Negative Regulation of Systemic Acquired Resistance by Replication Factor C Subunit 3 in Arabidopsis

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Summary:

Systemic acquired resistance (SAR) is a plant immune response induced by local necrotizing pathogen infections. Expression of SAR in plants correlates with accumulation of salicylic acid (SA) and up-regulation of Pathogenesis-Related (PR) genes. SA is an essential and sufficient signal for SAR. In a genetic screen to search for negative regulators of PR gene expression and SAR, we found a new mutant that is hypersensitive to SA and exhibits enhanced induction of PR genes and resistance against virulent oomycete *Hyaloperonospora arabidopsidis* Noco2. The enhanced pathogen resistance in the mutant is NPR1-independent. The mutant gene was identified by map-based cloning and it encodes a protein with high homology to the Replication Factor C, Subunit 3 (RFC3) of yeast and other eukaryotes, thus the mutant was named *rfc3-1*. *rfc3-1* mutant plants are smaller than wild type and have narrower leaves and petals. On the epidermis of true leaves, there are fewer cells in *rfc3-1* compared to wild type. Cell production rate is reduced in *rfc3-1* mutant roots, indicating that the mutated RFC3 slows down cell proliferation. As Replication Factor C is involved in replication-coupled chromatin assembly, our data suggest that chromatin assembly and remodeling may play important roles in the negative control of PR gene expression and SAR.

**Key words**: Systemic acquired resistance; PR genes; plant disease resistance; RFC3
Introduction

Plants have evolved inducible defense mechanisms to cope with infections by a wide range of microbial pathogens during plant pathogen co-evolutionary history (Jones and Dangl, 2006). In 1961, Ross found that tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed enhanced resistance to secondary infections in distal tissues (Ross, 1961). This spread of resistance throughout the plant was termed systemic acquired resistance (SAR). The response is long lasting, sometimes for the lifetime of the plant. SAR is also effective against a broad-spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Ryals et al., 1996; Durrant and Dong, 2004). During the onset of SAR, salicylic acid (SA) levels increase in both local and systemic tissues, accompanied by up-regulation of a set of Pathogenesis-Related (PR) genes. Because over-expression of a single PR gene is not sufficient to establish broad-spectrum resistance, it is believed that PR proteins enhance resistance by working in concert. In particular, PR-1 and β-1, 3-glucanase (BGL2, also known as PR-2) have been widely used as molecular markers for SAR (Uknes et al., 1992; Bowling et al., 1994; Cao et al., 1994).

SA was found to be a necessary and sufficient signal for SAR. SAR can be brought on by exogenous application of SA or its analogs such as 2, 6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH). On the other hand, removal of SA by exogenous SA hydroxylase (NahG) or genetic mutations in the SA biosynthetic pathway, such as EDS5 and SID2, disables SAR (White, 1979; Métraux et al., 1991; Ward et al., 1991; Friedrich, et al., 1996; Görlach et al., 1996; Lawton et al., 1996).

NPR1 is a central positive regulator of SAR downstream of SA. When NPR1 is mutated, plants can no longer mount an SAR response even with induction of SA or INA (Cao et al., 1994). It functions through associations with TGA transcription factors to regulate PR gene expression and pathogen resistance (Durrant and Dong, 2004). Triple knockout mutant of TGA2, TGA5 and TGA6 displayed compromised
SAR responses and increased basal PR gene expression, suggesting that TGA transcription factors have both positive and negative roles in regulating PR gene expression and SAR (Zhang et al., 2003).

As constitutive activation of defense is detrimental to plants, expression of PR genes is usually under tight control. In a previous study, an INA hypersensitive mutant, sni1 (suppressor of npr1-1, inducible), was found to be a transcriptional repressor of PR gene expression that negatively regulates SAR (Li, et al., 1999; Mosher et al., 2006). Mutant plants of sni1 exhibit basal level of PR gene expression that is independent of NPR1 and this expression is further enhanced upon SAR induction. SNI1 encodes a protein with structural similarity to Armadillo repeat proteins potentially involved in scaffolding or protein-protein interactions. Chromatin immunoprecipitation (ChIP) experiments indicated that histone modification could be involved in SNI1 function (Mosher et al., 2006). When a genetic screen was conducted to search for genetic suppressors of sni1, it was found that a loss-of-function mutation in RAD51D suppresses sni1 phenotypes completely. Both SNI1 and RAD51D were found to play roles in PR gene transcription and DNA recombination (Durrant et al., 2007). To identify additional negative regulators of PR gene expression and SAR, we screened for mutants that are hypersensitive to SA induction and found one with similar phenotypes to sni1. This mutant has higher expression levels of PR genes and displays enhanced resistance against virulent oomycete Hyaloperonospora arabidopsis (H. a.) Noco2. The gene was identified by map-based cloning and found to encode a protein with high homology to replication factor C subunit 3 (RFC3) proteins.
Results

