Plant Cell Physiol. 41(11): 1279–1285 (2000)

Molecular Characterization of cDNA Encoding Oxygen Evolving Enhancer Protein 1 Increased by Salt Treatment in the Mangrove *Bruguiera gymnorrhiza*

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Young plants of the common Okinawa mangrove species *Bruguiera gymnorrhiza* were transferred from freshwater to a medium with seawater salt level (500 mM NaCl). Two-dimensional gel electrophoresis revealed in the leaf extract of the plant a 33 kDa protein with pI 5.2, whose quantity increased as a result of NaCl treatment. The N-terminal amino acids sequence of this protein had a significant homology with mature region of oxygen evolving enhancer protein 1 (OEE1) precursor. The cloning of OEE1 precursor cDNA fragment was carried out by means of reverse transcription-PCR (RT-PCR) using degenerated primers. Both 3′- and 5′-regions were isolated by rapid amplification of cDNA ends (RACE) method. The deduced amino acid sequence consisted of 322 amino acids and was 87% identical to that of *Nicotiana tabacum*. In *B. gymnorrhiza*, the predicted amino acid sequence of the mature protein starts at the residue number 85 of the open reading frame. The first 84-amino acid residues correspond to a typical transit sequence for the signal directing OEE1 to its appropriate compartment of chloroplast. The expression of OEE1 was analyzed together with other OEE subunits and D1 protein of photosystem II. The transcript levels of all the three OEEs were enhanced by NaCl treatment, but the significant increase of D1 protein was not observed.

Key words: *Bruguiera gymnorrhiza* — Molecular cloning — Oxygen evolving enhancer protein 1 — Salt tolerance — Two-dimensional gel electrophoresis.

Introduction

Plant growth is severely limited by salt stress. Salt stress brings reduction of water potential, ion imbalance and toxicity. To cope with salt stress, plants respond with physiological and biochemical changes. These changes aim at the following, retention of water in spite of the high external osmoticum and the maintenance of photosynthetic activity while stomatal opening is reduced to counter water loss. Accumulation of low molecular weight compounds such as glycinebetaine, sugar alcohols and proline is a mechanism aimed at balancing water potential following increase in salinity. In addition to synthesis of these osmolytic compounds, specific proteins and translatable mRNA induced and increased by salt stress have been reported (Dure 1981, Singh et al. 1987, King et al. 1988, Claes et al. 1990, Hurkman et al. 1991, Reviron et al. 1992).

Mangroves develop in sheltered tidal zones exposed to varying degrees of salinity ranging from brackish to levels above seawater. Mangrove forest usually forms a unique zonation. *Bruguiera gymnorrhiza* is a characteristic element of the middle mangrove community in zonal distribution, extending into transitional landward communities (Tomlinson 1994). In addition to salinity, Tsukamoto and Nakanishi (1998) suggested that excess Mn in the leaves is one of restrictive factors in the zonation of this mangrove, since Mn content in the leaves showed correlation with electron conductivity of soil water. *Bruguiera gymnorrhiza* also has a specialized root system called “root knee”. Root knees are inundated by seawater during high tide, but fully or partially exposed during low tide. Such root system functions as a highly specialized ventilation mechanism, enabling this mangrove to survive in anaerobic soil.

Mangroves are divided into two distinct groups on the basis of their salt management strategies. One is “secreters” which have salt glands or salt hairs, and the other is “non-secreters” lacking such morphological features for excretion of excess salt. *Bruguiera gymnorrhiza* is included in the latter group. Although Scholander (1968) suggested that non-secreters including *B. gymnorrhiza* excluded 99% of the salt in surrounding seawater by ultrafiltration, Takemura et al. (2000) detected high level of sodium in xylem sap of *B. gymnorrhiza*. They also reported that superoxide dismutase activity increased as a result of an immediate increase of sodium content in the leaves after the plants were transferred from freshwater to high salinity. In this mangrove, it is suggested that cyclitols (Popp 1984) and organic acids such as malic acid and citric acid (Kato et al. 1986) act as compatible solutes to regulate water potential.

The aim of our study is to clarify the molecular mechanisms of salt tolerance in mangrove plants. *Bruguiera gymnorrhiza* is one of suitable materials for this purpose, since this mangrove is a member of non-secreters, which implies that biochemical or physiological mechanisms mainly contribute to

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salt tolerance. We have been approaching the mechanism using two-dimensional gel electrophoresis and differential display to find differentially expressed proteins and transcripts. In addition, we are constructing expressed sequence tag collection of this mangrove. This is our first report on molecular biological study of salt tolerance in *B. gymnorrhiza*. Here we present cloning of cDNA encoding oxygen evolving enhancer protein 1 (OEE1) differentially expressed in two-dimensional gel electrophoresis, and discuss an importance of OEE1 in salt tolerance of this mangrove.

