Identification of LOV KELCH PROTEIN2 (LKP2)-interacting Factors That Can Recruit LKP2 to Nuclear Bodies

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LOV KELCH PROTEIN2 (LKP2) is an F-box protein that has been postulated to function centrally, or near to the circadian clock oscillator. As a first step to determine which proteins act as substrates of LKP2, two hybrid screening was performed using LKP2 as bait, and two interaction factors, Di19 and COL1, were isolated. The transiently expressed Di19–GUS fusion protein was localized in the nucleus of Arabidopsis petiole cells. COL1 and other CO/COL family proteins could also interact with LKP1/ZTL, LKP2 or FKF1. The LKP2-binding site in CO or COL1 was near the center of each protein. The CCT motif in CO or COL1 was not sufficient for interaction with LKP2. LKP2 recognized CO with F-box and kelch repeat-containing regions, while it recognized COL1 with an LOV domain. When LKP2 was fused with cyan fluorescent protein (CFP) and transiently expressed in onion epidermal cells, CFP–LKP2 signals were localized in the nucleus and cytosol. Both yellow fluorescent protein (YFP)–CO and YFP–COL1 were located in the nucleus, forming nuclear bodies when they were transiently expressed. However, co-expression of CFP–LKP2 with YFP fused to either CO or COL1 resulted in the recruitment of CFP–LKP2 in nuclear bodies. Furthermore, the CFP–LKP2 and YFP–CO signals co-localized with signals for pUB2′′–mRFP, which is a marker for Cajal bodies. These results suggest the possibility that LKP2 functions with CO/COL family proteins in the nuclear bodies.

Keywords: Arabidopsis thaliana — CONSTANS — CONSTANS LIKE — Di19 — Nuclear bodies — Protein–protein interaction.

Introduction

Ubiquitin is a heat-stable, 76 amino acid peptide, consisting of two α-helix structures and five β-sheets, and is highly conserved among eukaryotes. The well-known function of ubiquitin is a signal for the proteasome-dependent protein degradation. However, the ubiquitination, covalent attachment of ubiquitin to another protein, regulates numerous cellular processes, such as viral budding, cell cycle regulation, DNA repair, regulation of transcription, kinase activity regulation, protein–protein interactions, intracellular localization of the ubiquitinated proteins, and so on. In the context of these regulations, polymerization sites and the state of ubiquitin are very important. K48-linked poly-ubiquitinations can be a targeting signal for the 26S proteasome, while mono-ubiquitinations and K63-linked poly-ubiquitinations can be a signal for a variety of intracellular processes (Deng et al. 2000, Hicke 2001, Pickart 2001, Hicke and Dunn 2003, Muratani and Tansey 2003, Pickart and Eddins 2004, Sakai et al. 2004, Shcherbik and Haines 2004, Sun and Chen 2004, Vodermaier 2004).

Ubiquitination occurs through the action of three enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). E1 forms a thiolester bond with the ubiquitin via ATP-dependent adenylation. E2 receives the activated ubiquitin and transfers it to target proteins. Although E2 can catalyze isopeptide bond formation between the ubiquitin and the target proteins, it requires E3 in most cases. E3 accepts the ubiquitin from ubiquitin-conjugated E2 and transfers it to the substrate (HECT type), or acts as a bridging factor that binds the ubiquitin-conjugated E2 and the substrate and then catalyzes the ligation reaction of the activated ubiquitin to the substrate (RING, PHD and U-box types). Since E3 is involved in the determination of substrate specificity, there is a large and diverse group of these enzymes in eukaryotes (Hegde 2004, Johnston and Madura 2004, Pickart and Eddins 2004).