Novel genetic screen to search for mutants that are hypersensitive to SA induction

While exogenous application of SA induces high expression of PR genes and SAR, wild type plants are almost non-responsive to very low concentrations of SA. Since the previously reported sni1 mutant plants are hypersensitive to SA or INA induction, we reasoned that if similar negative regulators are mutated, we should be able to find mutants having similar phenotypes as sni1. We first built an individually harvested M2 population of EMS-mutagenized Col wild type carrying the pBGL2-GUS reporter gene (Cao et al., 1994). Subsequently we searched for mutants showing strong GUS staining when grown on MS plates supplemented with 10 μM of SA. This concentration of SA does not induce visible GUS staining on wild type plants. Except for additional alleles of sni1 (Xia and Li, unpublished data), one mutant with enhanced inducible GUS staining was obtained and it was later named rfc3-1 after we found its identity (Figure 1A).

Mutant rfc3-1 plants are smaller than wild type and have similar morphology like sni1 (Figure 1B). Real-time PCR analysis showed that the endogenous PR-1 (Figure1C) and PR-2 (Figure1D) expression levels in rfc3-1 were slightly higher than wild type without SA induction, and the expression levels of PR-1 and PR-2 are much higher under SA induction. In addition, the rfc3-1 mutant plants are more resistant to the virulent oomycete pathogen Hyaloperonospora arabidopsis (H. a.) Noco2 (Figure 1E). Thus, like SNI1, RFC3 plays a negative regulatory role in PR gene expression and SAR. When RFC3 is mutated, plants exhibit enhanced PR gene expression and hypersensitivity to SA induction.

When rfc3-1 (with pBGL2-GUS) was backcrossed with a wild type pBGL2-GUS line, the F1 progeny exhibited wild type morphology, indicating that rfc3-1 is a recessive mutation. Among 46 F2 progeny, 10 showed rfc3-1-like pBGL2-GUS
staining, suggesting that the defect in rfc3-1 is caused by a single recessive mutation (3:1, $\chi^2=0.26$, p>0.1).

rfc3-1 npr1 double mutant exhibits similar enhanced resistance phenotypes as rfc3-1

SNI1 is a repressor of PR gene expression and double mutant sni1 npr1 exhibits phenotypes similar to that of sni1 (Li et al., 1999). Since rfc3-1 has phenotypes very similar to sni1, we tested the relationship between RFC3 and NPR1 through generating a double rfc3-1 npr1-3 mutant. As shown in Figure 2A, the morphology of rfc3-1 npr1-3 is similar to that of the rfc3-1 single mutant. The rfc3-1 npr1-3 double mutant plants also exhibit enhanced resistance to H. p. Noco2 to the same level as rfc3-1 (Figure 2B). Quantitative RT-PCR analysis showed that with or without SA induction, both the endogenous PR-1 (Figure 2C) and PR-2 (Figure 2D) expression levels in the rfc3-1 npr1-3 double mutant were more similar to those of rfc3-1 single mutant. Thus, RFC3 probably also functions as a repressor to regulate PR gene expression. When RCF3 is mutated, the ability to express PR genes no longer requires the function of NPR1.

Map-based cloning of rfc3-1 mutant

To map the rfc3-1 mutation, rfc3-1 (in the Columbia ecotype with pBGL2-GUS) was crossed with Landsberg erecta (Ler) (with no pBGL2-GUS) to generate a segregating population. For crude mapping, 30 plants homozygous at the rfc3-1 locus were identified in the F2 progeny on the basis of rfc3-1 morphology. Linkage was found on the bottom of chromosome 1 between the markers T4O12 and T8K14. To avoid the possibility that the mutation causing the morphological phenotypes associated with rfc3-1 might be closely linked with the one causing the sni1-like defense phenotypes, lines that were homozygous for pBGL2-GUS reporter gene and had wild type morphology were genotyped with T4O12 and T8K14. Lines that are
heterozygous at both markers should be heterozygous for rfc3-1, seeds of which were used for fine mapping. The presence of the homozygous pBGL2-GUS reporter gene enabled confident phenotyping of recombinants with GUS staining.

For fine mapping, 720 random F3 plants derived from F2 plants homozygous for pBGL2-GUS reporter gene were genotyped with markers T4O12 and T8K14. A total of 53 recombinants between these two markers were identified. Further analysis of the 53 recombinants with additional markers in the region indicated that rfc3-1 was flanked by F2P24 and T5M16, with a 70 kb distance in between (Figure 3A).