Materials and Methods

Source of plants and growth conditions

Seeds of the viviparous *B. gymnorrhiza* were collected from Iriomote Island, Okinawa, Japan, at the end of May 1998 and mid of September 1999. Five viviparous seeds were planted in each culture pot (15 cm in diameter and 14 cm in height) with vermiculite. The pots were irrigated every 2–5 d with water. Liquid fertilizer was first added after the cotyledons had developed. These pots were placed in a culture room controlled at 25°C with a 12 h photoperiod. The photon irradiance at the top of leaf level was approximately 500 μmol m^{-2} s^{-1}. After 8–10 months growth under these conditions, several pots were supplied with NaCl solution adjusted to 500 mM.

Protein extraction and two-dimensional gel electrophoresis

Second leaves (approximately 5 g FW) from the top in both freshwater controls and salt (500 mM) exposed plants were collected, and immediately frozen in liquid nitrogen. The leaves were then broken and ground to powder by a mortar and pestle. The powder was homogenized in an aqueous extraction buffer containing 0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2 mM PMSF and 3% (v/v) 2-mercaptoethanol. Soluble protein was extracted from the homogenate according to a phenol extraction procedure (Hurkman and Tanaka 1986).

The isoelectric focusing (IEF) gel electrophoresis was done using a gel (Immobiline Dry Strip, pH 4–7, Amersham Pharmacia, Freiburg, Germany), to which the extract containing 100 μg protein was applied. The first gel electrophoresis was carried out at 300 V for 4 h, and then at linear gradient to 3,500 V for 5 h followed by 3,500 V for 20 h. The temperature of the gel was controlled at 15°C. After the first dimensional gel electrophoresis, the gel was removed and equilibrated for 10 min using SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 1% SDS and 0.05% DTT. Second equilibration was done for 10 min using the same buffer but contained 0.9% 2-iodoacetamide instead of DTT. After equilibration, the gel was layered on the top of a SDS-polyacrylamide gel (Excel Gel XL SDS 12–14, Amersham Pharmacia) for the second dimension gel electrophoresis. This electrophoresis was carried out at 1,000 V, 25 mA for 45 min followed by 1,000 V, 40 mA for 5 min, and then for another 2.5 h at 1,000 V, 40 mA. The temperature of the gel was controlled at 15°C. The gel was subsequently silver-stained for detection. The experiments were repeated ten times using leaves from different plants in the conditions of both freshwater and salt treatment.

Electroblotting and amino terminal sequencing

The two dimensional gels were electroblotted to polyvinylidene difluoride (PVDF) membranes (Sequi-Blot PVDF Membrane, Bio-Rad Laboratories, CA, U.S.A.) at 100 V for 30 min, and the membranes were stained with Coomassie Blue R250 (Bio-Rad Laboratories). The protein spots were excised from the membranes (20 membranes per each spot) and destained with methanol. The 20 excised spots were placed into the reaction chamber of the gas-phase protein sequencer (PSSQ-10, Shimadzu Corp., Kyoto, Japan). Homology search was performed using the ADVANCED BLAST program.

Extraction of RNA

Second leaves (approximately 5 g) from the top were ground in liquid nitrogen, and incubated at 65°C for 1 h in 25 ml extraction buffer containing 20% CTAB, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.05% (w/v) spermidine and 4% 2-mercaptoethanol. The suspension was centrifuged at 2,500×*g* for 10 min. An equal volume of chloroform : isoamylalcohol (24 : 1, v/v) was added to the supernatant, and centrifuged at 2,500×*g* for 5 min. RNA was selectively precipitated and purified by overnight LiCl (2M LiCl) precipitation at –20°C, and recovered by centrifugation at 18,000×*g* for 20 min. The RNA sample pellet was resuspended in 500 μl SSTE buffer containing 1 M NaCl, 0.5% (w/v) SDS, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0) and then precipitated with ethanol at –20°C for 2–3 h. RNA was recovered by centrifugation at 18,000×*g* for 30 min, and rinsed with 1 ml of 80% ethanol, gently desiccated. The RNA was suspended in diethyl pyrocarbonate (DEPC)-treated water (approximately 5 μg μl^{-1}).

