Multiple Mode Regulation of a Cysteine Proteinase Gene Expression in Rice[1]

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In many plants, cysteine proteinases play essential roles in a variety of developmental and physiological processes. In rice (*Oryza sativa*), REP-1 is a primary cysteine proteinase responsible for the digestion of seed storage proteins to provide nutrients to support the growth of young seedlings. In the present study, the gene encoding REP-1 was isolated, characterized, and designated as *OsEP3A*. An *OsEP3A*-specific DNA probe was used to study the effect of various factors on the expression of *OsEP3A* in germinating seeds and vegetative tissues of rice. The expression of *OsEP3A* is hormonally regulated in germinating seeds, spatially and temporally regulated in vegetative tissues, and nitrogen-regulated in suspension-cultured cells. The *OsEP3A* promoter was linked to the coding sequence of the reporter gene, *gusA*, which encodes β-glucuronidase (GUS), and the chimeric gene was introduced into the rice genome. The *OsEP3A* promoter is sufficient to confer nitrogen regulation of GUS expression in suspension-cultured cells. Histochemical studies also indicate that the *OsEP3A* promoter is sufficient to confer the hormonal regulation of GUS expression in germinating seeds. These studies demonstrate that in rice the REP-1 protease encoded by *OsEP3A* may play a role in various physiological responses and processes, and that multiple mechanisms regulate the expression of *OsEP3A*.

Protein degradation is an essential component in plant growth, development, and environmental responses. Selective proteolysis provides a mechanism for protein turnover and reutilization of nitrogen for maintaining cellular homeostasis and growth. Although plants can synthesize all amino acids de novo, a substantial portion of new proteins is derived from recycled amino acids (Huffaker and Peterson, 1974; Vierstra, 1993). Amino acids can be generated from the degradation of proteins or they can be derived from specialized storage proteins in seeds or vegetative tissues (Staswick, 1994). Under nutrient stress conditions, protein degradation is accelerated to maintain the supply of amino acids (Davies, 1982; Dice, 1987). Proteolysis in plants is a complex process involving many enzymes. The Cys proteinase (CysP) enzymes, with a Cys residue at the active center, are extensively studied because they appear to play a central role in a wide range of proteolytic functions in higher plants.

CysP are recognized as the major enzymes for the catabolism of the majority of reserve proteins in seeds (Shutov and Vaintraub, 1987; Ryan and Walker-Simmons, 1991). In cereal grains, nutrients are stored in the endosperm in the form of starch and storage proteins. Early in seed germination, the embryo synthesizes gibberellins (GAs), which diffuse to the aleurone cells surrounding the starchy endosperm and act as signals to activate the synthesis and secretion of several groups of hydrolytic enzymes, including α-amylases and proteases (Fincher, 1989; Ho and Hagen, 1993). Sugars, amino acids, and small peptides resulting from the hydrolysis of endosperm starch and proteins are absorbed by the embryo to support the growth of young seedlings. In barley seeds, CysPs are the primary enzymes responsible for the hydrolysis of the major storage proteins, hordeins, to small peptides (Rostogi and Oaks, 1986).

Two barley CysPs, EPA and EPB, have been purified and shown to be capable of hydrolyzing hordeins in vitro (Koehler and Ho, 1988, 1990a; Zhang and Jones, 1996). The rice (*Oryza sativa*) REP-1 has also been purified and characterized as a major protease for the digestion of the main seed storage protein, glutelin (Kato and Minamikawa, 1996). The expression of barley and rice CysP genes in aleurone layers is induced by gibberellic acid (GA₃) and suppressed by abscisic acid (ABA) (Koehler and Ho, 1990b; Shintani et al., 1997). Recently, the promoters of two barley *EPB* genes were characterized and shown to confer GA induction and ABA suppression of a reporter gene expression in barley aleurone cells using transient gene expression assays (Mikkonen et al., 1996).

CysPs have also been postulated to play essential roles in many developmental and physiological processes, such as senescence and programmed cell death. mRNA levels of two CysP-related genes, *SAG2* and *SAG12*, are significantly higher in degreening tissues than in green tissues during senescence of Arabidopsis leaves, and was proposed to be involved in the progression of senescence in somatic tissues (Hensel et al., 1993; Lohman et al., 1994). Two CysP cDNAs, *tpp* and *TPE4A*, were isolated from senescent pea ovaries. Expression of *tpp* significantly increases 2 to 3 DPA in unpollinated pea ovaries and was proposed to be involved in the senescence of the unpollinated ovaries

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(Granell et al., 1992). Expression of *TPE4A* is induced during the senescence of unpollinated pea ovaries and the germination of pea seeds, indicating that the same CysP may be involved in remobilization of stored nutrients in these two different physiological processes (Cercós et al., 1999). The mRNA level of a CysP gene significantly increases during in vitro tracheary element differentiation in isolated mesophyll cells of zinnia, and CysP was suggested to be involved in autolysis of cytoplasm during xylogenesis (Ye and Varner, 1996). Recently, the role of CysP in modulating programmed cell death triggered by an avirulent strain of pathogen or by oxidative stress was demonstrated by ectopic expression of a CysP inhibitor gene in cultured soybean cells (Solomon et al., 1999).

Degradation of proteins is accelerated when the tissue becomes nitrogen-limited or senescent or when the stored amino acids are needed by sink tissues, suggesting that plants have signaling pathway(s) linking the supply of free amino acids or nitrogen to the rate of intracellular proteolysis (Staswick, 1994). An interesting question is thus raised regarding how the degradation of proteins is regulated. In the present study, the control mechanism of protease gene expression in plants was explored. As a first step, we investigated various factors that regulate the expression of a CysP in rice. We found that expression of a CysP gene, *OsEP3A*, in rice is hormonally regulated in germinating seeds, spatially and temporally regulated in vegetative tissues, and nitrogen regulated in suspension-cultured cells. We also found that the promoter of *OsEP3A* confers GA$_3$ induction of reporter gene expression in transformed germinating rice seeds. The same promoter also confers nitrogen-deprivation-induced expression of reporter gene expression in transformed rice suspension-cultured cells. To our knowledge, this is the first report of multiple modes of regulation of a single proteinase gene expression in plants.

MATERIALS AND METHODS

Plant Material

The rice variety used in this study was *Oryza sativa* L. cv Tainung 67. Immature seeds were dehulled, sterilized with 2.4% NaOCl for 1 h, washed extensively with sterile water, and placed on N6D agar medium (Toki, 1997) for callus induction. After 1 month, callus derived from scutella was subcultured in fresh N6D medium for transformation or to a liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% Suc and 10 μM 2,4-dichlorophenoxyacetic acid to establish a suspension cell culture as previously described (Yu et al., 1991).

