

# Molecular and Physiological Responses to Abscisic Acid and Salts in Roots of Salt-Sensitive and Salt-Tolerant Indica Rice Varieties<sup>1</sup>

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The Indica rice (*Oryza sativa* L.) varieties Pokkali and Nona Bokra are well-known salt tolerance donors in classical breeding. In an attempt to understand the molecular basis of their tolerance, physiological and gene expression studies were initiated. The effect of abscisic acid (ABA) on total proteins in roots from 12-d-old seedlings of Pokkali, Nona Bokra, and the salt-sensitive cultivar Taichung N1 were analyzed on two-dimensional gels. The abundance of ABA-induced proteins was highest in the most tolerant variety, Pokkali. Three ABA-responsive proteins, present at different levels in roots from tolerant and sensitive varieties, were further characterized by partial amino acid analysis. A novel histidine-rich protein and two types of late embryogenesis abundant (LEA) proteins were identified. Protein immunoblotting revealed that the levels of dehydrins and group 3 LEA proteins were significantly higher in roots from tolerant compared with sensitive varieties. Endogenous ABA levels showed a transient increase in roots exposed to osmotic shock (150 mM NaCl). Peak ABA concentrations were 30-fold higher for Nona Bokra and 6-fold higher for Pokkali compared with Taichung N1. Both the salt-induced endogenous ABA levels and a greater molecular response of root tissue to ABA were associated with the varietal differences in tolerance.

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Coastal salinity and accumulation of salts in irrigated land are primary factors depressing yield in rice crop production. Among lowland rice genotypes, the Indica varieties Pokkali and Nona Bokra are classified as highly tolerant on the basis of various physiological parameters (Akbar et al., 1986a). Genetic studies revealed that salt tolerance of these varieties is principally due to additive gene effects (Akbar et al., 1986b). The underlying molecular mechanism for their salt tolerance has never been studied.

In recent years, some progress has been made in the study of molecular processes involved in the physiological and metabolic adaptations of plants subjected to desiccation, salt stress, or cold. A large set of genes that are transcriptionally activated in vegetative plant tissue during

these stresses has been identified (Skriver and Mundy, 1990). It is assumed that stress-induced proteins might play a role in tolerance, but direct evidence is generally lacking. Furthermore, some stress-induced changes in gene expression might also be associated with deleterious stress effects. Therefore, linking the expression of a gene to a higher degree of tolerance within a genotype provides an important argument for a role in adaptation.

The phytohormone ABA is implicated in the control of physiological and molecular processes involved in the development of desiccation tolerance in seeds as well as vegetative tissue (Skriver and Mundy, 1990; Bray, 1991; Hetherington and Quatrano, 1991; Chandler and Robertson, 1994). Endogenous ABA concentrations increase in different plant tissues during a drought-, salinity-, or cold-induced reduction in water availability (Zeevaert and Creelman, 1988). However, only a few studies have compared either stress-induced endogenous ABA levels in tolerant and sensitive varieties (Walker-Simmons, 1987; Lee et al., 1993) or gene expression in response to exogenous ABA in genotypes differing in tolerance (Mohapatra et al., 1988; Galvez et al., 1993).

The salt, desiccation, or cold tolerance mechanisms from natural ecotypes appear to be complex. Until now, unique salt or desiccation tolerance genes have not been identified, but sets of proteins, or in vitro translation products, present at different levels in tolerant versus sensitive genotypes have regularly been observed, e.g. salt-induced proteins in *Lophopyrum*, a salt-tolerant wheat relative (Gulick and Dvořák, 1987), and in a salt-tolerant barley cultivar (Ramagopal, 1987; Hurkman et al., 1989), and ABA- or cold-induced proteins in a freezing-tolerant alfalfa cultivar (Mohapatra et al., 1988). Identification of these differentially regulated proteins is seldom included. The expression level of a number of specific genes has been reported to be correlated with the salt, desiccation, or cold tolerance of varieties or cell lines, e.g. HAL 1 overproduction in yeast (Gaxiola et al., 1992), the salt induction of osmotin in tobacco (LaRosa et al., 1989), the early salt-induced levels from 11 genes, including a group 2 LEA gene from *Lophopyrum* (Gulick and Dvořák, 1992; Galvez et al., 1993), the chilling induction of a Gly-rich protein from alfalfa

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Abbreviations: LEA, late embryogenesis abundant; Me-ABA, methyl-ABA.

(Mohapatra et al., 1989), and a group 2 LEA protein of wheat (Houde et al., 1992).

LEA proteins are suggested to be involved in desiccation survival, although their exact function is still unclear (Baker et al., 1988). Arguments for this role include their high abundance in desiccation-tolerant seed embryos (Roberts et al., 1993), the on-call water-stress inducibility of specific LEA genes in vegetative tissue (Close et al., 1989; Piatowski et al., 1990), and particular structural features (Baker et al., 1988; Dure, 1993). LEA genes are responsive to ABA (reviewed by Bray, 1991), which triggers their induction during tissue dehydration (Pla et al., 1991), although ABA-independent parallel induction pathways also exist (Chandler and Robertson, 1994, and refs. therein). LEA proteins fall into groups based on amino acid sequence similarities (Dure et al., 1989).

Group 2 LEA/Rab/dehydrin proteins generally accumulate in plants in response to dehydration caused by desiccation, salts, or chilling. Dehydrin-encoding cDNA clones have been identified from rice, barley, maize (Mundy and Chua, 1988; Close et al., 1989; Vilardell et al., 1990), and several dicots (Baker et al., 1988; Piatowski et al., 1990; Robertson and Chandler, 1992, and refs. therein). The four members of a rice Rab/group 2 LEA family, tandemly arrayed in a single locus, are coordinately expressed in response to ABA, drought, and NaCl in various rice tissues (Yamaguchi-Shinozaki et al., 1989).