cDNA cloning

Oligonucleotide primers used in this study are listed in Table 1. Single-strand cDNAs were synthesized from total RNA using Not1-$d(t)_{18}$ primer. To isolate OEE1 precursor cDNA, degenerated sense primers SP1 and SP2 were designed on the basis of N-terminal amino acid sequence. Degenerated antisense primer AP1 was designed on the basis of consensus amino acid sequence (GGRGDEEEL of the position 262–270 in *Arabidopsis thaliana*) of OEE1. When reverse transcription-PCR (RT-PCR) was conducted using primer set SP2-AP1, single amplified cDNA was obtained. To determine the entire nucleotide sequence of OEE1 precursor cDNA, rapid amplification of cDNA ends (RACE) method (Frohman et al. 1986) was applied. For this method, four specific primers, OEE1SP1 and OEE1SP2 for 3′-RACE, OEE1AP1 and OEEAP2 for 5′-RACE, were designed on the basis of nucleotide sequence obtained from RT-PCR product. Single-strand cDNAs for templates of 3′- and 5′-RACE were synthesized using Not1-$d(T)_{18}$ and OEE1AP1, respectively. Double-strand cDNA of the 3′-region was obtained by RT-PCR using primer sets of OEE1SP1-Not1. Primer set of OEE1SP2-Not1 was used for nested-PCR. To clone the 5′-region, 5′-RACE System version 2.0 (Gibco-BRL) was used according to the manufacturer's instruction. The gene specific primer OEE1AP2 was combined with dC-tail-specific primer AUAP.

The conditions for PCR were designated 30 cycles per 30 s at 94°C, 60 s at 55°C and 90 s at 72°C with a final elongation step of 5 min at 72°C for primer sets of SP1-Not1 and SP2-AP1, and 25 cycles per 30 s at 94°C, 60 s at 59°C and 90 s at 72°C with a final elongation step of 5 min for primer sets of OEE1SP1-Not1, OEE1SP2-Not 1, and AUAP-OEE1AP2. The PCR product was directly sequenced using a DNA autosequencer SQ-5500 (Hitachi Co., Ltd., Tokyo, Japan).

The cDNA fragments of OEE2 and OEE3 were obtained from RT-PCR using primer sets of OEE2SP-OEE2AP and OEE3SP-OEE3AP, respectively. These primers were designed on the basis of the conserved regions of known sequences of OEE2 and OEE3. The cDNA fragment of D1 protein was obtained from our expressed sequence tag (EST) collection of *B. gymnorrhiza*. The sequences of OEE2, OEE3 and D1 protein have been deposited in the DDBJ/EMBL database under the accession numbers of AB043961, AB043962 and AB043963, respectively.

Table 1 Oligonucleotide primers used for reverse transcription and PCR

Primer name	Sequence [a] (5′→3′)
Reverse transcription	
Not1-d(T)$_{18}$	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTT
OEE1AP1	GCATAATCAATCCCATCTTTCTCCAC
PCR	
Sense primers	
SP1	GARGGIGTICCIAARMG
SP2	GARGGIGTICCIAARMGIYTIACITAYGA
OEE1SP1	CGTGTGCCATTCCTCTTCACAG
OEE1SP2	AAGGGACGGGGTGGATCAAC
AUAP	GGCCACGCGTCGACTAGTAC
OEE2SP	GGTAAGCCAAAGGAAAACACAG
OEE3SP	CCTTCCGGTGGTGGICTICCIGGIAC
Antisense primers	
Not1	TGGAAGAATTCGCGGCCGCAG
AP1	ARYTCYTCYTCRTCICCICKICCIC
OEE1AP2	AGCCTTGACGGTGAATGAAGTTGG
OEE2AP	TTAAACCATCTCTTGTCACCAGC
OEE3AP	GCCTYTAICCIAGCTTGGCIAGAAC

[a] Ambiguous nucleotides are abbreviated according to the IUB-standard: M, A/C; R, A/G; Y, C/T; I, inosine.

Northern analysis

Total RNA (20 μg) was applied to formaldehyde-agarose gel electrophoresis and blotted to Hybond-XL (Amersham Pharmacia). The PCR product obtained from primer set of SP2-AP1 was used as probe for OEE1. The cDNA fragments obtained from RT-PCR was used as probes for OEE2 and OEE3, and cDNA fragment encoding D1 protein obtained from the EST collection was also used as probe. Probe was labeled with [^{32}P]dCTP using random primed kit (Megaprime DNA Labelling system, Amersham Pharmacia). Membrane was hybridized with the probe at 42°C for 16 h in 0.01% (w/v) salmon sperm DNA, 2.5% SDS, 5× SSPE, 5× Denhard's solution, and 10% dextran sulfate with 50% deionized formamide. Membrane was washed twice with 2× SSC, 0.1% SDS at 42°C for 5 min, and twice more in 0.1× SSC, 0.1% SDS at 60°C for 20 min. The membrane was exposed to Hyperfilm-MP (Amersham Pharmacia) for 15 h.