Screening of Rice Genomic Library and DNA Sequence Analysis

Approximately 2×10^6 recombinant λ phage clones derived from a rice (cv Japonica) genomic DNA library (CLONTECH Laboratories, Palo Alto, CA) were screened. Plaques were lifted on nylon transfer membranes (MSI, Westborough, MA) and probed with ^{32}P random-primer-labeled cDNA of EP-B, pHVEP4 (Koehler and Ho, 1990b). One positive clone containing a 2.6-kb insert was selected and subcloned into the *Eco*RI site of pBluescript KS+ (Stratagene, La Jolla, CA) and designated as pOsEP3A. The complete nucleotide sequence of the rice Cys endoprotease gene *OsEP3A* was determined from both orientations with an automated laser fluorescent DNA sequencer (Pharmacia, Piscataway, NJ) and by the manual dideoxy chain-termination method (Sequenase version 2, United States Biochemical, Cleveland). DNA sequence analysis was performed using the University of Wisconsin Genetics Computer Group software package, version 9.1.

Plasmid Constructions

Plasmid pRY18 carries a 3.8-kb DNA fragment that contains a rice genomic rDNA cluster, including the 3′ half portion of the 17S rRNA gene, the complete 5.8 S rRNA gene, and the 5′ half portion of the 25S rRNA gene in pUC13 (Sano and Sano, 1990). A 1.1-kb DNA fragment containing the 5′ flanking region and the first 28 amino acids of *OsEP3A* was PCR-amplified using the T7 primer (Stratagene) and the primer 5′-GATATCTGCAG<u>GGTAT CGCGGCGCACAG</u>-3′ (sequence complementary to positions +195 to +211 of *OsEP3A* underlined). This DNA fragment was cleaved with *Pst*I at both ends and cloned into the *Pst*I site of pBX-2 in such a way as to allow an in-frame fusion with the *gusA* (Jefferson, 1987) coding region. pBX-2, a pBluescript KSII+ (Stratagene)-derived plasmid, containing the *gusA* gene and a nopaline synthase gene (*Nos*) polyadenylation site between the *Bam*HI and *Xba*I sites was kindly provided by Dr. Ray Wu (Cornell University, Ithaca). A DNA fragment containing the cauliflower mosaic virus 35S RNA gene (35S) promoter-hygromycin phosphotransferase coding region (Hph)-tumor morphology large gene terminator (Tml 3′) was excised from pTRA151 (Zheng et al., 1991) with *Pst*I and *Eco*RI and inserted into the same sites in pPZP200 (Hajduk-iewicz et al., 1994) to generate pPZP200-H. The *virG* gene of *Agrobacterium tumefaciens* pTiBo542 (Chen et al., 1991) was PCR-amplified with blunt ends and inserted into the *Sca*I site of pPZP200-H to generate pSMY1H. The *OsEP3A* promoter-*gusA*-*Nos*3′ chimeric gene was excised from pBX-2 with *Hin*dIII and inserted into the *Hin*dIII site of pSMY1H to generate pEPGS1.

Primer Extension Analysis

5′-Primer extension analysis was performed according to the method of Sambrook et al. (1989). Total RNA was isolated from germinating embryos 3 d after germination. Poly(A$^+$) RNA was purified from the total RNA and hybridized with ^{32}P-labeled primer P2 (5′-ATCGATCGATC GCCACT-3′) (Fig. 1). The polymerization reaction was conducted with reverse transcriptase (RT) (SuperScript, GIBCO-BRL, Cleveland). Dideoxynucleotide sequencing of *OsEP3A* was also performed using the P2 primer and served as the sequence reference. The extension product and the sequence references were electrophoresed and visualized by autoradiography.

