Responsiveness and adaptation to salt stress of the REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1) gene are controlled by its promoter

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Running title: Promoter adaptation to salt stress

Keywords: promoter/ RRTF1/ salt stress/ roots/Arabidopsis thaliana

Word count: 3548

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Summary

The REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1) gene encodes a member of the ERF/AP2 transcription factor family involved in redox homeostasis. The RRTF1 gene shows tissue-specific responsiveness to various abiotic stress treatments including a response to salt stress in roots. An interesting feature of this response is an adaptation phase that follows its activation, when promoter levels revert to a base line level, even if salt stress is maintained. It is unclear if adaption is controlled by a switch in promoter activity or by changes in transcript levels. Here we show that the RRTF1 promoter is sufficient for the control of both activation and adaptation to salt stress. As constitutive expression of RRTF1 turned out to be detrimental to the plant, we propose that promoter-regulated adaptation evolved as a protection mechanism to balance the beneficial effects of short-term gene activation and the detrimental effects of long-term gene expression.

Introduction

Transcription is the first step in a number of processes that regulate the conversion of genetic information into a phenotype. Induced promoters, promoters induced by environmental signals, during defined developmental stages or in certain cell types have been widely used to control the expression of transgenes, and an extensive collection of promoters has been assembled [1]

. While promoter activity regulates primary transcript levels, RNA steady state levels are also significantly influenced by RNA stability and turnover [2]. Transcript synthesis and degradation are often dynamic processes when feedback loops alter the concentration of transcription control factors [3] or when the induction of natural antisense transcription generates small RNAs that induce transcript degradation [4]. In addition, promoter regions can undergo epigenetic changes that alter their transcriptional competence due to changes in accessibility for regulatory factors [5].

The REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 (RRTF1) gene in Arabidopsis thaliana, encodes a key regulator in a redox network that is required to achieve redox homeostasis following exposure to photosynthetic perturbations as inactivation of RRTF1 makes plants sensitive to high light stress [6]. RRTF1 shows an unusual activation profile in roots where its transcript levels increase when exposed to high NaCl levels but revert to basic levels even when the salt concentration remains high. This suggested that prolonged exposure to salt stress causes changes in promoter activity or that it induces a transcript turnover mechanism. The latter assumption was supported by the observation that the gene partially

overlaps with a gene in antisense orientation, which could be the source for antisense transcripts that alter RRTF1 transcript levels. To differentiate between transcriptional and post-transcriptional effects, we analysed the activity of the RRTF1 promoter as part of a transgene reporter construct. When linked to a transgene, the RRTF1 promoter still displayed the same expression features as shown by the RRTF1 gene, which makes it likely that induction and adaptation activity are regulated by the promoter and not by antisense-mediated RNA turnover. The unusual transient promoter activity of the RRTF1 gene under continuous stress may have evolved to address two conflicting features: the beneficial effects of short-term RRTF1 activation and the detrimental effects of prolonged RRTF1 over-expression.

Results

When we screened the expression profiles of Arabidopsis genes with overlapping antisense genes [7] on the Genevestigator platform [8], we identified an interesting adaptation feature for the RRTF1 gene (At4g34410). The gene was activated in roots under salt stress [9] reaching a peak of expression after around 6 hours, which, however, reverted to similar low transcript levels that it displays before stress application although the salt stress was maintained (Figure 1). This suggested that the gene was activated in response to salt stress to which it quickly adapted by reverting to pre-stress level.