Group 3 LEA proteins are composed principally of tandem repeats of an 11-mer amino acid motif, forming an amphiphilic helix that readily binds ions (Dure et al., 1989; Dure, 1993). cDNA clones encoding type I group 3 LEA proteins have been isolated from cotton, barley, rape, wheat, carrot, and *Craterostigma* (Dure, 1993, and refs. therein); group 3 (type II) LEA proteins have been studied for soybean, carrot, and wheat (Curry and Walker-Simmons, 1993, and refs. therein). In barley seedlings, group 3 (type I) LEA mRNAs are induced by dehydration, cold, exogenous ABA, and, to a lesser extent, salts in an organ-specific manner (Hong et al., 1992). Dehydration-induced group 3 (type I) LEA mRNA levels were correlated with increases in endogenous ABA levels in wheat seedlings (Curry et al., 1991).

In this paper, we present a comparison of molecular and physiological responses to salt stress and ABA in tolerant and sensitive rice genotypes with emphasis on the role of ABA. Growth inhibition, endogenous ABA levels, and changes in root protein patterns were studied. Three ABA-induced proteins were identified by partial protein sequence analysis. In addition, expression levels of group 2 and group 3 LEA proteins were analyzed by western detection.

## MATERIALS AND METHODS

### Plant Material, Plant Growth, and ABA Treatments

Seeds of Pokkali and Nona Bokra, two salt-tolerant Indica rice varieties (*Oryza sativa* ssp. Indica var Nona Bokra and var Pokkali) and seeds from a salt-sensitive Indica rice variety (*O. sativa* ssp. Indica cv Taichung Native 1), were

supplied by the International Rice Research Institute (Manila, Philippines).

The rice seeds were sown on autoclaved vermiculite and grown on control medium: half-strength Hoagland solution (pH 5.6), 2.5 mM Ca<sup>2+</sup>, enriched with nitrogen (Hoagland and Arnon, 1950), at 27°C, 16 h of light, 8 h of dark for 9 d prior to stress treatment. Nine-day-old seedlings were transferred to hydroponic cultures of either control solution or control solution supplemented with 20 μM ABA or 100 μM ABA, adjusted to pH 5.6. Plants were incubated on these media for 3 d.

### Measuring Stress Tolerance

Seeds of each variety were sown (d 0), germinated, and grown for 10 d on vermiculite soaked with control medium, composed of half-strength Hoagland solution, pH 5.6, or with control medium supplemented with 50 mM NaCl. After 10 d, the lengths of the first leaf and the primary root were measured for 100 seedlings of each variety and for each growth condition in three independent experiments. The SE on the mean length of shoot and root was calculated. The percentage of relative growth inhibition of shoot and root and the percentage of relative increase of the root to shoot growth ratio were derived from these data.

### Measuring Endogenous ABA levels

Approximately 50 seeds of each variety were germinated and grown on grids, placed above pots containing control solution. After 10 d of growth, the grids holding the seedlings were placed on top of pots containing control solution supplemented with 150 mM NaCl for 2, 4, 8, 12, 24, 48, and 72 h. Root material was harvested and immediately frozen at -196°C. From shoot samples of these salt-stressed seedlings, fresh weight and dry weight (24-h drying at 100°C) were determined, and the percentage of shoot water content ( $100 \times [\text{fresh weight} - \text{dry weight}] / \text{fresh weight}$ ) was calculated. ABA was extracted from frozen root material and purified as described by Prinsen et al. (1991). DL-*cis,trans*-[G-<sup>3</sup>H]ABA (300 Bq, 2.26 TBq/mmol; Amersham) and D<sub>6</sub>-ABA (200 ng; for preparation, see Milborrow, 1971) were initially added as tracers for localization and isotope dilution purposes, respectively. After the samples were methylated with diazomethane (Schlenk and Gellerman, 1960), Me-ABA was analyzed by GC-MS (HP 5890 series II coupled to a VG TRIO 2000 quadrupole mass spectrophotometer; column 15 m BD5, 0.25 mm i.d.; gas phase He, temperature gradient from 120–240°C, 15° min<sup>-1</sup>; 250°C injection temperature; retention time of Me-ABA, 5.59 min) following the method of Rivier et al. (1977). For the detection of Me-ABA and Me-D<sub>6</sub>-ABA, 190 and 194 were used as selective diagnostic ions, respectively (Milborrow, 1971).

### Protein Analysis

After an ABA treatment for 3 d, roots were harvested. Protein extraction was performed as described by Hurkman and Tanaka (1986). Two-dimensional gel electrophoresis was carried out as described by Bravo (1984).

Proteins were successively separated by IEF and SDS-PAGE. For the IEF, an ampholine mixture composed of ampholines 3.5–10, 5–8, and 8–9.5 (LKB) in a 2:8:1 ratio was used, extending the pI range to basic values. The first-dimension mini-gels were polymerized into capillary pipettes of 100  $\mu$ L (127 mm long, 1 mm i.d.). For the second dimension, the separation gel (90  $\times$  100 mm) contained 15% (w/v) acrylamide. The mini-gels were stained with Coomassie blue. All inductions and two-dimensional protein gel analyses were performed at least three times.

#### Amino Acid Analysis

The first dimension of the preparative gels was poured into glass tubes of 150 mm in length and 2.5 mm i.d. Isolated protein spots were combined from four to six separation gels (150  $\times$  150 mm). Peptides were either generated by in situ proteolytic digestion of proteins immobilized on Immobilon (Bauw et al., 1989) or by limited acid hydrolysis in the polyacrylamide gel (Vanfleteren et al., 1992). Generated peptides were separated by reversed-phase HPLC, and the amino acid sequence analysis was performed using a 473A protein sequenator (Applied Biosystems, Foster City, CA). With the peptide sequences obtained, the Protein Identification Resource Data Bank (PIR, release No. 41) and the University of Geneva Protein Sequence Data Bank (Swissprot, release No. 28.1) were screened using the software supplied by Genetic Computer Group, University of Wisconsin (version 7.3).