To identify the mutation in rfc3-1, primers were designed to sequence the coding regions of the genes between the final two flanking markers. A single G to A mutation was found in At1g77470. At1g77470 consists of nine exons. The mutation in rfc3-1 occurred in the second exon (Figure 3B), changing the nonpolar aliphatic GLY84 to a negatively charged ASP.

To confirm that the mutation found in rfc3-1 indeed causes the enhanced disease resistance phenotypes, we carried out complementation analysis using a genomic clone containing RFC3. As rfc3-1 homozygotes are partially sterile, the wild type RFC3 clone was transformed into the rfc3-1/RFC3 heterozygous plants. Transgenic lines homozygous for rfc3-1 were identified by PCR. As shown in Figure 3C, transgenic plants carrying RFC3 in the rfc3-1 background displayed wild type morphology. Expression of the pBGL2-GUS reporter gene was also restored to wild type levels (Figure 3D). Furthermore, enhanced resistance against H. p. Noco2 in rfc3-1 was also lost in the transgenic plants (Figure 3E). All these data suggest that the wild type RFC3 can complement the rfc3-1 mutation, and the G to A mutation found in rfc3-1 causes the SA hypersensitivity and enhanced resistance phenotypes in rfc3-1.

rfc3-1 is a partial loss-of-function allele of RFC3

To determine whether the rfc3-1 mutation is a complete or partial loss-of-function mutation, we obtained an additional mutant allele of At1g77470 from the
Arabidopsis Biological Resource Center (ABRC). rfc3-2 (SAIL_401_E05) contains a T-DNA insertion in the third intron of At1g77470 (Figure 3B). We could not identify any rfc3-2 homozygotes among 31 plants carrying the T-DNA insertion, indicating that the homozygotes of rfc3-2 are lethal. To test whether it is allelic to rfc3-1, heterozygous rfc3-2/RFC3 plant was crossed with homozygous rfc3-1. The F1 plants with rfc3-1/rfc3-2 genotype all had morphology similar to that of rfc3-1 (data not shown), indicating that rfc3-2 did not complement rfc3-1, and they carry mutations in the same gene. Since rfc3-2 is lethal whereas rfc3-1 is not, we deduced that the G to A mutation in rfc3-1 most likely is a partial loss-of-function mutation.

RFC3 localizes to the nucleus and functions in cell replication and proliferation

RFC3 encodes a protein of 369 amino acids with a molecular weight of 41.4 kD. It is highly similar to RFC3 in yeast and other eukaryotic species (Figure 4). In eukaryotes, a replication factor C complex resides in the nucleus and contains one large subunit and four small subunits. RFC3 is one of the small subunits. There is only one copy of RFC3 gene in the Arabidopsis genome. In most other eukaryotes, RFC3 also appears to be a single copy gene. To determine the localization of RFC3, Arabidopsis mesophyll protoplasts were transfected with a construct expressing the RFC3-GFP fusion protein. Consistent with its localization in other organisms, the RFC3-GFP fusion protein localized to the nucleus (Figure 5A), suggesting that RFC3 is a nuclear protein.

Since RFC3 encodes a putative replication factor, we tested whether the rfc3-1 mutant exhibits replication-related phenotypes. rfc3-1 plants are dwarfed and have smaller and narrower leaves compared to wild type plants (Figure 5B). The leaf blades of the mutant are almost half the size of wild type (Figure 5C). The flower petals are also smaller (Figure 5B). To determine if the smaller leaves of the rfc3-1 mutant is caused by the smaller size of the cells, the epidermal cells of the true leaves were examined under a microscope. Surprisingly, the epidermal cells of the true leaves in rfc3-1 were much bigger than those of the corresponding wild type true
leaves (Figure 5C). With larger sized cells and smaller sized leaves, the number of cells in rfc3-1 mutant leaves is likely lower than that of the wild type. Thus smaller leaves of rfc3-1 are caused by reduced cell numbers rather than smaller sizes of the cells, indicating a crucial function of RFC3 in cell replication.

