ROXY1, a member of the plant glutaredoxin family, is required for petal development in *Arabidopsis thaliana*

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Summary

We isolated three alleles of an *Arabidopsis thaliana* gene named *ROXY1*, which initiates a reduced number of petal primordia and exhibits abnormalities during further petal development. The defects are restricted to the second whorl of the flower and independent of organ identity. *ROXY1* belongs to a subgroup of glutaredoxins that are specific for higher plants and we present data on the first characterization of a mutant from this large *Arabidopsis* gene family for which information is scarce. *ROXY1* is predominantly expressed in tissues that give rise to new flower primordia, including petal precursor cells and petal primordia. Occasionally, filamentous organs with stigmatic structures are formed in the second whorl of the *roxy1* mutant, indicative for an ectopic function of the class C gene *AGAMOUS* (*AG*). The function of *ROXY1* in the negative regulation of *AG* is corroborated by premature and ectopic *AG* expression in *roxy1-3 ap1-10* double mutants, as well as by enhanced first whorl carpeloidy in double mutants of *roxy1* with repressors of *AG*, such as *ap2* or *lug*. Glutaredoxins are oxidoreductases that oxidize or reduce conserved cysteine-containing motifs. Mutagenesis of conserved cysteines within the ROXY1 protein demonstrates the importance of cysteine 49 for its function. Our data demonstrate that, unexpectedly, a plant glutaredoxin is involved in flower development, probably by mediating post-translational modifications of target proteins required for normal petal organ initiation and morphogenesis.

Key words: *ROXY1*, Glutaredoxin, Flower development, Perianth, *Arabidopsis*

Introduction

Flower formation in higher plants serves as a model system with which to understand the regulatory processes controlling initiation and morphogenesis of organs that form a complex reproductive structure. In *Arabidopsis*, flowers develop four different organ types that are arranged in concentric whorls. In the first whorl, four sepals are formed; in the second whorl, four petals are initiated alternately. As the reproductive organs, six stamens develop in the third whorl and two carpels fuse in the fourth whorl, generating the gynoecium. The control of floral organ identity has been intensively studied in the past 15 years. Analysis of floral homeotic mutants led to the proposal of a simple genetic model, explaining how three groups of regulatory genes (class A, B and C genes) control alone or by interaction the organ identity of the four floral whorls (Coen and Meyerowitz, 1991). Class A genes control sepal organogenesis, class A and B genes together petal formation. Class B and C genes govern stamen and class C alone, carpel development. Recently, newly identified genes were incorporated encoding proteins that interact with the class A, B and C proteins in larger complexes (Honma and Goto, 2001). Interactions between the class A genes *APETALA2* (*AP2*) and *APETALA1* (*AP1*), the latter also functioning in the specification of floral meristem identity, and the class C gene *AGAMOUS* (*AG*), refine their expression pattern during later stages. *AG* represses *AP1* in whorl 3 and 4 and *AP2* functions in repressing *AG* in the first and second whorl, as indicated by ectopic *AG* expression in *ap2* mutants (Gustafson-Brown et al., 1994; Drews et al., 1991). Additionally, further genes participate in the repression of *AG*, among which *LEUNIG* (*LUG*) and *SEUSS* (*SEU*) encode for proteins that act also as transcriptional repressors in yeast and animals (Conner and Liu, 2000; Franks et al., 2002).

Whereas the ABC genes control the identity of the organs, initiation of organ primordia is determined before the onset of the class A, B and C activity. For example, in mutants from the class B gene *PISTILLATA* (*PI*), feminization of the third whorl organs becomes visible only after initiation of the respective primordia (Hill and Lord, 1989). Primordia formation requires the proper allocation of progenitor cells and regulated control of cell divisions, processes that are presumably under genetic control. Petal primordia are initiated when cells in the L2 layer of the floral primordium divide periclinally, rather than anticlinally (Hill and Lord, 1989). Several mutants have recently been characterized in *Arabidopsis* that disturb primordia initiation and thus develop an either increased or decreased floral organ number. In *clavata* mutants, floral organ number is increased because of an enlargement of the floral meristem (Clark et al., 1993). In *perianthia* mutants, five organs are formed in the first three whorls (Running and Meyerowitz, 1996), indicating that *PAN* normally establishes a tetramerous whorl architecture. *UFO* encodes a F-box protein

required for the activation of the class B gene expression, and strong *ufo* mutants resemble class B mutant flowers (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Additionally, *UFO* is also involved in the early control of petal outgrowth, probably by counteracting inhibitory effects non-autonomously exerted by *AG* (Durfee et al., 2003; Laufs et al., 2003). Two other genes have been shown to affect predominantly initiation of petal development: *PETAL LOSS* (*PTL*), a trihelix transcription factor (Brewer et al., 2004); and *RABBIT EARS* (*RBE*), a SUPERMAN-like zinc finger protein (Takeda et al., 2004). In *ptl* mutants, the number of petals is reduced and if they develop, the orientation is altered and the size reduced. *PTL* is not expressed in petal primordia, but between sepal primordia, indicating a rather indirect effect on petal development (Brewer et al., 2004). Similarly, petal initiation in *rbe* mutants is disturbed and in a strong mutant only a reduced number of filamentous organs is formed in the second whorl. *RBE* is expressed in petal precursor cells and petal primordia and controls second whorl organ development independently of organ identity (Takeda et al., 2004). As *ptl* and *rbe* mutants still form aberrant petals, primordia initiation seems to be a complex process that is regulated by additional factors.

Here, we report on *roxy1* mutants that displays defects in petal initiation and also abnormalities during later petal development. *ROXY1* encodes a glutaredoxin (GRX), of which many isoforms exist in different organisms, such as *E. coli*, yeast, animals, humans and plants. GRXs are oxidoreductases that are involved in many different cellular processes, mainly in the response to oxidative stress (Fernandes and Holmgren, 2004). Our data show a novel function during the regulation of flower development for a GRX that belongs to a subgroup specific for higher plants.

Materials and methods

Plant materials and growth conditions

The three recessive alleles of *roxy1-1*, *1-2* and *1-3* were obtained from the GABI-KAT collection (Rosso et al., 2003). Mutants and wild-type plants are all Columbia (Col) ecotypes. Seeds for *ag-1*/+ in Landsberg *erecta* (L*er*); *ap3-3*/+ in L*er*; *ap1-10* in Col; *ap2-5* in Col; *lug-1* in L*er* and *ptl-1* in Col (Griffith et al., 1999) were obtained from the Nottingham Arabidopsis Stock Centre. The *rbe-2* allele is from the sequence-indexed T-DNA insertion library from the Salk Institute Genomic Analysis Laboratory (accession number SALK_037010). Seeds of *AG-I::GUS* transgenic plants contain the pAG-I::GUS plasmid, driving *GUS* reporter expression identical to wild-type *AG* expression (Sieburth and Meyerowitz, 1997). Seeds were sown on soil and treated at 4°C for 2-4 days. Plants were grown in the greenhouse at 21-23°C under long day conditions (16 h light).

Microscopy

Inflorescences or flowers from GFP transgenic plants were observed under the binocular microscope LEICA MZ-FLIII. Images were made using a digital camera (KY-F70). For scanning electron microscopy (SEM), we followed the protocol described by Zachgo et al. (Zachgo et al., 1995).

