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Nuclear Import of the LIKE HETEROCHROMATIN PROTEIN1 is redundantly mediated by Importinα−1, Importinα−2, and Importinα−3

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SUMMARY

LIKE HETEROCHROMATIN PROTEIN1 (LHP1) encodes the only plant homologue of the metazoan HETEROCHROMATIN PROTEIN1 (HP1) protein family. LHP1 is necessary for proper epigenetic regulation of a range of developmental processes in plants. LHP1 is a transcriptional repressor of flowering related genes, such as *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS C* (*FLC*), *AGAMOUS* (*AG*), and *APETALA 3* (*AP3*). We found that LHP1 interacts with importin [alpha]-1 (IMPα−1), importin [alpha]-2 (IMPα−2), and importin [alpha]-3 (IMPα−3), both *in vitro* and *in vivo*. A genetic approach revealed that triple mutation of *impα−1*, *impα−2*, and *impα−3* resulted in *Arabidopsis* plants with a rapid flowering phenotype similar to that of plants with mutations in *lhp1* due to the up-regulation of *FT* expression. Nuclear targeting of LHP1 was severely impaired in the *impα* triple mutant, resulting in the de-repression of LHP1 target genes *AG, AP3*, and *SHATTERPROOF 1* as well as *FT*. Therefore, the importin proteins IMPα−1, IMPα−2, and IMPα−3 are necessary for the nuclear import of LHP1.

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AUTHOR CONTRIBUTIONS

C.C. and H.Y. performed most of the experiment. D.K. analyzed LHP1-GFP in protoplasts and primary root. B.Y and Z.B carried out transcriptome analysis. Y.L., H.K., Y.R., J. M., and H.K. performed the phenotype observations, measurements, and the gene cloning. S.S. and J.H. designed experiments and wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Keywords

TFL2; LHP1; Flowering; Importin

INTRODUCTION

LIKE HETEROCHROMATIN PROTEIN1 (LHP1), also known as Terminal Flower 2 (TFL2), is the only *Arabidopsis* protein that shows the homology to HP1 of metazoans and *Schizosaccharomyces pombe*. In general, HP1 proteins are enriched in the heterochromatin region, and are involved in not only heterochromatin formation and maintenance, but also in the regulation of heterochromatic and euchromatic genes in animals and yeast (Gaudin *et al.*, 2001, Li *et al.*, 2002). These proteins associate with target regions via the interaction of their chromodomain with di- or tri-methylated lysine 9 residues of histone 3 (H3K9me2 or H3K9me3, respectively) (Mateescu *et al.*, 2004, Fischle *et al.*, 2005). LHP1 is widely conserved as a single copy gene in plants (Gaudin *et al.*, 2001, Kotake *et al.*, 2003). Mutations in *lhp1* exhibit a diverse range of developmental phenotypes, including photoperiod-independent early-flowering, reduced sensitivity to photoperiod, termination of the inflorescences, and dwarfism (Larsson *et al.*, 1998).

LHP1 is localized to the nucleus, and it is enriched within euchromatin regions but not in constitutively active heterochromatin regions in *Arabidopsis* (Libault *et al.*, 2005). In *Arabidopsis*, LHP1 acts as a transcriptional repressor of genes in euchromatic regions. For instance, LHP1 is involved in the maintenance of *FLOWERING LOCUS C* (*FLC*) repression after vernalization, repression of *FLOWERING LOCUS T* (*FT*), and regulation of *AGAMOUS* (*AG*), *APETALA 3* (*AP3*), *PISTILLATA* (*PI*), and *SHATTERPROOF 1* (*SHP1*) through the function of the polycomb repressive complex 1 (PRC1) (Kotake *et al.*, 2003, Mylne *et al.*, 2006, Sung *et al.*, 2006, Calonje *et al.*, 2008, Liu and Mara, 2009). Interestingly, it has been reported that LHP1 may also act as a transcriptional activator. For example, auxin levels are lower in the *lhp1* mutant than in wild-type plants due to the down-regulation of *YUCCA* genes related to auxin biosynthesis (Rizzardi *et al.*, 2011), suggesting that LHP1 is involved in auxin biosynthesis through the positive regulation of *YUCCA* genes. In addition in soybean, LHP1 regulates the expression of genes for salt tolerance by interacting with Plant Homeodomain 6 (GmPHD6) to form a transcriptional activation complex (Wei *et al.*, 2017). Thus, LHP1 can serve as both a transcriptional repressor and an activator in plants.