To determine whether the numbers of cells in other part of the plant are also reduced in the rfc3-1 mutant, root cortex cells of 10-day-old plants grown on MS plates were examined under the microscope. The root length of rfc3-1 mutant seedlings is slightly longer than that of the wild type root (data not shown). The average root elongation rate of rfc3-1 is also higher than that of the wild type root during the first three days, but from the fourth day, there is no significant difference between rfc3-1 and the wild type plant (Figure 6A). Further observation inside the root reveals that the root cortex cells in the root hair zone of rfc3-1 are significantly longer than those of the wild type, and quantitative data shows that the length of cortex cells in rfc3-1 is twice the size of the wild type cortex cells (Figure 6B). As a result, the root cell production rate (number of cells produced per hour) of rfc3-1 is only half of that of the wild type plants (Figure 6C), indicating that mutation in RFC3 leads to defects in cell proliferation. Together with data from the leaf epidermal cells, our data suggest that the Arabidopsis RFC3 functions in the process of cell proliferation and replication and most likely is an ortholog of the yeast RFC3.
Discussion

Since constant defense is costly to plants without pathogen infection, tight negative control must be present to prevent the over-activation of the resistance mechanisms. SNI1 was identified earlier as a negative regulator of systemic acquired resistance (SAR; Li et al., 1999; Mosher et al., 2006). A mutation in SNI1 causes up-regulation of defense genes and hypersensitivity to the defense signal molecule salicylic acid (SA). To identify more negative regulators of SAR like SNI1, a genetic screen was performed to search for mutants that are hypersensitive to SA induction. One recessive mutant, rfc3-1, was identified that displays similar morphology to sni1. rfc3-1 plants have higher PR gene expression than wild type without SA induction and under SA application; they exhibit much higher sensitivity to the defense hormone. Mutation in SNI1 restored SAR in the npr1-1 background, and SNI1 was found to encode a leucine-rich nuclear transcriptional repressor protein (Li et al., 1999; Mosher et al., 2006). Similarly, rfc3-1 npr1 double mutant plants exhibit similar phenotypes as rfc3-1, suggesting that, like SNI1, RFC3 is a negative regulator of SAR and this function of RFC3 does not require NPR1.

DNA replication is essential for all organisms with DNA genomes. RFC is a protein complex that can bind to a DNA template-primer junction and load the proliferating-cell nuclear antigen (PCNA) clamp onto DNA with the assistance of ATP. This allows recruitment of DNA polymerase to the site of DNA synthesis. RFC plays essential roles in DNA replication and damage repair (reviewed by Mossi and Hübser, 1998). As a protein complex, RFC is conserved in all eukaryotes with one large subunit and four small subunits. AtRFC3 is highly homologous to the yeast RFC3 and the corresponding human RFC5 (Figure 4). The Arabidopsis RFC3 was found to localize to the nucleus and is essential for plant survival as a null mutant of RFC3 is lethal. Consistent with RFC3’s function in replication, plants with the partial loss-of-function rfc3-1 allele exhibit smaller and narrower leaves due to reduced number of the cells, suggesting defects in replication. Moreover, the root cell
production rate in rfc3-1 is only half of the wild type plants (Fig.6C), further indicating that rfc3-1 mutation slows down cell proliferation, most likely due to defects in replication.

One important question is how RFC3 regulates pathogen resistance in plants. Since the phenotypes of rfc3-1 are highly similar to those of snil, one possibility is that RFC3 may negatively regulate pathogen resistance through an interaction with SNI1. This interaction is probably not a direct protein-protein interaction, since we did not detect interactions between SNI1 and RFC3 in the yeast two-hybrid assays we performed (Xia and Zhang, unpublished data). It has been suggested that SNI1 represses transcription through affecting chromatin modification (Mosher et al., 2006). Loss of SNI1 function leads to increased abundance of activating histone modifications such as acetylated histone H3 (AcH3) and methylated lysine 4 on histone H3 (MeH3K4) at the PR-1 promoter, which may make the chromatin at promoter region adopt a more accessible conformation and lead to elevated gene expression (Mosher et al., 2006). During cell division, epigenetically defined chromatin structure is often propagated with high fidelity through replication-coupled chromatin assembly. Failure to transmit epigenetic modifications such as histone modifications and DNA methylations would lead to changes of gene expression patterns in the daughter cells. The rfc3-1 mutation probably causes defects in this process and leads to alterations of chromatin structure in the promoters of PR genes. In rfc3-1 mutant plants, promoters of PR genes may adopt more accessible conformations, which result in elevated gene expression. We have performed rigorous ChIP experiments to test whether there is a detectable difference in TGA2 binding to the PR1 promoter between wild type and rfc3-1. As expected, we were able to detect a clear difference between TGA binding to the PR1 promoter before and after INA induction. However, we did not observe a difference in binding between wild type and rfc3-1 (data not shown). We believe that this could be due to the fact that the method we used is not sensitive enough to detect small differences in chromatin accessibility of the PR1 promoter between wild type and rfc3-1 during defense.
The characterization and cloning of *rfc3-1* suggest that RFC3 is required for DNA replication and cell proliferation in plants and probably contributes to chromatin assembly and remodeling that are required for negative regulation of *PR* gene expression and SAR. Further careful investigations on chromatin modifications in *rfc3-1* may provide more detailed mechanistic insights in the future.
Experimental procedures

