cell-specific overexpression was successful in avoiding off-target perturbations. In fact, the transcriptome of these plants showed very little change compared with the corresponding nulls. A contributing factor to this outcome is likely that bundle sheath only makes up approximately 15% of chloroplast-containing leaf cells (Leegood, 2008). It was also noticeable that these lines showed little to no change in net photosynthesis or seed yield, again possibly due to the small proportion of bundle sheath cells in the leaf.

Exploiting multiple master regulators to manipulate chloroplast development

The work presented here investigated the potential of manipulating brassinosteroid signalling to increase the chloroplast compartment in rice bundle sheath cells to mimic a more C_4-like leaf anatomy. The percentage increases in bundle sheath chloroplast area of the ubiquitous and cell-specific *OsBZR1* overexpression lines reported here are comparable with other work describing master regulators of chloroplast development in rice. For example, overexpression of *ZmGLK* resulted in increases in chloroplast area of approximately 30% (Wang et al., 2017) and bundle sheath cell-specific overexpression of *OsCGA1* resulted in a 3.5-fold increase in chloroplast size and a significant increase in proportion of the bundle sheath cell occupied by chloroplasts (Lee et al., 2021). Based on these similarities in changes in chloroplast size, BZR1 could be considered as a promising candidate for engineering the chloroplast compartment in rice. In the controlled conditions we used we observed no changes to photosynthesis or yield when *OsBZR1* was overexpressed, which contrasts with overexpression of *ZmGLK* that resulted in a 30-40% increase in vegetative biomass and grain yield in the field (Li et al., 2020). It may therefore be interesting to assess these *OsBZR1* overexpression lines in the field. In contrast to Arabidopsis, where BZR1 has been shown to modulate *GLK* expression (Yu et al., 2011) our transcriptome data showed no consistent changes to *OsGLK* when *OsBZR1* was overexpressed. Additionally, the expression of other master regulators of chloroplast development (including *OsCGA1*, *OsGNC* as well as *OsMYBS* genes putatively orthologous to MYB-related transcription factors that were recently shown to control chloroplast biogenesis in *Marchantia polymorpha* and Arabidopsis) were unchanged when *OsBZR1* was overexpressed (**Supp fig. 14**). Thus, one could consider combining these regulators to test for additive or synergistic effects. To conclude, although work is still needed to elucidate precisely how brassinosteroids and BZR1 manipulate chloroplast size and number, the results shown here present a promising candidate to be harnessed to manipulate chloroplast development in rice and, potentially, other important crop species.

Materials and methods

Plant material and growth conditions

For seed propagation and phenotyping experiments, seeds of wild-type rice (*Oryza sativa spp japonica* cv. Kitaake) and transgenic rice lines (UBQ Null, UBQ OE, BS Null and BS OE) were imbibed in sterile Milli-Q water and incubated at 28°C in the dark for two days. Seeds were transferred to Petri plates with moistened Whatman filter paper and germinated in the growth cabinet at 28°C with a 16/8-hour light/dark cycle for a further two days. Germinated seedlings were placed into 9 by 9 cm pots (two plants per pot) filled with Profile Field and Fairway soil amendment (www.rigbytaylor.com) and grown in a walk-in plant growth chamber under a 12-hour photoperiod at a photon flux density of 400 µmol m^{-2} s^{-1} at 28°C day and 20°C night. Plants were fed once a week with Peters Excel Cal-Mag Grower fertiliser solution (LBS Horticulture, Clone, UK) at a concentration of 0.33 g/L with additional iron (Fe7 EDDHA regular, Gardening Direct, UK) at a concentration of 0.065 g/L. Once fully expanded, leaf 4 and/or leaf 8 was harvested for phenotyping.

Production of transgenic rice lines

All constructs were generated using the Golden Gate cloning system, with *Bpi*I and *Bsa*I enzymes used to create level 1 and level 2 modules respectively (Engler et al., 2009; Weber et al., 2011). The full-length cDNA sequence of *OsBZR1* (LOC_Os07g39220/Os07g0580500) was rice codon optimized for ease of detection against the endogenous *OsBZR1* gene and domesticated to remove internal *Bpi*I and *Bsa*I restriction sites (**Supp. dataset S5**). This sequence was then synthesized for cloning. For ubiquitous *OsBZR1* overexpression, the rice codon optimized *OsBZR1* sequence was cloned downstream of the maize (*Zea mays*) *UBIQUITIN* promoter (p*ZmUBI*) and upstream of a nos terminator (nost) (**Supp. fig. S6 A**). The p*ZmUBI* was made up of 983 bp upstream of the transcription start site and 1014 bp of the first intron (**Supp. dataset S5**). For the bundle sheath cell-specific *OsBZR1* line, the rice codon optimized *OsBZR1* sequence was cloned downstream of the rice *SULFITE REDUCTASE* promoter (p*OsSIR*) (Hua et al., 2024) and upstream of a nos terminator (nost) (**Supp. fig. S10 A**). The level 1 Golden Gate modules were confirmed through sequencing using backbone specific primers; pL1-F: GCGGACGTTTTTAATGTACTG , pL1-R: CCAATATATCCTGTCAAACACTG. Confirmed level 1 constructs were then cloned with a hygromycin resistance level 1 module which contained the hygromycin resistance gene (**Supp. dataset S5**) downstream of the rice *ACTIN* promoter (p*OsACT)* (**Supp. dataset S5**) and upstream of a nos terminator. This module was used for selection of transformants at T_0 stage and selection of homozygous lines at T_2 stage.

For the *OsBZR1* RNAi lines, the native *OsBZR1* coding sequence was submitted to WeigelWorld WMD amiRNA design website 3.1.18 (http://wmd3.weigelworld.org/), designing against the rice cDNA dataset (v6.1 MLU). The top 2 microRNA sequences were selected and cloned, along with specific stem loop sequences, downstream of the maize *UBIQUITIN* promoter (*pZmUBI).* These level 1 modules were then cloned with a hygromycin resistance level 1 module (p*OsACT-HYG)* and upstream of a nos terminator (**Supp. fig. S5 A**).

MicroRNA sequences used:

bzr1-1: TTTAACGGTACGTCACAGCGA
bzr1-2: TACAAGATTAACCTAAGGCTG

Once cloning of each construct was complete, *Oryza sativa spp. japonica* cv. Kitaake was transformed using *Agrobacterium tumefaciens* as described previously (Hiei & Komari, 2008) with several modifications. Seeds were de-husked and sterilized with 10% (v/v) bleach for 15 min before placing them on nutrient broth (NB) callus induction media containing 2 mg/L 2,4-dichlorophenoxyacetic acid for 4 weeks in the dark at 28°C. Calli were co-incubated with *A. tumefaciens* strain LBA4404 carrying the expression plasmid of interest in NB inoculation medium containing 40 µg/ml acetosyringone for 3 days in the dark at 22°C. Calli were transferred to NB recovery medium containing 300 mg/L timentin for 1 week in the dark at 28°C. They were then transferred to NB selection medium containing 35 mg/L hygromycin B for 4 weeks in the dark at 28°C. Proliferating calli were subsequently transferred to NB regeneration medium containing 100 mg/L myo-inositol, 2 mg/L kinetin, 0.2 mg/L 1-naphthaleneacetic acid, and 0.8 mg/L 6-benzylaminopurine for 4 weeks in the light at 28°C. Plantlets were transferred to NB rooting medium containing 0.1 mg/L 1-naphthaleneacetic acid and incubated in Magenta pots for 2 weeks in the light at 28°C. Finally, plants were transferred Profile Field and Fairway soil amendment (www.rigbytaylor.com) and grown in a walk-in plant growth chamber under a 12-hour photoperiod at a photon flux density of 400 μmol m^{-2} s^{-1} at 28°C day and 20°C night. DNA was isolated from individual T_0 plants and DNA blot analysis performed to determine insertion copy number. Lines with single insertions in different locations of the genome were used for phenotyping experiments.