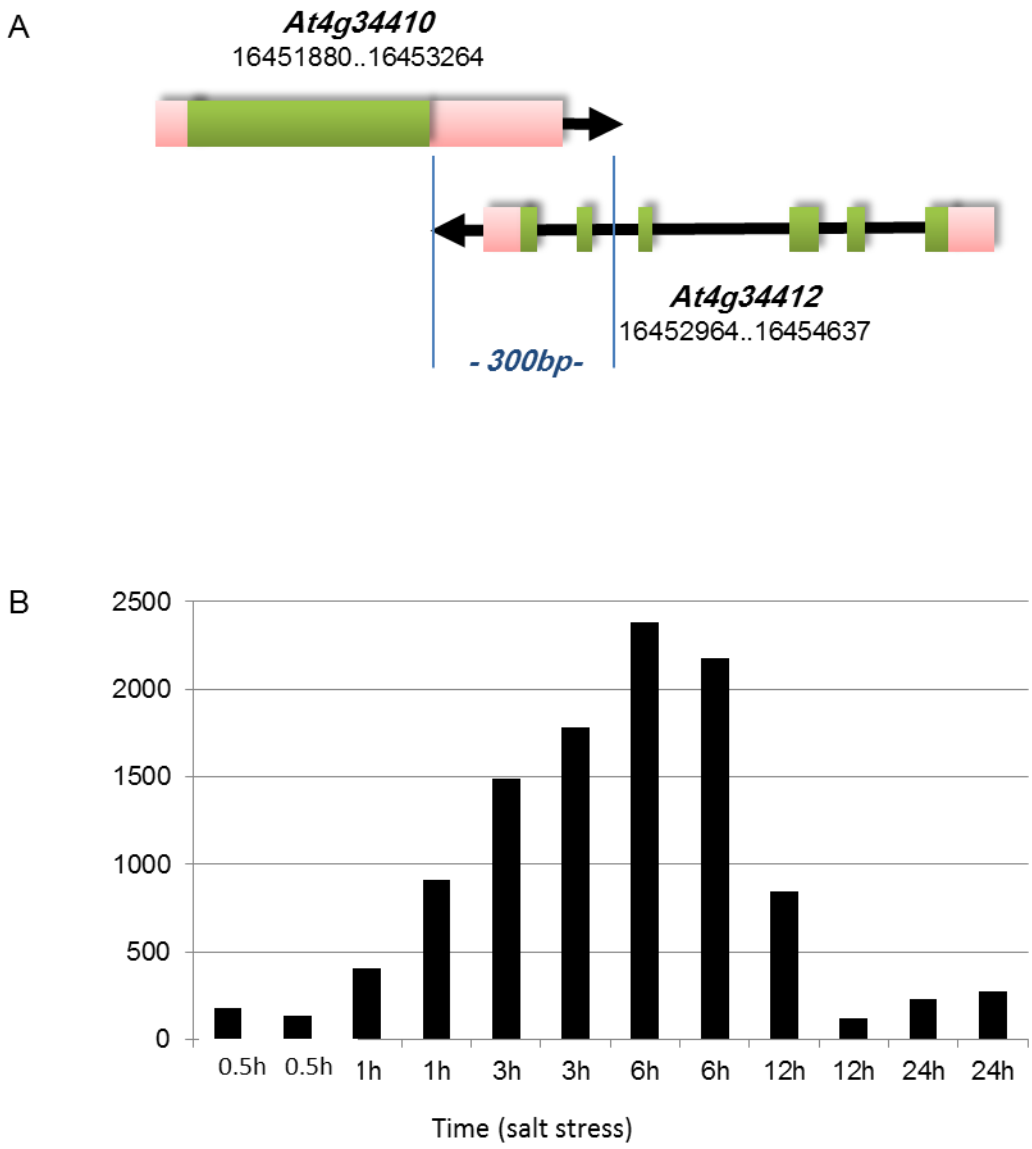

Figure 1: Structure and salt induction of RRTF1 (At4g34410)

A. Schematic map of At4g34410 and its partial overlap with At4g34412. Numbers refer to the positions on chromosome 4 and mark start and end points of genes. Exons are shown as boxes with coding regions in green. B. Salt response profiles of At4g34410 extracted from AtGenExpress salt stress experiment NASCARRAYS-140 [9] with two replica values shown for each time point. Activation peaks after 6h, after which transcript levels return to pre-activation levels.

