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SHORT COMMUNICATION

METALLOTHIONEIN genes encoding ROS scavenging enzymes are down-regulated in the root cortex during inducible aerenchyma formation in rice

Takaki Yamauchi[a,†], Aya Fukazawa[a], and Mikio Nakazono[a,b]

[a]Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, Japan; [b]UWA School of Agriculture and Environment, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, Australia

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 aided by downregulation of *MT1* genes, is needed for aerenchyma formation in rice roots.

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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.[1,2] In rice roots, lysigenous aerenchyma is constitutively formed under aerobic conditions (constitutive aerenchyma formation), and its formation is further induced under oxygen-deficient conditions (inducible aerenchyma formation).[3]

Programmed cell death (PCD), which is energy-dependent cell death, plays essential roles in development and in stress responses of multicellular organisms.[4] Cell collapse during lysigenous aerenchyma formation is characterized as a type of PCD.[5] The gaseous phytohormone ethylene stimulates PCD during inducible aerenchyma formation.[6] Under waterlogged conditions, ethylene accumulates in roots due to its low diffusion rate to the rhizosphere.[7] Moreover, expression of ethylene biosynthetic genes is induced, thereby increasing the activities of ethylene biosynthetic enzymes, during inducible aerenchyma formation.[8–10]

Reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), have a role in ethylene-dependent inducible aerenchyma formation in roots of gramineous plants.[11,12] As further evidence of the importance of ROS in aerenchyma formation, the expression of respiratory burst oxidase homolog (RBOH), a plant enzyme that generates $O_2^{\cdot-}$, increases in the cortex of rice roots under stagnant conditions that mimic waterlogged soil.[13] Moreover, knockout of one RBOH isoform (RBOHH) in rice reduces H_2O_2 accumulation and aerenchyma formation under stagnant conditions.[13]

Metallothionein (MT) is a small, cysteine-rich protein that plays a role in metal homeostasis.[14] Plant MTs were classified into four subfamilies (type/class 1, 2, 3 and 4) based on a study of MTs in Arabidopsis.[15] Several lines of evidence show that plant MTs act as ROS scavenging enzymes. Indeed, rice MT2b[17] and cotton (*Gossypium hirsutum*) MT3a[18] have high antioxidative activities against $O_2^{\cdot-}$ and hydroxyl radicals *in vitro*. Moreover, H_2O_2 accumulation in leaves, as well as growth retardation of tobacco (*Nicotiana tabaccum*) plants in response to NaCl treatment is alleviated by overexpression of rice MT1a (OsMT1e).[19] MTs have also been implicated in PCDs in plants. In rice, knockdown of *MT2b* expression was found to promote epidermal cell death in stems[20] and to accelerate H_2O_2-mediated aerenchyma formation in the internodes.[21] In maize, the expression level of *MT1* in the root cortex was found to decrease during aerenchyma formation under waterlogged conditions.[11] 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[17], 11 MT genes[22] and 13 *MT* genes[19] in the rice genome, for a total of 14 unique 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.[15] 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.

CONTACT Mikio Nakazono nakazono@agr.nagoya-u.ac.jp Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, 464–8601, Japan.
[†]Present address: Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo, Tokyo, Japan.
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Table 1. List of genes encoding metallothionein in rice.

Group	Gene name	Wong (2004)[*1]	Zhou (2006)[*2]	Kumar (2012)[*3]	RAP_Os IDs[*4]	MSU LOC_Os IDs[*5]	RAPDB_Description[*6]
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.
	MT1La		OsMT-I-1b	OsMT1b	Os03g0288000	LOC_Os03g17870	Similar to Metallothionein.
	MT1Lb			OsMT1f	Os12g0567800	LOC_Os12g38010	Plant metallothionein, family 15 protein.
	MT1Lc		OsMT-I-4b	OsMT1c	Os12g0568200	LOC_Os12g38051	Metallothionein-like protein type 1.
	MT1Ld				Os12g0568500	LOC_Os12g38051	Metallothionein-like protein type 1.
	MT1Le			OsMT1g	Os12g0571000	LOC_Os12g38290	Metallothionein-like protein type 1.
	MT2a	OsMT2a	OsMT-I-2a	OsMT2a	Os01g0149800	LOC_Os01g05650	Metallothionein-like protein type 2.
	MT2b	OsMT2b	OsMT-I-2c	OsMT2b	Os05g0111300	LOC_Os05g02070	Similar to Metallothionein.
	MT2c	OsMT2c	OsMT-I-2b	OsMT2c	Os01g0974200	LOC_Os01g74300	Metallothionein-like protein.
	(MT2La)[*7]			OsMT2d	Os01g0149200	LOC_Os01g05585	Hypothetical gene.
Type 3							
	MT3a	OsMT3a	OsMT-I-3a	OsMT3a	Os01g0200700	LOC_Os01g10400	Similar to Metallothionein-like protein type 3.
	MT3b	OsMT3b	OsMT-I-3b		Os05g0202800	LOC_Os05g11320	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.

