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Deletion of the RS domain of RRC1 impairs phytochrome B signaling in Arabidopsis

Hiromasa Shikata,[1] Moeko Nakashima,[1] Ken Matsuoka[1,2] and Tomonao Matsushita[1,*]

[1]Faculty of Agriculture; Kyushu University; Fukuoka, Japan; [2]Biotron Application Center; Kyushu University; Fukuoka, Japan

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Abbreviations: CBB, coomassie brilliant blue; cFR, continuous far-red light; cR, continuous red light; cW, continuous white light; phyB, phytochrome B; PIFs, phytochrome-interacting factors; RRC1, reduced red-light responses in *cry1cry2* background 1; RRC1-FLAG, full length RRC1 with a C-terminal 3xFLAG tag; RRC1ΔRS-FLAG, truncated RRC1 lacking the RS domain with a C-terminal 3xFLAG tag; RS domain, arginine/serine-rich domain; RT-PCR, reverse transcription-PCR

Phytochrome B (phyB), a major photoreceptor in plants, interacts with transcription factors to regulate gene expression and induce various light responses. Recently, we identified an SR-like splicing factor, RRC1 (reduced red-light responses in *cry1cry2* background 1), as a novel component of phyB signaling in Arabidopsis. RRC1 has a C-terminal arginine/serine-rich (RS) domain that is generally important for the regulation of alternative splicing. Whereas *rrc1* hypomorphic mutant alleles produce truncated RRC1 proteins that lack the C-terminal region, including the RS domain, and exhibit splicing defects and reduced phyB signaling, the *rrc1–4* null allele additionally displays pleiotropic developmental abnormalities with more severe splicing defects. Here, we show that transgenic Arabidopsis plants that express truncated RRC1 lacking the RS domain in the *rrc1–4* null allele background exhibited the same phenotype as the hypomorphic alleles. Hence, we conclude that deletion of the RS domain of RRC1 reduces phyB signaling, probably due to aberrant regulation of alternative splicing of target genes.

Phytochrome is a red/far-red light-absorbing photoreceptor that regulates various developmental processes throughout the life cycle of plants. After perception of red light, phytochrome is converted from its physiologically inactive red light-absorbing form (Pr) to the active far-red light-absorbing form (Pfr), and is then translocated from the cytoplasm to the nucleus.[1,2] In nuclei, Pfr interacts with basic helix-loop-helix transcription factors named phytochrome-interacting factors (PIFs).[3,4] This light-dependent interaction induces the proteasome-mediated degradation of PIFs to modulate transcription of light-responsive genes, which has been suggested to trigger light responses of plants.[5-7]

In Arabidopsis, phytochrome B (phyB) is a major molecular species of phytochrome, and mutants deficient in phyB display obvious morphological phenotypes, such as elongated hypocotyls under continuous red light (cR).[8,9] Recently, we identified an SR-like splicing factor, RRC1 (reduced red-light responses in *cry1cry2* background 1), as a novel component of the phyB signaling pathway in Arabidopsis. Moreover, we showed that phyB controls the alternative splicing of several target genes in response to cR, and that RRC1 is involved in phyB-dependent alternative splicing.[10] All of the hypomorphic alleles examined, i.e., *rrc1-1*, *rrc1-2* and *rrc1-3*, resulted in truncation of the C-terminal region of RRC1 and specifically inhibited phyB signaling. The deleted C-terminal region of RRC1 includes a serine/arginine-rich (RS) domain, which is thought to be important for the regulation of alternative splicing in response to environmental stimuli.[11] Based on these findings, we proposed that the regulation of alternative splicing by the RS domain of RRC1 is required for phyB signal transduction.[10] In this study, we expressed truncated RRC1 protein lacking the RS domain in the *rrc1-4* null allele background to test this hypothesis. We provide evidence that deletion of the RS domain of RRC1 reduces phyB signaling.

