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Effect of Exogenous Glucose and Abscisic Acid on Physiological and Morphological Performances of *in vitro* Indica Rice (*Oryza sativa* L. sp. *indica*)

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Abstract: The aim of this research is to investigate on the osmolarity changes, sugar profiles, chlorophyll fluorescence and growth performances of indica rice cultured under exogenous glucose and abscisic acid (ABA). Osmolarity in the root tissues of rice was positively related to increase glucose concentration in the media, while ABA application does not affected. Endogenous soluble sugars, sucrose, glucose and fructose in rice seedlings were slightly increased when exposure to glucose and ABA. High osmolarity in glucose treated seedlings was negatively related to total chlorophyll concentration. Chlorophyll a, chlorophyll b and total carotenoid concentrations in the rice seedlings were significantly damaged by high glucose and ABA application in the media. The chlorophyll a, total chlorophyll and total carotenoid degradation in the leaf tissues was positively correlated to maximum quantum yield of PSII (Fv/Fm), quantum efficiency of photosystem II (Φ_{PSII}) and non-photochemical quenching (NPQ), respectively. The low efficiency of light capture in photosystem II should be caused to low growth, in term of root length, plant height and dry matter. Induced water deficit or drought stress by exogenous glucose application and ABA treatment in this investigation would be further applied to drought tolerant screening in rice breeding program.

Key words: Photosystem II, osmolarity, pigment, soluble sugar, water deficit

INTRODUCTION

Drought or water deficit is one of the most important barriers to reduce on rice crop growth and development (Cabuslay *et al*., 2002; Pieters and Souki, 2005), leading to low productivity (O'Toole 2004; Pantuwan *et al*., 2004; Yang and Zhang, 2006; Kumar *et al*., 2006). There are many reports that demonstrated on adverse effects of drought to higher plants, in term of osmotic stress (Jongdee *et al*., 2002; Sanchez *et al*., 2004; Blum, 2005), oxidative stress (Wang *et al*., 2005; Nayyar and Gupta, 2006) and hormonal regulation (Riccardi *et al*., 2004; Perales *et al*., 2005; Zhang *et al*., 2006). Compatible solute accumulation including soluble sugar (Sancez *et al*., 2004), proline (Uyprasert *et al*., 2004; Yoshiba *et al*., 1997), glycinebetaine (Ma *et al*., 2006) and polyamines (Liu *et al*., 2004) in the plant cells is mainly function as osmoregulation defensive mechanism to drought stress. Soluble sugar is a member of neutral compatible solutes to play a central role in the biosynthetic pathway of primary and secondary metabolites, building blocks of macromolecules and controlling developmental processes (Gibson, 2000; Smeekens, 2000; Price *et al*., 2004) as well as drought tolerant mechanism (Gupta and

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Kaur, 2005). Normally, the sugar in the green plants is produced by photosynthesis system as a main energy sources for growth and development (Rolland *et al*., 2002) and enhanced photosynthesis using CO_2 enrichment delays drought stress (Lin and Wang, 2002; Widodo *et al*., 2003). There are a large number of gene(s) to induce by sugars as a complex regulatory web or signal transduction metabolisms, relating to environmental stresses (Gibson, 2000; Price *et al*., 2004). Abscisic acid (ABA) is a plant hormone that plays a role in plant response to drought stress (Seo and Koshiba, 2002; Wang *et al*., 2003; Alves and Setter, 2004; Lopez-Carbonell and Jauregui, 2005; Rook *et al*., 2006; Wan and Li, 2006) as cellular signaling (Verslues and Zhu, 2005) in water movement from root to leaf (Jia *et al*., 2001; Alves and Setter, 2004) and sugar sensing cascades (Toyofuku *et al*., 2000; Leon and Sheen, 2003; Li *et al*., 2004a), resulting in whole plant physiological and morphological adaptations (Jia *et al*., 2001; Li *et al*., 2004b; Yin *et al*., 2004). In addition, ABA is a stress signal generated in root tissues, carried up through xylem to shoot by transpiration stream and function as stomatal closure to reduce the water loss in transpiration (Steudle, 2000; Hartung *et al*., 2002; Seo and Koshiba, 2002). In present study, the exogenous glucose and ABA were applied as drought signaling stressors for physiological and morphological changes in indica rice seedlings.