#### Western Blot Analysis

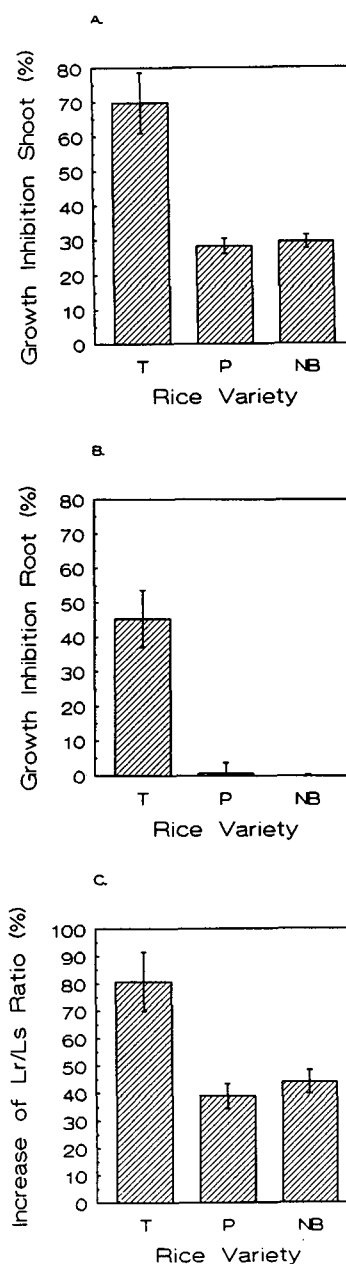
A polyclonal antibody from rabbit, produced against a fusion protein of the group 3 (type I) LEA protein from wheat (Ried and Walker-Simmons, 1993), was kindly supplied by J.L. Ried and M.K. Walker-Simmons (Washington State University, Pullman). An antiserum raised against maize dehydrins was generously supplied by P.M. Chandler (Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia). SDS-PAGE mini-gels or two-dimensional mini-gels were blotted onto nitrocellulose membranes (Hybond-C; Amersham) and stained with amido black prior to western blot analysis. Subsequently, the filters were destained in 10 mM NaOH and western blot analysis was performed essentially following the procedure of Harlow and Lane (1988) using an alkaline phosphatase conjugate (Boehringer and Sigma) and the color development reagents 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride (Bio-Rad) in the detection reaction.

## RESULTS

### Evaluation of the Stress Tolerance of Two Salt-Tolerant Varieties and a Salt-Sensitive Variety of Rice

The salt tolerance of the three varieties was compared using the salt-induced growth inhibition of fast-growing, young seedlings as a parameter. Seeds were germinated and grown for 10 d in mild salt-stress conditions. NaCl at

50 mM significantly reduced rice seedling growth but did not cause other visible stress symptoms such as wilting, bleaching or browning of the leaf tips, or chlorosis. The relative growth inhibition of Pokkali and Nona Bokra seedlings was significantly less than for Taichung N1 (Fig. 1A). Root growth of the tolerant varieties was not inhibited by 50 mM NaCl, but a rather strong inhibition was observed for the salt-sensitive variety Taichung N1 (Fig. 1B). The observed increase of the root to shoot ratio was more



**Figure 1.** Effect of 50 mM salt on the growth of Taichung N1 (T), Pokkali (P), and Nona Bokra (NB) seedlings. Percentage of relative growth inhibition of the shoot (A) and the root (B) of each variety is shown; percentage of relative increase of the root to shoot ratio (Lr/Ls) of each variety is shown in C. The seedlings were germinated and grown for 10 d.

pronounced for Taichung N1 than for the tolerant varieties (Fig. 1C).

Higher concentrations of NaCl (150 mM) provoked visible wilting of rice seedlings. Salt-induced wilting, quantified by fresh weight/dry weight measurements, was used as another parameter to compare salt tolerance of the three varieties. Ten-day-old, hydroponically grown seedlings were subjected to salt stress (150 mM NaCl) for increasing durations (2, 4, 8, 12, 24, 48, and 72 h; Fig. 2A). A biphasic decrease in shoot water content was observed for Taichung N1 in contrast to less pronounced decreases for both tolerant varieties. Wilting was always considerably less severe for Pokkali and Nona Bokra than for Taichung N1, which showed dramatic symptoms after a prolonged stress period (>24 h). Whereas Pokkali and Nona Bokra seedlings showed rather comparable symptoms of wilting within the

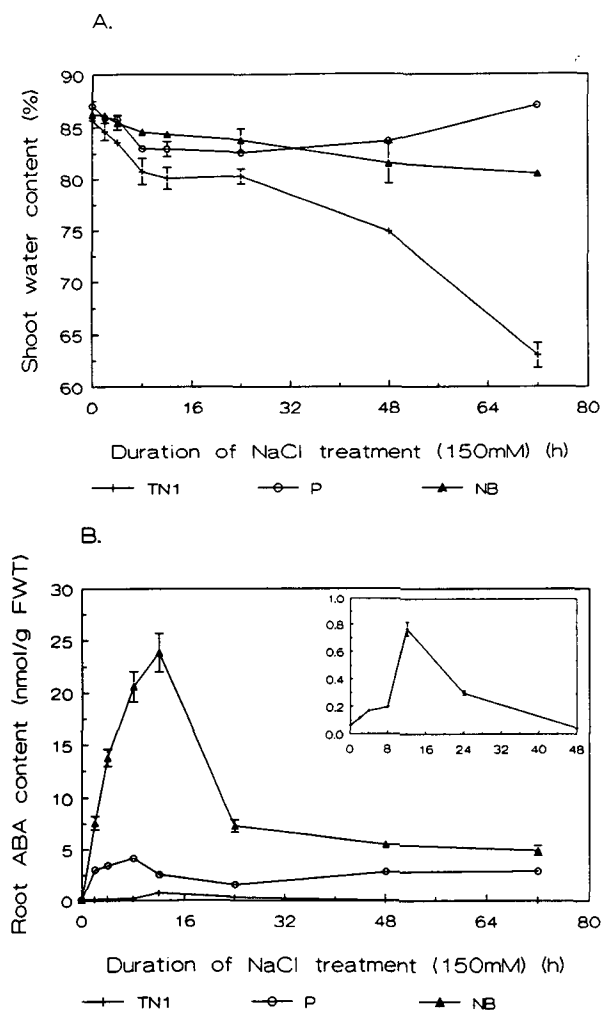
first 12 h after salt stress was imposed, Pokkali seedlings recovered remarkably about 36 h after the onset of the stress, in contrast to Nona Bokra seedlings. Pokkali consistently showed less severe salt-induced wilting after a prolonged stress period (>48 h) for vegetative plants at all stages studied (2, 3, 4, and 6 weeks; data not shown), suggesting that the salt tolerance mechanisms of Pokkali are apparently more efficient.