This unusual change in gene activity during a relatively short period of abiotic stress may have evolved to ensure efficient RRTF1 induction while preventing potentially detrimental effects of high RRTF1 concentration. To test if a prolonged increase in RRTF1 levels was detrimental to the plant, we expressed the RRTF1 gene in Arabidopsis under the control of the 35S promoter and a heat responsive promoter, respectively. In tissue culture, 35S-RRTF1 transformants grew slowly displaying a yellow leaf colour (Figure 2A), while in soil the transformants died before producing seeds (Figure 2B). The yellow leaf phenotype was also detectable one week after activation of RRTF1 transcription using a heat shock promoter (Figure 2C and D). These data imply that Arabidopsis is sensitive to prolonged increases in RRTF1 levels, and that the quick reduction of expression levels is required to prevent detrimental RRTF1 expression effects.

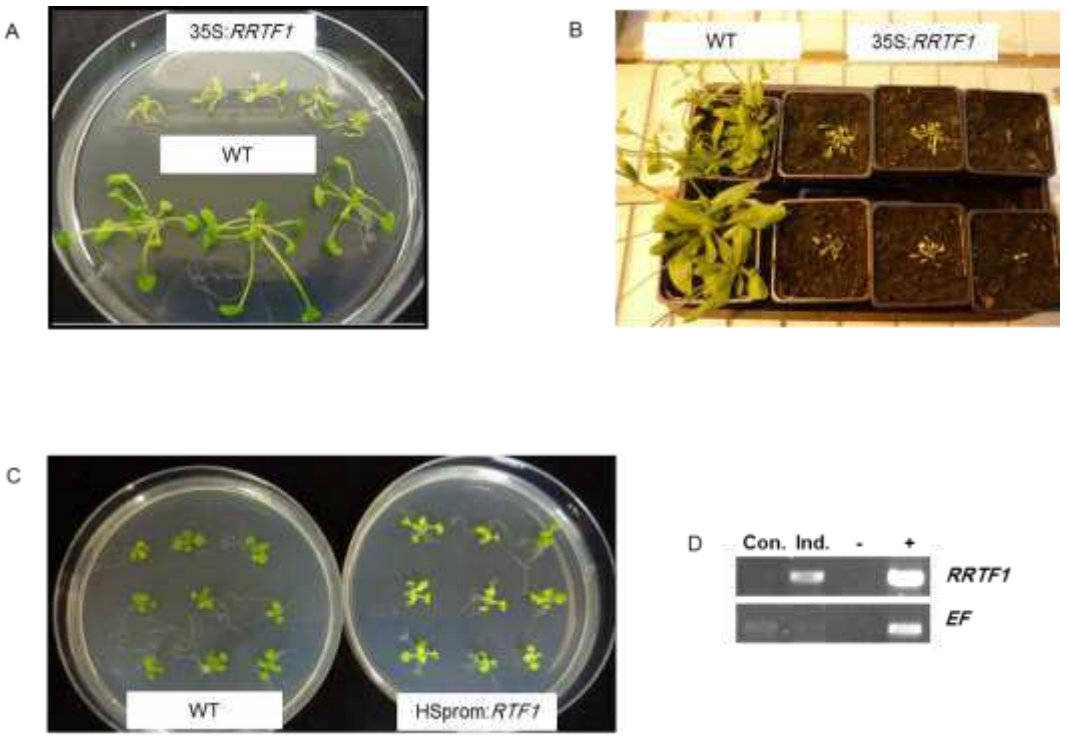

Figure 2: Over-expression of the RRTF1 gene.

A. Phenotypes of 30-day-old seedlings of wildtype and 35S:RRTF1 transformants in tissue culture. B. Phenotypes of 35-day-old seedlings of wildtype and 35S:RRTF1 transformants in soil. C. Phenotypes in WT and HSprom: RRTF1 transformants six days after 2h induction at 40^{o}C. D. Semi-quantitative RT-PCR analysis of RRTF1 induction after 2h heat treatment. Con.– control without induction, Ind.- 2h induction at 40oC, -/+ negative and positive PCR controls, EF - EF1a expression control.