Mutant Screen and Characterization

All plants were grown in a growth room under a 16 h day (23°C)/8 h night (21°C) regime. The pBGL2-GUS seeds (Bowling et al., 1994) were treated with ethylmethane sulfonate (EMS) at a dose of 20 mM for 16 hours. For the primary screen, individually harvested seeds from about 1,000 M1 plants were grown on MS medium supplemented with 10μM INA. Half of the seedlings were tested for expression of the pBGL2-GUS reporter gene by GUS staining. Since the GUS staining procedure kills the seedlings, the remaining siblings of potential mutants were transplanted and their progeny were tested on MS with 10μM SA for confirmation of SA hypersensitivity.

RNA used for gene expression analysis was extracted from 15-day-old plants grown on MS medium using the Totally RNA™ kit from Ambion. Reverse transcription was carried out using M-MLV reverse transcription kit (Takara). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit from Qiagen (Valencia, CA). All data were normalized with Actin1. The primers used for amplification of Actin1, PR1 and PR2 were described previously (Zhang et al., 2003). Infection of wild type and mutant plants with H. p. Noco2 was performed on two-week-old seedlings as previously described (Li et al., 1999).

To obtain the rfc3-1 npr1-3 double mutant, an rfc3-1 homozygous plant was crossed with npr1-3 mutant, and the specific PCR primers used to screen for double mutant in the F2 are as follows: 5’ tatggtctcccggtactca 3’ and 5’ ttgaecgcatatgccgttga 3’ for rfc3-1 mutation, 5’ tatggtctcccggtactcg 3’ and 5’ ttgaecgcatatgccgttga 3’ for wild type RFC3; 5’ gactcggatgatattgagttag 3’ and 5’ tgttctcgtttgtcttctga 3’ for npr1-3 mutation, 5’ gactcggatgatattgagttag 3’ and 5’ tgttctcgtttgtcttctgg 3’ for wild type NPR1.

Mapping of rfc3-1

To map the rfc3-1 mutation, rfc3-1 (in Col background) was crossed with wild
type Landsberg erecta (Ler). Crude mapping was performed on F2 plants homozygous for rfc3-1 and fine mapping was carried out on F3 plants derived from F2 plants that were heterozygous for rfc3-1 while carrying homozygous pBGL2-GUS reporter gene. Both morphology and pBGL2-GUS reporter GUS staining of the progeny were used to confirm the phenotypes of the recombinants obtained.

The markers used for mapping were designed according to the Monsanto Arabidopsis polymorphism and Ler sequence collections (Jander et al., 2002). Marker T4O12 was amplified with primers 5’ctgaagaatcgagcattgcatc3’ and 5’ctgaaacagactgttaggcaag3’, and the Col fragment is 27 bp shorter than the Ler fragment. Marker F28K19 was amplified with primers 5’cttaataaagttgtgtaaccg3’ and 5’gtgccattagcaagtgc3’, and the Col fragment is 29 bp shorter than the Ler fragment. Marker F22K20 was amplified with primers 5’atgatcctgagggaccatac3’ and 5’etctacctcaaggcagc3’, and the Col fragment is 17 bp shorter than the Ler fragment. Marker F2P24-2F2R was amplified with primers 5’caagaaagaagtcatattggtc3’ and 5’aactggtcgccaaacctgc3’, and the Col fragment is 79 bp shorter than the Ler fragment. Marker T5M16-2 was amplified with primers 5’caccaagtaattacttccgaag3’ and 5’ttagtagttggctgcatc3’, and the Col fragment is 5 bp shorter than the Ler fragment. Marker T5M16-3 was amplified with primers 5’gctcgctgacgttgaccg3’ and 5’gttatctgtcagaccctacc3’, and the Col fragment is 6 bp longer than the Ler fragment.

Transgene complementation analysis of rfc3-1

Since the RFC3 gene is relatively large, the full-length genomic sequence of RFC3 was divided into two fragments (fragment 1 and fragment 2) for PCR amplification using Phusion™ high-fidelity PCR master mix (Finnzymes). The primer pairs used for amplifying fragment 1 and fragment 2 were 5’ cgcggatccgtcctgcaaatgctgatg 3’ with 5’ cgggagctcacctatatgctcactgaagg 3’, and 5’ cggggtaccacatggctggaccagcagag 3’ with 5’ cgcgtcgacagctcacgcccatcacaatg 3’, respectively. The gel-purified PCR products were digested with BamHI and SacI, and
KpnI and SalI, respectively. The digested fragments were ligated into pG229 (4.5 kp). The final constructs were confirmed by sequencing and transformed into Agrobacterium tumefaciens strain GV3101 together with the helper plasmid pSoup. Plants heterozygous with rfc3/RFC3 genotype were transformed with Agrobacterium containing RFC3 using the floral-dip method (Clough and Bent, 1998). The transformants were selected by the herbicide Basta. Transformants homozygous for rfc3-1 were identified by PCR.