The nuclear transport of macromolecules is an important process for the regulation of various signal transduction pathways in plants (Kaffman and O'Shea, 1999, Cyert, 2001, Johnson *et al.*, 2004). In general, certain newly synthesized proteins must be transported from the cytoplasm to the nucleus to ensure proper cellular function (Stewart, 2007). Transport across the nuclear envelope requires transport complex machinery that is highly conserved from yeast to mammals (Hicks and Raikhel, 1995, Tzfira *et al.*, 2000, Wirthmueller *et al.*). Nuclear pore complexes (NPCs), which are embedded in the nuclear envelope, act as gateways between the nucleus and cytoplasm. Nuclear localization signals (NLSs), which have mostly lysine (K) amino acid residues, tag proteins for rapid transport

through these gates. Signal-mediated translocation into the nucleus requires soluble factors such as the importins (which are part of the larger karyopherin family) and small GTPase Ras-related nuclear (Ran) proteins (Pemberton and Paschal, 2005, Stewart, 2007). Importins consist of importin [alpha] (IMPα) and importin [beta] (IMPβ) subunits. IMPα and IMPβ form co-operative complexes with NLS-containing proteins in the cytoplasm, which then dock to the cytoplasmic side of the NPC via IMPβ (Gorlich *et al.*, 1995). Following nuclear translocation of this triple complex through the nuclear pore, the complex is dissociated in the nucleus with the help of GTP-bound Ran GTPases (Izaurralde *et al.*, 1997, Kutay *et al.*, 1997). There are 10 members of the IMPα family and 18 IMPβ orthologues in *Arabidopsis* (Merkle, 2011, Tamura and Hara-Nishimura, 2014). The functional redundancies and/or specificities of importin family proteins are poorly understood.

In this study, we identified components of the NPC that is responsible for the nuclear localization of LHP1 in *Arabidopsis*. Using a yeast two−hybrid screen, we identified IMPα−1, IMPα−2, and IMPα−3 as interacting proteins of LHP1. Consistent with the previously described role of IMPα subunits, the nuclear targeting of LHP1 was severely impaired in the *impα−1, impα−2, and impα−3* triple mutant plants. In addition, the triple mutant plants exhibited severe growth defects, dwarfism, and very early flowering, which phenocopies *lhp1* loss−of−function mutants. Our work identifies the members of the IMPα family that are mainly responsible for the nuclear import of LHP1.

RESULTS

LHP1 interacts with IMPα−1, IMPα−2, and IMPα−3

LHP1 is involved in various events during plant growth and development (Larsson *et al.*, 1998, Kotake *et al.*, 2003). Given the diverse functions of LHP1 in plant development, we expect that LHP1 functions through various combinations of interactions with other nuclear proteins. To address the functions of LHP1, we performed yeast two-hybrid screening to identify interacting proteins. Yeast-two hybrid screens using LHP1 as a bait identified several putative interacting proteins including IMPα−1, IMPα−2, and IMPα−3 (also known as MODIFIER OF SNC1 6) (Palma *et al.*, 2005) (Figure 1a). Moreover, LHP1 did not interact with the other seven IMPα proteins (Figure S1). To confirm our findings *in planta*, we generated three transgenic plants co-expressing LHP1 tagged with green fluorescent protein (GFP) and flag-tagged IMPα−1, LHP1-GFP and flag-IMPα−2, or LHP1-GFP and flag-IMPα−3. Co-immunoprecipitation assays were performed using extracts from these transgenic lines. All flag-IMPαs were co-precipitated with LHP1-GFP (Figure 1b), indicating that LHP1 indeed interacts with IMPα−1, IMPα−2, and IMPα−3 *in planta*.

The *impα* triple mutant plants flower rapidly and photoperiod-independently due to the up-regulation of *FT* expression

To address the biological implications of IMPαs in the function of LHP1, we analyzed *impα* mutants. First, we identified T-DNA insertion mutant lines for all three *impα* mutants and determined that they are loss-of-function alleles (Figure 2a and 2b). The single mutant plants exhibited macroscopically normal phenotypes and flowering times (Figure 2c and 2d), suggesting redundant function among IMPαs. The *impα−3* (*MOS6*) gene was previously

identified as a genetic modifier of the *snc1* mutant (Palma *et al.*, 2005). *impα−3* mutants partially suppress *snc1* mutants, resulting in enhanced disease susceptibility to pathogens; however, the *impα−3* single mutant does not show any noticeable morphological phenotype when compared to the wild-type plants (Palma *et al.*, 2005). This finding prompted us to create higher order mutants to confirm functional redundancy among LHP1-interacting IMPαs. None of the double mutant combinations showed any significant phenotypic differences compared to the wild-type Col-0 plants (Figure S2), whereas *impα* triple mutant plants exhibited pleiotropic phenotypes including small and narrow rosette leaves, reduced plant height, early flowering, and small siliques compared to those of wild-type plants (Figure 3a and 3b, Figure S3). These phenotypes are also observed in *lhp1* mutant (Figure 3a and 3c). These results suggest that the function of LHP1 is impaired in the *impα* triple mutant. To confirm whether the phenotypes observed in the *impα triple* mutant are indeed due to the functional losses of IMPα−1, −2 and −3, wild-type copies of IMPα−1, −2 and −3 driven by 35S promoter were introduced into the *impα triple* mutant. Any single IMPα−1, −2 and −3 transgene could rescue the *impα triple* mutant phenotypes (Figure S4). We also obtained *lhp1* mutation in *impα triple mutant* via the CRISPR-CAS9 system (Figure S5). These quadruple mutant plants flowered very early similar to the *impα triple* and *lhp1–3* mutants under both LD and SD (Figure 3a–3d). Taken together, our data indicate that *lhp1–3* and the *impα triple* mutant function in the same genetic pathway to regulate flowering time.

