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Zaxinone Synthase overexpression modulates rice physiology and metabolism, enhancing nutrient uptake, growth and productivity

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Abstract

The rice *Zaxinone Synthase (ZAS)* gene encodes a carotenoid cleavage dioxygenase (CCD) that forms the apocarotenoid growth regulator zaxinone in vitro. Here, we generated and characterized constitutive *ZAS*-overexpressing rice lines, to better understand *ZAS* role in determining zaxinone content and regulating growth and architecture. *ZAS* overexpression enhanced endogenous zaxinone level, promoted root growth and increased the number of productive tillers, leading to about 30% higher grain yield per plant. Hormone analysis revealed a decrease in strigolactone (SL) content, which we confirmed by rescuing the high-tillering phenotype through application of a SL analogue. Metabolomics analysis revealed that *ZAS* over-expressing plants accumulate higher amounts of monosaccharide sugars, in line with transcriptome analysis. Moreover, transgenic plants showed higher carbon

(C) assimilation rate and elevated root phosphate, nitrate and sulphate level, enhancing the tolerance towards low phosphate (Pi). Our study confirms *ZAS* as an important determinant of rice growth and architecture and shows that *ZAS* regulates hormone homoeostasis and a combination of physiological processes to promote growth and grain yield, which makes this gene an excellent candidate for sustainable crop improvement.

KEYWORDS
apocarotenoids, strigolactone, tiller number, zaxinone

1 | INTRODUCTION

Carotenoids are a class of tetraterpenes (C_{40}) pigments, which are characterized by their long hydrocarbon chains containing a conjugated double-bond system (Moise et al., 2014). They are responsible for the vibrant colours seen in fruits and flowers and have an essential role in photosynthesis (Bouvier et al., 2003; Hashimoto et al., 2016; Nisar et al., 2015; Zheng et al., 2020). Apocarotenoids, formed through the oxidative cleavage of double bonds in carotenoids, play a crucial role within the plant kingdom, as they serve as precursors for hormones and as pigments, aroma, scent constituents and regulatory molecules (Moreno et al., 2021; Zheng et al., 2021). Notably, abscisic acid (ABA) and strigolactones (SLs) are well-studied apocarotenoid hormones that play crucial roles in various aspects of plant growth, development and adaptation (Al-Babili & Bouwmeester, 2015; Cutler et al., 2010). The formation of apocarotenoids is primarily catalyzed by a family of carotenoid cleavage dioxygenases (CCDs), which exhibits divergent cleavage properties leading to the formation of various apocarotenoids with unique features and functions (Ahrazem et al., 2016; Auldridge et al., 2006). The genome of *Arabidopsis thaliana* encodes nine CCD members, which are designed as CCD1, CCD4, CCD7, CCD8 and five 9-*cis*-epoxycarotenoid cleavage dioxygenases (NCED2, 3, 5, 6 and 9). CCD1 and CCD4 enzymes possess a broader substrate specificity, allowing them to cleave a diverse range of carotenoids and apocarotenoids, which results in various volatiles and/or apocarotenoid pigments (Zheng et al., 2021). CCD7 is a SL biosynthetic enzyme cleaving 9-*cis*-β-carotene at positions 9, 10 or 9′,10′ to produce 9-*cis*-β-apo-10-carotenal and β-ionone (Alder et al., 2012). Then, CCD8 mediates this intermediate into carlactone (CL), the precursor of SLs, by cleaving at positions 13, 14 of 9-*cis*-β-apo-10-carotenal (Alder et al., 2012). NCEDs are involved in ABA biosynthesis, which cleaves the 11, 12 (11′, 12′) double bond of 9-*cis*-epoxy carotenoids to yield xanthoxin, the precursor of ABA (Schwartz et al., 1997; Tan et al., 2003). Recent studies revealed a bypass in ABA biosynthesis, leading to nonepoxydated β-apo-11-carotenoids to xanthoxin (Jia et al., 2022).

ZAS, a recently discovered member of the CCD family, cleaves apo-10′-zeaxanthinal, at the C13–C14 double bond, leading to zaxinone (apo-13-zeaxanthinal) (Wang et al., 2019). Zaxinone is a growth regulator and an apocarotenoid hormone candidate, which is required for normal rice growth and development and negatively regulates SL biosynthesis (Wang et al., 2019; Wang, Jamil, et al., 2020). Corresponding *ZAS* loss-of-function mutant in rice exhibited reduced zaxinone content in the roots, accompanied by severe growth defects, such as reduced root and shoot biomass, lower tiller number and increased SL level (Wang et al., 2019). Exogenous application of zaxinone partially rescued the growth defects in *zas* mutant and also promoted root growth in wild-type seedlings and repressed SL biosynthesis (Wang et al., 2019). Moreover, exogenous application of zaxinone mimics led to enhanced growth and productivity of major agricultural crops, such as capsicum, potato, strawberry and other crops in the field conditions (Aguliar Perez et al., 2023; Wang, Jamil, et al., 2022).

Interestingly, there are no *ZAS* orthologues in nonmycorrhizal species, such as Arabidopsis, which indicates a role of this gene in the arbuscular mycorrhizal (AM) symbiosis (Fiorilli et al., 2019; Wang et al., 2019). This association between AM fungi and host plants plays a crucial role in providing essential minerals such as phosphorus (P) and nitrogen (N) to the plant, while the AM fungi receive carbohydrates and lipids in return (Gutjahr & Parniske, 2013). In fact, the *zas* mutant exhibited a lower level of AM colonization compared to wild-type plants. Conversely, overexpression of *ZAS* under the control of the *OsPT11* promoter, which is known to be active in arbusculated cells, resulted in increased mycorrhization (Votta et al., 2022). Furthermore, *ZAS2* (homolog of *ZAS*) knock-out plants showed impaired AM colonization (Ablazov et al., 2023). These findings indicate that *ZAS* gene family members play an essential role in ensuring the successful establishment of AM symbiosis in rice.

Previous studies demonstrate that rice requires a functional *ZAS* gene and a certain level of zaxinone in its roots to maintain normal growth and symbiosis with AM fungi. However, the effect of increasing zaxinone biosynthesis on rice growth, physiology and architecture remained elusive. In this study, we addressed this question by generating and characterizing constitutive *ZAS*-overexpressing rice lines.

2 | RESULTS AND DISCUSSION

2.1 | Overexpression of *ZAS* enhanced zaxinone level, increased number of productive tillers and total grain weight per plants

We set out to explore the effect of increasing *ZAS* expression on rice growth and architecture, particularly tillers number and root size and on its productivity. To achieve this, we generated transgenic rice plants overexpressing *ZAS* under the control of the constitutive *CaMV 35S* promoter (Supporting Information S1: Figure S1A). For further studies, we selected three independent transgenic lines (designed as *OX1*, *OX6* and *OX9*) with around 90- and 1000-fold higher expression levels, compared to wild type, in shoots and roots, respectively (Figure 1a). Selected lines were grown until the T4 generation, with transgenicity confirmed through growth on hygromycin-containing media and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Southern blot analysis using the *hygromycin phosphotransferase* (*HPT*) probe confirmed the presence of a single copy of the inserted *T-DNA* (Supporting Information S1: Figure S1B). Zaxinone quantification of hydroponically grown *ZAS*-overexpression plants showed a significant increase in the range of 20% and 85% in shoot and root tissues, respectively (Figure 1b), suggesting the role of ZAS in determining internal zaxinone levels. Next, we characterized the lines during the maturing stage under greenhouse (GH) conditions. Compared to wild type, the overexpression lines developed significantly more tillers (~27%), which were mostly productive at the heading stage (Figure 1c,e). With respect to panicles and grains, we observed a slight reduction in panicle length and number of grains per panicle, compared to wild type, with the exception of *OX6* (Figure 1i,k,l). We also detected a slight decrease in grain size (Figure 1j,m,n), but no significant change in the 1000-grain weight (Figure 1h). In summary, *ZAS*-overexpressing lines produced about 30% higher grain weight per plant (Figure 1d,f). The total above-ground biomass also increased by an average of 20%–25% (Figure 1g), with a tendency to reduced height (Supporting Information S1: Figure S2A). With respect to the flowering time, we did not observe a significant difference to wild-type plants (Supporting Information S1: Figure S2B). The possible reason for the decrease in grain and panicle size could be attributed to the enhanced shoot growth and increased tillers in *ZAS* overexpression lines. This excessive growth might lead to a dilution of carbohydrates or other essential resources during the plant's vegetative stage. Consequently, the transgenic lines might have limited resources compared to the wild-type plants when it comes to allocating for the development of grains and panicles. However, overexpression of *ZAS* significantly enhanced the number of productive tillers, more than compensating for the reduction in the number and size of grains, ultimately resulting in higher yield under GH conditions.