In plants, many putative E3 genes have been annotated and identified as the result of genome sequencing projects. It was reported that the Arabidopsis genome encodes >500 RING-domain proteins and around 600–700 potential F-box proteins (Gagne et al. 2002, Kuroda et al. 2002). Some of these E3 genes have been shown to function in plant development,
morphogenesis, hormonal response, and so on (Moon et al. 2004, Schwechheimer and Villalobos 2004). UFO (UNUSUAL FLORAL ORGANS), which was the first F-box protein identified in plants, functions in floral meristem identity and floral organ development (Levin and Meyerowitz 1995, Samach et al. 1999). COP1 (Constitutive Photomorphogenesis 1), a photomorphogenic repressor, is a single subunit RING-type E3 which is involved in degradation of HY5 (Long Hypocotyl 5) (Osterlund et al. 2000, Saigo et al. 2003), phyA (phytochrome A) (See et al. 2004) and LAF1 (Long After Far Red Light 1) (Seo et al. 2003). SINAT5 (SEVEN IN ABSENTIA IN ARABIDOPSIS THALIANA 5) is also a single subunit RING type E3 which is involved in the ubiquitination of NAC1 (NAM/CUC-like protein 1), a transcriptional activator of lateral root formation in response to auxin (Xie et al. 2002).

TIR1 (TRANSPORT INHIBITOR RESPONSE 1) regulates the stability of AUX/IAA family proteins that can act as both transcriptional repressors for ARFs (auxin response factors) (Gray et al. 2001, Tiwari et al. 2001, Tiwari et al. 2004). SLY1 (SLEEPY1) regulates the stability of DELLA family proteins and is involved in gibberellin signaling (Dill et al. 2004, Fu et al. 2004). EBF1 (EIN3-binding F-box 1) and EBF2 regulate the level of transcriptional activator EIN3 (Ethylene Insensitive 3) in response to ethylene (Guo and Ecker 2003, Potuschak et al. 2003, Gagne et al. 2004). COI1 (Coronatine Insensitive 1) is involved in jasmonic acid signaling (Xu et al. 2002).

FKF1/LKP/ZTL family proteins are F-box proteins that affect the circadian clock and flowering time in Arabidopsis. This small family consists of three members: LKP1/ZTL, LKP2/FKL and FKF1 (Kiyosue and Wada 2000, Nelson et al. 2000, Somers et al. 2000). Each member of this protein family possesses an LOV, an F-box and kelch repeat domains. Their LOV domain structures resemble those of the PHOT1 and PHOT2 blue light receptors (Briggs et al. 2001, Briggs and Christie 2002, Cheng et al. 2003), and can bind FMN (Imaizumi et al. 2003). Their F-box domains have been shown to interact with several ASK (Arabidopsis SKP1) proteins (Kuroda et al. 2002, Risseeuw et al. 2003, Yasuhara et al. 2004). Kelch repeats are thought to form a WD40 repeat-like β-propeller structure and a protein–protein interaction module (Li et al. 2004). ZTL was shown to function in the circadian clock and to be involved in degradation of TOC1 (Timing of CAB Expression 1), which has been postulated as a component of a central circadian oscillator (Mas et al. 2003, Somers et al. 2004). FKF1 (Flavin binding, KELCH repeat, F-box 1), the gene expression for which is regulated by the circadian clock, was reported to be a blue light receptor that regulates the expression of the flowering time gene CO (CONSTANS) (Nelson et al. 2000, Imaiizumi et al. 2003). As regards LOV KELCH PROTEIN2 (LKP2), plants overexpressing the LKP2 gene exhibited a late-flowering phenotype when grown under long day light conditions, and showed arrhythmic promoter activities of the clock-controlled genes for CAB2 or CCR2 in either continuous light or dark conditions, suggesting that LKP2 functions close to the circadian oscillator (Schultz et al. 2001). The LKP2 protein could also interact with LKP1/ZTL, TOC1 and PRR5 (Yasuhara et al. 2004). PRR5 is a member of the PRR/TOC1 family of proteins that has been postulated to function in circadian clock regulation (Sato et al. 2002, Yamamoto et al. 2003). To understand further the function of LKP2 in circadian clock regulation and flowering time control, we isolated protein factors that interacted with LKP2 via yeast two-hybrid screening. In this study, we report the isolation, molecular interactions and localization of factors that exhibit active interaction with LKP2.