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 ~851                                                gaattccgtg
                                                    EcoRI
 -840  ggtagcggtgcgtgaaattggagcagcctgaagccctgaaccgtgaaaaactgggctgtg
 -780  agactcagagcgcgaaccaaatctgtcatggagaagatactccctccgtactgtaaaga
 -720  aaatcgtttagaacaatgtttaagtcaaaccttgagaatataaatcatgaataactatca
 -660  agttgttgagtttttaaaatataaaaattatatgaatagatttgtcttgaaaaataacata
 -600  catatatoacttttttaatgaatattttttataaaaataagaagttaaagttgtgtttaga
 -540  gaccgtgtcgctgtcctaaataagttactttacgagtatggagggggtaatgagctagcc
 -480  gatcaaccgacgtatcaacaacgcggtggtctctgcggctctgctcatcgcaaccaattc
 -420  ctacacgccgcgcaggcagccctcggcctccacgaccgccattaataccgtcctagccaa
 -360  gctacgatcaatcacagagcaaagcagccattcctcacgtcacgtagccgaccaccag
 -300  tgataaacttcccagcaaccagtactagcatccataaaattgcacacctaagctagcta
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 -120  actccctagctgaatctgacaccatcgcacgcatctttctgttaccccccatttaaatccg
  -60  tctcgtttcccgccgcaaatcccagctataaatacgtctcctccccttctccttcctccto
    1  ATGCGCCTCACCCTCCAATTCCAATCCCTCCCAAGAACTAGCTGATCACGACGAGCAGCT
          P1
   61  AGTCCACTGCTACTGCGATCGATCGGTTGCAAAGTTAATTAATTAGTGGCCGATCGATCGA
                                                     P2
  121  TCATGGGGAGGGTTATTAGCAGCTGGAGGGTTCTTGCGGTGGTGGCTGCTTTGATGGCCA
          M  G  R  V  I  S  S  W  R  V  L  A  V  V  A  A  L  M  A  M
  181  TGGCGGCGGTGGAGCTGTGCGCCGCGATACCGTTCGACGAGAGGGATTGGAGTCCGACG
          A  A  V  E  L  C  A  A  I  P  F  D  E  R  D  L  E  S  D  E
  241  AGGCGCTGTGGGATCTGTACGAGCGGTGGCAGGAGCACCACCACGTGCCGGCGCCACCACG
          A  L  W  D  L  Y  E  R  W  Q  E  H  H  H  V  P  R  H  H  G
  301  GCGAGAAGCACCGCCGGTTCGGCGCGTTCAAGGACAACGTGAGGTACATCCACGAGCACA
          E  K  H  R  R  F  G  A  F  K  D  N  V  R  Y  I  H  E  H  N
  361  ACAAGCGCGCCGCGGGCTACCCTCCGCTCAACCGCTTCGGCGACATGGGCAGAGAGGAGT
          K  R  A  P  G  Y  P  P  L  N  R  F  G  D  M  G  R  E  E  F
  421  TCCGGCGCCACGTTCGCCGGGTCCCACGCCCAACGACCTCCGCCGCGACGGCCTCGCCGCG
          R  A  T  F  A  G  S  H  A  N  D  L  R  R  D  G  L  A  A  P
  481  CGCCGGCTCCCGGGGTTCATCTACGAGGGCGTCCGCGACCTCCCCCGCGCCGTCGACTGGC
          P  L  P  G  F  M  Y  E  G  V  R  D  L  P  R  A  V  D  W  R
  541  GCCGCAAGGGCGCGGTCACCGGCGTCAAGGACCAGGGCAAGTCCGGCAGCTGCTGGGCGT
          R  K  G  A  V  T  G  V  K  D  Q  G  K  C  G  S  C  W  A  F
  601  TCTCCACGGTGGTGTCCGTGGAGGGCATCAACGCGATCCGGACGGGGCGGCTGGTGTCGC
          S  T  V  V  S  V  E  G  I  N  A  I  R  T  G  R  L  V  S  L
  661  TGTCGGAGCAGGAGCTGATCGACTGCGACACGGCCGACAACAGCGGCTGCCAGGGCGGGC
          S  E  Q  E  L  I  D  C  D  T  A  D  N  S  G  C  Q  G  G  L
  721  TCATGGAGAACGCCGTTCGAGTACATCAAGCACAGCGGCGGCATCACCACCGAGTCCGCCT
          M  E  N  A  F  E  Y  I  K  H  S  G  G  I  T  T  E  S  A  Y
  781  ACCCGTACCGGCCGCCGCAACGGAACGTGCCGACGCCGTCCGCGCCGCGGCGGGCTGGTGG
          P  Y  R  A  A  N  G  T  C  D  A  V  R  A  R  G  G  L  V  V
  841  TGATCGACGGGCACCAGAACGTGCCGGCCAACAGCGAGGCCGCGCTCGCCAAGGCCGTCG
          I  D  G  H  Q  N  V  P  A  N  S  E  A  A  L  A  K  A  V  A
  901  CCAACCAGCCCGTCTCCGTCGCCATCGACGCCGGCGACCAGTCCTTCCAGTTCTACTCCG
          N  Q  P  V  S  V  A  I  D  A  G  D  Q  S  F  Q  F  Y  S  D
  961  ACGGCGTCTTCGCCGGCGACTGCGGCACCGACCTCGACCACGGCGTCGCGGTGGTCGGCT
          G  V  F  A  G  D  C  G  T  D  L  D  H  G  V  A  V  V  G  Y
 1021  ACGGCGAGACCAACGACGGCACGGAGTACTGGATCGTCAAGAACTCGTGGGGCACAGCCT
          G  E  T  N  D  G  T  E  Y  W  I  V  K  N  S  W  G  T  A  W
 1081  GGGGCGAGGGCGGCTACATCCGGATGCAGCGCGACTCCGGCTACGACGGCGGCCTCTGCG
          G  E  G  G  Y  I  R  M  Q  R  D  S  G  Y  D  G  G  L  C  G
 1141  GCATCGCCATGGAAGCCTCCTACCCCGTCAAGTTCTCGCCCAAATCGTGTCACGCCAAGGC
          I  A  M  E  A  S  Y  P  V  K  F  S  P  N  R  V  T  P  R  R
 1201  GGCGCCCTTGGGCCAAAGGAAACCCAGTGATCGAGCCGGCGCCATCTCCGCCGTACACGTG
          A  L  G  P  K  E  T  Q  *          P3
 1261  CCCGCCTACGAGCCTGGATCAACGGCTCTGGTCGCGTCTCCTCCTCCAGCTTGCAAAATG
 1321  GTGCTATTATTGTTGCTGCTCTTTGTTAATTGCCTTGTGTTTATGTAATTCGTGTGTGAGGC
 1381  GATCAGGGAAAAGAATTACCTGAAATCGTCTAAGTTACTAGTGTTATTTCGTGTGTGCTA
 1441  AATAAGTTTTAGATGTGAGGTGAGGTCGATCTATCATGTAATGTACTGAACTTATATGTG
 1501  GTGTTCTTCGTGGCACCGGGAAATATATATAAGAAGGTTGTTGTTATGACTaaggttatg
 1561  ccaaatttcatccaaaaaagactaaggttatgccaaatttcatccaaaaaaaagttatct
 1621  caattgcttcagtttttagcttgtctcgctgggcgttcctatgacttatgtgagttacga
 1681  ctcgtgggattcgcaaccaaaatcgaagacgagagctttgcaaccggagaggaattc
                                                         EcoRI
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Figure 1. Nucleotide and deduced amino acid sequences of *OsEP3A*. The 5′- and 3′-flanking regions are shown in lowercase letters. The coding region is shown in uppercase letters. Nucleotide +1 is the mapped transcription start site. The predicted signal peptide sequence is underlined. The putative TATA box and polyadenylation signals are double-underlined. The putative GARE is labeled with bold underline. The positions of PCR primers used for examination of intron (P1 and P3) and for synthesis of *OsEP3A*-specific probe (P1 and P2) are labeled with arrows. The nucleotide sequence of *OsEP3A* is in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number AF099203.

PCR

DNA PCR of genomic clone pOsEP3A was performed using oligonucleotides P1 (5′-ATCGCCCTCACCCTCCA-3′, positions +1 to +17) and P3 (5′-TGTACGGCGGAGAT GGC-3′, positions +1240 to to +1256) (Fig. 1) as primers. The PCR product, P_{1-3}, containing the coding region of *OsEP3A* was later used as a probe in genomic DNA Southern-blot analysis. Seed mRNA RT-PCR was performed using poly(A$^+$) RNA isolated from germinating embryos as a template and oligonucleotides P1 and P3 as primers according to methods previously described (Chan and Yu, 1998). PCR using pOsEP3A DNA as a template and oligonucleotides P1 and P2 as primers generated a 120-bp DNA fragment P_{1-2}. P_{1-2}, which contains the 5′-untranslated region (5′UTR) of *OsEP3A*, was later used as a gene-specific probe in genomic DNA Southern-blot and RNA northern-blot analyses.

Genomic DNA Southern-Blot Analysis

Genomic DNA was isolated from wild-type or transformed calli according to the method of Sheu et al. (1996). Ten micrograms of genomic DNA was digested with restriction enzymes, fractionated in a 0.8% agarose gel, and transferred to a nylon membrane (MSI). Hybridization was performed at 42°C using ^{32}P random-primer-labeled *OsEP3A* cDNA (P_{1-3}) or gene-specific DNA (P_{1-2}) as a probe.

Northern-Blot Analysis

Total RNA was isolated from various tissues of germinating seeds according to the method of Yu et al. (1996) and isolated from suspension-cultured cells using TRIZOL reagent (GIBCO-BRL). RNA gel-blot analysis was performed as described by Thomas (1983). Ten micrograms of total RNA was electrophoresed in 1% agarose gel containing 10 mM sodium phosphate buffer (pH 6.5), transferred to a nylon filter, and hybridized with ^{32}P random-primer-labeled P_{1-2} or rDNA probe. The blot was visualized using autoradiography and quantified using phosphor-imaging software (PhosphoImager, Molecular Dynamics, Sunnyvale, CA).

Transformation

Plasmid pEPGS1 was introduced into *A. tumefaciens* strain EHA101 (Hood et al., 1986) with an electroporator (BTX, San Diego) according to the manufacturer's instructions. Calli induced from immature rice seeds were co-cultured with *A. tumefaciens* according to the methods described by Hiei et al. (1994) and Toki (1997).

