RESEARCH PAPER

Two SLENDER AND CRINKLY LEAF dioxygenases play an essential role in rice shoot development

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Received 29 July 2019; Editorial decision 28 October 2019; Accepted 27 November 2019

Editor: Dabing Zhang, Shanghai Jiao Tong University, China

Abstract

It is clear that 2-oxoglutarate-dependent dioxygenases have critical functions in salicylic acid (SA) metabolism in plants, yet their role in SA biosynthesis is poorly understood. Here, we report that two dioxygenase-encoding genes, SLENDER AND CRINKLY LEAF1 (SLC1) and SLC2, play essential roles in shoot development and SA production in rice. Overexpression of SLC1 (SLC1-OE) or SLC2 (SLC2-OE) in rice produced infertile plants with slender and crinkly leaves. Disruption of SLC1 or SLC2 led to dwarf plants, while simultaneous down-regulation of SLC1 and SLC2 resulted in a severe defect in early leaf development. Enhanced SA levels in SLC1-OE plants and decreased SA levels in slc1 and slc2 mutants were observed. Accordingly, these lines all showed altered expression of a set of SA-related genes. We demonstrated that SLC1 interacts with homeobox1 (OSH1), and that either the knotted1-like homeobox (KNOX1) or glutamate, leucine, and lysine (ELK) domain of OSH1 is sufficient for accomplishing this interaction. Collectively, our data reveal the importance of SLC1 and SLC2 in rice shoot development.

Keywords: Homeobox1, OsGA20ox, 2-oxoglutarate-dependent dioxygenase, rice, salicylic acid, shoot development.

Introduction

The 2-oxoglutarate-dependent dioxygenase (2OGD) superfamily is the second largest enzyme family in plants, and its members are involved in various oxygenation/hydroxylation reactions. They require 2-oxoglutarate (2OG) and molecular oxygen as co-substrates, and ferrous iron Fe(II) as a cofactor to catalyse the oxidation of different substrates (Prescott and John, 1996). Previous studies have shown that 2OGDs have oxidative activity on some compounds involved in plant hormone metabolism. For instance, in later reactions of gibberellin (GA) metabolism, activities of several 2OGDs, including Gibberellin 20-Oxidases (GA20oxs), Gibberellin 3-Oxidases (GA3oxs), and Gibberellin 2-Oxidases (GA2oxs), are essential (Hedden and Thomas, 2012; Hedden and Sponsel, 2015). The Green Revolution gene Semidwarf-1 (Sd-1), identified by analysing a loss-of-function mutant of OsGA20ox2, has been utilized for breeding and has achieved great success in increasing grain production (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). Enhancing expression of Grain Number per Panicle1 (GNP1)/OsGA20ox1 in inflorescence meristems results in higher grain number and grain yield (Wu et al., 2016). The rice DIOXYGENASE FOR AUXIN OXIDATION (DAO) and its Arabidopsis homologues, AtDAO1 and AtDAO2, encode 2OGDs that convert active indole-3-acetic acid (IAA) into biologically inactive OxIAA, serving as a mechanism to fine tune auxin levels in rice and Arabidopsis (Zhao et al., 2013; Porco et al., 2016; Zhang et al., 2016). 2OGDs also play critical roles in...
the inactivation of salicylic acid (SA) and jasmonic acid (JA). In Arabidopsis, SA 5-HYDROXYLASE/DOWNY MILDEW RESISTANT 6 (SSH/DMR6) and SA 3-HYDROXYLASE/DMR6-LIKE OXYGENASE 1 (SSH/DLO1) are 2OGDs that both hydroxylate SA (Zhang et al., 2013, 2017). In addition, the JA oxidases (JAOs) or jasmonate-induced oxygenases (JOXs) belong to the 2OGD superfamily and catalyse specific oxidation of JA to 12OH-JA in Arabidopsis (Caerls et al., 2017; Smirnova et al., 2017). GERMINATION INSENSITIVE TO ABA MUTANT 2 (GIM2), another 2OGD member in Arabidopsis, oxidizes GA12 and influences GA-mediated seed germination (Xiong et al., 2018).

Salicylic acid (2-hydroxybenzoic acid) is a phenolic plant hormone that plays an essential role in plant growth and development, including seedling establishment, leaf senescence, and disease resistance (Vlot et al., 2009; Rivas-San Vicente and Plasencia, 2011). Functioning as an endogenous signal mediating local and systemic plant defensive responses against pathogens, the function of SA is significant. Application of exogenous SA activates plant pathogenesis-related (PR) genes and induces systemic acquired resistance (SAR) in plants (Ward et al., 1991).

The biosynthesis of SA in plants is not yet fully understood, however two pathways have been proposed: the phenylalanine ammonia lyase (PAL) pathway and the isochorismate synthase (IC) pathway. In the PAL pathway, plants synthesize SA from cinnamate produced by PAL (Lee et al., 1995; Ribickicky et al., 1998). In the IC pathway, SA is synthesized from chorismate through two reactions catalysed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Strawn et al., 2007; Mustafa et al., 2009). To maintain optimal SA levels for growth and development, plants adopt a direct and efficient approach by controlling hormone biosynthesis and catabolism.