**Subcellular localization of RFC3**

To fuse RFC3 to the green fluorescent protein (GFP) gene, full length RFC3 cDNA without the stop codon was amplified by PCR and cloned into the pG229-GFP vector. The resulting plasmid was sequenced to confirm that the fusion gene was in-frame without PCR errors. For transient expression of the RFC3-GFP fusion proteins, pG229-RFC3-GFP was transfected into Arabidopsis mesophyll protoplasts according to a previously described protocol (Sheen, 2001). Green fluorescence was observed by using confocal microscopy. pG229-GFP was used as control.

**Leaf epidermal cell and root cortex cell examination**

Plant parts were photographed under a Leica dissecting microscope with a Canon Powershot s70 digital camera. The first to the fourth true leaves of 21-day-old plants of the rfc3-1 mutant and the wild type were collected separately in Eppendorf tubes, and treated with a chloral hydrate/glycerol/water solution (8:1:2) to clear the cells (Ohad et al. 1996). The epidermal cells on both the abaxial and adaxial leaf surfaces were photographed with a Zeiss Axiovert 200M microscope with Axiocam HRm digital camera that permits visualization of optical sections within the leaf.

To examine cortex cells, seeds of rfc3-1 and the wild type were sown on the same MS plate according to Chen et al., 2003. After germination, the length of each root was measured and marked every 24 hr. The roots of 10-day-old seedlings were harvested separately in Eppendorf tubes, treated with a chloral hydrate/glycerol/water
solution (8:1:2), and the root cortex cells were examined with a Zeiss Axiovert 200M microscope.

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References:


Figure Captions:

**Figure 1. Characterization of the rfc3-1 mutant**
(A) GUS staining of wild type Col and rfc3-1 plants, both with the pBGL2-GUS reporter gene. Two-week-old seedlings grown on MS with or without 10 μM SA were stained for GUS activity as previously described (Bowling et al., 1994).
(B) Morphology of wild type, rfc3-1 and snl1 plants. All plants were grown on soil and photographed when they were four weeks old.
(C) Relative PR1 and (D) PR2 expression in wild type and rfc3-1 plants. Two-week-old seedlings grown on MS with or without 50 μM SA were collected for RNA extraction and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real time PCR with Actin 1 (multiplied by 1000 for clarity). Shown are means of three replicates ± SD. The PR1 expression of rfc3-1 are significantly higher than Col wild type, with or without SA treatment (P<0.01, t test). The expression level of PR2 in rfc3-1 with 50μM SA is significantly higher than that of the wild type under the same conditions (p<0.01), but there is no significant difference in PR2 expression between wild type and rfc3-1 plants without SA induction (p>0.05). The experiment was repeated once with similar results.
(E) Growth of H. a. Noco2 on wild type, rfc3-1, and snl1 plants. Two-week-old seedlings were sprayed with H. p. Noco2 at a concentration of 5,000 spores per ml of water. The infection was scored 7 days after inoculation. The values presented are averages of four replicates ± standard deviation. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated three times with similar results.

**Figure 2. Enhanced pathogen resistance and PR gene expression in rfc3-1 npr1-3 double mutant**
(A) Morphology of npr1-3, rfc3-1 and rfc3-1 npr1-3 double mutant plants. All plants were grown on soil and photographed when they were four weeks old.
(B) Growth of H. a. Noco2 on npr1-3, rfc3-1 and rfc3-1 npr1-3 double mutant plants. The experiment was carried out as described in Figure 1E. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated three times with similar results.
(C) PR1 and (D) PR2 expression in npr1-3, rfc3-1 and rfc3-1 npr1-3 double mutant with or without SA induction. The experiment was carried out as described in Figure 1C. The PR1 and PR2 expression of rfc3-1 or rfc3-1 npr1-3 are significantly higher than npr1-3, with or without SA (P<0.01, t test). There is no significant difference in PR1 or PR2 expression between rfc3-1 and rfc3-1 npr1-3 upon SA induction (p>0.05). The experiments were repeated once with similar results.

**Figure 3. Map-based cloning of rfc3-1**
(A) Map of the rfc3-1 locus on chromosome 1. Positions of the markers used for
mapping are indicated. The two final flanking markers were F2P24 and T5M16. The rfc3-1 mutation is marked as an asterisk (*).

