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# Overexpression of *GSK3-like Kinase 5 (OsGSK5)* in rice (*Oryza sativa*) enhances salinity tolerance in part via preferential carbon allocation to root starch

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**Abstract.** Rice (*Oryza sativa* L.) is very sensitive to soil salinity. To identify endogenous mechanisms that may help rice to better survive salt stress, we studied a rice GSK3-like isoform (OsGSK5), an orthologue of a *Medicago* GSK3 previously shown to enhance salinity tolerance in *Arabidopsis* by altering carbohydrate metabolism. We wanted to determine whether OsGSK5 functions similarly in rice. *OsGSK5* was cloned and sequence, expression, evolutionary and functional analyses were conducted. *OsGSK5* was expressed highest in rice seedling roots and was both salt and sugar starvation inducible in this tissue. A short-term salt-shock (150 mM) activated *OsGSK5*, whereas moderate (50 mM) salinity over the same period repressed the transcript. *OsGSK5* response to salinity was due to an ionic effect since it was unaffected by polyethylene glycol. We engineered a rice line with 3.5-fold higher *OsGSK5* transcript, which better tolerated cultivation on saline soils (EC = 8 and 10 dS m<sup>-2</sup>). This line produced more panicles and leaves, and a higher shoot biomass under high salt stress than the control genotypes. Whole-plant <sup>14</sup>C-tracing and correlative analysis of *OsGSK5* transcript with eco-physiological assessments pointed to the accelerated allocation of carbon to the root and its deposition as starch, as part of the tolerance mechanism.

**Additional keywords:** carbohydrate, carbon allocation, carbon partitioning, salinity, salt stress, starch metabolism.

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## Introduction

Rice is the most important staple worldwide (Papademetriou 2000). Yields, however, are significantly challenged by soil salinity, and the magnitude of this problem is predicted to increase in the next 30 years (Smith *et al.* 2009; Thitisaksakul *et al.* 2012, 2015). Yield losses vary, but can be as high as 50% in soils with an electrical conductivity (EC) of 5 dS m<sup>-2</sup>, exerting pressure on efforts to increase global rice supply (Yeo 1999). Understanding the multitude of strategies rice uses to mitigate against salinity stress is therefore necessary, as they could help to genetically engineer plants with better tolerance to salinity (Mittler and Blumwald 2010).

One mechanism plants may employ to adapt to saline soils is to reconfigure their use of carbohydrates to directly counter the effects of osmotic or ionic stress, while presumably allowing the most judicious use of limited carbon and energy reserves (Rosa *et al.* 2009; Stitt *et al.* 2010; Lemoine *et al.* 2013; Osorio *et al.* 2014). Variation in starch and sugar levels may occur over differing timescales, and in distinct tissues during the stress

response, to enhance survival (Kerepesi and Galiba 2000; Ainsworth and Bush 2011). It is possible to hypothesise that salinity-induced changes in starch metabolism may permit it to act as a 'sugar sink' (Balibrea *et al.* 2000; Kempa *et al.* 2007; Pattanagul and Thitisaksakul 2008; Cha-um *et al.* 2009; Rosa *et al.* 2009; Amirjani 2011; Theerawitaya *et al.* 2012; Henry *et al.* 2015) to relieve sugar feedback inhibition of sensitive processes, or as a 'sugar source' (Chen *et al.* 2008; Kempa *et al.* 2008; Rosa *et al.* 2009), to provide carbon depending on plant metabolic needs.

Salt-induced spatio-temporal changes in carbohydrates can also alter whole plant source–sink partitioning for better adaptation (Gao *et al.* 1998; Balibrea *et al.* 2000; Lemoine *et al.* 2013; Osorio *et al.* 2014). Salinity can impair root sink strength, reducing assimilate demand and leading to a build-up of sugars in the source (Balibrea *et al.* 2000). Some salt-tolerant cultivars accumulated less sugars in the leaf at high salinity, in part by increasing the flux to starch – mechanisms that are not prominent in the sensitive cultivars tested (Dubey and Singh

1999; Balibrea *et al.* 2000; Pattanagul and Thitisaksakul 2008). In mature tomato with established sinks, high salinity enhanced fruit starch biosynthesis from imported assimilates (Gao *et al.* 1998; Yin *et al.* 2010). This amplified the sucrose gradient between source and sink, thus supporting higher export of sugars from source leaves and sustaining photosynthesis (Gao *et al.* 1998). How widespread these mechanisms are in crop plants is unknown, as there have not been many investigations of changes in whole-plant carbon partitioning in response to salinity (Gao *et al.* 1998).

Few genes that regulate carbohydrate levels under salinity stress in plants have been identified. The *Medicago sativa* L. Glycogen Synthase Kinase 3 (GSK3)/Shaggy-like isoform, MsK4, appears to have such a function (Kempa *et al.* 2007). Ectopic expression of MsK4 in *Arabidopsis* enhanced survival on high (100 and 200 mM) NaCl and this was coincident with a 4–5-fold increase in leaf starch and higher sugar accumulation (Kempa *et al.* 2007). It is not known whether higher rosette carbohydrate accumulation led to greater partitioning of assimilates to the non-photosynthetic sinks in *Arabidopsis*. Although the MsK4 isoform regulated carbohydrates in *Arabidopsis*, it is also not known whether this is a feature of all GSK3 isoforms. There are 9 GSK3 paralogues in rice and 10 in *Arabidopsis* (Youn and Kim 2015), whereas mammals and algae have two and a single isoform respectively (Saidi *et al.* 2012). The evolution of multiple paralogues in land plants compared with aquatic algae may have been an adaptation to terrestrial environment and subfunctionalisation (Qi *et al.* 2013). However, a wider survey of diverse unicellular organisms has not yet been conducted to establish a baseline understanding of GSK3 gene duplication and occurrence.

There were two aims of this work. The first aim was to identify and characterise a rice orthologue of MsK4 and to use comparative sequence analysis to determine its possible evolutionary and functional implications. The second aim was to determine (i) whether transgenic rice expressing higher levels of the native rice MsK4 orthologue would condition a better response to salinity as found in *Arabidopsis* (Kempa *et al.* 2007), and (ii) whether there would be alterations in plant carbohydrate status with ensuing effects on source-to-sink partitioning, thereby enhancing survival, in the transgenic rice.

## Materials and methods

### Plant material

#### Plant growth conditions

Rice was grown in 10 cm pots under controlled greenhouse conditions (20–25°C; 14/10 h day/night) in Davis, CA, USA. The potting mixture was composed of UC potting mix (peat moss: coarse sand, 1:1) and perlite 9:1 (v/v), supplemented with Osmocote 17-7-12 controlled release fertilizer (B&T Grower Supply Inc.). The pots were flooded with water or with the NaCl solution at various concentrations in the salt stress experiments.

#### Development of transgenic rice ectopically expressing OsGSK5

A myc-epitope-tagged *OsGSK5* (GenBank locus *Os03g0841800*) full-length cDNA was amplified from the

cDNA library of 150 mM NaCl treated rice root as a *Bam*HI/*Kpn*I fragment. The fragment was cloned into the multiple cloning sites located between the *Ubi1* promoter and the *Nos* terminator of the Ubi-pCAMBIA-1300 vector (Park *et al.* 2010). Rice (*Oryza sativa* L. cv. Kitaake) was then transformed using *Agrobacterium* with either an *OsGSK5* overexpressing construct or the Ubi-pCAMBIA-1300 empty vector at The Plant Transformation Facility, UC Davis (<http://ucdptf.ucdavis.edu>, accessed 20 October 2012). MS medium supplemented with 25 mg L<sup>-1</sup> hygromycin B (Sigma-Aldrich) was used to identify the putative transgenic rice. Ten lines were recovered after transformation, of which only two produced fertile seeds after selfing. These two lines were 'selfed' until stable lines homozygous for the construct (T<sub>4</sub>) were identified by PCR.

### Locus and accession numbers

TAIR and TGIR locus numbers and GenBank accession numbers are listed in the parenthesis following the protein names: AtSK11 (At5g26751), AtSK12 (At3g05840), AtSK13 (At5g14640), AtSK21 (At4g18710), AtSK22 (At1g06390), AtSK23 (At2g30980), AtSK31 (At3g61160), AtSK32 (At4g00720), AtSK41 (At1g09840), AtSK42 (At1g57870), OsSK11/OsGSK2 (Os01g14860), OsSK12/OsGSK3 (Os01g19150), OsSK13/OsGSK6 (Os05g04340), OsSK21/OsGSK1 (Os01g10840), OsSK22/OsGSK7 (Os05g11730), OsSK23/OsGSK4 (Os02g14130), OsSK24/OsGSK8 (Os06g35530), OsSK31/OsGSK9 (Os10g37740), OsSK41/OsGSK5 (Os03g62500), MsK1 (P51137.1), MsK2 (P51138.1), MsK3 (P51139.2), WIG (CAC08564.1), MsK4 (AAN63591.1), VvSK1 (XP\_002272112.1), VvSK2 (XP\_002279596.1), VvSK3 (XP\_002280673.1), VvSK4 (XP\_010660896.1), VvSK6 (XP\_010657061.1), VvSK7 (XP\_002263398.1), TaGSK1 (AAM77397.1), TaGSK2 (BAF36565.1), TaGSK3 (ABG29422.1).

### Methods

#### Protein and DNA sequence analysis

A phylogenetic analysis using maximum likelihood (ML) estimation with the RAxML software was performed (Stamatakis 2006), as previously described (Danchin *et al.* 2010). We systematically ran 100 bootstrap replicates followed by a ML search for the best scoring tree. Trees were generated using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>, accessed 18 May 2013). MEGA 5 (Tamura *et al.* 2011) was used to perform a phylogenetic reconstruction of *Arabidopsis*, *Medicago*, grape, wheat and rice GSK3 homologues using the neighbour-joining method. The gene structure of three rice candidate MsK4 orthologues, *OsGSK1*, *OsGSK2* and *OsGSK5*, was predicted using FancyGene ver. 1.4 (Rambaldi and Ciccarelli 2009).

#### Quantitative real-time RT-PCR

RNA extraction was done with the TRIzol reagent (Invitrogen). Quantification was done using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and integrity assessed by agarose gel electrophoresis. Total RNA of 1 µg was used to synthesise first strand cDNA using the GeneAmp RNA PCR Kit (Applied Biosystems) and 2.5 µM random hexamers (Fermentas) in a 20 µL reaction. The primers for

*OsGSK5* transcript amplification were 5'-TGGACAGGTTT CATTTCATGC-3' and 5'-GGAGTACCCAAAACCTGCAA-3'. To amplify rice *Actin1*, the primers used were 5'-TGTATG CCAGTGGTTCGTACCA-3' and 5'-CCAGCAAGGTTCGAGACGAA-3'. One  $\mu\text{L}$  of cDNA was amplified in a 20  $\mu\text{L}$  PCR reaction containing 1X iTaq Universal SYBR Green Supermix (Bio-Rad) and 300 nM sequence specific primers for *OsGSK5* or *Actin1*. The real-time PCR was performed using an Applied Biosystems 7300 Real-Time PCR System with the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C in MicroAmp Optical 96-well reaction plates with Barcode (Applied Biosystems). Melt-curve analysis was conducted according to the instrument default setting. Three technical replicates were analysed per each biological replicate. The comparative Ct ( $2^{-\Delta\Delta\text{Ct}}$ ) method was used to calculate changes in *OsGSK5* gene expression as a relative gene expression between a treated and an untreated sample (Wong and Medrano 2005).

