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Biochemical and Physiological Responses of Thai Jasmine Rice (*Oryza sativa* L. ssp. *indica* cv. KDML105) to Salt Stress

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ABSTRACT: The aim of this research was to investigate glycinebetaine (Glybet) accumulation in rice seedlings *via* the activity of betaine aldehyde dehydrogenase (BADH) and to characterize the physiological responses of Glybet-accumulated seedlings to salt stress. The BADH activity of Thai jasmine rice seedlings cultured photoautotrophically showed a progressive elevation during the first four days after growth under salt-stress condition (342 mM NaCl), but gradually decreased thereafter (days 6-8). This increase in BADH activity was accompanied by an accumulation of Glybet. In contrast, the BADH activity and Glybet content of control seedlings (0 mM NaCl) remained at a low and constant level during the same 8-day interval. Significant reductions in the concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid of salt-stressed seedling were observed with increasing exposure time to salt-stressed conditions. The decrease in total chlorophyll concentrations of salt-stressed seedlings was positively related to reductions in the net photosynthetic rate (r = 0.89), and dry weight (r = 0.89). The dry weight of *in-vitro* salt-stressed seedling was positively related to their survival percentage (r = 0.94). The survival percentage of salt-stressed seedlings was significantly decreased with increasing exposure time to the salt-stressed conditions. The Glybet accumulation in salt-stressed rice seedlings is consistent with a defense response to salt-stressed conditions. These results suggest that assaying BADH activity and/or Glybet accumulation may be further used in screening for salt-tolerant varieties of indica rice.

KEYWORDS: betaine aldehyde dehydrogenase, carotenoid, chlorophyll, glycinebetaine, net photosynthetic rate, salt stress, survival percentage.

INTRODUCTION

An estimated $4 \times 10^8 - 9.5 \times 10^8$ hectares of the Earth's surface is afflicted by high salinity, an area roughly three time larger than the land currently devoted to agriculture. Moreover, the use of low quality irrigation water and unsustainable agricultural processes may lead to an increase in the amount of land affected by salinity problems.[1] A primary response of plants exposed to increased salt concentrations is a decrease in plant water potential, which is detrimental to water use efficiency. Halophyte (salt-tolerant) species possess a high capacity to resist salt-stress through the biosynthesis and accumulation of compatible solutes. These substances raise the overall osmotic pressure within the cytoplasm, thereby enabling plant cells to maintain both turgor and the driving gradient for water uptake.[2] Thus, compatible solutes, which include a variety of proteins, carbohydrates, amino acids and quaternary ammonium compounds, serve important roles as osmotic balancing agents and plant cell stabilizers.[3-4]

The compatible solute Glycinebetaine (Glybet) is involved in the defensive responses of living organisms to extreme conditions of salt-, drought-, temperature- and light-stresses.[2-5] Numerous experiments have indicated that Glybet acts as an osmoprotective agent by stabilizing both the quaternary structures of proteins and the highly ordered structure of membranes against the adverse effects of salinity and water-deficit.[5] The main role of Glybet is probably to prevent the plant cells against the ravages of salt stress by preserving the osmotic balance[6], by stabilizing the structure of key proteins such as Rubisco[7], by protecting the photosynthetic apparatus such as reaction center complexes[8], and by functioning as an oxygen radical scavenger[9], and thereby to promote the photosynthetic

capacity during salt- and drought-stresses.

In higher plants, Glybet is synthesized *via* the two-step oxidation of choline. In the first step, choline is oxidized to betaine aldehyde by choline monooxygenase (CMO). CMO is an unusual Fe-dependent monooxygenase with a motif characteristic of Rieske-type ion-sulfur proteins.[10-11] The *CMO* gene has been cloned in both spinach (*Spinacia oleracea* L.) and sugar beet (*Beta vulgaris* L.), and has been shown to be induced by high salinity and water-deficit conditions.[11-12] The second step of betaine synthesis is catalyted by betaine aldehyde dehydrogenase (BADH), an NAD-dependent dehydrogenase that has been characterized and cloned from spinach[13] and sorghum.[14] BADH is considered to be a key enzyme of the Glybet biosynthesis pathway. In previous studies, the relative concentration of Glybet and BADH activity varied both among and within species. Near-isogenic maize lines displaying Glybet accumulation are more tolerant to salt-stress than lines without Glybet accumulation(ref.). In japonica rice (*Oryza sativa* L. ssp. *japonica* cv. nipponbare), both Glybet accumulation and BADH expression are very low.[15] Therefore, the aim of this research was to investigate the Glybet accumulation in indica rice subspecies *via* BADH activity and the physiological responses of Glybet-accumulated seedlings to salt stress.