Western-Blot Analysis

Total proteins were extracted from suspension-cultured cells with an extraction buffer (50 mM Tris-HCl, pH 8.8, 1 mM EDTA, 10% [v/v] glycerol, 1% [v/v] Triton X-100, 10 mM β-mercaptoethanol, and 0.1% [w/v] sarkosyl). The

culture medium was collected and centrifuged at 18,000g at 4°C for 15 min to remove cell debris. Western-blot analysis was performed as described by Yu et al. (1991). The GUS polyclonal antibodies (Molecular Probes, Eugene, OR) were diluted 10,000-fold prior to use.

Histochemical Localization of GUS Activity

The dehulled seeds with or without embryos were sterilized with 2.4% NaOCl and placed on two pieces of sterile Whatman No. 1 filter paper in a Petri dish. A solution containing 1 μM GA$_3$ and 10 mM CaCl$_2$ or sterile water was applied to the filter paper to imbibe the seeds. The seeds were incubated at 28°C in darkness for various time periods. After incubation, seeds were hand-cut longitudinally in half and stained with 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) at 37°C in darkness for 12 h. The stained seeds were reserved in 70% ethanol and rinsed with water before photography.

RESULTS

Cloning and Characterization of a Rice CysP Gene, *OsEP3A*

To elucidate the hormonal, developmental, and metabolic factor regulating rice CysP gene expression, we cloned and characterized a rice CysP gene. A rice gene that hybridized strongly with the barley CysP cDNA clone pHVEP4 was isolated and designated as *OsEP3A*. The *OsEP3A* clone containing 2588 bp was sequence analyzed. The complete coding and the 5'- and 3'-flanking sequences of *OsEP3A* are shown in Figure 1. Comparison with sequence data in GenBank showed that the coding sequence of *OsEP3A* matches perfectly with rice pRP60 cDNA, which encodes REP-1 (Kato and Minamikawa, 1996). The intronless feature of *OsEP3A* is similar to the barley *EPB* genes (Mikkonen et al., 1996). To verify that *OsEP3A* is intronless, oligonucleotides P1 and P3 (Fig. 1) were used as primers for genomic DNA PCR and seed mRNA RT-PCR analyses. A 1.26-kb DNA fragment was amplified from both genomic DNA and mRNA, which confirmed that *OsEP3A* was intronless (data not shown). The transcription start site was mapped to be located at the adenosine nucleotide 122 bp upstream from the initiation codon (data not shown) and designated as +1. A typical TATA box is located at −34 of the transcription start site (Fig. 1).

DNA-blot analysis was performed to determine the copy number of CysP gene in the rice genome. By using DNA fragment P$_{1–3}$ as a probe, under very low hybridization stringency, one strong hybridization band and some minor bands were observed irrespective of the restriction enzymes used (Fig. 2, lanes 1–3). This finding indicated that the rice CysPs are encoded by a multigene family. By using DNA fragment P$_{1–2}$ as a probe, only a single band was hybridized (Fig. 2, lanes 4–6). Nucleotide sequence analysis revealed that no significant homology was present among the 5'UTR of *OsEP3A* and four other rice CysP cDNAs (Watanabe et al., 1991; Shintani et al., 1997). It is very likely that *OsEP3A* exists as one-copy gene in the rice genome.

Therefore, the 5'UTR of *OsEP3A* was served as a gene-specific probe.

Temporal and Spatial Regulation of *OsEP3A* Expression in Rice

To examine the expression pattern of *OsEP3A* in germinating seeds, total RNA was purified from embryos and endosperms and subjected to gel-blot analysis using P$_{1–2}$ as a probe. As shown in Figure 3A, *OsEP3A* mRNA was barely detectable in the embryos of dry seeds (lane 1), became detectable 1 h after imbibition (lane 2), and then gradually increased with incubation time (lanes 3–7). Gel-blot analysis of RNA accumulated in the embryos and endosperms showed that levels of *OsEP3A* mRNA fluctuated during a 10-d seed germination period. The levels of *OsEP3A* mRNA in embryos (Fig. 3B) and endosperms (Fig. 3C) reached their first peaks at d 4, declined from d 5 to d 7, then increased again and reached their second peaks at d 8 to 9 after the onset of seed germination.

Total RNA was also purified from various vegetative tissues of rice at different growth stages and subjected to gel-blot analysis using P$_{1–2}$ as a probe. As shown in Figure 4, the *OsEP3A* mRNA was barely detectable in the shoot (lane 1) and root (lane 2) of a 20-d-old seedling and in the stem (lane 4) and sheath (lane 7) of a 3-month-old mature plant. *OsEP3A* mRNA level was low in the root (Fig. 4, lane 3) but high in the green leaves (Fig. 4, lane 5) and senescing
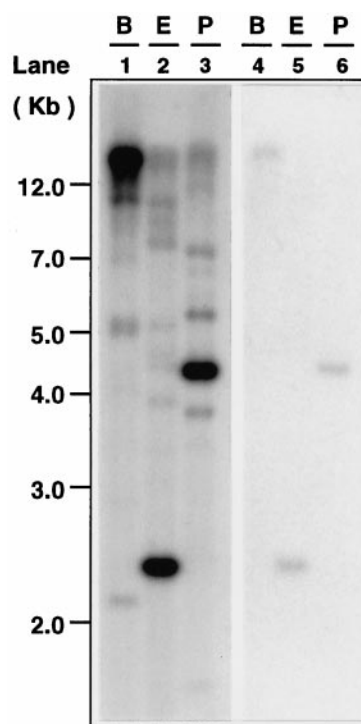
Figure 2. Gel-blot analyses of rice genomic DNA showing that CysP are encoded by a gene family. Rice genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), or *Pst*I (P) and subjected to gel-blot analysis using the full-length *OsEP3A* cDNA (lanes 1–3) or P$_{1–2}$ (lanes 4–6) as a probe. The molecular mass markers are shown in kb on the left of the autoradiogram.