MATERIALS AND METHODS

Plant Material

Seeds of *indica* rice (*Oryza sativa* L. sp. *indica* cv. Pathumthani 1) were obtained from the Pathumthani Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). Seeds were dehusked by hand, sterilized once in 5% Clorox® (5.25% sodium hypochlorite, The Clorox Co, US) 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 (Murashige and Skoog, 1962) in a 250 mL glass jar vessel. The media were adjusted to pH 5.7 before autoclaving. Seedlings were cultured *in vitro* under condition of 25±2°C ambient temperature, 60±5% relative humidity (RH) and 60±5 μmol m^{-2} sec^{-1} photosynthetic proton flux (PPF) provided by fluorescence lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) with 16 h day^{-1} photoperiod. Fourteen-day-old rice seedlings were aseptically transferred to MS-liquid media, containing 0, 111, 222, 333 or 444 mM glucose combined with 0, 20, 40, 60 or 80 μM abscisic acid (ABA) using vermiculite as supporting material. The number of air-exchanges in the glass vessels was adjusted to 2.32 h^{-1} by punching a hole on plastic cap (⌀ 1 cm) and covering the hole with a microporous filter (0.20 μM of pore size). All seedlings were continuously cultured under the same conditions as during the seed germination process for 7 days. Physiological characteristics, root osmolarity, soluble sugar content, pigment concentration and chlorophyll a fluorescence and morphological characteristics, root length, plant height and dry matter were measured.

Physiological Characteristics

Root osmolarity of rice seedlings was measured, according to Lanfermeijer *et al*. (1999). Fresh root tissues of rice were debris in 1.5 mL micro tube by glass rod. A 20 μL of extracted solution was directly dropped on a disc filter paper in osmometer chamber (Wescor, USA) and then measured.

Endogenous soluble sugars, sucrose, glucose and fructose in rice seedlings were evaluated following by Karkacier *et al*. (2003). A 100 mg frozen material was grinded in 1.5 mL micro tube with a small pestle. One milliliter of nanopure water was added and then sonicated for 15 min. The extracted material was centrifuged at 12,000 rpm for 10 min and then the supernatant was collected. The supernatant was directly filtered through 0.45 μM pore size prior to HPLC injection. Total soluble sugar, including sucrose, glucose and fructose was analyzed using 410 differential refractometer (RI) detector and Waters 600 gradient controller pump (Waters, Milford, MA, USA) with Metacarb 87C analytical column (300×6.5 mM). Nanopure water was used as mobile phase with 0.6 mL min^{-1} flow rate. The sucrose, glucose and fructose sugar classes were used as standard.

Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and carotenoid (C_{x+c}) concentrations were analyzed following the methods of Shabala *et al*. (1998) and Lichtenthaler (1987), respectively. The Chl_a and Chl_b concentrations were measured using an UV-visible spectrophotometer (DR/4000, HACH, USA) at wavelengths 662 and 644 nm. The C_{x+c} concentration was measured spectrophotometrically at 470 nm. A solution of 95.5% acetone was used as a blank. The Chl_a, Chl_b and C_{x+c} (μg g^{-1} FW) concentrations in the leaf tissues were calculated according to the following equations:

$$[Chla] = 9.784D_{662} - 0.99D_{644}$$
$$[Chlb] = 21.42D_{644} - 4.65D_{662}$$
$$[C_{x+c}] = \frac{1000D_{470} - 1.90[Chl_a] - 63.14[Chl_b]}{214}$$

where D_i is the optical density at wavelength i.

