Previously, we have isolated a novel circadian clock mutant (called *rs24*) in *Arabidopsis thaliana*. The mutant displayed short period rhythms of the *CAB2:LUC* bioluminescent reporter in continuous red light. Genetic mapping followed by complementation tests have demonstrated that the circadian phenotype is caused by a missense mutation in a gene coding for a putative acyltransferase. Preliminary data also indicated that the induction of salicylic acid (SA)-dependent pathogen-related genes is highly elevated in the mutant suggesting a role for the protein (RS24) in the regulation of SA accumulation or signalling.

The aim of our project was (1) to characterize the *RS24* gene and RS24 protein in details at molecular and biochemical levels, (2) to integrate this novel function, represented by RS24, in the molecular clockwork of Arabidopsis and (3) to investigate the possibility if RS24 could mediate crosstalk between SA and circadian clock signalling pathways.

CHARACTERIZATION OF THE RS24 MUTANT AND THE RS24 GENE/PROTEIN

We found that *RS24* is expressed in all tissues and cells, except for the root and inflorescence of adult plants. Neither the expression of *RS24* nor the stability of the RS24 protein was affected by treatments of different qualities and quantities of light or temperature. We found that *RS24* transcription shows a low-amplitude circadian rhythm, which could not be detected at the level of RS24 protein accumulation. The RS24-YFP fusion protein is localized in the nucleus and the cytoplasm and the localization pattern was unaffected by environmental conditions.

In order to test the proposed biochemical function (acyltransferase) of RS24 we have set up an *in vitro* transferase assay system. We demonstrated that the bacterially expressed and purified RS24 protein is able to transfer common acyl substrates (benzoyl-, cinnamoyl- or 4-cumaroyl-CoA) to anthranilate, although at a lower efficiency than the bona fide acyltransferase HCBT1. RS24 and HCBT1 belong to the family of acyltransferases with a HXXXD-type catalytic site. Indeed, mutation of this motif in RS24 (D179E) eliminated catalytic activity of the enzyme. However, ectopic expression of RS24[D179E] in the *rs24* mutant background fully rescued the short period phenotype. This indicates that the acyltransferase activity of the protein is not required for its circadian function.

Photoperiodic induction of flowering depends on a functional circadian clock. This prompted us to test this process in *rs24*. The mutant showed early flowering phenotype in short day (8h light/16h dark) conditions, but behaved as wild-type in long days (16h light/8h dark). We tested accumulation of *CO* and *FT* mRNAs under the conditions above. As expected, *CO* and *FT* levels/patterns in *rs24* were identical to that of the wild type in long days. However, in short days *CO* showed slightly earlier phase and *FT* showed significantly higher levels in the mutant. This suggested that the flowering phenotype is caused by the altered clock function primarily and RS24 does not regulate flowering directly. To provide ultimate evidence for this, flowering time was determined in T22 short day cycles (7.33h light/14.67h dark). This light/dark cycle matches the period of *rs24* and the phase of rhythmic processes is the same as in wild-type plants in T24 (normal) cycles. As expected, no flowering phenotype was observed under these conditions, demonstrating that early flowering was indeed caused by the altered clock function in *rs24*.

Most clock mutants show defects in light responses, although it is not always clear if the photomorphogenic phenotype arises from altered clock functions, or the particular clock component is directly involved in light signal transduction. To see if *rs24* mutants respond differently to light treatments, hypocotyl elongation was tested at different fluences of monochromatic red, far-red or blue light. Mutant plants displayed hypocotyls longer than wild-type specifically in red light. These results indicated that light signalling pathways initiated by phytochrome B are impaired in the mutants. This was verified by the analysis of *rs24 phyb* double mutants, which phenocopied the *phyb* single mutant.

INTEGRATION OF RS24 IN THE STRUCTURE OF THE PLANT CIRCADIAN CLOCK

The (plant) circadian system can be visualized as a functional combination of three units. The central oscillator, where clock genes and the encoded clock proteins regulate each other's expression and thus produce the primary 24h-oscillation; the input pathway providing environmental light and temperature signalling and thus synchronization to the central oscillator; and the output pathway through which the oscillator governs the rhythmic expression of 15-25% of the Arabidopsis genome. Using targeted and specific assays, we demonstrated that the circadian phenotype of