2.2 | ZAS regulates the tillering by suppressing the SL biosynthesis

Tillering is a trait controlled by different growth conditions and regulatory genes and is determined by phytohormones (Barbier et al., 2019; Li et al., 2003; Umehara et al., 2008; Wang & Li, 2011). SLs are a major inhibitor of axillary bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008; Waters et al., 2017). Mutants disrupted in SL biosynthesis or perception, such as *ccd7* (*d17*), *ccd8* (*d10*) or *d14*, are characterized by a high-tillering and dwarf phenotype, accompanied by a significant reduction in yield due to the dominance of nonproductive tillers (Al-Babili & Bouwmeester, 2015). Previously, we showed that zaxinone is a negative regulator of SL biosynthesis in rice. Loss-of-function of *ZAS* and its orthologue *ZAS2* led to increased SL content and a reduction in the number of tillers (Ablazov et al., 2023; Wang et al., 2019). Based on the phenotypes observed in *zas* mutants, we hypothesized that high tillering in *ZAS*-overexpression lines might be due to SL deficiency. To check this assumption, we quantified SLs in the roots where they are synthesized by using liquid chromatography/mass spectrometry; however, their content under optimal growth conditions, that is, sufficient Pi supply, was below detection limit. Therefore, we used the more sensitive *Striga* seed germination bioassay. Results obtained showed a drastic decrease in *Striga* seed germinating activity of root extracts of *ZAS*-overexpression lines, compared to wild type (Figure 2a), indicating a lower SL content. We obtained similar results with root exudates that showed around 50% reduction in *Striga* seed germinating activity (Figure 2b). Consistent with the lower SL content, the transcript level of two main SL biosynthetic genes, that is, *CCD7* and *CCD8*, was lower in *ZAS*-overexpression lines, compared to wild type (Figure 2c,d). To further confirm that the high-tillering phenotype was a result of a decrease in SL content, we applied the SL analogue methyl phenlactonoates 3 (MP3) (Jamil et al., 2018) to soil-grown *ZAS*-overexpression lines. As anticipated, the *ZAS*-overexpressing plants exhibited a greater number of tillers in their early growth stages compared to the wild-type plants (Figure 2e,f). Upon treatment with MP3, the *ZAS*-overexpressing lines displayed inhibition in the growth of secondary tillers, reverting back to the wild-type phenotype (Figure 2e,f). The inhibition observed upon MP3 treatment indicates that SL deficiency in *ZAS*-overexpression lines is a reason for their increased tiller number. Taken together, our findings suggest a correlation between a higher content of zaxinone in *ZAS*-overexpression lines and a reduction in SL biosynthesis and consequences thereof (Figure 2g).

2.3 | ZAS promotes the root growth by regulating the meristem activity

Besides being an anchorage in soil, roots are crucial for proper nutrient and water uptake, which ultimately determines the growth and overall development of plants. Overexpression of

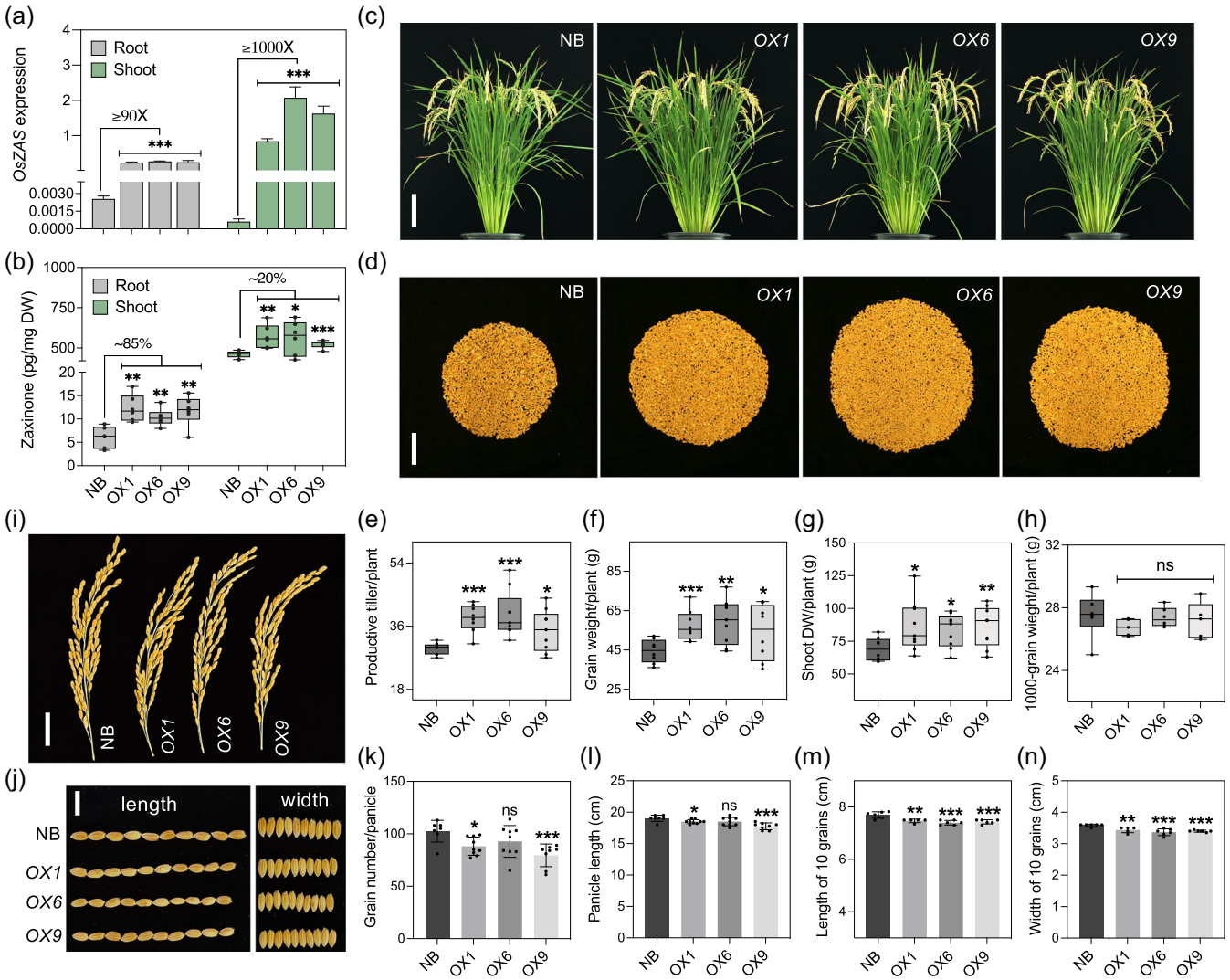

FIGURE 1 Overexpression of *ZAS* promotes rice tillering and yield. (a) Normalized expression value of *ZAS* analysed with qRT-PCR in transgenic rice lines roots and shoots, respectively. Data represent mean ± SD ($n \geq 3$). (b) Quantification of zaxinone content in *ZAS* overexpression lines in the shoots and roots, respectively. Data represent mean ± SD ($n \geq 4$). (c) Plant architecture of Nipponbare wild type (WT) and transgenic lines overexpressing *ZAS* (*OX1*, *OX6*, *OX9*) at maturing stage. Bar graph is 10 cm. (d) Presentation of total grains per plant. Bar graph is 5 cm. (e) Productive tiller number per plant. (f) Grain weight per plant. (g) Shoot dry weight (DW) per plant. (h) 1000-grain weight per pant. (I) Representation of main panicles per plants. Bar graph is 5 cm. (j) Length and width of 10 seeds from selected replicas. Bar graph is 1 cm. (k) Grain number per main panicle. (l) Length of main panicle. (m) Length of 10 grains separated from main panicle. (n) Width of 10 grains separated from main panicle. Boxes in boxplots represent the median, first and third quartile. The minimum and maximum values are shown with the length of the whiskers. Dots in the boxplots represent the biological replicates. Values in (k)–(n) are means ± SD ($n \geq 9$). Student's *t* test used for the statistical analysis (*$p < 0.05$; **$P < 0.01$; ***$P \leq 0.001$; ns, not significant). qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