In the Arabidopsis genome there are two ICS genes, ICS1, also known as SA INDUCTION DEFICIENT 2 (SID2), and ICS2 (Wildermuth et al., 2001). In rice (Oryza sativa), one ICS gene has been identified and its role in SA biosynthesis is characterized (Choi et al., 2015). The most studied SA biosynthetic pathway in rice is the PAL pathway (Sawada et al., 2006). The SA glucosyltransferases, which convert SA into SA β-glucoside (SAG), have been identified in both Arabidopsis and rice (Dean and Delaney, 2008; Unemura et al., 2009). SA is hydroxylated at the C5 or C3 position of its phenyl ring, leading to the formation of 2,5-Dihydroxybenzoic acid (2,5-DHBA) or 2,3-Dihydroxybenzoic acid (2,3-DHBA), a process catalysed by DMR6 or DLO1, respectively (Zhang et al., 2013, 2017). Usually, disruption of SA homeostasis leads to changes in pathogen resistance and plant development. Mutants that over-accumulate SA, such as constitutive expresser of PR genes 1 (cpr1), cpr5, cpr6-1, defense no death 1 (dnd1), and the s5hs3h double mutant, display a significant reduction in rosette leaf size and have constitutive activation of SAR (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998; Yu et al., 1998; Jirage et al., 2001; Zhang et al., 2017). Although many metabolites in the conjugation and catabolic pathways of SA in plants have been identified, the mechanism for controlling SA homeostasis in rice remains elusive.

In this study, we determined that SLC1 and SLC2 are homologous genes in the dioxygenase family and play important roles in the growth and development of rice. Altered expression of SLC1 and/or SLC2 produced a significant perturbation in shoot development. Increases in SA levels were achieved by overexpression of SLC1 in rice, and the involvement of SLC1 and SLC2 in maintaining the balance of SA levels was demonstrated. The interaction between SLC1 and OSH1 suggests a role for SLC1 as part of an important regulatory mechanism in rice development.

Materials and methods
Phylogenetic analysis
The rice GA20ox protein sequences (Han and Zhu, 2011) were obtained from the Rice Genome Annotation Project (https://rice.plantbiology.msu.edu) and aligned using the Clustal W multiple sequence alignment program (Larkin et al., 2007). The phylogenetic tree of the alignment was generated by the MEGA software (Molecular Evolutionary Genetic Analysis, version 6.0) (Tamura et al., 2013), using the neighbour-joining method with bootstrapping based on 1000 replicates.

Plant material and growth conditions
Except for the sk2 mutants which were generated in the Oryza sativa L. japonica cv. Nippounire (Nip) background, other rice plants were in the O. sativa L. japonica cv. Heising 19 (HJ) background. Rice transgenic lines were generated via Agrobacterium-mediated transformation and the transfectants were selected by screening for resistance to Hygromycin B (H397, Phytoech) (Nishimura et al., 2006). The sk1 and sk2 mutants were generated by BioRun (https://www.biorun.net), using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 targeted-genome editing (Feng et al., 2013). All rice plants were grown in pots and placed either in a controlled growth chamber at 24–32 ºC with photoperiod 12 h light/12 h dark, or outdoors from April to October in Wuhan, China. Arabidopsis transgenic lines (expressing race genes) were generated in the Columbia-0 (Col) background using the floral dip method (Clough and Bent, 1998) and the transfectants were selected by screening for resistance to Glufosinate-ammonium (45520, Sigma-Aldrich). All selected plants were grown in a growth room at 23 ºC with photoperiod 16 h light/8 h dark.

Plasmid construction
The control (empty) vector pCAMBIA1300-YFP was made by inserting the yellow fluorescent protein (YFP) fragment into pCAMBIA1300 (Addgene, https://www.addgene.org), driven by a maize (Zea mays) ubiquitin promoter. The plasmids pCAMBIA1300-YFP-SLC1 and pCAMBIA1300-YFP-SLC2 were constructed by cloning the full-length coding sequences (CDS) of SLC1 and SLC2 into pCAMBIA1300-YFP, respectively. To generate the transgenic rice lines expressing SLC1-N or SLC1-C, the fragments SLC1-N (+1 bp to +120 bp) and SLC1-C (+121 bp to +1152 bp) were inserted into pCAMBIA1300-YFP, respectively. The plasmids pBA002-SLC1, pBA002-SLC2, and pBA002-OsGA20ox2 were made by cloning the full-length CDS of SLC1, SLC2, and OsGA20ox2, respectively, into the binary vector pBA002 (Kost et al., 1998). To generate the RNAi transgenic lines co-silencing SLC1 and SLC2, pCAMBIA1300-SLC1-SLC2-RNAi was constructed by inserting the dsRNAi cassette containing the 500 bp fragment (+1 bp to +500 bp) of SLC1 and 420 bp fragment (+211 bp to +630 bp) of SLC2, into pCAMBIA1300 in frame. To generate transgenic plants carrying pCAMBIA1300-SLC1pro-GUS, the promoter fragment consisting of 1.9 kb upstream of the start codon of SLC1 was amplified from the genomic DNA of Hj, fused to the β-glucuronidase (GUS) gene, and then inserted into pCAMBIA1300. For analysing subcellular localization via transient expression assays in rice protoplasts, p3SS-YFP-SLC1, p3SS-YFP-SLC2, and p3SS-CFP-DLT were constructed by following the method previously described (Lu et al., 2013; Zhao et al., 2015; Yin...
et al., 2019). For the Binomolecular Fluorescence Complementation (BiFC) assay, CDS fragments of OSH1, OSH6, OSH15, OSH71, or SLC1 were cloned into the vector p3SSS-MCS-YN or p3SSS-MCS-YC (Zhao et al., 2015).