#### Plant growth conditions and salinity stress experiment

**Expression analysis.** Rice (*O. sativa* cv. Nipponbare) seedlings (14 days post germination) were exposed to 150 mM NaCl for 0, 0.5, 1, 6 and 24 h. Root tissues from two plants were pooled as one biological replicate to obtain enough tissue for analysis. Four biological replicates were harvested and snap frozen in liquid nitrogen at each time point. The experiment was repeated with two other treatments including 50 mM NaCl and 0.129 g g<sup>-1</sup> PEG-8000 (the concentration empirically tested to give equal iso-osmotic potential as 50 mM NaCl in half-strength Hoagland's nutrient solution). Another set of seedlings was subjected to extended dark conditions for 12 and 36 h, respectively, whereas the control plants were kept in the normal 14/10 h light/dark conditions.

**Salinity stress experiment.** Rice cv. Kitaake was grown as described above except that at the four-leaf stage (V4) (Counce *et al.* 2000), NaCl treatments of 0, 40, 80 and 100 mM were added to achieve EC of 0, 4, 8 and 10 dS m<sup>-2</sup> respectively. Each salt treatment was replicated in eight blocks and each genotype was randomised in each block. The EC values of growing solutions were maintained throughout the development (see Fig. S5, available as Supplementary Material to this paper). At the onset of the reproductive stage (R2) (Counce *et al.* 2000), leaves of the main stems and the third tillers, and roots were harvested for analyses of relative water content and amount of carbohydrates accumulated. A parallel experiment was set up whereby the plants were harvested for physiological and agronomical measurements including shoot and root length, fresh weight, dry weight, shoot-to-root ratio, days of panicle initiation, days of anthesis, panicle number, total grain weight and grain fertility.

#### Carbohydrate measurement

Approximately 500 mg tissue was boiled 3 times successively in 1.5 mL 80% (v/v) ethanol for 10 min each. The ethanol was then evaporated, and reconstituted with 300  $\mu\text{L}$  of distilled water. To measure sucrose, 50  $\mu\text{L}$  of sample was added to the 96 well plate containing a 200  $\mu\text{L}$  assay mix (1 U of glucose-6-phosphate dehydrogenase, 1 U of hexokinase, 1 U of phosphoglucosomerase, 20  $\mu\text{L}$  of 1M HEPES pH 6.9, 1  $\mu\text{L}$

of 1M MgCl<sub>2</sub>, 4  $\mu\text{L}$  of 50 mM ATP, and 4  $\mu\text{L}$  of 100 mM NAD. The A340 absorbance was recorded, and then 35 U of alkaline invertase was added to the mixture. The A340 nm absorbance was recorded after the reaction completed, and the difference in absorbance before and after invertase addition was used to calculate for sucrose content using glucose as a standard. The ethanol insoluble residue was digested to glucose and the amount of starch was assayed as previously described (Luengwilai and Beckles 2010).

#### Construction of <sup>14</sup>C single leaf labelling chamber

A <sup>14</sup>CO<sub>2</sub>-labelling device was created following the *Arabidopsis* prototype (Kölling *et al.* 2013) with some modifications (Fig. S7). The gas reservoir chamber of 24 × 24 × 31 cm<sup>3</sup> and a leaf chamber of 0.5 × 0.5 × 10 cm<sup>3</sup> were made with Plexiglas. The beaker containing <sup>14</sup>C sodium bicarbonate was placed on the stand mounted right below the acid inlet, allowing for acidification of the bicarbonate. The 12V small electronic fan (Sunon Inc.) and the 6V miniature air pump (Koge Electronics) were installed to disperse and pump the generated <sup>14</sup>CO<sub>2</sub> into the tube, which was connected to the leaf chamber. The <sup>14</sup>CO<sub>2</sub> travelled back to the reservoir chamber through another tube. These two tubes were connected in the middle via 2 Discifix 3-way stopcocks (B. Braun Medical Inc.) to allow for bypassing of the <sup>14</sup>CO<sub>2</sub> back to the chamber during the replacement of the plant sample.

#### <sup>14</sup>CO<sub>2</sub> labelling and fractionation

**<sup>14</sup>CO<sub>2</sub> labelling.** Two sets of null segregants (NS), overexpressing (OE) and co-suppressing (CS) plants were kept in 0 or 100 mM NaCl for 6 h before the labelling. After 6 h into the light period, a single mature leaf of a three-leaf stage seedling plant (V3 stage) (Counce *et al.* 2000) was placed in the leaf chamber. <sup>14</sup>CO<sub>2</sub> was generated from 0.08 MBq NaH<sup>14</sup>CO<sub>3</sub> (Sigma-Aldrich) acidified with 200  $\mu\text{L}$  of 10% (v/v) lactic acid in the reservoir chamber. After 15 min, the <sup>14</sup>CO<sub>2</sub> bypassed the leaf chamber and shunted back to the reservoir chamber. New plants were replaced successively after each pulse. Labelled plants were left to chase in light for 1, 2 and 3 h. At the end of the feeding, 500  $\mu\text{L}$  of 10% (v/v) KOH was added to the <sup>14</sup>CO<sub>2</sub> generating beaker for 30 min to stop its generation and to capture the <sup>14</sup>CO<sub>2</sub> in the chamber.

**Tissue fractionation.** The tissues were homogenised in liquid nitrogen, boiled successively for 10 min each in 80, 50 and 20% (v/v) ethanol, and then separated into the soluble and insoluble fractions. The insoluble fractions were digested with amyloglucosidase (10 U per 200  $\mu\text{L}$  of insoluble fraction, Roche Biosciences) to analyse for starch (Centeno *et al.* 2011). The ethanol soluble fractions were separated into the sugar, amino acid, and organic acid fractions using ion-exchange column chromatography (Luengwilai *et al.* 2010). <sup>14</sup>C activity was measured by liquid scintillation counting.

#### Statistical analysis

Descriptive statistics were calculated for all variables, and the test for significant changes among different genotypes was done using one-way ANOVA on the SPSS statistics software (IBM Corp.). The differences were deemed significant at  $P < 0.05$  and Tukey's *post-hoc* test was done where significant differences



were found. Spearman correlative analysis was performed using *OsGSK5* transcript levels and all physiological data from the transgenic plants on Metabox (Wanichthanarak *et al.* 2017). The correlation was deemed significant at  $P < 0.05$ . The correlative network was drawn using Cytoscape (Shannon *et al.* 2003).

## Results

### Sequence and expression analyses of *OsGSK5*

#### *Phylogenetic analysis of GSK3s in eukaryotic microorganism and plants*

GSK3 homologues are implicated in several signalling pathways in mammals, plants, fungi and amoebae (Saidi *et al.* 2012). To elucidate the evolutionary history of the GSK3-like proteins, a phylogenetic reconstruction was performed (Fig. 1). GSK3-like genes were identified in all eukaryotic taxa studied independently of the type of their storage carbohydrate,  $\alpha$ - or  $\beta$ -glucans. In contrast to the low copy numbers observed in most eukaryotic lineages, GSK3-like genes had expanded into a multi-gene family (Fig. 2a) in land plants (Qi *et al.* 2013).

#### *Identification of a rice Msk4 orthologue*

A *Medicago* GSK3 homologue, MsK4, was previously shown to confer salinity tolerance when ectopically expressed in *Arabidopsis*, by regulating levels of starch and other carbohydrates (Kempa *et al.* 2007). The phylogenetic reconstruction of known plant GSK3 homologues (see Table S1, available as Supplementary Material to this paper) was thus performed to identify rice MsK4 orthologues (Fig. 2a). A sequence denoted OsGSK5 (Fig. 2a) was the closest homologue (81% identity). They were grouped into clade IV (Jonak and Hirt 2002), whereas the other two candidates, OsGSK2 and OsGSK1 (>75% similarity to MsK4), fell into clade I and II (Fig. 2a). Gene structure analysis also showed that like MsK4, *OsGSK5* and *OsGSK1* had an additional exon in the 5' variable regions (Fig. 2b). Semiquantitative RT-PCR indicated that *OsGSK5* was preferentially expressed in the root compared with leaf (data not shown).

#### *Expression pattern of rice OsGSK5*

The cis-regulatory elements in the promoter region of *OsGSK5* inferred a putative role for this gene in stress responsive pathways and carbohydrate gene regulation (Table S2) (Verslues and Zhu 2005). *OsGSK5* may also be inducible by sugar starvation (Lu *et al.* 2002), since its promoter contained elements required for the transcriptional regulation of  $\alpha$ -amylase (Table S2). In addition, *OsGSK5* transcript was higher in mature root, immature panicle and *Xanthomonas oryzae* infected leaf (Fig. S4) (Nobuta *et al.* 2007), supporting a role in sink organs and stressed tissue.

Changes in *OsGSK5* transcript levels in response to salinity, osmotic stress and carbohydrates were measured in the root of the treated plants compared with a control, to determine if this gene may have shared functionality to *MsK4* (Kempa *et al.* 2007).

**Salt and osmotic stress.** *OsGSK5* expression was induced by 150 mM NaCl, 6 h post exposure (Fig. 3a). However, its transcript was downregulated by moderate (50 mM) salt

concentration, 30 min and 6 h post exposure. It was unknown if the salinity response was due solely to an osmotic or ionic effect, or a combination of the two, as high salt triggers both (Munns and Tester 2008). Therefore, the osmotic potential of the rice-growing solution was lowered using polyethylene glycol (PEG) in order to differentiate between these effects (Castillo *et al.* 2007). The data showed that there was no *OsGSK5* transcriptional response to changes in osmotic potential due to PEG (Fig. 3b).

**Sugar starvation.** Fourteen-day-old seedlings were grown in an extended dark period (12 h) in order to induce sugar starvation in roots. This lowered sucrose 3-fold in the root (Fig. 3d) and was accompanied by 35-fold higher *OsGSK5* transcript level (Fig. 3c).

