Enhanced Resistance to Seed-Transmitted Bacterial Diseases in Transgenic Rice Plants Overproducing an Oat Cell-Wall-Bound Thionin

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Submitted 30 November 2001. Accepted 5 February 2002.

Bacterial attack is a serious agricultural problem for growth of rice seedlings in the nursery and field. The thionins purified from seed and etiolated seedlings of barley are known to have antimicrobial activity against necrotrophic pathogens; however, we found that no endogenous rice thionin genes alone are enough for resistance to two major seed-transmitted phytopathogenic bacteria, *Burkholderia plantarii* and *B. glumae*, although rice thionin genes constitutively expressed in coleoptile, the target organ of the bacteria. Thus, we isolated thionin genes from oat, one of which was overexpressed in rice. When wild-type rice seed were germinated with these bacteria, all seedlings were wilted with severe blight. In the seedling infected with *B. plantarii*, bacterial staining was intensively marked around stomata and intercellular spaces. However, transgenic rice seedlings accumulating a high level of oat thionin in cell walls grew almost normally with bacterial staining only on the surface of stomata. These results indicate that the oat thionin effectively works in rice plants against bacterial attack.

In rice-growing areas such as Japan, the method for raising rice seedlings for machine transplantation into paddy fields has recently changed from the anaerobic conditions of paddy fields to the aerobic conditions of upland fields. This change to upland fields from paddy fields promises more vigorous growth of rice seedlings in the nursery and subsequent enhanced growth in the paddy fields after transplantation, resulting in a stable mass production of rice seed. However, seedlings grown in upland areas at a higher temperature are susceptible to attack by bacterial pathogens. Such infections have not been reported in seedlings grown in paddy fields. Thus, a change from anaerobic to aerobic growing conditions likely altered the kinds of infectious bacteria afflicting the rice plant and induced a new agricultural problem, seedling blight disease, caused by seed-transmitted bacteria such as *Burkholderia* (previously classified to *Pseudomonas*) *plantarii* and *B. glumae*. The grain rot phenotype caused by *B. glumae*, in particular, has become a major topic of research.

Generally, attempts to control diseases rely on the use of agrochemicals. However, it repeatedly has been shown that bacteria easily acquire resistance to bactericides. Furthermore, enhanced chemical injury by the use of bactericides together with fungi-

cides has become another problem in rice seedlings grown in upland fields. Another orthodox method for obtaining resistant plants is crossbreeding. However, we could not find suitable genetic resources to cross with practical Japonica rice cultivars.

Thus, the generation of transgenic rice plants resistant to bacterial attack has been awaited. Among the genes available for this purpose, thionin genes are of particular interest because they are reported to have antimicrobial activity as follows: (i) a barley leaf thionin induced by *Septoria nodorum* (Stevens et al. 1996) and *Drechslera graminea* (Vale et al. 1994) has been reported to be toxic to phytopathogenic bacteria and fungi in vitro (Bohlmann et al. 1988; Molina et al. 1993); (ii) α -holdothionin from barley seed conferred enhanced resistance to *Pseudomonas syringae* when overproduced in tobacco plants (Carmona et al. 1993); and (iii) Epplé and associates (1997) reported that overexpression of the *Arabidopsis thi2.1* gene in *Arabidopsis* plants resulted in enhanced resistance to *Fusarium oxysporum*.

We first evaluated the role of the endogenous rice thionins, concluding that they alone are not enough to protect against bacterial infection. Then, we newly isolated five thionin genes from oat plants originally grown in aerobic conditions and report here that overproduction of an oat thionin in Japonica rice plants resulted in a strong resistance to infection by two major seed-transmitted soilborne bacteria.

RESULTS

Evaluation of the endogenous thionin genes for resistance to bacterial attacks in rice plants.

