THE ROLE OF A SMALL GTPASE IN REGULATING THE PLANT CIRCADIAN CLOCK, STRESS RESPONSES AND THE LIGHT DEPENDENT ENDOREDUPLICATION

Thesis of Ph.D. dissertation

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INTRODUCTION

The optimal growth and development of plants are mediated by various signaling pathways that enable plants to modulate their molecular and physiological reactions in response to changes of the environment. To monitor changes in ambient light conditions, plants evolved several families of photoreceptors. The red/far-red light-absorbing phytochromes (PhyA–PhyE) and the blue light-absorbing cryptochromes (CRY1 and CRY2) are considered to mediate the majority of physiological and developmental responses to visible light. These photoreceptors also play an essential role in entraining/synchronizing the circadian clock to the daily light/dark cycles (Devlin and Kay, 2000). Circadian clocks are biochemical timers regulating many physiological and molecular processes according to the day/night cycle. The central clockwork generates the primary oscillation in the expression of clock components with a period of about 24 h.

The rhythm generating module of the *Arabidopsis thaliana* clock consists of at least three interconnected feedback loops (McWatters and Devlin, 2011). The “central loop” functions through the reciprocal regulation between the *CCA1*, *LHY* and *TOC1* genes (Alabadí et al., 2001). The morning-expressed CCA1/LHY Myb transcription factors repress the *TOC1* gene; conversely, the evening-expressed TOC1 positively regulates the transcription of *CCA1/LHY*. The “evening loop” is formed by TOC1 and a hypothetical factor Y, both expressed in the evening. Y positively regulates *TOC1*, whereas TOC1 represses *Y* transcription, which is also inhibited by CCA1/LHY. TOC1 promotes *CCA1/LHY* transcription via another hypothetical component; X. GI (a nuclear protein with unknown biochemical function) is an essential contributor to Y function (Locke et al., 2005; Zeilinger et al., 2006). The “morning loop” is formed by CCA/LHY and PRR7/9 (Makino et al., 2000).

CCA1/LHY activates *PRR7/9* expression in the morning; conversely, PRR7/9 inhibit *CCA1*/*LHY* during the rest of the day.

Circadian regulation is clearly overrepresented among the genes that are implicated in light, hormonal, or stress signaling, suggesting a molecular basis for the temporal modulation of these pathways (Covington et al., 2008). Adaptation to the changing environment also requires plasticity of the developmental program both at the organism and cell levels.

Previously, we identified the small GTPase LIGHT INSENSITIVE PERIOD1 (LIP1) as a circadian clock associated factor in *Arabidopsis* (Kevei et al., 2007). Loss of LIP1 function in the *lip1-1* mutant severely reduced the effect of light on the shortening of freerunning period.

RESEARCH OBJECTIVES

Detailed analysis of the plant circadian clock revealed, that most of the clock components and clock associated components have pleiotrop functions and play important role in mediating different cell processes. It has been shown recently that many clock components affect the hormone system or take part in regulation of metabolic processes.

It is known based on our previous results, that LIP1 plays role in

- regulating the circadian clock,
- the photomorphogenesis,
- and LIP1 protein is localized in the nucleus and the cytosol as well.

Recently, we have observed, that *lip1* mutant plants are sensitive to salt and the cotyledon pavement cells show abnormal morphology.

Our aim was, to get more information about the above mentioned functions and to understand how LIP1 takes part in these processes. We wanted to know

- how LIP1 affects the plant circadian clock, which clock components are responsible for the LIP1 function,
- which photoreceptors take part in the LIP1 mediated regulation of the photomorpfogenesis,
- how does LIP1 take part in mediating stress responses,
- what is the reason for the abnormal pavement cell morphology;
- how does the localization of LIP1 affect these phenotypes.

RESEARCH METHODS

- Molecular cloning techniques
- Creation and maintenance of transgenic *Arabidopsis thaliana* plants
- *In vivo* luciferase enzyme activity measurements in intact seedlings
- Determination of period length of circadian rhytms by using BRASS software
- Plant genomic DNA extraction
- Plant total RNA extraction
- Quantitative Real-Time PCR assay
- Total plant protein isolation, Western-blotting
- Flow citometry
- Light, fluorescence, confocal and laser scanning microscope

RESULTS

Microscopic analysis of young *lip1-1* and *lip1-2* mutant seedlings (Kevei et al., 2007) revealed defects in cell development. In wild-type *Arabidopsis* plants, pavement cells have a characteristic jigsaw puzzle shape with lobes, whereas in *lip1-1* and *lip1-2* mutants the cell shape is more rounded and much less complex. Pavement cells in the first true leaves of *lip1-1* and *lip1-2* mutants had wild-type morphology.