For expressing His-tagged recombinant SLC1 in Escherichia coli (E. coli), the CDS of SLC1 was cloned into pET28a (Novagen, USA). For expressing GST-tagged recombinant OSH1, the CDS of OSH1 was inserted into pGEX-4T1 (GE Healthcare, USA). For expressing the MBP-tagged recombinant proteins for enzyme assays, the CDS of SLC1, SLC2 or OsGa20ox2 were cloned into pET28a (Novagen, USA).

For yeast two-hybrid (Y2H) assays, the CDS of OSH1, OSH6, OSH10, OSH15, or OSH71 were cloned into pGADT7 (AD, Clontech). Truncated-fragments of OSH1 were cloned into pGADT7. The CDS of SLC1, SLC2, or OsGa20ox2 were cloned into pGBK T7 (BD, Clontech). The truncated-fragments of SLC1 were cloned into pGBK T7. The primer sequences used for analysing gene expression are listed in Supplementary Table S1 at JXB online.

RNA extraction and gene expression analysis
Total RNA was extracted using the EASYspin Plus Plant RNA Kit (RN38, Aidlab) or TRIzol reagent (Invitrogen, USA) by following the manufacturer's instructions. RNA samples were reverse-transcribed with ReverTra Ace-® (Toyobo, Japan). Real-time-quantitative PCR (RT-qPCR) was performed using Advanced SYBR Green supermix (Bio-Rad) with the CFX connect real-time PCR detection system 185–5201 (Bio-Rad), and relative gene expression was analysed using the CFX manager software (Bio-Rad). For semi-quantitative RT-PCR, initial denaturation was conducted at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 20 s; annealing at 56 °C for 30 s and elongation at 72 °C for 90 s. The primer sequences used for analysing gene expression are listed in Supplementary Table S2.

Enzyme assay and measurement of hormone and metabolite levels
The enzyme assay was performed according to a previously described method (Zhao et al., 2013; Xiong et al., 2018). The substrate GA12 or GA53 (OilCheml, Czech Rep) was incubated with the cell lysate of E. coli expressing MBP-SLC1, MBP-SLC2, or OsGa20ox2, in a total reaction volume of 100 μL. The reaction was incubated at 30 °C for 3 h with gentle agitation. The reaction solution contained 100 μM Tris–HCl (pH 7.0) and cofactor mixture (5 mM 2-oxoglutarate, 5 mM L-ascorbate, and 0.5 mM FeSO4). Acetic acid (10/150, v/v) was added to Tris-HCl (pH 7.0) and cofactor mixture (5 mM 2-oxoglutarate, 5 mM L-ascorbate, and 0.5 mM FeSO4) with elution buffer containing 510 μL H2O/ACN (acetonitrile) (10/500, v/v). After elution, the sample was evaporated under nitrogen gas and re-suspended in 50 μL SDS-PAGE sample buffer. Samples were boiled, briefly spun down, and the supernatants separated on a 12% SDS–PAGE gel with subsequent immunoblotting with anti-His or anti-GST antibodies (1:3000) (ABclonal, China).

Results
Overexpression of SLC1 or SLC2 in rice perturbs shoot development
To elucidate the function of dioxygenase-encoding genes involved in rice development, we analysed the phylogenetic relationship of putative GA20oxs in rice (Supplementary Fig. S1A), and investigated further the divergent trait of OsGa20ox7 (LOC_Os08g44590). We used the protein sequence of OsGA20ox7 in a BLAST search of the MSU Rice Genome Annotation Project Release 7 (https://rice.plantbiology.msu.edu) and found that the Os09g0570800 (LOC_Os09g39720) locus shares 54.6% amino acid sequence identity with OsGA20ox7, contains a conserved Fe(II) 2OG dioxygenase domain (Supplementary Fig. S1B), and both are classified into the same clade of DOXC37 (Kawai et al., 2014).

We generated transgenic rice lines overexpressing OsGA20ox7 or Os09g0570800 in the Hejiang 19 (HJ) background (Fig. 1). The growth of T0 plants regenerated from calli overexpressing OsGA20ox7 was severely perturbed (Fig. 1A–F). After 14 d of regeneration, the leaves of these plants were slender and crinkly (Fig. 1B), therefore we renamed OsGA20ox7 as SLENDER AND CRINKLY LEAF1 (SLC1). After 30 d of regeneration, shoot growth of SLC1 overexpressing (SLC1-OE) plants was substantially promoted but root elongation was repressed (Fig. 1C). After 40 d of regeneration, peculiar leaf blades and crinkly leaves were prominent; in severe cases, no leaf was formed in the leaf sheath (Fig. 1D). After 60 d of regeneration (heading stage), the SLC1-OE plants retained slender and taller architecture (Fig. 1E); young panicles were wrapped in the leaf sheath and seeds did not develop in any of these T0 plants (Fig. 1F). As a result, we performed additional rounds of transformation, and in total, over 100 T0 SLC1-OE plants were obtained and examined. Unfortunately, all of these plants developed abnormally and no seeds were produced. Similar phenotypes were also produced in the plants overexpressing Os09g0570800, including the slender and crinkly leaves (Fig. 1G, H). Hence, we named Os09g0570800 as SLENDER AND CRINKLY LEAF2 (SLC2). Together, these results suggest an important role for SLC1 and SLC2 in the growth and development of rice.
Fig. 1. Overexpression (OE) of SLENDER AND CRINKLY LEAF1 (SLC1) or SLC2 in rice produces plants with slender and crinkly leaves. (A) Expression level of SLC1 in SLC1-OE#1 and SLC1-OE#2 T0 plants was quantified and compared with that of control (Ctrl) plants expressing the empty vector (Ctrl#1 and Ctrl#2). All plants were in the Hejiang 19 background. The expression of OsACTIN1 (Os03g0718100) was used as the internal control. (B) Slender and crinkly leaves were observed in SLC1-OE T0 plants but not in Ctrl, after 14 d of regeneration. Scale bars: 1 cm. (C) Taller shoots and shorter roots were observed in SLC1-OE T0 plants compared to Ctrl, after 30 d of regeneration. Scale bar: 1 cm. (D) Crinkly leaves were observed in SLC1-OE T0 plants, after 40 d of regeneration. Scale bar: 1 cm. (E) After 60 d of regeneration, slender but taller SLC1-OE T0 plants were observed. Scale bar: 10 cm. (F) After 60 d of regeneration, twisted leaves and twisted young panicles enclosed in the leaf sheath of SLC1-OE T0 plants were observed. To visualize the young panicles, the leaf sheath was opened slightly. Scale bars: 1 cm. (G) Expression of SLC2 was quantified in regenerated plants overexpressing SLC2 (SLC2-OE#1, T0). The expression of OsACTIN1 was used as the internal control. (H) Phenomena such as crinkly, slender and twisted leaves were observed in the SLC2-OE plants, after 14 d of regeneration. The control (Ctrl) plantlet harboring the empty vector displayed a normal growth phenotype. Scale bars: 1 cm.
Expression of SLC1 or SLC2 in Arabidopsis produces dwarf plants