Molecular cloning of *ROXY1* gene

To identify the flanking sequence adjacent to the T-DNA in *roxy1-1*, genomic DNA was prepared from the mutant and digested with *Dra*I. An adaptor (a mix of two oligonucleotides: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and 5′-ACCAGCCC-3′) was ligated to the ends of digested genomic DNA using ligase from Promega (Mannheim, Germany). First, PCR was carried out with adaptor primer 1 (5′-GTAATACGACTCACTATAGGG-3′) and T-DNA left border primer (5′-ACGACGGATCGTAATTTGTCG-3′); then, a second PCR was performed with the nested adaptor primer 2 (5′-ACTATAGGGCACGCGTGGT-3′) and a nested left border primer (5′-ATATTGACCATCATACTCATTGC-3′). Expand High fidelity polymerase (Roche, Mannheim, Germany) was used in the reaction. PCR products were gel purified and sequenced. The cDNA cloning was performed using the 5′/3′ cDNA RACE kit (Roche, Mannheim, Germany) as indicated in the manufacturer's instructions.

Generation of double mutants

To obtain double mutants, *roxy1-3* was crossed either with homozygous (*ptl-1*; *rbe-2*; *ap1-10*; *ap2-5*; *lug-1*; *ap3-3* and *ufo-2*) or heterozygous (*ag-1*) lines. All F1 plants produced wild-type flowers. Novel phenotypes were identified in segregating F2 populations and genotypes of double mutants were confirmed by PCR.

RNA isolation and RT-PCR analysis

Total RNA was extracted with the RNeasy kit (Qiagen). Different organs of 6-week-old plants were used, except for the root, which originates from a three-week liquid culture. First strand cDNA was synthesized from 2 μg of total RNA using 200 U SuperScript™ II Reverse Transcriptase (Invitrogen) according to the supplier's instructions. PCR reactions (58°C annealing temperature with 40 seconds extension for 31 cycles) were carried out using *ROXY1*-specific primers from the 5′ UTR (100 bp upstream of the start codon; 5′-TCGCGAATTCCCAACAAACTTTAGCCAATCCCTC-3′) and from the 3′ UTR (220 bp downstream of the stop codon; 5′-ACGCGAATTCTGTTTACTATTATGATTTAATGAGAGC-3′). PCR products were loaded on an ethidium bromide-stained 1.2% (w/v) agarose gel. Images were made with the PhosphorImager Typhoon 8600 (Amersham Biosciences). 18S rRNA (Katz et al., 2004) was used as an internal control.

Complementation experiment

A 4468 bp *ROXY1* genomic fragment was amplified using the Expand High fidelity PCR system (Roche) with the gene-specific primers 5′-GCGTAGATCTCAATAGTCGAGGATCATTCGGAGTGC-3′ and 5′-GTACGCTAGCCTTCAAGCTTCACCTATCTCACTCATAGTC-3′, digested using *Bgl*II and *Nhe*I and subcloned into pGSA1252 (www.chromdb.org/plasmids/pGSA1252.html). A recombinant plasmid containing the correct coding sequence was transformed into the *Agrobacterium tumefaciens* strain GV3101, and introduced into *roxy1-3* mutants using the floral infiltration method (Clough and Bent, 1998). T1 transformants were obtained by BASTA selection.

In situ RNA hybridization and histochemistry

RNA in situ hybridization was performed as previously described by transcription from PCR templates containing a T7 polymerase binding site at the 3′ end (Zachgo, 2002). To avoid cross-hybridization with other GRXs, the *ROXY1* antisense probe was prepared using a unique 245 bp fragment from the *ROXY1* 5′ end (5′-GCGGAATTAACCCTCACTAAAGGGCCAACAAACTTTAGCCAATC-3′ and 5′-GCTCGTAATACGACTCACTATAGGGCACGTGCTCACGCTGAAGATC-3′) and a unique 225 bp fragment from the *ROXY1* 3′ end (5′-GCGGAATTAACCCTCACTAAAGGGTCTGATCCCTTCCTCTGCTTTC-3′ and 5′-GCGCGTAATACGACTCACTATAGGGCTGTTTACTATTATGATTTAATG-3′) as templates. Control experiments on *roxy1-3* inflorescences confirmed probe specificity. The *AG* antisense probe was generated by PCR with the primer pair: 5′-GTGGAATTAACCCTCACTAAAGGGAGCTTACGAGCTCTCTGTTCTTTG-3′ and 5′-GTGCGTAATACGACTCACTATAGGGCAATTCACTGATACAACATTCATGG-3′. T7 RNA polymerase (Roche) was used for

in vitro transcription. GUS staining was done as described by Müller et al. (Müller et al., 2001).

ROXY1::ROXY1-GFP plasmid construction

The *GFP* ORF with stop codon was amplified using primer 5′-TAGGCGCGCCCATGGGTAAAGGAGAAGAACTTTTC-3′ and 5′-GAACGAGCTCGTCGACCGGTGAGGATCCTTATTTGTATAG-3′, and subcloned into the binary vector pGPTV-HPT. The regions 3912 bp upstream from the *ROXY1* stop codon TGA and 445 bp downstream of the stop codon were further amplified using primer pairs: 5′-TGCGTCTAGAACTACATAAAAGCCTTTCAG-3′ and 5′-TAGGCGCGCCGAGCCAGAGAGCGCCGGCGTCTTTGAGAA-3′; and 5′-GCCGACCGGTTCCCTTCCTCTGCTTTCTTTTTTCTTTTC-3′ and 5′-GTACGAGCTCCTTCAAGCTTCACCTATCTCACTCATAGTC-3′. The two fragments were fused up- and downstream to the GFP ORF, respectively. The clone, selected for transformation into plants, was confirmed by sequencing.

Site-directed mutagenesis

Point mutations in specific amino acids of ROXY1 were introduced by a PCR-mediated mutagenesis strategy as described by Rouhier et al. (Rouhier et al., 2002). To introduce a serine/cysteine exchange mutation, pairs of mutagenic oligomers (mutagenic bases are written in lowercase) were used: 5′-TGATCTTCAGCGTGAGCACGTcCTGC-3′ and 5′-GCAGgACGTGCTCACGCTGAAGATCA-3′ (C49S); 5′-TGATCTTCAGCGTGAGCACGTGCTGCATGTcCCA-3′ and 5′-TGGgACATGCAGCACGTGCTCACGCTGAAGATCA-3′ (C52S); and 5′-CCTCATTCGTCTCCTCGGCTcCT-3′ and 5′-AGgAGCCGAGGAGACGAATGAGG-3′ (C90S). After sequencing, the three mutated *ROXY1* genes were cloned into the pBAR-35S vector (Müller et al., 2001) and transformed into the *roxy1-3* mutant. Complementation was analyzed in transgenic T1 plants, obtained by BASTA selection.