#### Endogenous ABA Accumulation in Roots of Pokkali, Nona Bokra, and Taichung N1 upon Salt Stress

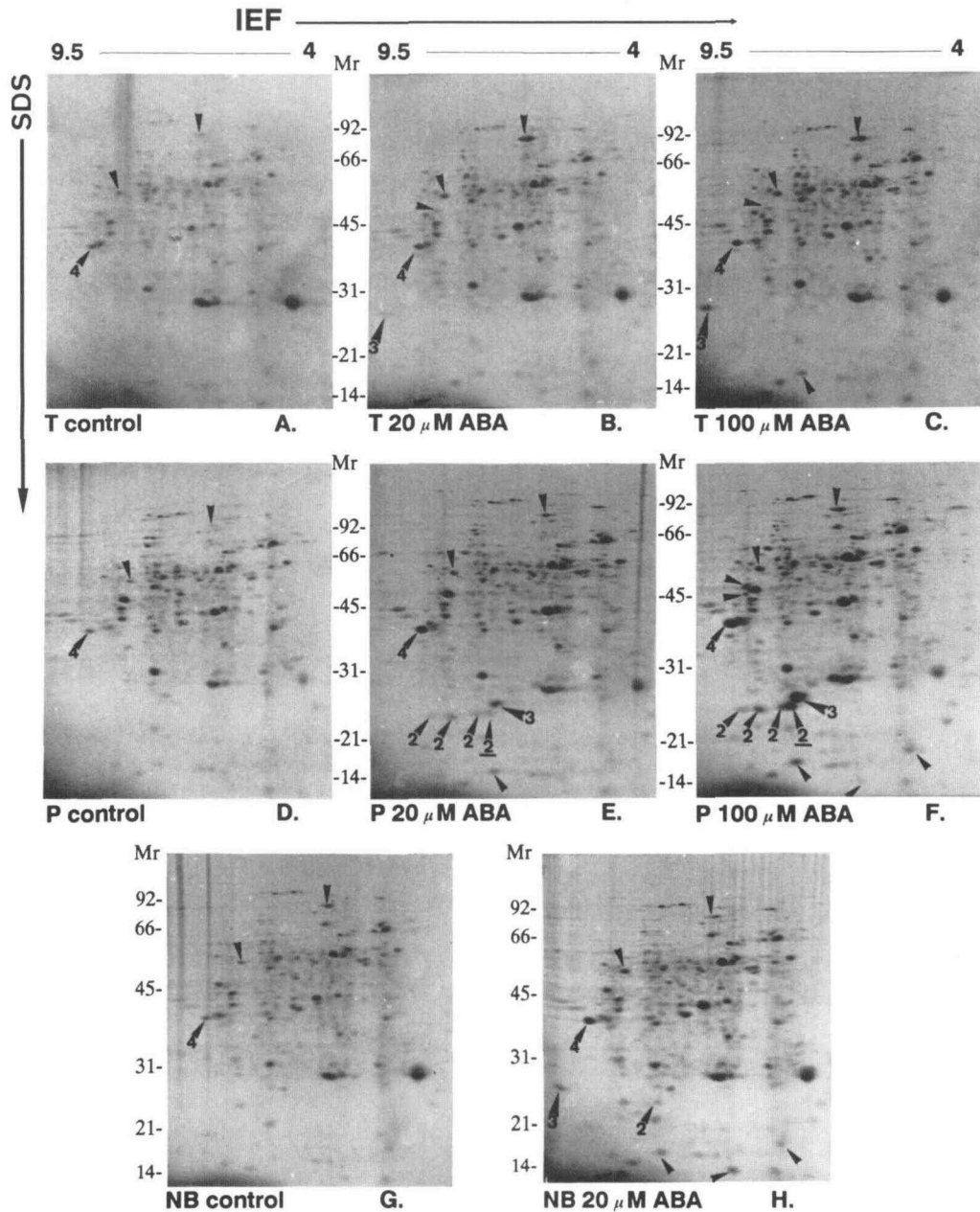
In view of the clear differences in salt tolerance, the first question that arose was whether differences in ABA contents would exist among the three varieties. The rate of ABA increase, as well as the absolute levels of ABA, might be important in establishing an efficient adaptive response. Therefore, time-course measurements of endogenous ABA levels in roots of 10-d-old seedlings were performed after imposition of an osmotic shock (150 mM NaCl) (Fig. 2B). In parallel, the decrease in shoot water content was measured for the same plants. Salt stress was found to induce a transient increase in the ABA content in roots of all three varieties compared with well-watered controls, exhibiting peak levels after 8 to 12 h of stress. However, in the root of salt-stressed Taichung N1 seedlings, only a minor increase in ABA level was found, to a maximal level of  $0.77 \text{ nmol ABA g}^{-1}$  fresh weight at 12 h and returning to control levels after 48 h of stress. Both salt-tolerant varieties exhibited a considerably larger, more rapid, and seemingly also less transient increase in ABA contents, reaching peak levels of  $4.03 \text{ nmol g}^{-1}$  fresh weight for Pokkali and  $23.80 \text{ nmol g}^{-1}$  fresh weight for Nona Bokra. Of the two salt-tolerant varieties, Nona Bokra consistently accumulated the highest levels of ABA.