As the RRTF1 gene partially overlaps with a gene in antisense orientation, it was possible that adaptation was due to the synthesis of antisense transcripts causing double strand RNA formation and its breakdown into small RNAs. Alternatively, the adaptation phase could have been caused by inactivation of the RRTF1 promoter in response to prolonged exposure to salt stress. To differentiate between these alternatives, we cloned the RRTF1 promoter in front of a reporter gene and we tested the expression profile of the reporter construct, and of six deletion constructs (A-F) with different parts of the promoter region removed (Figure 3). The reporter gene showed a similar expression profile as the endogenous RRTF1 gene with an activation phase that peaked around 6h after salt application, followed by an adaptation phase. A similar salt response profile was retained in transformants that contained deletion construct F, while activation was inhibited in transformants with construct A, and was significantly reduced in transformants carrying constructs B and C. A, B and C don't share a common deletion region, which suggests that more than one promoter region contributes to promoter activation. Plants with deletion constructs D and E showed a moderate activation level providing further support for the assumption that activation levels result from cooperative effects of different promoter regions. The adaptation response was retained among all transformants, as all lines with an activation peak at 6h post salt application showed a

reduction in transcript levels at 24h. This also applied to plants expressing deletion construct D but these had slightly increased pre-stress levels, which suggests that the region between position -2047 and -1822, which is present in all deletion constructs except construct D, contains elements that contribute to the repression of the promoter in the absence of salt stress.