**Results**

Isolation of Di19 and COL1 by yeast two-hybrid screening

We used yeast two-hybrid screening to isolate proteins that interact with LKP2. Yeast cells were co-transformed with the pGBKT7 vector carrying LKP2 full-length cDNA, and with a 2.5×10⁶ clone cDNA library in the pGADT7 vector that had been prepared with RNA from Arabidopsis rosette leaves. Two hundred and twenty candidate clones were selected by complementation on SD medium amended with 5 mM 3-amino-1H-1,2,4-triazole (3-AT) but lacking adenine, histidine, leucine and tryptophan. As a second screening, plasmids from candidate clones were isolated and re-co-transformed into yeast cells with pGBK7-LKP2. Among the 220 plasmid pools isolated, 90 gave auxotrophic complementation, of which 48 colonies could grow in the presence of 5 mM 3-AT. Twenty of these colonies could grow at 10 mM 3-AT and 14 colonies could grow at 15 mM 3-AT. As a third screening, we sequenced all of the pGADT7-cDNA isolates from the 14 colonies that grew at 15 mM 3-AT.

Most of the co-transformed clones isolated after three rounds of screening were rRNA, chloroplast or vacuole proteins, and out-of-frame clones. Clones for chloroplast and vacuole proteins were eliminated since LKP2 is thought to reside mainly in nuclei (Yasuhara et al. 2004). Other clones were a DnaJ-like protein, drought-induced unknown proteins (two clones of different size), a serine/threonine protein kinase and a zinc finger protein. As a fourth screening, we co-transformed yeast cells with each one of five pGADT7-cDNA plasmids and with pGBK7 empty vector. As a result, two clones activated the two-hybrid system without pGBK7-LKP2. Therefore, we concluded that the drought-induced unknown protein, Di19, and the zinc finger protein, COL1, could be LKP2-binding proteins.

Interaction of Di19 with LKP2 and its intracellular localization

Di19 was initially isolated as a drought-inducible cDNA clone (Gosti et al. 1995), but there is no more information about this gene. Both of our Di19 clones were partial and corresponded to a truncated peptide either from amino acids 53–206 or 63–206, so we performed the two-hybrid assay with a full-length Di19 reverse transcription (RT)-PCR cDNA. Full-
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length Di19 interacted with LKP2 and other members of the LKP/ZTL protein family (Fig. 1). Interactions also occurred when the DNA-binding and activation domains of GAL4 were exchanged (data not shown). There were no effects of light on two-hybrid interactions in the red, far-red, blue, green or white light spectra at 40 µmol m\(^{-2}\) s\(^{-1}\) (data not shown).

To visualize the intracellular localization of Di19, the cauliflower mosaic virus (CaMV) 35S promoter-derived β-glucuronidase (GUS)–Di19 fusion protein construct and a vector control were introduced into Arabidopsis petiole cells by bombardment. Di19–GUS activity was localized in the nucleus (Fig. 2A, B), but vector control GUS activity was not limited to any cellular region or organelle (Fig. 2C). Thus, subcellular Di19 co-locates with LKP2 (Yasuhara et al. 2004).

Interaction of CO/COL family proteins with LKP2

COL1 is a member of the CO/COL1 family of proteins, and has two B-box-type zinc finger motifs and a CCT motif (Ledger et al. 2001). The CCT motif is conserved in all CO/COL and PRR/TOC1 family proteins, while some CO/COL proteins contain only one B-box motif. Since the COL1 cDNA isolated from yeast cells was truncated, we examined the molecular interactions between COL1 and LKP2 using a full-length COL1 cDNA and a series of deletions (Fig. 3A). Full-length COL1(1–355) activated the reporter genes in the absence of LKP2. An N-terminal deletion of COL1(145–355) could also activate the system without LKP2. However, both COL1(163–355) and COL1(195–355) could activate the reporter genes depending on the presence of LKP2. COL1(195–355) corresponded to the clone that had been isolated from two-hybrid screening. Neither COL1(228–355) nor COL1(286–327), having deletions that contained the CCT motif, could activate the reporter genes. These results suggested that the interaction between COL1 and LKP2 required the COL1 region containing amino acids 195–227. None of the interactions was affected by light at 40 µmol m\(^{-2}\) s\(^{-1}\) (data not shown).