The maximum quantum yield (F_V/F_m) of the adaxial surface on the leaves was directly measured using Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggini *et al*. (1999). A 30 min dark-adapted leaf of leaf-clips (PEA/LC, Hansatech Instrument Ltd., UK) was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (<0.5 μmol m^{-2} sec^{-1}) with 1.6 s pulse of saturating light (>6.8 μmol m^{-2} sec^{-1} PAR) and calculated by FMS software for Windows® (Fluorescence Monitoring System Software, Hansatech Instrument Ltd., UK). The variable fluorescence yield (F_v) was calculated by the equation of F_m-F_0. The PSII activity was calculated by the ratio of F_v/F_m. The photon yield of PSII (Φ_{PSII}) in the light was calculated by $\Phi_{PSII} = (F_m'-F)/F_m'$ after 45 sec of illumination, when steady state was achieved and NPQ by Fm-Fm')/Fm', respectively.

Morphological Characteristics

The root length, plant height and dry matter of rice seedlings were measured as described by Lutts *et al*. (1996). The seedlings were dried at 110°C in a hot-air oven (Memmert, Model 500, Germany) for 2 days, incubated in desiccators before measurement of dry weight and calculated the dry matter percentage.

Experimental Design

The experiment was designed as 5×5 factorials in Completely Randomized Design (CRD) with ten replicates and four plantlets per replication. The mean in each treatment was compared by Dancan's New Multiple Range Test (DMRT) at $p \leq 0.01$ and analyzed by SPSS software (SPSS for Windows, SPSS Inc., USA).

RESULTS AND DISCUSSION

Osmolarity in the root tissues of rice seedlings cultured in exogenous glucose application was gradually increased, relating to increase the concentration in the media (Fig. 1). High osmolarity in glucose-added seedlings (333-444 mM) was directly limited on water availability as water deficit or drought condition. Soluble sugar accumulation in rice seedlings, including sucrose, glucose and fructose was strongly exhibited by the combination factors of glucose and ABA in the media. In exogenous glucose application, the profile of endogenous sucrose, glucose and fructose was relatively increased when exposure to 40-60 μM ABA and then slightly dropped in 80 μM ABA application (Table 1).

Table: 1: Sucrose, glucose and fructose concentrations in indica rice seedlings treated by 0 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week

Glucose treatment (mM)	Abscisic acid (μM)	Sucrose (μM g^{-1} FW)	Glucose (μM g^{-1} FW)	Fructose (μM g^{-1} FW)
	0	1.56hi	11.21n	6.71j
	20	2.77hi	51.88hijk	26.38g
0	40	2.32hi	78.59de	63.50a
	60	4.12hi	51.26hijk	29.71f
	80	9.50f	57.51ghij	30.83f
	0	2.29hi	15.06mn	12.01i
	20	2.99hi	53.88ghijk	42.71c
111	40	8.29fg	45.71jk	20.84h
	60	5.35gh	63.16ghi	31.94f
	80	3.52hi	67.11efg	40.88c
	0	5.42gh	46.64jk	14.08i
	20	30.39bc	42.98k	20.64h
222	40	16.27e	28.98l	34.92de
	60	16.85e	77.51de	30.41f
	80	1.64hi	90.29bcd	36.81d
	0	4.45ghi	40.94k	21.89h
	20	23.03d	59.44ghij	30.98f
333	40	32.91b	81.59cd	30.12f
	60	27.67c	94.50bc	65.79a
	80	0.94i	78.66de	46.27b
	0	3.49hi	26.26lm	13.87i
	20	37.72a	50.91ijk	26.74g
444	40	20.56d	64.97fgh	30.68f
	60	33.11b	119.15a	35.08de
	80	1.56hi	94.93b	32.56ef
Significant level				
Glucose		**	**	**
ABA		**	**	**
Glucose×ABA		**	**	**

Different letter(s) in each column show significant difference at p≤0.01 by Duncan's New Multiple Range Test (DMRT).
**: Highly significant in statistics