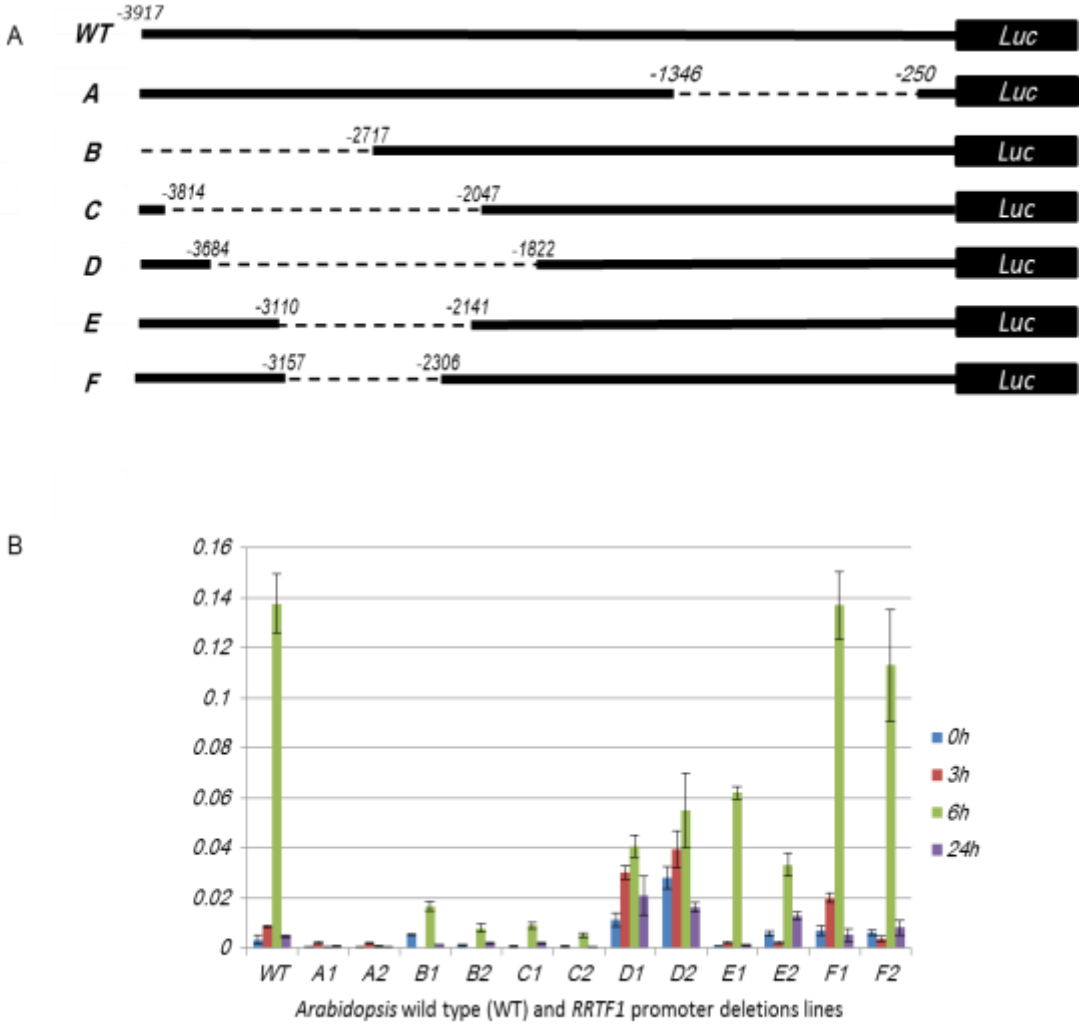

Figure 3: Analysis of deletion constructs of the RRTF1 promoter.

A. Schematic diagram of the RRTF1 promoter and its deletion derivatives. Dashed lines indicate deleted fragments. Numbers indicate positions relative to the translation start site.

B. Salt-activation of RRTF1 promoter wildtype and deletion constructs in roots. RT-qPCR

data are shown for two single copy transformants for each deletion construct. RNA was analysed 0, 3, 6, 24 hours after application of salt stress. Expression levels are shown relative to EF1a expression levels.

The expression characteristics of the RTTF1 promoter suggested that activation and adaptation were independent of potential siRNAs generated via antisense transcription from At4g34412. To test if siRNA-based mechanisms influenced RRTF1 expression, we tested the induction profile of a RRTF1-luc transgene and of the endogenous RRTF1 gene in a mutant for RNA DEPENDENT RNA POLYMERASE6 (RDR6), which contributes to several si-RNA pathways [10] [4] [11]. Interestingly, induction levels were reduced in rdr6 lines while adaption was not altered (Figure 4). A similar reduction in activation levels was observed in a mutant for DNA METHYLTRANSFERASE 1 (MET1), which suggests that epigenetic mechanisms are at least indirectly involved in the promoter activation of RRTF1, possibly by altering the availability or concentration of transcription factors or activators for the RRTF1 promoter. Interestingly, the deletion of the RRTF1 gene also resulted in reduced activation rates (Figure 4), which suggest an involvement of RRTF1 in the regulation of its own promoter. While activation levels were reduced in all three mutants, there was no indication that any of the mutation affected the reversion to pre-stress expression levels after prolonged salt stress.