Results

ROXY1 is required for petal initiation and morphogenesis

In *Arabidopsis*, four petals develop in the second whorl of the flower. Two petal primordia are initiated next to the two lateral stamen primordia and develop initially slightly slower than the stamens. Petal growth progresses rapidly when organ differentiation starts with the formation of a stalked base and flattened blade (Fig. 1E). We identified a mutant, named *roxy1*, displaying severe defects during petal development in the T-DNA GABI-KAT collection (Fig. 1B-D). Three different *roxy1* alleles were investigated that show similar phenotypes (Table 1). The petal organ number is decreased and varies between 0 and 4 petals. On an average, mutant flowers form about 2.5 petals instead of 4.0 petals (Fig. 1B-D; Table 1). SEM analysis shows that absence of petals is due to lack of petal primordia initiation. Either one or two of the petal primordia initiated next to the lateral stamens can be missing in *roxy1* mutants (Fig. 1G,H). Analysis of 100 mutant flowers did not reveal a positional bias: ab- and adaxial petal organogenesis were affected equally. Furthermore, strength of the defect was not dependent on the position of the flowers in the inflorescence. These observations indicate that the *ROXY1* function is required for the initiation of petal primordia. Further petal development is often delayed and abnormal. In wild-type flowers, petal primordia start to differentiate into a stalked structure at stage 9 (Smyth et al., 1990) and commence rapid growth that leads to the formation of a tongue-shaped petal. The size of *roxy1* petals is frequently reduced and blades often

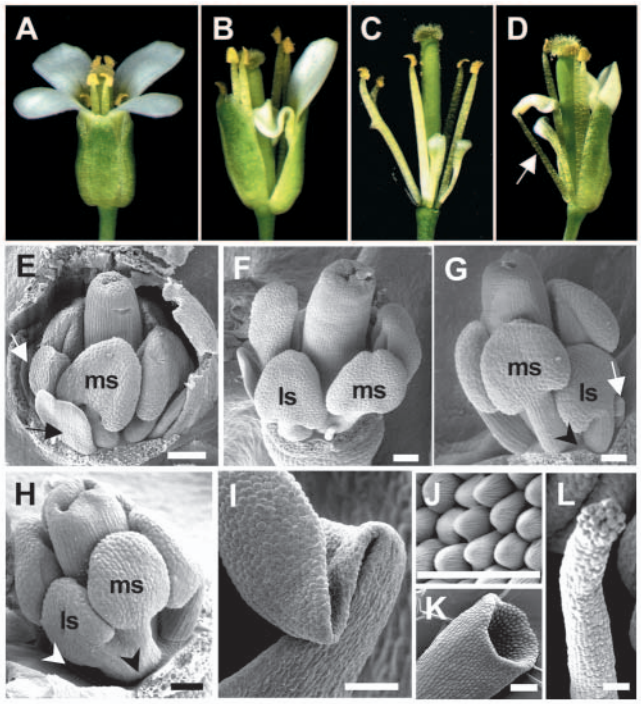

Fig. 1. The *roxy1* phenotype. (A) Wild-type flower. (B-D) Phenotypes of *roxy1-3* flowers. Petal number is reduced and the size often smaller, forming an abnormal blade and base. Arrow in D marks a filament-like stalk of a petal with a reduced blade. (E-L) SEM photographs of wild-type (E) and *roxy1-3* mutant flowers (F-L) after onset of organ differentiation (stages 8-10) (Smyth et al., 1990). Sepals were partially or completely removed to reveal inner organs. Arrows indicate wild-type and abnormal petals in the second whorl between the medial (ms) and lateral (ls) stamens. Petal primordia often fail to be initiated, indicated by arrowheads (G,H). (I) *roxy1-3* petal showing abnormal folding at the tip. (J) Conical cells, similar to wild-type cells, are formed in the inner petal epidermis at the tip of the folded petals. (K,L) Occasionally, *roxy1-3* second whorl organs form fused (K) or filamentous structures that carry cells resembling stigmatic papillae at their tips (L). Scale bar: 50 μm.

fold inwards such that petals are not protruding out of the flower (Fig. 1B,I). The identity of the differentiated epidermal cell layers of the folded petals is not affected by *roxy1* mutations. Conical cells, typical for the adaxial (inner) epidermal cell layer are formed at the tip of these petals (Fig. 1J). Occasionally, filamentous structures emerge in the second whorl that develop on their tips stigmatic papillae, a carpeloid feature (Fig. 1F,L). Rarely, petals form spoon- or tube-shaped organs (Table 1, Fig. 1K). These data demonstrate that *ROXY1* has an additional function during petal development by maintaining normal petal morphogenesis throughout later stages of petal development. As similar flower phenotypes are displayed by the *ptl* and *rbe* mutants (Griffith et al., 1999; Takeda et al., 2004), we excluded the possibility that *roxy1* is allelic to *ptl* and *rbe* (data not shown). To a lesser degree, *roxy1* mutations also affect stamen development. Rarely, fusions of lateral and medial stamens were observed predominantly occurring between their filaments (Fig. 1H). Overall, the number of stamens was reduced by about 10%, mainly caused by lack of lateral stamen formation (Table 1). Sepal

Table 1. Comparison of organ formation in mature wild-type and *roxy1* mutant flowers

Organ type	Wild type	*roxy1-1*	*roxy1-2*	*roxy1-3*
Normal petal	100%	46.6%	30.7%	39.3%
Folded petal	0%	27.4%	43.0%	43.0%
Small petal	0%	17.1%	13.1%	12.4%
Filamentous structure	0%	5.6%	7.8%	0.8%
Stamenoid petal	0%	0.8%	2.9%	2.5%
Others*	0%	2.6%	2.5%	2.2%
Number of second whorl organs	400	234	244	267
Number of stamens	590	528	523	509

100 mature flowers were analyzed for each genotype.
*Petals forming tube- or spoon-like organs.

development was normal, although at higher temperature (26°C), stigmatic structures were occasionally produced at the tips of these organs (data not shown). Fourth whorl organogenesis was not affected.

Isolation of the *ROXY1* gene

The *roxy1-1* mutant was identified in a T3 population of the GABI-KAT T-DNA tagging collection (Rosso et al., 2003). For isolation of the mutation causing the *roxy1-1* phenotype, genomic sequences flanking the T-DNA were obtained by adapter ligation. Blast searches using the whole *Arabidopsis* genome revealed that the T-DNA was inserted into the promoter region of a gene with the Accession Number At3g02000. At least two T-DNA borders connected and inserted in the same position, 175 bp upstream of the putative At3g02000 start codon (Fig. 2A). Thereupon, two other T-DNA lines, named *roxy1-2* and *roxy1-3*, were identified in the GABI-KAT collection. Reciprocal crosses confirmed that the three mutants are allelic (data not shown) and further work was focused on the *roxy1-2* and *roxy1-3* alleles, as they carry insertions in the coding region of At3g02000 (Fig. 2A). In the *roxy1-2* mutant, the T-DNA insertion was localized in the 5′ end of the coding region, which comprises a single exon. The right T-DNA border was detected at the 5′ end, but neither a left nor a right border could be detected at the 3′ end of the insert, indicating rearrangements. In the *roxy1-3* mutant, the T-DNA inserted in the 3′ end of the coding region and a left border could be identified at the 3′ insertion side (Fig. 2A).

Complementation studies were carried out employing a 4468 bp genomic sequence of At3g02000 spanning over 3600 bp of the promoter region (Fig. 2A). Binary vectors were transformed into *roxy1-3* mutants, and 20 independent transgenic T1 lines were analyzed. In total, 18 lines produced wild-type like flowers and two plants formed mutant-like flowers. These results show that the 4468 bp genomic fragment of At3g02000 is sufficient to complement the *roxy1-3* mutant phenotype.