As rice plants overexpressing SLC1 or SLC2 produced no seed, we generated transgenic Arabidopsis plants expressing rice SLC1 or SLC2, and expression of the transgenes was confirmed using semi-quantitative PCR (Fig. 2A). These Arabidopsis lines were morphologically defective, displaying shorter roots (Supplementary Fig. S2A), reduced stature (Fig. 2B, C), and greener leaves (Supplementary Fig. S2B). The chlorophyll content in leaves of 30-day-old plants of SLC1#1 and SLC2#1, was markedly higher than in the wild type control (Col) (Supplementary Fig. S2C). Considering that OsGA20ox2 catalyses the conversion of GA3 to GA20, promoting the biosynthesis of endogenous GA1 (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002), as a comparison we also generated transgenic Arabidopsis plants expressing OsGA20ox2. Initially, the growth of 40-day-old plants of OsGA20ox2#1 and OsGA20ox2#2 was morphologically similar to that of Col. At flowering stage (54-day-old), OsGA20ox2 plants were significantly taller than Col (Supplementary Fig. S2D). These results demonstrate that the role of SLC1 or SLC2 in plant development is distinct from that of OsGA20ox2.

slc1 and slc2 rice mutants have reduced stature

We generated slc1 rice mutants in the HJ background using CRISPR/Cas9 genome editing. Several mutant lines with a variety of edits were obtained, two of which, slc1-4 and slc1-7, were subjected to subsequent analysis. The homozygous slc1-4 mutant contained a G base deletion in the first exon of SLC1, resulting in a frame-shift and causing a premature stop codon (Supplementary Fig. S3A, B). The slc1-7 mutant had an A insertion, also leading to a frame-shift in the mRNA sequence of SLC1 (Supplementary Fig. S3A, B). In addition, we generated three homozygous lines of slc2 genome-edited rice mutants in the Nip background; slc2-27, slc2-61 and slc2-62. All three slc2 mutants contained a C base deletion in SLC2, introducing a stop codon (TAG) at 153 bp (Supplementary Fig. S3C, D). Development of T2 slc1 mutants 7 d after germination was significantly different from that of HJ. Height and leaf sheath length of slc1-4 and slc1-7 shoots were obviously shorter than those of HJ (Fig. 3A, B). Similarly, height and leaf sheath length of seven-day-old shoots of slc2-27, slc2-61 and slc2-62 were also reduced (Fig. 3C, D). When compared with HJ, slc1-4 and slc1-7 mutants remained shorter at all stages (Supplementary Fig. S3E, F). The length of the main culms in 90-day-old slc1-4

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Fig. 2. Ectopic expression of SLENDER AND CRINKLY LEAF1 (SLC1) or SLC2 in Arabidopsis produces dwarf plants. (A) SLC1 and SLC2 expression analysis in SLC1 (#1 and #2) and SLC2 (#1 and #2) lines (Columbia-0, Col, background). Total RNA was extracted from rosette leaves of 30-day-old plants. The expression of ACTIN2 (At3g18780) was used as the internal control. (B) SLC1#1 and SLC1#2 T2 plants were smaller than Col. Left panel: photo taken at 35 d after germination (DAG). Right panel: photo taken at 53 DAG. Scale bars: 1 cm. (C) SLC2#1 and SLC2#2 T2 plants were smaller than Col. Left panel: photo taken at 40 DAG. Right panel: photo taken at 54 DAG. Scale bars: 1 cm.
Fig. 3. Phenotype of slender and crinkly leaf1 (slc1) and slc2 rice mutants. (A) Seven-day-old slc1 mutants (slc1-4 and slc1-7, T2) were shorter than Hejiang 19 (HJ). Scale bar: 1 cm. (B) Plant height, second leaf sheath length, and root length in seven-day-old HJ and slc1 plants. Data represent the mean ±SD (n=15, **P<0.01, Student's t-test). (C) Seven-day-old slc2 mutants (slc2-27, slc2-61 and slc2-62, T2) were shorter than Nipponbare (Nip) plants. Scale bar: 1 cm. (D) Plant height, second leaf sheath length, and root length of seven-day-old Nip and slc2 plants. Data represent the mean ±SD (n=10, **P<0.01, Student's t-test). (E) SLC1 and SLC2 expression levels were significantly reduced in SLC1-SLC2-RNAi lines (#3 and #4). SLC1 or SLC2 expression level in HJ was taken as 1.0. Data represent the mean ±SD of three independent experiments. (F) Seven-day-old SLC1-SLC2-RNAi plants displayed altered growth. Scale bars: 1 cm. (G) Plant height, second leaf sheath length, and root length of seven-day-old HJ and SLC1-SLC2-RNAi plants. Data represent the mean ±SD (n=8, **P<0.01, Student's t-test).
The spatiotemporal expression of SLC1 and SLC2 in developing culms