We also examined the two-hybrid interactions of LKP/ZTL family members with other CO/COL family members (Fig. 3B). Since the zinc finger-containing region of COL1 activated the reporter genes without a prey insert, we eliminated the zinc finger motif(s) from all tested CO/COL proteins. Vector pGBK7T or pGADT7 for CO/COL proteins, was used to decrease background activation levels. As a result, CO and COL1-6, COL8 and COL13–15 interacted with LKP2, and some also interacted with LKP1/ZTL or FKF1. As in interactions with Di19, there was no observable effect of light on the two-hybrid interaction (data not shown).

Interaction between CO and LKP2 was examined using a series of N- and C-terminal deletion derivatives (Fig. 3C). CO(1–373) and a series of N-terminal-deleted mutants, CO(106–373), CO(140–373) and CO(157–373), interacted significantly with LKP2 in this assay. As for the C-terminal domain deletion mutant series, CO(175–326) interacted with LKP2, while CO(175–305), CO(175–275), CO(175–239) and CO(175–206) gave activation in the absence of LKP2. The CCT motif of CO(306–348) did not interact with LKP2, as seen in COL1 CCT.
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A

Zinc finger
8-Box1 8-Box2
CCT-domain

prey:
LKP2  Vector

bait:
COL1
COL(145-355)
COL(163-355)
COL(196-355)
COL(228-355)
COL(269-327)

SD-LW  SD-AHLW  SD-LW  SD-AHLW

B

prey: COL

LKP1
LKP2
FKF1
Vector

bait:
LKP1
LKP2
FKF1
Vector

SD-LW  SD-AHLW

C

Zinc finger
8-Box1 8-Box2
CCT-domain

prey:
LKP2  Vector

bait:
CO
CO(106-373)
CO(140-373)
CO(157-373)
CO(175-373)
CO(208-373)
CO(241-373)
CO(175-326)
CO(175-305)
CO(175-275)
CO(175-239)
CO(175-206)
CO(306-348)

SD-LW  SD-AHLW  SD-LW  SD-AHLW
The domains of LKP2 that are required for the interaction with CO or COL1 were investigated by the two-hybrid system (Fig. 3D). Either the F-box or kelch repeat-containing region was sufficient for interaction with CO, while the LOV domain-containing region was necessary and sufficient for interaction with COL1.

**Nuclear body formation of LKP2 in the presence of CO or COL1**

CO was reported to be localized in the nucleus (Robson et al. 2001). CO is a transcription factor protein, and nuclear partitioning is thought to be important for its transcriptional activity (Robson et al. 2001). LKP2 was also reported to be localized in the nucleus (Yasuhiro et al. 2004). To examine the subcellular co-localization of those proteins, cyan fluorescent protein (CFP)–LKP2, yellow fluorescent protein (YFP)–CO and YFP–COL1 fusions were constructed.

To confirm the reliability of our fluorescence detection system, we first bombarded YFP–CO alone and acquired an image through the CFP channel. The YFP signal of YFP–CO proteins was detected through the YFP channel in an exposure time of 200 ms (Supplementary Fig. S2D). However, YFP signals were not detected through the CFP channel in exposure times of 700 or 1,500 ms (Supplementary Fig. S2A, B). Even with an exposure time of 4000 ms, very faint YFP signals were detected through the CFP channel (Supplementary Fig. S2C). Therefore, we determined that the exposure time was <1,000 ms in this co-localization assay.

Each fusion gene was put under control of the CaMV 35S promoter and bombarded into onion epidermal cells. YFP–CO and YFP–COL1 proteins were localized in the nucleus and formed nuclear bodies when they were co-expressed with CFP (Fig. 4G, 5G). CFP–LKP2 was observed in the nucleus and cytosol when it was co-expressed with YFP (Fig. 4B, 5B). When CFP–LKP2 and YFP–CO or YFP–COL1 were co-bombarded, the nucleo-cytoplasmic partitioning ratio of CFP-associated fluorescence increased, and CFP–LKP2 formed nuclear bodies (Fig. 4I, 5I). The position of CFP–LKP2 nuclear bodies was perfectly co-incident with YFP–CO or YFP–COL1 nuclear bodies (Fig. 4L, 5L). Co-localization of CFP–LKP2 and YFP–CO was also confirmed by three-dimensional image reconstruction (Supplementary Fig. S4). These co-localization patterns were not affected by incubation illumination conditions that we tested (data not shown).