ROXY1 encodes a protein of the glutaredoxin family

For isolation of the full-length *ROXY1* cDNA, 5′ and 3′ Rapid Amplification of cDNA ends (RACE) reactions were conducted. The 5′ RACE allowed us to locate the transcription initiation site 100 bp upstream of the ATG start codon of translation. By 3′ RACE, three cDNAs with 3′ UTRs of different lengths were isolated. The longest 3′ UTR comprises 220 bp downstream of the TGA stop codon, the other two are

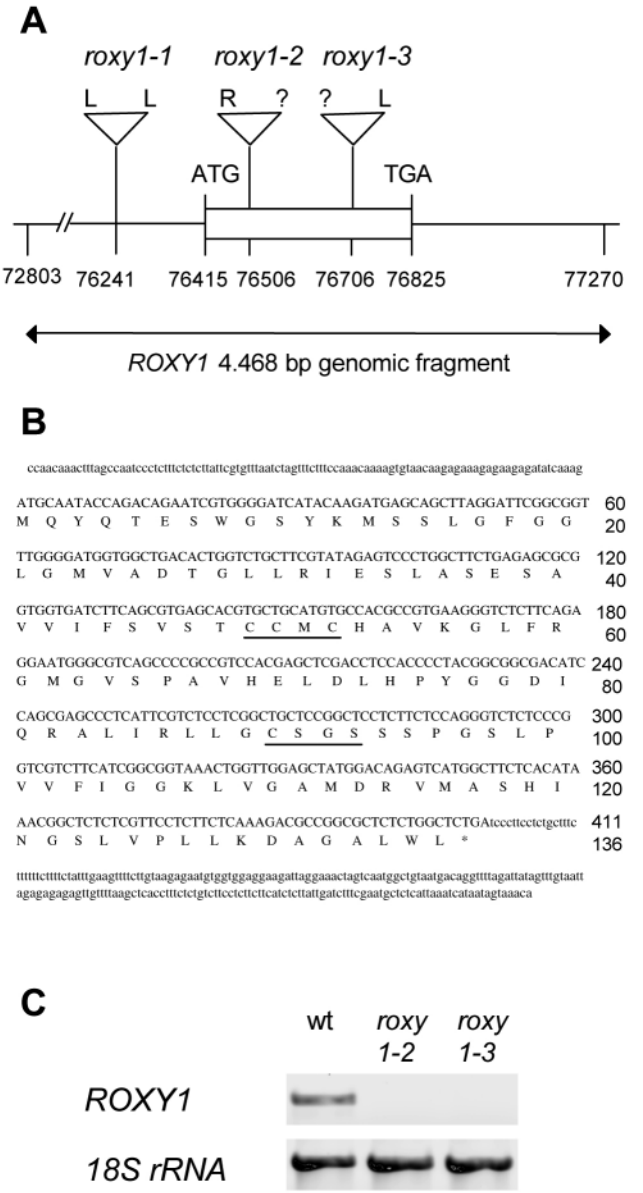

Fig. 2. Isolation of the *ROXY1* gene. (A) T-DNA insertion sites are shown for each allele. The T-DNA insert in *roxy1-1* is located in the promoter, 175 bp upstream of the start codon ATG. *roxy1-2* and *roxy1-3* carry insertions in the single coding exon, 91 and 291 bp downstream of the ATG, respectively. Numbering of coordinates is based on the *Arabidopsis* genomic clone BACF1C9 that contains the Col-0 *ROXY1* gene. Left and right (L and R) T-DNA borders are only indicated where they could be identified. (B) *ROXY1* cDNA and predicted amino acid sequence encoding a 136 amino acid protein (GenBank Accession Number AY910752). The dithiol CCMC and the monothiol CSGS motif are underlined. Locus name is At3g02000. (C) Comparison of *ROXY1* expression in wild-type and *roxy1-2* and *roxy1-3* mutants by RT-PCR. RNA was isolated from inflorescences and primers were used allowing specific cDNA amplification. As a control, reactions with 18S rRNA were carried out.

159 and 147 bp long. By RT-PCR analysis, no *ROXY1* gene expression could be detected in the *roxy1-2* and *roxy1-3* mutants (Fig. 2C), indicating that they are null mutants.

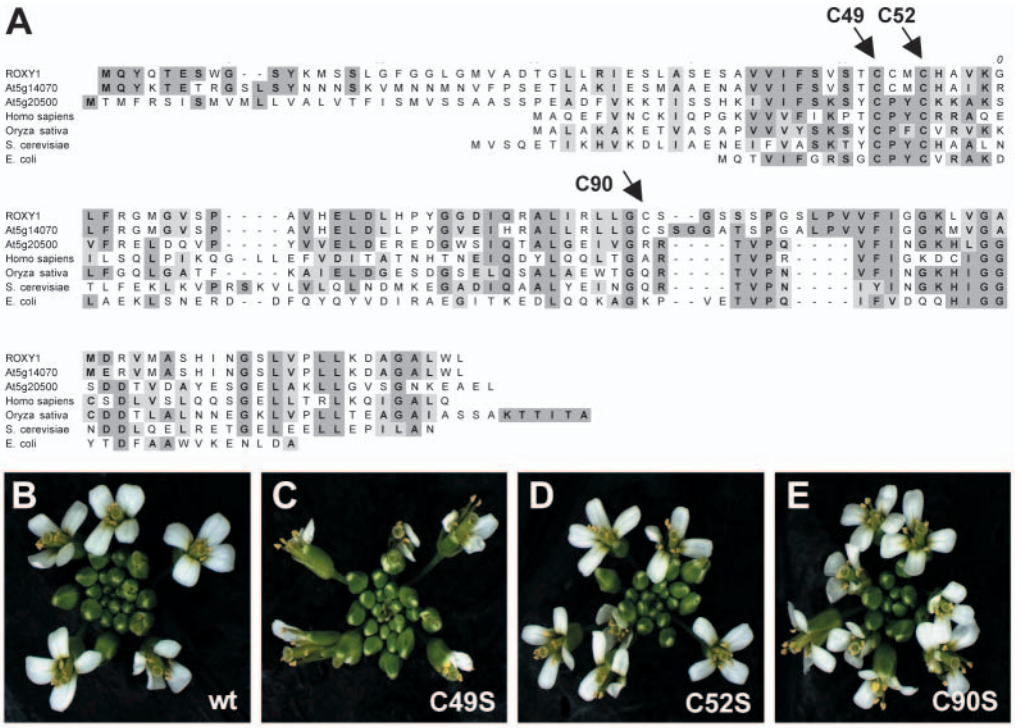

Fig. 3. Contribution of conserved cysteines to the *ROXY1* function in petal development. (A) Alignment of open reading frames from GRXs of different species. Identical amino acids are in dark shading, similar ones in light shading. Two cysteines in the active site CXXC, conserved among all GRXs, are indicated by arrows (C49, C52). Another cysteine at position 90 (C90) is part of a putative monothiol CXXS motif specific to ROXY1. (B-E) Complementation analysis of mutagenized ROXY1 cysteines. *roxy1-3* mutants were transformed with the wild-type (B) and mutagenized *ROXY1* genes, where the three cysteines C49, C50 and C90 were exchanged into serines (C-E) and expressed under the control of the 35S promoter. (B) Wild-type protein complements 41/64 transgenic T1 *roxy1-3* plants. (C) Mutagenesis of the N-terminal cysteine in the dithiol motif (C49S) disables the complementation capacity of the protein. C49S plants (88/92 T1 plants) resemble the *roxy1-3* mutant. (D,E) C52S and C90S proteins were able to restore over 50% of the T1 *roxy1-3* mutants (35/68 and 37/54, respectively), forming flowers with almost wild-type like petals. Photographs show representative inflorescences from transgenic T1 plants. Accession Numbers: *E. coli*, NP_415370; *S. cerevisiae*, AP_009895; *Homo sapiens*, AAH05304; *Oryza sativa*, CAA54397.

The *ROXY1* open reading frame consists of one single exon, encoding 136 amino acids (Fig. 2B). Homology searches revealed that the ROXY1 protein belongs to the family of glutaredoxins (GRXs), which are represented by a number of isoforms in different species, including procaryotes as well as lower and higher eukaryotes (see below). GRXs are small redox proteins that can reduce disulfides via a dithiol or monothiol mechanism by way of conserved cysteines. The dithiol mechanism depends on an active site that contains a conserved N- and C-terminal cysteine (CXXC), and can reduce both, protein disulfides and glutathione (GSH) mixed disulfides. Monothiol GRXs (CXXS) can only reduce GSH mixed disulfides (Vlamis-Gardikas and Holmgren, 2002; Lemaire, 2004). These oxidoreductases have thus far mainly been studied in *E. coli*, yeast and mammal cells, and play a major role in the response to oxidative stress (Fernandes and Holmgren, 2004). For plants, information on the function of GRXs is scarce. *roxy1* represents the first mutant revealing a function for a plant glutaredoxin during flower development.