The *impα* triple mutant plants flowered very early under long-day (LD) and short-day (SD) conditions, similar to *lhp1* mutants (Figure 3a–3d). Because LHP1 is necessary for the repression of *FT* expression through the direct association with *FT* chromatin (Turck *et al.*, 2007, Zhang *et al.*, 2007), we analyzed the expression of *FT* by quantitative RT-PCR (qRT-PCR) in wild-type, *impα* triple mutant, and *lhp1-3* mutant plants under both LD and SD conditions. qRT-PCR data showed that *FT* expression was highly up-regulated in both *impα triple* and *lhp1-3* mutant plants regardless of photoperiod (Figure 3e and S6). These results indicate that IMPα−1, −2, and −3 are all necessary for the proper function of LHP1.

IMPα−1, IMPα−2, and IMPα−3 are involved in the nuclear import of LHP1

To exclude the possibility of down-regulation of LHP1 transcripts in *impα* triple mutants, we performed qRT-PCR to compare the levels of *LHP1* transcripts between the wild-type and *impα* triple mutant plants and observed no significant difference (Figure S7). This result indicates that IMPα−1, IMPα−2, and IMPα−3 do not affect the transcription of *LHP1*. To confirm our hypothesis that IMPα−1, IMPα−2, and IMPα−3 are required for the LHP1 protein from the cytosol to the nucleus, we evaluated the subcellular localization of LHP1-GFP in protoplasts from wild-type and *impα* triple mutant plants in a transient expression assay. The control GFP proteins were mainly localized to the cytosol (Figure 4a). In Col-0 plants, LHP1-GFP proteins were localized in the nucleus, and the LHP1-GFP fluorescence perfectly overlapped with the NLS-RFP signal in protoplasts prepared from Col-0 plants (Figure 4b), indicating that LHP1-GFP is localized in the nucleus as expected (Libault *et al.*, 2005). In contrast, in the *impα* triple mutant plants fluorescence was observed throughout the cytosol the localization of LHP1-GFP in protoplasts prepared from the *impα* triple mutant plants lacked a clear pattern (Figure 4c), indicating that the nuclear import of LHP1-GFP is impaired in *impα* triple mutant plants. We also generated transgenic plants

expressing LHP1-GFP in the wild-type Col-0 and *impα* triple mutant plants and analyzed subcellular localization of LHP1-GFP in the primary roots. LHP1-GFP protein signals were clearly overlapped with DAPI, a nuclear marker in wild-type plants however LHP1-GFP protein signals were diffuse in *impα triple* mutant plants (Figure 4d). These results showed that IMPα−1, IMPα−2, and IMPα−3 are involved in the nuclear import of LHP1.

Target genes of LHP1 are highly expressed in the *impα* triple mutant plants

LHP1 controls expression of several transcription factors, and these loci are commonly enriched with H3K27me3, a repressive histone modification (Turck *et al.*, 2007). We employed RNA-Seq approaches to examine the differentially expressed genes (DEGs) in *impα triple* and *lhp1–3* mutants. A large number of DEGs was identified by comparison between the wild-type (Col-0) and the *impα triple* mutants (17,625) and between the wild-type (Col-0) and lhp1–3 mutants (16,893). Overall, 87.64% of DEGs (FDR <0.05) in *lhp1–3* is overlapped with DEGs in the *impα triple* mutants (Figure 5a), In addition, there is a very strong positive correlation between transcriptomes of *lhp1–3* and the *impα triple* mutants. Our transcriptome analysis further supports that the majority of LHP1 functions through IMPα−1, IMPα−2, and IMPα−3. To confirm the transcriptomic data, the levels of several known LHP1 target genes, *FT, AG*, *SEPALLATA 2* (*SEP2*), *SHP1*, and *FLC* were investigated using qRT-PCR in the Col-0, *impα* triple mutant, and *lhp1-3* mutant plants. Consistent with the role of LHP1 as a transcriptional repressor, these genes were up-regulated in *impα* triple mutant and *lhp1–3* mutant plants compared to the wild-type plants (Figure 6a). In addition, we analyzed enrichment of the H3K27me3 on *FT* and *AG* chromatin. H3K27me3 levels were significantly reduced at the transcription start sites and across the first introns of *FT* and *AG* chromatin in the *impα* triple and *lhp1-3* mutants compared to the wild-type plants (Figure 6b and 6c).