Truncated RRC1 Lacking the RS Domain Rescues Pleiotropic Developmental Defects but not Reduced phyB Signaling in *rrc1-4*

The null allele *rrc1-4* displayed pleiotropic developmental defects, such as dwarfism in the homozygous state and semi-sterility in the heterozygous state under normal growth conditions and reduced inhibition of hypocotyl elongation specifically under cR.[10] On the other hand, the *rrc1* hypomorphic alleles only exhibited evidence of reduced phyB signaling, such as elongated hypocotyls under cR, but did not show pleiotropic developmental defects.[10] It has been shown that all of these defects in *rrc1-4* were rescued by exogenously expressed RRC1 containing a C-terminal

*Correspondence to: Tomonao Matsushita; Email: mat@agr.kyushu-u.ac.jp
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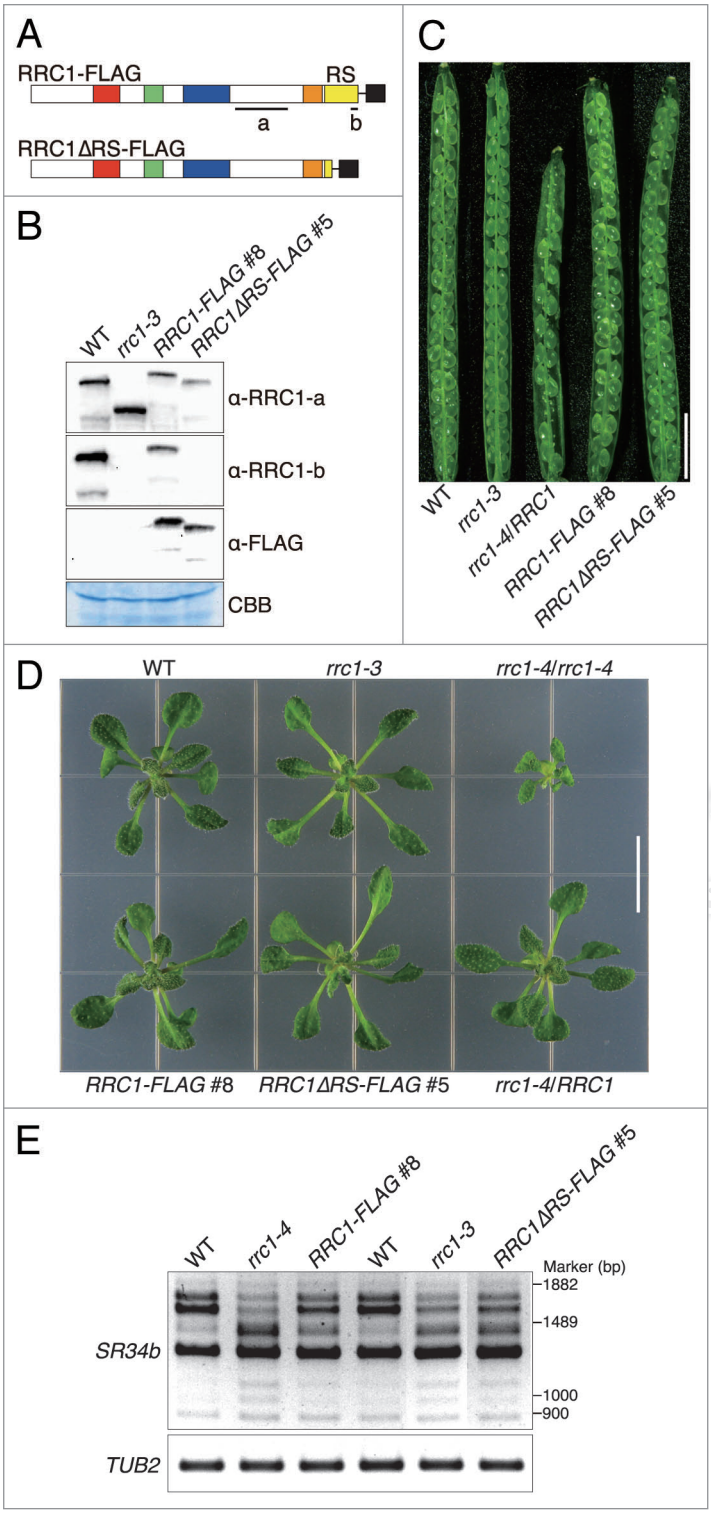