ZAS significantly increased the growth and number of crown roots in both solid (1/2 Murashige and Skoog [MS], 0.4% agarose) and liquid (hydroponic) medium, as determined at different time points in comparison to wild-type plants (Supporting Information S1: Figure S3A,B). Moreover, the *ZAS*-overexpression lines showed a significantly enhanced root length and biomass (Figure 3a–d). To gain insights at cellular level, we examined the meristem size in root tips, which indicates the rate of cell division, using bright-filed microscopy. *ZAS*-overexpression lines displayed larger root meristem size, compared to the wild type (Figure 3e,f). Confirming increased meristem size, the number of meristematic cells in *ZAS*-overexpression lines was higher than in wild type (Figure 3g). These data indicate that overexpression of *ZAS* promotes root elongation by enhancing the meristem activity and cell division rate. Our findings are consistent with results obtained by exogenous zaxinone treatment that led to an increase in meristem size and cell number (Wang et al., 2021) and suggest that the higher level of zaxinone in transgenic lines can stimulate the cell division rate, thereby promoting root growth and development.

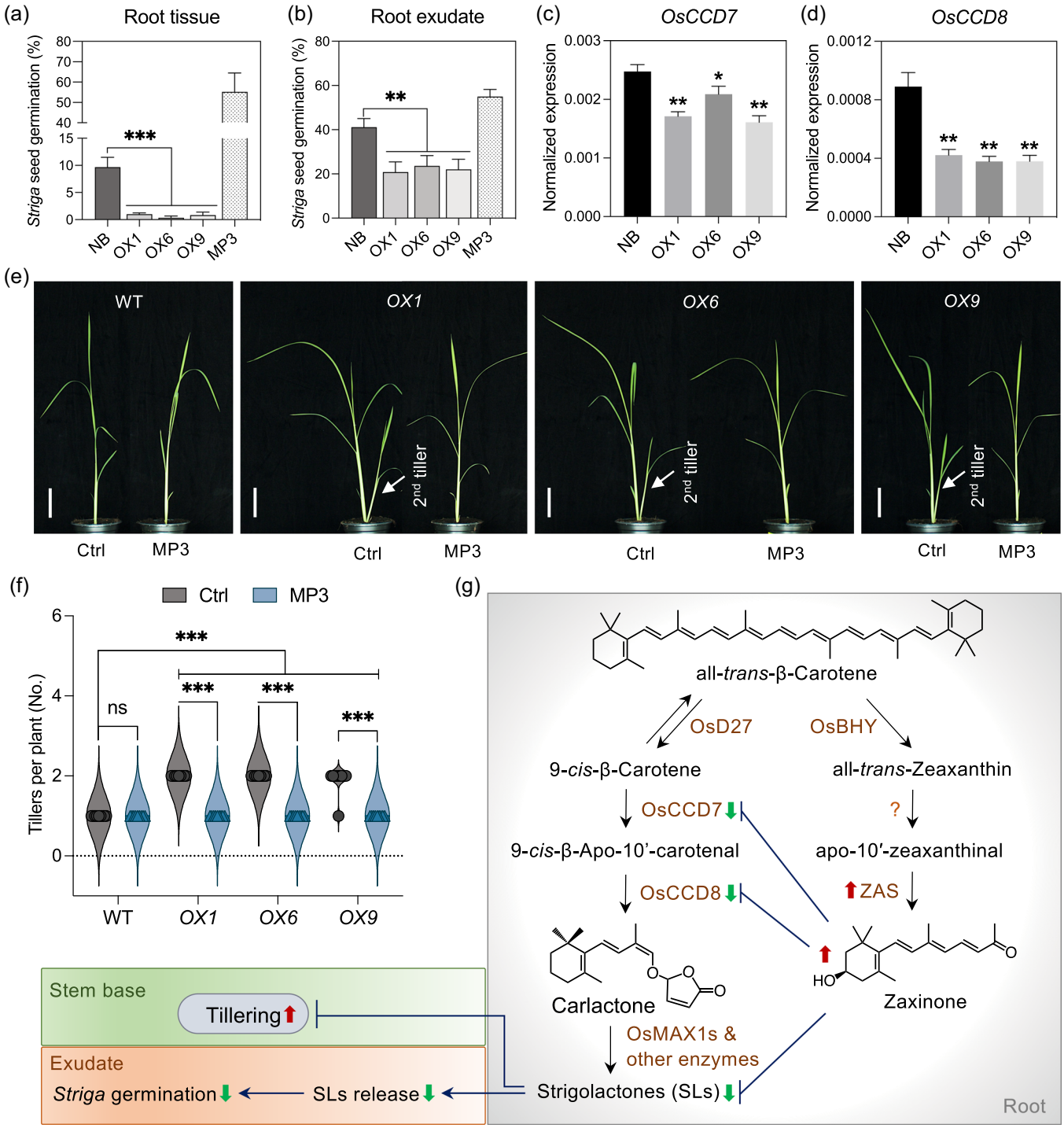

FIGURE 2 ZAS regulates tiller development by repressing the SL biosynthesis in rice. (a) and (b) SL quantification in roots tissue and exudates of wild type and *ZAS* overexpression lines with the *Striga* germination assay. 1 μM of MP3 (SL mimic) used as a control. Data represent mean ± SD ($n \geq 5$). (c) and (d), Two main SL biosynthetic genes (*CCD7* and *CCD8*) expression determined by qRT-PCR in the root tissue of wild type and *ZAS* overexpression lines. Data represent mean ± SD ($n \geq 3$). (e) Picture of tillering rescue experiment of *ZAS* overexpression transgenic lines which performed in the soil by applying MP3 (5 μM) for 1 week. Bar graph is 5 cm. (f) Quantification of tiller number in wild type and ZAS overexpression lines after MP3 application. Values in (f) are means ± SD ($n = 9$). Student's *t* test used for the statistical analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (g) Biosynthesis of zaxinone and SL in rice. Zaxinone represses SL biosynthesis, which leads to enhanced number of tillers and reduced SL content in root exudates. OsD27, Dwarf 27; OsMAX1s (OsMAX1-900, OsMAX1-400, OsMAX1-1500, OsMAX1-Os5100, OsMAX1-1900 (Yoneyama et al., 2018; Chen et al., 2023), more axillary growth; OsBHY, β-carotene hydroxylase (Zhu et al., 2018). qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; SL, strigolactone; ZAS, *Zaxinone Synthase*. [Color figure can be viewed at wileyonlinelibrary.com]