Rice seedlings grown in upland fields are susceptible to attack by soilborne bacteria. We first evaluated the contribution of endogenous rice thionins to disease resistance. In 8×10^4 clones of raw rice expressed sequence tags, we found more than 100 clones that encode the same amino acid sequence for the predicted thionin *Osthi1* with the three conserved domains characteristic of plant thionins: a signal peptide, a mature thionin, and an acidic protein region (Fig. 1A) (Bohlmann 1999; Bohlmann and Apel 1991; Broekaert et al. 1997). The mature rice thionin contains 45 amino acids, including 6 consensus cysteine residues with a predicted pI of 8.77 (Fig. 1A).

To elucidate the genomic organization of *Osthi1*, DNA from *Oryza sativa* cv. Nipponbare was subjected to Southern blot analysis, first, with a common probe for the thionin region and second, with the 3' untranslated region (UTR) of *Osthi1* as a specific probe (Fig. 1A). The common probe detected approximately 10 signals in all digests, indicating that thionin genes

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compose a multigene family in rice. The specific probe detected approximately four bands in high stringency conditions, suggesting the *Osthi1* gene and several quite similar genes are contained in the rice genome (Fig. 1B).

Transcripts of endogenous rice thionin genes were found to be abundant in coleoptiles and rare in roots but not detectable at all in leaf blade and panicle, using the common probe under low stringency conditions (Fig. 1C, left panel). In 2-week-old rice seedlings, no transcript was found in leaf blade and leaf sheath treated with defense signal molecules salicylic acid (SA) and ethephon (ET), an ethylene-releasing compound. The level of the transcript was only increased in the leaf sheath at 24 h after jasmonic acid (JA) treatment (Fig. 1D, left panel). When the 3' UTR was used as the probe (Fig. 1C and D, right panel), very similar results were obtained, suggesting that the *Osthi1* gene and related genes are the major thionin genes expressed in rice coleoptile, the target organ of phytopathogenic

bacteria. However, seedlings of Japonica rice cultivars, including Nipponbare and Chiyohonami, are known to be vulnerable to seed-transmitted bacteria such as *B. plantarii* and *B. glumae*, indicating that expression of rice thionin genes, including *Osthi1*, did not protect against the infection.

Cloning and characterization of oat thionin genes.

As described above, the expression of endogenous thionin genes alone is not enough to prevent bacterial attack in rice plants. To obtain more effective antibacterial thionin genes, we selected as a genetic source the oat plant, a secondary crop that has been bred from weed and originally grew under aerobic conditions in upland fields.

The target organ of the two bacteria is coleoptile; therefore, we prepared a cDNA library (1.5×10^5 clones) of etiolated oat (*Avena sativa* cv. Zensin) seedlings. Using degenerate primers deduced from the signal sequence and C-terminal sequence of mature barley thionin, the fragments for the thionin genes were amplified from oat genomic DNA by polymerase chain reaction (PCR). Thus, we could isolate five full-length cDNA clones for putative thionin genes (*Asthi1* ~ 5) using the fragments as probes. DNA sequence analysis showed that they encode proteins with the three conserved domains of plant thionins. For example, *Asthi1* has 28 amino acids (aa) for the signal peptide, 46 aa for the mature thionin, and 63 aa for the acidic protein domain. In the mature thionin region, six cysteine residues (Fig. 2) and the thirteenth tyrosine residue (asterisk) are conserved in oat and other plant species, and two cysteine residues are conserved in barley, wheat, and oat. The amino acid sequence homology of a barley leaf thionin (DB4) to *Asthi1*, *Asthi2*, *Asthi3*, *Asthi4*, and *Asthi5* and *Osthi1* is 76, 78, 54, 65, 61, and 59%, respectively. Thus, *Asthi1* and *Asthi2* are most similar to the cell wall-bound barley leaf thionin DB4. The predicted pI of *Asthi1* and *Asthi2* calculated by GENETIX (Software Development Co., Tokyo) is the same, 9.13, which is the highest value among leaf thionins reported to date.

Comparison of the C-terminal amino acid sequences of mature thionin regions (Fig. 2) showed a clear divergence between the leaf-type (DYPK, green) sequence and the seed-type (PK, orange) sequence, which lacks the twenty-first amino acid of the leaf-type. *Asthi4*, *Asthi5*, *Osthi1*, *thi2.1*, and *thi2.2* are classified as other types, in which only the amino acid sequences of *Osthi1* and *Asthi5* share high homology (67% identity), lacking the thirty-eighth residue (dark blue). Thus, *Osthi1* may have different characteristics from leaf and seed thionins in localization or the antimicrobial spectrum.

