Lysigenous aerenchyma, which is created by cell death and lysis of cells in the root cortex, is essential for the internal transport of oxygen from shoots to roots of rice (*Oryza sativa*) and other gramineous plants under waterlogged conditions. In rice roots, the expression of *MT1a*, *MT1b*, *MT1c* and *MT1Ld* were higher than those of the other *MT* genes. In the root cortex, where aerenchyma forms exclusively, the expression of *MT1a*, *MT1b* and *MT1Ld* was reduced prior to aerenchyma formation. These findings suggest that ROS accumulation in the cortex is induced by downregulation of *MT* genes, which is needed for aerenchyma formation in rice roots.

Metallothionein (MT) is a small, cysteine-rich protein that plays a role in metal homeostasis. Plant MTs were classified into four subfamilies (type/class 1, 2, 3 and 4) based on a study of MTs in Arabidopsis. Several lines of evidence show that plant MTs act as ROS scavenging enzymes. Indeed, rice MT2b and cotton (*Gossypium hirsutum*) MT3a have high antioxidative activities against O$_2^\cdot$ and hydroxyl radicals in *vitro*. Moreover, H$_2$O$_2$ accumulation in leaves, as well as growth retardation of tobacco (*Nicotiana tabacum*) plants in response to NaCl treatment is alleviated by overexpression of rice MT1a (OsMT1e). MTs have also been implicated in PCDs in plants. In rice, knockdown of *MT2b* expression was found to promote epidermal cell death in stems and to accelerate H$_2$O$_2$-mediated aerenchyma formation in the internodes. In maize, the expression level of *MT1* in the root cortex was found to decrease during aerenchyma formation under waterlogged conditions. These findings suggest that MTs have a role in determining the fate of cells in roots during inducible aerenchyma formation.

Previous studies reported 9 *MT* genes, 11 *MT* genes and 13 *MT* genes in the rice genome, for a total of 14 unique *MT* genes (Table 1). However, our search of the rice genome annotation databases revealed that rice genome has 15 *MT* genes, that is, we identified one more *MT* gene (*MT1Ld*) (Table 1). Our phylogenetic analysis of the predicted amino acid sequences of the 15 *MT* proteins (Fig. 1) classified them into four types (1–4) (Table 1). The four types were homologous to the four types described by Cobbett & Goldsbrough. One of the type 2 MTs (MT2La) lacks an N-terminal cysteine-rich motif, so that it might not have metal binding or ROS scavenging activities.

### ABSTRACT

Under waterlogged conditions, roots of gramineous plants form lysigenous aerenchyma (internal gas spaces) by inducing the death of cortical cells. Rice (*Oryza sativa*) roots induce aerenchyma formation through ethylene- and reactive oxygen species (ROS)-mediated signaling. Metallothionein (MT) is a small, cysteine-rich protein that acts as a ROS scavenger. In rice roots, the expression of *MT1a*, *MT1b*, *MT1c* and *MT1Ld* were higher than those of the other *MT* genes. In the root cortex, where aerenchyma forms exclusively, the expression of *MT1a*, *MT1b* and *MT1Ld* was reduced prior to aerenchyma formation. These findings suggest that ROS accumulation in the cortex, which is induced by downregulation of *MT* genes, is needed for aerenchyma formation in rice roots.

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### Keywords

Laser microdissection; lysigenous aerenchyma; metallothionein (MT); reactive oxygen species (ROS); rice (*Oryza sativa* L.)
In rice (cv. Shiokari) roots, inducible aerenchyma formation starts at 24 to 36 h and peaks at 48 h after the transfer to stagnant conditions.13 To identify MT genes highly expressed in rice roots during inducible aerenchyma formation, absolute transcript levels of the 15 MT genes at 10 mm (±2 mm) from the tips of adventitious roots were investigated. Among these genes, four type 1 MT genes (MT1a, MT1b, MT1c and MT1Ld) had the highest transcript levels under aerated conditions (Fig. 2A). Moreover, the transcript levels of each of these genes except MT1c dramatically decreased under stagnant conditions (Fig. 2A). In a time-course analysis, the expression levels of MT1a and MT1b started to decrease at 12 h under stagnant conditions (Fig. 2B, C). The expression level of MT1c was comparable between aerated and stagnant conditions (Fig. 2D), whereas that of MT1Ld started to decrease at 24 h under stagnant conditions (Fig. 2E). These results indicate that expression of MT1a, MT1b and MT1Ld in rice roots strongly decreased prior to inducible aerenchyma formation.