#### Protein Patterns in Roots of Pokkali, Nona Bokra, and Taichung N1 upon ABA Treatment

ABA-induced changes in total protein populations were compared for sensitive and tolerant genotypes. Changes in gene expression were studied in roots, the organ responsible for water uptake and mineral absorption and in direct contact with the saline environment. Proteins from roots of control seedlings and seedlings grown in the presence of 20 and  $100 \mu\text{M}$  ABA for 3 d were analyzed by two-dimensional gel electrophoresis, using IEF (pI 4–9.5) and 15% SDS-PAGE. Typically, protein spots of 90 kD (pI 7) and 40 kD (pI 8.5) increased and proteins of 26 kD (pI 7.5 or 9) and 24 kD (pI 7.5–8.5) were induced *de novo* in rice roots (Fig. 3). Low molecular mass proteins of 14 to 17 kD (pI 5.5–8.5), including a 14.5-kD protein (pI 5.5) (Claes et al., 1990), accumulated in roots of all three varieties in response to ABA (Fig. 3). Virtually no additional ABA-induced protein spots were apparent when IEF was extended from pH 3 to 10 by nonequilibrium pH gradient gel electrophoresis for seedlings of all varieties (data not shown). The number and



**Figure 2.** A, Time-course analysis of the shoot water content of 10-d-old Taichung N1 (TN1), Pokkali (P), and Nona Bokra (NB) seedlings exposed to osmotic shock (150 mM NaCl). B, Time-course analysis of the ABA levels (in  $\text{nmol g}^{-1}$  fresh weight [FWT]) in roots of seedlings exposed to osmotic shock (150 mM NaCl). Results are the average from two individual measurements. Inset, Root ABA content ( $\text{nmol g}^{-1}$  of fresh weight) of Taichung N1 with an expanded vertical scale.



**Figure 3.** Comparison of the changes in two-dimensional protein patterns in response to exogenously applied ABA in roots of seedlings from Indica rice varieties that differ in salt tolerance. Proteins were isolated from roots of seedlings from the salt-sensitive cv Taichung N1 (T) and the salt-tolerant varieties Pokkali (P) and Nona Bokra (NB) grown on control medium or on medium supplemented with 20 or 100  $\mu\text{M}$  ABA for 3 d. Gels have been stained with Coomassie blue. A, Taichung N1, control medium; B, Taichung N1, 20  $\mu\text{M}$  ABA; C, Taichung N1, 100  $\mu\text{M}$  ABA; D, Pokkali control; E, Pokkali, 20  $\mu\text{M}$  ABA; F, Pokkali, 100  $\mu\text{M}$  ABA; G, Nona Bokra control; H, Nona Bokra, 20  $\mu\text{M}$  ABA.

the extent of accumulation of ABA-responsive proteins was highest in Pokkali seedlings.

**Characterization of Three ABA-Induced Proteins Differentially Accumulating in Roots of Tolerant and Sensitive Varieties**

We further concentrated on three ABA-induced proteins present at different levels in roots of tolerant and sensitive

varieties: a 40-kD protein of pI 8.5, a 26-kD protein of pI 7.5 or 9, and 24-kD proteins of pI 7.5 to 8.5. The 40-kD (pI 8.5) ABA-responsive protein (Fig. 3, No. 4), accumulated in all three varieties proportionally to the applied ABA concentration. The extent of accumulation was higher in both salt-tolerant varieties compared to Taichung N1. Microsequencing was performed for three tryptic peptides from the 40-kD spot (Table I). A cDNA library constructed with mRNA isolated from ABA-treated rice roots was screened

**Table 1.** Partial amino acid sequence analysis of ABA-induced proteins from rice roots (*var Pokkali*)

Internal peptides were generated either by trypsin digestion or by partial acid hydrolysis. The amino acid sequences of some major, well-resolved peptides, isolated by reversed-phase HPLC, were determined. Unidentified positions are indicated with —. Identifications based on similarities with known proteins are given.

Molecular Mass (kD)	pI	No. <sup>a</sup>	Digestion	Peptide Sequence	Identification
40	8.5	4	Trypsin	LVPYNPGYQDESVLWTES LAPFNPEYPDESVLWTES LNFD AFHGDKDHGGVHDGT	Novel His rich
26	7.5	3	Acid hydrolysis	AVM—TLGMTE	Group 3 (type I) LEA
24	7.5	2	Trypsin Trypsin Acid hydrolysis	DQTGGFL TGSVLQQASEQV GMGGI—KKGIKE	Group 2 LEA

<sup>a</sup> Refers to numbers in the figures.

using oligonucleotide probes. The corresponding cDNA clone encoded a hitherto unknown protein rich in His residues (A. Moons, unpublished results).