[*1]Wong et al. Plant Physiol 2004; 135:1447-56.
[*2]Zhou et al. Biochem Mol Biol 2006; 39:595-606.
[*3]Kumar et al. BMC Plant Biol 2012; 12:107.
[*4]RAP Os IDs in Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp/).
[*5]MSU LOC_Os IDs in Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/).
[*6]Descriptions in RAP-DB (IRGSP-1.0).
[*7]OsMT2La lacks the N-terminal cystein-rich motif.

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.

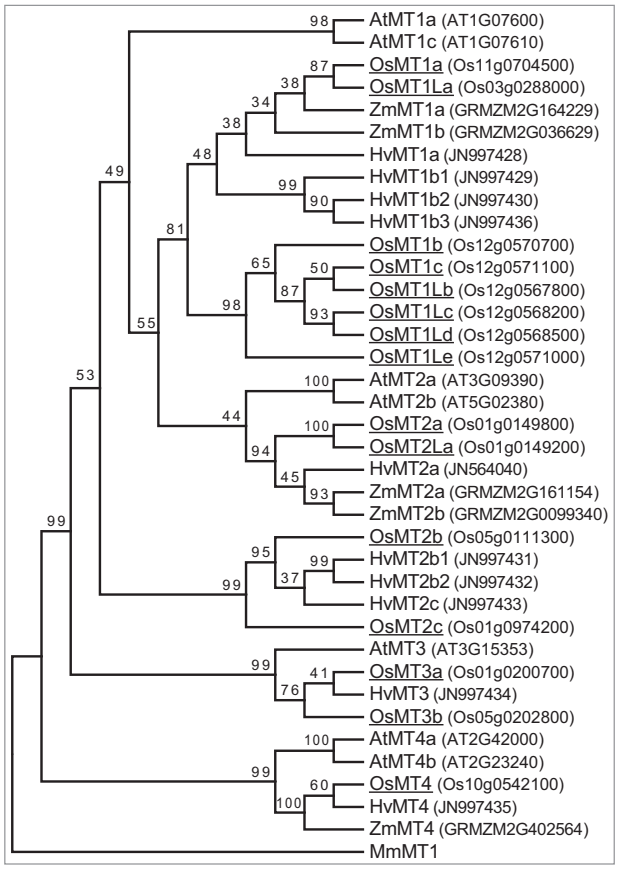

Figure 1. Phylogenetic analysis of MT proteins in plants. The amino acid sequences of the MT proteins were aligned by the ClustalW (MEGA6 package). MEGA6 (http://www.megasoftware.net) was used for construction of the neighbor-joining phylogenetic tree with bootstrap values calculated based on 1000 replicates. MT proteins from monocotyledonous plants [rice (*Oryza sativa*; Os), maize (*Zea mays* ssp. *mays*; Zm), and barley (*Hordeum vulgare*; Hv)], and dicotyledonous plants [Arabidopsis (*Arabidopsis thaliana*; At)] were used for the analysis. The rice OsMT proteins were indicated by underlines. MmMT1 from mouse (*Mus musculus*) was used as the out-group. Accession numbers or IDs of each protein were denoted in the parentheses. The amino acids sequences were obtained from each database as described by Yamauchi and colleagues.[10]