The central cylinder (CC), cortex (Co), and outer part of the roots (OPR) were isolated from sections at 10 mm (±2 mm) from the tips of adventitious roots by laser microdissection at 36 h under aerated or stagnant conditions. The expression levels of MT1a, MT1c and MT1Ld were highest at the OPR (Fig. 3A, C, D), and those of MT1b were highest at the Co (Fig. 3B). MT1a, MT1b and MT1Ld expression was significantly reduced in all the tissues examined under stagnant conditions (Fig. 3A, B, D), whereas MT1c expression was comparable between aerated and stagnant conditions (Fig. 3C). Under stagnant conditions, expression of MT1a and MT1Ld were higher in the OPR than in the CC and Co (Fig. 3A, D). Moreover, MT1c expression was specific to the OPR (Fig. 3C). These results suggest that ROS scavenging by MT1 proteins can still occur in the OPR, but that it is suppressed in the Co under stagnant conditions.

### Table 1. List of genes encoding metallothionein in rice.

| Group | Gene name | Wong (2004)† | Zhou (2006)‡ | Kumar (2012)§ | RAP_Os IDs * | MSU LOC_Os IDs † | RAPDB_Description †† |
|-------|-----------|-------------|-------------|--------------|--------------|-------------------|----------------------|
| Type 1| MT1a      | OsMT1a      | OsMT-I-1a   | OsMT1e       | Os11g0704500 | LOC_Os11g47809     | Metallothionein-like protein type 1. |
|       | MT1b      | OsMT1b      | OsMT-I-4a   | OsMT1a       | Os12g0570700 | LOC_Os12g38270     | Similar to Metallothionein-like protein type 1. |
|       | MT1c      | OsMT1c      | OsMT-I-4c   | OsMT1d       | Os12g0571100 | LOC_Os12g38300     | Similar to Metallothionein-like protein type 1. |
|       | MT1Ld     | Os12g0568500 | Os12g0568500 | Os12g0568200 | LOC_Os12g38501 | Metallothionein-like protein type 1. |
|       | MT1Lc     | Os12g0567800 | Os12g0568200 | LOC_Os12g38010 | Plant metallothionein, family 15 protein. |
|       | MT1Lb     | Os12g0567800 | Os12g0567800 | LOC_Os12g38010 | Plant metallothionein, family 15 protein. |
| Type 3| MT3a      | OsMT3a      | OsMT-I-3a   | OsMT3a       | Os10g0200700 | LOC_Os10g10400     | Similar to Metallothionein-like protein type 3. |
|       | MT3b      | OsMT3b      | OsMT-I-3b   | OsMT3b       | Os10g0200800 | LOC_Os10g11320     | Similar to Metallothionein-like protein 3B. |
| Type 4| MT4       | OsMT4       | OsMT-II-1a  | OsMT4        | Os10g0542100 | LOC_Os10g39610     | Plant EC metallothionein-like protein, family 15 protein. |

†Wong et al. Plant Physiol 2004; 135:1447-56.
‡Zhou et al. Biochem Mol Biol 2006; 39:595-606.
§Kumar et al. BMC Plant Biol 2012; 12:107.
*RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).
††MSU LOC_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).
*Descriptions in RAP-DB (IRGSP-1.0).
‡‡OsMT2La lacks the N-terminal cysteine-rich motif.
Expression of RBOHH, whose product converts O$_2$ to O$_2^{-}$, is induced in the Co and OPR under stagnant conditions, although its transcript levels are higher in the Co than in the OPR. In the Co, expression of MT1 genes (e.g., MT1a, MT1b, and MT1Ld) is suppressed under stagnant conditions. The 20 to 40 mm roots of the 10-d-old aerobically grown rice seedlings were subjected to the treatments. The gene encoding transcription initiation factor IIE, TFIIE, was used as a control. Values are means ± SD (n = 3). Significant differences between aerated and stagnant conditions at $P < 0.01$ and $P < 0.05$ (two sample t test) are denoted by ** and *, respectively. The methods are described in more detail by Yamauchi and colleagues.

The Arabidopsis and rice genomes have 7 and 15 MT genes (Fig. 1; Table 1), respectively. So far, there is little information about the subcellular localizations of MTs in plants possibly due to the instability of MT proteins. O$_2^{-}$ in the apoplast is thought to be spontaneously or enzymatically converted to H$_2$O$_2$, which then diffuses into the cytosol. It is thus likely that MTs act as scavengers of ROS in the cytosol, and would stall the ROS-mediated signal transduction that triggers PCD in the OPR. Overexpression of MT1a/OsMT1e reduced H$_2$O$_2$ accumulation in leaves of tobacco plants under high salinity conditions, thereby improving their growth. Alternatively, rice MT1a/OsMT1a has been proposed to indirectly enhance the activities of catalase and peroxidase, which detoxify H$_2$O$_2$. These antioxidant enzymes may also be involved in regulating ROS-mediated aerenchyma formation. Further studies are needed to understand how MT1s scavenge O$_2^{-}$ and/or H$_2$O$_2$ during inducible aerenchyma formation in roots of gramineous plants.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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