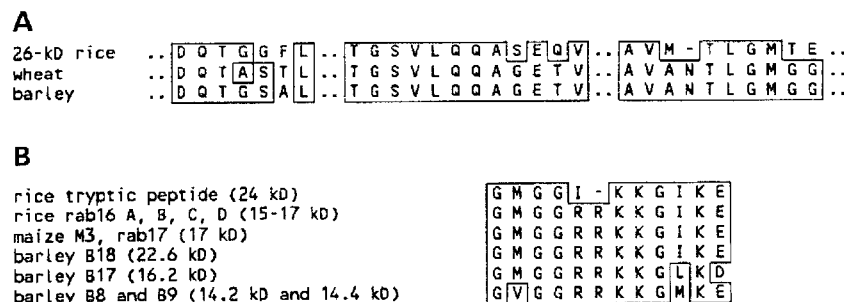
#### Group 3 LEA Proteins

A major spot of 26 kD (pI 7.5; Fig. 3, No. 3) was induced de novo to strikingly high levels in roots of Pokkali seedlings. Peptides were generated both by proteolytic cleavage with trypsin and by chemical degradation (Table I). The three determined peptide sequences had an overall similarity of 72% to the cDNA-deduced group 3 (type I) LEA proteins described for wheat (Curry et al., 1991) and barley (Hong et al., 1988) (Fig. 4A). All generated peptides were located outside the region formed by tandem repeats of the 11-mer, characteristic for this class of proteins. The rice, wheat, and barley group 3 LEA proteins have a similar electrophoretic mobility. In roots of Taichung N1 and Nona Bokra seedlings, no similar 26-kD ABA-induced protein spot of pI 7.5 was observed (Fig. 3).

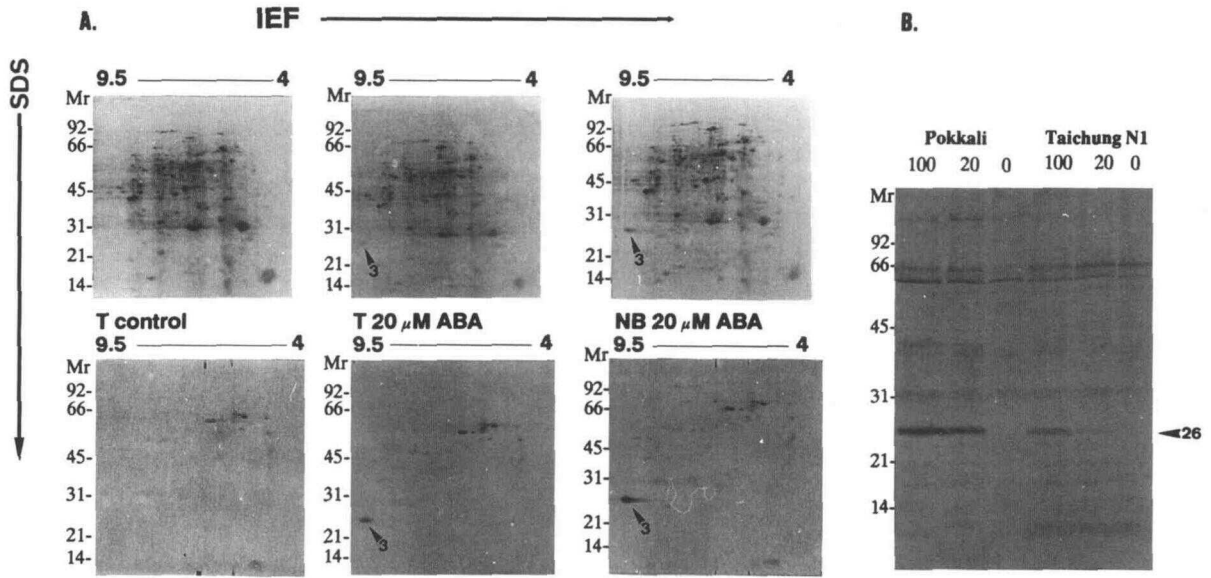
To identify group 3 LEA proteins in roots of the two other rice varieties, western blot analysis was performed on two-dimensional protein patterns using an antibody raised against a group 3 (type I) LEA protein from wheat (Curry

et al., 1991). Two-dimensional protein blots were stained with amido black dye prior to western detection, allowing the precise localization of the cross-reactive proteins. In roots of ABA-treated Taichung N1 and Nona Bokra seedlings, a de novo induced 26-kD protein spot (pI 8.5) was detected (Fig. 5A). Likewise, basic group 3 LEA proteins have been described for barley and wheat (Hong et al., 1988; Curry et al., 1991). In Pokkali roots, the abundant ABA-induced spot of 26 kD (pI 7.5) (Fig. 3, E and F, No. 3), identified before by microsequencing, was recognized by the antibody. In control roots not treated with ABA, no protein spot of 26 kD was detected, but cross-reactivity in the high molecular mass region was observed and is, therefore, thought to be nonspecific. Thus, only for the variety Pokkali did group 3 LEA proteins exhibit a neutral pI.

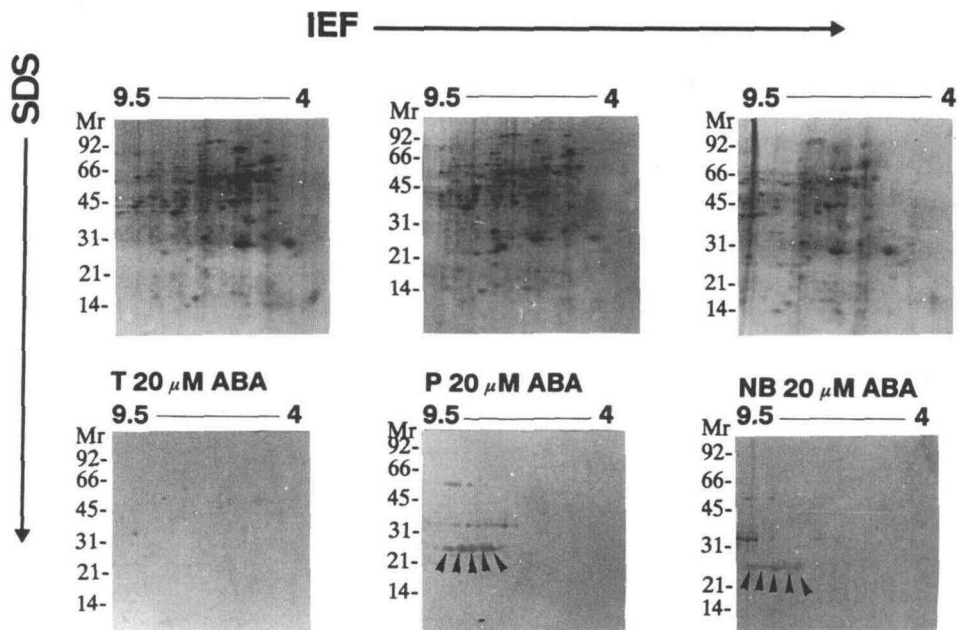
To compare the ABA-induced expression level of group 3 LEA proteins in tolerant and sensitive varieties, western blot analysis was performed on seedling root extracts separated by SDS-PAGE (Fig. 5B). In response to 20 and 100  $\mu$ M ABA applied for 3 d, group 3 LEA proteins accumulated to considerably higher levels in the salt-tolerant variety Pokkali compared with Taichung N1. Moreover, on two-dimensional protein patterns of the three varieties, the