IMPα−1, IMPα−2, and IMPα−3 recognize several classes of nuclear localization signals

Generally, IMPαs act as receptors that recognize the NLSs on karyophilic proteins in the cytoplasm at the first step of the protein nuclear import (Tamura and Hara-Nishimura, 2014). There are six classes of NLS consensus sequences, which are classified based on monopartite or bipartite clusters of basic residues (Chang *et al.*, 2013). *Arabidopsis* IMPαs can recognize three different classes of NLSs, and rice IMPα−1 preferentially binds to class 3, 4, and 5 NLSs (Smith *et al.*, 1997, Kosugi *et al.*, 2009). LHP1 possesses five classical NLSs (class 1 NLSs) and the partial LHP1 protein with the 3rd and 4th NLSs forming bipartite signal maintains the nuclear localization (Libault *et al.*, 2005). Consistent with this, only the 3rd and 4th NLSs of LHP1 interacted with IMPα−1, IMPα−2, and IMPα−3 in yeast, suggesting that the 3rd and 4th NLSs in LHP1 may be the major NLSs for nuclear targeting and mediate the interaction with IMPα−1, IMPα−2, and IMPα−3 (Figure S8). Because IMPα−1, IMPα−2, and IMPα−3 interact with LHP1, which has a class 1 NLS, they likely recognize class 1 NLSs but may also recognize NLSs of other classes. Subcellular targeting of proteins containing five different classes of NLSs (classes 1, 2, 4, 5, and 6; Table 1) was investigated in protoplasts prepared from the wild-type Col-0 and triple mutant plants (Figure 7). Classes 1, 2, and 5 NLS-GFPs were mis-localized to the cytoplasm or vacuole in triple mutants, whereas class 4 and 6 NLS-GFPs were localized to the nucleus

(Figure 7). These results indicated that IMPα−1, IMPα−2, and IMPα−3 are necessary for import of nuclear proteins with class 1, 2, and 5 NLSs.

Discussion

In this study, we analyzed relationship between LHP1 and its interacting proteins, IMPα−1, IMPα−2, and IMPα−3. The *Arabidopsis* genome encodes 10 IMPα proteins that belong to a large family of armadillo (ARM) repeat-containing proteins in plants (Mudgil *et al.*, 2004). Most IMPαs contain 8 to 10 ARM repeats, which both give the protein an elongated super-helical structure and form the NLS-binding sites (Gorlich *et al.*, 1995, Mudgil *et al.*, 2004). Because of similar protein structures among the IMPα family members, it was assumed that there is functional redundancy among them. Consistent with this prediction, our genetic analysis showed that none of the single mutants or double mutant combinations of *impα−1*, *impα−2*, and *impα−3* had significant differences in morphological and developmental phenotypes from wild-type plants (Figure 2d and Figure S2). The *impα−1*, *impα−2*, and *impα−3* triple mutant had a phenotype almost identical to that of *lhp1-3* mutant plants (Figure 3a and 3b). However, there was phenotypical difference between the *impα* triple and *lhp1–3* mutants. Root elongation was more compromised in the triple *impα* mutant than in *lhp1–3* (Figure S3a). A previous study showed that LHP1 acts to regulate the timing of middle cortex formation together with SCARECROW (SCR) required for the first cell division and longitudinal asymmetric cell divisions that generate cortex and endodermis in root. Indeed, *lhp1–3* mutant seedlings have nearly normal root length, but a second longitudinal asymmetric cell division occurs in the ground tissue earlier than the wild-type (Cui and Benfey, 2009). It is possible that some proteins with class 1, 2, or 5 NLSs that mediate the formations of cortex and endodermis in root may be mis-located in *impα* triple mutant plants, and therefore the root elongation of the triple mutant plants could be affected. Recently, it was reported that the nuclear import of Arabidopsis Poly (ADP-Ribose) Polymerase 2 is mediated by IMP2-α (Chen *et al.*, 2018). This suggests that several kinds of nuclear proteins, including chromatin regulators, that have class 1, 2, or 5 of NLSs might be mis-targeted in the triple mutant plants. This phenomenon may cause severe growth defect phenotypes in the *impα* triple mutant. Another paper reported that LHP1 associates with a number of the *YUCCA* genes involved in auxin biosynthesis and their expression is down-regulated in *lhp1* mutation, resulting in lower level of auxin (Rizzardi *et al.*, 2010), therefore affecting various plant growth habits. Taken together, IMPαs may be involved in several kinds of mechanism, including flowering, root elongation and auxin biosynthesis by importing of LHP1 and also other nuclear proteins. This is also consistent with our transcriptome analysis that shows a greater number of DEGs in the *impα* triple mutant than in *lhp1–3* mutants (Figure 5). In addition, LHP1 interacted with IMPα−1, IMPα−2, and IMPα−3 but not the other IMPαs in our yeast two-hybrid screen (Figure S1). These results indicate that IMPα protein may differ in their target specificities. Our work identified a subclass of IMPα proteins that dictates the nuclear localization of LHP1.