A

B

C
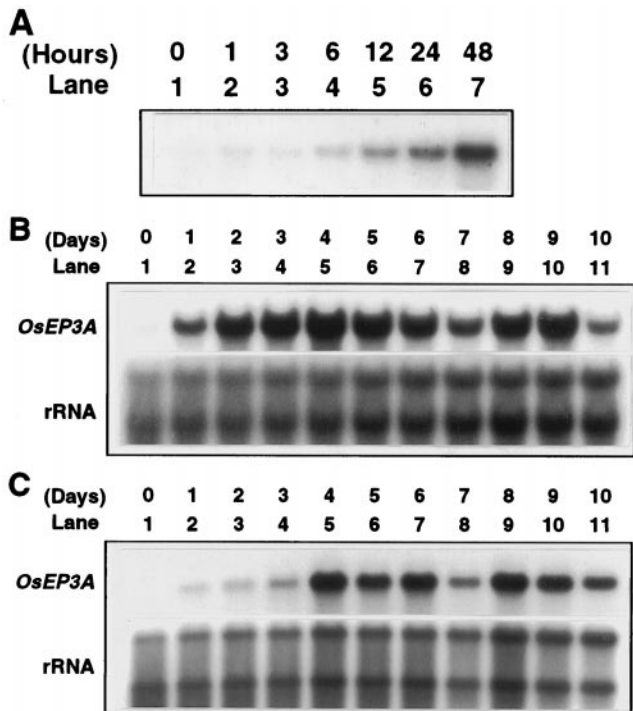
Figure 3. Developmental expression of *OsEP3A* in germinating rice seeds. Rice seeds were germinated for various time periods. Total RNA was purified from the embryos and endosperms and subjected to RNA gel-blot analysis using P_{1-2} or rDNA gene as a probe. Ten micrograms of total RNA was loaded in each lane. A, Levels of mRNA in embryos germinated for 48 h. B, Levels of mRNA in embryos germinated for 10 d. C, Levels of mRNA in endosperms of seeds germinated for 10 d. The experiment was performed twice producing similar result.

leaves (Fig. 4, lane 6) of the mature plant. Quantitation of mRNA indicates that the level of *OsEP3A* mRNA in the mature green leaves and senescing leaves was 3- and 9-fold, respectively, of that in mature root. These studies demonstrate that the expression of *OsEP3A* in vegetative tissues is developmentally and spatially regulated.

Metabolic Regulation of *OsEP3A* Expression in Suspension-Cultured Rice Cells

Plant cells store nitrogen in the form of proteins, and degradation of proteins allows recycling of amino acids and nitrogen. To investigate whether the expression of *OsEP3A* is regulated by nitrogen, rice cells were cultured in MS medium with or without nitrogen sources (NH_4NO_3 plus KNO_3) for various time periods. Total RNA was purified and subjected to gel-blot analysis using P_{1-2} as a probe. *OsEP3A* mRNA was not detectable in cells provided with nitrogen but accumulated in cells starved of nitrogen (Fig. 5A). The accumulation of *OsEP3A* mRNA was detected 1 d after nitrogen starvation and increased gradually up to 12 d, at which point the experiment was terminated. The accumulation of *OsEP3A* mRNA was suppressed by the addition of nitrogen sources into the culture medium and became undetectable within 48 h (Fig. 5B). These re-
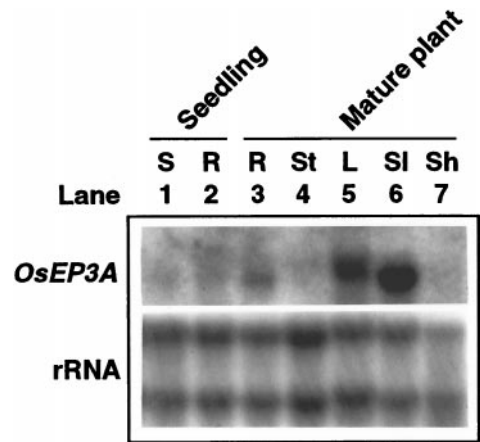
Figure 4. Developmental and spatial expression of *OsEP3A* in rice plants. Various tissues of young (20-d-old) and mature (3-month-old) plants were collected and total RNA was purified and subjected to RNA gel-blot analysis using P_{1-2} or rDNA gene as a probe. S, Shoot; R, root; St, stem; L, leaf; SL, senescencing leaf; Sh, sheath.

sults indicate that expression of *OsEP3A* is suppressed by nitrogen and activated by nitrogen starvation.

To determine whether the expression of *OsEP3A* was specifically induced by nitrogen starvation and affected by different forms of nitrogen source, rice suspension cells were cultured in medium with or without Suc or different sources of nitrogen. Within an 8-d culture period, the accumulation of *OsEP3A* mRNA was not or barely detectable

A

B
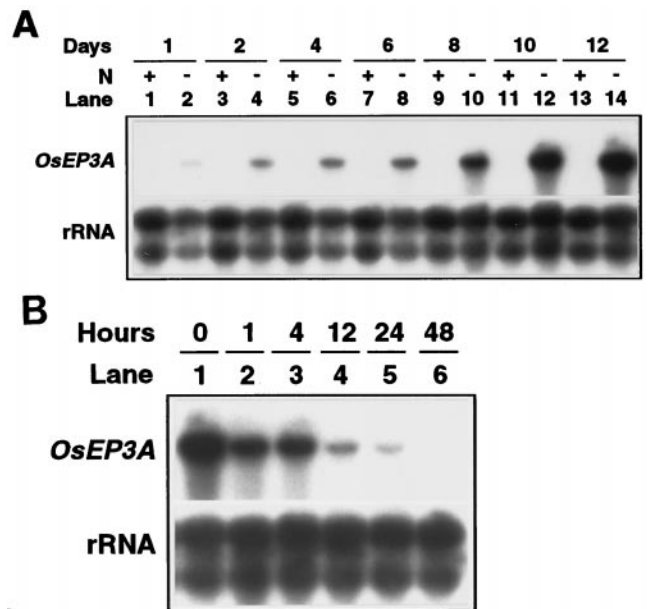
Figure 5. Nitrogen starvation activates expression of *OsEP3A* in rice suspension cells. A, Rice suspension cells were cultured in complete MS medium (containing 20 mM each of NH_4NO_3 and KNO_3) for 4 d and transferred to MS medium with (+) or without (−) nitrogen sources (N) and cultured for 12 d. B, Cells cultured in nitrogen-free medium for 12 d were transferred into complete MS medium for 48 h. Total RNA was purified from cells and subjected to gel-blot analysis using P_{1-2} or rDNA as a probe.