The presence of eight or more thionin-related genes in the oat genome was revealed by DNA blot analysis using the thionin domain of *Asthi1* as probe (Fig. 3A). The level of *Asthi1* transcript was high in oat coleoptile but negligible in the first and second leaves of 2-week-old oat plants (Fig. 3B). A transient small increase was found in leaves 6 h after cutting, but SA treatment seemed to have no effect. Alternatively, methyl jasmonate (MeJA), a wound signal compound, clearly induced an accumulation of the *Asthi1* transcript at 6 h, with a peak at approximately 24 to 48 h. Thus, *Asthi1* differs from rice *Osthi1* in structure and in the expression profile in leaves.

Generation of transgenic rice plants overproducing *Asthi1*.

For overproduction in rice plant, we selected *Asthi1*, with a predicted pI value higher than that of *Asthi3* and of *Asthi4*, because the thionin with higher pI value was suggested to exhibit greater antimicrobial activity (Thevissen et al. 1996). We constructed a binary vector, pMLH7133-thionin, containing seven tandem repeats of the 0.2-kb *Cauliflower mosaic virus* (CaMV) 35S enhancer (E7), the Ω sequence from *Tobacco mosaic virus*, and an intron of phaseolin (Mitsuhara et al. 1996) 5'

Fig. 1. Characterization of the rice thionin gene. **A**, The *Osthi1* gene encodes three conserved domains for plant thionins. **B**, Southern blot analysis was performed using genomic DNA (5 μ g) from *Oryza sativa* cv. Nipponbare after digestion with *EcoRI* (E), *HindIII* (H), and *PstI* (P), using a common probe corresponding to the mature thionin domain (left panel) and a specific probe corresponding to the 3' untranslated region (right panel). **C**, Total RNA (20 μ g) was subjected to RNA blot analysis using the common probe under low stringency conditions (two washes with $1\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 60°C for 20 min, left panel) and the specific probe under high stringency conditions (two washes with $0.1\times$ SSC and 0.1% SDS at 68°C for 20 min, right panel). Root from 2-week-old rice plant, coleoptile from 4-day-old etiolated plant, leaf blade from fully expanded leaf of the plant at each leaf stage, and panicle from 2-month-old plant. **D**, Total RNA (20 μ g) was subjected to RNA blot analysis as in C. Leaf blades and sheaths from the plant at 3.5 leaf stage were treated for 24 h with 3 mM salicylic acid (SA), 1 mM jasmonic acid (JA), and 1 mM ethephon (ET).

upstream of the *Asthl1* gene. The hygromycin resistance gene was used as a selectable marker (Mochizuki et al. 1999). The vector was reported to confer a 70 times higher constitutive expression than the 35S *CaMV* promoter in pBI 121 in rice protoplasts (Mitsuhashi et al. 1996).

Accumulation and localization of *Asthi1* gene products in transgenic rice plants.

immunoblot analysis. A similar level of Asth1l was detected in the insoluble fraction as in the total extract, while no Asth1l was found in the soluble fraction (Fig. 4F). Thus, the oat leaf thionin *Asth1l* produced in transgenic rice plants was secreted outside cells and ionically bound to cell walls.

Type	Gene	Plant	Mature thionin region										AAs	pI																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
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the presence of tropolone, only *B. plantarii* could multiply in the medium, indicating that the data reflected the actual number of *B. plantarii* not disturbed by other bacteria.