LHP1 was originally shown to function in inflorescence meristem development (Larsson *et al.*, 1998). *lhp1* is a single-copy gene in *Arabidopsis* that encodes a functional homolog of metazoan HP1 (Gaudin *et al.*, 2001, Kotake *et al.*, 2003). The LHP1 protein structure is conserved in many plants (Zemach *et al.*, 2006), and diverse functions have been reported

that depend on interacting proteins (Exner *et al.*, 2009). In *Arabidopsis*, LHP1 directly interacts with ASYMMETRIC LEAVES 1 (AS1) and AS2, which are involved in leaf development. This interaction contributes to establishing the histone modification at the chromatin regions of *KNOTTED1-like homeobox* genes (Li *et al.*, 2016). Transcription factor-encoding *SEP3* is repressed by a flowering time gene, SHORT VEGETATIVE PHASE (SVP), through the direct interaction of SVP with LHP1. This repression prevents premature differentiation of floral meristems and determines the proper timing of floral organ patterning (Liu and Mara, 2009). LHP1 also interacts with a replication complex to recruit other PRC2 components to targets after replication (Hyun *et al.*, 2013). EMBRYONIC FLOWER1 (EMF1) is also well-known as a key partner of LHP1 forming a PRC1-like complex for PcG silencing (Wang *et al.* 2014). Thus, LHP1 and its many interacting proteins work together to perform diverse gene regulatory roles in plant growth and development.

The results we describe here demonstrate that IMPα−1, −2, and −3 are all necessary for nuclear localization and subsequent functions of LHP1. Although functional and physical interactions among nuclear transport receptors, IMPαs and IMPβs, and nucleoporins and their NLS-containing cargo molecules is not fully understood, plant nuclear import is important for plant growth and development through specific cargo selection by IMPαs. In order to understand the nuclear transport mechanisms in plants, it will be necessary to identify the plant cargo molecules and to address the physical interaction between cargoes and receptors.

EXPERIMENTAL PROCEDURES

Plant materials and growth condition

Arabidopsis seeds were germinated on 0.8% agar plates with Murashige and Skoog Salt, 0.8% sucrose, stratified at 4 °C for 3 days, and grown for 14 days in long day conditions (16 h light at 22 °C, and 8 h dark at 21 °C) or short-day conditions (8 h light at 22 °C, 16 h dark at 21 °C) and then plants were transplanted to soil and transferred to growth chambers (21 °C ± 2 °C) under LD or SD conditions for flowering time tests. For RNA isolation plants were harvested on day 14.

Arabidopsis knock-out lines carrying T-DNA insertion in *impα*s and *lhp* genes were provided by the *Arabidopsis* Biological Resource Center (ABRC), and seeds were screened to identify homozygous mutants. The *impα−1* (Salk_001092), *impα−2* (Salk_099707), *impα−3* (mos6–2, Salk_025919), and *lhp1–3* (CS3797) lines were previously described (Sung *et al.*, 2006, Bhattacharjee *et al.*, 2008). Transgenic *Arabidopsis* plants over-expressing *LHP1-GFP* or *impα−1*, *impα−2*, and *impα−3* containing a Flag-tag were generated. Briefly, cDNA fragments of full-length *lhp1* and *impα*s were cloned into pK7WGF or pEarleyGate vector to generate in-frame fusion proteins with GFP or Flag sequences. Transformation of *Arabidopsis* was carried out by the floral dip method (Clough and Bent, 1998).

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out according to the manufacturer's protocol (Invitrogen). Briefly, the bait plasmid, *pDBD-LHP1*, and total cDNAs generated from *Arabidopsis* and fused to the pAD vector were used to sequentially transform *Saccharomyces cerevisiae* strain pJG69–4A. The resulting transformants were then plated on synthetic complete (SC) medium lacking tryptophane (Trp), leucine (Leu), and histidine (His) and/or SC lacking Trp^- and Leu^- and including 3 mM 3-amino-1,2,4-triazole. Histidine-positive colonies were analyzed for β-galactosidase activity according to the manufacturer's protocol (Invitrogen). The clones of cDNAs encoding candidate LHP1 interacting proteins were selected and isolated based on their growth as blue colonies in histidine-deficient medium. The each cDNA was isolated and sequenced. The DNA sequences were then subjected to BLAST analysis against GenBank.

Transient expression assay

LHP1 cDNA was fused upstream of *GFP* under the control of the CaMV 35S promoter (pUC::GFP) (Benfey and Chua, 1990). The resulting construct (*LHP1-GFP*) was co-transformed into *Arabidopsis* protoplasts along with the cDNA encoding red fluorescent protein (RFP) fused to a nuclear localization signal peptide (*NLS-RFP* (Cheong *et al.*, 2003)). Transient expression of GFP- and RFP-fused constructs in *Arabidopsis* protoplasts was performed according to the method described by Heo *et al.* (Heo *et al.*, 2005). Briefly, recombinant plasmids were introduced by polyethylene glycol-mediated transformation into *Arabidopsis* protoplasts that had been prepared from seedling plants. Expression of the fusion constructs was monitored after transformation by fluorescence microscopy (Olympus AX70 TR, Olympus).