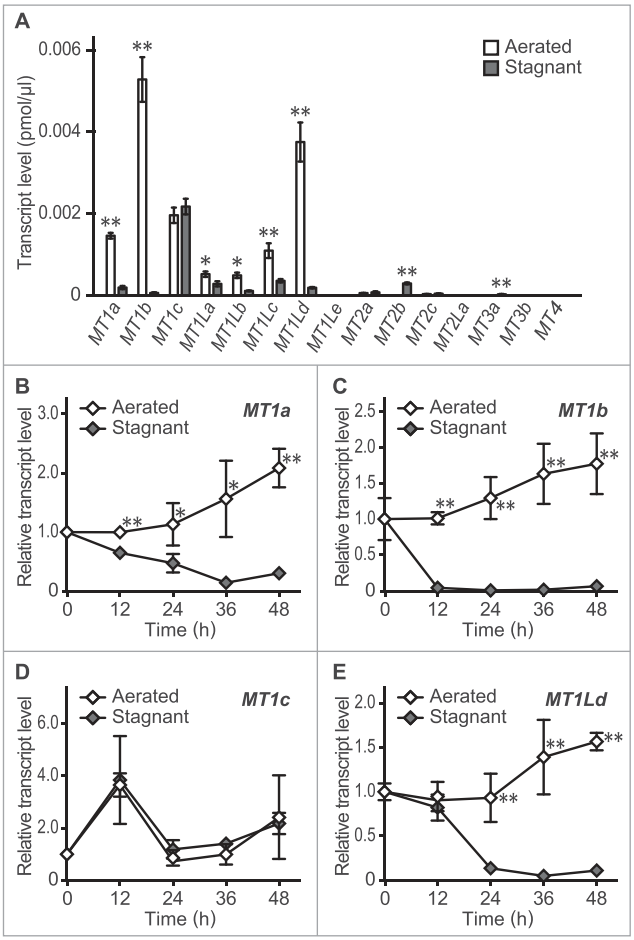

Figure 2. Expression of *MT* genes in rice roots during aerenchyma formation. (A) Absolute transcript levels of *MT* genes at 10 mm (± 2 mm) from the tips of adventitious roots of rice seedlings grown under aerated or stagnant conditions for 36 h. (B-E) Time-course relative transcript levels of *MT1a* (B), *MT1b* (C), *MT1c* (D), and *MT1Ld* (E) at 10 mm (± 2 mm) from the root tips under aerated or 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.[13]

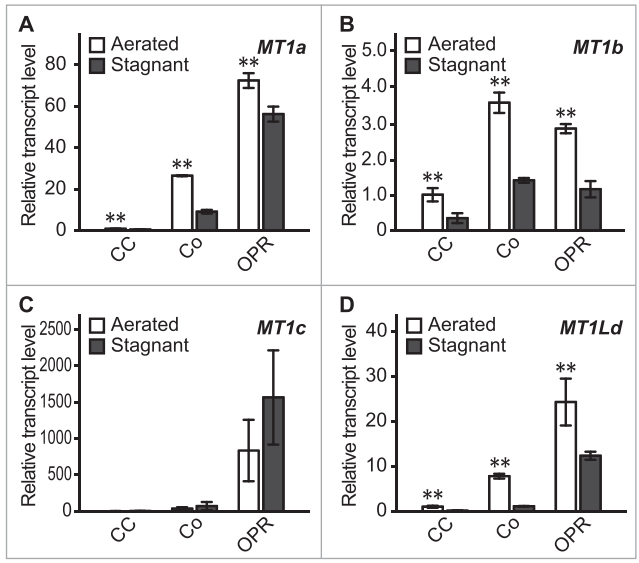

Figure 3. Tissue-specific expression of *MT1* genes in rice roots during aerenchyma formation. (A-D) Relative transcript levels of *MT1a* (A), *MT1b* (B), *MT1c* (C), and *MT1Ld* (D) in the central cylinder (CC), cortex (Co), and outer part of the root (OPR) at 10 mm (± 2 mm) from the root tips under aerated or stagnant conditions for 36 h. The 30 to 50 mm roots of the 20-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.[13]

Expression of *RBOHH*, whose product converts O_2 to $O_2^{\cdot-}$,[23] is induced in the Co and OPR under stagnant conditions, although its transcript levels are higher in the Co than in the OPR.[13] In the Co, expression of *MT1* genes (e.g., *MT1a, MT1b* and *MT1Ld*) is suppressed under stagnant conditions (Fig. 3A, B, D), and thus high levels of ROS generated by RBOHH may be conserved, thereby inducing the PCD (i.e., aerenchyma formation) in the cortical cells. By contrast, ROS generated by RBOHH in the OPR may be reduced by MT proteins. Previously, we found that *RBOHH* expression was induced in all cell types of maize primary roots, but the expression of a gene encoding an MT1 (ZmMT1a in Fig. 1), which is a close homologue of rice MT1a, was specifically reduced in the Co during inducible aerenchyma formation under waterlogged conditions.[11] These findings suggest that MT1-mediated ROS scavenging commonly underlies ROS-mediated inducible aerenchyma formation in gramineous plants.

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.[15] $O_2^{\cdot-}$ in the apoplast is thought to be spontaneously or enzymatically converted to H_2O_2, which then diffuses into the cytosol.[24] 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_2O_2 accumulation in leaves of tobacco plants under high salinity conditions, thereby improving their growth.[19] Alternatively, rice MT1a/OsMT1a has been proposed to indirectly enhance the activities of catalase and peroxidase, which detoxify H_2O_2.[25] These antioxidant enzymes may also be involved in regulating ROS-mediated aerenchyma formation. Further studies are needed to understand how MT1s scavenge $O_2^{\cdot-}$ and/or H_2O_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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