The localization of the bacteria in the infected seedlings was examined microscopically. Sections of rice coleoptiles infected with *B. plantarii* were fixed, cut into cross-sections 15 µm thick, and then subjected to Stoughton's double staining (Stoughton 1930), by which bacteria turn purple. In wild-type rice coleoptiles, the bacteria appeared to invade the inner tissue through stomata on the surface of the coleoptiles (red arrowhead in Fig. 5Ce and h), and accumulated in aerenchyma (black arrowheads in Fig. 5Cb) and the intercellular spaces (green arrowheads). However, in the coleoptiles of transgenic rice line 2, invasion of bacteria was completely blocked at the stomata (Fig. 5Cf and i), and no bacterium was found in the inner tissue (Fig. 5Cc). At higher magnification, we observed that the bacteria were likely restricted only on the surface of the stomata (Fig. 5Ci).

DISCUSSION

In this study, overproduction of an oat leaf thionin resulted in the generation of transgenic rice plants with enhanced resistance to seed-transmitted soilborne bacteria. To our knowledge, this is the first report to show that ectopic expression of a monocotyledonous leaf thionin enhanced resistance to necrotrophic bacterial infection in rice, an economically important monocotyledonous plant. The level of resistance was high, and the bacteria were likely stopped at the surface of stomata in coleoptiles, which is the invasion site of *B. plantarii* (Fig. 5). Seed transmission of bacteria to the next generation could be protected in transgenic plants, because infection and multiplication of *B. glumae* and *B. plantarii* were considerably suppressed at the seedling stage. Furthermore, the grain rot phenotype caused by *B. glumae* at flowering stage also may be

protected. Thus, overproduction of Asthi1 in rice plants would be useful for conferring enhanced resistance to bacterial diseases, especially in Asian countries where bacterial diseases are more serious because of the climate of high temperature and high humidity.

As the expressed gene, *Asthi1* would be suitable for our purposes. The oat thionin gene possibly has evolved in oat plants that grow in aerobic soils on upland fields, acquiring resistance

Fig. 3. Characterization of oat leaf thionin genes. **A**, Digested genomic DNA (5 µg) from *Avena sativa* cv. Zensin was subjected to DNA blot analysis using the mature thionin domain of *Asthi1* as probe. **B**, Poly(A) RNA (2 µg) was subjected to RNA blot analysis. Coleoptiles were prepared from 5-day-old etiolated oat seedling. First and second leaves, treated with water, 50 µM salicylic acid, and 50 µM methyl jasmonate for the indicated time period, were prepared from 10-day-old oat seedlings.

Fig. 4. Generation of transgenic rice plants overproducing *Asthi1*. **A**, Construct of the introduced gene. **B**, Southern blot analysis of the *Asthi1* gene in transgenic rice lines. Digested genomic DNA (5 µg) was hybridized with the thionin region of *Asthi1* as probe. **C**, Total RNA (20 µg) from the coleoptile (Co) and leaf blade (L) of transgenic rice lines 2, 77, and 91 (T3) was subjected to Northern blot analysis using the thionin region of *Asthi1* as probe under high stringency conditions (two washes with 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate at 68°C for 20 min, upper panel) and the common probe of *Osthi1* under low stringency conditions as shown in the legend of Figure 1C (lower panel). **D**, Levels of *Asthi1* transcript in the coleoptiles of 5-day-old wild-type oat seedlings. **E**, Accumulation of *Asthi1* protein in the coleoptiles of 5-day-old transgenic rice lines 2, 77, and 91. Synthetic *Asthi1* was used as the standard. Polyclonal antibody against synthetic *Asthi1* was prepared in rabbits and used as primary antibody. Crude protein extract corresponding to 1 mg of fresh coleoptile from transgenic lines was used in each lane. **F**, Localization of expressed *Asthi1* protein. Coleoptiles from 1-week-old transgenic line 2 were homogenized and fractionated into soluble (sol.) and insoluble (insol.). Insoluble fraction mainly included the cell walls. An aliquot corresponding to 1 mg of fresh coleoptile was subjected to Western blot analysis.

to phytopathogenic aerobic bacteria such as *B. plantarii* and *B. glumae*. Interestingly, we could not find any *Asthi1*-type thionin genes in rice plant. The major thionin gene expressed in rice coleoptiles is *Osthi1*, which encodes a type of thionin different from that found in *Asthi1* (Fig. 2). Alternatively, in rice plants that have grown in paddy fields with anaerobic soil, thionin genes have evolved to protect against anaerobic bacteria but not aerobic bacteria, resulting in the lack of an *Asthi1*-type thionin gene. Thus, introduction of the oat *Asthi1* gene into rice plants conferred additive resistance to aerobic bacteria, aiding the normal growth of rice seedlings in upland soil.