Quantitative real-time PCR (qRT-PCR)

Total RNAs were isolated from about 50 mg of *Arabidopsis* seedlings by Trizol reagent according to the manufacturer's protocol (Invitrogen). cDNAs were synthesized from about 400 ng of total RNAs by the PrimeScript RT-PCR Kit with gDNA Eraser (TaKaRa), and qPCR was performed using a Thermal Cycler Dice Real Time System with a SYBR *Primer-Ex Taq* II kit (TaKaRa), and the relative transcript level of each gene was determined by normalization of the resulting expression levels versus that of *PP2A*. Primers for qRT-PCR are listed in Table S1.

Chromatin Immuno-precipitation (ChIP) assay

ChIP assays were performed as described previously (Heo and Sung, 2011). After chromatin isolation, immunoprecipitation was performed using anti-H3K27me3 antibody (Abcam, Cat. Ab6002). Crosslinks were reversed by incubation at 65 °C for 6 h, and DNA was purified with QIAquick spin columns (Qiagen) and eluted in 50 μl of TE (pH 8.0). qRT-PCR was used to quantify fragments of *FT* and *AG*. Relative enrichment of each fragment was calculated based on comparison to the input sample (i.e., chromatin sample before immunoprecipitation). Reported are averaged percentages of input fraction relative to negative controls from at least two independent experiments.

Co-Immunoprecipitation assays

Seedlings expressing GFP or co-expressing LHP1-GFP and Flag-IMPα−1, LHP1-GFP and Flag-IMPα−2, LHP1-GFP and Flag-IMPα−3 were homogenized in extraction buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1% protease inhibitor cocktail (Sigma-Aldrich)). The protein extract was incubated with anti-GFP polyclonal antibody (Abcam, Cat. ab6556) in 2× IP buffer (25 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 3 mM $MgCl_2$) at 4 °C for 4 h followed by incubation at 4 °C for 6 h with protein A-Sepharose beads. The beads were washed five times in IP buffer with 1% Triton X-100. The proteins elutes were subjected to western blot analysis with anti-flag monoclonal antibody (Abcam, Cat. ab205606).

Transcriptomic analysis

Whole seedlings grew on ½ MS medium containing 0.8% sucrose under long-day (16 h light, 8 h dark) condition were collected at ZT12. Total RNAs were extracted by using TRIzol (Invitrogen) and treated with DNase I enzyme (Promega) to eliminate traces of genomic DNA. Sequencing libraries were prepared with 500 ng total RNA following NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7420). Quality of libraries was assessed on a Bioanalyzer (Agilent High Sensitivity DNA Assay). Reads generated by Illumina NextSeq 500 platform were checked for quality using FastQC. After quality assessment, reads were aligned on TAIR10 genome using HISAT2. SAM files generated from mapping were then converted into BAM files and sorted using Samtools. For the gene counting bedtools program was used which generates raw count using BAM files. Raw count was then fed into R for differential gene expression analysis using edgeR and data visualization. This transcriptional dataset has been submitted to the NCBI (https://www.ncbi.nlm.nih.gov) and it will be released with the reference GSE144849.

Classification of IMPα-dependent NLSs

The TAIR10 protein database (www.arabidopsis.org/) was searched using previously established consensus sequences of NLS classes (Kosugi *et al.*, 2009) to identify candidate proteins from each class. These candidates were analysed using the program PredictNLS (https://www.predictprotein.org/), and we selected one or two proteins of known function as representatives of each NLS class (Table 1). The cDNA fragments of these proteins were PCR-amplified and cloned into the smGFP-N vector.

Data Availability Statement

The datasets generated for this study can be found in the NCBI database https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144849