Materials and Methods

Plant Material

Seeds of Thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML105) were obtained from the Pathumthani Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). Rice seeds were dehusked by hand, sterilized once in 5% Clorox®(5.25% sodium hypochlorite, Clorox Co, USA) for 60 min, once in 30% Clorox®for 30 min, and then rinsed three times with sterile distilled water. Surface-sterilized seeds were germinated on 0.25% Phytagel®-solidified MS media in a 250 mL glass vessel. The media were adjusted to pH 5.7 before autoclaving. Seedlings were cultured *in-vitro* under conditions of 25 ± 2 °C room temperature, 60 ± 5% relative humidity (RH) and 60 ± 5 μmol $m^{-2}s^{-1}$ photosynthetic photon flux (PPF). The PPF was provided by fluorescent lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) under a 16 h d^{-1} photoperiod. Seven-day-old rice seedlings were aseptically transferred to MS-liquid media under a photoautotrophic system (CO_2 as a carbon source), with vermiculite used as a supporting material. The amount of air-exchange in the glass vessels were adjusted to 2.32 h^{-1} by punching a hole in the plastic cap (∅ 1 cm) and covering the hole with a gas-permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan). All seedlings were continuously cultured in a Plant Growth Incubator (EYELA, Model EYELATRON FLI-301LH, Japan) under temperature shift (25 ± 2 °C darkperiod /28 ± 2 °C photoperiod), 60 ± 5% RH and 100 ± 5 μmol $m^{-2}s^{-1}$ PPF. After 13 days of growth, the NaCl concentration of the media was maintained at 0 (control) or adjusted to 342 mM NaCl (salt-stress). The cultures were then grown for an additional 0, 2, 4 or 8 days.

Biochemical Responses

BADH enzyme extraction, purification and assay

To obtain crude protein extracts, 500 mg fresh weight of leaf tissues were ground in a mortar on ice with 900 μL of cold extraction buffer A (50 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM EDTA and 20 mM β-mercaptoethanol). Plant debris was removed by centrifugation at 14,500 g for 30 min at 4°C. The supernatant was added with 600 μL of buffer B (20% sucrose (w/v)) and then applied to a Hitrap™ Q-FF ion exchange column (Q-Sepharose Fast Flow, Amersham, Uppsala, Sweden), volume 5 mL, equilibrated with buffer B. Eluted fractions displaying BADH enzyme activity were pooled, aliquoted and then stored at –20 °C.

The BADH activity was assayed spectrophotometrically by monitoring the absorbance at 340 nm (NADH formation) of a mixture (0.5 mL) consisting of 1.0 mM betaine aldehyde, 0.3 mM NAD^+ in 100 mM potassium phosphate buffer, pH 8.0, and 500 μL crude protein. A reaction of betaine aldehyde and NAD^+ alone was used as a blank. Initial steady-state rate was determined from the initial, linear portions of reaction progress curves. Each determination was performed at least twice. One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min in standard assay.[16] Total protein concentration was determined by the Bradford Protein Assay, using bovine serum albumin as standard.[17]