(B) Gene structure of RFC3. Exons are indicated with boxes and introns are represented by lines. The region where the G to A mutation occurs in rfc3-1 is shown in detail.

(C) Morphology of wild type, rfc3-1 and rfc3-1 transformed with a genomic clone of RFC3 driven by its native promoter. Plants were grown on soil for four weeks before the picture was taken.

(D) pBGL2-GUS reporter gene expression in rfc3-1 and rfc3-1 transformed with a genomic clone of RFC3 driven by its native promoter. Two-week old seedlings grown on MS with or without 10 μM SA were stained for GUS activity as previously described (Bowling et al., 1994).

(E) Growth of H. a. Noco2 on wild type, rfc3-1 and two independent transgenic rfc3-1 lines carrying genomic RFC3 driven by its native promoter. The experiment was carried out as described in Figure 1E. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated at least three times with similar results.

Figure 4. Amino acid sequence alignments of AtRFC3 and its homologs in Oryza sativa (OsRFC3), Saccharomyces cerevisiae (ScRFC3), Drosophila melanogaster (DmRFC3), human (hRFC5), Mus musculus (MmRFC5), Xenopus laevis (XRFC5) and Danio rerio (DrRFC5).

Identical amino acids are shaded in black and similar amino acids are shaded in gray. Alignment was carried out using ebi ClustalW (http://www.ebi.ac.uk/clustalw/). AtRFC3, accession number NP_177871.1; OsRFC3, accession number XM_468050.1; ScRFC3, accession number NC_001146.3; DmRFC3, accession number NM_135555.2; hRFC5, accession number NM_007370.3; MmRFC5, accession number Q9D0F6; XRFC5, accession number BC072889.1; and DrRFC5, accession number NM_001003862.1.

Figure 5. Functional analysis of Arabidopsis RFC3

(A) Localization of RFC3-GFP. Arabidopsis mesophyll protoplasts expressing the pG229-RFC3-GFP fusion proteins were examined by confocal microscopy and the picture was taken from a representative protoplast. The red fluorescence reflects the chlorophyll auto-fluorescence. The nucleus is obvious from the bright field image of the protoplast. pG229-GFP alone was used as control. The experiment was repeated once with similar results.

(B) Morphology of mature plants, representative flowers, and petals of wild type and rfc3-1.

(C) Morphology of young seedlings and leaves of wild type and rfc3-1 plants. The first to the fourth true leaves of 20-day-old seedling were used to measure the epidermal cells under microscope. The scale bar is the same for both images.

Figure 6. Root growth and cell production in wild type and rfc3-1 mutant

(A) Root elongation rate in wild type and rfc3-1 seedlings. The values presented are
averages of 30 replicates ± standard deviation. Statistical analyses of root elongation rates of *rfc3-1* compared with Col on each date revealed no statistically significant difference (P>0.05, t test).

(B) Root cortex cell length in the root hair zone of wild type and *rfc3-1* plants. The values presented are averages of 50 replicates ± standard deviation. The root cortex cell length of *rfc3-1* is significantly longer than Col wild type (P<0.01, t test).

(C) Cell production rate (root elongation rate/root cortex cell length) of wild type and *rfc3-1* plants. Statistical analyses of cell production rates of *rfc3-1* compared with Col on each date revealed statistically significant difference except values at Day 1, 2 and 3 (P<0.01, t test).
**A**

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**H.a. Noco2**

![bar graph](image15)
Figure 1. Characterization of the *rfc3-1* mutant

(A) GUS staining of wild type Col and *rfc3-1* plants, both with the *pBGL2-GUS* reporter gene. Two-week-old seedlings grown on MS with or without 10μM SA were stained for GUS activity as previously described (Bowling et al., 1994).

(B) Morphology of wild type, *rfc3-1* and *sni1* plants. All plants were grown on soil and photographed when they were four weeks old.

(C) Relative *PR1* and (D) *PR2* expression in wild type and *rfc3-1* plants. Two-week-old seedlings grown on MS with or without 50μM SA were collected for RNA extraction and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real time PCR with *Actin 1* (multiplied by 1000 for clarity). Shown are means of three replicates ± SD. The *PR1* expression of *rfc3-1* are significantly higher than Col wild type, with or without SA treatment (P<0.01, t test). The expression level of *PR2* in *rfc3-1* with 50μM SA is significantly higher than that of the wild type under the same conditions (p<0.01), but there is no significant difference in *PR2* expression between wild type and *rfc3-1* plants without SA induction (p>0.05). The experiment was repeated once with similar results.