BL and Brz treatments and chlorophyll analysis

Oryza sativa spp. japonica cv. Kitaake seeds were de-husked and sterilized in 10% (v/v) bleach for 30 min. After washing several times with sterile water, seeds were imbibed in water and incubated at 28°C in the dark for two days. For de-etiolation experiments, seedlings germinated in the dark were transferred in a dark room equipped with a green light into ½ strength Murashige and Skoog (MS) medium (0.8% agar) supplemented with either 10 μM brassinolide (Santa Cruz Biotechnology, Inc.) or 10 μM brassinazole (Merck Life Science UK Ltd., Gillingham, UK). Magentas were covered in aluminium foil and placed in a growth cabinet set to a 28°C, 16-hour day and 20°C, 8-hour night cycle for a further three days. At the beginning of the fourth photoperiod, aluminium foil was removed from the magentas to expose the seedlings to light. Leaf tissue was harvested for chlorophyll quantification and confocal microscopy at 0, 4, 12 and 24 hours after exposure to light. For later stages, sterile germinated seedlings were transferred into ½ strength Murashige and Skoog medium (0.8% agar) supplemented with either 10 μM Brassinolide or 10 μM Brassinazole and placed in a growth cabinet set to a 28°C, 16-hour day and 20°C, 8-hour night cycle until leaf 4 had fully expanded, at which time it was harvested for chlorophyll quantification and confocal microscopy. The concentration of BL was selected after performing the de-etiolation and later time point experiments using a concentration gradient, including 0, 2 μM, 5 μM and 10 μM. The 10 μM concentration had the greatest and most consistent impact on plant morphology, bundle sheath chloroplast area and the number of chloroplasts per bundle sheath cell (**Supp. fig. S1**) and was thus used for downstream experiments. This concentration has also been used previously in rice (Bai et al., 2007).

Tissue for chlorophyll quantification (leaf 4, leaf 8 or seedlings during de-etiolation experiments) was harvested, weighed and immediately flash-frozen in liquid nitrogen. Frozen tissue was ground into a fine powder and suspended in 1 ml of 80% (v/v) acetone. After vortexing, the tissue was incubated on ice for 15 min with occasional mixing of the suspension. The tissue was spun at 13 000 rpm for 5 min at 4°C and supernatant removed. The extraction was repeated, and supernatants pooled before measuring absorbance at 663.6 nm and 646.6 nm. Total chlorophyll content was determined as described previously (Porra et al., 1989).

Chloroplast imaging and quantification

Chloroplasts in individual bundle sheath and mesophyll cells were imaged and quantified using light and confocal laser microscopy. To isolate single mesophyll and bundle sheath cells for light microscopy, cells from leaf 8 of wild-type Kitaake or transgenic rice lines (UBQ Null, UBQ OE, BS Null and BS OE) were isolated following the protocol of Khoshravesh & Sage (2018). Briefly, the middle region of fully expanded leaf 8 was cut into 5 mm-long x 2 mm-wide strips along the leaf proximodistal axis using a razorblade and immediately immersed in room temperature 4% w/v paraformaldehyde (pH 6.9) (Thermo Fisher Scientific Inc.). Fixed tissue was left in paraformaldehyde at 4°C for at least one hour (up to overnight). Cell walls were then digested by incubating in 0.2M sodium-EDTA (pH 9.0) at 55°C for 2 hours, rinsed in digestion buffer (0.15M Na_2HPO_4, 0.04M citric acid, pH 5.3) and then incubated in 2% w/v pectinase from *Aspegillus niger* (Merck Life Science UK Ltd., Gillingham, UK) in digestion buffer at 45°C for 2 hours. Digestion was stopped by incubation in empty digestion buffer twice for 30 minutes at room temperature. After digestion, individual cells were released by mechanical disruption using the bottom of an Eppendorf tube. Isolated mesophyll and bundle sheath cells were imaged by brightfield microscopy using an Olympus BX41 microscope (Olympus UK and Ireland, Southend-on-Sea, UK), recording each cell in the paradermal plane where most of the chloroplasts were in focus. Images were captured using an MP3.3-RTV-R-CLR-10-C MicroPublisher camera and QCapture Pro 7 software (Teledyne Photometrics, Birmingham, UK). The area of individual mesophyll and bundle sheath cell chloroplasts in each image was quantified using ImageJ version 1.53k.

To visualise and quantify chloroplasts in bundle sheath cells using a confocal laser microscope, the methods described in Billakurthi and Hibberd (2023) were followed. Briefly, the middle region of fully expanded leaf 4 and 8 was fixed with 1% (w/v) glutaraldehyde (Thermo Fisher Scientific Inc.) in 1X PBS buffer. Samples were left in fixative for two hours and then washed twice with 1X PBS buffer. Before confocal microscopy, the adaxial side of the fixed leaf material was ablated gently with a fine razor blade to remove mesophyll layers and then incubated in calcofluor white (0.1%; Sigma) for 5 mins to stain cell walls prior to rinsing twice with H_2O. A Leica SP8X confocal microscope upright system (Leica Microsystems) was used for fluorescence imaging of the bundle sheath cell chloroplasts and cell walls. The microscope has two continuous wave laser lines, 405 and 442 nm, a 460-670 nm super continuum white light laser (WLL), four hybrid detectors and one photomultiplier tube. Imaging was conducted using a 25X water immersion objective and Leica Application Suite X software (LAS X; version: 3.5.7.23225). Calcofluor white was excited at 405 nm and emitted fluorescence captured from 452 to 472 nm, gain was adjusted for each image. Chlorophyll autofluorescence was excited at 488 nm and emission captured 672 to 692 nm, gain was set at 100 for all images. For all lines, leaf 4 and 8 from 4 plants were assessed, with 3 different intermediate veins imaged in each leaf. The planar area of individual mesophyll and bundle sheath chloroplasts in each image was quantified using ImageJ version 1.53k.

Serial block-face scanning electron microscopy

To visualise the ultrastructure of individual chloroplasts, serial block-face scanning electron microscopy (SEM) was used. For this, the middle region of fully expanded leaf 8 was cut into 2 mm x 2 mm squares and fixed in 2% (v/v) glutaraldehyde and 2% (w/v) formaldehyde in 0.05 - 0.1 M sodium cacodylate (NaCac) buffer (pH 7.4) containing 2 mM calcium chloride. Samples were vacuum infiltrated overnight, washed 5 times in 0.05 – 0.1 M NaCac 557 buffer, and post-fixed in 1% (v/v) aqueous osmium tetroxide, 1.5% (w/v) potassium ferricyanide in 0.05 M NaCac buffer for 3 days at 4°C. After osmication, samples were washed 5 times in deionized water and post-fixed in 0.1% (w/v) thiocarbohydrazide for 20 min at room temperature in the dark. Samples were then washed 5 times in deionized water and osmicated for a second time for 1 hr. in 2% (v/v) aqueous osmium tetroxide at room temperature. Samples were washed 5 times in deionized water and subsequently stained in 2% (w/v) uranyl acetate in 0.05 M maleate buffer (pH 5.5) for 3 days at 4°C and washed 5 times afterwards in deionized water. Samples were then dehydrated in an ethanol series, transferred to acetone, and then to acetonitrile. Leaf samples were embedded in

Quetol 651 resin mix (TAAB Laboratories Equipment Ltd.) and cured at 60°C for 2 days. Ultra-thin sections of embedded leaf samples were prepared and placed on Melinex (TAAB Laboratories Equipment Ltd) plastic coverslips mounted on aluminium SEM stubs using conductive carbon tabs (TAAB Laboratories Equipment Ltd), sputter-coated with a thin layer of carbon (~30 nm) to avoid charging and imaged in a Verios 460 scanning electron microscope at 4 keV accelerating voltage and 0.2 nA probe current using the concentric backscatter detector in field free (low magnification) or immersion (high magnification) mode (working distance 3.5 – 4 mm, dwell time 3 μs, 1536 x 1024 pixel resolution). SEM stitched maps were acquired at 10,000X magnification using the FEI MAPS automated acquisition software. Greyscale contrast of the images was inverted to allow easier visualisation.