### Functional characterisation of *OsGSK5* transgenic plants

#### *Generation of *OsGSK5* transgenic plants*

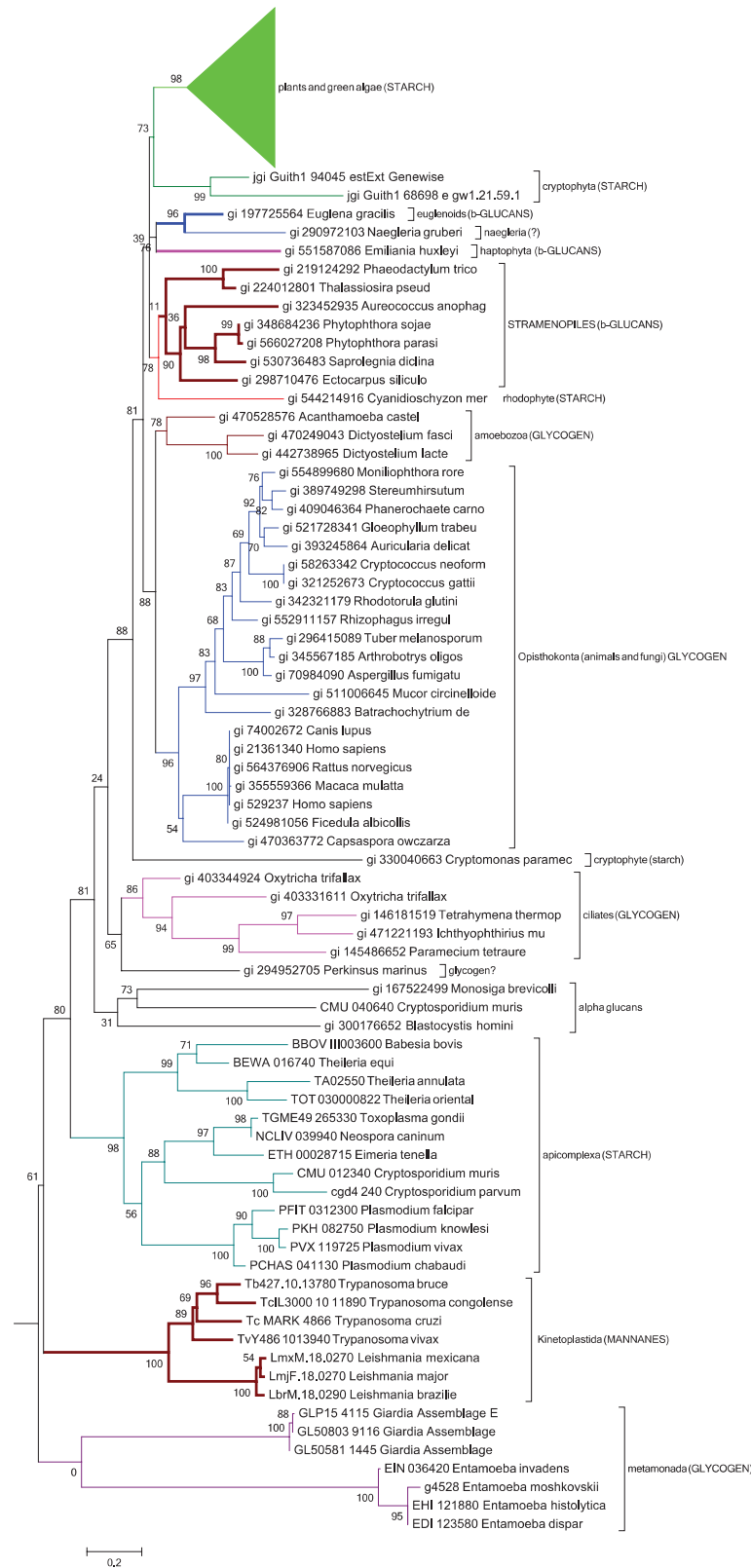
Transgenic rice lines homozygous for the presence of the empty vector (EV) and *OsGSK5*-overexpressing construct were identified. A null segregant (NS) that lost the construct through segregation was also selected as a control. Leaf *OsGSK5* expression in the EV was similar to that of the NS control. The overexpressing (OE) line had 3.5-fold higher *OsGSK5* transcript ( $P < 0.05$ ) compared with the NS, whereas the co-suppressing (CS) lines accumulated 1-fold lower transcript, but the difference was not significant ( $P = 0.511$ ; Fig. 4a). After initial physiological results showed that the EV did not differ significantly from the NS, in all subsequent experiments, the NS, OE and CS, which represented rice lines with normal, high and low *OsGSK5* expression were selected for further study.

#### *Contrasting phenotypes of *OsGSK5* overexpressor and *OsGSK5* co-suppressed lines under high salinity*

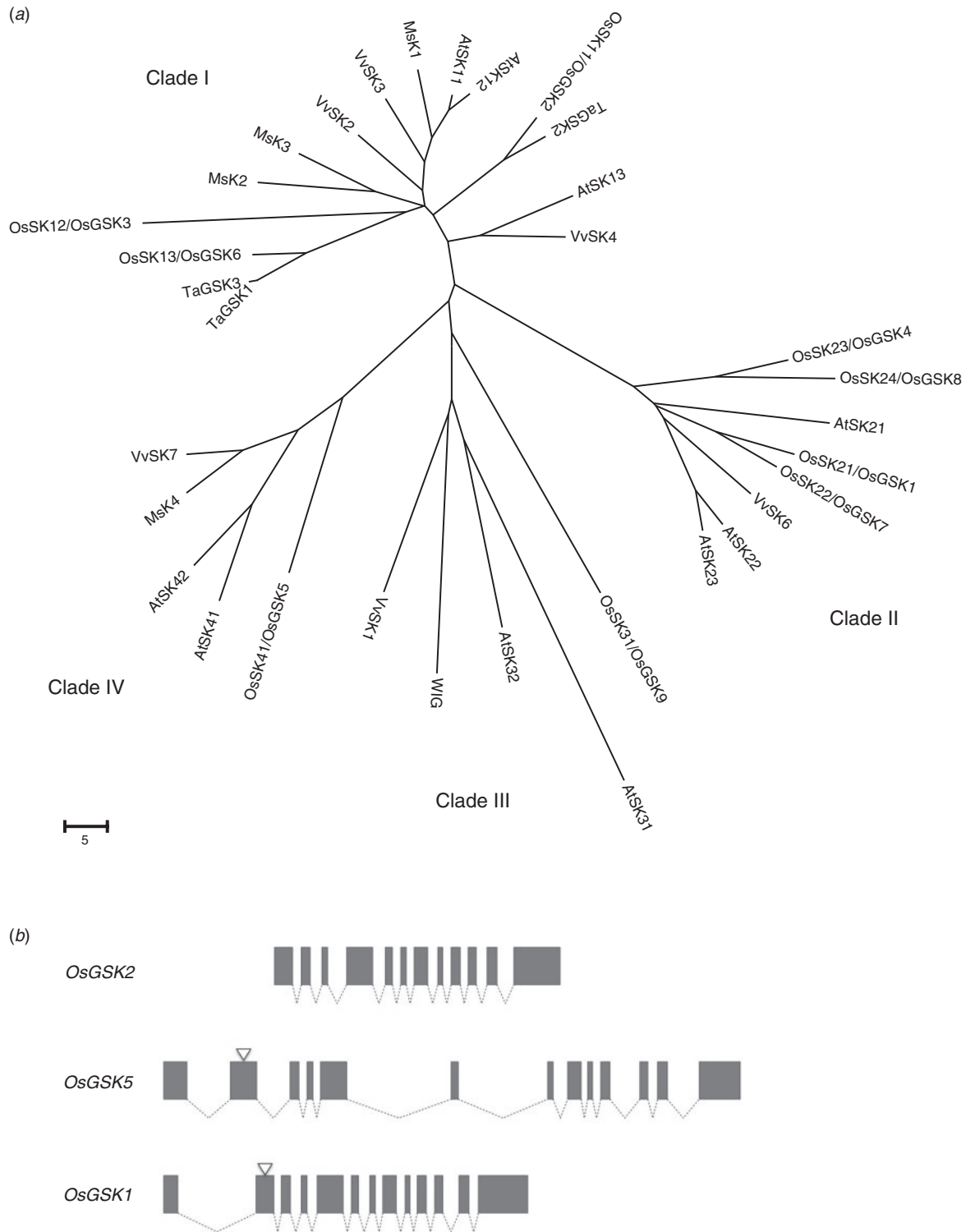
Under control conditions, there was no difference in growth between the OE line and the other genotypes (Table 1). However, shoot fresh and dry weights (SFW and SDW) and root fresh and dry weights (RFW and RDW) were higher in the OE under saline conditions (Table 1).

Rice plants were exposed to a range of saline conditions, the severity of which was classified based on the effect of the stress on 100-grain weight. There were no reductions in weight at 20 mM NaCl, and only small (18%) and non-significant reductions ( $P > 0.05$ ) at 40 mM NaCl, and whereas at 80 mM NaCl weight decreased by 50% (data not shown). Therefore, 40 and 80 mM were selected as mild and moderate stress respectively and a 100 mM NaCl treatment was added to impose a high stress. The EC of each salt treatment was maintained throughout the plant life cycle to ensure treatment stability (Fig. S5). Under 40 mM NaCl, the OE line had 48–122% higher SFW compared with the other genotypes (Table 1). Under 80 and 100 mM NaCl, SFW and SDW, was also higher (153 and 57% respectively) in the OE (Table 1). The OE line also showed better development: superior panicle growth under 80 mM NaCl, and greater leaf initiation under 100 mM NaCl (Fig. 4b). The relative water content (RWC) among genotypes, however, was similar when they were treated with salt (Table 1).

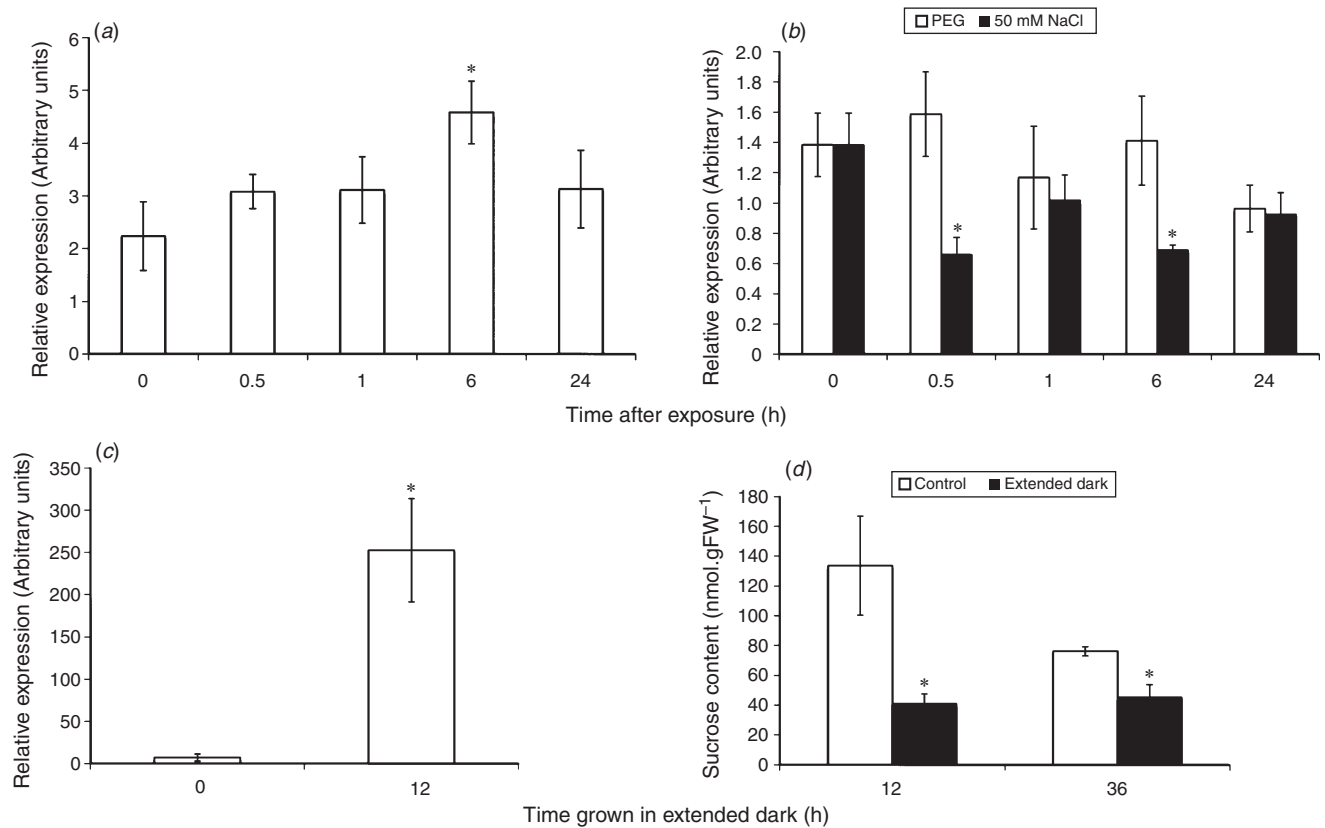
The reproductive capacity among different rice lines under mild salinity (40 mM) was also examined. This salinity level is more likely to occur in the field than is a non-agronomic salt



**Fig. 1.** Phylogenetic analyses of the GSK3-like protein sequences. Representation of the phylogenetic tree was generated by FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>, accessed 18 May 2013). The different branch colours correspond to the different taxa. Bootstrap support values from the maximum likelihood (ML) analysis are indicated at each node.