We observed a predominant accumulation of the Asthi1 protein in cell walls (Fig. 4) and restriction of the bacteria to the stomata of transgenic rice plants (Fig. 5), suggesting that *B. plantarii* first makes contact with the cell wall in rice coleoptiles and that cell wall-bound *Asthi1* effectively inhibits bacterial multiplication. Indeed, evidence indicating the importance of the cell wall in pathogen infection has been reported. In barley leaves resistant to powdery mildew infection, a localized accumulation of thionin in the cell wall at the infection site was detected immunoelectronically (Ebrahim et al. 1989; Ebrahim-Nesbat 1993), and Aldon and associates (2000) reported that contact between the bacteria and cell wall was necessary for the activation of bacterial virulence genes in *Arabidopsis* spp.

Use of the strong E7QI promoter resulted in accumulation of 100 µg or more of Asthi1 protein per g of fresh coleoptile in transgenic lines (Fig. 4). Asthi1 in transgenic rice plants was found to be ionically bound to the cell wall, which is the original target of the protein in oat plants. Cell walls would be the ideal place for the highly expressed Asthi1 protein to accumulate. Actually, ectopic mass production of a foreign antibacterial peptide sarcotoxin 1A from fresh fly interior cells resulted in distorted plant growth and form (Okamoto et al. 1998), whereas secretion of the peptide outside cells resulted in normal growth and normal phenotypes with bacterial resistance (Mitsuhara et al. 2000;

Ohshima et al. 1999). Thus, accumulation of Asthi1 in the cell wall resulted in normal growth and fertility of transgenic rice plants, in addition to conferring bacterial resistance.

It has been reported that high levels of transgene expression frequently induces gene silencing, and this was the case in our experiments; 22 out of 41 transgenic rice lines (T0) exhibited transgene silencing in the next generation. However, the remaining 19 lines produced high levels of Asthi1 protein in the later generations. The progeny of *Asthi1* homozygotes (lines 2, 77, and 91) stably accumulated high levels of Asthi1 at all time points tested. Thus, we could select stable Asthi1 over-producers, even if transgenic plants (T0) expressed a very high level of transgene transcript.

MATERIALS AND METHODS

Plant materials and bacterial strains.

Rice seed (*Oryza sativa* cvs. Nipponbare and Chiyohonami) soaked in water overnight at 31°C was sown in soil (Bonsol1, Sumitomo Chemical Corp., Tokyo) and was grown in a greenhouse at 20 to 30°C. Oat plants (*Avena sativa* cv. Zenshin) were grown in the dark on moist vermiculite at 25°C for 5 days and then in a greenhouse for a week. *B. plantarii* (MAFF 301723), the cause of rice bacterial seedling blight disease, and *B. glumae* (MAFF 302554), the cause of grain rot disease in seedlings, were obtained from the MAFF collection (Tsukuba, Japan).

RNA and DNA blot analysis.

Total RNA from rice plant materials was extracted by the aurin tricarboxylic acid method described by Nagy and associates (1988). Genomic DNA was isolated from oat and rice seedlings as described by Murray and Thompson (1980). RNA and DNA blot analysis were performed using the digoxigenin (DIG) nonradioactive nucleic acid labeling and detection sys-

