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# Conserved Functions of *Arabidopsis* and Rice CC-Type Glutaredoxins in Flower Development and Pathogen Response

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**ABSTRACT Glutaredoxins (GRXs) are ubiquitous oxidoreductases that play a crucial role in response to oxidative stress by reducing disulfides in various organisms. In planta, three different GRX classes have been identified according to their active site motifs. CPYC and CGFS classes are found in all organisms, whereas the CC-type class is specific for higher land plants. Recently, two *Arabidopsis* CC-type GRXs, *ROXY1* and *ROXY2*, were shown to exert crucial functions in petal and anther initiation and differentiation. To analyze the function of CC-type GRXs in the distantly related monocots, we isolated and characterized *OsROXY1* and *OsROXY2*—two rice homologs of *ROXY1*. Both genes are expressed in vegetative and reproductive stages. Although rice flower morphology is distinct from eudicots, *OsROXY1/2* floral expression patterns are similar to their *Arabidopsis* counterparts *ROXY1/2*. Complementation experiments demonstrate that *OsROXY1* and *OsROXY2* can fully rescue the *roxy1* floral mutant phenotype. Overexpression of *OsROXY1*, *OsROXY2*, and *ROXY1* in *Arabidopsis* causes similar vegetative and reproductive plant developmental defects. *ROXY1* and its rice homologs thus exert a conserved function during eudicot and monocot flower development. Strikingly, overexpression of these CC-type GRXs also leads to an increased accumulation of hydrogen peroxide levels and hyper-susceptibility to infection from the necrotrophic pathogen *Botrytis cinerea*, revealing the importance of balanced redox processes in flower organ development and pathogen defence.**

**Key words: *ROXY1*; glutaredoxin; *Arabidopsis*; rice; flower development; pathogen defence; *Botrytis cinerea*.**

## INTRODUCTION

Glutaredoxins (GRXs) are small (10∼15-kDa) oxidoreductases that catalyse the reduction of disulfide bonds of their substrate proteins in the presence of glutathione (GSH). All GRXs have a CXXC/S active site motif where the cysteines can be used in the reduction reaction via a dithiol (using the two cysteines) or monothiol (involving only the N-terminal cysteine) mechanism (Fernandes and Holmgren, 2004; Buchanan and Balmer, 2005). Several studies of GRX family genes have been conducted so far in *E. coli*, yeast, and mammals, documenting a direct or indirect function of these oxidoreductases in oxidative stress responses (Luikenhuis et al., 1998; Fernandes and Holmgren, 2004; Gallogly and Mieyal, 2007; Herrero et al., 2007).

Based on predicted amino acid sequences and the composition of active site motifs, plant GRXs were divided into three classes: namely the CPYC, CGFS, and CC-type classes. The CPYC and CGFS classes exist in all examined organisms from prokaryotes to eukaryotes, including all investigated plant species, whereas the CC-type class is specific for land plants (Lemaire, 2004; Rouhier et al., 2004). The CPYC class contains GRXs with C[P/G/S][Y/F][C/S] active site motifs, shared by the *E. coli* GRX1 and GRX3, yeast GRX1 and GRX2 as well as the human GRX1 and GRX2. GRXs from the CGFS class, such as GRX3 and GRX4 from *E. coli* and GRX5 from yeast, all contain the strictly conserved CGFS motif (Rouhier et al., 2007). Recent reports support functions for these two ubiquitously occurring GRX classes in responding to oxidative stress and iron–sulfur cluster

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biosynthesis (Cheng et al., 2006; Rouhier et al., 2007; Bandyopadhyay et al., 2008; Cheng, 2008; Rouhier et al., 2008).

The land plant-specific CC-type GRXs contain a CC[M/L][C/S] active site. Recent comparative analysis of evolutionary informative plant species, such as *Physcomitrella patens*, *Pinus taeda*, *Oryza sativa*, *Populus trichocarpa*, and *Arabidopsis thaliana*, revealed that the number of GRXs in the CC-type class expanded during plant evolution (Xing et al., 2006). Contrarily, the number of members of the CPYC and CGFS classes remained relatively constant. For instance, only two CC-type GRXs exist in *Physcomitrella*, whereas 21 were identified in *Arabidopsis*. This observation indicates a possible function for CC-type GRXs in contributing to the evolution of land plants that form organs of a higher complexity (Xing et al., 2006). The only functional data reported thus far for CC-type GRXs demonstrate their participation in two different processes, which do not seem to be connected with one another. Firstly, they exert a crucial role during flower development (Xing et al., 2005; Xing and Zachgo, 2008) and, secondly, they are active in the response to pathogens by affecting the salicylic acid/jasmonate cross-talk (Ndamukong et al., 2007). *ROXY1* and *ROXY2* are two characterized *Arabidopsis* CC-type GRXs genes that control floral organ initiation and differentiation. Mutation of *ROXY1* results in a reduced initiation of petal primordia; in mutant flowers, instead of 4.0, only 2.5 petals are formed on average (Xing et al., 2005). Lack of the *ROXY1* function together with that of *ROXY2*, its closest homolog, reveals their redundant activity during reproductive organ differentiation, as no normal anther lobes develop and microspore production is defective (Xing and Zachgo, 2008). These studies in the model eudicot plant species *Arabidopsis* document crucial functions of CC-type GRXs in floral organ development and indicate the importance of redox processes participating in reproductive development.