The alteration of cell morphology is frequently linked with changes in nuclear DNA content (Guimil and Dunand, 2007). Therefore, we analyzed ploidy patterns in the cotyledons of *lip1* mutants by flow cytometry and found that the proportion of nuclei having high DNA content was increased in the mutants. Ploidy patterns in isolated hypocotyls of the *lip1-2* mutant showed the same tendency as in the cotyledons, demonstrating that in the seedling stage the effect of LIP1 on endoreplication is not organ specific. Ploidy patterns in the matured first leaves of *lip1-2* and wild-type plants were not significantly different suggesting that LIP1 suppresses ploidy levels in a developmentally regulated manner.

Four cycles of endoreplication could occur in dark grown *Arabidopsis* seedlings, whereas the fourth cycle is inhibited in light (Gendreau et al., 1998). We analyzed ploidy patterns in *lip1* mutants grown under different light conditions. Elevated ploidy level was detected in *lip1* seedlings grown under light/dark cycles and the DNA content increased further when plants were grown in continuous white light. Ploidy levels were identical in etiolated *lip1* and wild-type seedlings. In red light, the light-induced inhibition of endoreplication depends on the photoreceptor PhyB (Gendreau et al., 1998).We tested the epistatic relation between LIP1 and PhyB in controlling red light-dependent endoreplication. In continuous red light (cR), both *lip1-2* and *phyB*-

9 mutants showed similarly increased ratios of 16C and 32C nuclei. The *lip1-2 phyB-9* double mutant phenocopied the *phyB-9* single mutant in cR. The DNA content of these mutants grown in darkness displayed patterns like in wild-type seedlings. Ploidy level of *lip1-2* mutant showed significant increases in cB, but it was indistinguishable from the wild type in cFR. The mutation in the *LIP1* gene was combined with the *cry1 cry2* and *Phy-A211*mutations. In cB, the ploidy pattern of the *lip1-2 cry1 cry2* triple mutant was most similar to that of the *cry1 cry2* double mutant. In cFR, the *lip1-2 phyA-211* double mutant phenocopied the *phyA-211* single mutant. It had been demonstrated that *lip1-1* mutants show elevated photomorphogenic responses to red and blue light but not to far-red light (Kevei et al., 2007). We characterized the responsiveness of *lip1-2 phyB-9, lip1-2 phyA-211,* and *lip1-2 cry1 cry2* multiple mutants to red, far-red, and blue light, respectively. In cR, the *lip1-2* mutant showed significantly shorter hypocotyls than the wild type; however, the *lip1-2 phyB-9* double mutant produced hypocotyls much longer than the wild type and was very similar to that of the *phyB-9* single mutant. In cB, the *lip1-2* mutant displayed significant hypersensitivity compared with the wild-type, but *lip1-2 cry1 cry2* produced long hypocotyls similar to the *cry1 cry2* double mutant. In cFR, *lip1-2* phenocopied wild-type plants, and the *lip1-2 phyA-211* double mutant showed hypocotyl lengths identical to those of the *phyA-211* mutant.

lip1 mutants grown under 12-h-light/12-h-dark cycles displayed an increased sensitivity to salt (NaCl). In addition to poor growth and development, the germination rate of *lip1-2* seedlings was also significantly reduced and the relative inhibition of root growth was significantly stronger in the *lip1* mutants under salt stress conditions as compared to the wild type. We have also analyzed germination rate in dark-grown plants. *lip1-2* mutants were more sensitive than wild-type plants, indicating that the salt stress phenotype is not caused by other light-dependent defects of *lip*. We tested the effect of salt

stress on the clock function in *lip1* mutants. There was no observable connection between the hypersensitivity to salt and the defect of the clock function.

Osmotic stress leads to the induction of early stress inducible genes like *RESPONSIVE TO DESICCATION29 A (RD29A)*, *RD29B*, or *RESPONSIVE TO ABA 18 (RAB18)*, whereas ionic stress induces the transcription of *SOS2* (*SALT OVERLY SENSITIVE2)*, an activator of the Na^+/H^+ transporter SOS1. Salt-induced expression of *RD29A*, *RD29B, RAB18,* and *SOS2* genes was monitored by quantitative reverse transcription (qRT)-PCR. There were no significant differences in the kinetics or the level of induction of these genes in the mutant. These results indicate that LIP1 plays a minor role, if any, in sensing salt stress signals and the transcriptional activation of the salt stress related genes tested.

The *lip1* mutants grown under 12-h-light/12-h-dark cycles displayed an increased sensitivity to K^+, Cs^{2+}, Li^{2+} and heat stress as well. These data indicate that LIP1 has a more general role in the regulation of responses to abiotic stress conditions.