Cysteine 49 is crucial for proper function of ROXY1 during petal development

In *Arabidopsis*, GRXs of the CPYC type exist (e.g. At5g20500), sharing the active site with other GRXs from *E. coli*, *S. cerevisiae* and human GRXs. However, *ROXY1* and its closest *Arabidopsis* homolog At5g14070 belong to the CC type of GRXs, which are specific for higher plants (Lemaire, 2004). In the ROXY1 protein, the putative active site is composed of two cysteines (C49 and C52; Fig. 3A), separated by another cysteine and methionine (CCMC). Furthermore, these two proteins have another cysteine in common (C90; Fig. 3A), which could represent (but only for ROXY1), a conserved N-terminal cysteine of an additional monothiol site (CSGS).

To investigate how the different cysteines identified in the ROXY1 protein contribute to its function during petal development, cysteines at the positions 49, 52 and 90 were converted into serines. Thereby, we aimed to remove the capacity to form dithiol bridges and thus to abolish the putative reducing power of the ROXY1 protein. Three mutated (C49S, C52S, C90S) and a wild-type cDNA were brought under the control of the cauliflower mosaic virus promoter, transformed into *roxy1-3* mutants and the complementation capacity of the mutagenized proteins was analyzed. Fig. 3B-E shows representative inflorescences from T1 transformants. The wild-type control construct was able to complement the *roxy1-3* mutant (Fig. 3B). Sixty-four percent of the transgenic T1 lines (41/64) formed wild-type flowers. Similarly, 51% from the C52S (35/68) and 68% from the C90S (37/54) transgenic *roxy1-3* T1 plants resembled wild-type flowers, respectively (Fig. 3D,E). However, only 4% C49S T1 plants (4/92) were complemented (Fig. 3C). These data suggest that the N-terminal C49 in the CXXC motif is crucial and less indispensable than the other investigated cysteines for the ROXY1 protein to exert its function as an oxidoreductase during petal development.

Expression pattern of *ROXY1*

Organ-specific expression of *ROXY1* was investigated by RT-

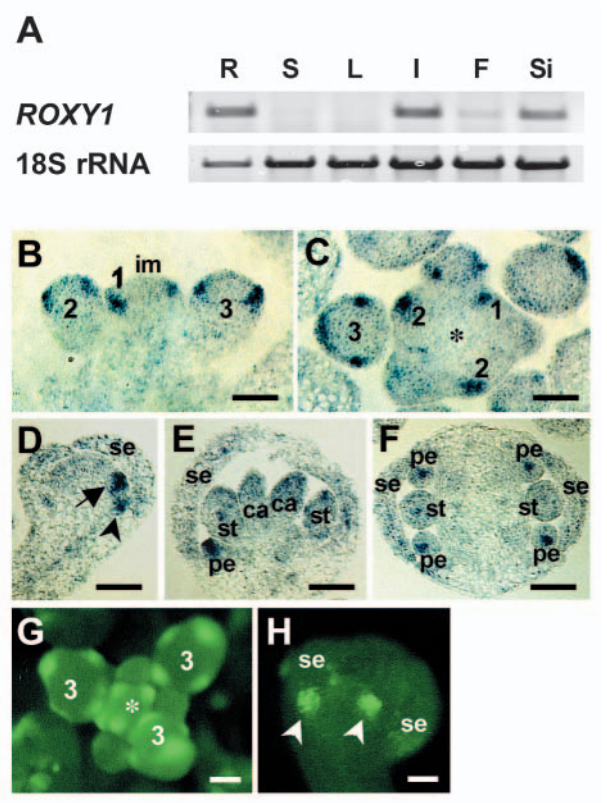

Fig. 4. *ROXY1* expression. (A) RT-PCR analysis of *ROXY1* in wild-type organs. For first-strand cDNA synthesis, RNA was isolated from roots (R), stems (S), leaves (L), inflorescences (I), mature flowers (F) and siliques (Si). 18S rRNA was used as a control. (B-F) In situ analysis of *ROXY1* expression in wild-type flowers. (B,C) Longitudinal (B) and transverse (C) sections through the tip of an inflorescence. Onset of *ROXY1* expression is visible in the inflorescence apex where a future primordium will be initiated (pre-stage 1). Then, signal is detectable when a flower primordium emerges (stage 1), in a flower primordium (stage 2) and in the area where the sepal primordia are formed (stage 3). (D,E) *ROXY1* expression in longitudinal sections of a wild-type flower at stage 4 (D) and stage 7 (E). Expression is detected in petal (arrowhead) and stamen primordia (arrow) that are just initiated (D). *ROXY1* mRNA is still expressed throughout young petals but confined to the vasculature in stamens (E). (F) Cross-section through a bud at stage 8 shows that the signal becomes restricted at a later stage to the central vasculature of both older petals and stamens. (G) Top view of a transgenic inflorescence meristem revealing expression of the ROXY1-GFP fusion protein comparable to the in situ staining shown in C. (H) Arrowheads indicate ROXY1-GFP expression in petal primordia in a flower bud at stage 8. se, sepal; pe, petal; st, stamen; ca, carpel. 1, 2, 3 indicate developmental stages (Smyth et al., 1990). Asterisks in C and G indicate the position of the inflorescence meristem (im). Scale bar: 50 μm.

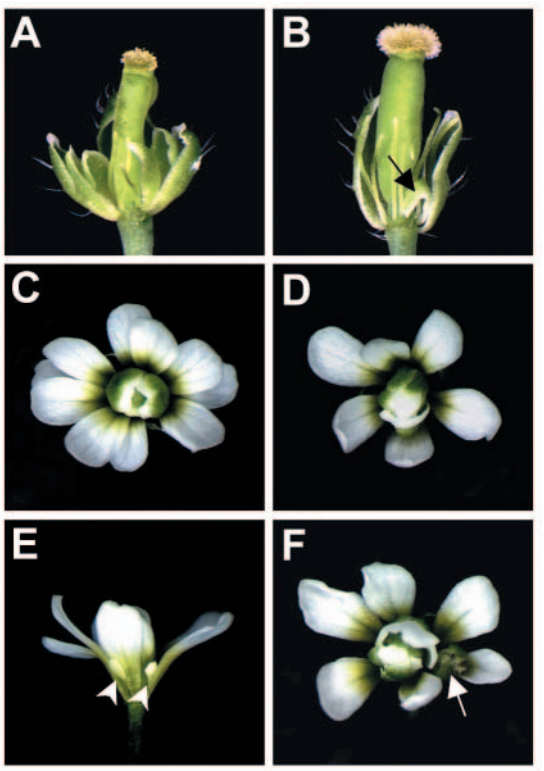

Fig. 5. Double mutant analysis with class B and C genes affecting organ identity. (A) *ap3-3* mutant flowers produce sepals instead of petals in the second whorl and stamens are replaced by carpeloid, filamentous organs. (B) In the *roxy1-3 ap3-3* double mutant, fewer sepals of a reduced size are formed in the second whorl. They are often bent (arrow) and resemble filamentous structures. (C) *ag-1* mutant flowers display a transformation of stamens into petals and a new mutant flower is initiated in the fourth whorl. (D-F) *roxy1-3 ag-1* double mutant flowers produce fewer petals in the second whorl that are often reduced in size (arrowheads in E). However, third whorl petals are not affected and occasionally formation of a new flower (arrow) can be observed in the second whorl (F).