The first monocot GRX, belonging to the CPYC subgroup, was isolated from rice (Sha et al., 1997), and, recently, it was shown that its expression is responsive to oxidative stress (Tsukamoto et al., 2005). However, the function of the plant-specific CC-type GRXs in monocots is unknown. For a comparative functional analysis, we chose the monocot model species rice. This grass species forms one lemma and one palea in the first floral whorl and two lodicules in the second whorl; each organ reveals a distinct, grass-specific morphology and function, and contributes to the formation of a grass flower differing from the typical eudicot flower. We analyzed the function of the two rice GRXs *OsROXY1* and *OsROXY2* that share the highest sequence similarity to *ROXY1*. Expression, complementation and overexpression studies support a conserved function for *ROXY1* homologs in floral organ development in these distantly related monocot and eudicot model species. Furthermore, overexpression plants allow an intriguing insight into a dual role of GRXs that most likely participate in signalling mechanisms crucial for flower development as well as for pathogen response.

# RESULTS

## Isolation of *ROXY1* Homologs from Rice

Recently, analysis of the *roxy1* and *roxy1 roxy2* mutants revealed a novel function for GRXs in flower development and showed that these GRXs participate in petal morphogenesis and anther development (Xing et al., 2005; Xing and Zachgo, 2008). *ROXY1* and *ROXY2*, together with 19 other GRXs, belong to the class of CC-type GRXs that have thus far only been identified in land plants (Xing et al., 2006). To investigate the function of the closest *ROXY1* homologs from a distantly related monocot model species, rice (*Oryza sativa*) was selected that comprises 17 CC-type GRXs (Xing et al., 2006).

Based on amino acid sequences of the CC-type GRXs from *Arabidopsis* and rice, a phylogenetic tree was constructed that shows that these GRXs cluster in four clades (Figure 1A). Clade I consists of eight *Arabidopsis* genes, including a five-gene cluster on chromosome 4. Clade II contains 10 rice genes, of which also four genes cluster together on chromosome 11. Clade III represents the *ROXY1* subgroup and includes the ROXY1 and ROXY2 proteins that share an overall amino acid identity of 71% (Xing and Zachgo, 2008). The two rice CC-type GRXs, Os04g32300 and Os02g30850, are the closest rice homologs to *ROXY1* and *ROXY2* and were named *OsROXY1* and *OsROXY2* in this study (Figure 1A). Clade IV comprises four *Arabidopsis* genes and five genes from rice.

The predicted ORFs of OsROXY1 and OsROXY2 are both 135 amino acids long and share 86% identity with each other. Amino acid identities with ROXY1 are 63 and 61%, respectively. Like ROXY1 and ROXY2, OsROXY1 and OsROXY2 are encoded by only one exon, and contain a CCMC active site motif and a highly conserved C-terminus. Contrarily, the N-termini are more divergent among ROXY1, ROXY2, OsROXY1, and OsROXY2 (Figure 1B). A conserved glycine (G110), shown to be indispensable for the ROXY1 activity and likely involved in GSH binding (Xing and Zachgo, 2008), is also found in OsROXY1 and OsROXY2 (Figure1B), suggesting a similar function in the rice homologs.

## *OsROXY1* and *OsROXY2* Are Expressed during Inflorescence and Floret Development

To determine the expression of *OsROXY1* and *OsROXY2* in different organs, RT–PCR experiments were performed. *OsROXY1* and *OsROXY2* transcripts can be detected at different levels in vegetative organs such as roots, stems and leaves as well as in rice inflorescences, the spikelets (Figure 2A). High levels of the *OsROXY1* transcript accumulate in roots and spikelets, with lower RNA levels being detectable in stems and leaves (Figure 2A).

To determine the expression in floral tissues in detail, in-situ hybridization experiments were performed with both rice genes. For comparison of the CC-type GRX expression in a monocotyledonous and eudicotyledonous species, schematic flower diagrams of *Arabidopsis* and rice are depicted in Figure 2B. The main rice inflorescence comprises many secondary