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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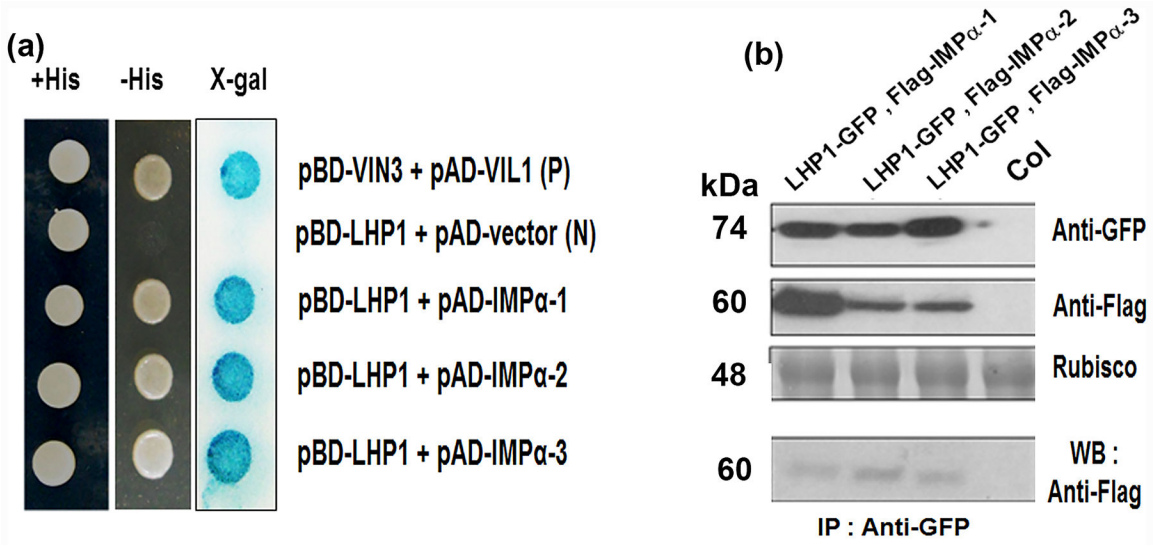

Figure 1.

LHP1 interacts with IMPαs *in vitro* and *in planta*. (a) Yeast two-hybrid assay between LHP1 and IMPαs. Each construct was co-transformed into pJG69–4A yeast cells, and their interaction evaluated based on β-galactosidase activity measured by the filter assay method (Invitrogen). VIN3 and VIL1 were used as a positive control for interaction. (b) Co-immunoprecipitation analysis. Total proteins were extracted from Col-0 and overexpression lines and then immunoprecipitated with anti-GFP antibody. The immunoprecipitates were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-flag antibody.

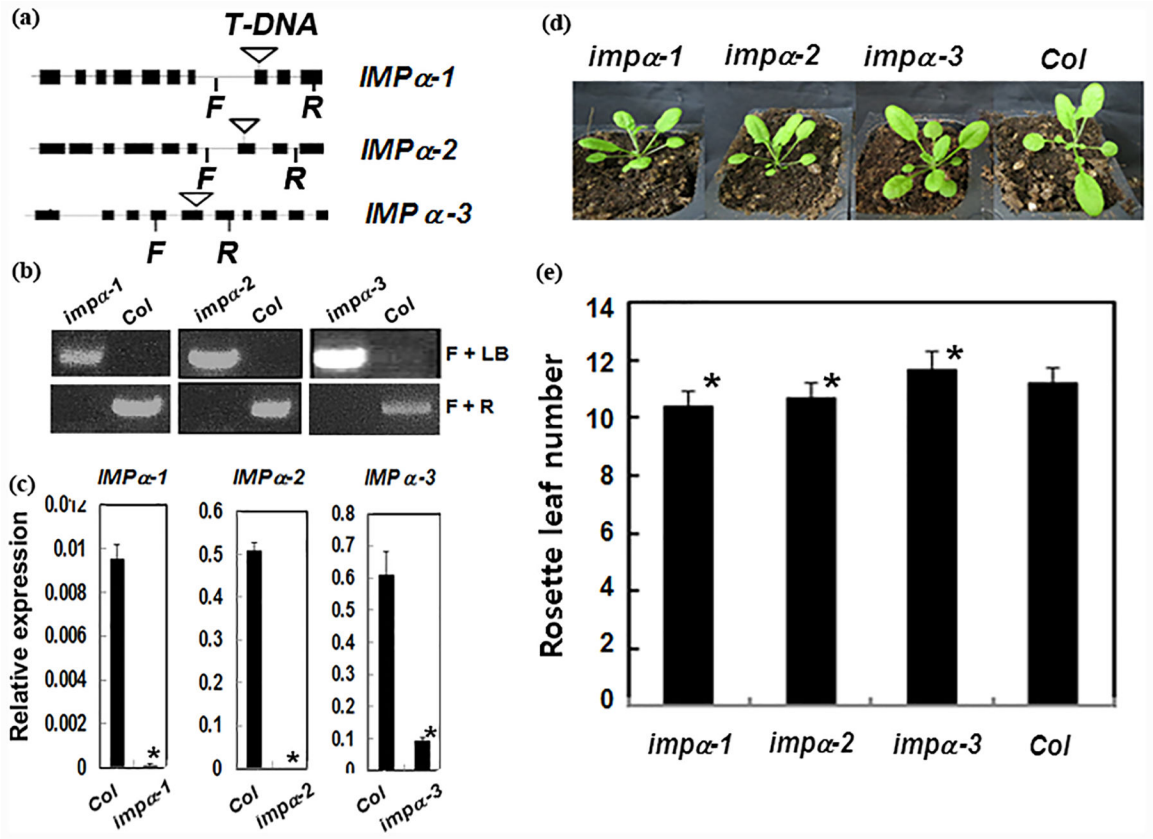

Figure 2.