in cells cultured in complete MS medium (containing NH_4NO_3 plus KNO_3) with Suc (Fig. 6A, lane 1) or MS medium without Suc (Fig. 6A, lane 2), but increased significantly in MS medium without any nitrogen source (Fig. 6A, lane 3). The accumulation of *OsEP3A* mRNA was also not detectable in MS medium with either NH_4NO_3 or KNO_3 (Fig. 6A, lanes 4 and 5) as the nitrogen source. These results demonstrate that expression of *OsEP3A* is specifically induced by nitrogen starvation and not by Suc starvation. The accumulation of *OsEP3A* mRNA in the nitrogen-starved cells (Fig. 6B, lane 1) could be significantly repressed by the addition of Asn, Gln, Glu, NH_4Cl, or NH_4NO_3 into the MS medium as a nitrogen source (Fig. 6B, lanes 2–6). The addition of KNO_3 into the MS medium as a nitrogen source partially suppressed the accumulation of *OsEP3A* mRNA (Fig. 6B, lane 7), further demonstrating that a metabolizable nitrogen source may suppress the expression of *OsEP3A*.
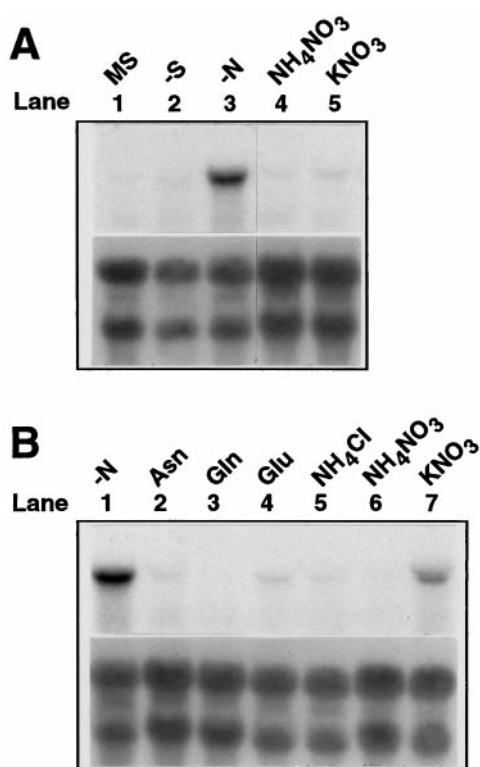
Figure 6. Nitrogen specifically suppresses the expression of *OsEP3A*. A, Rice suspension cells were cultured in complete MS medium (RNA in lane 1), MS medium without Suc (RNA in lane 2) or a nitrogen source (RNA in lane 3), or MS medium containing 20 mM of NH_4NO_3 (RNA in lane 4) or 20 mM KNO_3 (RNA in lane 5) as the sole nitrogen source for 8 d. B, Cells starved of nitrogen for 8 d were transferred to fresh MS medium without a nitrogen source (RNA in lane 1) or fresh MS medium containing 10 mM each of Asn, Gln, or Glu, or 20 mM each of NH_4Cl, NH_4NO_3, or KNO_3 (lanes 2–7) for 2 d. Total RNA was purified from cells and subjected to gel-blot analysis using P_{1-2} or rDNA gene as a probe.

## Analysis of *OsEP3A* Promoter Activity in Transformed Rice Suspension Cells

To investigate the role of the promoter in the regulation of *OsEP3A* expression in rice, a 1.1-kb DNA fragment containing the 5′ regulatory and putative signal peptide sequences of *OsEP3A* was fused in-frame at the 5′ end of *gusA* gene. The chimeric gene was inserted into a binary vector to generate pEPGS1 as shown in Figure 7A. pEPGS1 was introduced into *A. tumefaciens* for rice transformation. Thirty transgenic lines were regenerated and four lines containing a single copy of the *gusA* gene (data not shown) were selected for further study. Callus derived from scutella of transgenic seeds was cultured as suspension cells. The transformed suspension cells were then cultured in medium with or without nitrogen. Total RNA was purified and subjected to gel-blot analysis using the *gusA* cDNA plus P_{1-2} as probes. Accumulation of both *gusA* and *OsEP3A* mRNAs was detectable in cells starved of nitrogen but not in cells provided with nitrogen (Fig. 7B, lanes 3–10). No *gusA* mRNA was detected in the non-transformed cells (Fig. 7B, lanes 1 and 2).

The levels of *OsEP3A* mRNA in the four transgenic lines were fairly similar, while the levels of *gusA* mRNA varied from line to line, indicating a position effect on transgene expression. Suspension-cultured cells of transgenic line 14 were selected for further study of the function of the putative signal peptide sequence in protein secretion. Cells were grown in medium with or without nitrogen for 10 d and proteins were extracted from cells or collected from the culture medium and subjected to western-blot analysis using GUS antibodies. As shown in Figure 7C, GUS was detected in cells and medium only when cells were starved of nitrogen. This result indicates that the putative signal peptide of *OsEP3A* is capable of directing translocation of GUS through the secretory pathway of transformed cells into the culture medium.

Analysis of *OsEP3A* Promoter Activity in Transgenic Rice Seeds

To investigate the role of promoter in the temporal, spatial, and hormonal regulation of *OsEP3A* expression during rice seed germination, histochemical GUS assays of transgenic seeds carrying *OsEP3A-gusA* chimeric gene were performed. As shown in Figure 8A, in longitudinally cut germinating seeds, GUS activity was detected in the scutellar epithelium within 1 d after germination (panel I), spread into the adjacent aleurone layer by d 3 (panels II and III), and finally covered the entire aleurone layer by d 5 (panels IV and V). As shown in Figure 8B, in cross-cut germinating seeds, GUS activity was not detected in the non-transformed aleurone layer by d 3 (panel I), but was first detected in the ventral side of the aleurone layer within 1 d after germination (panel II), spread into the dorsal side of aleurone layer by d 3 (panel III), and finally almost the entire aleurone layer by d 5 (panel IV).

To examine the effect of GA_3 on the expression of *OsEP3A-gusA* gene in seeds, de-embryonated transgenic rice seeds were treated with GA_3 for various time periods
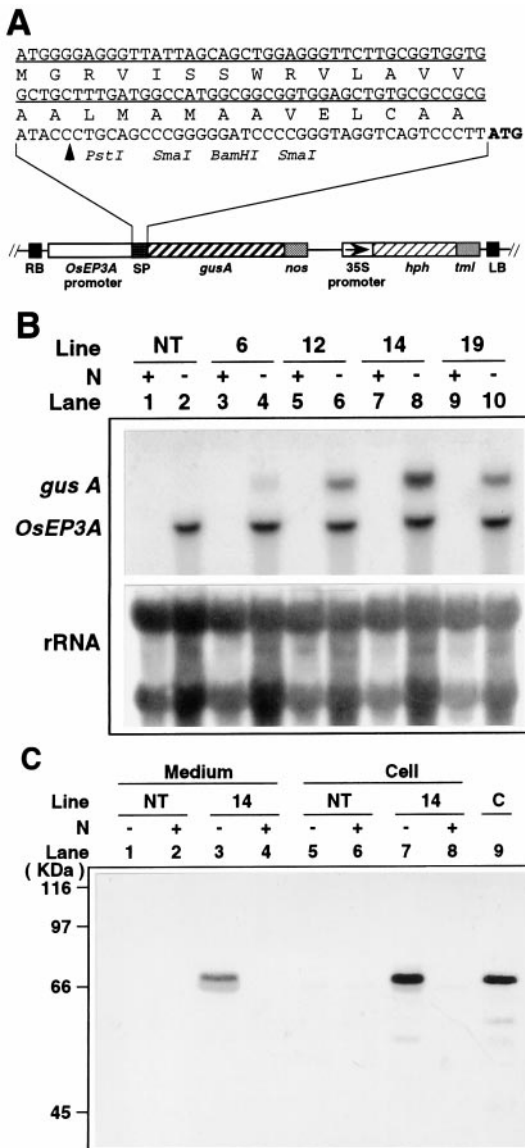
Figure 7. The *OsEP3A* promoter confers nitrogen-starvation-activated expression in transformed rice suspension cells. Calli were generated from T1 transgenic rice seeds containing the *OsEP3A-gusA* chimeric gene and cultured as suspension cells. The transformed suspension cells were cultured in MS medium with (+) or without (−) a nitrogen source (N) for 10 d. A, Schematic diagram of the plasmid pEPGS1 used for rice transformation. The promoter region and signal peptide (sp) sequence (the latter underlined) of *OsEP3A* were in-frame joined to the coding region of *gusA* with the polyadenylation signals of the nopaline synthase gene (*nos*). RB and LB, Right and left border of T-DNA, respectively; 35S, cauliflower mosaic virus 35S RNA promoter; *hph*, hygromycin phosphotransferase gene; *tml*, tumor morphology large gene terminator; ATG, start of the *gusA* coding region. B, Total RNA was purified from treated cells and subjected to gel-blot analysis using *gusA* cDNA plus $P_{1–2}$ or rDNA as probes. C, Total proteins were extracted from transformed cells or collected from the culture medium of transgenic line 14 and subjected to western-blot analysis using the GUS antibodies. One-hundred-twenty microliters of culture medium was applied in each of lanes 1 to 4. Twenty micrograms of total cellular proteins were applied in each of lanes 5 to 8. NT, Non-transformed control cell line. Lane 9 contains 200 ng of purified *Escherichia coli* GUS.