We quantified the spatiotemporal expression patterns of SLC1 and SLC2 in different rice tissues. The expression level of SLC1 in seven-day-old plants was much lower than in mature leaves and developing culms (Fig. 4A), with the highest expression level observed in the second node. A similar pattern of expression of SLC2 was also detected in developing culms (Fig. 4A). By fusing the SLC1 promoter to the GUS marker gene (SLC1pro-GUS), we demonstrated that SLC1 expression was detectable throughout the developmental stages tested (Fig. 4B), with predominant expression in the coleoptile, leaf sheath and blade, and root tip of seven-day-old plants. In mature leaves, GUS activity was restricted to vascular bundles of leaf blades. Strong GUS signal was detected in developing culms, especially in the second node.

To determine the subcellular localization of SLC1 and SLC2, we performed a transient expression assay in rice protoplasts and tobacco leaf epidermal cells (Fig. 4C, Supplementary Fig. S4). Consistent with the expression pattern of known dioxygenases (Xiong et al., 2018), the fusion proteins YFP-SLC1 and YFP-SLC2 were detected in the cytoplasm and in the nucleus, with the latter confirmed using co-localization with a known nuclear protein, DWARF AND LOW-TILLERING (DLT) (Tong et al., 2012).

SLC1 and SLC2 are involved in phytohormone homeostasis

To test our enzyme assay, we first determined the catalytic activity of OsGA20ox2 against its substrates GA_{12} and GA_{53}, and the products GA_{15} and GA_{44} were produced (Supplementary Fig. S5A). We then analysed the catalytic activity of SLC1 and SLC2 by incubating the cell lysate extracted from *E. coli* expressing either MBP-SLC1 or MBP-SLC2 with GA_{12} and GA_{53}. Neither reaction showed catalytic activity (Supplementary Fig. S5A), indicating that neither GA_{12} nor GA_{53} were substrates of either SLC1 or SLC2. The expression of either SLC1 or OsGA20ox2 in Arabidopsis created divergent growth phenotypes (Fig. 2B, Supplementary Fig. S2D), indicating that SLC1 might not function as a conventional GA20ox in rice. Therefore, we measured the levels of GA_{4}, GA_{19} and GA_{1} in SLC1-OE, slc1-4 and slc2-62 rice plants. GA_{4} was not detected, supporting the notion that the GA_{1} content in vegetative tissues is normally low (Hirano et al., 2008). The levels of GA_{19} and GA_{1} were not significantly affected compared to their appropriate controls (Fig. 5A). These results suggest that SLC1 and SLC2 might not function as conventional GA20-oxidases.

Numerous studies have demonstrated that, rather than acting as a GA20ox to metabolize a GA-intermediate, 2OGDs may catalyse substrates of other hormones (Zhang et al., 2013, 2016, 2017; Zhao et al., 2013; Brewer et al., 2016; Porco et al., 2016; Caars et al., 2017; Smirnova et al., 2017). To investigate how SLC1 and SLC2 function, we quantified ABA, IAA and SA levels in rice plants of various genetic backgrounds. The level of SA was significantly higher in *T_{0} SLC1-OE* plants (2586 ng g^{-1} FW), when compared to *T_{0} empty vector* control plants (1278 ng g^{-1} FW) (Fig. 5B). In contrast, a reduced level of SA was measured in *slc1-4* (2415 ng g^{-1} FW) compared to *HJ* (3817 ng g^{-1} FW), and in *slc2-62* plants (3879 ng g^{-1} FW) compared to *Nip* (4296 ng g^{-1} FW). There was little variation in IAA levels in all tested lines (Supplementary Fig. S5B). ABA levels were reduced in both SLC1-OE and *sk1-4* plants, but more severely in SLC1-OE. The ABA content in *slc2-62* plants was similar to *Nip*.

We also analysed the levels of several hormones in SLC1#1 and SLC2#1 Arabidopsis plants. In both lines, SA levels were almost 10-fold higher and JA levels were over 2-fold higher, compared to *Col* (208 ng g^{-1} FW) (Fig. 5C). There was little variation in IAA and ABA levels in all Arabidopsis lines tested (Supplementary Fig. S5C). Collectively, these results implicate a role for SLC1 and SLC2 in SA metabolism.