Gas exchange measurements

Fully expanded leaf 8 was used to measure photosynthetic rates using a LI-6800 photosynthesis system (LICOR Biosciences). For the UBQ OE plants, measurements were performed prior to the onset of early senescence. Four individual plants were sampled per line. Measurements were made at a constant airflow of 400 μmol.s^{-1}, leaf temperature of 30°C and relativity humidity of 60%. Leaves were acclimated in the chamber for approximately 10 mins before net photosynthesis (CO_2 assimilation rate - A) measurements were made at ambient conditions (light intensity of 400 μmol photons m^{-2} s^{-1} and intercellular CO_2 concentration (Ci) of 400 μmol.CO_2 mol^{-1} air). All measurements were performed on the midportion of the leaf blade.

Phylogenetic tree inference

To identify putative rice orthologs of transcription factors that mediate BR-responsive gene expression in Arabidopsis (*Arabidopsis thaliana*), the protein coding genes derived from representative gene models were downloaded from Phytozome (Goodstein et al., 2012). These proteomes were subject to orthogroup inference using OrthoFinder (Emms & Kelly, 2015, 2019). The orthogroup containing Arabidopsis BZR1, BES1, (BZR2), BEH1, BEH2, BEH3 and BEH4 was identified. The sequences from this orthogroup were subject to multiple sequence alignment using MergeAlign (Collingridge & Kelly, 2012) followed by bootstrapped maximum likelihood phylogenetic tree inference using IQTREE2 (Minh et al., 2020) with the best fitting model of sequence evolution (JTT+I+G4) inferred from the data.

Total RNA extraction, cDNA library preparation and transcriptome analysis

Total RNA was extracted from leaf 4 using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Genomic DNA was removed from each sample using the RNase-Free DNase Set (Qiagen, Germany). RNA degradation and contamination was checked on 1% (w/v) agarose gels, then RNA quality and concentration determined with the RNA600 Pico Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and ND-200 nanodrop (NanoDrop Technologies Inc., USA). RNA was sent to the Novogene Genomic Sequencing Centre (Cambridge) for library preparation and sequencing using the Illumina NovaSeq 600 PE150 sequencing platform and strategy. At least 6 Gb of raw data per sample was generated for subsequent transcriptome analysis. All transcriptome data has been deposited on NCBI in fasq format.

Quality of raw sequencing data was assessed and controlled using the FastQC platform version 0.11.4 (Andrew, S. 2010). Adapter trimming and filtering of all low quality reads was performed using BBDuk (https://www.geneious.com/plugins/bbduk/) with the following parameters: k=13, ktrim=r, useshortkmers=t, mink=5, qtrim=r, trimq=2- minlength=50, ftl=10, ftr=139. The *Oryza sativa* IRGSP-1.0 transcriptome was downloaded from Ensemble Plants (https://plants.ensemble.org) and used to build a Salmon reference index which was subsequently used to quantify the cleaned reads (Salmon version 1.5.2). For Salmon quantification (Patro et al., 2017), all parameters were left as default. The Salmon alignment and quantification results were checked using MultiQC (Ewels et al., 2016). To check that biological replicates clustered together and to visualise how the overexpression lines differed from corresponding null lines and from each other, a principal component analysis (PCA) was performed using the ggfortify package in R. Transcripts per million (TPM) counts from Salmon alignments were filtered for genes with at least 48 counts across all samples. The filtered data were then normalised to account for library size and transformed to a log scale using rlog transformation to allow for easier visualisation. For the Pearson's correlation heatmap, the filtered, rlog transformed TPM data was used.

To determine changes in expression of individual genes between OE lines and corresponding null lines, the DESeq2 package (version 4.2) in R was used (Love, Huber & Anders, 2014). The quant.sf file generated for each sample from the Salmon quantification was used as the input for the DESeq2 analysis. Independent DESeq2 analyses were performed for the two different overexpression lines (i.e. UBQ OE samples versus corresponding UBQ Null samples and BSC OE samples versus corresponding BSC Null samples). Lists of significantly differentially expressed genes for further investigation were developed by filtering for genes with an adjusted *p*-value less than 0.05. To gain further biological insight into DEG lists, Gene Ontology (GO) enrichment analyses were performed using AgriGO v2 (Tian et al., 2017) with the *Oryza sativa* MSU7.0 genome set as the background reference.

Reverse transcription quantitative PCR (RT-qPCR)

To check transgene expression levels, RNA was extracted from fully expanded leaf 4 as described above. The total RNA was used as a template to synthesize cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. RT-qPCR was carried out on the cDNA using SYBR Green JumpStart Taq ReadyMix (Merck Life Science UK Ltd., Gillingham, UK) on a CFX384 Real-Time System (Bio-Rad) with the following cycle parameters; 94°C for 2 min, (94°C for 15 sec, 60°C for 1 min) x 40. This was performed using primers specific to the endogenous *OsBZR1* sequence (*endOsBZR1*) or primers specific to the rice codon optimized *OsBZR1* sequence (*rcoOsBZR1*) and *OsEF-1*α and *OsUBQ* reference genes (Jain et al., 2006; Jain et al., 2018) with the following sequences.

endOsBZR1-F: ATGCTGCGATTTGGGCGATTTC,
endOsBZR1-R: ACACAGAGATGAACAGTGAAGCC
rcoOsBZR1-F*:* CGTACAACCTCGTGAACCC,
rcoOsBZR1-R*:* CGTCACCCTACCTTTGTCG,
OsEF-1α-F: TTTCACTCTTGGTGTGAAGCAGAT,
OsEF-1α-R: GACTTCCTTCACGATTTCATCGTAA,
OsUBI5-F: ACCACTTCGACCGCCACTACT,
OsUBI5-R: ACGCCTAAGCCTGCTGGTT

Data analyses

Unless otherwise stated, all statistical analyses were performed using StatSoft Statistica software. Statistical analyses included one-way ANOVA followed by Fisher LSD post-hoc analysis and independent t-tests. A *p*-value < 0.05 was considered significant. For instances where multiple hypothesis testing was performed (DEseq2 analyses), adjusted *p*-values (Benjamini-Hochberg) < 0.05 were considered significant. Refer to figure legends for specific statistical analyses conducted for each result presented.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under GeneIDs listed in Supplementary dataset S4.

Supplementary Data

Supplemental Figure S1: Optimization of BL treatment concentration.

Supplemental Figure S2: Bundle sheath cell area of untreated, BL-treated and Brz-treated seedlings during de-etiolation.