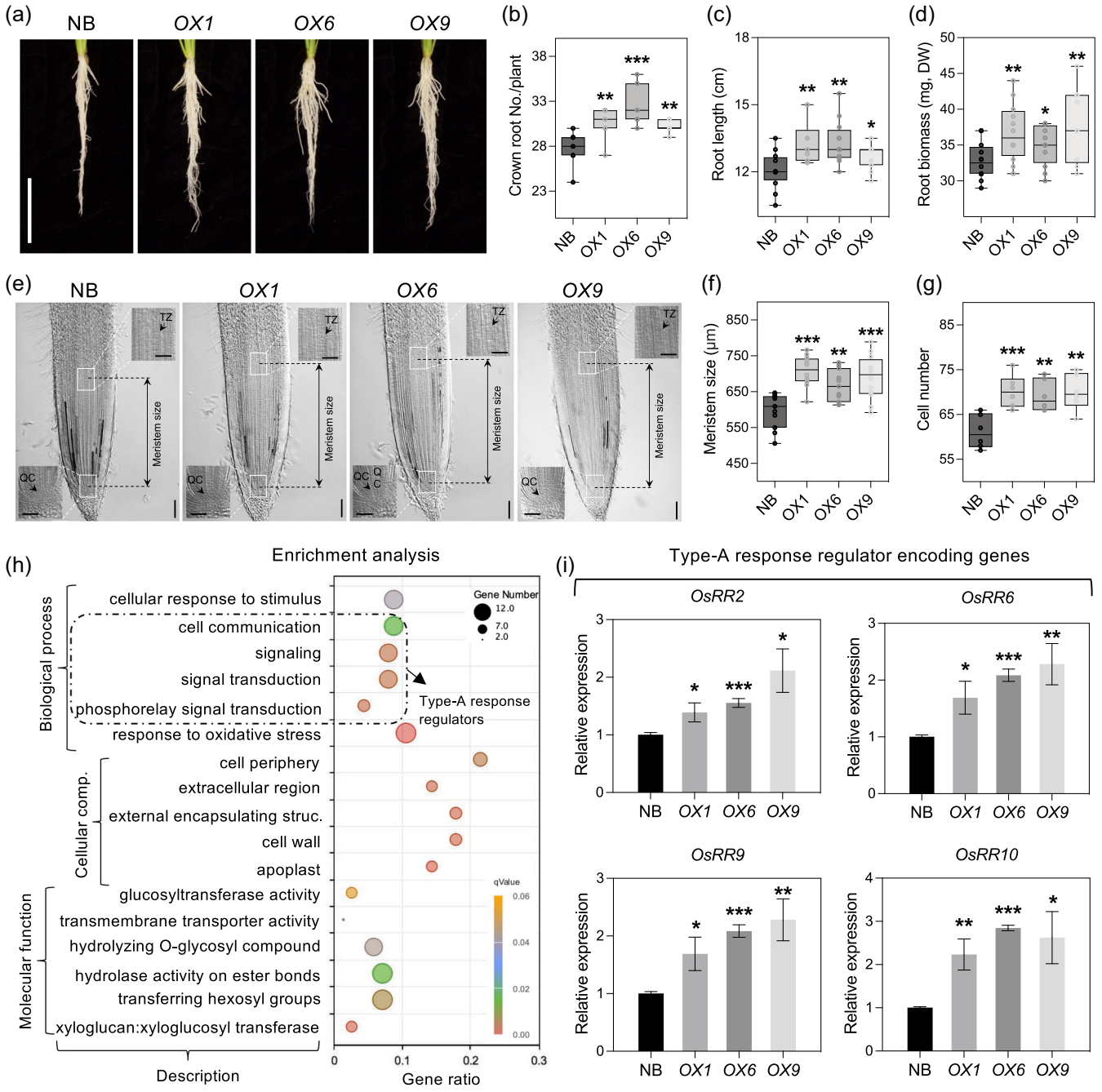

FIGURE 3 ZAS promotes root growth through regulating meristem activity. (a) Root phenotype of *ZAS* overexpression lines grown in the hydroponics medium for 3 weeks. (b) Crown root numbers (per plant) of *ZAS*-overexpression lines. (c) Root length of *ZAS* overexpression lines. (d) Root biomass (DW, dry weight) of *ZAS*-overexpression lines. (e) Root tips of 5-day-old *OsZAS* transgenic and wild-type seedlings were examined under light microscope with 10× and 20×. The distance between the quiescent centre (QC) and transition zone (TZ) represents the size of meristem (between two dash lines). Horizontal and vertical bars in the pictures represent 50 and 100 μm, respectively. (f) The meristem size was measured in the root tips of *ZAS*-overexpression and wild type with 10× (n = 11–12). (g) The number of central cylinder cells was counted in the meristem region of *ZAS*-overexpression and wild-type root tips with 20× (n = 6). (h) Gene Ontology (GO) term analysis of up-regulated genes in roots of *ZAS* overexpression (*OX6*) transgenic plants. GO terms with corrected p value ≤ 0.05 were considered significantly enriched by up-regulated genes. (i) Relative expression level of rice type-A cytokinin response regulator encoding genes in 3-week-old rice seedlings ($n \geq 3$). Boxes in boxplots represent the median, first and third quartile. The minimum and maximum values are shown with the length of the whiskers. Dots in the boxplots represent the biological replicates. Student's t test used for the statistical analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant). [Color figure can be viewed at wileyonlinelibrary.com]

The regulation of root meristem activity and cell proliferation is predominantly controlled by the plant hormones auxin and cytokinins (CKs) (Dello Ioio et al., 2007, 2008; Durbak et al., 2012; Kieffer et al., 2010), which have opposing roles in root development, with auxin being a promoter of cell proliferation while CKs negatively impacts meristem size. Therefore, we hypothesized that *ZAS* overexpression may enhance cell division rate by modulating auxin/cytokinin homoeostasis. To test this hypothesis, we measured the levels of the auxin indole-3-acetic acid and active CKs (Isopentenyl adenine, *trans*- and *cis*-Zeatin) in the roots of *ZAS*-overexpression lines. Compared to wild type, we did not detect a change in auxin or *cis*-zeatin (cZ) content (Supporting Information S1: Figure S4A,B), but observed a moderate decrease in the level of isopentenyl adenine (iP) and a significant enhancement in *trans*-zeatin (tZ) content (Supporting Information S1: Figure S4B). We also determined the level of other hormones. We did not observe a change in abscisic acid (ABA) and gibberellic acid (GA) level, but detected an increase in salicylic acid (SA) content in *ZAS-OX* lines, compared to wild type (Supporting Information S1: Figure S4C–E). In conclusion, quantification of hormones did not provide a clear explanation for the root phenotype. Therefore, we assumed that the overexpression of *ZAS* might influence the signalling pathways of these hormones. To test this hypothesis, we carried out RNAseq experiment on roots of the *ZAS*-overexpression line *OX6*. There was no significant enrichment in GO (Gene Ontology) terms related to auxin and other hormones signalling, but we observed an enrichment of CK signalling-associated pathways, including the phosphorelay signal transduction and cell communication (Figure 3h). In particular, there was an upregulation of genes encoding type A response regulators (RRs) that act as inhibitor of CK signal transduction in plants (Hwang & Sheen, 2001; Kakimoto, 2003; To et al., 2004). As confirmed by quantitative real-time polymerase chain reaction (qRT-PCR), transcript level of *OsRRs* (*OsRR2*, *6*, *9*, *10*) was significantly higher in *ZAS*-overexpression lines (Figure 3i), which may explain the observed root phenotype. Indeed, previous studies demonstrated that overexpression of *OsRR2* increased the number of crown roots (Zhao et al., 2015), while *OsRR3*- and *OsRR5*-overexpressing lines exhibited reduced sensitivity to exogenous CK application (Cheng et al., 2010), indicating their role in regulating CK signalling and root development. To confirm the reduced CK-sensitivity in the roots of our transgenic lines, we germinated rice *OX1* and wild-type seeds on 1/2 MS medium in the presence or absence of the CK analogue 6-Benzylaminopurine (BA, 1 µM concentration). Supporting our hypothesis, *OX1*, *ZAS*-overexpressing plants showed, upon BA treatment, around 45% lower root growth inhibition, compared to wild type (Supporting Information S1: Figure S5A,B). Taken together, our findings indicate that *ZAS* likely enhances root elongation by increasing *OsRRs* activity and thus inhibiting CK signalling, which results in a higher cell division rate in root meristem (Supporting Information S1: Figure S5C).

2.4 | Overexpression of *ZAS* led to an increase of monosaccharide sugars accumulation and enhances the rate of carbon (C) assimilation

Sugars play a significant role as universal carbon source for building cellular components in living organisms, in addition to their function in providing the energy required for growth and development. We previously demonstrated that exogenous zaxinone application promoted rice growth by enhancing sugar metabolism (Wang et al., 2021). Therefore, we hypothesized that increased zaxinone content that is caused by overexpressing *ZAS* may improve rice growth through enhancing sugar accumulation. To test this hypothesis, we measured the levels of different sugars in both shoot and root tissues of the *ZAS*-overexpressing lines *OX1* and *OX6*, using gas chromatography–mass spectrometry (GC–MS). Our results showed a significant increase in different monosaccharides, such as glucose, fructose and myo-inositol, in roots and shoots of the overexpression lines (Figure 4a). However, disaccharide sugars, such as sucrose, maltose, galactinol and trehalose, remained generally unchanged or decreased in overexpression lines (Supporting Information S1: Figure S6), indicating a dynamic sugar metabolism where disaccharide sugars might be quickly hydrolyzed into simple sugars or utilized for building up polysaccharides. Analysis of hexose phosphates, such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), revealed a significant increase in both roots and shoots, with exception of glucose-1-phosphate (G1P) that was slightly reduced in the shoots of the two *ZAS*-overexpressing lines (Figure 4b). Previous studies suggest that sugar phosphates act as signalling molecules repressing the activity of SnRK1 (SUCROSE-NON-FERMENTING1-RELATED KINASE1), a key negative regulator of plant growth that promotes catabolic processes while inhibiting energy-using anabolic processes (Baena-González & Hanson, 2017; Barbier et al., 2019; Margalha et al., 2016; Van Leene et al., 2022). It could be speculated that *ZAS* promotes rice growth through suppressing SnRK1 activity by increasing the levels of sugar phosphates. Consistent with the sugar measurements, GO enrichment analysis of up-regulated genes, deduced from our RNA sequencing (RNA-Seq) experiment, revealed a significant increase in terms related to sugar metabolisms (carbohydrates, polysaccharides, disaccharides and trehalose) in *ZAS* overexpression lines (Figure 4c). Taken together, our findings suggest that *ZAS* promotes sugar accumulation, particularly of monosaccharides, likely through increasing zaxinone content.