and histochemical GUS assays were performed. As shown in Figure 8C, in the GA_3-treated and longitudinally cut seeds, GUS activity was not detected in the non-transformed de-embryonated seed by d 3 (panel I), but was first detected in the ventral side of the aleurone layer within 1 d (panel II), in both the ventral and dorsal sides of the aleurone layer by d 3 (panels III and IV), and finally in the entire aleurone layer by d 5 (panel V).

DISCUSSION

The rice REP-1 cDNA has been isolated and characterized and the deduced amino acid sequence shares 75% similarity with that of the barley EPB (Kato and Minamikawa, 1996). Since REP-1 plays an essential role in the degradation of seed storage proteins during germination and early seedling growth, it is important to study how the expression of REP-1 is regulated. As a first step, we isolated the rice gene, *OsEP3A*, encoding REP-1. Genomic DNA-blot analysis showed that the rice CysP gene family consists of only one member of *OsEP3A* and that at least five other CysP gene family members have sequences similar to *OsEP3A* (Fig. 2). Three other rice CysP cDNAs encoding oryzains α, β, and γ (Watanabe et al., 1991) and another rice CysP cDNA pRP80 (Shintani et al., 1997) have also been isolated from the cDNA library of germinating rice seeds. The *OsEP3A* (pRP60) and pRP80 DNAs do not cross-hybridize to each other (Shintani et al., 1997), and the nucleotide sequence of *OsEP3A* coding region shares 58%, 56%, and 50% homology with oryzains α, β, and γ cDNAs, respectively. Comparison of nucleotide sequences in promoter regions reveals that sequence +18 to −120 of *OsEP3A* shares 86% homology with sequence +18 to −180 (containing 60-bp extra non-conserved sequences within this region) of the barley *EPB-1* and *EPB-2* (Mikkonen et al., 1996), but sequences upstream of this regions show very low homology between *OsEP3A* and *EPB*.

The *OsEP3A*-specific DNA probe was useful for studying the expression pattern of *OsEP3A* in germinating seeds and other tissues of rice. In germinating rice seeds, the amount of *OsEP3A* mRNA fluctuate in both the embryos (Fig. 3B) and endosperms (Fig. 3C). The temporal and fluctuant expression pattern of *OsEP3A* is similar to that of α-amylase genes (Yu et al., 1996), suggesting that expression of *OsEP3A* and α-amylase genes may be subject to similar regulation by both GA_3 and osmotica in the rice embryo and endosperm (Yu et al., 1996). *OsEP3A* is also expressed in other rice tissues, particularly in the roots and leaves of mature plant and in senescing leaves (Fig. 5). Nitrogen remobilization is one of the major functions of senescence and is achieved through the action of proteolytic enzymes (Staswick, 1994). Expression of CysP genes increases in senescent leaves of various plant species and is considered to be responsible for protein degradation (Hensel et al., 1993; Lohman et al., 1994; Xu and Chye, 1999). In the present study, accumulation of OsEP3A mRNA in mature and senescing leaves is consistent with the role of OsEP3A in protein turnover and nitrogen remobilization. Interestingly, OsEP3*A* seems to degrade both seed and vegetative storage proteins.
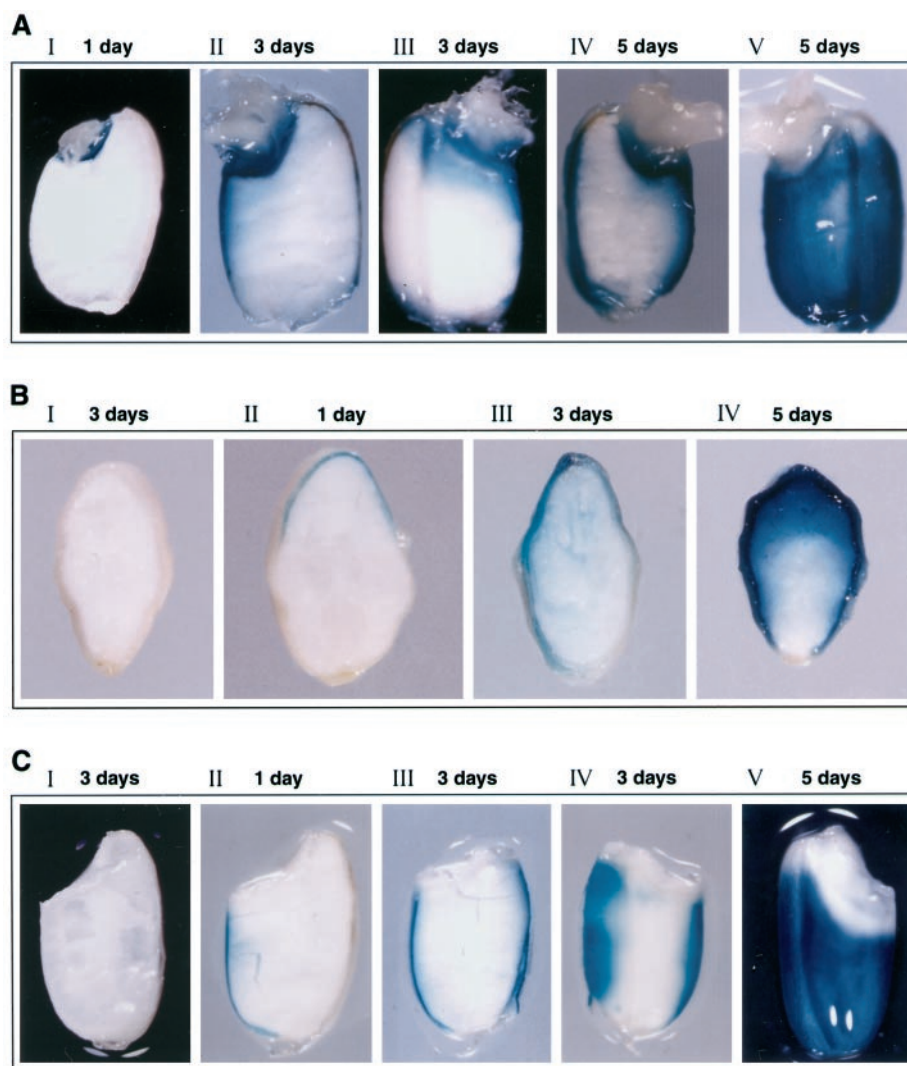
Figure 8. Histochemical analysis of temporal, spatial, and hormonal regulation of *OsEP3A-gusA* expression in transgenic rice seeds. A, Transgenic seeds were germinated in water at 28°C for various time periods, longitudinally cut in half, and stained for GUS activity. Shoots were removed before staining. I, II, and IV are viewed from the cutting surface and III and V are viewed from the grain surface. II and III are the same half-seed and IV and V are the same half-seed. B, Germinating transgenic seeds were cross-cut in half and stained for GUS activity. I, Non-transformed seed; II to IV, transformed seeds. The dorsal side of the half-seeds is positioned up. C, Transgenic rice seeds were de-embryonated and incubated in a solution containing 1 μM GA$_3$ and 10 mM CaCl$_2$ at 28°C for various time periods. The non-transformed seed (I) was similarly treated for 3 d. The treated seeds were longitudinally cut in half, then stained for GUS activity. I, II, and III are viewed from the cutting surface, and IV and V are viewed from the grain surface. III and IV are the same half-seed and V is an uncut seed.