PCR, showing that *ROXY1* is strongly expressed in inflorescences, roots and siliques. Weaker expression was detected in mature flowers, and no expression was observed in stems and leaves (Fig. 4A). Inflorescence-specific expression was further analyzed by in situ hybridization. The earliest detectable *ROXY1* signal was localized in the inflorescence apex, delineating the area where future floral primordia will emerge (pre-stage 1). After formation of young floral primordia (stage 1; Fig. 4B,C), *ROXY1* expression is restricted to young floral organ primordia (stage 2 and early stage 3). Signal was detected in the areas where sepal primordia will be initiated (Fig. 4B,C) and vanishes once sepal primordia start to overgrow the flower primordium (Fig. 4D). Then, at stage 4 and 5, *ROXY1* mRNA appears in the petal and stamen primordia. Again, onset of expression starts slightly before the respective organ primordia are formed (Fig. 4D). In early stages, *ROXY1* is expressed throughout the whole petal and stamen primordia. After onset of stamen differentiation, *ROXY1* expression is restricted to the vascular tissue of these two organs (Fig. 4E,F) and was also detected in young ovule primordia (data not shown).

For analysis of the in vivo protein expression pattern, a fusion-protein of ROXY1 and GFP was produced, driven by a 3560 bp long promoter fragment and comprising 445 bp located downstream of the *ROXY1* stop codon. Comparison of Fig. 4C,D with Fig. 4G,H shows that the ROXY1 protein and mRNA expression patterns overlap. We therefore conclude that the GFP construct contains all regulatory elements required for the proper expression of ROXY1.

Reduction of petal organs in *roxy1* mutants is position-dependent

To determine whether the reduction of petal organs in *roxy1* mutants is whorl or organ specific, double mutants with class B and C mutants were constructed. Owing to loss of the class B gene function in the *APETALA3* (*AP3*) mutant *ap3-3*, petals are transformed into sepals and stamens into carpeloid organs or filamentous structures (Fig. 5A). In the *roxy1-3 ap3-3* double mutant, an additive flower phenotype was observed. In the second whorl, the organ number and size of sepals is reduced and some are folded inwards, as observed in *roxy1-3* single mutants (compare Fig. 1B with Fig. 5B). In the class C mutant *ag-1*, third whorl stamens are transformed into petals and another mutant flower is produced in the place of fourth whorl carpels (Fig. 5C). In the *roxy1-3 ag-1* double mutant, the number of second whorl petals is reduced and their shape altered (Fig. 5D,E). However, petals formed in the third whorl are not affected in their development, resembling *ag-1* third whorl organs. Additionally, some double mutant flowers produced a secondary flower in the second whorl, a feature not observed in the single mutants under our growth conditions (Fig. 5F). Overall, these data show that *ROXY1* exerts its function in a position- and not organ-dependent mode.

AGAMOUS is ectopically expressed in *roxy1-3 ap1-10* double mutants

In the *roxy1* single mutants, occasional appearance of stigmatic papillae on filamentous structures formed in the second whorl (Fig. 1L) indicated that loss of the *ROXY1* function might cause ectopic expression of the class C gene *AG*. Analysis of *AG-I::GUS* expression in *roxy1-3* mutants led to an occasional weak detection of *GUS* expression in second whorl petals (data not shown). However, when we analyzed double mutants with the class A mutant *ap1-10*, the phenotype differed dramatically from both single mutants. In the *ap1-10* single mutant, first whorl sepals are transformed into leaf-like organs or form rarely sepal-carpel intermediate organs (Schultz and Haughn, 1993). Second whorl organs are absent or reduced and often, secondary flowers are produced at the base of the first whorl organs (Fig. 6A). In the double mutant, first whorl organs are carpeloid, an effect that was strongest in younger flowers no longer producing secondary flowers (Fig. 6B). Correlation of the double mutant phenotype with ectopic *AG* expression was investigated by in situ hybridization and histochemical staining of *roxy1-3 ap1-10 AG-I::GUS* flowers. In *ap1-10*, as in wild-type flowers, *AG* is expressed in the center of the floral meristem from stage 3 on and is later restricted to the center of the flower (Fig. 6C,E,G). Loss of *AP1* function does not cause ectopic *AG* expression (Gustafson-Brown et al., 1994). By contrast, in the double mutant onset of *AG* expression occurs prematurely in the inflorescence meristem and in young flower primordia (Fig. 6D). During further development, ectopic *AG* expression extends into first and second whorl organs (Fig. 6D), where it is detectable until late stages, as indicated by GUS staining in mature feminized first whorl organs of *roxy1-3 ap1-10 AG-I::GUS* flowers (Fig. 6F,H).

In summary, ectopic *AG* expression is not strongly detectable in *roxy1-3* or *ap1-10* single mutants. However, the combination of *roxy1-3* with *ap1-10* results in a synergistic defect revealed by premature onset and ectopic expression of *AG*, which is likely to be responsible for carpeloid transformation of first whorl organs.

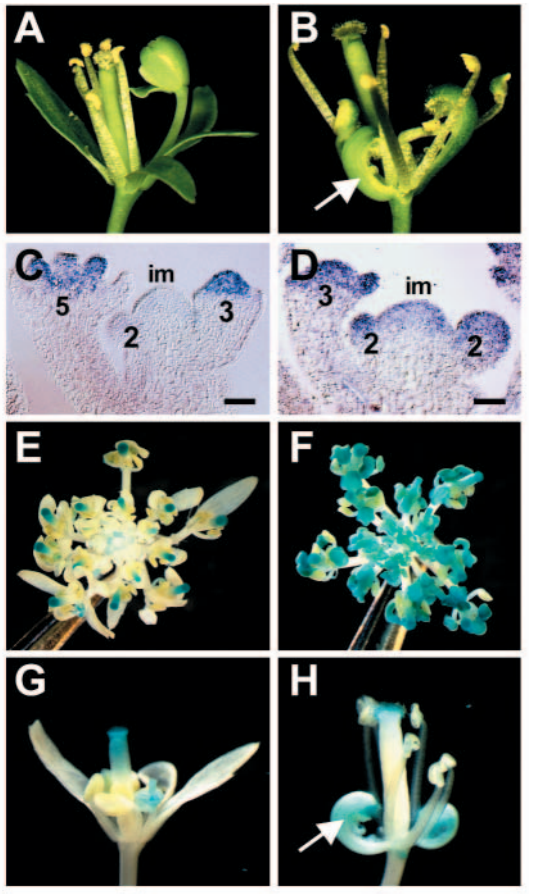

Fig. 6. Changes in *AG* expression in *roxy1-3 ap1-10* double mutants. (A) *ap1-10* mutants develop leaf-like first whorl organs; petals are absent in the second whorl, but an additional flower is formed in flowers arising on the inflorescence at later stages. (B) Strong feminization of the first whorl organs in the *roxy1-3 ap1-10* double mutant (arrow). (C) In situ analysis of *AG* expression in young *ap1-10* mutant inflorescences. Onset of *AG* expression is detectable at stage 3 and is confined to the two inner floral whorls. (D) *AG* is prematurely expressed in the *roxy1-3 ap1-10* double mutant and expression is already detectable in the inflorescence meristem (im). Furthermore, *AG* is ectopically expressed in sepal primordia at stage 3. (E,G) *AG-I::GUS* expression in *ap1-10* flowers resembles wild-type *AG* expression, being confined to the third and fourth whorl organs. (F,H) *AG-I::GUS* analysis in inflorescences and mature flowers of *roxy1-3 ap1-10* mutants shows continued ectopic *AG* expression in the feminized first whorl organs (arrow).