the *rs24* mutant is independent of the light or temperature conditions and is detected at the level of several different overt rhythms, implying that RS24 affects the function of the rhythm-generating central oscillator. In order to reveal the mechanism by which RS24 affects the clock, first we monitored mRNA accumulation of all oscillator and oscillator-associated genes (37 in total) under free-running conditions, where the mutant phenotype is clearly detected. The short period phenotype was apparent for the accumulation pattern of all rhythmically expressed genes, but we found no significant alterations in the overall level of any of the transcripts tested. This suggested that the primary effect of RS24 on the circadian oscillator is exerted at post-transcriptional (most probably at protein) levels. In order to test if RS24 affects protein levels or subcellular localization of central clock proteins, CCA1/LHY/TOC1/GI/PRR5/PRR7/PRR9/ELF4/LUX-YFP fusion proteins were expressed in the *rs24* mutant and WT backgrounds. Protein levels were tested by Western analysis, whereas localization was investigated by fluorescent microscopy. Our data demonstrate that neither the protein levels nor the distribution of the tested clock components changed in the *rs24* mutant. These results left us with the conclusion that RS24 affects the function of one or more clock proteins. In order to identify these proteins, we have generated plants expressing RS24-HIS-FLAG proteins in the *rs24* mutant background. The HIS-FLAG tag served as an affinity tag facilitating the isolation of RS24 complexes *ex planta*. This approach was aimed to identify potential interactions that could depend of plant-specific post-translational modification of RS24 or its partners. MALDI-TOF mass spectrometry analysis of the samples identified RS24, IPCS2 (INOSITOL PHOSPHOCERAMIDE SYNTHASE 2, directly involved in salicylic acid (SA) accumulation), and ELONGATED HYPOCOTYL 5 (HY5) among the proteins constituting the complex. The HY5 transcription factor is involved UV-B and visible light signalling pathways and has function in the circadian clock as well. The above interactions were verified by Bimolecular Fluorescence Complementation *in planta*. Several double mutant Arabidopsis lines were produced by crossing *rs24* to mutants affecting the core components of the circadian clock. Monitoring circadian rhythms in these double mutants revealed genetic interaction between *CCA1* and *RS24*. We have shown that RS24 do not affect expression level or protein stability of CCA1. However, CCA1 has been shown to physically interact with

HY5, which is the way HY5 affects the clock. Collectively, our results indicated that RS24 may regulate the clock by modulating the CCA1-HY5 interaction. Epitope tagged RS24(-FLAG), CCA1(-YFP) and HY5(-HA) proteins were co-expressed transiently in *Nicotiana benthamiana* leaves, and a complex containing all the 3 proteins could be co-immunoprecipitated with any of the 3 proteins. Pairwise test showed no binding between RS24 and CCA1 and demonstrated that RS24-bound HY5 has lower affinity to CCA1. In other words, RS24 negatively regulates binding of HY5 to CCA1 by directly interacting with HY5.

CCA1 is a Myb-domain transcription factor with well-known target genes and binding DNA motifs. Using Electrophoretic Mobility Shift Assays (EMSAs) we showed that the DNA binding ability of CCA1 is inhibited by HY5 via direct interactions. However, RS24-bound HY5 was less inhibitory, thus, RS24 positively regulates the DNA binding activity of the central clock component CCA1.

These results identify the molecular mechanism by which a novel clock-associated factor, RS24, regulates the central circadian oscillator.

THE ROLE OF RS24 IN SALICYLIC ACID (SA) SIGNALLING

Our preliminary results showed that UV-C light induced expression of the PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1) genes is higher in the *rs24* mutant than in WT plants. Since UV-C induced expression of PCC1 absolutely depends on increased SA biosynthesis, our results suggested that RS24 could affect SA accumulation. In order to elucidate the function of RS24 in SA regulated responses, the *rs24* mutation was combined with mutants impaired in SA biosynthesis (*sid1*, *sid2*), SA accumulation (*eds1*, *ndr1*, *pbs3*, *eps1*) or SA signalling (*npr1*) and the double mutants were analysed for the induction of SA-dependent gene expression. We showed that *rs24* is epistatic to all SA biosynthesis and accumulation mutants, but is hypostatic to SA signalling mutants. These results demonstrate that RS24 affect a late step of SA accumulation (or inactivation). This is consistent with our finding that RS24 interacts with IPCS2, an inhibitor of SA accumulation (see above). Notably, none of the single SA mutants produced any period phenotypes, whereas all the double mutants displayed a short period phenotype indistinguishable from that of the single *rs24* mutant. Moreover, expression of the RS24[D179E] mutant derivative in the *rs24*

mutant background rescued the circadian phenotype (see above), but failed to complement the SA-related phenotype indicating that the acyltransferase activity of RS24 is essential to fulfil its role in the regulation of SA accumulation.

On the one hand, our results demonstrate that SA signalling has no significant effect of the pace of the clock. On the other hand, we showed that RS24 has separable pleiotropic functions: (i) it modulates the function of the clock by enhancing DNA binding of CCA1 as a co-factor for HY5 and (ii) inhibits SA accumulation by interacting with IPCS2 as an acyltransferase enzyme.