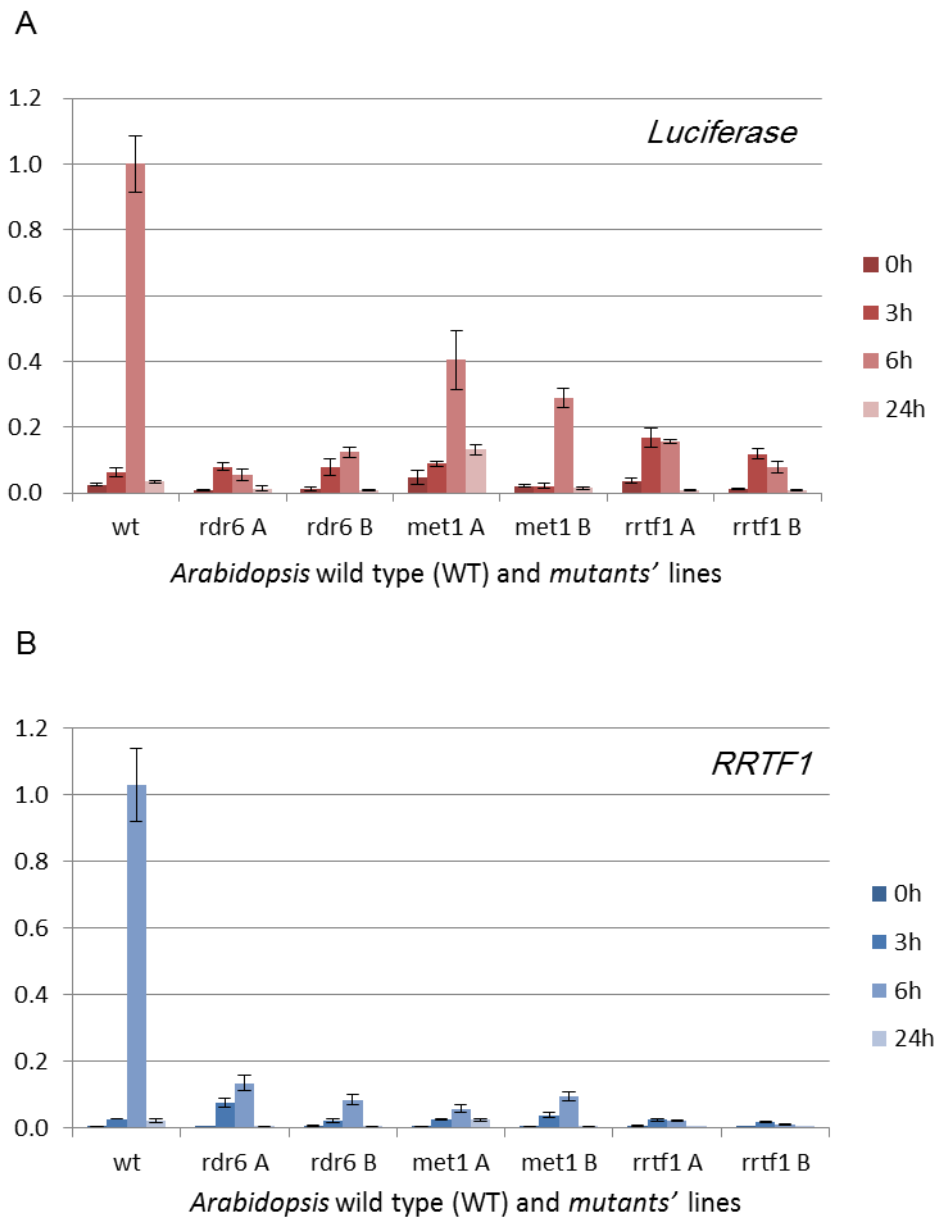

Figure 4: Comparing RRTF1 promoter activity in mutant lines.

Transcript levels were compared for the luciferase transgene (A) and for the endogenous RRTF1 gene (B) in single copy RRTF1prom:luc transformants in wildtype background (wt) and in mutant backgrounds for RNA-DEPENDENT RNA POLYMERASE 6 (rdr6), DNA METHYLTRANSFERASE 1 (met1) or RRTF1 (rrtf1). RT-qPCR data are shown for two single copy transformants for each mutant line. RNA was analysed 0, 3, 6, 24 hours after application of salt stress. Expression levels are shown relative to EF1a expression.

Discussion

The RRTF1 gene shows a complex tissue- and stress-specific induction profile. In roots, its response to salt stress can be divided into an activation phase over about 6 hours followed by an adaptation phase with transcript levels reaching pre-induction values within 12-24 hours post induction. Activation and adaptation phases are conserved for a marker transgene linked to the RRTF1 promoter, which suggest that both phases are controlled by the promoter.

Deletion analysis of the promoter identifies several regions that contribute to the efficiency of activation, as deletion of different regions result in loss or reduction of activation levels. In contrast, adaptation was unchanged in all deletion constructs, which makes it unlikely that activation and adaptation mechanisms are mediated by the same promoter elements. This assumption is also supported by the analysis of the three mutant lines rdr6, met1 and rrtf1, which all show a reduction in activation levels but don't alter the adaptation characteristics.

Our data do not allow us to define the mechanisms responsible for the adaptation response but they argue against post-transcriptional effects mediated by antisense expression as they have been described for other overlapping gene pairs [4], [12]. It is more likely that adaptation involves changes in the availability of regulatory factors that control transcription, or that the RRTF1 promoter undergoes structural changes that alter its accessibility to regulatory factors. An example for a repressive mechanism based on the change of a regulatory factor is the WRKY transcription factor whose synthesis is induced in roots under salts stress. WRKY8 interaction with the VQ protein, which is also produced in response to salt stress, reduces its

binding efficiency to DNA , and it has been suggested that VQ acts as a repressive factor to balance WRKY8 activity [13].