**Fig. 2.** (a) Evolutionary relationship of known *Arabidopsis* (At), *Medicago* (Ms), grape (Vv), wheat (Ta), and rice (Os) GSK3-like homologues. The evolutionary history was inferred using the neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. (b) Exon and intron structure of 3 rice candidate MsK4 orthologous genes. The grey boxes represent exons and dotted lines represent introns. Open triangles indicate additional exons only present in *OsGSK5* and *OsGSK1*.



**Fig. 3.** Transcriptional response of *OsGSK5* to salt and osmotic stresses and extended dark in rice roots. Relative expression of *OsGSK5* in roots under (a) 150 mM NaCl, (b) 50 mM NaCl and osmotic stress ( $0.129 \text{ g g}^{-1}$  PEG-8000) and (c) under 12 h extended dark. (d) Sucrose content in rice roots grown under 12 and 36 h extended dark. The treated plants were moved to dark chamber after the end of 10 h dark period for an additional 12 and 36 h, whereas the control plants were kept in its normal 14/10 h light/dark photoperiod. Significant differences among means as indicated by Student's *t*-test are indicated: \*,  $P \leq 0.05$  ( $n = 4$ ).

shock ( $>100 \text{ mM NaCl}$ ) (Shavrukov 2013). However, no difference in panicle initiation, anthesis time and grain fertility was observed among genotypes (Table S3). Panicle number and total grain weights among genotypes were similar, although both were compromised by salt (Table S3).

#### *Accumulation of starch and sucrose in OsGSK5 overexpressor under high salinity*

If *OsGSK5* is a key regulator of plant salt stress response and carbohydrate metabolism, similar to its homologue *Msk4* (Kempa *et al.* 2007), then the starch contents of sink and source organs should differ among the genotypes when grown on salt. The mature leaf on the main stem (source), the young leaf on the third tiller (sink) and root (sink) tissues were harvested and assayed for these carbohydrates in order to test this hypothesis.

Under control condition, the starch in the OE source leaf was 38 and 43% higher than that of the NS and CS lines respectively (Fig. 5a). Salinity did not change starch levels in source leaves of any of the genotype except in the NS where it was reduced to levels seen in the CS. In contrast, there were no changes in starch in sink leaves, under either control or saline conditions (Fig. 5b).

The starch content of non-photosynthetic tissues (i.e. root and grain) also showed important differences. Under non-saline conditions, starch content in the root was 68.1% higher in the

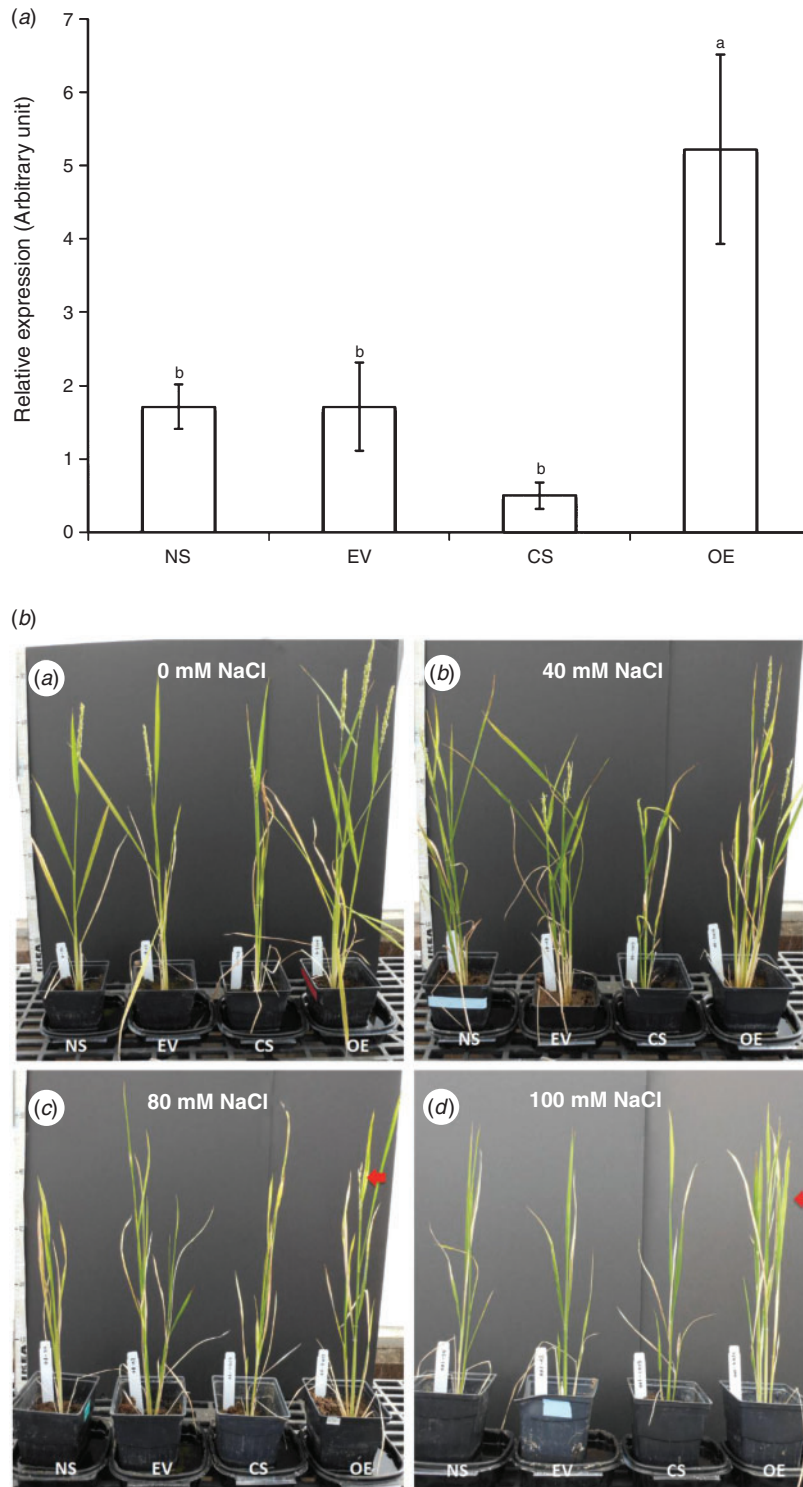
OE compared with the NS control. Starch content was unaffected by salinity in all of the genotypes so that the levels in the OE root were still comparatively higher (Fig. 5c). Altering *OsGSK5* levels did not affect grain starch content under control conditions. Under the mild salinity tested ( $40 \text{ mM NaCl}$ ) starch was reduced in the CS plants only (Table S4).

Sucrose is the primary assimilate that is exported from source to sink in rice (Scofield *et al.* 2007). Its accumulation was stable and did not vary as a result of genotype or salt treatment in any tissue tested i.e. mature leaf, main stem leaf, root or grain (Table S4; Fig. S6).

#### *OsGSK5 transcript level positively correlates with tolerant characteristics*

Spearman's rank correlative analysis was performed to determine if varying *OsGSK5* transcript levels were statistically associated with any of the physiological parameters assayed especially under salinity. This was important to determine especially when plant-to-plant variability reduced statistical differences among genotypes (Table 1). There was a strong positive correlation between *OsGSK5* transcript and (i) grain starch ( $r^2 = 0.713$ ), and (ii) source leaf starch ( $r^2 = 0.608$ ) under salinity stress (Fig. 6). However, the parameters that showed the strongest correlation with *OsGSK5* transcript levels were root fresh and dry weights from plants cultured in both control





**Fig. 4.** Effect of salinity stress on the development of *OsGSK5* transgenic plants. (a) Transcript levels of *OsGSK5* overexpression (OE), co-suppression (CS), null segregant (NS), and empty vector (EV) rice lines. Values are means  $\pm$  s.e. Means of each column with different letters are significantly different by Tukey's test ( $P \leq 0.05$ ;  $n = 4$ ). (b) Rice plants at harvest under (a) 0, (b) 40, (c) 80 and (d) 100 mM NaCl treatments. Red arrows indicate panicle formation and a greater number of leaves as seen in the *OsGSK5* OE lines in (c) and (d) respectively.

**Table 1. Physiological characteristics of different rice lines under salinity stress**

Values are means  $\pm$  s.e. For each level of electrical conductivity (EC), means within each column with different letters are significantly different according to Tukey's test. Values that significantly higher than all other genotypes are indicated: \*,  $P \leq 0.05$  ( $n = 8$ ). Abbreviations: S-to-R, shoot-to-root ratio; OE, OsGSK5 overexpression line; CS, co-suppression line; NS, null segregant line; EV, empty vector line