We showed previously that the yellow fluorescent protein YFP-LIP1 fusion protein is detectable both in the cytosol and in the nucleus. This distribution pattern is not affected by light conditions or the circadian clock (Kevei et al., 2007). To test if any of the pleiotropic functions of LIP1 require specific subcellular localization, we generated transgenic *lip1* plants expressing the LIP1-YFP fusion protein with or without nuclear localization signal (NLS) or nuclear export signal (NES). Transgenic lines with comparable expression levels were selected and localization of the different LIP1 fusion proteins was analyzed by fluorescence microscopy. As expected, YFP-LIP1 was detectable both in the cytoplasm and in the nuclei, whereas YFP-LIP1-NLS and YFP-LIP1-NES were clearly restricted to the nucleus and the cytoplasm.

Complementation of the ploidy, salt stress, photomorphogenic, and circadian phenotypes of the *lip1* mutants was tested in the selected transgenic lines. Expression of YFP-LIP1, YFP-LIP1-NLS, or YFP-LIP1-NES in the *lip1* mutant background restored ploidy levels to the wildtype level. The jigsaw shape of cotyledon pavement cells was also restored in all complemented lines. Furthermore, *lip1-2* plants expressing either of the LIP1 fusion proteins were able to tolerate 100 mM NaCl and develop similarly to wildtype plants. Hypocotyl lengths of either of the complemented lines grown in red, blue, or far-red light were also indistinguishable from that of the wild type. The expression of YFP-LIP1 or YFP-LIP1-NLS restored wild-type circadian rhythms, whereas YFP-LIP1-NES-expressing transgenic *lip1-2* seedlings displayed rhythms very similar to that of *lip1-2*. Period estimates quantitatively demonstrated full complementation or the complete lack of complementation as indicated. We conclude that for the regulation of the circadian clock, a significant portion of LIP1 needs to be present in the nucleus. These observations suggest that the function of LIP1 in the circadian clock can be separated from its role in the control of cell development, endoreplication, stress tolerance, and photomorphogenesis.

The lack of LIP1 causes short period phenotype with all tested output marker genes (*CCR2, CAB, PRR9, CCA1, LHY, GI*), proving its importance in the plant circadian network. We measured the mRNA of core clock components in *lip1* mutants grown under 12-h-light/12-h-dark cycles and found that the peak level of *CCA1* and *TOC1* mRNA decreased significantly compared to the wild type. Examination of genetic interactions between *LIP1* and *TOC1*, *CCA1, PRR5, PRR9* and *GI* genes via double mutant analysis revealed that GI is a clock component mediating LIP1 action. *GI* has epistatic effect on *LIP1*, regarding the circadian, photomorphogenic and the stress

phenotype as well. The relation between GI and LIP1 is probably responsible for the decrease in the mRNA levels of *CCA1* and *TOC1*, based on the positive transcriptional regulation between *GI* and *TOC1* and between *TOC1* and *CCA1*. *cca1* and *toc1* mutants have short period phenotype. The decrease in CCA1 and TOC1 level can contribute to the short period phenotype of the *lip1* mutant in part.

CONCLUSIONS

We have provided physiological and molecular data demonstrating the following remarks:

1. LIP1 is required for the normal development of pavement cells in young seedlings.
2. LIP1 is a component of the PhyB-controlled red light and CRY-controlled blue light signaling cascade inhibiting endoreplication and LIP1 is involved in mediating PhyB- and CRY-controlled photomorphogenesis.
3. LIP1 function is needed for stress tolerance.
4. By manipulating the subcellular localization of LIP1, we have shown that the circadian function of LIP1 can be separated from its other functions, not only at the physiological level but also at the cellular level. The localisation of LIP1 is needed in the nucleus for proper clock function.
5. The cell shape, ploidy, hypocotil and stress phenotype could be complemented with LIP1-YFP, LIP1-YFP-NLS and LIP1-YFP-NES fusion constructs as well.
6. The lack of LIP1 causes short period phenotype of all tested output marker genes (*CCR2, CAB, PRR9, CCA1, LHY, GI*). At the mRNA level, we could observe decrease in the maximum level of the *CCA1* and *TOC1* expression.
7. Examination of clock gene and *lip1* double mutants showed, that *gi* mutation has epistatic effect above *lip1* mutation regarding the circadian, hypocotil and stress phenotype as well.

LIST OF PUBLICATIONS

Publication used in the thesis:

Terecskei K, Tóth R, Gyula P, Kevei É, Bindics J, Coupland G, Nagy F, Kozma-Bognár L. 2013. The Circadian Clock-Associated Small GTPase LIGHT INSENSITIVE PERIOD1 Suppresses Light-Controlled Endoreplication and Affects Tolerance to Salt Stress in Arabidopsis. Plant Physiol 161(1):278-90.

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