An alternative to the expression of inhibitor proteins is the establishment of epigenetic changes that render a promoter inactive or less active. There are several examples for stress-induced epigenetic changes that influence gene expression levels or the expression competence of genes. In rice, salts stress is accompanied by variation in body methylation, and it has been suggested that this plays a role in regulating gene expression in a organ and genotype specific manner under salinity stress [14]. Histone deacetylases HDA6 and HDA19 are induced by jasmonic acid (JA) and ethylene, and have been proposed to contribute to stress-responsive repressive states at certain target genes [15]. As body methylation requires MET1 [16] and as RRTF1 adaptation is not altered in a met1 mutant, it is unlikely that body methylation is involved in adaptation but other epigenetic marks cannot be ruled out. A ChIP analysis of histone marks associated with the RRTF1 promoter at the beginning and end of the adaptation phase should help to narrow down the mechanistic models behind RRTF1 adaptation, if the technical challenges can be overcome to isolate chromatin from Arabidopsis roots.

Experimental procedures

Mutant lines

All mutants used in this study are homozygous mutants in a Columbia (Col) background. Genotyping was performed to confirm the homozygous state of mutations as described for rdr6-11 [17] , met1-1 [18] and rrtf1 (Salk_150614) [6].

Design of the RRTF1promo:luc vector and its deletion derivatives.

A 4218 bp fragment upstream of the RRTF1 translation start site was amplified using forward and reverse primers 1 and 2 (Table 1), the later containing a NcoI restriction site. The amplified 4218 bp fragment was cut with EcoRV, producing a 3917bp NcoI-EcoRV promotor fragment that was inserted into a pGreen 0179 vector in front of a luciferase gene [19]. For deletion construct A, a 250 bp region of the proximal end of the RRTF1 promoter was amplified using primer 3 containing an AgeI restriction site and a reverse primer 2 containing a NcoI restriction site. The amplified fragment was digested with AgeI and NcoI and was ligated to the corresponding sites of RRTF1promo:luc previously digested with both enzymes. For deletion construct B, RRTF1promo:luc was digested with BamHI and AflII and religated after Klenow (Invitrogen) treatment. For deletion construct C, RRTF1promo:luc was digested with HindIII and religated. For deletion construct D-F, RRTF1promo:luc was digested with AflII at position -2717, followed by digestion with 2-3 units/µg BAL 31 for 0.5 or 1h, respectively. Deletion endpoints for constructs D-F were determined by sequencing.

Design of plasmids HSprom:RRTF1 and 35S:RRTF1

The HSprom:RRTF1 construct was prepared by replacing the GUS gene in pGreenII 0029 (http://www.pgreen.ac.uk/a_pls_fr.htm) with a RRTF1-FLAG fragment. The FLAG tag sequence was prepared by annealing F-flag and R-flag oligonucleotides (table 1) followed by Klenow (Invitrogen) extension, which produced a 47 bp FLAG fragment with BamHI, XbaI, AatII restriction sites at the 5' end and a SacI restriction site at the 3' end. The FLAG tag fragment was digested with BamHI and SacI and was ligated to a BamHI/SacI digested pGreenII 0029 vector with a hsp18.2 heat shock promoter [20] that had been inserted as a BstXI-BamHI fragment. The coding sequence of the RRTF1 gene was amplified using primer 4 that contained a BamHI restriction site and primer 5 that contained an AatII restriction site. The amplified 820bp fragment was digested with BamHI and AatII and was cloned into the corresponding sites of the HSpromo:Flag vector digested with both enzymes.

The 35S:RRTF1 construct was prepared by isolating the RRTF1-FLAG:nos terminator cassette from HSprom:RRTF1 as a 1126 bp BamHI–EcoRV fragment. This cassette was ligated into the complementary ends of the pGreen0179:35S cassette vector (http://www.pgreen.ac.uk/a_pls_fr.htm) in front of the 35S promoter.