As for Di19, YFP–Di19 and CFP–LKP2 signals were also co-localized when they were co-expressed in onion cells. We examined 26 cells. In 21 cells, YFP–Di19 signals were detected only in nuclei while CFP–LKP2 signals were detected in nuclei and cytosol (Supplementary Fig. S3A). However, both CFP–LKP2 and YFP–Di19 signals were localized in nuclear bodies of five of the examined cells (Supplementary Fig. S3B).

Two markers were employed to define further the nature of the nuclear bodies. One is U2B′′ [U2 small nuclear ribonucleoprotein (snRNP)-specific B′′ protein], which is a molecular marker of Cajal bodies, and the other is SCL28 (SC35-like protein with an M, of 28), which is a marker for spliceosomal speckles (Lorkovic et al. 2004, Shaw and Brown 2004). In onion cells, CFP–LKP2, YFP–CO and U2B′′–mRFP signals were co-localized, while SCL28–RFP signals were not coincident with those for CFP–LKP2 or YFP–CO (Fig. 6).

**Discussion**

In this study, we identified and characterized Di19 and CO/COL family proteins as potential factors that can interact with LKP2 domains.
Identification of interacting factors of LKP2 with FKF/LKP/ZTL family proteins. Di19 cDNA was first isolated as a drought-induced clone (Gosti et al. 1995). The Di19 gene was strongly expressed in both the roots and leaves of Arabidopsis plants during progressive drought. At present, 23 sequences containing the Di19 domain are listed in a database, though the precise function of Di19 or the Di19 domain is still unknown (Geer et al. 2002). Therefore, the biological significance of the interaction of LKP family proteins with Di19 is not clear, but may indicate a function in stress responses.

COL1 protein has two adjacent zinc finger motifs at its N-terminus and a basic region CCT motif at its C-terminus (Putterill et al. 1995, Putterill et al. 1997). CCT motifs are conserved in CO, COL and PRR/TOC1 family proteins (Robson et al. 2001). The zinc finger regions resemble B-boxes that regulate protein–protein interactions in several animal transcription factors (Borden 1998, Torok and Elkin 2000). Gene expression for COL1 is regulated by the circadian clock, and its temporal pattern is similar to that of CCA1 and LHY (Schaffer et al. 1998, Wang and Tobin 1998). Ectopic expression of COL1 in Arabidopsis leads to a shortened expression rhythm of the CAB2 promoter and showed fluence rate-dependent circadian defects, which suggests that COL1 has an effect on a light...
input pathway (Ledger et al. 2001). Antisense plants with reduced expression of the COL1 gene showed no circadian effects on rhythm, suggesting a functional redundancy of the COL family proteins (Ledger et al. 2001). Since both LKP1/ZTL and LKP2 could be blue light receptors with an LOV domain (Kiyosue and Wada 2000, Somers et al. 2000, Imaizumi et al. 2003) and function within or very close to the circadian oscillator (Schultz et al. 2001, Somers et al. 2004), the interaction of COL1 or other COL family proteins with LKP1/ZTL and LKP2 may reflect their function in clock regulation.

CO is a key regulator for flowering in the photoperiodic pathway in Arabidopsis. CO levels in plants in the presence of light are postulated to be the key factor that determines flowering time (Schultz and Kay 2003, Yanovsky and Kay 2003). CO-deficient mutants were isolated by their late-flowering phenotypes under long day photoperiod (LD) and their biological functions have been genetically characterized (Putterill et al. 1995, Kobayashi et al. 1999, Onouchi et al. 2000, Samach et al. 2000, Robson et al. 2001, Suarez-Lopez et al. 2001). CO protein levels are determined not only by its transcriptional regulation, but also by post-transcriptional mechanisms such as protein degradation (Valverde et al. 2004). Since LKP2-OX plants had a late flowering phenotype under LD, it would not be surprising if FKF/LKP/ZTL family proteins are involved in CO degradation. Indeed, CO protein has been shown to be degraded by a ubiquitin–proteasome system (Valverde et al. 2004). As the overall amino acid sequences of CO and COL1 are very similar (64.2% identity), it may be reasonable that LKP family proteins recognize CO as well as COL proteins.