EC (dS m <sup>-2</sup> )	Genotypes	RWC (%)	SL (cm)	RL (cm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)	S-to-R (length basis)	S-to-R (DW basis)
0	NS	94.2 $\pm$ 2.4a	60.9 $\pm$ 2.0a	23.1 $\pm$ 1.1a	6.8 $\pm$ 0.9ab	3.5 $\pm$ 0.5b	2.7 $\pm$ 0.4ab	0.8 $\pm$ 0.1ab	2.7 $\pm$ 0.1a	3.4 $\pm$ 0.3a
	EV	98.0 $\pm$ 0.7a	63.7 $\pm$ 2.2a	23.3 $\pm$ 1.3a	7.4 $\pm$ 1.0ab	4.5 $\pm$ 0.7ab	3.4 $\pm$ 0.5a	0.8 $\pm$ 0.2ab	2.8 $\pm$ 0.2a	3.1 $\pm$ 0.4a
	CS	97.3 $\pm$ 0.4a	56.7 $\pm$ 2.1a	25.4 $\pm$ 1.8a	4.7 $\pm$ 0.6b	2.3 $\pm$ 0.3b	1.7 $\pm$ 0.2b	0.5 $\pm$ 0.1b	2.3 $\pm$ 0.2a	3.5 $\pm$ 0.4a
	OE	96.8 $\pm$ 0.2a	65.4 $\pm$ 2.9a	27.7 $\pm$ 1.6a	9.8 $\pm$ 0.9a	6.2 $\pm$ 1.0a	4.0 $\pm$ 0.4a	1.2 $\pm$ 0.2a	2.4 $\pm$ 0.2a	3.1 $\pm$ 0.4a
4	NS	87.4 $\pm$ 2.7a	48.1 $\pm$ 2.9a	25.9 $\pm$ 1.7a	4.5 $\pm$ 0.8b	1.7 $\pm$ 0.4b	1.6 $\pm$ 0.3b	0.4 $\pm$ 0.1ab	2.1 $\pm$ 0.2a	4.6 $\pm$ 0.5a
	EV	87.0 $\pm$ 2.1a	46.6 $\pm$ 0.8ab	26.6 $\pm$ 1.6a	4.8 $\pm$ 0.5b	2.0 $\pm$ 0.3ab	1.8 $\pm$ 0.2ab	0.4 $\pm$ 0.1ab	1.8 $\pm$ 0.1a	3.8 $\pm$ 0.6a
	CS	88.5 $\pm$ 2.4a	39.8 $\pm$ 1.7b	23.8 $\pm$ 1.1a	3.2 $\pm$ 0.3b	1.1 $\pm$ 0.2b	1.2 $\pm$ 0.1b	0.3 $\pm$ 0.0b	1.7 $\pm$ 0.1a	5.1 $\pm$ 0.6a
	OE	88.5 $\pm$ 1.7a	48.4 $\pm$ 1.8a	26.9 $\pm$ 1.5a	7.1 $\pm$ 0.6a*	3.2 $\pm$ 0.4a	2.6 $\pm$ 0.3a	0.6 $\pm$ 0.1a	1.7 $\pm$ 0.1a	4.6 $\pm$ 0.5a
8	NS	86.5 $\pm$ 2.5a	40.8 $\pm$ 1.6a	19.9 $\pm$ 2.1a	1.7 $\pm$ 0.3b	0.5 $\pm$ 0.2b	0.7 $\pm$ 0.1b	0.1 $\pm$ 0.1b	2.2 $\pm$ 0.2a	5.4 $\pm$ 1.0a
	EV	85.9 $\pm$ 1.8a	42.4 $\pm$ 2.3a	26.2 $\pm$ 2.4a	3.0 $\pm$ 0.3ab	0.9 $\pm$ 0.2b	1.1 $\pm$ 0.1ab	0.2 $\pm$ 0.0ab	1.7 $\pm$ 0.2ab	5.6 $\pm$ 0.5a
	CS	84.5 $\pm$ 3.3a	36.4 $\pm$ 2.4a	23.3 $\pm$ 0.8a	1.7 $\pm$ 0.4b	0.5 $\pm$ 0.1b	0.7 $\pm$ 0.1b	0.1 $\pm$ 0.0b	1.6 $\pm$ 0.1b	4.4 $\pm$ 0.5a
	OE	91.1 $\pm$ 0.8a	43.8 $\pm$ 1.5a	23.4 $\pm$ 1.1a	4.3 $\pm$ 0.6a	2.0 $\pm$ 0.4a*	1.7 $\pm$ 0.3a	0.3 $\pm$ 0.0a	2.0 $\pm$ 0.1ab	4.7 $\pm$ 0.4a
10	NS	83.4 $\pm$ 3.4a	39.9 $\pm$ 1.4a	23.1 $\pm$ 2.2a	2.6 $\pm$ 0.2b	0.6 $\pm$ 0.2b	1.0 $\pm$ 0.1ab	0.2 $\pm$ 0.0ab	2.1 $\pm$ 0.3a	6.5 $\pm$ 1.0a
	EV	76.3 $\pm$ 3.5a	38.4 $\pm$ 0.9a	20.7 $\pm$ 1.2a	2.5 $\pm$ 0.4b	0.4 $\pm$ 0.1b	1.0 $\pm$ 0.2ab	0.1 $\pm$ 0.0b	1.9 $\pm$ 0.1a	5.7 $\pm$ 0.7a
	CS	81.4 $\pm$ 2.4a	38.5 $\pm$ 1.4a	19.5 $\pm$ 1.8a	2.3 $\pm$ 0.1b	0.3 $\pm$ 0.1b	0.9 $\pm$ 0.0b	0.1 $\pm$ 0.0b	2.1 $\pm$ 0.2a	6.1 $\pm$ 0.5a
	OE	84.6 $\pm$ 1.8a	39.0 $\pm$ 1.1a	23.1 $\pm$ 1.5a	3.6 $\pm$ 0.3a*	1.5 $\pm$ 0.3a*	1.4 $\pm$ 0.1a	0.3 $\pm$ 0.0a	1.7 $\pm$ 0.1a	5.1 $\pm$ 0.6a

and saline solutions ( $\rho$  values ranging from 0.692 to 0.832; Fig. 6). These results suggested that OsGSK5 may be attributed to better overall plant growth through biochemical and physiological changes in the root. Therefore, a contributing role for OsGSK5 in altering carbon partitioning and allocation in plant adaptation to high salinity was determined.

#### Time-course <sup>14</sup>C distribution in transgenic plants under high salinity

A single leaf <sup>14</sup>CO<sub>2</sub>-labelling system (Fig. S7) was used to investigate photoassimilate partitioning between source (<sup>14</sup>C fed mature leaf) and sinks (young leaf and root) in NS, OE and CS. OsGSK5 is likely part of an early response to high salinity stress (Fig. 3a); therefore, plants were exposed to high salinity (100 mM) for 1 h, to identify short-term adaptive changes in whole-plant <sup>14</sup>C partitioning and intra-organ allocation into different metabolic pools. After <sup>14</sup>C pulsing, the radioactivity in different organs and fractions was monitored over different time periods (1, 2 and 3 h post <sup>14</sup>C labelling) i.e. the chase, the details of which are in Figs S8–10. The data showed great variability and only salient features of those data were discussed.

**<sup>14</sup>C whole-plant partitioning.** High salt caused a reduction in the percentage of <sup>14</sup>C remaining in OE source leaves after the 3 h chase, which was paralleled with a corresponding increase in the total <sup>14</sup>C in the OE roots ( $P = 0.19$ ) (Fig. S8). The gradient was greater in OE compared with the CS, suggesting higher mobilisation of assimilates to the root in the former. The rate of change in <sup>14</sup>C in each organ induced by salinity stress was calculated (Fig. 7). There was a higher rate of <sup>14</sup>C loss in the OE source leaf and a higher rate of total <sup>14</sup>C accumulation in the root compared with NS.

**<sup>14</sup>C allocation into different metabolic pools.** Next, we tracked changes in amino acids, organic acids and especially, sugars and starch in source and sink tissues. The observed higher <sup>14</sup>C mobilisation between source and sink in the OE

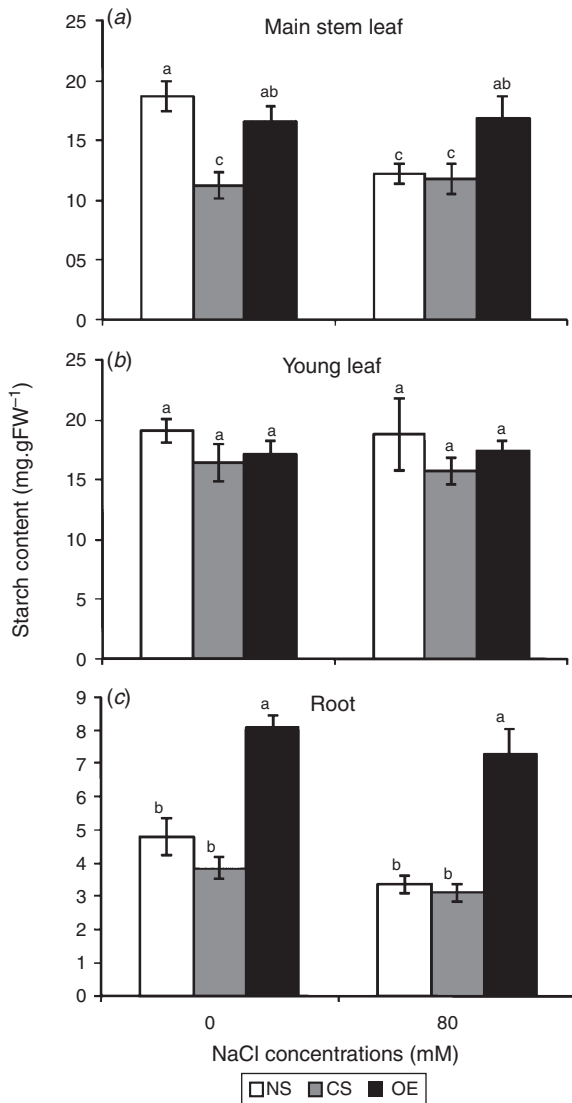
may be attributed to relative changes in sugar and starch in these tissues (Fig. 7). In the absence of salt, more <sup>14</sup>C was allocated to sugars in the OE source leaf compared with the NS ( $P = 0.06$ ) and CS ( $P = 0.13$ ) after the 1 h chase (Fig. S9a). However, salt treatment reduced the proportion allocated to sugars ( $P < 0.05$ ) in the OE and increased it in the amino ( $P = 0.06$ ) and organic acids pools ( $P < 0.05$ ). In contrast to the OE, salinity increased the <sup>14</sup>C in the sugar fraction of the CS source leaves after 3 h ( $P < 0.05$ ; Fig. S9a).

In parallel with decreased <sup>14</sup>C incorporation into sugars in the OE source leaf, we observed increased allocation of label into sugars in the sinks ( $P = 0.15$ ) at high salt after the 1 h chase (Fig. S9b). After 3 h, <sup>14</sup>C in sugars decreased in the OE root compared with initial levels ( $P = 0.11$ ) such that it was even lower than that in the other two genotypes ( $P = 0.06$ ) (Fig. S9c). In accordance, label in the starch fraction in this tissue was greater than that of CS 3 h post-labelling (36%;  $P = 0.14$ ; Fig. S10c). This suggests that decreases in root sugars at this time-point were due to their conversion to starch (Fig. S9c).

## Discussion

### Evolutionary history of plant GSK3s and the identification of MsK4 orthologue in rice

GSK3 is an ancient gene present in all eukaryotic species studied (Fig. 1) (Saidi *et al.* 2012). In line with previous studies, only one copy of a GSK3-like gene was found in the eukaryotes studied with the exception of *Guillardia theta*, yeast, green plants and humans. This is consistent with the idea that multiple duplications and subsequent expansion of the GSK3 family played a significant role in the stress tolerance adaptation to the terrestrial environment of early land plants (Saidi *et al.* 2012). GSK3 in mammals regulates glycogen and a few plant GSK3 isoforms have been shown to regulate starch and sugars (Kempa *et al.* 2007; Lecourieux *et al.* 2010). We identified GSK3 sequences in organisms that synthesise a diverse array



**Fig. 5.** Starch content in (a) the main stem leaf (b) the third tiller (young) leaf and (c) the roots of different rice lines under 80 mM NaCl treatments. Values are means  $\pm$  s.e. Means of each column with different letters are significantly different according to Tukey's test ( $P \leq 0.05$ ;  $n = 6$ ). OE is *OsGSK5* overexpression line; CS is co-suppression line; NS is null segregant line; EV is empty vector line.

of storage polysaccharides (Fig. 1), i.e. both  $\alpha$ -glucans (Kempa *et al.* 2007) or  $\beta$ -glucans (Cohen and Frame 2001). This large distribution suggests that GSK3 family is implicated in the carbon allocation independently of the type of glucans metabolised.