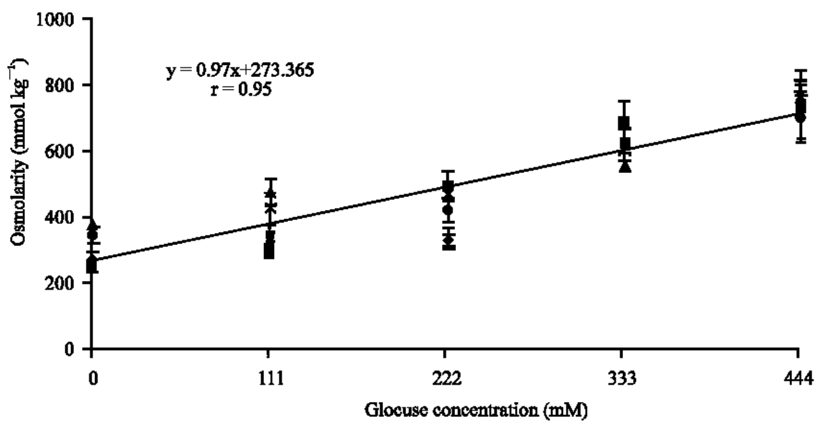

Fig. 1: Osmolarity in the root tissues of indica rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0 (•), 20 (▪), 40 (▴), 60 (×) or 80 (♦) μM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

In sugar-free media, the glucose and fructose profiles were similarly expressed to sugar adding media, except sucrose profile was positively accumulated. Moreover, the endogenous soluble sugar was directly stimulated by exogenous glucose application (Fig. 2). The accumulation soluble sugars in rice

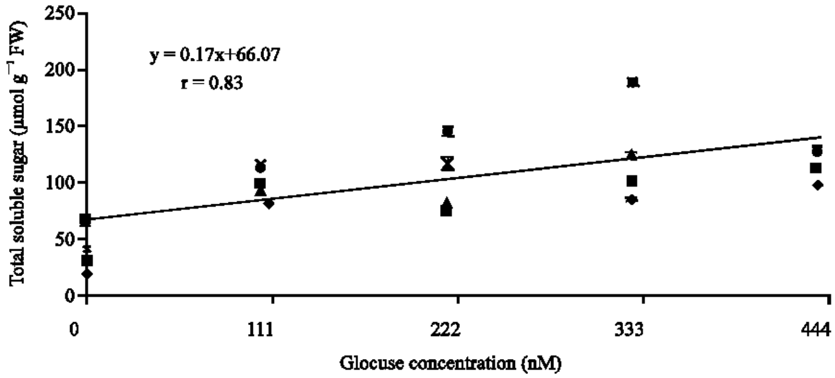

Fig. 2: Total soluble sugars, sucrose glucose and fructose, in *indica* rice seedlings treated by 0 111, 222, 333 or 444 mM glucose and 0 (•), 20 (▪), 40 (▴), 60 (×) or 80 (♦) µM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

Table 2: Chlorophyll a, chlorophyll b and total carotenoid concentrations in *indica* rice seedlings treated by 0 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 µM abscisic acid (ABA) in the media for 1 week

Glucose treatment (mM)	Abscisic acid (µM)	Chlorophyll ($\mu g\ g^{-1}$ FW)	Chlorophyll ($\mu g\ g^{-1}$ FW)	Total carotenoid ($\mu g\ g^{-1}$ FW)
	0	329.0ab	89.3b	110.7a
	20	207.6cd	68.1c	94.5b
0	40	154.2de	70.4c	58.0e
	60	96.3efg	51.6c	23.6fg
	80	102.1efg	30.2cd	19.8fg
	0	324.6b	88.2b	107.3ab
	20	105.7ef	32.4cd	64.3de
111	40	74.4fgh	62.8c	17.5g
	60	60.6fgh	19.8d	15.8g
	80	64.4fgh	20.3d	13.9g
	0	383.2a	152.6a	97.2ab
	20	71.6fgh	22.8cd	21.5fg
222	40	79.6fgh	25.8cd	17.5g
	60	44.5fgh	13.7d	14.4g
	80	43.9fgh	14.9d	14.8g
	0	199.8cd	55.4c	77.7cd
	20	184.3cd	109.6b	34.2f
333	40	80.3fgh	25.4cd	20.6fg
	60	63.9fgh	29.3cd	16.4g
	80	59.4fgh	48.8c	12.8g
	0	232.0c	62.4c	80.0c
	20	74.7fgh	19.5d	18.2g
444	40	48.1fgh	13.7d	16.8g
	60	37.1gh	11.3d	13.7g
	80	29.9h	10.5d	13.1g
Significant level				
Glucose		**	**	**
ABA		**	**	**
Glucose×ABA		**	**	**