**Figure 4.** A, Alignment of the internal peptides from a 26-kD (pI 7.5) ABA-responsive protein from rice roots (Pokkali) to cDNA-deduced sequences of group 3 LEA proteins from wheat (Curry et al., 1991) and barley (Hong et al., 1988). B, Alignment of the peptide sequence from a 24-kD (pI 7.5) ABA-responsive protein from rice roots (Pokkali) to the central, K-rich region of Rab/dehydrin/group 2 LEA proteins from different monocots: rice rab16 A, B, C, and D (Yamaguchi-Shinozaki et al., 1989; Mundy and Chua, 1988); maize rab17 (Vilardell et al., 1990); maize M3 and barley B18, B17, B8, and B9 (Close et al., 1989).



**Figure 5.** A, Immunodetection of group 3 LEA proteins on two-dimensional blots of root extracts from Taichung N1 (T) and Nona Bokra (NB). Seedlings were incubated either on control medium or on a 20-  $\mu\text{M}$  ABA solution for 3 d. The top panels show the amido black staining; the bottom panels show the immunodetection of the same blot. Western blot analysis was performed using an antiserum raised against a wheat group 3 LEA protein. B, Comparison of ABA-induced group 3 LEA protein levels in roots from Taichung N1 and Pokkali by immunodetection. Seedlings were incubated either on control medium (0), on a 20- $\mu\text{M}$  ABA solution (20), or on a 100- $\mu\text{M}$  ABA solution (100) for 3 d. Equal amounts of protein extracts were loaded in the lanes. Western blot analysis was performed using an antiserum raised against a wheat group 3 LEA protein. Arrowheads show the positions of the identified LEA 3 proteins.



**Figure 6.** Immunodetection of group 2 LEA proteins on two-dimensional blots of root extracts from Taichung N1 (T), Pokkali (P), and Nona Bokra (NB). Seedlings were incubated on 20  $\mu\text{M}$  ABA for 3 d. The top panels show the amido black staining; the bottom panels show the immunodetection of the same blot. Western blot analysis was performed using an antiserum raised against maize dehydrins. Arrowheads show the positions of the identified dehydrins.



identified group 3 LEA proteins (Figs. 3 and 5, No. 3) showed a clearly stronger accumulation in response to 20  $\mu\text{M}$  ABA in both salt-tolerant varieties compared with Taichung N1.

### Dehydrins

In addition, a set of de novo ABA-induced protein spots of 24 kD, ranging in pI from 7.5 to 8.5, was detected in roots of Pokkali (Fig. 3, E and F, No. 2). A peptide sequence from the major 24-kD (pI 7.5) spot (Fig. 3, No. 2) showed 91% similarity to the central, Lys-rich region of group 2 LEA/Rab/dehydrin proteins (Table I; Fig. 4B). This protein seems to differ in pI, electrophoretic mobility, and amino acid sequence from the four gene products of the rice *rab* gene family (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989).

An antiserum raised against maize (*Zea mays*) dehydrins (Close et al., 1989) cross-reacted with the complete set of 24-kD (pI 7.5–8.5) proteins in Pokkali roots (Fig. 6). Sets of ABA-induced protein spots of 35 and 50 kD, not present in control roots, were also detected immunologically (Fig. 6). This indicated that for rice also different molecular mass classes of group 2 LEA proteins exist, as reported for barley (Close et al., 1989), maize, and pea (Robertson and Chandler, 1992). In response to 20  $\mu\text{M}$  ABA, the dehydrins of 24 kD (pI 7.5–8.5) accumulated to strikingly high levels in Pokkali roots, were also clearly induced in Nona Bokra, but were not detectable in the sensitive variety as shown by western blot analysis (Fig. 6). Furthermore, a set of dehydrins of 24 kD (pI 7.5–8) and the high molecular mass dehydrins were demonstrated in roots of Taichung N1 seedlings only when treated with 100  $\mu\text{M}$  ABA (data not shown). Immunodetection allowed the precise localization of the most abundant 24-kD dehydrins on protein patterns of Pokkali and Nona Bokra (Fig. 3, No. 2). Thus, group 2 LEA proteins of 24 kD are more strongly induced by ABA in the two tolerant varieties compared with the sensitive variety.

## DISCUSSION

We were interested in studying the role of ABA in salt tolerance of rice. Therefore, we characterized a model system consisting of the salt-tolerant Indica varieties Pokkali and Nona Bokra and the salt-sensitive cv Taichung N1 to which many Indica cultivars are related. Rice is categorized among the moderately salt-sensitive crops. However, the Indica varieties Pokkali and Nona Bokra are relatively highly salt-tolerant ecotypes. The underlying mechanism conferring their salt tolerance is largely unknown.