Results

Two-dimensional gel electrophoresis

Ten-month-old plants grown with water were supplied with 500 mM NaCl solution for 5, 7 and 14 d. Proteins were extracted from leaves harvested from NaCl-untreated and NaCl-treated plants, and separated by two-dimensional gel electrophoresis (Fig. 1). Several protein spots induced and increased by salt treatment were detected. Two proteins of about 17 and 18 kDa with a pI of 6.1 were induced after 7 d of NaCl treatment. The level of a 33 kDa protein with a pI of 5.2 increased by NaCl treatment within 5 d.

The ten residues of amino acid sequence from N-terminus of protein with a 33 kDa (pI 5.2) were EGVPKRLTYD. Homology search revealed that the N-terminal sequence of this protein had a significant homology with oxygen evolving enhancer protein 1 (OEE 1) precursors starting at amino acid 86, 86, 80, and 82 of *A. thaliana*, *Solanum tubersum* (potato), *Triticum aestivum* (wheat) and *Pisum sativa* (pea), respectively. Two polypeptides with 17 and 18 kDa (pI 6.1) could not be sequenced, since they had a blocking group at N-terminus.

Characterization of OEE1 precursor cDNA

To isolate cDNA fragment of OEE1 precursor, RT-PCR was carried out using primer set of SP1-Not1. However, we could not distinguish OEE1 precursor cDNA fragment because of contamination of unspecific products. New primer SP2 that is longer in base number than SP1 was, thus, designed, and combined with AP1. Using this primer set, single product with 550 base pairs (bp) in size was obtained in RT-PCR. The nucleotide sequence of this product had a homology with other known sequences of OEE1, but this approach does not result in isolation of full-length clone. To determine the entire nucleotide sequence of OEE1 precursor cDNA, 3′- and 5′-regions were cloned by RACE method. Both products of 3′- and 5′-RACE were about 350 bp in size, and shared partial nucleotide sequences with that of RT-PCR product using primer set of SP2-AP1.

Nucleotide sequence of the full-length OEE1 precursor cDNA determined from assembling of these sequences is shown, together with the deduced amino acid sequences, in Fig. 2. This sequence has been deposited in the DDBJ/EMBL database under the accession number of AB043960. In the un-

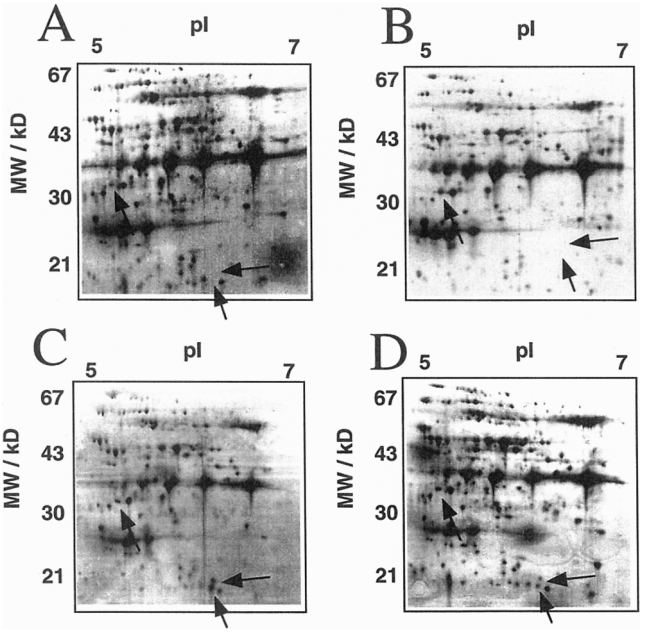

Fig. 1 Effect of NaCl treatment on protein profile separated by two-dimensional gel electrophoresis. Proteins were extracted from leaves grown under water for 10 months (A) and then another for 5 d (B), 7 d (C) and 14 d (D) with NaCl treatment. Proteins that were induced and increased by NaCl treatment are marked by arrows.