**PR genes are up-regulated by enhanced expression of SLC1 or SLC2**

One of the major roles of SA in plant development is the induction of *PR* gene expression (Stintzi et al., 1993; Hoffmann-Sommergruber, 2000). In Arabidopsis, expression of *PR1*, *PR2* and *PR5* is stimulated by SA (Selitrennikoff, 2001; Zhang et al., 2010); the induction of *PR3* and *PR4* is independent of SA-signaling but dependent on JA-signaling (Thomma et al., 1998); and activation of the SA-signaling pathway suppresses a large set of JA-responsive genes including *PLANT DEFENSIN1.2 (PDF1.2)* and *VEGETATIVE STORAGE PROTEIN2* (VSP2) (van Wees et al., 1999; Van der Does et al., 2013). We analysed the expression of a set of SA- and JA-related genes in SLC1#1 and SLC2#1 Arabidopsis plants compared to *Col*. Expression of the SA-responsive genes, *PR1*, *PR2* and *PR5*, was enhanced significantly (Fig. 6A). The JA-responsive gene *PR4* was up-regulated, while the expression of *VSP2* was reduced.

In rice, *OsPR1a*, *OsPR1b*, *OsPR2* and *OsPR5* are induced by SA treatment (Jwa et al., 2006), thus we examined the expression of these *OsPR* genes in *T_{0} SLC1-OE* and found their expression was markedly increased compared to control plants (Fig. 6B). In the *sk1-4* mutant, although *OsPR1a* expression was higher.
than that of HJ, the expression of both OsPR1b and OsPR5 was reduced (Supplementary Fig. S6A). In the slc2-62 mutant, all of these OsPR genes were significantly down-regulated. In addition, we observed that in seven-day-old shoots, SLC1 expression was higher than SLC2 expression in HJ, while the opposite was true in Nip (Supplementary Fig. S6B).

Two pathways for SA biosynthesis in plants have been proposed (Lee et al., 1995; Ribnicky et al., 1998; Strawn et al., 1995).
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To explore the role of SLC1 and SLC2 in SA metabolism, we examined the expression of SA biosynthesis and catabolism genes in SLC1#1 and SLC2#1 Arabidopsis plants. There are four genes encoding PAL and two genes encoding ICS in the Arabidopsis genome (Ohl et al., 1990; Wanner et al., 1995; Wildermuth et al., 2001). Both DMR6 and DLO1 encode SA-hydroxylase which fine-tunes SA homeostasis (Zhang et al., 2013; Zhang et al., 2017). In SLC1#1 and SLC2#1, ICS1, DMR6 and DLO1 were up-regulated, whereas ICS2 and PAL4 were down-regulated (Fig. 6C). We then analysed the levels of potential SA precursors in SLC1#1 and SLC2#1. In both lines, the level of

![Fig. 5. The relationship between expression of SLENDER AND CRINKLY LEAF1 (SLC1) or SLC2 and salicylic acid (SA) levels. (A) Level of two gibberellins (GAs), GA_{19} and GA_{1}, in plants overexpressing SLC1 (SLC1-OE), and in slc1-4 and slc2-62 mutants. Control (Ctrl) plants contain the empty vector. Samples were collected from shoots of SLC1-OE plants after 14 d of regeneration, or from seven-day-old shoots of slc1-4 and slc2-62 mutants (T_{2} homozygous). (B) Levels of SA in SLC1-OE, slc1-4 and slc2-62 plants. For measuring SA levels in SLC1-OE plants, samples were collected from shoots of SLC1-OE or Ctrl after 14 d of regeneration. For measuring SA levels in slc1-4 and slc2-62 mutants, samples were collected from seven-day-old shoots of Hejiang 19 (HJ) and slc1-4 (T_{2} homozygous), or Nipponbare (Nip) and slc2-62 (T_{2} homozygous). HJ and Nip control plants were grown from seed. Data represent the mean ±SD of three independent experiments (*P<0.05, **P<0.01; Student's t-test). (C) Increased SA and JA levels in SLC1#1 and SLC2#1 Arabidopsis plants. Samples were collected from aerial tissues of 30-day-old plants. Data represent the mean ±SD of three independent experiments (*P<0.05, **P<0.01; Student's t-test).]
L-phenylalanine was higher and the chorismate level remained unchanged compared to Col. Due to their low abundance in Arabidopsis, trans-cinnamic acid and o-coumaric acid were not detected (Supplementary Fig. S6C).

SLC1 interacts with OSH1 and its N-terminus has transcriptional activation activity

The knotted1-like homeobox (KNOX) transcription factors are crucial for establishment and maintenance of the shoot apical meristem (SAM). Since overexpression of SLC1 and SLC2 in rice led to aberrant shoot development, we sought to explore the biochemical relationship of SLC1 or SLC2 with the KNOX proteins. Seven KNOX proteins, OSH1, OSH3, OSH6, OSH10, OSH15, OSH43, and OSH71, function together to establish the SAM in rice (Sentoku et al., 1999). Using a Y2H assay, we screened OSH proteins to determine which, if any, interacted with SLC1. Interactions were observed for SLC1 with OSH1, OSH6, OSH15, and OSH71, but not OSH10 (Fig. 7A, Supplementary Fig. S7A). We verified these interactions in rice protoplasts using BiFC (Fig. 7B), and further confirmed the interaction between SLC1 and OSH1 in a protein pull-down assay (Fig. 7C).

Similarly to SLC1, OsGA20ox2 interacted with OSH1, OSH6, and OSH15, but did not interact with OSH10 or OSH71 (Supplementary Fig. S7B). OSH1 is expressed in a specific region during early embryogenesis and its function may be related to a regulatory process before or independent of organ determination in rice (Sato et al., 1996). The full-length OSH1 protein (361 amino acids) is composed of five conserved domains: KNOX1 and KNOX2 (MEINOX), GSE, ELK, and homeodomain (HD) (Sentoku et al., 1999; Nagasaki et al., 2001). To distinguish the binding specificity of SLC1 and OsGA20ox2 to OSH1, we analysed the interaction of SLC1 with various truncations of OSH1 in Y2H assays (Fig. 7D, E). Interactions were detected between SLC1 and OSH1 (242–361), OSH1 (1–241), OSH1 (1–144), and OSH1 (145–263).