Glycinebetaine extraction and quantification

Five hundred milligrams of leaf tissue were frozen in liquid nitrogen for sap extraction and then homogenized in methanol. Glycinebetaine (Glybet) was extracted with hot methanol (95 °C) for 2 h, after which the methanol evaporated off in an air stream.[18] The Glybet was then dissolved in water, centrifuged for 15 min at 9,000 *g*, and the liquid phase filtered using a 0.45 μm membrane filter (Minisart SRP15, Sartorius, Germany). The clear extract solution was purified by a HiTrap™ Q-FF ion exchange column prior to High Performance Liquid Chromatography (HPLC). The

column used was a stainless steel (250 mm × 4.6 mm) packed with Partilsil 10 SCX. The mobile phase was nanopure-water containing 5% (v/v) methanol buffered with 0.05 M KH_2PO_4, pH 4.6, and the flow rate was set at 1.0 mL min^{-1} by a Waters 2690 Pump Delivery System. Detection was carried out using a Waters 996 Photodiode Array (PDA) detector with the wavelength set at 195 nm. Betaine (Merck, USA) was used as a standard and mixed with the sample as an internal standard.[19] An injection loop volume of 50 µL was applied.

Physiological Responses

Concentrations of chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b), total chlorophyll and total carotenoid (C_{x+c}) was analyzed following the Shabala[20] and Lichtenthaler[21] methods, respectively. One hundred milligrams of leaf material were collected from the second and third nodes of the shoot tip. The leaf samples were placed in a 25 mL glass vial (Opticlear®KIMBLE, USA), added with 10 mL of 95.5% acetone, and blended with a homogenizer (T25 basic ULTRA-TURRAX®, IKA, Malaysia). The glass vials were sealed with parafilm to prevent evaporation and then stored at 4 °C for 48 h. The Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer (DR/4000, HACH, USA) at wavelengths 662 nm and 644 nm. The C_{x+c} concentration was measured by absorbance at 470 nm. A solution of 95.5% acetone was used as a blank. The Chl_a, Chl_b, total chlorophyll and C_{x+c} (µg g^{-1} FW) concentrations in the leaf tissues were calculated according to the following equations.

$$[Chl_a] = 9.784D_{662} - 0.99D_{644}$$

$$[Chl_b] = 21.42D_{644} - 4.65D_{662}$$

$$\text{Total chlorophyll} = [Chl_a] + [Chl_b]$$

$$[C_{x+c}] = \frac{1000D_{470} - 1.90\,[Chl_a] - 63.14\,[Chl_b]}{214}$$

where D_i is the optical density at the wavelength *i*.

Net-photosynthetic rate (*NPR*) was calculated by measuring the different concentrations of CO_2 inside and outside of the glass vessels containing the seedlings. The CO_2 concentrations inside and outside the glass vessel (C_{in} and C_{out}) at steady state were measured by Gas Chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The detector (TCD; Thermal Conductivity Detector) and injector were set at 250 °C. The temperature program of GC capillary column (GS-Q, J&W Scientific ®, Germany) was set at 30 °C for 1 min the initial state and increased to 100 °C at a rate of 20 °C per min and kept for 1 min. The *NPR* was calculated according to Fujiwara[22], as follows:

$$[NPR] = \frac{K' \times E' \times V' \times (C_{out} - C_{in})}{L}$$

where *K* is the conversion factor converting CO_2 amount from volume to mole (40.9 mol m^{-3} at 28 °C); *E* is the number of air exchanges per hour of the vessel (2.32 h^{-1}); *V* is the air volume of the glass vessel (0.0025 m^3); C_{in} and C_{out} are CO_2 concentrations (µmol mol^{-1}) inside and outside the glass vessels at steady state, respectively; and *L* is the leaf area (m^2).

Growth Measurement

Leaf area, shoot height, root length, fresh weight, and dry weight of seedlings were measured as described by Lutts.[23] The seedlings were dried at 110 °C in a hot-air oven (Memmert, Model 500, Germany) for 2 days, and then incubated in a desiccator before measurement of dry weight. The leaf area of seedlings was measured using a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., UK).

Survival Percentage Assay

The survival percentage of rice seedlings was checked by the following growth on Phytagel®-solidified MS media at day 0, 4, 8, 16 and 30 according to Gangopadhyay.[24]

Experimental Design

The experiment was designed as Completely Randomized Design (CRD) with ten replicates and four plantlets per replicate. The mean values obtained were compared by *t*-test and analyzed by SPSS software (SPSS for Windows, SPSS Inc., USA). The correlations between BADH activity and Glybet content, total chlorophyll concentration and net photosynthetic rate, net photosynthetic rate and dry weight, as well as dry weight and survival percentage, were evaluated by Pearson's correlation coefficients.

