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Effect of Salinity on Lignin and Hydroxycinnamic Acid Contents in Rice

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ABSTRACT: The lignin contents between IR-29 and Pokkali were not significantly different in the absence of NaCl, but they were slightly increased at 40 mM NaCl. Although lignin contents were not relatively significantly different between salt treated and control plants, the total yields of alkaline nitrobenzene oxidation ranged from 17.4 - 20.0 mg/g of cell wall residue at 40 mM NaCl were significantly different compared with control plants (11.8 - 12.2 mg/g). The total amounts of ester-linked hydroxycinnamic acids in IR-29 were decreased from 14.5 to 9.9 mg/g, while Pokkali is almost same levels (14.9 - 15.0 mg/g) under treated and control with 40 mM NaCl. In contrast, the total amounts of ether-linked hydroxycinnamic acids were increased from 9.4 to 13.9 mg/g together with an opposite trend in Pokkali as a decrease 10.9 to 8.8 mg/g under treated and control with 40 mM NaCl. These results revealed that IR-29 is more sensitive in response to 40 mM NaCl in terms of hydroxycinnamic acids than Pokkali.

Keywords: salinity, lignin, hydroxycinnamic acids, IR-29, Pokkali

Traditionally, lignins are considered to be the polymers of three alcohol monomers: *p*-coumaryl alcohol (H unit of lignin), coniferyl alcohol (G unit of lignin), and sinapyl alcohol (S unit of lignin) (Fig. 1). These complex polymers are believed to contribute to the compressive strength, resistance to degradation by microbial attack, and water impermeability for the polysaccharide-protein matrix of the cell wall (Whetten *et al.*, 1998). Moreover, lignin polymer forms with a significant different degree of homogeneity during lignin polymerization through oxidative radical-radical coupling reactions (Yamamoto *et al.*, 1989) For example, all the condensed types of lignin such as β-5 (phenylcoumaran), β-1 (diarypropane), β-β (pinoresinol) and 5-5 (biphenyl) are highly formed at immature stage of plant growth, compared to the mature stage, at which arylglycerol-β-aryl ether (β-*O*-4) linkage is mainly formed (Terashima *et al.*, 1998; Chung & Iiyama, 2003; Jin *et al.*, 2003) Thus, the structural features of lignin and recovery of alkaline nitrobenzene oxidation (NBO) products based on the lignin content at the immature stage are significantly different from those of the mature stage. According to previous papers (Terashima *et al.*, 1993; Jin *et al.*, 2003), the maximum NBO recovery from arylglycerol-β-aryl ether is about 50%, whereas those from condensed types are about 10-15%.

Prior to lignification, during cell elongation, hydroxycinnamic acids such as ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric acid (PCA, 4-hydroxy-cinnamic acid) are incorporated into the developing pectin-rich middle lamella and primary cell wall. As bifunctional molecules with carboxylic and phenolic bonding sites, these hydroxycinnamic acids can be involved in both ester and ether linkages. For example, in the cell walls of cereal straws, FA is ester-linked to arabinoxylans, and as lignification is initiated, FA ether links with lignin to allow its intricate incorporation with the polysaccharides that make up the pectins, hemicelluloses, and cellulose (Sun *et al.*, 2002). Such a cross-linking can have a dramatic influence on the wall mechanical properties, extensibility, and biodegradability. In addition, ferulate polysaccharides esters in grasses were found to be acting as initiation or nucleation sites for lignification and are critical entities in directing cell wall cross linking during plant growth and development (Ralph *et al.*, 1995).

The content and composition of hydroxycinnamic acids are dependent on the morphological location and the differentiation stage. It has been reported that FA rapidly deposits in the cell walls at the immature stage of lignification, subsequently PCA residue deposits continuously throughtout the lignification and become a predominant constituent of

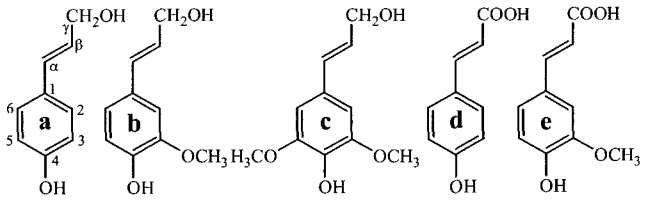

Fig. 1. The chemical structures of monolignols (a-c) and hydroxycinnamic acids (d and e) a, *p*-coumaryl alcohol, b, coniferyl alcohol; c, sinapyl alcohol, d, *p*-coumaric acid, e, ferulic acid

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hydroxycinnamic acids. All the cell walls of sugarcane and rice straw involve 5 - 15% hydroxycinnamic acid moieties based on total lignin, except the vessel of protoxylem which contains only about 3 - 6% (Sun *et al.*, 2002).