Supplemental Figure S3: Number of veins in leaf 4 from untreated, BL-treated and Brz-treated plants.

Supplemental Figure S4. Maximum likelihood phylogenetic tree of the *BZR1* gene family in plants.

Supplemental Figure S5: Development of *OsBZR1* knockout lines through RNA interference (RNAi).

Supplemental Figure S6: Development of rice lines constitutively overexpressing rice codon-optimized *OsBZR1* (UBQ OE).

Supplemental Figure S7: Constitutive overexpression of *OsBZR1* does not impact the number of chloroplasts per bundle sheath cell.

Supplemental Figure S8: Total chlorophyll content in leaf 8 of UBQ Null and UBQ OE lines.

Supplemental Figure S9: Bundle sheath cell size in UBQ Null and UBQ OE lines.

Supplemental Figure S10: Development of rice lines overexpressing rco*OsBZR1* specifically in bundle sheath cells (BS OE).

Supplemental Figure S11: Bundle sheath cell-specific overexpression of *OsBZR1* does not impact chloroplast number per bundle sheath cell.

Supplemental figure S12: Bundle sheath cell-specific overexpression of *OsBZR1* does not impact bundle sheath cell size.

Supplemental Figure S13: Brassinazole suppresses the positive effect of bundle sheath cell-specific overexpression of *OsBZR1*.

Supplemental figure S14: Expression of other master regulators of chloroplast development in the constitutive and bundle sheath cell-specific *OsBZR1* overexpressors.

Funding

The work was funded by BBSRC grant BBP0031171 to J.M.H**.** S.K. was supported by a Royal Society University Research Fellowship. Work in S.K.'s laboratory was supported by the European Union's Horizon 2020 research and innovation programme under grant agreement no. 637765 and by the Wellcome Trust under grant number 226598/Z/22/Z. R.W.H. was supported by a BBSRC studentship through BB/J014427/1.

Acknowledgments

We acknowledge Dr. Karin Müller from the Cambridge Advanced Imaging Centre and Dr. Tina Shreier for their significant help with SEM imaging.

Author contributions

S.K. and R.-W.H. conducted the phylogenetic analysis and identified BZR1 as a potential regulator of chloroplast biogenesis. R.W.H designed the coding sequences for the overexpression and RNAi constructs. A.R.G.P cloned the DNA constructs for stable rice transformation. S.S carried out the stable rice transformation. N.W carried out the Southern blot analyses of transformed rice. L.H. identified the bundle sheath cell-specific promoter. L.H.L grew and genotyped the ubiquitous overexpression lines and contributed intellect and discussion throughout the project. L.C. conducted all experiments and analysed the data. L.C. and J.M.H wrote the manuscript with input from all authors. J.M.H guided execution of experiments and oversaw the project.

Declaration of interests

The authors declare no competing interests.

Figure legends

Figure 1: Brassinosteroids modulate chlorophyll accumulation and bundle sheath chloroplast area and number during de-etiolation. Seeds were germinated in water and transferred in the dark to ½ MS-agar media with or without 10 µM brassinolide (BL) or 10 µM brassinazole (Brz). After 4 days seedlings were transferred to light and shoot tissue harvested 0, 4, 12 and 24 hours (hrs) later for chlorophyll quantification and imaging using confocal laser scanning microscopy. **A:** Representative images of control and BL/Brz treated

seedlings during de-etiolation. **B:** Mean chlorophyll content during de-etiolation normalised to amount of fresh weight (FW) harvested. Data are from 5 biological repeats for each timepoint in each treatment. Error bars represent standard error. **C:** Confocal images of bundle sheath cells and chloroplasts during de-etiolation. Red and blue channels indicate chloroplasts and cell walls respectively. White dotted lines highlight bundle sheath cells. **D** and **E:** Number of chloroplasts per bundle sheath cell (BS) in control and BL/Brz treated seedlings 4 (**D**) and 12 (**E**) hours after exposure to light. Data are derived from confocal microscopy and from at least thirty bundle sheath cells for each timepoint in each treatment. **F** and **G:** Bundle sheath cell chloroplast area in control and BL/Brz treated seedlings 4 (**F**) and 12 (**G**) hours after exposure to light. Data are derived from confocal microscopy and from at least 150 chloroplasts for each timepoint in each treatment. For D, E, F and G Individual data points are overlaid as dots, where each dot represents a single observation. Dots are stacked symmetrically along the y-axis to visualize data density within each group. Letters above violins represent statistically significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following a one-way ANOVA.

Figure 2: Brassinosteroids modulate chlorophyll accumulation and bundle sheath chloroplast area and number in mature leaves. Seeds were germinated in water and transferred to ½ MS-agar media with or without 10 µM brassinolide (BL) or 10 µM brassinazole (Brz). Leaf 4, once fully expanded, was harvested for chlorophyll quantification and imaging using confocal laser scanning microscopy. **A:** Representative images of control and BL/Brz treated plants. White numbers indicate leaf number in order of appearance. **B:** Chlorophyll content of leaf 4 from control and BL/Brz treated plants, data are from 6 biological repeats for each treatment normalised to amount of fresh weight (FW) harvested. **C:** Confocal images of bundle sheath cells and chloroplasts in leaf 4 of controls and BL/Brz treated plants. Red and blue channels indicate chloroplasts and cell walls respectively. White dotted lines highlight bundle sheath cells. **D:** Number of chloroplasts per bundle sheath cell (BS) in leaf 4 from controls and BL/Brz treated plants. Data are derived from confocal microscopy and from at least 70 bundle sheath cells for each treatment. **E:** Bundle sheath cell chloroplast area in leaf 4 from controls and BL/Brz treated plants. Data are derived from confocal microscopy and from at least 550 chloroplasts for each timepoint in each treatment. For B, D and E Individual data points are overlaid as dots, where each dot represents a single observation. Dots are stacked symmetrically along the y-axis to visualize

data density within each group. Letters above violins represent statistically significant differences ($p \leq 0.05$) in mean values as determined by Fisher LSD post-hoc analysis following one-way ANOVA.

Figure 3: Ubiquitous overexpression of *OsBZR1* increases the area of chloroplasts in rice bundle sheath cells but impacts plant health. The rice codon optimized sequence for *BZR1* (rco*OsBZR1*) was cloned upstream of the maize *UBIQUITIN* promoter (p*ZmUBI*) and transformed into rice to generate constitutive overexpression lines (UBQ OE). **A:** Representative images from brightfield microscopy of individual bundle sheath and mesophyll cells from leaf 8. White dotted lines highlight individual chloroplasts within each bundle sheath cell. **B** and **C:** Chloroplast area in bundle sheath (BS) (**B**) and mesophyll (M) (**C**) cells from leaf 8. Chloroplast areas are from quantification of the brightfield microscopy. The center dot within each violin represents the mean chloroplast area. Percentages above violins indicate the change in chloroplast area of overexpressor lines compared with corresponding nulls. No statistically significant change in chloroplast area is represented by “ns”. The value below each violin is the mean chloroplast area calculated for that line and n represents the number of chloroplasts quantified. Four biological replicates were used for each line. **D:** Images derived from confocal laser scanning microscopy of bundle sheath cells and chloroplasts in leaf 4. Red and blue channels indicate chloroplasts and cell walls respectively. White dotted lines highlight bundle sheath cells. **E** and **F:** Bundle sheath cell chloroplast area in leaf 4 (**E**) and leaf 8 (**F**). Chloroplasts quantified are from confocal microscopy. The center dot within each violin represents the mean chloroplast area. Percentages above violins indicate the change in chloroplast area of overexpressor lines compared with corresponding nulls. No statistically significant change in chloroplast area is represented by “ns”. The value below each violin is the mean chloroplast area calculated for that line and n represents the number of chloroplasts assessed. Four biological replicates were used for each line. **G:** Scanning electron microscope (SEM) images of bundle sheath and mesophyll cell chloroplasts from leaf 8. **H:** Rate of net photosynthesis under conditions of growth for overexpressor lines compared with corresponding nulls. Data are from 4 biological replicates for each line. **I:** Representative images of leaf 8 from null and overexpressor plants of the same age depicting increased senescence. **J:** Number of seeds produced by each line. Data are from 4 biological replicates for each line. For figures B, C, E, F, H and J, stars above violins or boxes indicate a statistically significant difference