Results and Discussion

As shown in Fig 1, the betaine aldehyde

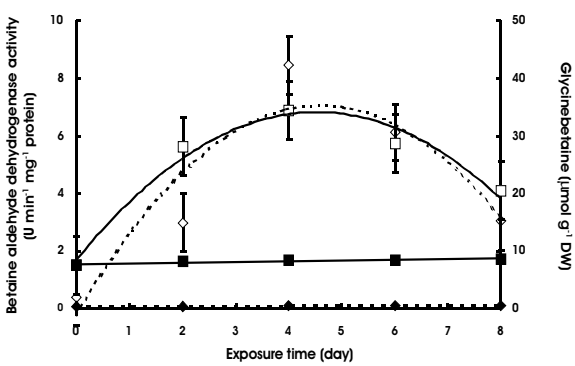

Fig 1. Betaine aldehyde dehydrogenase (BADH) enzyme activity (-----) and glycinebetaine (Glybet) content (——) of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl (light) or without NaCl (dark) for 0, 2, 4, 6 and 8 days. Error bars represent ± SE.

dehydrogenase (BADH) activity of Thai jasmine rice seedlings cultured photoautotrophically *in vitro* under salt stress progressively increased during the first few days of NaCl exposure, and gradually decreased from day 6 onward. The BADH activity of salt-stressed rice seedlings was highest on day 4 at 17.3 times the BADH activity of control (0 mM NaCl) seedlings. Similarly, the glycinebetaine content (Glybet) of salt-stressed seedlings also peaked at day 4, and was 4.6 times higher than the Glybet content of control samples. The BADH

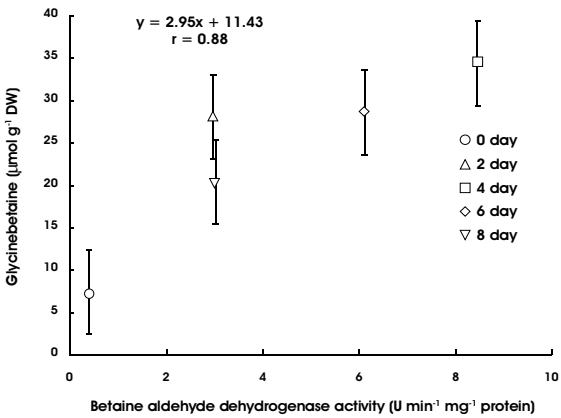

Fig 2. Relationship between betaine aldehyde dehydrogenase (BADH) and glycinebetaine (Glybet) of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl for 0, 2, 4, 6 and 8 days. Error bars represent ± SE.

enzyme activity in salt-stressed seedlings was positively related to Glybet accumulation (r = 0.88) (Fig 2). Throughout the 8 day interval, both the BADH activity and Glybet content in control seedlings remained low (Fig 1).

Glybet accumulation in higher plants under salt-stress conditions has been reported for many halophyte species (*e.g. Atriplex*[25-26], *Suaeda fruticosa*, *Haloxylon recurvum* and *Halopyrum macronatum*[27]), crop species (*e.g.* sorghum[28], barley[29], maize[30] and wheat[31]) and grass species (*e.g. Bouteloua cutipendula*, *Buchloe dactyloides*, *Cynodon dactylon*, *Distichlis spicata*, *Sporobolus airoides*, *S. cryptandrus* and *Zoysia japonica*[32]). The accumulation of Glybet in these species depended on salt inducers[26,29], salt concentrations[25,32], exposure time to salt stress[14] as well as genotypic variations.[28-33] Normally, the higher of salt concentrations and longer exposure times to salt-stress led to a greater accumulation of Glybet in these plants. Similar observations have been made for BADH enzyme abundance and/or activity in response to salt stress.[14, 34-35] The Glybet biosynthesis of rice seedlings cultured under salt stress may be positively related to BADH expression.[15] It should be noted that the BADH enzyme plays as an important role in Glybet biosynthesis of salt-stressed rice seedlings.

The chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid concentrations of salt-stressed seedlings were sharply decreased with increasing exposure time to salt-stressed conditions. Total chlorophyll concentrations were reduced in salt stressed seedlings to 68%, 82% and 95% reduction relative to control seedlings after exposure to salt stress for 2, 4 and 8 days, respectively (Fig 3A). Total carotenoid concentrations exhibited a similar reduction upon seedling growth under salt-stressed conditions (Fig 3B). The degradation of pigments in salt-stressed seedlings was positively related to low net

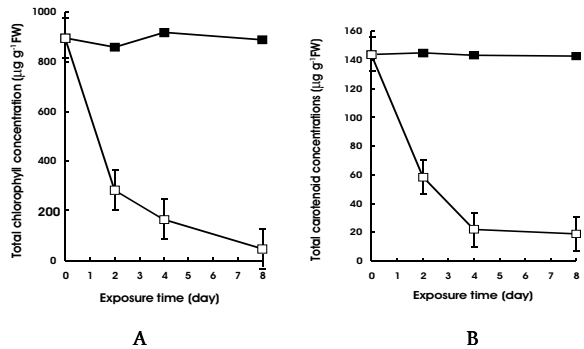

Fig 3. Total chlorophyll (A) and total carotenoid concentrations (B) of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl (□) or without NaCl (■) for 0, 2, 4 and 8 days. Error bars represent ± SE.

Table 1. Leaf area, shoot height, root length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl or without NaCl for 4 days.

Photoautotrophic system	Leaf area (cm^2)	Shoot Height (cm)	Root Length (cm)	Fresh weight (mg)		Dry weight (mg)	
				Shoot	Root	Shoot	Root
0 mM NaCl (Control)	5.13 a	44.19 a	9.96 a	150 a	57 a	30 a	9 a
342 mM NaCl	2.92 b	27.94 b	5.90 b	60 b	22 b	14 b	3 b
Significant level	**	**	**	**	**	**	**

Means within a row followed by the different letters in each column are significantly different at $P \leq 0.01$ by *t*-test.

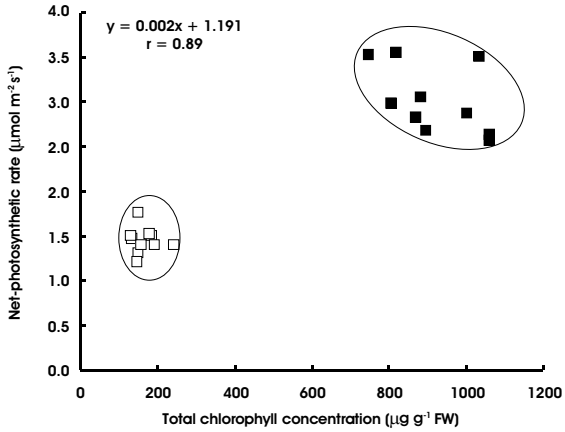

Fig 4. Relationship between total chlorophyll concentration and net-photosynthetic rate of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl (□) or without NaCl (■) for 4 days.

photosynthetic rates (*NPR*) (r = 0.89). The *NPR* of salt-stressed seedlings was lower than those of control seedlings by a factor of 2.1 times on day 4 (Fig 4). The *NPR* of rice seedlings was not only used in the production of carbohydrate, but was also directly related to growth, as determined by dry weight (r = 0.89) (Fig 5A). Moreover, the leaf area, shoot height, root length, fresh weight and dry weight of salt-stressed seedlings were significantly reduced in comparison to those of control seedlings by factors of 1.8, 1.6, 1.7, 2.5 and 2.3 times, respectively (Table 1). The reduction in dry weight of salt-stressed seedlings was closely related to survival percentage (r = 0.94) (Fig 5B). The survival percentage of salt-stressed seedlings was significantly decreased with increasing exposure time to salt stress (Fig 6).