Different letter(s) in each column show significant difference at $p \le 0.01$ by Duncan's New Multiple Range Test (DMRT).
**: Highly significant in statistics

seedlings may be played a central role as osmoregulation in the high osmolarity or drought stress. Chlorophyll a, chlorophyll b and total carotenoid concentrations in the leaf tissues of rice seedlings were gradually reduced by both high glucose and ABA application (Table 2). In addition, total

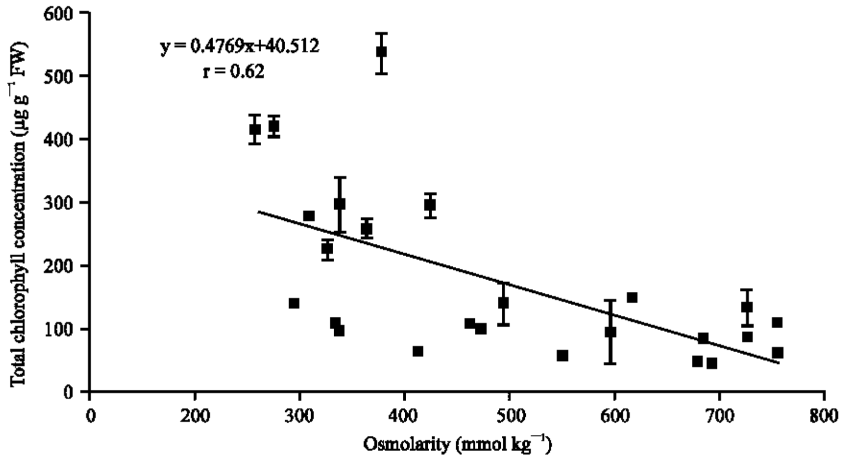

Fig. 3: Relationship between osmolarity and total chlorophyll concentration in *indica* rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

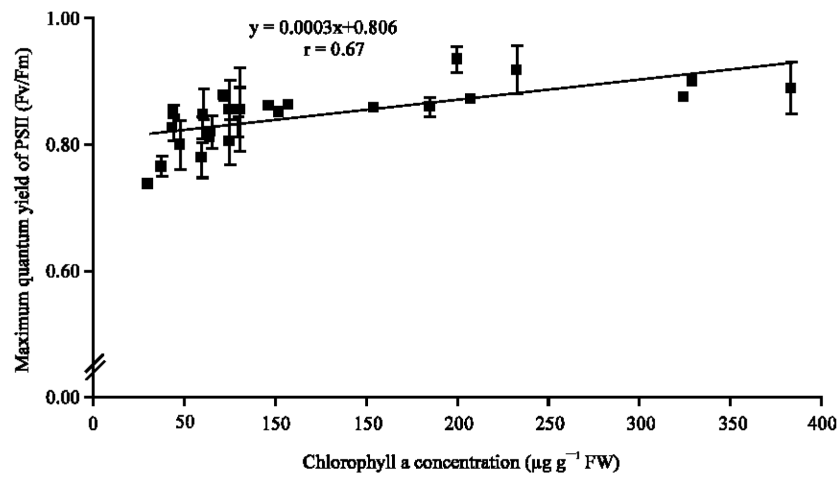

Fig. 4: Relationship between chlorophyll a concentration and maximum quantum yield of PSII (Fv/FV) in *indica* rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

chlorophyll contents were directly damaged by water deficit with high osmolarity (Fig. 3). The degradation of chlorophyll a, total chlorophyll and total carotenoid was positively related to maximum quantum yield of PSII (FV/FV) (Fig. 4), quantum efficiency of PSII (Φ_{PSII}) (Fig. 5) and non-photochemical quenching (NPQ) (Fig. 6), respectively. It should be noted that the pigment concentration in glucose and ABA treated seedlings was damaged and inhibited the function of light capture ability. The reduction of photosynthetic ability was a main cause to decrease on growth performances, root length, plant height and dry matter (Table 3). The growth parameters of rice seedlings cultured on high concentrations of glucose (333-444 mM) and/or ABA (40-80 μM) were powerfully inhibited in a week treatment.