between overexpressor lines compared with corresponding null as determined by independent t-test, where $p \leq 0.05$ is flagged with one star (*), $p \leq 0.01$ is flagged with 2 stars (**) and $p \leq 0.001$ is flagged with three stars (***). No statistically significant change is represented by “ns”. For figures H and J, the box plots show the median and the interquartile range (IQR) between the first and third quartiles, whiskers indicate the smallest and largest values within 1.5 × IQR from the quartiles, while points beyond this range are considered outliers

Figure 4: Bundle sheath cell-specific overexpression of *OsBZR1* increases the area of chloroplasts in rice bundle sheath cells with no impact on plant health. The rice codon optimized sequence for *BZR1* (rco*OsBZR1*) was cloned upstream of the rice bundle sheath cell-specific *SULPHITE REDUCTASE* promoter (p*OsSiR*) and transformed into rice to generate cell-specific overexpression lines (BS OE). **A:** Representative images from brightfield microscopy of individual bundle sheath and mesophyll cells from leaf 8. White dotted lines highlight individual chloroplasts within each bundle sheath cell. **B** and **C:** Chloroplast area in bundle sheath (BS) (**B**) and mesophyll (M) (**C**) cells from leaf 8. Chloroplast areas are from quantification of the brightfield microscopy. The center dot within each violin represents the mean chloroplast area. Percentages above violins indicate the change in chloroplast area of overexpressor lines compared with corresponding nulls. No statistically significant change in chloroplast area is represented by “ns”. The value below each violin is the mean chloroplast area calculated for that line and n represents the number of chloroplasts quantified. Four biological replicates were quantified for each line. **D:** Images derived from confocal laser scanning microscopy of bundle sheath cells and chloroplasts in leaf 4. Red and blue channels indicate chloroplasts and cell walls respectively. White dotted lines highlight bundle sheath cells. **E** and **F:** Bundle sheath cell chloroplast area in leaf 4 (**E**) and leaf 8 (**F**). Chloroplasts quantified are from the confocal microscopy. The center dot within each violin represents the mean chloroplast area. Percentages above violins indicate the change in chloroplast area of overexpressor lines compared with corresponding nulls. No statistically significant change in chloroplast area is represented by “ns”. The value below each violin is the mean chloroplast area calculated for that line and n represents the number of chloroplasts quantified. Four biological replicates were used for each line. **G:** Scanning electron microscope (SEM) images of bundle sheath and mesophyll cell chloroplasts from leaf 8. **H:** Total chlorophyll in leaf 8 of overexpressor lines compared with corresponding

nulls. Data are from 4 biological repeats for each line. **I:** Rate of net photosynthesis under conditions of growth for overexpressor lines compared with corresponding nulls. Data are from 4 biological replicates for each line. **J:** Representative images of leaf 8 from null and overexpressor plants of the same age. **K:** Number of seeds produced by each line. Data are from 4 biological replicates for each line. For figures B, C, E, F, H, I and K stars above violins or boxes indicate a statistically significant difference between overexpressor and corresponding null as determined by independent t-test, where $p \leq 0.05$ is flagged with one star (*), $p \leq 0.01$ is flagged with 2 stars (**) and $p \leq 0.001$ is flagged with three stars (***). No statistically significant change is represented by "ns". For figures H, I and k, the box plots show the median and the interquartile range (IQR) between the first and third quartiles, whiskers indicate the smallest and largest values within 1.5 × IQR from the quartiles, while points beyond this range are considered outliers.

Figure 5: Transcriptome analyses of constitutive and bundle sheath cell-specific *OsBZR1* overexpressors. RNA from leaf 4 of 4 biological replicates from all of the UBQ Null, UBQ OE, BS Null and BS OE lines was used for cDNA library construction and subsequent transcriptome sequencing. **A:** Principal component analysis (PCA) indicates the transcriptome data separated primarily based on genotype. **B:** Heatmap showing relatedness of all samples based on Pearson's correlation performed on log transformed data. The colour of each block indicates the Pearson's correlation value. **C:** Numbers of significantly (*p*-adj. < 0.05) differently expressed genes (DEGs) were determined by DESeq2 analysis which compared samples from an overexpressor with its corresponding null. Up-regulated genes are represented by black bars, and down-regulated genes by white bars. Numbers above each bar indicate the count of DEGs represented by the bar. **D:** Gene ontology (GO) terms enriched in the significantly differentially expressed gene lists from the overexpressors. Circle size depicts percentage, which is the number of genes in the given DEG list with the GO term divided by the number of genes in the reference genome with the GO term. The colour of each circle indicates the statistical significance of enrichment for each GO term, with FDR representing false discovery rate. **E:** Heatmaps showing the expression of genes of interest significantly differentially expressed in all three lines of constitutive overexpressors. The colour of each block indicates the Log2 fold change (FC). A black dot within each block indicates a statistically significant change in Log2 FC. **F:** Log2 FC in expression of genes involved in brassinosteroid (BR) signalling, BZR1 downstream

genes, BR biosynthesis and catabolism and chloroplast development. Stars above bars indicate a statistically significant difference in Log2 FC expression where *p*-adj. ≤ 0.05 is flagged with one star (*), *p*-adj. ≤ 0.01 is flagged with 2 stars (**) and *p*-adj. ≤ 0.001 is flagged with three stars (***). Error bars represent standard error. For A, B and F, UBQ Null, UBQ OE, BS Null and BE OE samples are represented in light grey, blue, dark grey and purple respectively. For E and F, Log2 FC was determined by DESeq2 analyses using four biological replicates for each line. Statistical significance was determined using the Wald test for pairwise comparisons and *p*-values adjusted for multiple testing using the Benjamini-Hochberg (FDR) correction. Refer to Supp. dataset S4 for full gene names and gene IDs.

Supplementary Figure legend titles

Supplemental Figure 1: Optimization of BL treatment concentration.

Supplemental Figure 2: Bundle sheath cell area of untreated, BL-treated and Brz-treated seedlings during de-etiolation.

Supplemental Figure 3: Number of veins in leaf 4 from untreated, BL-treated and Brz-treated plants.

Supplemental Figure 4. Maximum likelihood phylogenetic tree of the *BZR1* gene family in plants.

Supplemental Figure 5: Development of *OsBZR1* knockout lines through RNA interference (RNAi).

Supplemental Figure 6: Development of rice lines constitutively overexpressing codon-optimized *OsBZR1* (*UBQ* OE).

Supplemental Figure 7: Constitutive overexpression of *OsBZR1* does not impact the number of chloroplasts per bundle sheath cell.

Supplemental Figure 8: Total chlorophyll content in leaf 8 of *UBQ* Null and *UBQ* OE lines.