Salt tolerance of the three varieties was compared in our experimental conditions using the salt-induced decrease in shoot water content (wilting) or the relative growth inhibition of young seedlings as parameters. Pokkali and Nona Bokra proved to be significantly more tolerant to salinity than Taichung N1 (Fig. 1, A and B, and Fig. 2A). It was also noted that the increase of root to shoot ratio (Sharp and Davies, 1979; Fig. 1C) was the highest for the sensitive variety, suggesting no contribution of this growth response in the varietal differences in tolerance. In later stages of vegetative development, Pokkali consistently appeared to be more efficient than Nona Bokra in establishing an adaptive salt response (Fig. 2A; data not shown), a difference not observed at the seedling stage (Fig. 1, A and B). Salt tolerance is known to vary differently with growth and development for different salt-tolerant varieties (Akbar et al., 1986a).

To examine whether differences in stress-induced ABA concentrations occurred that could be related to varietal differences in tolerance, a time-course analysis of root ABA levels was performed for seedlings exposed to osmotic shock (150 mM NaCl; Fig. 2B). The sensitive cv Taichung N1 showed only a minor, delayed, and transient increase of ABA levels in roots after salt treatment (Fig. 2B, inset). Both tolerant varieties showed a large increase, which was seemingly also more rapid and less transient than for Taichung. Of both salt-tolerant varieties, Nona Bokra showed a greater capacity to accumulate ABA. In parallel, hardly any increase in the endogenous shoot ABA levels was found in 10-d-old Taichung N1 seedlings exposed to osmotic shock (120 mM NaCl) for 8 h, whereas there was a 1.6-fold increase in Pokkali shoots and a 5.8-fold increase in Nona Bokra shoots compared to nonstressed controls (data not shown). These data demonstrate that varietal differences in salt-induced ABA accumulation are consistent in root and shoot, adding to the relevance of their impact on salt-stress responses. Chilling-induced ABA levels also showed a greater and more rapid transient increase in the root of a chilling-tolerant rice cultivar compared with Taichung N1, which is chilling sensitive (Lee et al., 1993).

Because salt-tolerant varieties showed greater accumulation of ABA in response to salt stress compared with Taichung N1, we decided to compare ABA-induced differences in the levels of individual proteins. Application of ABA (20 and 100  $\mu\text{M}$ ) to seedling roots from the three varieties led to de novo synthesis of specific proteins and increased the net synthesis of some other proteins (Fig. 3). Both the number and level of induction of ABA-responsive proteins were the highest for the most tolerant variety

**Table II.** Differences in level of three proteins in three rice varieties following ABA treatment

Protein			Level in Response to ABA <sup>b</sup>		
Molecular Mass (kD)	pI	No. <sup>a</sup>	Pokkali	Nona Bokra	Taichung N1
40	8.5	4	+++	+++	++
26	7.5 or 9	3	+++	++	+
24	7.5–8.5	2	++++	++	+

<sup>a</sup> The numbers refer to those indicated in the figures. <sup>b</sup> +++++, Very high; +++, high; ++, medium; +, low.



Pokkali. Three types of proteins, identified as an ABA-responsive His-rich, 40-kD protein (Fig. 3, No. 4) and group 2 and group 3 LEA proteins (Fig. 3, No. 2 and No. 3, respectively) coordinately accumulated to higher levels in roots from both tolerant varieties compared with Taichung N1 (Tables I and II). In a comparative analysis of gene expression in *Lophopyrum* and wheat, a set of 11 mRNAs was found to be induced to higher levels in roots from the halotolerant wheat relative and the amphiploid both upon osmotic shock and in response to exogenous ABA (Gulick and Dvořák, 1992; Galvez et al., 1993).

LEA genes are suggested to be involved in tolerance to cellular dehydration upon desiccation, salt stress, or cold, although direct evidence is still lacking. Dehydration-induced group 3 LEA protein levels were associated with the desiccation tolerance of wheat tissues (Ried and Walker-Simmons, 1993). In contrast, during cold acclimation a greater level of group 3 LEA expression was found in the less freeze-resistant barley cultivar (Sutton et al., 1992). We found an association of ABA-induced group 3 LEA gene expression and salt tolerance in rice (Table II, No. 2). NaCl also induced these group 3 LEA proteins in rice roots as detected immunologically (data not shown). The observed discrepancy in pI of the Pokkali group 3 LEA protein compared with the basic group 3 LEA proteins of Taichung N1, Nona Bokra (Fig. 3, No. 3), barley, and wheat might be a consequence of differences in primary sequence and/or differences in posttranslational modifications.

The expression of group 2 LEA genes has been associated with cold acclimation (Houde et al., 1992). No differences in dehydrin levels were found in desiccation-tolerant (*O. sativa*) and recalcitrant (*Zizania palustris*) seeds (Bradford and Chandler, 1992). Overproduction of a chimeric group 2 LEA protein of *Craterostigma* in tobacco did not confer osmotic tolerance (Iturriaga et al., 1992). However, upon osmotic shock dehydrin mRNA levels were much higher in roots of *Lophopyrum* than in those of wheat (Galvez et al., 1993). In our model system, ABA-induced dehydrin levels were associated with varietal differences and salt tolerance (Table II).

Salt stress is a complex stress imposing a water deficit, because of the osmotic effects of the salts, and exerting toxic effects, because of the ion excess, thus affecting a variety of metabolic activities (Greenway and Munns, 1980). Salt-tolerance mechanisms of natural ecotypes appear to be complex as well as diverse. Higher endogenous ABA levels and greater levels of ABA-induced proteins were found to be characteristic of the tolerant varieties. Yet, Nona Bokra and Pokkali exhibited mutual differences. Nona Bokra showed a greater capacity to accumulate endogenous ABA upon salt stress and exhibited a less pronounced induction of ABA-responsive proteins. In contrast, the most tolerant variety, Pokkali, showed a greater induction of the same proteins and only a moderate increase in endogenous ABA levels. Most likely quantitative and qualitative aspects of a number of traits are correlated with salt tolerance in rice. Two of these traits, both concerning the role of ABA in response to salt stress, were identified in this study. In summary, varietal evidence

showed that not only the accumulation of ABA upon stress but also the level of induction of ABA-responsive proteins were associated with salt tolerance of rice seedlings.

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