Genetic interactions with other floral regulatory genes

As our data indicate that *ROXY1* is involved in the negative control of *AG* expression we tested genetic interactions of *roxy1-3* with two other negative regulators of *AG, AP2* and *LUG*. Single *ap2-5* and *lug-1* mutants (Fig. 7A,C) show premature and ectopic *AG* expression (Jofuku et al., 1994; Liu and Meyerowitz, 1995), and their phenotypes resemble the *roxy1-3 ap1-10* double mutant forming carpeloid first whorl organs. Double mutants of *roxy1-3* with the intermediate *ap2-5* and the weak *lug-1* alleles reveal that a lack of the *ROXY1*

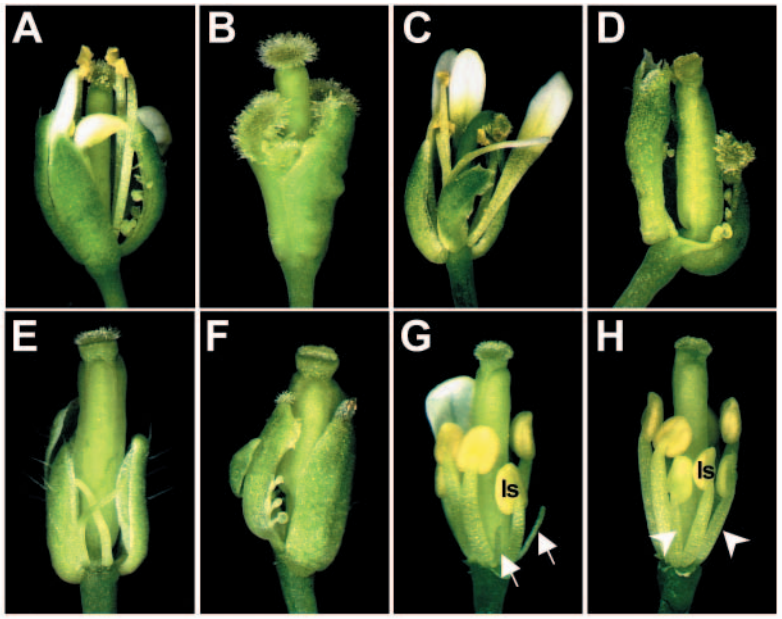

Fig. 7. Phenotypes of double mutants between *roxy1-3* and *ap2-5*, *lug-1*, *ufo-2* and *rbe-2*. (A-D) Enhancement of first whorl carpeloidy of *roxy1* in combination with negative regulators of *AG*. (A) *ap2-5* mutant flower showing transformation of sepals into carpeloid organs. (B) In *roxy1-3 ap2-5* double mutants, first whorl carpeloidy is strongly enhanced and fused organs are topped with stigmatic papillae. (C) The *lug-1* single mutant forms feminized first whorl organs. (D) In the *roxy1-3 lug-1* double mutant carpeloidy of the first whorl organs is enhanced. (E) Mutants of the strong *ufo-2* allele display defects in all floral whorls, affecting most severely second and third whorl organs resembling sepaloid and stamenoid structures, respectively. (F) *roxy1-3 ufo-2* double mutant flowers form a reduced number of carpeloid second whorl organs. One first whorl sepal was removed to reveal inner organs. (G) *rbe-2* single mutant, where sepals were removed. Arrows indicate filamentous structures formed instead of petals in the second whorl. (H) *roxy1-3 rbe-2* double mutant flower, sepals were removed. Extra stamens develop in the second whorl (arrowheads), instead of petals or filamentous structures. ls, lateral stamen.

function strongly enhances single mutant phenotypes. In the *roxy1-3 ap2-5* double mutant, first whorl organs are fused at their base and stigmatic papillae are formed at the tips (Fig. 7B). No petals are formed and occasionally abnormal stamens develop in the third whorl (data not shown). Similarly, first whorl carpeloidy is enhanced in the *roxy1-3 lug-1* double mutant and rarely second whorl organs develop (Fig. 7D).

We also investigated genetic interactions with mutants affecting petal development. *UFO* has a function in controlling the identity of whorl 2 and whorl 3 organs, probably by degrading a repressor of the class B genes *AP3* and *PI* (Levin and Meyerowitz, 1995). Recently, it was shown that *UFO* controls also petal initiation in a dose-dependent manner (Durfee et al., 2003; Laufs et al., 2003). We tested whether the defects during petal initiation and morphogenesis represent a disruption of the same process as in *roxy1-3* by scoring *roxy1-3 ufo-2* double mutant flowers. In *ufo-2*, second whorl organs are often absent or transformed into sepaloid organs and the third whorl produces carpeloid or filamentous structures (Fig. 7E). In the *roxy1-3 ufo-2* double mutant, a reduced number of carpeloid second whorl organs forms, again indicating ectopic class C activity (Fig. 7F). Additionally, inflorescence growth in the double mutant terminates prematurely in a pistil or in a cluster of carpeloid structures (data not shown).

The *rbe-2* mutant is very similar to the *roxy1* mutant, as its defects are restricted to petal initiation and morphogenesis (Fig. 7G) (Takada et al., 2004). Double mutants between *roxy1-3* and *rbe-2* were generated to determine whether *ROXY1* and *RBE* function in the same pathway during petal development. *RBE* is expressed in petal precursor cells and petal primordia, overlapping with *ROXY1* expression (Takeda et al., 2004). In *roxy1-3 rbe-2* double mutant flowers, instead of filamentous petals, stamens develop in the second whorl (Fig. 7H), producing fertile pollen (data not shown). First whorl organs are not affected. The double mutant phenotype might correlate with ectopic *AG* expression, affecting only the second whorl. As ectopic stamen formation depends on class B function (Jack et al., 1997), it seems that sufficient class B activity either remains in the *roxy1-3* mutant or is enhanced in the double mutant to control, together with ectopic C function, stamen organogenesis in the second whorl.

Taken together, double mutant analyses indicate a function for *ROXY1* in the temporal and spatial expression regulation of the key floral regulator *AG*, as all observed organ transformations are probably caused by ectopic class C function. However, double mutants differ with respect to the degree of the ectopic *AG* function, such that double mutants with *ap1-10*, *ap2*-5 and *lug-1* indicate *AG* activity in the first whorl, whereas this activity is restricted to the second whorl in double mutants with *ufo-2* and *rbe-2*.

Discussion

Although much research has been conducted on the function of homeotic genes that control flower organogenesis, still little is known about the genes required for the initiation of the flower organ primordia, an event that is not controlled by these key regulatory transcription factors. Here, we demonstrate that a novel gene, named *ROXY1,* is required for proper petal initiation and organogenesis. Furthermore, double mutant analysis shows that it functions in restricting *AG* activity to the third and fourth whorl. Surprisingly, the gene encodes for a GRX and belongs to a family of genes that have thus far been associated with the response to oxidative stress.