(E) Growth of *H. a. Noco2* on wild type, *rfc3-1*, and *sni1* plants. Two-week-old seedlings were sprayed with *H. p. Noco2* at a concentration of 5,000 spores per ml of water. The infection was scored 7 days after inoculation. The values presented are averages of four replicates ± standard deviation. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated three times with similar results.
A

B

H. a. Noco2

Spores/plant (x10 )

C

PR1

Relative PR1/Actin1

D

PR2

Relative PR2/Actin1

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Figure 2. Enhanced pathogen resistance and PR gene expression in *rfc3-1 npr1-3* double mutant

(A) Morphology of *npr1-3, rfc3-1* and *rfc3-1 npr1-3* double mutant plants. All plants were grown on soil and photographed when they were four weeks old.

(B) Growth of *H. a. Noco2* on *npr1-3, rfc3-1* and *rfc3-1 npr1-3* double mutant plants. The experiment was carried out as described in Figure 1E. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated three times with similar results.

(C) *PR1* and (D) *PR2* expression in *npr1-3, rfc3-1* and *rfc3-1 npr1-3* double mutant with or without SA induction. The experiment was carried out as described in Figure 1C. The *PR1* and *PR2* expression of *rfc3-1* or *rfc3-1 npr1-3* are significantly higher than *npr1-3*, with or without SA (P<0.01, t test). There is no significant difference in *PR1* or *PR2* expression between *rfc3-1* and *rfc3-1 npr1-3* upon SA induction (p>0.05). The experiments were repeated once with similar results.
Figure 3. Map-based cloning of \textit{rfc3-1}

(A) Map of the \textit{rfc3-1} locus on chromosome 1. Positions of the markers used for mapping are indicated. The two final flanking markers were F2P24 and T5M16. The \textit{rfc3-1} mutation is marked as an asterisk (*).

(B) Gene structure of \textit{RFC3}. Exons are indicated with boxes and introns are represented by lines. The region where the G to A mutation occurs in \textit{rfc3-1} is shown in detail.

(C) Morphology of wild type, \textit{rfc3-1} and \textit{rfc3-1} transformed with a genomic clone of \textit{RFC3} driven by its native promoter. Plants were grown on soil for four weeks before the picture was taken.

(D) \textit{pBGL2-GUS} reporter gene expression in \textit{rfc3-1} and \textit{rfc3-1} transformed with a genomic clone of \textit{RFC3} driven by its native promoter. Two-week old seedlings grown on MS with or without 10μM SA were stained for GUS activity as previously described (Bowling et al., 1994).

(E) Growth of \textit{H. a. Noco2} on wild type, \textit{rfc3-1} and two independent transgenic \textit{rfc3-1} lines carrying genomic \textit{RFC3} driven by its native promoter. The experiment was carried out as described in Figure 1E. Statistical differences among the samples are labelled with different letters (P<0.01, ANOVA). The experiment was repeated at least three times with similar results.
### Figure 4. Amino acid sequence alignments of AtRFC3 and its homologs in *Oryza sativa* (OsRFC3), *Saccharomyces cerevisiae* (ScRFC3), *Drosophila melanogaster* (DmRFC3), human (hRFC5), *Mus musculus* (MmRFC5), *Xenopus laevis* (XRFC5) and *Danio rerio* (DrRFC5).

Identical amino acids are shaded in black and similar amino acids are shaded in gray. Alignment was carried out using ebi ClustalW (http://www.ebi.ac.uk/clustalw/). AtRFC3, accession number NP_177871.1; OsRFC3, accession number XM_468050.1; ScRFC3, accession number NC_001146.3; DmRFC3, accession number NM_135555.2; hRFC5, accession number NM_007370.3; MmRFC5, accession number Q9D0F6; XRFC5, accession number BC072889.1; and DrRFC5, accession number NM_001003862.1.
Figure 5. Functional analysis of Arabidopsis RFC3

(A) Localization of RFC3-GFP. Arabidopsis mesophyll protoplasts expressing the pG229-RFC3-GFP fusion proteins were examined by confocal microscopy and the picture was taken from a representative protoplast. The red fluorescence reflects the chlorophyll auto-fluorescence. The nucleus is obvious from the bright field image of the protoplast. pG229-GFP alone was used as control. The experiment was repeated once with similar results.

(B) Morphology of mature plants, representative flowers, and petals of wild type and rfc3-1.

(C) Morphology of young seedlings and leaves of wild type and rfc3-1 plants. The first to the fourth true leaves of 20-day-old seedling were used to measure the epidermal cells under microscope. The scale bar is the same for both images.
A

Root elongation rate (µm/h)

B

Root cortex cell length (µm)

C

Cell production rate (cell/h)
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