The CCT domain is thought to be involved in nuclear localization of CO/COL and PRR/TOC1 family proteins (Makino et al. 2000, Strayer et al. 2000, Robson et al. 2001), though it has an additional role. The co-7 mutant has a single amino acid change in its CCT domain, R340Q. The co-7 mutant flowers later, though a green fluorescent protein (GFP)–co-7 fusion protein was shown to localize correctly in the nucleus of onion cells (Robson et al. 2001). LKP2 interacts with PRR1/TOC1 and PRR5 (Yasuhara et al. 2004), and with CO and COL1 (Fig. 3B), suggesting that the CCT motif might control these interactions. However, the CCT motif was not sufficient for LKP2 binding. The LKP2 interaction sites for CO and COL1 are apparently located at the N-terminal region adjacent to the CCT motif. The binding site of LKP2 with CO was different from that with COL1 (Fig. 3D). This interaction site difference may be due to differences in the primary structures of those proteins, since the binding sites for CO and COL1 are less similar in comparison with the conserved regions, the CCT motif and B-boxes.

Arabidopsis plants overexpressing GFP–LKP2 fusion proteins show late flowering and elongated hypocotyls (Yasuhara et al. 2004), which are also seen in LKP2-overexpressing plants (Schultz et al. 2001), suggesting that GFP does not affect LKP2 function. CFP is a derivative of GFP and only a few amino acids are changed, which suggests that the CFP–LKP2 fusion protein is also biologically active. When we expressed LKP2 as a fusion protein with CFP, CFP–LKP2 localization in onion cells was not completely co-incident with GFP–LKP2 in transgenic Arabidopsis plants. In onion cells, CFP–LKP2 signals are localized in both cytosol and nuclei, resembling naked CFP fluorescence (Fig. 4, 5), whereas in Arabidopsis GFP–LKP2 signals are seen in nuclei but not in nucleoli (Yasuhara et al. 2004). This disparity may be due to the differences between the cells expressing the protein fusions. Arabidopsis cells may possess some additional factor(s) that retains LKP2 in nuclei and/or transports LKP2 to nuclei. Our co-expression results support the former possibility.

The fusion of GFP with CO retained biological activity. Overexpression of GFP–CO complemented the co-2 mutation and plants flowered early, as do CO-overexpressing plants (Robson et al. 2001). The fusion protein was localized in nuclei in both Arabidopsis and onion cells (Robson et al. 2001). YFP is also a derivative of GFP, suggesting that the YFP–CO fusion protein is also biologically active. To date, there have been no published reports on the biological activity of GFP– or YFP–COL1 fusions. Since the overall primary structures of CO and COL1 are similar, COL1 fusions may also retain biological activity.

CFP–LKP2 formed nuclear bodies only when it was co-expressed with YFP–CO or YFP–COL1 (Fig. 4I, 5I), which supports the likelihood of interaction of LKP2 with CO and COL1 in plant cells. Several nuclear structures or domains have been reported (Shaw and Brown 2004). ‘Speckles’ is a nuclear domain wherein splicing factors and small RNP particles localize (Misteli 2000, Lamond and Spector 2003). Phytochromes accumulate in nuclei to form speckle-like structures when they receive light signals, though the biological function of the structures is also unknown (Yamaguchi et al. 1999, Nagatani 2000, Kircher et al. 2002). YFP–TOC1 was distributed in nuclei in a distinctive speckled pattern, which implied that TOC1 functions in transcriptomes, spliceosomes or proteasomes (Strayer et al. 2000). Among these possibilities, proteasome localization in nuclear bodies is very attractive, as LKP2 is an F-box protein that can interact with TOC1 (Yasuhara et al. 2004). However, we did not see any decrease in YFP–CO- or YFP–COL1-related signals when they were co-expressed with CFP–LKP2 under white light or dark conditions (data not shown).