Supplemental Figure 9: Bundle sheath cell size in *UBQ* Null and *UBQ* OE lines.

Supplemental Figure 10: Development of lines overexpressing rco*OsBZR1* specifically in bundle sheath cells (BS OE).

Supplemental Figure 11: Bundle sheath cell-specific overexpression of *OsBZR1* does not impact chloroplast number per bundle sheath cell.

Supplemental figure 12: Bundle sheath cell-specific overexpression of *OsBZR1* does not impact bundle sheath cell size.

Supplemental Figure 13: Brassinazole suppresses the positive effect of bundle sheath cell-specific overexpression of *OsBZR1*.

Supplemental figure 14: Expression of other master regulators of chloroplast development in the constitutive and bundle sheath cell-specific *OsBZR1* overexpressors.

References

Ahmar, S., & Gruszka, D. (2022). *In-Silico* Study of Brassinosteroid Signalling Genes in Rice Provides Insight Into Mechanisms Which Regulate Their Expression. *Frontiers in Genetics*, *13*. https://doi.org/10.3389/fgene.2022.953458

Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Asami, T., Min, Y. K., Nagata, N., Yamagishi, K., Takatsuto, S., Fujioka, S., Murofushi, N., Yamaguchi, I., & Yoshida, S. (2000). Characterization of Brassinazole, a Triazole-Type Brassinosteroid Biosynthesis Inhibitor 1. *Plant Physiology, 123,* 93-99

Azpiroz, R., Wu, Y., Locascio, J. C., & Feldmann, K. A. (1998). An Arabidopsis Brassinosteroid-Dependent Mutant Is Blocked in Cell Elongation. *The Plant Cell* (Vol. 10).

Bai, M.-Y., Zhang, L.-Y., Gampala, S. S., Zhu, S.-W., Song, W.-Y., Chong, K., & Wang, Z.-Y. (2007). Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signalling in rice. *PNAS, 104*(34)

Billakurthi, K., & Hibberd, J. M. (2023). A rapid and robust leaf ablation method to visualize bundle sheath cells and chloroplasts in C3 and C4 grasses. *Plant Methods*, *19*(1). https://doi.org/10.1186/s13007-023-01041-x

Cackett, L., Luginbuehl, L. H., Schreier, T. B., Lopez-Juez, E., & Hibberd, J. M. (2022). Chloroplast development in green plant tissues: the interplay between light, hormone, and transcriptional regulation. *New Phytologist, 233(5),* 2000-2016

Chaiwanon, J., & Wang, Z. Y. (2015). Spatiotemporal brassinosteroid signalling and antagonism with auxin pattern stem cell dynamics in Arabidopsis roots. *Current Biology*, *25*(8), 1031–1042. https://doi.org/10.1016/j.cub.2015.02.046

Chory, J., Nagpal, P., & Petob, C. A. (1991). Phenotypic and Genetic Analysis of det2, a New Mutant That Affects Light-Regulated Seedling Development in Arabidopsis. *The Plant Cell, 3*

Clouse, S. D., Langford, M., & Mcmorris, T. C. (1996). A Brassinosteroid-Insensitive Mutant in *Arabidopsis thaliana* Exhibits Multiple Defects in Growth and Development'. *Plant Physiol (11)*. https://academic.oup.com/plphys/article/111/3/671/6070319

Collingridge, P. W., & Kelly, S. (2012). MergeAlign: improving multiple sequence alignment performance by dynamic reconstruction of consensus multiple sequence alignments. *BMC Bioinformatics 13(117)*

Emms, D. M., & Kelly, S. (2015). OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biology*, *16*(1). https://doi.org/10.1186/s13059-015-0721-2

Emms, D. M., & Kelly, S. (2019). OrthoFinder: Phylogenetic orthology inference for comparative genomics. *Genome Biology*, *20*(1). https://doi.org/10.1186/s13059-019-1832-y

Engler, C., Gruetzner, R., Kandzia, R., & Marillonnet, S. (2009). Golden gate shuffling: A one-pot DNA shuffling method based on type ils restriction enzymes. *PLoS ONE*, *4*(5). https://doi.org/10.1371/journal.pone.0005553

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Fàbregas, N., Lozano-Elena, F., Blasco-Escámez, D., Tohge, T., Martínez-Andújar, C., Albacete, A., Osorio, S., Bustamante, M., Riechmann, J. L., Nomura, T., Yokota, T., Conesa, A., Alfocea, F. P., Fernie, A. R., & Caño-Delgado, A. I. (2018). Overexpression of the vascular brassinosteroid receptor BRL3 confers drought resistance without penalizing plant growth. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06861-3

Frangedakis, E., Yelina, N. E., Billakurthi, K., Hua, L., Schreier, T., Dickinson, P. J., Tomaselli, M., Haseloff, J., & Hibberd, J. M. (2024). MYB-related transcription factors control chloroplast biogenesis. *Cell*. https://doi.org/10.1016/j.cell.2024.06.039

González-García, M. P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E., & Caño-Delgado, A. I. (2011). Brassinosteroids control meristem size by promoting cell cycle progression in Arabidopsis roots. *Development*, *138*(5), 849–859. https://doi.org/10.1242/dev.057331

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., & Rokhsar, D. S. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research*, *40*(D1). https://doi.org/10.1093/nar/gkr944

Hacham, Y., Holland, N., Butterfield, C., Ubeda-Tomas, S., Bennett, M. J., Chory, J., & Savaldi-Goldstein, S. (2011). Brassinosteroid perception in the epidermis controls root meristem size. *Development*, *138*(5), 839–848. https://doi.org/10.1242/dev.061804

Hibberd, J. M., Sheehy, J. E., & Langdale, J. A. (2008). Using C4 photosynthesis to increase the yield of rice-rationale and feasibility. *Current Opinion in Plant Biology, 11(2)*, 288-231. https://doi.org/10.1016/j.pbi.2007.11.002

Hiei, Y., & Komari, T. (2008). Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols*, *3*(5), 824–834. https://doi.org/10.1038/nprot.2008.46

Hong, Z., Ueguchi-Tanaka, M., Shimizu-Sato, S., Inukai, Y., Fujioka, S., Shimada, Y., Takatsuto, S., Agetsuma, M., Yoshida, S., Watanabe, Y., Uozu, S., Kitano, H., Ashikari, M., & Matsuoka, M. (2002). Loss-of-function of a rice brassinosteroid biosynthetic enzyme, C-6 oxidase, prevents the organized arrangement and polar elongation of cells in the leaves and stem. *Plant Journal*, *32*(4), 495–508. https://doi.org/10.1046/j.1365-313X.2002.01438.x

Hua, L., Wang, N., Stanley, S., Donald, R., Billakurthi, K., & Rita Borba, A. (n.d.). *A transcription factor quartet orchestrating bundle sheath expression in rice 1 2*. https://doi.org/10.1101/2024.06.17.599020

Hudson, D., Guevara, D. R., Hand, A. J., Xu, Z., Hao, L., Chen, X., Zhu, T., Bi, Y. M., & Rothstein, S. J. (2013). Rice cytokinin GATA transcription factor1 regulates chloroplast development and plant architecture. *Plant Physiology*, *162*(1), 132–144. https://doi.org/10.1104/pp.113.217265

Iliev, E. A., Xu, W., Polisensky, D. H., Oh, M. H., Torisky, R. S., Clouse, S. D., & Braam, J. (2002). Transcriptional and posttranscriptional regulation of Arabidopsis TCH4 expression by diverse stimuli. Roles of cis regions and brassinosteroids. *Plant Physiology*, *130*(2), 770–783. https://doi.org/10.1104/pp.008680