Sequence comparisons of various plant GSK3 isoforms showed that a putative protein *OsGSK5* in rice was closely related to *MsK4* and thus may show conserved functionality (Fig. 2a). All plant GSK3 homologues in the same clade as *MsK4*, including *OsGSK5*, have an additional exon in the structurally variable 5' region (Yoo *et al.* 2006) (Fig. 2b). In addition, *OsGSK5* and *OsGSK1* were both transcriptionally responsive to 150 mM NaCl treatment (Fig. S1a, b). However, *OsGSK1* is an orthologue of the *Arabidopsis BIN2* gene and a negative

regulator of the rice salt responsive pathway (He *et al.* 2002; Koh *et al.* 2007). In contrast, *MsK4* was a positive regulator of the salt adaptive trait in transgenic *Arabidopsis* (Kempa *et al.* 2007). Therefore, *OsGSK1* is not likely to be an orthologue of *MsK4*.

Because GSK3 evolved from a common ancestor, there should be conserved motifs and domains within the plant and mammalian homologues related to their functionality. Plant GSK3 isoforms would also be expected to show subfunctionalisation (Jonak and Hirt 2002). Multiple sequence analysis and comparative protein homology modelling of plant homologues revealed key amino acid changes, especially those located in the N-terminal regions that may contribute to the putative unique functionality of *MsK4* and *OsGSK5*, relative to the other plant homologues (Figs S2, S3).

#### *OsGSK5* is responsive to ionic stress and sugar starvation

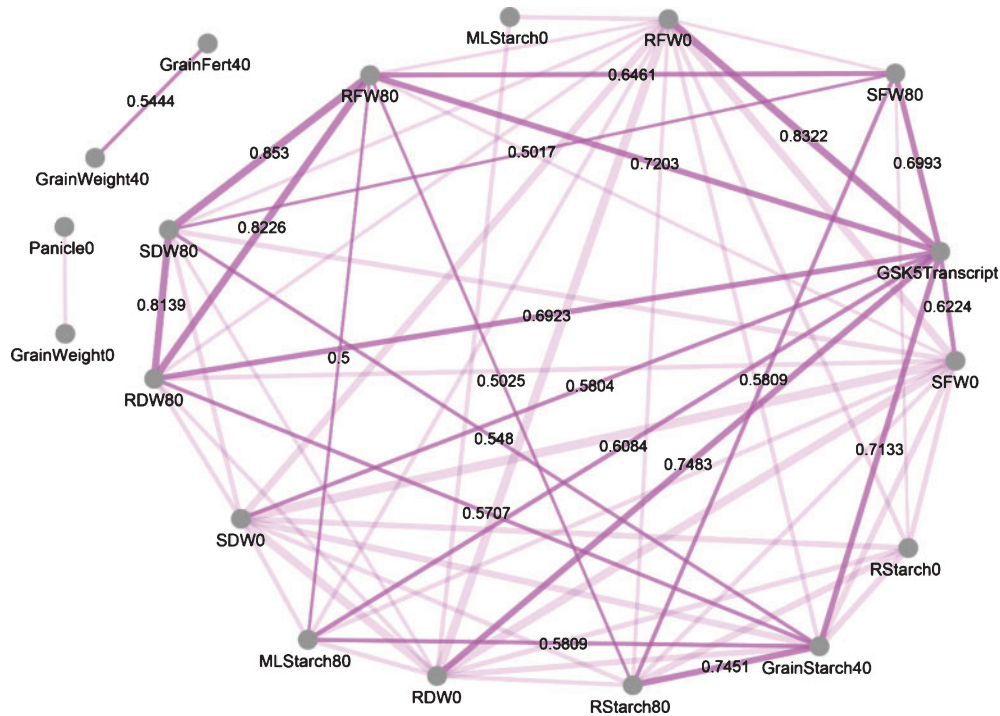
High salinity changes the ionic and osmotic status of the cell, however, the *OsGSK5* transcript only responded to changes in ionic concentration (Fig. 3b) (Lefèvre *et al.* 2001). It also responded to a salt shock (150 mM) and salt stress (50 mM) in opposite ways i.e. being stimulated and repressed respectively (Fig. 3a, b). The kinase activity of the ectopically expressed *MsK4* was induced by NaCl, KCl and sorbitol in transgenic *Arabidopsis* (Kempa *et al.* 2007). It is possible that *OsGSK5* may regulate a salinity stress response in rice at the post-translational level similar to *MsK4* (Kempa *et al.* 2007).

*OsGSK5* transcript in the root was upregulated when sugar starvation was induced by growing rice seedlings in an extended dark period (Fig. 3c). This response potentially differs from *VvSKI*, which was transcriptionally upregulated by sugar in its native grape cell suspension (Lecourieux *et al.* 2010). The presence of  $\alpha$ -amylase elements in the *OsGSK5* promoter (Table S2) may facilitate triggering of transcriptional activity by sugar starvation (Lu *et al.* 2002), which may be connected to *OsGSK5* functionality. However, this gene may also be upregulated by dark conditions rather than the reduction in sucrose levels induced by darkness (Sato-Nara *et al.* 2004).

*OsGSK5* expression was induced by a short-term salt shock, which supports its role in a salt stress pathway. Its upregulation also coincided with the extended dark-induced sugar starvation, suggesting its possible function in carbohydrate metabolic pathways. The transcriptional response of this *OsGSK5* to ionic stress might be transduced through crosstalk between hormonal signalling pathways (Yaling and Lizhong 2012), consistent with the presence of the AUX and ABA responsive elements in its promoter region (Table S2). Potential interactors of *OsGSK5* were queried at the Rice Kinase Database (<http://ricephylogenomics.ucdavis.edu/kinase/interactome>, accessed 8 March 2015). Two candidates, both uncharacterised proteins, were predicted to interact with *OsGSK5* (Dardick *et al.* 2007). Whether they are involved in this hormonal crosstalk is unknown.

#### Overexpressing *OsGSK5* confers salinity tolerance in rice

*OsGSK5* transcript levels correlated with better growth through enhanced shoot and root biomass, leaf number and grain parameters under both salinity stress and control conditions



**Fig. 6.** Network analysis showing the relationships between *OsGSK5* transcript levels and some physiological characteristics of the transgenic lines. Each node (grey circle) represents a variable used in the analysis. The number shown on each edge (line) is the Spearman's rank correlation coefficient ( $\rho$ ) between the two connecting nodes. Only correlations that met the  $P < 0.05$  threshold for statistical significance are shown, and higher correlations values are indicated with a thicker line. Abbreviations: GSK5 Transcript, *OsGSK5* transcript level; SFW0 and SFW80, shoot fresh weight at 0 and 80 mM NaCl; RFW0 and RFW80, root fresh weight at 0 and 80 mM NaCl; SDW0 and SDW80, shoot dry weight at 0 and 80 mM NaCl; RDW0 and RDW80, root dry weight at 0 and 80 mM NaCl; MLStarch0 and MLStarch80, mature leaf starch at 0 and 80 mM NaCl; RStarch0 and RStarch80, root starch at 0 and 80 mM; Panicle0, panicle number at 0 mM NaCl; GrainWeight0 and GrainWeight40, grain weight at 0 and 40 mM NaCl; GrainStarch40, grain starch at 40 mM NaCl; GrainFert0 and GrainFert40, grain fertility at 0 and 40 mM NaCl.

(Table 1; Figs 4, 6). Higher *OsGSK5* transcript also supported the transition from the vegetative to reproductive phase under salinity stress. This was also seen in *Arabidopsis* plants overexpressing *MsK4*, which had the capacity to flower under very high (200 mM NaCl) salinity, whereas the wild type did not (Kempa *et al.* 2007). Similarly, better panicle development was seen in the OE line grown under 80 mM NaCl (Fig. 4b). No difference in yield components among the rice lines under mild salinity was observed (Table S3). As indicated by the lack of activation of the *OsGSK5* transcript by PEG, *OsGSK5* overexpression likely facilitated a better response to cellular ionic changes, rather than through osmotic adjustments under high salt. This was further supported by the observation that RWC was unchanged when different *OsGSK5* genotypes were salt-treated (Table 1; Fig. 3b).

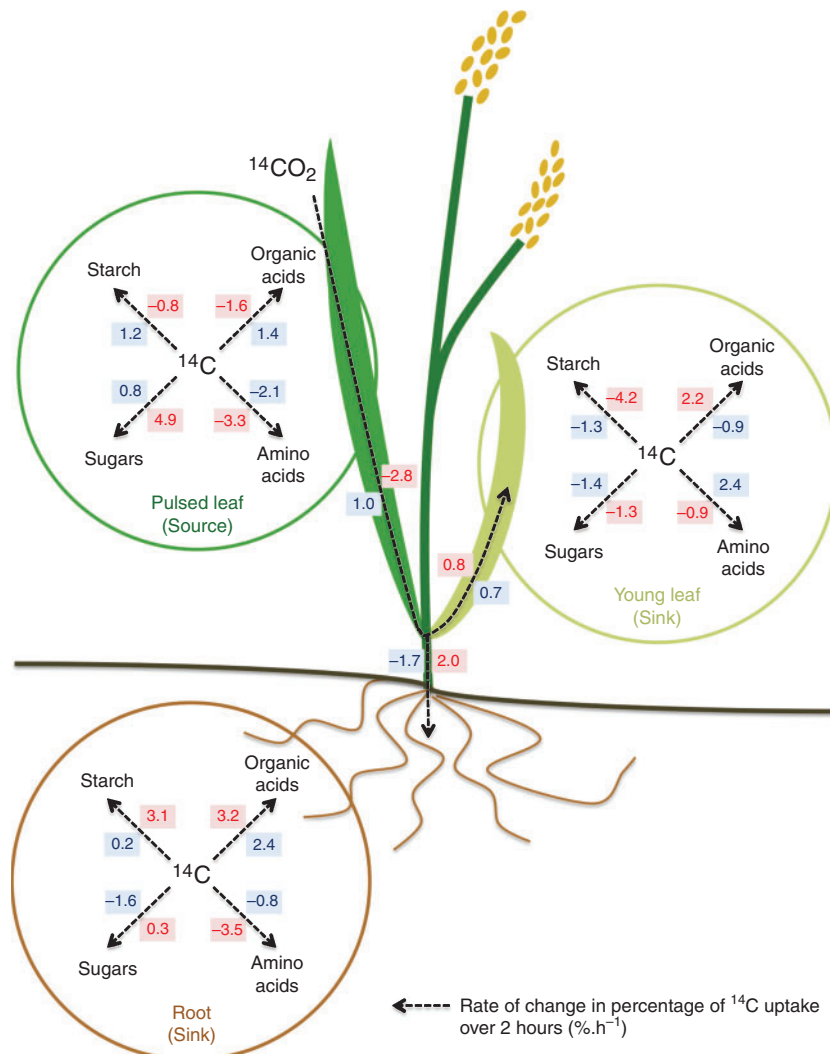
#### Changes in carbon partitioning and allocation is key to salinity tolerance

Salinity reduced transitory starch accumulation and metabolism in NS source leaves, whereas starch levels were maintained in the OE. Moreover, CS leaf starch was compromised under both normal and salt stress conditions (Fig. 5a). This suggests that *OsGSK5* is required for normal transitory starch accumulation,

and that its ectopic abundance could maintain leaf starch reserve during salinity stress. This may be a conserved feature of this GSK3 homologue, as starch accumulation in *Arabidopsis* leaf overexpressing *MsK4* was 4–5-fold higher than the wild-type grown under 150 mM NaCl (Kempa *et al.* 2007). Both the function of the kinase and its interacting counterparts appears to be retained even after the divergence of monocots and dicots 150 MYA. An interaction between *MsK4* protein and starch synthase was previously suggested (Kempa *et al.* 2007). However, most GSK3s function as a negative regulator of their respective target proteins in both plants (He *et al.* 2002) and animals (Cohen and Frame 2001). It is therefore possible that *OsGSK5* overexpression impaired starch degradation, thus leading to higher starch accumulation.