Based on the increased monosaccharides level, we assumed that overexpression of the *ZAS* gene may enhance the photosynthetic activity. To check this assumption, we analysed C assimilation and transpiration rate, stomatal conductance and relative chlorophyll content of the youngest fully expanded leaves of 4-week-old rice plants, using the LICOR-6800 Gas Exchange system and SPAD-502 Chlorophyll metre. Results obtained indicated that *ZAS*-overexpression lines have a higher net rate of CO_2 uptake and relative chlorophyll content per unit of projected leaf area, compared to the wild type (Figure 4d,g). However, we did not observe significant difference in the stomatal conductance or transpiration

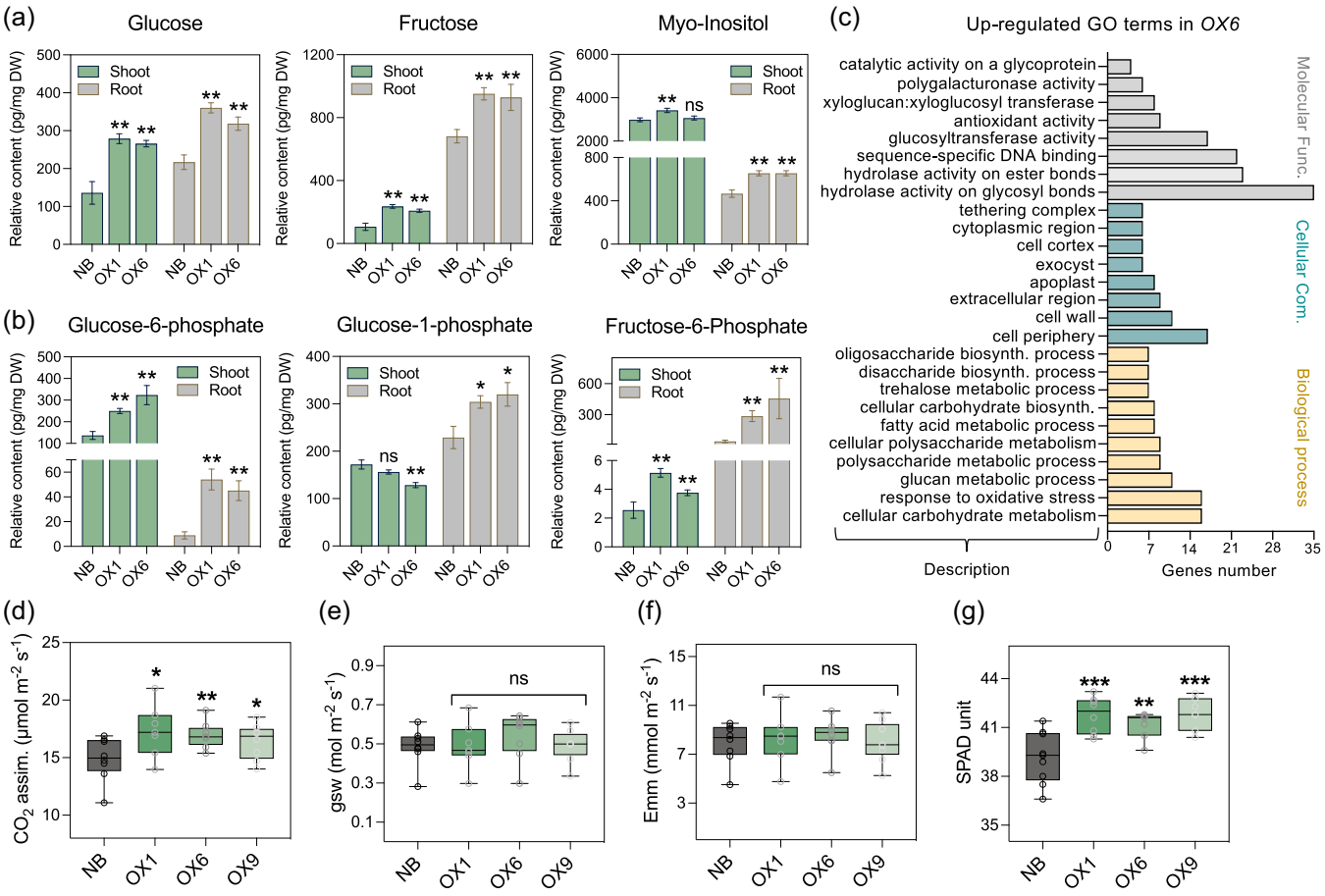

FIGURE 4 ZAS increases monosaccharide sugars accumulation and carbon dioxide (CO_2) assimilation. (a) SL monosaccharaide sugars were quantified in both root and shoot of 3-week-old rice seedlings grown in hydroponics. (b) Hexose sugars were quantified in both root and shoot of 3-week-old rice seedlings. Values (a)–(b) represent mean ± SD ($n \geq 5$). (c) Gene Ontology (GO) term analysis of up-regulated genes in shoots of *ZAS* overexpression (*OX6*) from RNA-seq. GO terms with corrected *p* value ≤ 0.05 were considered significantly enriched by up-regulated genes. (d) Carbon dioxide (CO_2) assimilation rate per unit of projected leaf area. (e) Stomatal conductance to water vapour (gsw) per unit of projected leaf area. (f) Transpiration rate (Emm) per unit of projected leaf area. (g) The relative chlorophyll content was measured using SPAD-502Pus (Konica Minolta). Values (d)–(g) represent mean ± SD ($n \geq 8$). Boxes in boxplots represent the median, first and third quartile. The minimum and maximum values are shown with the length of the whiskers. Dots in the boxplots represent the biological replicates. Student's *t* test used for the statistical analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). ns, not significant; SD, standard deviation; SL, strigolactone; ZAS, *Zaxinone Synthase*. [Color figure can be viewed at wileyonlinelibrary.com]

rate between the *ZAS*-overexpression plants and wild type (Figure 4e,f). From these findings, we conclude that *ZAS* fosters photosynthesis by facilitating C assimilation and increasing chlorophyll content, which can explain the higher sugar accumulation in overexpression lines.

2.5 | *ZAS* overexpression improved major macronutrients uptake in roots

Considering the overall improved performance of *ZAS-OX* lines and increased root growth, we assumed that *ZAS* overexpression might improve nutrient uptakes. To test this assumption, we quantified phosphates (PO_4^{3-}), nitrate (NO_3^-) and sulphate (SO_4^{2-}) in roots and shoots of *ZAS* overexpressing lines. We observed a striking increase in phosphate (~60%) and sulphate (~90%) content in roots of the overexpression lines, compared to wild type (Figure 5a; Supporting Information S1: Figure S7A). We also detected an increase in nitrate content, but to a less pronounced extent (Supporting Information S1: Figure S7B). However, we did not observe any significant change in all measured nutrients in shoots of *ZAS*-overexpression lines (Figure 5a; Supporting Information S1: Figure S7A,B). It is possible that the reason for the higher levels of nutrients found in the roots, rather than the shoots, of the *ZAS*-overexpression lines could be a decrease in nutrient utilization within the roots. This phenomenon may be a result of altered physiological process caused by the overexpression of the *ZAS* gene.