Hudson, D., Guevara, D., Yaish, M. W., Hannam, C., Long, N., Clarke, J. D., Bi, Y. M., & Rothstein, S. J. (2011). GNC and CGA1 modulate chlorophyll biosynthesis and glutamate synthase (GLU1/FD-GOGAT) expression in Arabidopsis. *PLoS ONE*, *6*(11). https://doi.org/10.1371/journal.pone.0026765

Jain, M., Nijhawan, A., Tyagi, A. K., & Khurana, J. P. (2006). Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, *345*(2), 646–651. https://doi.org/10.1016/j.bbrc.2006.04.140

Jain, N., Vergish, S., & Khurana, J. P. (2018). Validation of house-keeping genes for normalization of gene expression data during diurnal/circadian studies in rice by RT-qPCR. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-21374-1

Kang, Y. H., Breda, A., & Hardtke, C. S. (2017). Brassinosteroid signalling directs formative cell divisions and protophloem differentiation in Arabidopsis root meristems. *Development 144*(2), 272–280. https://doi.org/10.1242/dev.145623

Khoshravesh R, & Sage T. (2018). *Creating Leaf Cell Suspensions for Characterization of Mesophyll and Bundle Sheath Cellular Features* (S. Covshoff, Ed.; Vol. 1770). Springer New York. https://doi.org/10.1007/978-1-4939-7786-4

Kim, Y., Park, S. U., Shin, D. M., Pham, G., Jeong, Y. S., & Kim, S. H. (2020). ATBS1-INTERACTING FACTOR 2 negatively regulates dark-and brassinosteroid-induced leaf senescence through interactions with INDUCER of CBF EXPRESSION 1. *Journal of Experimental Botany*, *71*(4), 1475–1490. https://doi.org/10.1093/jxb/erz533

Kobayashi, K., Baba, S., Obayashi, T., Sato, M., Toyooka, K., Keränen, M., Aro, E. M., Fukaki, H., Ohta, H., Sugimoto, K., & Masudaa, T. (2012). Regulation of root greening by light and auxin/cytokinin signalling in Arabidopsis. *Plant Cell*, *24*(3), 1081–1095. https://doi.org/10.1105/tpc.111.092254

Kobayashi, K., Sasaki, D., Noguchi, K., Fujinuma, D., Komatsu, H., Kobayashi, M., Sato, M., Toyooka, K., Sugimoto, K., Niyogi, K. K., Wada, H., & Masuda, T. (2013). Photosynthesis of root chloroplasts developed in Arabidopsis lines overexpressing GOLDEN2-LIKE transcription factors. *Plant and Cell Physiology*, *54*(8), 1365–1377. https://doi.org/10.1093/pcp/pct086

Komatsu, T., Kawaide, H., Saito, C., Yamagami, A., Shimada, S., Nakazawa, M., Matsui, M., Nakano, A., Tsujimoto, M., Natsume, M., Abe, H., Asami, T., & Nakano, T. (2010). The chloroplast protein BPG2 functions in brassinosteroid-mediated post-transcriptional accumulation of chloroplast rRNA. *Plant Journal*, *61*(3), 409–422. https://doi.org/10.1111/j.1365-313X.2009.04077.x

Lee, D. Y., Hua, L., Khoshravesh, R., Giuliani, R., Kumar, I., Cousins, A., Sage, T. L., Hibberd, J. M., & Brutnell, T. P. (2021). Engineering chloroplast development in rice through cell-specific control of endogenous genetic circuits. *Plant Biotechnology Journal*, *19*(11), 2291–2303. https://doi.org/10.1111/pbi.13660

Lee, M. S., Boyd, R. A., Boateng, K. A. & Ort, D.R. (2023) Exploring 3D leaf anatomical traits for C4 photosynthesis: chloroplast and plasmodesmata pit field size in maize and sugarcane. *New Phytologist, 239*: 506-517.

Leegood, R. C. (2008). Roles of the bundle sheath cells in leaves of C_3 plants. *Journal of Experimental Botany*, *59*(7), 1663–1673. https://doi.org/10.1093/jxb/erm335

Li, J., Hee Nam, K., Vafeados, D., & Chory, J. (2001). *BIN2,* a New Brassinosteroid-Insensitive Locus in Arabidopsis*. Plant Physiology (127),* 14-22.

Li, X., Wang, P., Li, J., Wei, S., Yan, Y., Yang, J., Zhao, M., Langdale, J. A., & Zhou, W. (2020). Maize GOLDEN2-LIKE genes enhance biomass and grain yields in rice by improving photosynthesis and reducing photoinhibition. *Communications Biology*, *3*(1). https://doi.org/10.1038/s42003-020-0887-3

Lim, C., Kim, Y., Shim, Y., Cho, S. H., Yang, T. J., Song, Y. H., Kang, K., & Paek, N. C. (2024). Rice OsGATA16 is a positive regulator for chlorophyll biosynthesis and chloroplast development. *Plant Journal*, *117*(2), 599–615. https://doi.org/10.1111/tpj.16517

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12). https://doi.org/10.1186/s13059-014-0550-8

Luo, X. M., Lin, W. H., Zhu, S., Zhu, J. Y., Sun, Y., Fan, X. Y., Cheng, M., Hao, Y., Oh, E., Tian, M., Liu, L., Zhang, M., Xie, Q., Chong, K., & Wang, Z. Y. (2010). Integration of Light- and Brassinosteroid-Signalling Pathways by a GATA Transcription Factor in Arabidopsis. *Developmental Cell*, *19*(6), 872–883. https://doi.org/10.1016/j.devcel.2010.10.023

Manghwar, H., Hussain, A., Ali, Q., & Liu, F. (2022). Brassinosteroids (BRs) Role in Plant Development and Coping with Different Stresses. *International Journal of Molecular Sciences, 23(3)*. MDPI. https://doi.org/10.3390/ijms23031012

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Von Haeseler, A., Lanfear, R., & Teeling, E. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530–1534. https://doi.org/10.1093/molbev/msaa015

Mori, M., Nomura, T., Ooka, H., Ishizaka, M., Yokota, T., Sugimoto, K., Okabe, K., Kajiwara, H., Satoh, K., Yamamoto, K., Hirochika, H., & Kikuchi, S. (2002). Isolation and characterization of a rice dwarf mutant with a defect in brassinosteroid biosynthesis. *Plant Physiology*, *130*(3), 1152–1161. https://doi.org/10.1104/pp.007179

Nakamura, A., Shimada, Y., Goda, H., Fujiwara, M. T., Asami, T., & Yoshida, S. (2003). AXR1 is involved in BR-mediated elongation and SAUR-AC1 gene expression in Arabidopsis. *FEBS Letters*, *553*(1–2), 28–32. https://doi.org/10.1016/S0014-5793(03)00945-1

Nakamura, H., Muramatsu, M., Hakata, M., Ueno, O., Nagamura, Y., Hirochika, H., Takano, M., & Ichikawa, H. (2009). Ectopic overexpression of the transcription factor *Osglk1* induces chloroplast development in non-green rice cells. *Plant and Cell Physiology*, *50*(11), 1933–1949. https://doi.org/10.1093/pcp/pcp138

Nolan, T. M., Vukašinović, N., Hsu, C. W., Zhang, J., Vanhoutte, I., Shahan, R., Taylor, I. W., Greenstreet, L., Heitz, M., Afanassiev, A., Wang, P., Szekely, P., Brosnan, A., Yin, Y., Schiebinger, G., Ohler, U., Russinova, E., & Benfey, P. N. (2023). Brassinosteroid gene regulatory networks at cellular resolution in the Arabidopsis root. *Science*, *379*(6639). https://doi.org/10.1126/science.adf4721

Nolan, T. M., Vukasinović, N., Liu, D., Russinova, E., & Yin, Y. (2020). Brassinosteroids: Multidimensional regulators of plant growth, development, and stress responses. *Plant Cell*, *32*(2), 298–318. https://doi.org/10.1105/tpc.19.00335

Oh, E., Zhu, J., Wang, Z. (2012) Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nature Cell Biology, 14*: 802–809.