Fig. 5. Enhanced resistance to infection by necrotrophic bacteria in transgenic rice lines overproducing Asthi1. **A**, Phenotype of transgenic rice lines at 10 days after inoculation with *Burkholderia glumae*. **B**, Transgenic line 2 phenotype 10 days after inoculation with *B. plantarii*. The number of bacteria recovered from infected seedlings is shown at the bottom of the picture; crude extract of inoculated seedlings was spread on agar medium containing tropolone, the toxin produced by *B. plantarii*, at 50 µg/ml (Sigma-Aldrich, St. Louis) and colonies were enumerated 2 days after incubation at 27°C. **C**, Microscopic observation of *B. plantarii* in cross sections (15 µm thick) of the inoculated rice seedlings. (a), (d), and (g): Coleoptiles in healthy wild-type seedlings. (b), (e), and (h): Wild-type coleoptiles inoculated with 1×10^6 CFU/ml. (c), (f), and (i): Coleoptiles from inoculated transgenic line 2. Black arrowheads show the aerenchyma. Red arrowheads show the immature stomata on the surface of coleoptiles. Green arrowheads show bacteria accumulated in the intercellular spaces. White arrowheads show the nonspecific absorption of the bacteria at the cut sites that developed with coleoptile splits at the growth stage. All sections were stained by Stoughton's double staining method (Stoughton 1930), making the bacteria appear purple.

tem (Roshe, Mannheim, Germany) and following the manufacturer's instructions. Sequences of the primers for the *Osthi1* common probe (Fig. 1) are 5'-GCAAAGAGTTGTTGCCCCAT-CC-3' and 5'-GGTATGGTGAACGTAAGG-3'. Those for the 3' UTR probe of *Osthi1* are 5'-AGCAAGTGCAACATGCAAGC-3' and 5'-CTTACGCAATGATATCATCA-3'.

Cloning of oat leaf thionin genes.

The DNA fragment corresponding to the thionin gene was amplified from oat genomic DNA by PCR using the primers 5'-GTTCTCTGCAGTCATACTGGGTTTAGTTCTGGAGC-3' and 5'-GTAGTCTAGAATTCAGTTTAGGATAGTC(A,C)CT-AG(G,A)GC-3' under the following conditions: denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and polymerase extension at 72°C for 2 min, for 30 cycles. The PCR fragments were subcloned into pUC18 by a standard method and were used for cDNA screening as probe. Poly (A) RNA was isolated from dark-grown etiolated oat seedlings using the FastTrack 2.0 kit (Invitrogen, Groningen, The Netherlands). A cDNA library was constructed from 5 µg of poly (A) RNA using the TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotec, Buckinghamshire, U.K.). cDNAs were ligated into λgt10 vector and then packaged into phage. Plaque hybridization was performed with the DIG nonradioactive nucleic acid labeling and detection system (Roshe), following the manufacturer's instructions.

Construction of the introduced gene.

We amplified the oat thionin cDNA by PCR using the primers 5'-CCAAGGATCCATGGGAAGTATCAAAGGTCTTAA-G-3' and 5'-GCATGAGCTCTTAGGCTGCAACTGATGCA-CG-3' in which *Bam*HI and *Sac*I sites, respectively, were included. A binary vector, pMLH7133 (Mitsuhara et al. 1996), was digested with *Bam*HI and *Sac*I and the products ligated with the PCR product.

Generation of transgenic rice plants.

Agrobacterium tumefaciens EHA101 (a gift from E. Hood, Prodigene, TX, U.S.A.) was transformed with pMLH7133 as described by Holsters and associates (1978). Transformation of rice (cv. Chiyohonami) was performed as described by Tanaka and associates (2001) or Hiei and associates (1994), with some modifications.

Immunoblot analysis.

For localization of expressed Asthi1 protein, coleoptiles from 1-week-old transgenic line 2 were homogenized and fractionated to soluble and insoluble fractions after centrifugation at 10,000 × g for 5 min. Insoluble fraction was washed three times with 50 mM phosphate buffer, pH 7.0, containing 0.1% Triton-X100 to remove thylakoid membranes, and soluble proteins were washed according to Reimann-Philipp and associates (1989). The resulting insoluble fraction mainly included the cell walls. Immunoblot analysis was performed as described by Eppele and associates (1997).

ACKNOWLEDGMENTS

This work was partly supported by the research project COE (center of excellence) program of the National Institute of Agrobiological Science by Japan Science and Technology Corporation, and a grant-in-aid for scientific research on priority area (A) by the Ministry of Education, Science and Culture.

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