Salt stress plays an important role in the plant growth, and the formation of lignins and hydroxycinnamic acids would be related to the plant growth (Rodriguez *et al.*, 1997; Sun *et al.*, 2002; Kim *et al.*, 2004). Therefore those two factors would probably have co-relationship that is, salt stress will affect the formation of lignins and hydroxycinnamic acids. Even though many researchers reported that the inhibition of plant growth by salt stress was due to various chemical, physiological, and molecular responses (Moons, *et al.*, 1995; Lefèvre *et al.*, 2001; Kswasaki *et al.*, 2002), there is no report that the effect of salt stress on formation of lignins and hydroxycinnamic acids.

The present research was undertaken to improve understanding of the response of lignins and hydroxycinnamic acids in rice to salt stress. The emphasis is to quantitatively determine both ester- and ether-linked hydroxycinnamic acids, e.g. FA, PCA, and characteristics of lignins under salt stress.

MATERIALS AND METHODS

Plant material, growth conditions and stress application

Seeds of the rice (*O. sativa* L.) cvs IR-29 (salt-sensitive) and Pokkali (salt-resistant) were obtained from International Rice Research Institute (IRRI; Philippines). Seeds were put polystyrene plates with a plant-to-plant distance of 15 cm and grown in the Chungnam National University green house facilities.

Stress (40 mM NaCl, three tanks per dose) was applied on 21-day-old plants and 20 plants each tank were harvested, measured, and analyzed after 30 days of exposure to stress.

Extraction and determination of lignin content

The samples were manually ground with liquid nitrogen, and extracted twice with ethanol-toluene (1:1, v/v), ethanol, and distilled water for 12 h with constant stirring at an ambient temperature in the dark. The resulting cell wall residues (CWR) were dried *in vacuo*. A modified method of Iiyama & Wallis (1990) was used for the determination of the acetyl bromide (AcBr) lignin. The sample (5 mg) was suspended in 2.5 ml of AcBr solution (25% AcBr in glacial acetic acid), and added 0.1 ml of perchloric acid (70%). The mixtures were placed in an oven at 70°C for 30 min, with each mixture shaken at 10 min intervals. After 30 min, the mixture was immediately placed in iced water and transferred to a 50 ml volumetric flask containing 10 ml of 2 M NaOH and 12 ml of glacial acetic acid. The mixture volume was adjusted to 50 ml with glacial acetic acid. Lignin contents were determined by a Shimadzu UV 200 spectrophotometer (Tokyo, Japan) at 280 nm.

Alkaline nitrobenzene oxidation (NBO)

The chemical structure of lignin was examined by alkaline nitrobenzene oxidation, according to a procedure modified by Iiyama & Lam (1990). The CWR (40 mg) was put in a 10 ml stainless steel reactor with 4 ml of 2 M NaOH and 0 25 ml of nitrobenzene. The products were quantified using 3-ethoxy-4-hydroxybenzaldehyde (0.1 ml of a 250 mg /50 ml) as an internal standard by the Shimadzu GC-18A (Tokyo, Japan) gas chromatograph (NB-capillary column, 25 m × 0.25 mm id) equipped with a flame-ionization detector. The injector and detector temperatures were 280 °C. The column temperature was kept at 150 °C for 10 min, then programmed at 5 °C min^{-1} to 250 °C, and maintained for 5 min at 250 °C.

Determination of hydroxycinnamic acids

Esterified and total hydroxycinnamic acids were determined using the procedures of Sun *et al.* (2002). For esterified hydroxycinnamic acids, the CWR (50 mg) were suspended in 5 ml of 0.5 M NaOH and, after hydrolysis at 37 °C overnight, *m*-coumaric acid (0.1 ml of a 4 mg/ml MeOH) was added as internal standard. The mixture was acidified with 4 M HCl to pH 1 after filtration, then extracted twice with 30 ml dichloromethane, followed by 30 ml diethyl ether The combined organic extracts were evaporated and the residue was dissolved in small amount of diethyl ether. The products were detected by gas chromatograph as trimethylsilyl (TMS) derivatives The column temperature was kept at 180 °C for 10 min, then programmed to 250 °C with 5 °C min^{-1}. The injector and detector temperatures were 250 °C. For the determination of total (ester- and ether-linked) hydroxycinnamic acids, the CWR (50 mg) was hydrolyzed with 5 ml of 4 M NaOH at 170 °C for 2 hr. After being filtered and washed twice with 5 ml of water, the filtrate was adjusted to pH 1 with 4 M HCl. The acidified solution was extracted and detected by same procedure as mentioned above.