broken reading frame of OEE1 precursor protein cDNA the first ATG codon is at nucleotide position 10 and the open reading frame is terminating with a TAA stop codon at position 1,006, thereby coding for 332 amino acids. This sequence contains 77 bp 3′ non-coding region and a poly(A) tail. The 3′-untranslated region includes a putative polyadenylation signal (AATGAA). The N-terminal sequence of the protein obtained from two-dimensional gel electrophoresis can be found at the residue number 85–94, which shows that the first 84-amino acid residues is a chloroplast import leader sequence and the mature protein starts at 85-amino acid residues. The comparison of the deduced amino acid sequence with the available sequences revealed that the OEE1 precursor protein of *B. gymnorrhiza* was 87, 85, 84, 82, and 78% identical to those of *N. tabacum* (tobacco, with accession number CAA45701), *Lycopersicon esculentum* (tomato, CAA78043), *S. tuberosum* (potato, P26320), *P. sativum* (pea, P14226), and *A. thaliana* (P23321), respectively (Fig. 3). The discrepancies are observed in the N-terminal region for a transit peptide to import to chloroplast.

Northern analysis

The gene expression of OEE1 precursor was analyzed together with OEE2, OEE3 and D1 protein using Northern hybridization (Fig. 4). Although the transcripts of three OEEs expressed in untreated plant, NaCl treatment led to an increase in

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                9                  18                  27                  36                  45                  54
5' AAA TTA ACA ATG GCA GCC TCA CTA CAA GCG GCA GCT ACA CTC ATG CAA CCC GCC
       M   A   A   S   L   Q   A   A   A   T   L   M   Q   P   A

               63                  72                  81                  90                  99                 108
   AAG GTG GGT GTG CCT TCT AGG ACT GGC CTT CAG CTG AGG TCT TCT CAG ATT GTT
   K   V   G   V   P   S   R   T   G   L   Q   L   R   S   S   Q   I   V

              117                 126                 135                 144                 153                 162
   TCC AGG GCT TTT GGG TTG GAG CCT GCC GCT GCC AGG ATC ACT TGC TCT TCG CAA
   S   R   A   F   G   L   E   P   A   A   A   R   I   T   C   S   S   Q

              171                 180                 189                 198                 207                 216
   ACT GAC TTT AAG GAC TTG GCC CAA AAG TGT GTA GAT GCT ACT AAG ATT GCT GGT
   T   D   F   K   D   L   A   Q   K   C   V   D   A   T   K   I   A   G

              225                 234                 243                 252                 261                 270
   TTT GCT CTC GCT ACT TCA GCT CTT GTT GTC TCG GGA GCA GGA GCT GAA GGG GTC
   F   A   L   A   T   S   A   L   V   V   S   G   A   G   A   E   G   V

              279                 288                 297                 306                 315                 324
   CCA AAG AGG CTA ACC TAT GAC GAA ATC CAG AGC AAG ACA TAT CTG GAA GTA AAG
   P   K   R   L   T   Y   D   E   I   Q   S   K   T   Y   L   E   V   K

              333                 342                 351                 360                 369                 378
   GGA ACC GGA ACT GCT AAC CAA TGC CCA ACA ATC GAA GGT GGG GTT GAC TCA TTT
   G   T   G   T   A   N   Q   C   P   T   I   E   G   G   V   D   S   F

              387                 396                 405                 414                 423                 432
   GCC TTC AAG CCA GGC AAG TAC AAT GTG AAG AAA TTA TGC CTG GAA CCA ACT TCA
   A   F   K   P   G   K   Y   N   V   K   K   L   C   L   E   P   T   S

              441                 450                 459                 468                 477                 486
   TTC ACC GTC AAG GCT GAA GGT GTA AAC AAG AAT GCC CCA CCC GAG TTC CAG AAC
   F   T   V   K   A   E   G   V   N   K   N   A   P   P   E   F   Q   N

              495                 504                 513                 522                 531                 540
   ACC AAG CTC ATG ACT CGT TTA ACC TAC ACA CTC GAC GAG ATA GAG GGA CCT TTT
   T   K   L   M   T   R   L   T   Y   T   L   D   E   I   E   G   P   F

              549                 558                 567                 576                 585                 594
   GAA GTC TCT CCT GAT GGA ACA GTC AAG TTT GTG GAG AAA GAT GGG ATT GAT TAT
   E   V   S   P   D   G   T   V   K   F   V   E   K   D   G   I   D   Y

              603                 612                 621                 630                 639                 648
   GCT GCG GTC ACC GTG CAG CTG CCT GGT GGC GAA CGT GTG CCA TTC CTC TTC ACA
   A   A   V   T   V   Q   L   P   G   G   E   R   V   P   F   L   F   T