![Fig. 6. Up-regulation of pathogenesis-related (PR) genes in plants overexpressing SLENDER AND CRINKLY LEAF1 (SLC1) or SLC2.](image)

(A) Expression levels of salicylic acid (SA)- and jasmonic acid (JA)-related genes in SLC1#1 and SLC2#1 Arabidopsis lines. Total RNA was extracted from rosette leaves of 30-day-old plants. The expression of ACTIN2 was used as the internal control. Data represent the mean ± SD of three independent experiments (*P<0.05, **P<0.01; Student’s t-test). (B) Expression levels of OsPR genes in regenerated plants overexpressing SLC1 (SLC1-OE, T0). The expression of OsACTIN1 was used as the internal control. Data represent the mean ± SD of three independent experiments (*P<0.05, **P<0.01; Student’s t-test). (C) Expression of SA biosynthesis and catabolism genes in SLC1#1 and SLC2#1. Total RNA was extracted from rosette leaves of 30-day-old plants. Data represent the mean ± SD of three independent experiments (*P<0.05; Student’s t-test).
However, OsGA20ox2 only interacted with OSH1 (242–361), which contained the ELK and HD domains. Hence, either the KNOX1 or ELK domain of OSH1 was sufficient to allow interaction with SLC1. Additionally, we examined the interaction of SLC2 and OSH1 by Y2H, and no interaction was detected (Supplementary Fig. S7C).

To examine the functional specificity of the N- and C-termini of SLC1, we assessed the truncations SLC1-N (1–40) and SLC1-C (41–383) in a Y2H assay. The yeast cells carrying BD–SLC1-N and AD (empty vector) were able to grow on the selection medium (Supplementary Fig. S7D), suggesting that SLC1-N had transcriptional activation activity in SLC1 interacts with knotted1-like homeobox (KNOX) proteins. (A) Yeast two-hybrid (Y2H) assay to assess the interaction of SLC1 with homeobox1 (OSH1), OSH6, OSH15, and OSH71. Controls: AD (pGADT7) and BD (pGAL7); -LWHA, high-stringency selective medium (SD/Leu−/Trp−/His−/Ade−). (B) Bimolecular Fluorescence Complementation (BiFC) assay to assess the interaction of SLC1 (SLC1–YC) with OSH1 (OSH1–YN), OSH6 (OSH6–YN), OSH15 (OSH15–YN), and OSH71 (OSH71–YN). Protoplasts isolated from seven-day-old Hejiang 19 seedlings. YN, N-terminal YFP fragment; YC, C-terminal YFP fragment. Scale bars: 10 μm. (C) Pull-down assay to confirm the protein interaction between SLC1 and OSH1. Empty vector (GST) and GST–OSH1 immunoblotted with anti-GST antibody. His–SLC1 immunoblotted with anti-His antibody (Input). (D) Diagram of the full-length and truncated proteins of OSH1 (not to scale). (E) Y2H assay to assess the interaction between truncated proteins of OSH1 with SLC1 and OsGA20ox2. Control: AD (pGADT7); -LWHA, high-stringency selective medium (SD/Leu−/Trp−/His−/Ade−).
the assay. To confirm this, we performed the transcriptional activation assay by solely expressing BD-SLC1-N in yeast cells. The yeast cells expressing BD-SLC1-N, but not BD-SLC1-C, grew well in the selection medium, indicating strong transcriptional activation activity of SLC1-N (Supplementary Fig. S7E). To further characterize the impact of SLC1-N on the function of SLC1, we generated transgenic rice plants overexpressing SLC1-N (SLC1-N-OE) and SLC1-C (SLC1-C-OE), respectively. The SLC1-N-OE plants showed a similar growth phenotype to Hj, whereas the SLC1-C-OE plants mimicked those shown in the SLC1-OE plants (Supplementary Fig. S7F-G).