Our co-bombardment results suggested that LKP2 and CO could localize in Cajal bodies. Cajal bodies are spherical structures ranging in size from 0.2 to 2.0 µm in plant cells, and are frequently associated with the nucleolus. The function of Cajal bodies is not clear, though experimental evidence is accumulating that they function in the assembly of transcriptomes, snRNP assembly, modification of spliceosomal snRNAs and trafficking of snRNPs involved in modifying RNA in the nucleolus (Ogg and Lamond 2002, Gall 2000, Gall 2003, Lorkovic and Barta 2004).
Further investigation is necessary to determine whether LKP family proteins function as F-box proteins for the modification of Di19, CO or COL proteins, and to determine the mechanism that leads to the relocation of LKP2 in nuclear bodies.

Materials and Methods

DNA sequence analysis

Plasmid DNA templates for sequencing were prepared and DNA sequences were determined as described previously (Yasuhara et al. 2004). The GENETYX (Software Development, Tokyo, Japan) and Sequencher (Gene Codes Co., Ann Arbor, MI, USA) software systems were used for the DNA sequence analysis.

cDNA library construction and yeast two-hybrid assay

The two-hybrid assay was performed as described (Yasuhara et al. 2004). cDNA library construction and subsequent two-hybrid screening were according to the manufacturer’s instructions (MATCH-MAKER Library Construction & Screening Kit, Clontech, Palo Alto, CA, USA) with RNA isolated from rosette leaves of Arabidopsis (Columbia ecotype), which had been grown under LD conditions of 16 h light and 8 h dark at 22°C, and harvested at 4 h intervals (0, 4, 8, 12, and 20 h) from the onset of illumination. A full-length LKP2 cDNA with the GAL4 activating domain was used as bait.

cDNA clones

Columbia versions of cDNA for Di19, CO, COL1-10 and COL13–16 were obtained by RT–PCR with ReverTra Dash (Toyobo, Japan) and sequenced and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCGTCGTCCTAGGAAGGTTAGATAAG-3′, 5′-AGGATCCGTCCTAGGAAGGTTAGATAAG-3′, and 5′-AGGATCCGTAAGATAGATAAG-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

For CO deletions, each construct was PCR-amplified, subcloned, and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

Immunoblot analysis

For immunoblot analysis, yeast cells were grown in 50 ml ofYPD (yeast extract/peptone/dextrose) medium for 4–8 h (OD600 = 0.6), pelleted by centrifugation, and frozen in liquid nitrogen. The pellets were resuspended in 400 µl of extraction buffer (40 mM Tris–HCl, pH 6.8, 8, 5 mM sodium dodecyl sulfate (SDS), 10% glycerol, 0.8% 2-mercaptoethanol, 1.86 µg ml-1 pepstatin A, 1.86 µg ml-1 leupeptin, 9.0 µg ml-1 benzamidine, 23.0 µg ml-1 aprotinin, 0.77 mg ml-1 phenylmethylsulfonyl fluoride), and homogenized. Crude cell extract (12 µl) was loaded onto each lane, and proteins were separated by SDS–PAGE and electro-transferred onto nylon membranes.

For CO deletions, each construct was PCR-amplified, subcloned, and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

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For COL deletions, each construct was PCR-amplified, subcloned, and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

For COL deletions, each construct was PCR-amplified, subcloned, and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

For COL deletions, each construct was PCR-amplified, subcloned, and ligated into two-hybrid vectors. The forward primers used for PCR were 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, 5′-AGGATCCCCGGATCAATTACGAGGCTGTTGTA-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and the reverse primers were 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, 5′-AGGATCTAGTAATGAAGGTTATGCT-3′, and 5′-AGGATCTAGTAATGAAGGTTATGCT-3′.

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YFP and RFP signals were detected with a BX51 microscope (Olympus, Tokyo, Japan) with a CCD camera, Retiga EXi fast-cooled Mono 12 bit (Qimaging, Burnaby, BC, Canada) and standard CFP or YFP filter (Olympus). The images were analyzed with MetaMorph Imaging Software Version 6.1 (Universal Imaging Corporation, Downingtown, PA, USA). Three-dimensional deconvoluted images were constructed using AutoDeblur/AutoVisualize (version 9.3) software (AutoQuanl Imaging, New York, NY, USA).

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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References
Identification of interacting factors of LKP2


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