To understand the potential cause behind the higher concentration of major macronutrients in the root but not in the shoots, we analysed our transcriptome data in search of clues that could shed

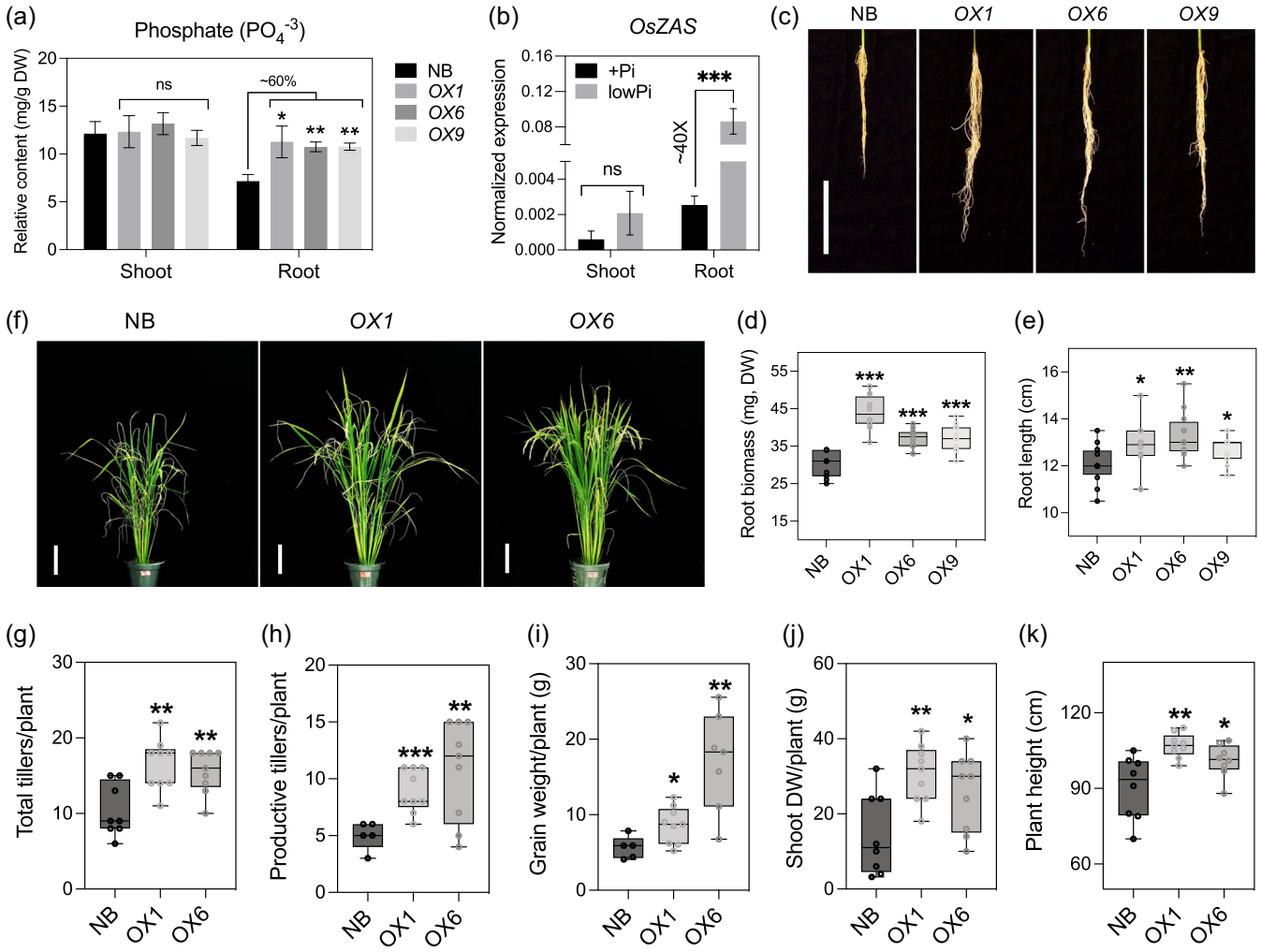

FIGURE 5 ZAS enhances phosphate (Pi) uptake and low Pi tolerance. (a) Phosphate content was quantified in both roots and shoots of wild type (NB) and *ZAS* overexpression lines grown for 3 weeks in hydroponics (mean ± SD; $n \geq 4$). (b) Normalized expression level of *OsZAS* in 3-week-old rice seedlings grown under +Pi (400 μM $K_2HPO_4.2H_2O$) and low Pi (4 μM $K_2HPO_4.2H_2O$) conditions for 2-weeks (mean ± SD; $n \geq 3$). (c) Root phenotype of *ZAS*-overexpression lines grown under low Pi conditions for 2 weeks after 1-week pretreatment growth. (d) Quantification of root biomass (DW, dry weight) shown in (c). (e) Measurement of root length shown in (c). Values represented in d and e are means ± SD ($n \geq 11$). (f) Plant architecture of wild type and transgenic lines overexpressing *ZAS* at maturing stage grown in low Pi soil under GH conditions. (g)–(k), Total tiller number per plants (g), productive tiller number per plants (h), grain weight per plants (i), shoot biomass (j), plant height (k) of NB and *ZAS*-overexpression lines (*OX1*, *OX6*) represented in (f). Values in (g)–(k) are means ± SD ($n \geq 5$). Boxes in boxplots represent the median, first and third quartile. The minimum and maximum values are shown with the length of the whiskers. Dots in the boxplots represent the biological replicates. Student's *t* test used for the statistical analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). ns, not significant; SD, standard deviation; ZAS, *Zaxinone Synthase*. [Color figure can be viewed at wileyonlinelibrary.com]

light on this mechanism. In support of the root's phosphate (Pi) accumulation, we observed the upregulation of two genes responsible for encoding Pi transporters: *OsPT2* (LOC_Os03g05640) and *OsPT3* (LOC_Os10g30770) in the roots of transgenic plants (OX6) (Supporting Information S1: Figure S7C). Previous studies have shown that OsPT2 plays a vital role as a Pi transporter in rice, where overexpression of the *OsPT2* gene substantially boosts Pi accumulation in both roots and shoots under normal conditions (Li et al., 2019; Liu et al., 2010). This implies that ZAS likely facilitates Pi uptake by upregulating the expression level of *OsPTs*. Besides, we observed reduction of nitrate reductase (*OsNia1*; LOC_Os08g36480) encoding gene's expression in the roots of transgenic plants (*OX6*) (Supporting Information S1: Figure S7D). This enzyme governs rate limiting step in conversion of nitrate (NO_3^-) into the nitrite (NO_2^-) (Han et al., 2022; Pelsy & Caboche, 1992; Yu et al., 1998). Thus, indicate that higher accumulation of nitrate could be result of reduced activity of *OsNia1*. Surprisingly, the level of *OsNia1* transcript was up-regulated in shoots, suggesting that N assimilation rate could be higher in the shoot of transgenic lines compared to wild type. This may explain why the content of nitrate remained unaltered in the shoots while it enhanced in the roots of *ZAS-OX* lines. Taken together, our data suggest that the overexpression of *ZAS* enhances the uptake of major nutrients by likely regulating the genes responsible for transporting and assimilation of nutrients. Furthermore, an increase in phosphate

content may explain why overexpression of *ZAS* lead to a decrease in SL level, given the inverse relationship between phosphate content and SL biosynthetic rate in many plant species, including rice (Barbier et al., 2019; Li et al., 2003; Umehara et al., 2008; Wang, Han, et al., 2020; Yoneyama et al., 2012, 2020).