The maintenance of the OE transitory starch in source leaves may explain the larger biomass and higher number of leaves under moderate and high salinity (Table 1; Fig. 4b). The conversion of sugars into transitory starch may prevent a negative feedback of accumulated sugars on photosynthesis as previously suggested, (Pattanagul and Thitisaksakul 2008), thereby allowing the OE line to sustain growth of photosynthetic tissues. Additionally, greater starch content in the leaf during the day may act as a superior carbon reservoir for remobilisation of sugars to the





**Fig. 7.** A schematic diagram of  $^{14}\text{C}$  flux in the *OsGSK5* overexpressor line under high salinity. Arrows indicate the rate of change in  $^{14}\text{C}$  uptake over the 2 h chase period. The values in blue boxes are from the null segregant (NS) plants, and the value in pink boxes are from the *OsGSK5* overexpression (OE) plants.

non-photosynthetic organs during the night. This could possibly result in a better growth phenotype seen in the OE line (Table 1; Fig. 4b) (Stitt and Zeeman 2012). Conversely, the NS plants showed greater mobilisation of leaf starch when salt treated (Fig. 5a). A similar mechanism was seen in salt-sensitive tomato (Khelil *et al.* 2007), *Arabidopsis* (Kempa *et al.* 2008), quinoa (Rosa *et al.* 2009) and rice (Amirjani 2011; Theerawitaya *et al.* 2012), which exhibited starch degradation and mobilisation when exposed to a prolonged and high salt stress.

Unlike the source leaf, no difference in starch content in the young leaf on the third tiller was observed among different rice lines (Fig. 5b). These leaves were exposed to salinity stress ~3 weeks later than the main stem leaf. Therefore they may not have experienced sodium ion-toxicity stress (Munns 2002), which resulted in no difference in starch accumulation.

The OE lines accumulated higher starch in the root under both control and saline conditions (Fig. 5c). *OsGSK5* was highly

expressed in rice roots (Fig. S4), and its interactors were thus likely present in this organ. Therefore, the high root starch content of the OE line could be due to the constitutive overexpression of *OsGSK5*. This means that *OsGSK5* protein targets must be capable of responding to its higher amounts.

Higher root starch (167%) may be beneficial for the OE line in numerous ways. Firstly, it may explain the greater root biomass (50%) seen under high salt conditions in this line compared with the control (Table 1). Secondly, as previously mentioned, the ability to metabolise sugars into starch in the OE root may enhance sink strength, thus facilitating source-sink carbon translocation (Balibrea *et al.* 1996; Geiger *et al.* 2000). This could eventually lead to the upkeep of photosynthesis in the source by minimising feedback inhibition by sugars (Geiger *et al.* 2000). Further, higher starch could also influence statolith production, and thereby root growth in response to gravity (Leitz *et al.* 2009). Enhanced root growth under salinity stress is an



adaptive mechanism to low water potential (Pérez-Alfocea *et al.* 1996) and here, greater root biomass was found in the OE line (Table 1).

Although our data suggest that OsGSK5 may mediate tolerance to salinity stress through alterations in carbohydrates, it is notable that overexpression of another GSK3 isoform *ASK3* in *Arabidopsis* plants conferred salt tolerance through a remediation of redox stress (Dal Santo *et al.* 2012). It was thus possible that our OE line also gained salinity tolerance through reduced reactive oxygen species (ROS) accumulation. It should also be noted that we did not examine translocation of carbon to the culm, which accumulates significant amount of carbon reserves (Okawa *et al.* 2002). Thus our conclusions should be taken with caution.

The <sup>14</sup>C-labelling experiment was designed to examine an early response to salinity stress in Kitaake. Accordingly, there were no drastic adjustments in plant metabolism. However, steady-state measurements and dynamic flux assessments of carbohydrates together revealed that the OE plants partitioned newly incorporated <sup>14</sup>C into the root tissue during the first 2 h of the chase (Fig. S8) and in accordance, there was less residual <sup>14</sup>C in the source leaf compared with the CS plants under salinity an hour later (Fig. S8). Higher OsGSK5 transcript may have improved plant ability to export <sup>14</sup>C assimilates to sink organs. Enhanced <sup>14</sup>C transport from a pulsed leaf to adjacent fruits was shown in tomatoes grown under moderate salinity (50 mM) (Gao *et al.* 1998). Therefore, better mobilisation of resources to the sink in the OE line might be another early adaptive response to salt stress (Fig. 7). We note that an early NaCl response suggested that the OE plant did not use starch as a sucrose–sink in the leaf, since the rate of <sup>14</sup>C accumulation in starch fraction declined, whereas that of sucrose increased over time (Fig. 7). However, there is evidence to suggest that starch acted as a sucrose–sink in the root (Sonnewald and Willmitzer 1992). Thus this may be a novel mechanism for coping with salinity stress.

In conclusion, both end-point and dynamic investigation of carbohydrates in the transgenic plants grown on sustained and short-term high salinity stress revealed the preferential starch accumulation in the root of *OsGSK5* overexpressing plants compared with other genotypes. This enhanced starch accumulation in the sink organs may lead to the maintenance of carbon supply, which eventually contributed to the improved fitness of rice under salt stress conditions. The findings from this study also suggested the potential importance of a kinase in the regulation of starch metabolism, and the possibility of modulating it to improve plants' fitness in response to salinity stress.

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## References

- Ainsworth EA, Bush DR (2011) Carbohydrate export from the leaf: a highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiology* **155**, 64–69. doi:10.1104/pp.110.167684
- Amirjani MR (2011) Effect of salinity stress on growth, sugar content, pigments and enzyme activity of rice. *International Journal of Botany* **7**, 73–81. doi:10.3923/ijb.2011.73.81
- Balibrea ME, Santa Cruz AM, Bolarín MC, Pérez-Alfocea F (1996) Sucrolytic activities in relation to sink strength and carbohydrate composition in tomato fruit growing under salinity. *Plant Science* **118**, 47–55. doi:10.1016/0168-9452(96)04424-X
- Balibrea ME, Dell'Amico J, Bolarin MC, Perez-Alfocea F (2000) Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. *Physiologia Plantarum* **110**, 503–511. doi:10.1111/j.1399-3054.2000.1100412.x
- Castillo E, Tuong TP, Ismail A, Inubushi K (2007) Response to salinity in rice: comparative effects of osmotic and ionic stresses. *Plant Production Science* **10**, 159–170. doi:10.1626/pp.10.159
- Centeno DC, Osorio S, Nunes-Nesi A, Bertolo ALF, Carneiro RT, Araújo WL, Steinhäuser M-C, Michalska J, Rohrmann J, Geigenberger P, Oliver SN, Stitt M, Carrari F, Rose JKC, Fernie AR (2011) Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *The Plant Cell* **23**, 162–184. doi:10.1105/tpc.109.072231
- Cha-um S, Trakulyingcharoen T, Smitamana P, Kirdmanee C (2009) Salt tolerance in two rice cultivars differing salt tolerant abilities in responses to iso-osmotic stress. *Australian Journal of Crop Science* **3**, 221–230.
- Chen HJ, Chen JY, Wang SJ (2008) Molecular regulation of starch accumulation in rice seedling leaves in response to salt stress. *Acta Physiologiae Plantarum* **30**, 135–142. doi:10.1007/s11738-007-0101-y
- Cohen P, Frame S (2001) The renaissance of GSK3. *Nature Reviews. Molecular Cell Biology* **2**, 769–776. doi:10.1038/35096075
- Counce PA, Keisling TC, Mitchell AJ (2000) A uniform, objective, and adaptive system for expressing rice development. *Crop Science* **40**, 436–443. doi:10.2135/cropsci2000.402436x
- Dal Santo S, Stampfl H, Krasensky J, Kempa S, Gibon Y, Petutschnig E, Rozhon W, Heuck A, Clausen T, Jonak C (2012) Stress-induced GSK3 regulates the redox stress response by phosphorylating glucose-6-phosphate dehydrogenase in *Arabidopsis*. *The Plant Cell* **24**, 3380–3392. doi:10.1105/tpc.112.101279
- Danchin EGJ, Rosso M-N, Vieira P, de Almeida-Engler J, Coutinho PM, Henrissat B, Abad P (2010) Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17651–17656. doi:10.1073/pnas.1008486107
- Dardick C, Chen J, Richter T, Ouyang S, Ronald P (2007) The rice kinase database. A phylogenomic database for the rice kinome. *Plant Physiology* **143**, 579–586. doi:10.1104/pp.106.087270
- Dubey RS, Singh AK (1999) Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolising enzymes in rice plants. *Biologia Plantarum* **42**, 233–239. doi:10.1023/A:1002160618700
- Gao Z, Sagi M, Lips SH (1998) Carbohydrate metabolism in leaves and assimilate partitioning in fruits of tomato (*Lycopersicon esculentum* L.) as affected by salinity. *Plant Science* **135**, 149–159. doi:10.1016/S0168-9452(98)00085-5
- Geiger DR, Servaites JC, Fuchs MA (2000) Role of starch in carbon translocation and partitioning at the plant level. *Functional Plant Biology* **27**, 571–582. doi:10.1071/PP99128
- He J-X, Gendron JM, Yang Y, Li J, Wang Z-Y (2002) The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proceedings*