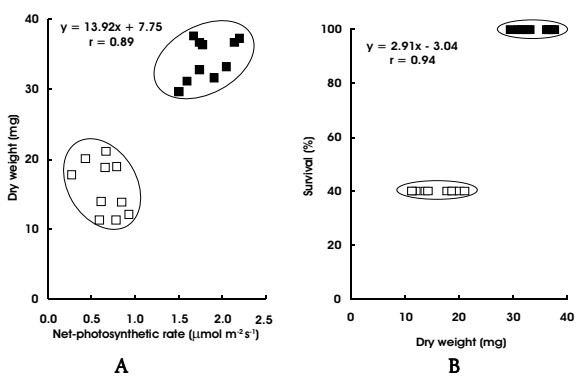

Fig 5. Relationship between net-photosynthetic rate and dry weight (A), dry weight and survival percentage (B) of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl (□) or without NaCl (■) for 8 days.

Thai jasmine rice seedlings cultured under salt-stressed conditions typically showed evidence of chlorosis or chlorophyll degradation after exposure to salt stress for 4 days. The salt-stressed seedlings accumulated the highest levels of Glybet on day 4, while their chlorophyll concentration decreased by 80% of the initial level. Normally, the Glybet accumulation in plant cells under salt stress plays a role as stabilizing the ultrastructure of macromolecules. In this work, the Glybet in rice seedlings cultured under salt stress may have been at low concentration for maintaining the ultrastructures of pigments such as chlorophyll and carotene. Since chlorophyll pigments convert electronic energy into chemical energy by transferring electrons from reaction centers to acceptor molecules in photosystem II (PSII) of light reaction, this chlorophyll degradation in salt-stressed seedlings presumably contributed to the observed reduction in

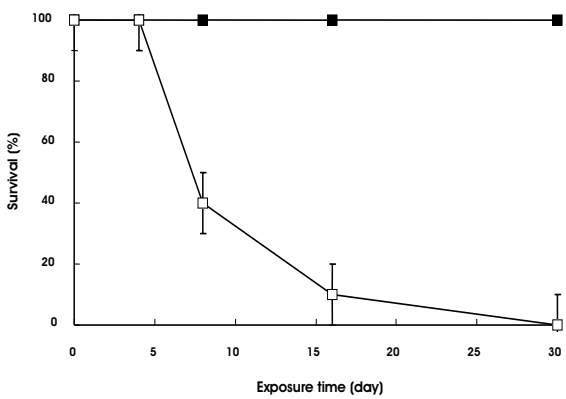

Fig 6. Survival percentage of Thai jasmine rice seedlings cultured under photoautotrophic system with 342 mM NaCl (□) or without NaCl (■) for 0, 4, 8, 16 and 30 days. Error bars represent ± SE.

NPR.[7] Allakverdiev *et al* (2002) [36] nicely showed that salt stress alone barely affects PSII activity. However, salt stress in combination with light significantly impairs the activity of PSII. Their work also showed that high concentrations of NaCl also inhibited the recovery of PSII from photodamage. In this work, the rice seedlings grew in 342 mM NaCl under 100 μmol m^{-2} s^{-1} PPF, the observed chlorosis symptoms) low NPR, and poor growth could be the consequence of extensive photoinhibition. Moreover, the reduction of *NPR* in salt-stressed seedlings directly decreases growth.[20] However, Glybet accumulation in rice seedlings cultured under salt-stress conditions was 4-10 fold lower than those reported for halophyte or salt-tolerant species.[25-27] The low level of Glybet content in salt-stressed indica seedlings may not be sufficient to

prevent plant cells, organelles and tissues from being damaged by prolonged exposure to salt stress. There are many reports that Glybet accumulation under salt stress depends on salt concentrations [25,33] and plant species *i.e.* sorghum[28], grasses[32] and maize.[33]

In conclusion, Thai jasmine rice seedlings cultured under salt stress exhibit a significant increase in Glybet biosynthesis and accumulation in the leaf tissues. This Glybet accumulation immediately following the initial exposure of rice seedlings to salt stress may function as a defense response to unfavorable growth conditions. Thus, Glybet content in salt-stressed seedlings may potentially be further utilized as a rapid biochemical index for the screening of new salt-tolerant lines of rice.

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