              657                 666                 675                 684                 693                 702
   GTC AAG CAG CTA GTA GCA ACA GGC AAG CCA GAA AGC TTT AGC GGA GAG TAT CTG
   V   K   Q   L   V   A   T   G   K   P   E   S   F   S   G   E   Y   L

              711                 720                 729                 738                 747                 756
   GTG CCA TCC TAC CGT GGA TCA TCC TTC TTG GAC CCC AAG GGA CGG GGT GGA TCA
   V   P   S   Y   R   G   S   S   F   L   D   P   K   G   R   G   G   S

              765                 774                 783                 792                 801                 810
   ACC GGT TAT GAC AAT GCG GTG GCA CTG CCT GCG GGA GGC AGA GGA GAT GAG GAG
   T   G   Y   D   N   A   V   A   L   P   A   G   G   R   G   D   E   E

              819                 828                 837                 846                 855                 864
   GAG CTC ACC AAG GAG AAC ATC AAG AAC ACA TCA TCG TCA ACT GGG AAA ATC ACC
   E   L   T   K   E   N   I   K   N   T   S   S   S   T   G   K   I   T

              873                 882                 891                 900                 909                 918
   CTG GGT GTC ACC AAG AGC AAG CCT GAG ACC GGA GAG GTT ATT GGA GTG TTC GAG
   L   G   V   T   K   S   K   P   E   T   G   E   V   I   G   V   F   E

              927                 936                 945                 954                 963                 972
   AGC CTG CAA CCA TCT GAC ACT GAT TTG GGA GCA AAG GTC CCT AAG GAT GTC AAG
   S   L   Q   P   S   D   T   D   L   G   A   K   V   P   K   D   V   K

              981                 990                 999                1008                1017                1026
   ATC CAG GGC ATC TGG TAT GCC CAG CTT GAC TCA TAA ATC TCT CTT GTA CGA ATG
   I   Q   G   I   W   Y   A   Q   L   D   S   *

             1035                1044                1053                1062                1071                1080
   AAC TTG TAT TTA TTC TTA CTT CAG GTT TTA CTT AAT TAT AAG TTC AAG ATG TTG

             1089                1098
   CCC AAA AAA AAA AAA AAA AAA A 3'
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Fig. 2 Nucleotide sequence and deduced amino acid sequence of OEE1 precursor cDNA. The stop codon is marked with an asterisk. Underline indicates the identical residues obtained from N-terminal amino acid sequence. A putative polyadenylation signal is double underlined.

the level of transcripts corresponding to OEEs. The level of OEE1 transcript increased within 3 d after NaCl treatment, and the elevated level was maintained during 14 d of treatment. The transcript of OEE2 also began to increase within 3 d and reached maximum level in 7 d. The transcript of OEE3 at 3 d after the treatment was almost the same level as that of non-treated plant, but the level increased in 5 d and was maintained