Our data showed that expression of *OsEP3A* in suspension-cultured rice cells was induced under conditions of nitrogen deprivation. Questions were thus raised regarding the in vivo role of OsEP3A in the nitrogen-starved cells. Cells store nitrogen in the form of proteins and nitrogen deprivation may trigger degradation of cellular proteins. The amino acids and nitrogen released then contribute to the pool of soluble nitrogen for re-utilization and synthesis of new proteins under nitrogen starvation. Consequently, the synthesis of proteinase such as OsEP3A could be crucial for cell survival. On the other hand, under normal growth conditions with an adequate supply of nitrogen source, expression of OsEP3A is subject to metabolic repression. For various metabolizable nitrogen

sources tested, all except KNO$_3$ significantly suppressed the accumulation of *OsEP3A* mRNA. Provision of KNO$_3$ to the nitrogen-starved cells only partially suppressed the accumulation of *OsEP3A* mRNA (Fig. 6B, lane 7), which could be due to a slower rate in the uptake of NO$_3^-$ by the nitrogen-starved cells. It has been shown that roots of intact rice plants have a similar affinity for NH$_4^+$ and NO$_3^-$, however, the uptake rate is somewhat higher for NH$_4^+$ than for NO$_3^-$ (Youngdahl et al., 1982). In suspension-cultured rice cells, the expression pattern of *OsEP3A* induced by nitrogen starvation is similar to that of α-amylase genes induced by sugar starvation (Sheu et al., 1994). One major difference is that cultured cells die within 5 d after Suc starvation (Chen et al., 1994), whereas cells

remain alive and continue to express *OsEP3A* 10 to 12 d after nitrogen starvation (Fig. 5A). Such phenomena reflect a much higher demand for carbohydrates than nitrogen in the maintenance of cell life.

Our study demonstrates that expression of the *OsEP3A-gusA* chimeric gene in transformed rice suspension cells is induced by nitrogen starvation. This finding indicates that the 1.2-kb *OsEP3A* promoter region contains the necessary cis-regulatory sequences for conferring metabolic regulation of downstream gene expression. This promoter sequence also confers GA_3 regulation of *OsEP3A* expression in germinating seeds. These results suggest that the nitrogen and GA_3 regulations both act at the level of transcription. The putative 28-amino acid signal sequence predicted with a statistical method (Von Heijne, 1986) is capable of directing extracellular targeting of GUS into the culture medium of suspension cells, as well as secretion of GUS from aleurone cells into the starchy endosperm.

Histochemical GUS assays of germinating transgenic seeds revealed patterns of temporal and spatial regulation of the *OsEP3A-gusA* gene, which is consistent with studies using RNA gel-blot analysis (Fig. 3). The temporal and spatial expression of *OsEP3A-gusA* is similar to that of the *Ramy1A* (an α-amylase gene)-*gusA* gene in germinating transgenic rice seeds (Itoh et al., 1995). Furthermore, these two chimeric genes show a similar pattern of response to GA_3 in aleurone layers of embryoless seeds. The absence of GUS activity in the region of the aleurone layer that surrounds the wound site where embryos and scutella were removed (Fig. 8C) was probably due to death of cells in this region during sterilization of the embryoless transgenic seeds by NaOCl prior to treatment with GA_3. It is interesting to note that accumulation of GUS is first detected in the scutellar epithelium of embryo, then the proximal region of the ventral side of aleurone layer, and finally the entire aleurone layer. Such pattern of GUS accumulation indicates progression pattern of storage protein breakdown in the endosperm of germinating rice seeds.

It appears that *OsEP3A* and α-amylase genes are coordinately regulated by GA_3 and the promoters of the two sets of genes may share common features essential for regulation of gene expression. A GA-response element (GARE) is essential for GA-dependent expression of several α-amylase genes in barley aleurone cells (Skriver et al., 1991; Gubler and Jacobsen, 1992; Lanahan et al., 1992). An MYB transcription factor, HvGAMYB, binds to the GARE of a barley high-pI α-amylase gene promoter and is able to transactivate the high pI α-amylase gene promoter in transient expression assays (Gubler et al., 1995). Two sequences resemble the consensus HvGAMYB binding site (Gubler et al., 1999) are located at positions between −163 to −156 and −143 to −136 of the *OsEP3A* promoter (Fig. 1) and one or both may function as a GARE. Identification of the nitrogen- and senescence-responsive cis-regulatory elements in the *OsEP3A* promoter is yet to be determined, and will be required to elucidate the mechanisms underlying the regulation of *OsEP3A* expression under different physiological conditions.

In conclusion, the transgenic approach will be useful in studying functions and interactions of cis-regulatory ele-ments in the *OsEP3A* promoter that are essential for GA and temporal and spatial regulation in germinating cereal grains. In addition, nitrogen-starved suspension cells express *OsEP3A* in a manner similar to senescencing leaves in terms of nitrogen demand and re-utilization. Because the expression of CysP and the degradation of proteins in cultured cells can be easily manipulated by altering the nitrogen level in the medium, the rice cell culture system represents an ideal tool for studying the biochemical and molecular biological aspects of CysP gene regulation, nitrogen metabolism, and senescence in plants.

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