- of the National Academy of Sciences of the United States of America **99**, 10185–10190. doi:10.1073/pnas.152342599
- Henry C, Bledsoe SW, Griffiths CA, Kollman A, Paul MJ, Sakr S, Lagrimini LM (2015) Differential role for trehalose metabolism in salt-stressed maize. *Plant Physiology* **169**, 1072–1089. doi:10.1104/pp.15.00729
- Jonak C, Hirt H (2002) Glycogen synthase kinase 3/SHAGGY-like kinases in plants: an emerging family with novel functions. *Trends in Plant Science* **7**, 457–461. doi:10.1016/S1360-1385(02)02331-2
- Kempa S, Rozhon W, Samaj J, Erban A, Baluska F, Becker T, Haselmayer J, Schleiff E, Kopka J, Hirt H, Jonak C (2007) A plastid-localized glycogen synthase kinase 3 modulates stress tolerance and carbohydrate metabolism. *The Plant Journal* **49**, 1076–1090. doi:10.1111/j.1365-3113X.2006.03025.x
- Kempa S, Krasensky J, Dal Santo S, Kopka J, Jonak C (2008) A central role of abscisic acid in stress-regulated carbohydrate metabolism. *PLoS One* **3**, e3935. doi:10.1371/journal.pone.0003935
- Kerepesi I, Galiba G (2000) Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Science* **40**, 482–487. doi:10.2135/cropsci2000.402482x
- Khelil A, Menu T, Ricard B (2007) Adaptive response to salt involving carbohydrate metabolism in leaves of a salt-sensitive tomato cultivar. *Plant Physiology and Biochemistry* **45**, 551–559. doi:10.1016/j.plaphy.2007.05.003
- Koh S, Lee SC, Kim MK, Koh JH, Lee S, An G, Choe S, Kim SR (2007) T-DNA tagged knockout mutation of rice *OsGSK1*, an orthologue of *Arabidopsis BIN2*, with enhanced tolerance to various abiotic stresses. *Plant Molecular Biology* **65**, 453–466. doi:10.1007/s11103-007-9213-4
- Kölling K, Müller A, Flütisch P, Zeeman SC (2013) A device for single leaf labelling with CO<sub>2</sub> isotopes to study carbon allocation and partitioning in *Arabidopsis thaliana*. *Plant Methods* **9**, 45. doi:10.1186/1746-4811-9-45
- Lecourieux F, Lecourieux D, Vignault C, Delrot S (2010) A sugar-inducible protein kinase, VvSK1, regulates hexose transport and sugar accumulation in grapevine cells. *Plant Physiology* **152**, 1096–1106. doi:10.1104/pp.109.149138
- Lefèvre I, Gratia E, Lutts S (2001) Discrimination between the ionic and osmotic components of salt stress in relation to free polyamine level in rice (*Oryza sativa*). *Plant Science* **161**, 943–952. doi:10.1016/S0168-9452(01)00485-X
- Leitz G, Kang B-H, Schoenwaelder MEA, Staehelin LA (2009) Statolith sedimentation kinetics and force transduction to the cortical endoplasmic reticulum in gravity-sensing *Arabidopsis* columella cells. *The Plant Cell* **21**, 843–860. doi:10.1105/tpc.108.065052
- Lemoine R, La Camera S, Atanassova R, Deedaldechamp F, Allario T, Pourtau N, Bonnemain JL, Laloi M, Coutos-Theevenot P, Maurouset L, Faucher M, Girousse C, Lemonnier P, Parrilla J, Durand M (2013) Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science* **4**, 272. doi:10.3389/fpls.2013.00272
- Lu C-A, Ho T-hD, Ho S-L, Yu S-M (2002) Three novel MYB proteins with one DNA binding repeat mediate sugar and hormone regulation of  $\alpha$ -amylase gene expression. *The Plant Cell* **14**, 1963–1980. doi:10.1105/tpc.001735
- Luengwilai K, Beckles MD (2010) Climacteric ethylene is not essential for initiating chilling injury in tomato (*Solanum lycopersicum*) cv. Ailsa Craig. *Journal of Stored Products and Postharvest Research* **1**, 1–8.
- Luengwilai K, Fiehn OE, Beckles DM (2010) Comparison of leaf and fruit metabolism in two tomato (*Solanum lycopersicum* L.) genotypes varying in total soluble solids. *Journal of Agricultural and Food Chemistry* **58**, 11790–11800. doi:10.1021/jf102562n
- Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: challenges and perspectives. *Annual Review of Plant Biology* **61**(1), 443–462. doi:10.1146/annurev-arplant-042809-112116
- Munns R (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment* **25**, 239–250. doi:10.1046/j.0016-8025.2001.00808.x
- Munns R, Tester M (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**, 651–681. doi:10.1146/annurev-arplant.59.032607.092911
- Nobuta K, Venu RC, Lu C, Belo A, Vemaraju K, Kulkarni K, Wang WZ, Pillay M, Green PJ, Wang GL, Meyers BC (2007) An expression atlas of rice mRNAs and small RNAs. *Nature Biotechnology* **25**, 473–477. doi:10.1038/nbt1291
- Okawa S, Makino A, Mae T (2002) Shift of the major sink from the culm to the panicle at the early stage of grain filling in rice (*Oryza sativa* L. cv. Sasanishiki). *Soil Science and Plant Nutrition* **48**, 237–242. doi:10.1080/00380768.2002.10409196
- Osorio S, Ruan YL, Fernie AR (2014) An update on source-to-sink carbon partitioning in tomato. *Frontiers in Plant Science* **5**, 516. doi:10.3389/fpls.2014.00516
- Papademetriou MK (2000) Rice production in the Asia-Pacific region: issues and perspectives. In 'Bridging the rice yield gap in the Asia-Pacific region'. (Eds MK Papademetriou, FJ Dent, EM Herath) pp. 4–25. (FAO: Bangkok)
- Park C-J, Bart R, Chern M, Canlas PE, Bai W, Ronald PC (2010) Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. *PLoS One* **5**, e9262. doi:10.1371/journal.pone.0009262
- Pattanagul W, Thitisaksakul M (2008) Effect of salinity stress on growth and carbohydrate metabolism in three rice (*Oryza sativa* L.) cultivars differing in salinity tolerance. *Indian Journal of Experimental Biology* **46**, 736–742.
- Pérez-Alfocea F, Balibrea ME, Cruz AS, Estañ MT (1996) Agronomical and physiological characterization of salinity tolerance in a commercial tomato hybrid. *Plant and Soil* **180**, 251–257. doi:10.1007/BF00015308
- Qi X, Chanderbali AS, Wong GK-S, Soltis DE, Soltis PS (2013) Phylogeny and evolutionary history of glycogen synthase kinase 3/SHAGGY-like kinase genes in land plants. *BMC Evolutionary Biology* **13**, 143. doi:10.1186/1471-2148-13-143
- Rambaldi D, Ciccarelli FD (2009) FancyGene: dynamic visualization of gene structures and protein domain architectures on genomic loci. *Bioinformatics* **25**, 2281–2282. doi:10.1093/bioinformatics/btp381
- Rosa M, Hilal M, Gonzalez JA, Prado FE (2009) Low-temperature effect on enzyme activities involved in sucrose-starch partitioning in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa* Willd.) seedlings. *Plant Physiology and Biochemistry* **47**, 300–307. doi:10.1016/j.plaphy.2008.12.001
- Saidi Y, Hearn TJ, Coates JC (2012) Function and evolution of 'green' GSK3/Shaggy-like kinases. *Trends in Plant Science* **17**, 39–46. doi:10.1016/j.tplants.2011.10.002
- Sato-Nara K, Nagasaka A, Yamashita H, Ishida J, Enju A, Seki M, Shinozaki K, Suzuki H (2004) Identification of genes regulated by dark adaptation and far-red light illumination in roots of *Arabidopsis thaliana*. *Plant, Cell & Environment* **27**, 1387–1394. doi:10.1111/j.1365-3040.2004.01241.x
- Scofield GN, Hirose T, Aoki N, Furbank RT (2007) Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. *Journal of Experimental Botany* **58**, 3155–3169. doi:10.1093/jxb/erm153
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* **13**, 2498–2504. doi:10.1101/gr.1239303
- Shavrukov Y (2013) Salt stress or salt shock: which genes are we studying? *Journal of Experimental Botany* **64**, 119–127. doi:10.1093/jxb/ers316
- Smith AM, Coupland G, Dolan L, Harberd N, Jones J, Martin C, Sablowki RAA (2009) 'Environmental stress.' (Garland Press: New York)
- Sonnewald U, Willmitzer L (1992) Molecular approaches to sink-source interactions. *Plant Physiology* **99**, 1267–1270. doi:10.1104/pp.99.4.1267

- Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690. doi:10.1093/bioinformatics/btl446
- Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. *Current Opinion in Plant Biology* **15**, 282–292. doi:10.1016/j.pbi.2012.03.016
- Stitt M, Lunn J, Usadel B (2010) *Arabidopsis* and primary photosynthetic metabolism – more than the icing on the cake. *The Plant Journal* **61**, 1067–1091. doi:10.1111/j.1365-313X.2010.04142.x
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739. doi:10.1093/molbev/msr121
- Theerawitaya C, Boriboonkaset T, Cha-um S, Supaibulwatana K, Kirdmanee C (2012) Transcriptional regulations of the genes of starch metabolism and physiological changes in response to salt stress rice (*Oryza sativa* L.) seedlings. *Physiology and Molecular Biology of Plants* **18**, 197–208. doi:10.1007/s12298-012-0114-x
- Thitisaksakul M, Jiménez RC, Arias MC, Beckles DM (2012) Effects of environmental factors on cereal starch biosynthesis and composition. *Journal of Cereal Science* **56**, 67–80. doi:10.1016/j.jcs.2012.04.002
- Thitisaksakul M, Tananuwong K, Shoemaker CF, Chun A, Tanadul OU, Labavitch JM, Beckles DM (2015) Effects of timing and severity of salinity stress on rice (*Oryza sativa* L.) yield, grain composition, and starch functionality. *Journal of Agricultural and Food Chemistry* **63**, 2296–2304. doi:10.1021/jf503948p
- Verslues PE, Zhu JK (2005) Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. *Biochemical Society Transactions* **33**, 375–379. doi:10.1042/BST0330375
- Wanichthanarak K, Fan S, Grapov D, Barupal DK, Fiehn O (2017) Metabox: A toolbox for metabolomic data analysis, interpretation and integrative exploration. *PLoS One* **12**, e0171046. doi:10.1371/journal.pone.0171046
- Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. *BioTechniques* **39**, 75–85. doi:10.2144/05391RV01
- Yaling S, Lizhong X (2012) Systematic analysis of glycogen synthase kinase 3 genes in rice reveals their differential responses to phytohormones and abiotic stresses. *Journal of Huazhong Agricultural University* **31**, 1–9.
- Yeo A (1999) Predicting the interaction between the effects of salinity and climate change on crop plants. *Scientia Horticulturae* **78**, 159–174. doi:10.1016/S0304-4238(98)00193-9
- Yin YG, Kobayashi Y, Sanuki A, Kondo S, Fukuda N, Ezura H, Sugaya S, Matsukura C (2010) Salinity induces carbohydrate accumulation and sugar-regulated starch biosynthetic genes in tomato (*Solanum lycopersicum* L. cv. ‘Micro-Tom’) fruits in an ABA- and osmotic stress-independent manner. *Journal of Experimental Botany* **61**, 563–574. doi:10.1093/jxb/erp333
- Yoo MJ, Albert VA, Soltis PS, Soltis DE (2006) Phylogenetic diversification of glycogen synthase kinase 3/SHAGGY-like kinase genes in plants. *BMC Plant Biology* **6**, 3. doi:10.1186/1471-2229-6-3
- Youn J-H, Kim T-W (2015) Functional insights of plant GSK3-like kinases: multi-taskers in diverse cellular signal transduction pathways. *Molecular Plant* **8**, 552–565. doi:10.1016/j.molp.2014.12.006