Figure 1. RRC1ΔRS-FLAG rescues pleiotropic developmental defects in *rrc1–4*. (A) Schematic diagrams of the RRC1-FLAG and RRC1ΔRS-FLAG proteins, which represent full-length RRC1 (946 amino acid) and truncated RRC1 lacking the RS domain (870 amino acid), respectively, with a C-terminal 3x FLAG tag. Each construct was introduced into *rrc1–4* and driven by the native *RRC1* promoter. These plants are referred to as *RRC1-FLAG* and *RRC1ΔRS-FLAG* plants, respectively. Red boxes, RNA recognition motif; green boxes, SWAP/Surp domain; blue boxes, RPR or ENTH/VHS domain; orange boxes, cwf21 domain; yellow boxes, RS domain; and black boxes, 3x FLAG tag. (B) Immunoblot analysis of RRC1 in wild-type (WT), *rrc1–3*, *RRC1-FLAG* and *RRC1ΔRS-FLAG* plants. Each lane was loaded with 50 μg of total protein that was extracted from 2-week-old plants grown under continuous white light (cW; 40 $\mu molm^{-2}s^{-1}$). Anti-RRC1-a, anti-RRC1-b and anti-FLAG were used as primary antibodies.[10] The regions in the RRC1 polypeptide that are recognized by the anti-RRC1-a and anti-RRC1-b antibodies are underlined and labeled with letters a and b, respectively in (A). The Coomassie brilliant blue (CBB)-stained gel image is shown as a loading control. (C) Opened siliques resulting from the self-fertilization of each line specified in the part. Scale bar: 2 mm. (D) Mature plants grown under cW for 3 weeks. Scale bar: 1 cm. (E) Alternative splicing pattern of *SR34b*. Total RNA was isolated from 2-week-old plants grown under cW and RT-PCR analysis was performed as previously described in reference 10. *TUB2* was used as an internal control.

3x FLAG tag (RRC1-FLAG).[10] In the current study, we generated the construct *RRC1ΔRS-FLAG*, in which the DNA fragment encoding the C-terminal 77 amino acid residues of RRC1 was deleted (**Fig. 1A**). The encoded protein is predicted to lack most of the RS domain and resemble the mutant RRC1 protein in *rrc1-1*.[10] This construct was introduced into *rrc1–4* plants, and RRC1 accumulation in the T3 homozygous plants was confirmed by immunoblotting (**Fig. 1B**). The *RRC1ΔRS-FLAG* plants, as well as the wild-type (WT), *rrc1-3* and *RRC1-FLAG* plants, did not exhibit any obvious developmental defects under normal growth conditions (**Fig. 1C and D**). These results indicate that RRC1ΔRS-FLAG retains the function of RRC1, which is fundamental to various developmental processes under normal conditions.

The hypomorphic *rrc1* alleles display aberrant alternative splicing patterns of several SR protein genes, such as *SR34b*, and the splicing defects are even more severe in the null allele *rrc1–4*.[10] We examined the alternative splicing pattern of *SR34b* to determine if RRC1-FLAG or RRC1ΔRS-FLAG could complement the splicing defects in *rrc1–4*. *RRC1-FLAG* plants exhibited a similar splicing pattern to that in WT, whereas *RRC1ΔRS-FLAG* plants displayed the same pattern as that in the hypomorphic allele *rrc1-3* (**Fig. 1E**). This result demonstrated that the splicing defects in *rrc1-3* resulted from deletion of the RS domain of RRC1. Note that RRC1ΔRS-FLAG restored the splicing pattern in the null allele *rrc1–4* to that in *rrc1-3,* suggesting that truncated RRC1 lacking the RS domain retains some residual splicing activity, as previously discussed in reference 10.