Sugar in the culture media of heterotrophic and photomixotrophic tissue cultures is generally applied as a carbon source of *in vitro* culture practice. It is directly absorbed by plant cells from the

Table 3: Root length, plant height and dry matter in indica rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week

Glucose treatment (mM)	Abscisic acid (μM)	Root length (cm)	Plant height (cm)	Dry matter (%)
	0	60.0a	13.0ab	86.5a
	20	37.9cd	12.8ab	82.1a
0	40	29.1d	11.5b	82.2a
	60	28.1de	10.0bc	81.8a
	80	22.0de	10.0bc	80.8a
	0	63.1a	13.5ab	85.7a
	20	26.4de	13.0ab	79.9ab
111	40	21.6de	12.0ab	77.6ab
	60	19.3e	11.8b	76.1ab
	80	18.9e	10.8bc	75.4ab
	0	63.8a	15.5a	81.2a
	20	44.6bc	14.8a	78.9ab
222	40	28.3de	13.0ab	76.2ab
	60	28.1de	12.3ab	75.0ab
	80	24.1de	12.0ab	70.9b
	0	55.4ab	12.3ab	80.4a
	20	32.3cd	11.3b	78.2ab
333	40	30.6cd	11.0b	74.2ab
	60	29.1d	10.5bc	72.3b
	80	23.3de	8.0c	71.9b
	0	51.7bc	10.5bc	75.4ab
	20	46.3bc	10.3bc	73.4b
444	40	28.8de	10.0bc	72.5b
	60	26.5de	9.8bc	70.3b
	80	20.8de	9.0bc	69.4b
Significant level				
Glucose		**	**	**
ABA		**	*	**
Glucose×ABA		**	**	*

Different letter(s) in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT). **,* Highly significant and significant in statistics, respectively

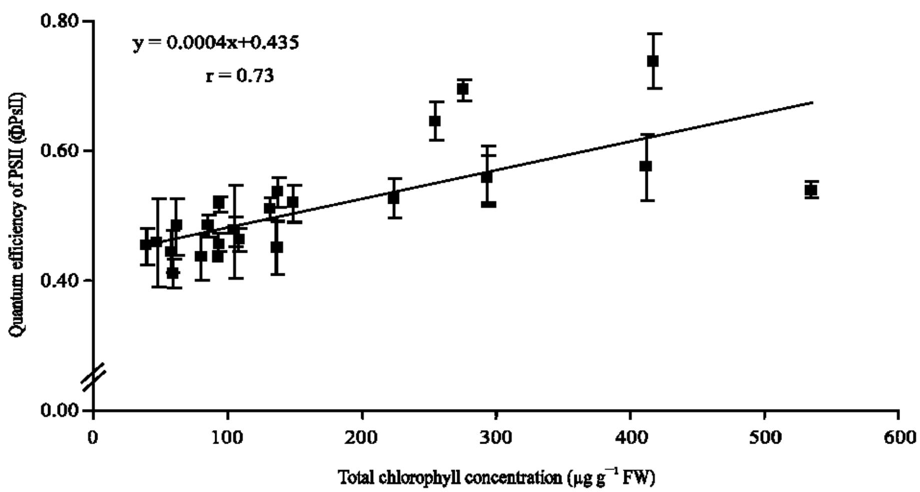

Fig. 5: Relationship between total chlorophyll concentration and quantum efficiency of PSII (ϕ_{PSII}) in indica rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

extracellular space through the plasma membrane, moving from cell to cell via plasmodesmata (symplastic loading) (Roitsch, 1999; Williams *et al*., 2000). In present data, there is evident information