**Discussion**

**SLC1 and SLC2 are essential for shoot development**

In plants, 2OGDs are involved in a wide range of biological processes, including DNA demethylation, proline hydroxylation, plant hormone biosynthesis, and the biosynthesis of various specialized metabolites. Due to their agricultural significance, the role of 2OGDs in the biosynthesis of GAs has been extensively studied. We previously reported that the function of OsGA20ox2 has been extensively analysed since it acts as a typical GA20ox. In this study, we used OsGA20ox2 as a control for analysing the catalytic activity of SLC1 and SLC2. Our data indicated that neither SLC1 nor SLC2 converts GA12 or GA53 (Supplementary Fig. S5A), suggesting that neither protein possesses GA20-oxidase activity. Furthermore, the levels of GA19 and GA1 were hardly affected in SLC1-OE, slc1-4 and slc2-62 plants. Instead, SA levels were dramatically altered (Fig. 5B), demonstrating that the activity of SLC1 and SLC2 correlates with SA homeostasis in rice. The effect of SA on plant growth and development has been documented in numerous reports. For instance, the elevation of SA in the Arabidopsis mutants cpr1, cpr5, cpr6-1 and dud1, as well as the s5hs3h double mutant, results in reduced plant stature (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998; Yu et al., 1998; Jirage et al., 2001; Zhang et al., 2017). JA levels were affected in SLC1#1 and SLC2#1 plants (Fig. 5C), indicating the influence of SLC1 and SLC2 expression on JA levels in Arabidopsis. SA and JA play interactive roles in plant development and immune signaling networks (Mur et al., 2006; Koornneef et al., 2008). Endogenously accumulating SA antagonizes JA-dependent defense responses, thereby prioritizing SA-dependent defense (Pieterse et al., 2012). In SLC1#1 and SLC2#1 Arabidopsis plants, the SA-responsive genes PR1, PR2 and PR5 were strongly induced, whereas among four JA-responsive genes (PR3, PR4, PDF1.2 and VSP2), only PR4 expression was up-regulated (Fig. 6A). Although JA levels were elevated in SLC1#1 and SLC2#1, VSP2 expression was drastically suppressed (Fig. 6A), demonstrating a consequence of the antagonism between SA- and JA-signaling. Thus, an improved SA-response occurred in SLC1#1 and SLC2#1. Increased expression of OsPR genes in SLC1-OE rice plants (Fig. 6B) indicated a link between SLC1 and SA homeostasis. In contrast to the low basal level of SA in Arabidopsis and tobacco (less than 100 ng g⁻¹ FW), rice has a level of SA two orders of magnitude higher (5000–30 000 ng g⁻¹ FW) and appears to be insensitive to exogenous SA treatment (Chen et al., 1997; Yang et al., 2004). We confirmed that SA levels in rice and Arabidopsis are dramatically different (Fig. 5B, C). Thus, the differential phenotypes resulting from expression of SLC1 or SLC2 in rice or in Arabidopsis are closely associated with the basal level of SA in each species. Two distinct pathways have been proposed for the biosynthesis of SA in plants: the ICS pathway and the PAL pathway. By analysing the expression of SA biosynthesis and catabolism genes, as well as the abundance of SA precursors (Fig. 6C, Supplementary Fig. S6C), we found that expression of PAL4 was down-regulated, and the level of L-phenylalanine was elevated in SLC1#1 and SLC2#1 Arabidopsis plants. Although the expression of both ICS1 and ICS2 was altered in SLC1#1 and SLC2#1, the level of chorismate was unaffected. In support of the opinion that, in Arabidopsis, SA is synthesized via isochorismate and not through the PAL pathway (Tamaoki, 2008), trans-cinnamic acid and o-coumaric acid were not detected. Enhanced expression of DMR6 and DLO1 in SLC1#1 and SLC2#1 was attributed to the increased SA level in these lines, suggesting the presence of negative feedback regulation. Studies are now required to
elucidate precisely how SLC1 and SLC2 are involved in SA homeostasis, and in which steps of the SA biosynthesis pathway SLC1 and SLC2 implement their functions.

**SLC1 interacts with OSH1 and may have diverse functionality**

KNOX genes encode HD-containing transcription factors that are expressed in the SAM, but excluded from lateral organ primordia (Tsuda et al., 2014). Loss-of-function mutants of KNOX genes, such as SHOOT MERISTEMLESS (STM) in Arabidopsis and OSH1 in rice, show defects in SAM formation and/or maintenance (Long et al., 1996; Tsuda et al., 2011). OSH1 is important for specifying cell identity and regionalization for the formation of the shoot and its adjacent tissues in rice (Sato et al., 1996). In our Y2H assay, OSH1 interacted with SLC1 and OsGA20ox2 (Fig. 7A, Supplementary Fig. S7B). However, SLC1 and OsGA20ox2 exhibited distinct binding specificity to OSH1 (Fig. 7E), indicating functional specialization of 2OGD in rice. The conserved MEINOX and ELK domains in OSH1 are considered to be crucial for protein–protein interaction (Vollbrecht et al., 1991; Bürglin, 1997). Our results indicated that either the KNOX1 or ELK domain of OSH1 is sufficient for the interaction between SLC1 and OSH1 (Fig. 7E). Although our results suggest that SLC1 and SLC2 are functionally redundant, SLC1 may have characteristics that differ from SLC2. For example, SLC1 can interact with OSH1, whereas SLC2 cannot (Fig. 7A, Supplementary Fig. S7C). The N-terminus of SLC1 (SLC1-N) showed transcriptional activation activity in yeast cells. Overexpression of SLC1-N in rice did not affect growth of SLC1-N-OE plants, but abnormal growth, similar to that shown in SLC1-OE lines (Fig. 1), was observed in the plants of SLC1-C-OE (Supplementary Fig. S7G). SLC1 appears to be functionally diverse; it may act as a dioxygenase, and also work together with its associated proteins, such as OSH1, to modulate shoot development in rice.

In this study, we propose that SLC1 and SLC2 are homologous genes belonging to the 2OGD family that both play important roles in rice shoot development, and we demonstrate the impact of SLC1 and SLC2 on SA homeostasis. Future studies to determine the enzymatic activity of SLC1 and SLC2, identify their substrates, elucidate the biochemical mechanisms of the interaction between SLC1 and OSH1, and clarify the redundancy and specificity of SLC1 and SLC2, will further our knowledge on the association between SA homeostasis and shoot development in rice.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. The relationships of eight 2-oxoglutarate-dependent dioxygenases in rice.

Fig. S2. Expressing either SLC1 or SLC2 in Arabidopsis alters plant development.

Fig. S3. SLC1 and SLC2 genome-edited mutants were generated using the CRISPR/Cas9 system.

Fig. S4. Subcellular localization of YFP–SLC1 and YFP–SLC2 in tobacco leaf epidermal cells.
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