2.6 | Overexpression of *ZAS* enhanced the growth and productivity under low Pi supply

Supporting the role of ZAS in improving Pi uptake, a recent study revealed the presence of the Pi starvation-responsive binding site (P1BS) in the *ZAS* promoter, and that the expression of this gene is transactivated by PHOSPHATE STARVATION RESPONSE 2 (PHR2), a key regulator of Pi signalling and arbuscular mycorrhizal (AM) symbiosis, upon Pi deficiency in rice (Das et al., 2022). Consistently, the expression of *ZAS* significantly increased (by about 40-fold) under low Pi conditions in roots, but remained unaffected in shoots (Figure 5b). These data indicate that *ZAS* could enhance low Pi tolerance in rice. To test this hypothesis, we evaluated the performance of our overexpression lines under low Pi conditions, when grown under controlled conditions in both hydroponic medium and soil. The overexpression of *ZAS* significantly improved root growth, as evidenced by increased root biomass and elongation, compared to the wild type (Figure 5c–e). Moreover, overexpression lines grown in low Pi soil under GH conditions maintained their high-tillering phenotype observed under Pi-sufficient conditions (Figure 5f–h). Furthermore, grain weight and above-ground biomass were significantly higher in *ZAS*-overexpression lines, compared to wild type (Figure 5i,j). Interestingly, *ZAS*-overexpression lines were taller than wild type under low Pi conditions, in contrast to what we observed under normal conditions (Figure 5f,k). Overall, these findings suggest that ZAS enhances Pi uptake and hence improves the tolerance to low Pi conditions.

2.7 | Overexpression of *ZAS* did not affect AM fungi colonization

Recently, we showed that overexpression of *ZAS* under the control of the *OsPT11* promoter, that is known to be active in arbusculated cells led to increased mycorrhization (Votta et al., 2022). Therefore, we investigated the mycorrhization capability of our constitutive *ZAS* overexpressing lines. As shown in (Supporting Information S1: Figure S8A,B), we did not detect any significant alteration in AM colonization. This inconsistency could be explained by the different activity of the promoters used to generate the overexpressing lines. Indeed, *CaMV 35S* is a strong constitutive promoter which exhibits high transcriptional activity and stability compared to *OsPT11* promoter, that is exclusively active in arbuscule-containing cells and in rice root apexes. This is mirrored in zaxinone content since in the root of *35S prom::OsZAS* lines, zaxinone reaches a significant increment (~85%), while in the root of *OsPT11prom::OsZAS* lines zaxinone increment was lower (~45%) (Votta et al., 2022). Moreover, contrarily to *35S prom::OsZAS* lines, *OsPT11prom::OsZAS* lines displayed an increment of SLs content compared to WT, which is in agreement with the increased mycorrhization level observed in these lines.

3 | CONCLUSION

In summary, we show here that constitutive overexpression of *ZAS* caused a novel combination of effects, which promotes rice growth and increases its productivity by enhancing the number of productive tillers and improving root growth, nutrient uptake and photosynthesis, without having negative impact on mycorrhization. Importantly, *ZAS* overexpression decreased the level of SLs, which explains the increased the high-tillering and may be a result of better Pi uptake. The fact that *ZAS* overexpressing lines, in contrast to SL-deficient mutants, developed productive tillers may be a result of an increased shoot biomass, photosynthetic capacity and nutrient uptake, which make the resources available, which are required for the observed significant increase in grain productivity. The increase in sugar content also supports this assumption. In addition, our findings mostly align with the exogenous zaxinone effect on rice growth and development (Aguliar Perez et al., 2023; Wang et al., 2019; Wang, Jamil, et al., 2020; Wang, Jamil, et al., 2022). Thus, our study offers an alternative strategy to enhance the rice growth performance and productivity by manipulation of *ZAS* expression, negating the need for exogenous zaxinone application. Although we are still at the beginning of understanding zaxinone biology, our study demonstrates the importance of *ZAS* as a target for sustainable enhancement of rice performance and for reducing the demand for Pi fertilisers.

4 | MATERIALS AND METHODS

4.1 | Plant material and growth conditions

In this study, we utilized *Oryza sativa* L. cv. Nipponbare rice as experimental model. Rice seeds were first incubated in 2% sodium hypochlorite (v/v) for 15 min and rinsed five times in sterile double-distilled water. Seeds were then germinated on ½ MS (0.4% Agarose) medium for 2 days under dark conditions at 30°C. After 2 days, seeds were moved to a growth chamber (Conviron) with 28°C day/22°C night rhythm and a photoperiod of 12-h light/12-h dark. The photon irradiance was set at 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the relative humidity at 70%. Then, the 7-day-old seedlings were hydroponically grown in Hoagland's nutrient solution for 2 weeks, according to (Ablazov et al., 2023). For metabolite and molecular analysis, root and shoot samples were harvested and stored at −80°C until analysis. For the low Pi hydroponics experiment, 7-day-old seedlings were subjected low

Pi (4 μM $K_2HPO_4 \times 2H_2O$) deficiency for 2 weeks, and root and shoot phenotypes were recorded. To characterize the yield-related phenotypes of *ZAS* overexpression transgenic rice lines, 7-day-old seedlings were transferred into soil-filled pots in a greenhouse (GH) with specific conditions (temperature 28°C day/25°C night; photoperiod 12-h-light/12-h-dark; light intensity ~800 μM $m^{-2}s^{-1}$ and 70% relative humidity). Rice plants were watered twice a week with modified half-strength Hoagland's nutrient solution. For the low Pi soil experiment, a single plant was grown in a 2-liter pot filled with 1 kg of stender soil (153 mg Pi/kg as P_2O_5) and 1 L of sand mixture. The amount of Pi was defined and calculated as low Pi based on (Dai et al., 2016). The rice plants were supplemented twice a week with −Pi (without $K_2HPO_4 \times 2H_2O$) modified half-strength Hoagland's nutrient solution. At maturity, agronomic traits were recorded. The AM fungi experiment was performed as described by (Ito et al., 2022).

4.2 | *Striga hermonthica* seed germination bioassay and SL quantification

Striga seeds were preconditioned following the procedure described by (Jamil et al., 2020). Root extracts and exudates were prepared and applied as described by (Ablazov et al., 2023). Then, SL-containing samples were applied on preconditioned Striga seeds and kept at 30°C for 24 h in the dark. Moreover, the SL analogue MP3 (1 μM) and MillQ water were applied as positive and negative control, respectively. Germinated and nongerminated seeds were scanned with a light microscope and analysed by using the software SeedQuant (Braguy et al., 2021). SLs in rice root exudates were enriched as described by (Wang, Chen, et al., 2022). SLs in root tissues were extracted following the protocol described by (Wang et al., 2019). SL samples were analysed by using UHPLC-Triple-Stage Quadrupole Mass Spectrometer (TSQ-Altis) with parameters as mentioned in (Wang, Chen, et al., 2022).

4.3 | Tillering rescue bioassays

Seven-day-old seedlings of the Nipponbare wild type and *ZAS* overexpressing lines (*OX1*, *OX6*, *OX9*), were planted in soil (500 mL plastic pots) supplemented with the synthetic SL analogue MP3 (5 μM) and acetone (0.05%) as a control. MP3 solution was applied every 3 days for 2 weeks and tillers per plant were counted and pictured with digital camera.

4.4 | Quantitative analysis of zaxinone

Zaxinone content was quantified using the previously described protocol (Wang et al., 2019). Approximately 20 mg of lyophilized rice samples were extracted twice with 2 mL of ethyl acetate containing 2 ng of D3-zaxinone (customized synthesis; Buchem B.V.). After that, samples were sonicated for 15 min in an ultrasonic bath and centrifuged at 3800 rpm for 8 min at 4°C. The two supernatants were combined and then dried under vacuum. The dried extract was dissolved in 100 μL of ethyl acetate and 2 mL of hexane before being purified. Silica gel SPE column (500 mg/3 mL) were used for purification, preconditioned with 3 mL of ethyl acetate and 3 mL of hexane. After washing with 3 mL of hexane, zaxinone was extracted using 3 mL of ethyl acetate and evaporated to dryness under vacuum. The residue was then redissolved in a mixture of 120 μL acetonitrile:water (25:75, v/v) for zaxinone and filtered through a 0.22 μm filter for UHPLC-Triple-Stage Quadrupole Mass Spectrometer (TSQ-Altis) with parameters as described in (Wang, Chen, et al., 2022).

4.5 | Meristem size measurement and cell number quantification

Seeds of wild type (NB) and *ZAS*-overexpression lines were placed on a 0.8% Agarose (1/2 MS) plate and allowed to germinate in complete darkness at 28°C for 2 days. The plates were later moved to a growth chamber (Conviron) and kept under specific conditions: a 12-h light/12-h dark cycle at a temperature of 28°C during the day and 22°C at night, with photon irradiance set at 500 μmol·m^{-2}·s^{-1}, and a relative humidity of 70%. After 96 h, roots were cut, and seedlings were subjected to chloral hydrate solution for 5 days at a temperature of 4°C. Root tips were then fixed onto a slide and photographed under a light microscope with 10× and 20× magnification. Using the ImageJ software, Meristem size and cell number were analysed, using the ImageJ software.