RESULTS AND DISCUSSION

The first experiment on shoot length measurement exposed to 40 mM NaCl was performed (Table 1). In the absence of stress, the average shoot lengths of two rice cultivars as IR-29 and Pokkali were 32.4 and 48.5 cm, respectively. There was significantly decreased in shoot length as 23.4 cm in IR-29,

while Pokkali (45.2 cm) was negligibly different from the control plant at 40 mM NaCl. The reduction in shoot length at 40 mM NaCl was remarkably pronounced in IR-29 as 28.4%, together with relatively low reduction rate in Pokkali as 6.4%. No significant difference of lignin content as 15.4 15.5% between two cultivars in the absence of NaCl, but it was slightly increased in lignin content ranged from 17.5 - 18.7% at 40 mM NaCl probably owing to high activity of salt-induced peroxidase (Fig. 2). In general, peroxidase produces phenoxy radicals, which can act as an initiator for making lignin polymer through oxidative radical-radical coupling reactions (Yamamoto *et al.*, 1989). This result suggests that the effect of two different cultivars on lignin content was not significant in both absence and presence of 40 mM NaCl.

To determine the building units of lignin, alkaline nitrobenzene oxidation (NBO) was performed (Table 2). Although lignin contents were not relatively significantly different between salt treated (17.5 - 18.7%) and untreated (15.4 - 15.5%) plants, the total yields of NBO ranged from 17.4 - 20.0 mg/g of CWR at 40 mM NaCl were significantly different compared with control plants (11.8 - 12.2 mg/g of CWR). Therefore, these results suggest that the lignin from control plants would relatively highly contain several condensed structures of lignin such as β-5 (phenylcoumaran), β-1 (diarylpropane), β-β (pinoresinol), and 5-5 (biphenyl), while arylglycerol-β-aryl ether (uncondensed type) is the predominant structural units that from salt treated ones. Because NBO recovery from uncondensed lignin is generally much higher than condensed types (Terashima *et al.*, 1993; Jin *et al.*, 2003). In addition, Pokkali exposed to 40 mM NaCl contained much higher vanillin as the main G units of lignin (8.3 mg/g of CWR) and syringaldehyde as the main S units than those of IR-29, but *p*-hydroxybenzaldehyde as the main H units in Pokkali (3.5 mg/g of CWR) is relatively less than IR-29 (4.6 mg/g of CWR). Thus the recovery of NBO products in Pokkali as 11 5% (based on lignin content) at 40 Mm NaCl is relatively higher than IR-29 as 9.3% (based on lignin content), owing to high amounts of G and S units (Fig. 3). These results imply that each unit of lignin can contribute to make condensed or uncondensed structures in lignin polymers. Indeed H unit has the highest possibility among three lignin units to make condensed types of lignin at *C*-3,5 positions during radical coupling

Table 1. Effect of salinity on shoot growth of IR-29 and Pokkali cultivars The plants were exposed to salt treatment for 30 days

Shoot length (cm)	IR-29	Pokkali	Inhibition (%)
0 mM	32.7±3 7	48.5±5.3	28.4±0.6
40 mM	23.4±4.3	45.2±4.0	6 4±1.8

Data are the means ±S.D (n = 20)

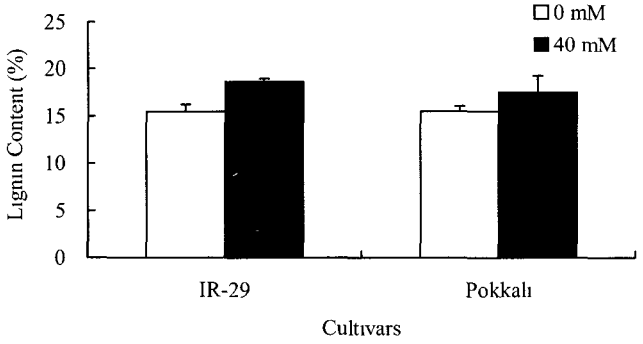

Fig. 2. Lignin contents of IR-29 and Pokkali cultivars exposed to 40 mM NaCl for 30 days The bars represent S.D. (n=3)

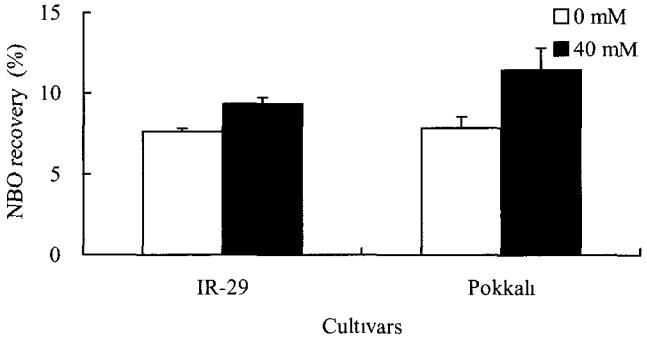

Fig. 3. Recovery of alkaline nitrobenzene oxidation based on lignin contents of IR-29 and Pokkali cultivars exposed to 40 mM NaCl for 30 days The bars represent S D. (n=3).