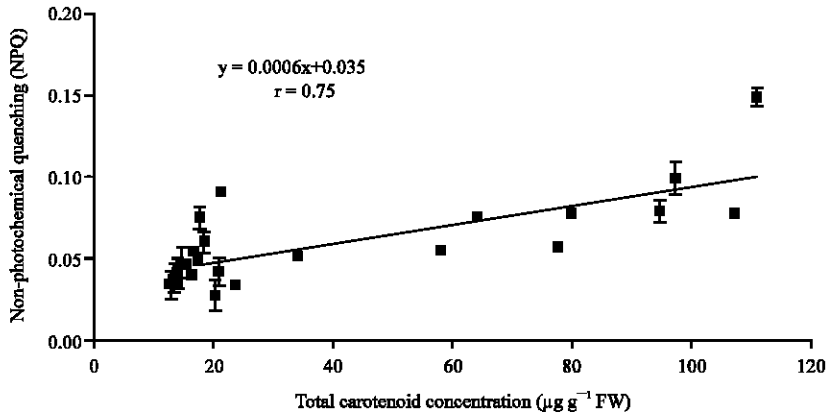

Fig. 6: Relationship between total carotenoid concentration and non-photochemical quenching (NPQ) in indica rice seedlings treated by 0, 111, 222, 333 or 444 mM glucose and 0, 20, 40, 60 or 80 μM abscisic acid (ABA) in the media for 1 week. Error bars represent by ±SE

on the sugar accumulation in rice seedlings treated by a low level of exogenous glucose in the media. Glucose is an important carbohydrate in respiratory metabolism and acts as a substrate for the biosynthesis of complex carbohydrates as starch and cellulose as well as provides building blocks for the biosynthesis of primary and secondary metabolites during plant growth and development (Koch, 1996; Smeekens and Rook, 1997; Sheen *et al*., 1999; Smeekens, 2000; Rolland *et al*., 2002). On the other hand, high concentration of sugar and/or sugar alcohols namely mannitol and sorbitol, in the media or nutrient solution has been used as osmotic stress or water deficit condition because the water available is limited or high osmotic pressure. The osmotic adjustment in plant cells is required for plant grown in water deficit or drought stress (Taybi and Cushman, 2002; Li *et al*., 2004b; Perales *et al*., 2005; Morsy *et al*., 2006). There are many reports to highlight on drought stress effects i.e., osmotic adjustment (da Silva and Arrabaca, 2004; Sanchez *et al*., 2004; Blum, 2005), antioxidants (Reddy *et al*., 2004; Nayyar and Gupta, 2006), hormonal regulation (Nan *et al*., 2002; Perales *et al*., 2005) and physiological responses (Cabuslay *et al*., 2002; Reddy *et al*., 2004; Pieters and Souki, 2005). Abscisic acid (ABA) is one of endogenous hormonal regulations in drought stressed plant, which is attractively investigated in many crop species (Neill and Burnett, 1999; Jia *et al*., 2001; Nan *et al*., 2002; Alves and Setter, 2004; Lopez-Carbonell and Jauregui, 2005; Perales *et al*., 2005; Nayyar and Gupta, 2006; Wan and Li, 2006; Zhang *et al*., 2006). An exogenous ABA is an alternative way to enhance on water-deficit defense mechanisms. The ABA accumulation in drought stressed plants is plays an important role on water use efficiency, stomata closure, CO_2 assimilation and photosynthesis system, leading to maintain on plant growth (Blasi *et al*., 1998; Blomstedt *et al*., 1998; Taybi and Cushman, 2002; Nan *et al*., 2002; Wang *et al*., 2003; Li *et al*., 2004b; Yin *et al*., 2004; Perales *et al*., 2005; Wan and Li, 2006). On the other hand, endogenous ABA in rice seedlings treated by high exogenous ABA may be increased, causing on negative effects on plant growth and development or senescence stimulation. In addition, the sugar and ABA have been reported as combinatorial control in plant defense mechanisms to environmental stresses (Toyofuku *et al*., 2000; Leon and Sheen, 2003; Rook *et al*., 2006).

In conclusion, the osmolarity in rice seedlings was adjusted to high value when cultured in glucose and ABA. The sugar accumulation in rice seedlings was directly promoted by low concentrations of exogenous glucose and ABA application in the media. Alternatively, the pigments in rice seedlings cultured on high osmolarity were directly degraded, causing on light capture limitation in photosystem II. The reduction on light reaction of photosynthetic system was positively related to decrease on growth and development of rice seedlings. The responsive gene(s) regulation of rice exposed to glucose and/or ABA should be further investigated as a novel defense mechanism.

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