To investigate the effect of deleting the RS domain of RRC1 on phyB-mediated light responses, we examined the inhibition of hypocotyl elongation under cR in the transgenic lines. In our previous study, the homozygous *rrc1–4* plants exhibited reduced inhibition of hypocotyl elongation specifically under cR, although they had shorter hypocotyls even in darkness, probably due to general growth defects.[10] In this study, *RRC1-FLAG* plants displayed similar hypocotyl lengths to those in WT plants under all light conditions examined (**Fig. 2**), as previously reported in reference 10. By contrast, *RRC1ΔRS-FLAG* plants, as well as *rrc1-3* mutants, displayed elongated hypocotyls under cR, but not under cFR or in darkness, similarly to but to a lesser extent than the *phyB-9* null mutant (**Fig. 2**).

This indicates that both RRC1-FLAG and RRC1ΔRS-FLAG rescued the general growth defects of *rrc1–4*, and that deletion of the RS domain of RRC1 could mimic the reduced phyB signaling that was observed in *rrc1-3*. From these data, we conclude that deletion of the RS domain of RRC1 reduced phyB signaling in *rrc1* mutants, probably due to aberrant regulation of alternative splicing.

In our previous and present studies, we demonstrate that RRC1 functions in phyB signal transduction via its RS domain.[10] However, it is unclear how the RS domain of RRC1 is involved in phyB signaling. The RS domain is known to be present in many splicing factors and to facilitate the protein-protein interactions among these splicing factors.[12] Furthermore, the RS domain is reversibly phosphorylated in response to various signals.[11] RRC1 is an ortholog of human SR140,[10] which was initially identified as a protein associated with the 17S U2 small nuclear ribonucleoprotein (snRNP) complex.[13] Thus, one can speculate that phyB alters the phosphorylation status of the RS domain of RRC1 to modulate the interaction between RRC1 and other splicing factors, and thereby regulates the composition of the U2-type spliceosome in response to red light. Further biochemical experiments need to be performed to investigate this possibility.

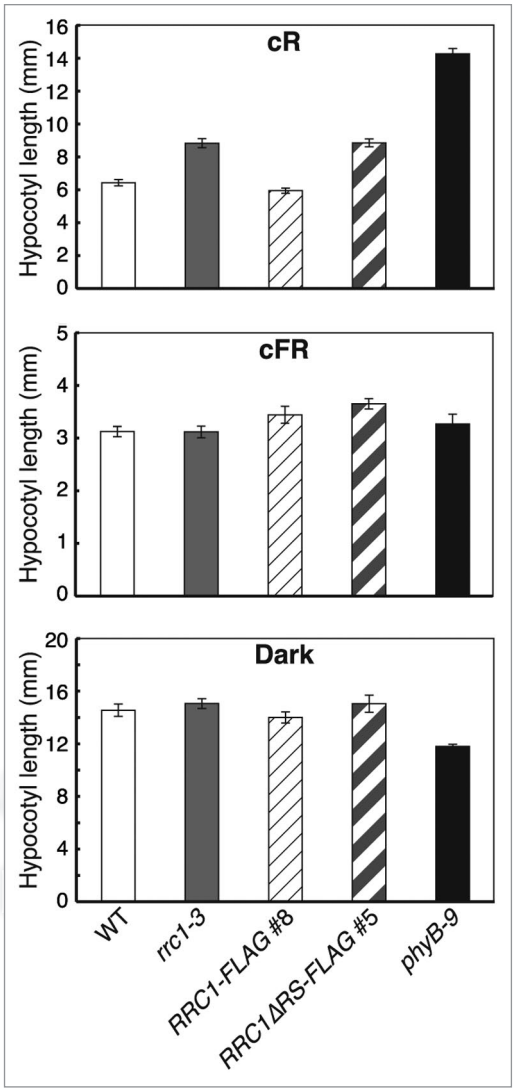

Figure 2. *RRC1ΔRS-FLAG* plants show elongated hypocotyls specifically under continuous red light. Wild-type (WT), *rrc1-3*, *RRC1-FLAG*, *RRC1ΔRS-FLAG* and *phyB-9* seedlings were grown for 5 d under continuous red light (cR; 7.7 μmol $m^{-2}s^{-1}$, top part), continuous far-red light (cFR; 31 μmol$m^{-2}s^{-1}$, middle part) or in darkness (Dark, bottom part), and hypocotyl lengths were measured. Data represent the mean ± SE (n = 30).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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