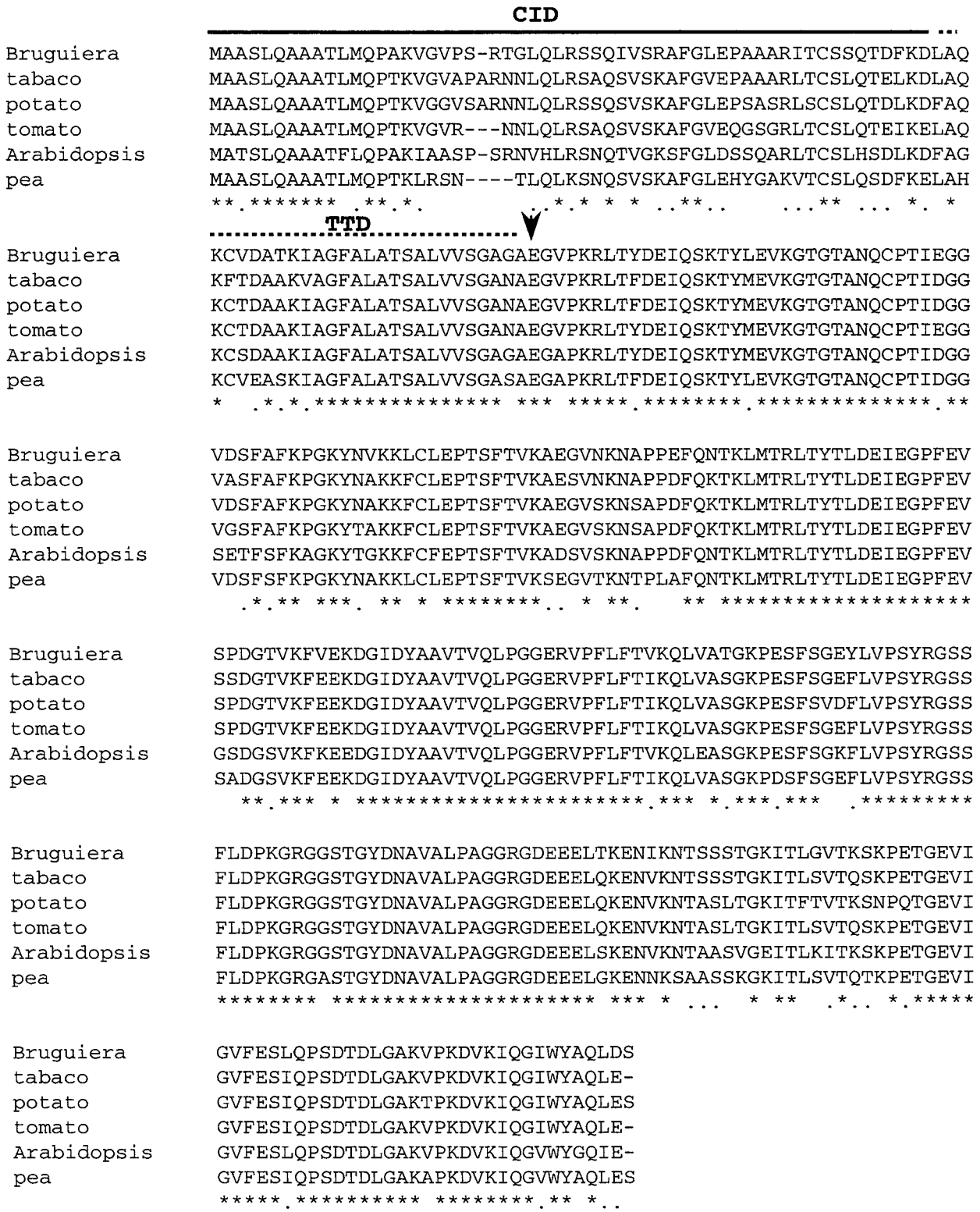

Fig. 3 Comparison of the amino acid sequences of OEE1 precursor from *Bruguiera gymnorrhiza* with those of known proteins. Asterisks indicate amino acids that are conserved among the different sequences and chemically similar residues are denoted by dots on the bottom line. Solid and broken lines show chloroplast import domain (CID) and thylakoid transfer domain (TTD) on the top, respectively. Mature region starts at the arrowhead position.

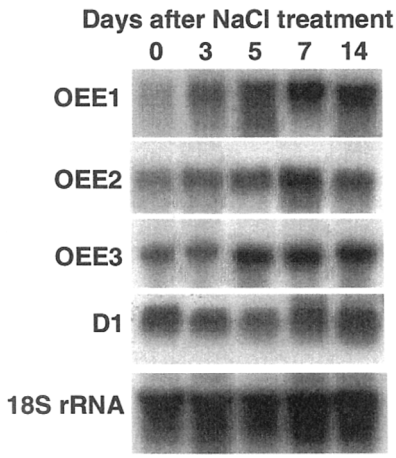

Fig. 4 Expression of the transcripts of OEE1, OEE2, OEE3 and D1 protein. Total RNA was prepared from leaves grown under freshwater for 8 months and then another for 3, 5, 7 and 14 d with NaCl treatment. The same blots were reproved with 18S rDNA.

up to 14 d. In contrast to these results, the level of transcript corresponding to D1 protein was not affected by NaCl treatment.

Discussion

We detected several proteins increased or induced by NaCl treatment in *B. gymnorrhiza*. The 33 kDa protein with pI 5.2 was identified with oxygen evolving enhancer protein 1 (OEE1). Oxygen evolving enhancer proteins (OEEs) consist of three subunits, OEE 1 (33 kDa), OEE 2 (23 kDa) and OEE 3 (16 kDa). These are nuclear-encoded chloroplast proteins, and peripherally bound to photosystem II (PSII) on the lumenal side of the thylakoid membrane. The transit peptide which is the signal directing OEEs to their appropriate compartment of chloroplast resides in the N-terminal region. The transit peptide region is composed of two domains, one with a chloroplast import domain (CID) and the other with a thylakoid transfer domain (TTD). OEEs synthesized in the cytosol translocate across the chloroplast by CID, and intermediate OEEs are yielded. These intermediate proteins subsequently translocate across the thylakoid membrane by TTD (Robinson and Klosgen 1994), resulting in mature portion of OEEs are located on lumenal side of PSII.