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, *14*(4), 417–419. https://doi.org/10.1038/nmeth.4197

Porra R. J., Thompson W A, & Kriedemann P E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta, 957*.

Qiao, S., Sun, S., Wang, L., Wu, Z., Li, C., Li, X., Wang, T., Leng, L., Tian, W., Lu, T., & Wang, X. (2017). The RLA1/SMOS1 transcription factor functions with OsBZR1 to regulate brassinosteroid signaling and rice architecture. *Plant Cell*, *29*(2), 292–309. https://doi.org/10.1105/tpc.16.00611

Sage, T. L., & Sage, R. F. (2009). The functional anatomy of rice leaves: Implications for refixation of photorespiratory CO_2 and efforts to engineer C_4 photosynthesis into rice. *Plant & Cell Physiology. 50(4)*, 756-777.

Sage, R. F., Sage, T.L., Kocacinar, F. (2012). Photorespiration and the evolution of C4 photosynthesis. *Annual Review of Plant Biology,* 63, 19–47.

Savaldi-Goldstein, S., Peto, C., & Chory, J. (2007). The epidermis both drives and restricts plant shoot growth. *Nature*, *446*(7132), 199–202. https://doi.org/10.1038/nature05618

Smith, E. N., van Aalst, M., Tosens, T., Niinemets, Ü., Stich, B., Morosinotto, T., Alboresi, A., Erb, T. J., Gómez-Coronado, P. A., Tolleter, D., Finazzi, G., Curien, G., Heinemann, M., Ebenhöh, O., Hibberd, J. M., Schlüter, U., Sun, T., & Weber, A. P. M. (2023). Improving photosynthetic efficiency toward food security:

Strategies, advances, and perspectives. *Molecular Plant, 16*(10), 1547-1563, https://doi.org/10.1016/j.molp.2023.08.017

Sun, Y., Fan, X. Y., Cao, D. M., Tang, W., He, K., Zhu, J. Y., He, J. X., Bai, M. Y., Zhu, S., Oh, E., Patil, S., Kim, T. W., Ji, H., Wong, W. H., Rhee, S. Y., & Wang, Z. Y. (2010). Integration of Brassinosteroid Signal Transduction with the Transcription Network for Plant Growth Regulation in Arabidopsis. *Developmental Cell*, *19*(5), 765–777. https://doi.org/10.1016/j.devcel.2010.10.010

Szekeres, M. S., & Né, K. (1996). Brassinosteroids Rescue the Deficiency of CYP90, a Cytochrome P450, Controlling Cell Elongation and De-etiolation in Arabidopsis. *Cell, 85*.

Tachibana, R., Yamagami, A., Miyagi, S., Nakazawa-Miklasevica, M., Matsui, M., Sakuta, M., Tanaka, R., Asami, T., & Nakano, T. (2022). BRZ-INSENSITIVE-PALE GREEN 1 is encoded by chlorophyll biosynthesis enzyme gene that functions in the downstream of brassinosteroid signaling. *Bioscience, Biotechnology and Biochemistry*, *86*(8), 1041–1048. https://doi.org/10.1093/bbb/zbac071

Tachibana, R., Abe, S., Marugami, M., Yamagami, A., Akema, R., Ohashi, T., Nishida, K., Nosaki, S., Miyakawa, T., Tanokura, M., Kim, J. M., Seki, M., Inaba, T., Matsui, M., Ifuku, K., Kushiro, T., Asami, T., & Nakano, T. (2024). BPG4 regulates chloroplast development and homeostasis by suppressing GLK transcription factors and involving light and brassinosteroid signaling. *Nature Communications*, *15*(1). https://doi.org/10.1038/s41467-023-44492-5

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W., & Su, Z. (2017). AgriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*, *45*(W1), W122–W129. https://doi.org/10.1093/nar/gkx382

Yu, X., Li, L., Zola, J., Aluru, M., Ye, H., Foudree, A., Guo, H., Anderson, S., Aluru, S., Liu, P. et al. (2011) A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in *Arabidopsis thaliana*. *The Plant Journal, 65*, 634–646.

Vragović, K., Selaa, A., Friedlander-Shani, L., Fridman, Y., Hacham, Y., Holland, N., Bartom, E., Mockler, T. C., & Savaldi-Goldstein, S. (2015). Translatome analyses capture of opposing tissue specific brassinosteroid signals orchestrating root meristem differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(3), 923–928. https://doi.org/10.1073/pnas.1417947112

Wang, L., Tian, Y., Shi, W., Yu, P., Hu, Y., Lv, J., Fu, C., Fan, M., & Bai, M. Y. (2020). The miR396-GRFs module mediates the prevention of photo-oxidative damage by brassinosteroids during seedling de-etiolation in Arabidopsis. *Plant Cell*, *32*(8), 2525–2542. https://doi.org/10.1105/tpc.20.00057

Wang, P., Khoshravesh, R., Karki, S., Tapia, R., Balahadia, C. P., Bandyopadhyay, A., Quick, W. P., Furbank, R., Sage, T. L., & Langdale, J. A. (2017). Re-creation of a Key Step in the Evolutionary Switch from C_3 to C4 Leaf Anatomy. *Current Biology*, *27*(21), 3278-3287.e6. https://doi.org/10.1016/j.cub.2017.09.040

Wu, C. Y., Trieu, A., Radhakrishnan, P., Kwok, S. F., Harris, S., Zhang, K., Wang, J., Wan, J., Zhai, H., Takatsuto, S., Matsumoto, S., Fujiok, S., Feldmann, K. A., & Pennell, R. I. (2008). Brassinosteroids regulate grain filling in rice. *Plant Cell*, *20*(8), 2130–2145. https://doi.org/10.1105/tpc.107.055087

Xiong, M., Yu, J., Wang, J., Gao, Q., Huang, L., Chen, C., Zhang, C., Fan, X., Zhao, D., Liu, Q. Q., & Li, Q. F. (2022). Brassinosteroids regulate rice seed germination through the BZR1-RAmy3D transcriptional module. *Plant Physiology*, *189*(1), 402–418. https://

Xu, W., Purugganan, M. M., Polisensky, D. H., Antosiewicz, D. M., A9', S. C., & Braamay2, J. (1995). Arabidopsis TCH4, Regulated by Hormones and the Environment, Encodes a Xyloglucan Endotransglycosylase. *The Plant Cell* (Vol. 7)

Zhang, D., Tan, W., Yang, F., Han, Q., Deng, X., Guo, H., Liu, B., Yin, Y., & Lin, H. (2021). A BIN2-GLK1 Signaling Module Integrates Brassinosteroid and Light Signaling to Repress Chloroplast Development in the Dark. *Developmental Cell*, *56*(3), 310-324.e7. https://doi.org/10.1016/j.devcel.2020.12.001