Table 2. Alkaline nitrobenzene oxidation (NBO) products of IR-29 and Pokkali cultivars exposed to 40 mM NaCl for 30 days

	NaCl (mM)	NBO products (mg/g of CWR)			Total (mg/g)	Recovery (%)
		H	V	S		
IR-29	0	2 5±0.3	4 7±0.4	4 6±0 2	11 8±0.8	7 6±0.2
	40	4 6±0.2	6 5±0.3	6.3±0 5	17 4±0 7	9.3±0 4
Pokkali	0	2 3±0.2	5 1±0 5	4.8±0.1	12.2±0.7	7.9±0.7
	40	3.5±0 2	8 2±0 2	8.3±0.3	20.0±0.4	11.5±1.4

Data are the means ±S D. of triplicate experiments. CWR = cell wall residue
H = *p*-hydroxybenzaldehyde, V = vanillin, S = syringaldehyde.

Table 3. The contents of total and ester- and ether-linked *p*-coumaric (PCA) and ferulic acids (FA) of IR-29 and Pokkali cultivars exposed to 40 mM NaCl for 30 days.

	NaCl (mM)	Ester-linked HA (mg/g of CWR)			Ether-linked HA[a] (mg/g of CWR)			Total HA (mg/g of CWR)		
		PCA	FA	total	PCA	FA	total	PCA	FA	total
IR-29	0	5.9±0.2	8.6±0.3	14.5±0.5	1.7±0.2	7.8±0.3	9.4±0.4	7.5±0.3	16.3±0.3	23.8±0.5
	40	4.8±0.1	5.1±0.2	9.9±0.3	4.4±0.5	9.6±0.6	13.9±0.8	9.1±0.4	14.6±0.5	23.7±0.6
Pokkali	0	6.4±0.1	8.5±0.1	14.9±0.1	2.0±0.3	8.9±0.4	10.9±0.5	8.5±0.3	17.4±0.4	25.9±0.4
	40	6.6±0.3	8.4±0.2	15.0±0.3	1.7±0.4	7.1±0.4	8.8±0.9	8.1±0.2	15.5±0.5	23.6±0.6

Data are the means ±S.D of triplicate experiments [a], Content of ether-linked PCA and FA were calculated as the difference between total and ester-linked PCA and FA. HA=hydroxycinnamic acids

reactions, whereas G (at *C*-5) and S units have relatively low possibility (Marita *et al.*, 1999).

The results of hydroxycinnamic acid analyses are shown in Table 3. The concentrations of the total hydroxycinnamic acids, *p*-coumaric acid (PCA), and ferulic acid (FA), in all samples were similar levels (23.6 - 25.9 mg/g of CWR). All the cell walls of samples involve 13 - 17% hydroxycinnamic acid moieties based on total lignin, and these levels were in good agreement with those of previous papers (He & Terashima, 1991; Lam & Iiyama, 2000). However, the amounts of hydroxycinnamic acids bound by specific linkages (ester- and ether-linked) to cell wall components are remarkably changed between two cultivars at 40 mM NaCl. The total amounts of ester-linked hydroxycinnamic acids in IR-29 were decreased from 14.5 to 9.9 mg/g of CWR, while Pokkali is almost same levels (14.9 - 15.0 mg/g of CWR) under treated and untreated 40 mM NaCl. In contrast, the total amounts of ether-linked hydroxycinnamic acids were increased from 9.4 to 13.9 mg/g of CWR together with an opposite trend in Pokkali as a decrease 10.9 to 8.8 mg/g of CWR under treated and untreated 40 mM NaCl. This opposite tendency suggests that IR-29 rapidly turn over from immature to mature stage, while Pokkali is growing continuously throughout normal development of cell wall under salt stress. In fact, the hydroxycinnamic acids bonding between components of the cell wall shifts from ester linkage at the early stage of lignification to become predominantly etherified at the late stage (Sun *et al.*, 2002). For example, ester-linked FA from IR-29 was drastically decreased from 8.6 to 5.1 mg/g of CWR, together with an increase in ether linkage from 7.8 to 9.6 mg/g of CWR. In conclusion, IR-29 is more sensitive in response to 40 mM NaCl in terms of hydroxycinnamic acids than Pokkali.

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