Northern analysis showed that the transcript of OEE1 increased in amount by NaCl treatment. This result supports that the level of the protein increased after NaCl treatment in two-dimensional gel electrophoresis. The transcript levels of OEE2 and OEE3 also increased by NaCl treatment. OEE1 is bound to the chlorophyll-*a*-binding protein CP47 and a small unidentified intrinsic subunit of PSII (Seidler 1996). OEE1 provides the binding site for OEE2 (Kavelaki and Ghanotakis 1991), and OEE3 is bound to PSII via OEE2 (De Vitry et al. 1989). Although removal of OEE2 and OEE3 led to lose oxygen evolution, the function of these proteins could be replaced by 5 mM Ca^{2+} and 30 mM Cl^{-} (Miyao and Murata 1984). However, removal of OEE1 together with OEE2 and OEE3 caused significant loss of oxygen evolving activity and unstablility of Mn cluster. The function of OEE1 could not be replaced by Ca^{2+} and Cl^{-}. Rebinding OEE1 to PSII restored activity of oxygen evolution and stability of the Mn cluster. These results indicate that OEE1 is the most important protein for oxygen evolution and PSII stability.

Takemura et al. (2000) showed that gross photosynthesis of *B. gymnorrhiza* was decreased in response to increase salinities. The depression was attributable to restriction to CO_2 access due to reduction of transpiration. Kawamitsu et al. (1995) also reported the decrease of photosynthesis and leaf conductance under elevated salinities in *B. gymnorrhiza*. The relationship between the rate of photosynthesis and leaf conductance was linear proportional, and no change of the slope was observed in any salinity. This result implies that the depression of photosynthesis in *B. gymnorrhiza* results from not depression of photosynthetic capacity but reduction of stomatal conductance. It is possible that the level of OEEs concomitantly increase as the result of increasing the number of PSII center. However, the number of PSII center seems not to increase, since the transcript level of D1 protein did not change after NaCl treatment. Two other possibilities on the increase of these levels can be considered. The increases of their levels are due to making up the protein levels what these proteins lack in dissociation, or what the proteins are damaged by NaCl. It is known that OEE2 and OEE3 can be easily removed from PSII complex in the presence of NaCl. Sodium concentration in leaves of *B. gymnorrhiza* increased and reached steady state value in several days after NaCl treatment (Takemura et al. 2000). The enhancement of transcript levels of OEE2 and OEE3 might be due to an acceleration of association with PSII complex to overcome the dissociation. In *Erythrina variegata* seedling, salt stress had a detrimental effect on PSII (Muthuchelian et al. 1996). This impairment was caused by damage of OEEs. Also in *B. gymnorrhiza*, prompt turnover of OEEs might be required as the result of NaCl damaged the OEEs.

Murota et al. (1994) pointed out the importance of OEE2 in salt adaptation process in photoautotrophically cultured green tobacco cells. According to them, the dissociation of the OEE2 from thylakoid membranes in NaCl-adapted cells was not observed, but observed in unadapted cells. They suggested that the stronger association of OEE2 in adapted cells be due to changes in the protein or changes in other proteins interacted with OEE2. Our data suggest that not only the OEE2 but also the OEE1 and OEE3 play an important role in maintenance of PSII activity under NaCl stress condition. In particular, OEE1 is an essential for oxygen evolving activity and PSII stability. The expression of OEE1 is also considered to be the rate-limiting step in the assembly of PSII subunit (Mizobuchi and

Yamamoto 1989). Therefore, it is considered that the recovery or turnover of OEE1 is one of the mechanisms to maintain the capacity of PSII under NaCl treatment.

The increase of transcript level of D1 protein was not observed, in spite of those of OEEs were increased. This shows that the expression of nuclear-encoded OEEs is not coupled to the expression of the chloroplast-encoded D1 protein. It is not necessary to increase the number of PSII center including D1 protein because of depression of photosynthesis under salt stress condition. This study suggested that *B. gymnorrhiza* copes with NaCl stress by means of not increase of the number of PSII complex but maintenance of PSII complex.

Acknowledgements

We thank Dr. Yukiko Iwadate, Ms. Maina Yoshida and Minako Kaga, Mrs. Hitoshi Ito, Kentaro Saito, Tomohiro Togo and Kazuyuki Seki for their technical assistance. An Endowed Chair in Environmental Biotechnology funded by EBARA Corporation, Japan supported this work.

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(Received March 31, 2000; Accepted September 4, 2000)