ROXY1: a plant glutaredoxin of the CC type

Sequence analysis revealed that *ROXY1* codes for a GRX. GRXs are small (~12 kDa) disulfide oxidoreductases that possess a typical glutathione-reducible dithiol CXXC or monothiol CXXS active site, required for the reduction of target protein disulfides. Together with thioredoxins (TRXs), GRXs form the large thioredoxin superfamily, whose members share one or several common thioredoxin folds, defined successions of β-sheets and α-helices (Martin, 1995). GRXs, like TRXs, may operate as dithiol reductants, and perform fast and reversible thiol-disulfide exchange reactions between their active site cysteines and cysteines of their disulfide substrates. Additionally, GRXs uniquely reduce mixed disulfides in a

monothiol mechanism (Fernandes and Holmgren, 2004). In most organisms, TRXs and GRXs are the major reducing molecules, and are involved in many cellular processes. Very recently, comparative studies including phylogenetic tree analysis were conducted with oxygenic photosynthetic organisms, comprising *Arabidopsis*, *Chlamydomonas* and *Synechocystis*. Plant GRXs can be grouped into three classes that differ in their active site composition (Lemaire, 2004; Rouhier et al., 2004). In *Arabidopsis*, 30 GRX genes have been identified. Six belong to the classical CPYC type, intensively studied in *E. coli* and yeast; and four to the CGFS type that have thus far been analyzed in yeast and humans. *ROXY1*, together with 19 other *Arabidopsis* GRXs, forms a third and novel CC type group, defined by the presence of CCMC or CCMS motifs. These GRXs have thus far been identified only in higher plants, suggesting a specifically evolved function (Lemaire, 2004). In addition to the 49CCMC52 site, *ROXY1* contains another motif, 90CSGS93, that could represent an active site of the CXXS monothiol type. To investigate the contribution of the different cysteines to the *ROXY1* function during flower development, site-directed mutagenesis experiments were carried out. Mutation of C49 to S49 caused a strongly reduced capacity of the protein to complement the *roxy1-3* mutant. Similar results were obtained with *E. coli*, human and recently also a poplar GRX, showing that the catalytic cysteine was found to be located at the N-terminus of the active site (Foloppe et al., 2001; Padilla et al., 1996; Rouhier et al., 2002). Given that C52 and C90 are less important, our data indicate that C49 might function in the monothiol pathway, even though it is part of a CCMC motif.

In the monothiol mechanism, GRXs catalyze the reduction of glutathione (GSH)-mixed disulfides by using only the N-terminal cysteine thiol, a process known as deglutathionylation (Bushweller et al., 1992). This reversible mechanism is considered to be the more general function of GRXs (Fernandes and Holmgren, 2004; Cotgreave and Gerdes, 1998). However, little is known about glutathionylation of proteins in photosynthetic organisms. In bacteria, yeast and animal cells, several proteins have been identified as being glutathionylated, especially in response to oxidative stress, including chaperones, cytoskeletal proteins, metabolic enzymes and kinases (Lind et al., 2002). Glutathionylation of transcription factors, as shown for NF-κB and Jun reveals a mechanism for a redox-induced inhibition of DNA-binding (Pineda-Molina et al., 2001; Klatt et al., 1999). Our data indicate a novel function of GRXs in flower development, probably the modification of factors involved in the regulation of floral organogenesis post-translationally.

ROXY1 is the first glutaredoxin shown to play a role in petal development

In *roxy1* mutants, petal development is affected at very early and also during later stages, as revealed by initiating a reduced number of petal primordia and abnormally bended mature petals. SEM analysis suggests that lack of primorida initiation does not seem to be caused by a reduced size of the flower meristem in the area where petal primordia are normally initiated. Rather, it might be due to a lack of initiation of proper cell divisions required for primordia outgrowth. Similar phenotypes have been observed in mutants of the *RBE* and *PTL* genes (Takeda et al., 2004; Brewer et al., 2004). Only the *RBE* and *ROXY1* expression domains overlap in young petal primordia and second whorl organ number is reduced similarly in the single mutants and in the double mutant. This suggests that *ROXY1* and *RBE* might have a common function during second whorl organ initiation. A combination of both *roxy1-3* or *rbe-1* with the class B *ap3* mutants results in additive effects, demonstrating that the function of both genes is organ identity independent. Interestingly, the RBE protein contains two cysteines in the zinc-finger motif and one further cysteine close to the C terminus that could represent putative target sites for modification by ROXY1.

During later petal differentiation, abnormal petal bending was observed in over 40% of the remaining *roxy1* petals, a phenotype not observed in *rbe* mutants. The organ identity of folded petals is not altered, as they still form conical cells typical for adaxial wild-type epidermal cell layer. Curvature of otherwise flat organs can be caused by deregulation of cell division processes, as demonstrated by leaf mutant analysis (Nath et al., 2003). In the *roxy1* mutant, disturbance of cell division regulation could be responsible for both, lack of primordia initiation at early stages and altered curvature of petals at later stages. As *ROXY1* expression is confined to the vasculature in older petals, this effect would probably be exerted non cell-autonomously. Two observations indicate redundancy for the *ROXY1* function. First, *roxy1* mutants still produce a reduced number of normal petals. Second, we observed only a defect in the second whorl, although early *ROXY1* expression is not restricted to this whorl. As *ROXY1* belongs to the CC type group of GRXs, which comprises 20 genes, redundancy could be due to the activity of related proteins. Ectopic *ROXY1* expression does not disturb wild-type flower development (Fig. 3B), which could be due to high substrate specificity of ROXY1 and/or lack of ubiquitous target gene expression.

ROXY1 participates in the negative regulation of *AG* expression in the first and second whorl

Several genes are known to be involved in repression of *AG*, including *AP2*, *LUG*, *SEU*, *AINTEGUMENTA*, *STERILE APETALA*, *BELLRINGER* and *CURLY LEAF* (Drews et al., 1991; Conner and Liu, 2000; Franks et al., 2002; Elliott et al., 1996; Byzova et al., 1999; Bao et al., 2004; Goodrich et al., 1997). All these genes restrict the expression of *AG* to the reproductive organs of the flowers. Occasional formation of filamentous structures topped with stigmatic papillae in the second whorl of *roxy1* flowers indicated that *ROXY1* is an additional component in this repressive mechanism. This effect is enhanced in double mutants of *roxy1-3* with *ap2-5*, *lug-1* and also *ap1-10*, where flowers develop strongly feminized organs in the first whorl and largely lack second whorl formation. Surprisingly, the influence of *ROXY1* on *AG* expression is also revealed in combination with *ufo-2* and *rbe-2* mutants that do not obviously control *AG* activity in single mutants. In *roxy1 ufo-2* double mutants lack of class B activity in the second whorl (due to mutation in *UFO*) (Levin and Meyerowitz, 1995), along with an ectopic *AG* activity (due to the lack of *ROXY1* function) explains second whorl transformation into carpeloid structures. In double mutants with *rbe-2*, enough residual class B activity probably still resides in the second whorl that causes together with an ectopic *AG* activity transformation of second whorl organs into stamens. ROXY1

could participate in *AG* regulation by modifying its repressors post-translationally. In fact, GRXs have been shown in animals to glutathionylate transcription factors and thereby alter their DNA-binding activity (Pineda-Molina et al., 2001; Klatt et al., 1999). Recently, also a redox-sensitive plant transcription factor, *TGA1*, has been reported, the reduced form of which displays enhanced DNA-binding during systemic acquired resistance (Després et al., 2003).

Conclusion

Our data indicate for the first time that a GRX plays a crucial role during flower development, probably by means of a conserved cysteine that mediates posttranslational modifications. The spectrum of *ROXY1* target genes seems to be rather broad, as indicated by the *roxy1* mutants displaying distinctive early and late defects throughout petal development, and by diverse genetic interactions involved in repressing ectopic *AG* expression. Characterization of the *roxy1* mutants can now serve as a starting point to investigate the function of the plant GRX family in more detail, aiming for an understanding of their biochemical properties, the identification of target proteins and unraveling the intriguing connection between redox regulation and flower development.

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