Agrobacterium-mediated transformation

Recombinant constructs were transformed into Agrobacterium tumefaciens GV3101 by electroporation using a BioRAD Gene pulser cell-porator with C= 25μF; R= 400Ω; 8-9ms delay and pulsed at V=1.8KV. Arabidopsis thaliana (Col-0) was transformed using the floral dip method [21]. The transformed seeds were collected and surface sterilized and grown on MS

media supplemented with 25µg/ml hygromycin except for the selection of the HSpromo:RRTF1 transformants, which were selected on 50µg/ml kanamycin.

Stress treatment

Salt stress experiments were carried out according to Kilian et al. 2007 [22]. Seedlings were grown in a phytochamber under a long day regime (16h light and 8h darkness) under sterile conditions. Sterilized seeds were grown for 13 days on the surface of rafts in growth boxes that contained MS medium with 2% sucrose and 5.5% agar. The boxes were closed with a membrane-vented lid. The rafts were moved to boxes containing 100ml liquid MS medium without sucrose. Root samples were collected 0, 3, 6 and 24h after 18 days old seedlings were transferred into boxes with150mM NaCl.

Expression analysis

Total RNA was extracted from Arabidopsis roots using the PureLink® RNA Mini Isolation Kit (cat.# 12183018A, Life technologies-Invitrogen). DNA was removed from isolated RNA samples using the PureLink® DNase Kit (cat.# 12185010, Life technologies-Invitrogen). 2µg DNAse-treated RNA was used for cDNA synthesis with SuperScript™ II reverse transcriptase and oligo $(dt)_{18}$ primers (cat.# 18064-014, Life technologies-Invitrogen) following the manufacturer's instructions. For semi-quantitative RT-PCR analysis we used Mytaq™ red mix DNA polymerase (cat.# BIO-25043, Bioline) according to the manufacturer's protocol. Primer sequences are listed in table 1. For quantitative PCR analysis we used the brilliant III ultrafast SYBR green qPCR master mix (cat.# 600882, Agilent Technologies-Stratagene) and a Bio-Rad CFX96 cycler (Bio-Rad) according to the manufacturer's protocol. Transcript levels were calculated relative to *EF1α* expression using the ΔCT method [23].

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Table 1: Primer names and sequences

Primer ID	Sequence 5' to 3'	Description
1	AGGTATACTGGTTTCCACGGAATCTCC	Full RRTF1 promoter forward primer
2	AGGATAATCCATGGCCGACTCTCGATTCC	Full RRTF1 promoter reverse primer with NcoI restriction site (undelined)
3	GGGGACCGGTGTGTTTCCAAAAACACAGAT	RRTF1 promoter forward primer with AgeI restriction site (underlined).
4	CCCGGATCCTCGGGTATGCATTATCCTAACAACAG	RRTF1 forward primer with BamHI restriction site (underlined).
5	CCCGACGTCCTGGAACATATCAGCAATTGT	RRTF1 reverse primer with AatII restriction site (underlined).
6	CGTGTCAGGGTTTTTCCAGT	Forward RRTF1 gene specific primer
7	CCTCCTCCTCCGTTCCATTGC	Reverse RRTF1 gene specific primer
8	GCGTGTCATTGAGAGGTTCG	Forward *EFα1* gene specific primer
9	GTCAAGAGCCTCAAGGAGAG	Reverse *EFα1* gene specific primer
10	CGTCTTCAGTTTCATCTCCTGTTGC	q-RRTF1 forward primer
11	GAAGAGATACGCCCTGGTTCCTG	q-luciferase forward primer
12	ACTGCATACGACGATTCTGTGATTTG	q-luciferase reverse primer
13	CTCTCCTTGAGGCTCTTGACCAG	q- *EFα1* forward primer
14	CCAATACCACCAATCTTGTAGACATCC	q- *EFα1* reverse primer

F-FLAG	GGGGGATCCTCTA**GACGTC**GATTACAAGGATGACGAC	Forward FLAG oligonucleotides. It contains the restriction site for BamHI (underlined), XbaI (italic) and AatII (bold) respectively.
R-FLAG	CTAATGTTCCTACTGCTGCTATTCACTCGAGGGG	Reverse FLAG oligonucleotides with SacI restriction site (underlined)