4.6 | Generation of *ZAS* overexpression transgenic lines in rice

The full-length cDNA of *ZAS* (*LOC_Os09g15240*) was PCR-amplified using primers *ZAS-OX-F* and *ZAS-OX-R* (Supporting Information S1: Table S1) and cloned into the corresponding site downstream of the *35S* promoter in the binary vector *pCAMBIA1300*, yielding the plasmid *pCAMBIA1300-ZAS*. The plasmid was introduced into Nipponbare wild type *Japonica* rice cultivar by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation as previously described (Hiei & Komari, 2008). The transgenic seedlings were selected on half MS medium containing 50 mg L^{-1} hygromycin and grown in GH till *T1* generation. Independent transgenic lines were selected, and 12 seedlings per transgenic lines were grown to identify homozygote lines at the *T2* generation. Forty seeds from each transgenic lines were germinated again on half MS medium containing 50 mg L^{-1} hygromycin and transgenic lines that showed 90%–100% germination rate considered as homozygote lines. Then, *T2* homozygote transgenic *ZAS* overexpression plants were propagated to obtain *T3* generation seeds.

4.7 | Southern-blot analysis

Rice genomic DNA was isolated as previously described (Xu et al., 2005). Southern blot was performed with the digoxigenin (DIG)-labelled PCR-amplified gene fragment of *HPT* probe as described in the DIG System Manual (Roche, Inc.). Genomic DNA (20 µg) was digested overnight with *HindIII*/*BamHI* (only one cut site in the T-DNA region), fractionated on a 0.8% agarose gel by electrophoresis and transferred onto an Amersham Hybond N^+ nylon membrane (GE Healthcare). The 840 bp *HPT* probe was synthesized by PCR DIG Probe Synthesis Kit (Roche) using primers *HPT-DIG-F* and *HPT-DIG-R* (Supporting Information S1: Table S1). The blotted membrane was prehybridized in DIG Easy Hybridization solution (Roche) at 42°C for 2 h. Afterwards, a denatured DIG-labelled *HPT* probe was added, and hybridization was performed overnight at 42°C. After hybridization, the membrane was washed at 65°C, two times with 2 × saline-sodium citrate (SSC) buffer and 0.1% sodium dodecyl sulphate (SDS) for 15 min and two times with 0.1 × SSC and 0.1% SDS for 15 min. Furthermore, probe hybridization signal was examined by digoxigenin chemiluminescence detection.

4.8 | Photosynthesis parameters measurement

Leaf gas exchange was measured on the youngest fully expanded leaf of 4-week-old rice plants using the LICOR-6800 Gas Exchange system and the 6800-12A chamber. Gas exchange was determined on a 1 × 3 cm leaf aperture or if the leaf was thinner, leaf width was measured, and gas exchange measurements were extrapolated for a 1 × 3 cm aperture. The following instrument settings were programmed: leaf vapor pressure deficit 1.5 kPa, Txchg 28°C, CO_2 410 ppm, flow 500 µmol s^{-1}, ΔPcham 0.2 KPa, fan speed of 7000 rpm, PARi 400 µmol m^{-2} s^{-1} using the light-emitting diode light source. The relative chlorophyll content was measured using SPAD-502Pus (Konica Minolta).

4.9 | Gene expression analysis by quantitative real-time PCR

Total RNA was extracted from rice tissues, using TRI-Reagent with the Direct-zol RNA MiniPrep Kit according to the manufacturer's instruction (Zymo Research). RNA concentration, quality and integrity were checked using a NanoDrop 2000 ultraviolet-visible Spectrophotometer (Thermo Scientific). Reverse transcription reaction was performed with the iScript cDNA Synthesis Kit, using 1 µg of total extracted RNA and following the manufacturer's instructions (BIO-RAD Laboratories). Primers used for qRT-PCR analysis are listed in Supporting Information S1: Table S1. qRT-PCR was performed in a StepOne™ Real-Time PCR Systems (Applied Biosystems), using SYBR Green Supermix to monitor double-stranded DNA (dsDNA) synthesis following the manufacturer's instructions. Relative expression levels of genes were determined using a comparative C_t method as previously described (Livak & Schmittgen, 2001) and the rice *Ubiquitin* (*UBQ*) gene was used as the internal control to normalize target gene expression.

4.10 | Sugar quantification

Sugars were extracted based on the protocol described by (Drapal et al., 2018; Enfissi et al., 2010) with a slight modification. Briefly, 15 mg of freeze-dried powder in 2 mL tube (Eppendorf) was extracted by adding methanol (400 µL)/water (400 µL), and the samples were kept on the continuous shaker for 1 h at room temperature. Next, 800 µL of chloroform added to the tubes and vortexed, then proceeded with 5 min centrifugation at 14 000 rpm (Revolutions Per Minute). In addition, 20 µL of aliquot from the upper phase of extract was transferred to new HPLC tube and 10 µL of ribitol (10 mg/mL) added as internal standard, and the samples were evaporated to dryness under vacuum. Derivatization was initiated with 30 µL of methoxyamine hydrochloride (Sigma-Aldrich) at a concentration of 20 mg/mL in pyridine. The samples were incubated at 40°C for 1 h, and then 70 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Sigma-Aldrich) was added to the samples and kept again at 40°C for 45 min. The samples ran on GC–MS (Agilent HP6890) with a 5973MSD and a 10:1 split injector. For retention index external calibration, a mixture of alkanes, ranging from 10 to 30 carbons, was ran together with samples.

4.11 | RNAseq experiment and data analysis

Total RNA was extracted from rice roots and shoots of the *OX6* line, using TRI-Reagent with Direct-zol RNA MiniPrep Kit according to the manufacturer's instruction (Zymo Research). RNA quality and quantity were checked using RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). Library construction, sequencing and data analysis were performed by Novogene Technology. The Illumina Novaseq platform was used for sequencing and 150 bp paired-end reads were generated. Paired-end clean reads were mapped to the Nipponbare reference genome (http://rice.uga.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/) by Hisat2 v2.0.5. Gene quantity was calculated by featureCounts v1.5.0-p3, and then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differentially expressed genes were identified using the DESeq. 2R package (1.20.0) with the following conditions; an adjusted p value ≤ 0.05 and fold change (FC) ≥ 0.3 (Supporting Information S1: Figure S9A,B). Principal component analysis (PCA) and heatmap of DEGs performed using Heatmaper (http://www.heatmapper.ca/expression/) to show the biological replicas consistency (Supporting Information S1: Figure S9C,D). GO enrichment analysis of DEGs were implemented by the clusterProfiler R package via NovoMagic online platform (https://ap-magic.novogene.com/). GO terms with corrected

p value ≤ 0.05 were considered significantly enriched by differential expressed genes (Supporting Information S1: Figure S9E,F).

4.12 | Inorganic anions analysis by capillary electrophoresis (CE)

Anions were extracted from freeze-dried root and leaf tissues with ultrapure water using a mortar and pestle. The extracts were filtered using 0.22 µm filters and assayed by capillary electrophoresis (Agilent 7100; Agilent Technologies). Sample injection was at 50 mbar for 5 s with +30 kV voltage and a detection wavelength of 310/20 nm. Sulphate (SO_4^{2-}), nitrate (NO_3^-) and phosphate (PO_4^{3-}) were analysed through a bare fused silica capillary column with an extended light path BF3 (i.d. = 50 µm, l = 72 cm, L = 80.5 cm). Sample injection was followed by 50 mbar pressure for 4 s with −30 kV voltage and detection at the 350/380 nm wavelength. All anions were identified using pure standards. The final anion contents in each sample were calculated as $\mu g\, g^{-1}$ DW (dry weight). For each genotype, at least four biological replicated were considered for each organ (root and shoot).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The materials utilized in this study are accessible under an MTA agreement with the King Abdullah University of Science and Technology (KAUST). All data are available in the main figures or supplementary materials. The RNA sequencing data produced in this study deposited in NCBI's Gene Expression Omnibus (GEO) with accession number GSE269765, which can be accessed at the following web address: https://www.ncbi.nlm.nih.gov/bioproject/.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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