Single *impα* mutants are phenotypically normal. (a) Schematic representation of the *impα−1*, *impα−2*, and *impα−3* alleles with the T-DNA insertions shown as inverted triangles. Shaded bars indicate coding regions. Gene-specific (forward, F, and reverse, R) and T-DNA specific (LB) primers used in the genotyping and RT-PCR are shown. (b) Genotyping of the *impα−1*, *impα−2*, and *impα−3* mutant plants. The gene-specific primers used for genomic PCR are indicated at right. (c) Relative expression of indicated *impα* mRNAs in *impα* mutant plants. Total RNA was extracted from Col-0 and each *impα* mutant plant, and after cDNA synthesis each transcript level was measured by qRT-PCR. *PP2A* was used as a control. Statistical significance was determined by Student's *t* test (*, $p < 0.05$) (d) Flowering phenotypes of *impα* mutant plants. When a first bud of *impα−1* was open, pictures of all plants were taken (e) Flowering times of *impα* mutant plants determined by rosette leaf numbers at the time of bolting under LD (16 h light, 8 h dark) condition. At least 12 plants of each genotype were used. Error bars indicate SD. Statistical significance was determined by Student's *t* test (*, $p < 0.001$)

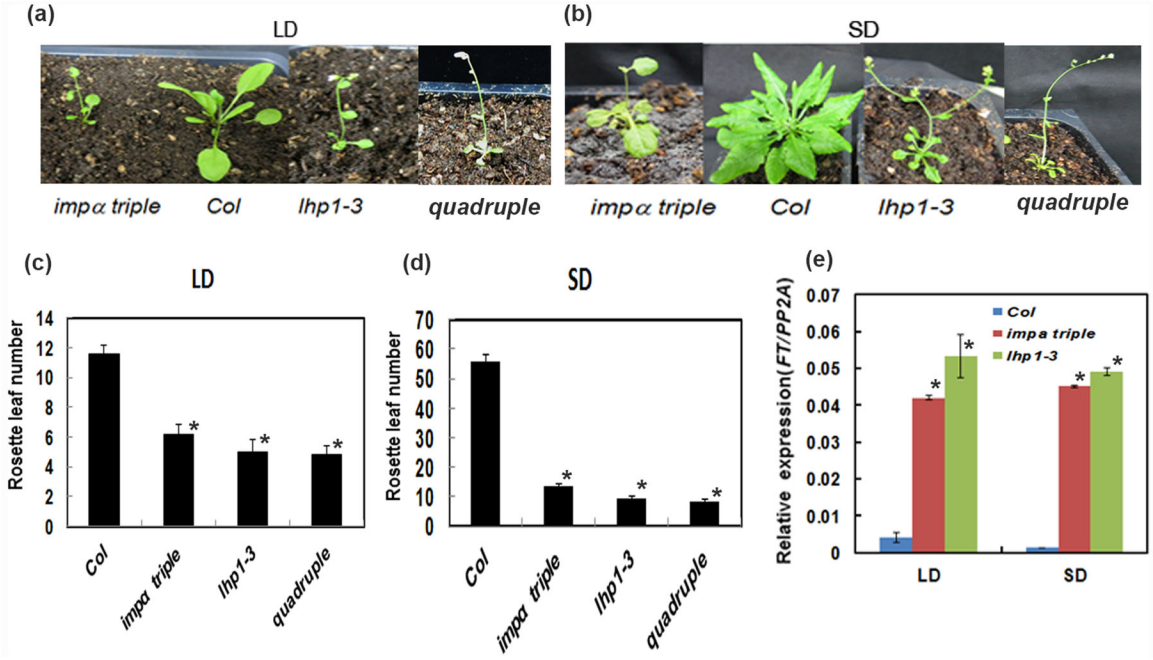

Figure 3.

The *impα* triple mutant plants flower rapidly. (a) Flowering phenotypes of *impα* triple mutant, Col-0, and *lhp1–3* mutant plants under LD. When a first bud of *lhp1–3* was open, pictures of all plants were taken (b) Flowering times of *impα* mutant plants determined by rosette leaf numbers at the time of bolting under LD condition. At least 12 plants of each genotype were used. (c) Flowering phenotypes under SD. When a first bud of *lhp1–3* was open, pictures of all plants were taken (d) Flowering times under SD. At least 12 plants of each genotype were used. Statistical significance was determined by Student's *t* test (*, $p < 0.001$). (e) The levels of *FT* mRNA under LD (16 h light, 8 h dark) and SD (8 h light, 16 h dark) conditions determined by qRT-PCR of samples from two-week-old plants. Samples were taken at ZT12. Each bar represents an average of three independent replicate experiments. Error bars indicate SD. Statistical significance was determined by Student's *t* test (*, $p < 0.05$)

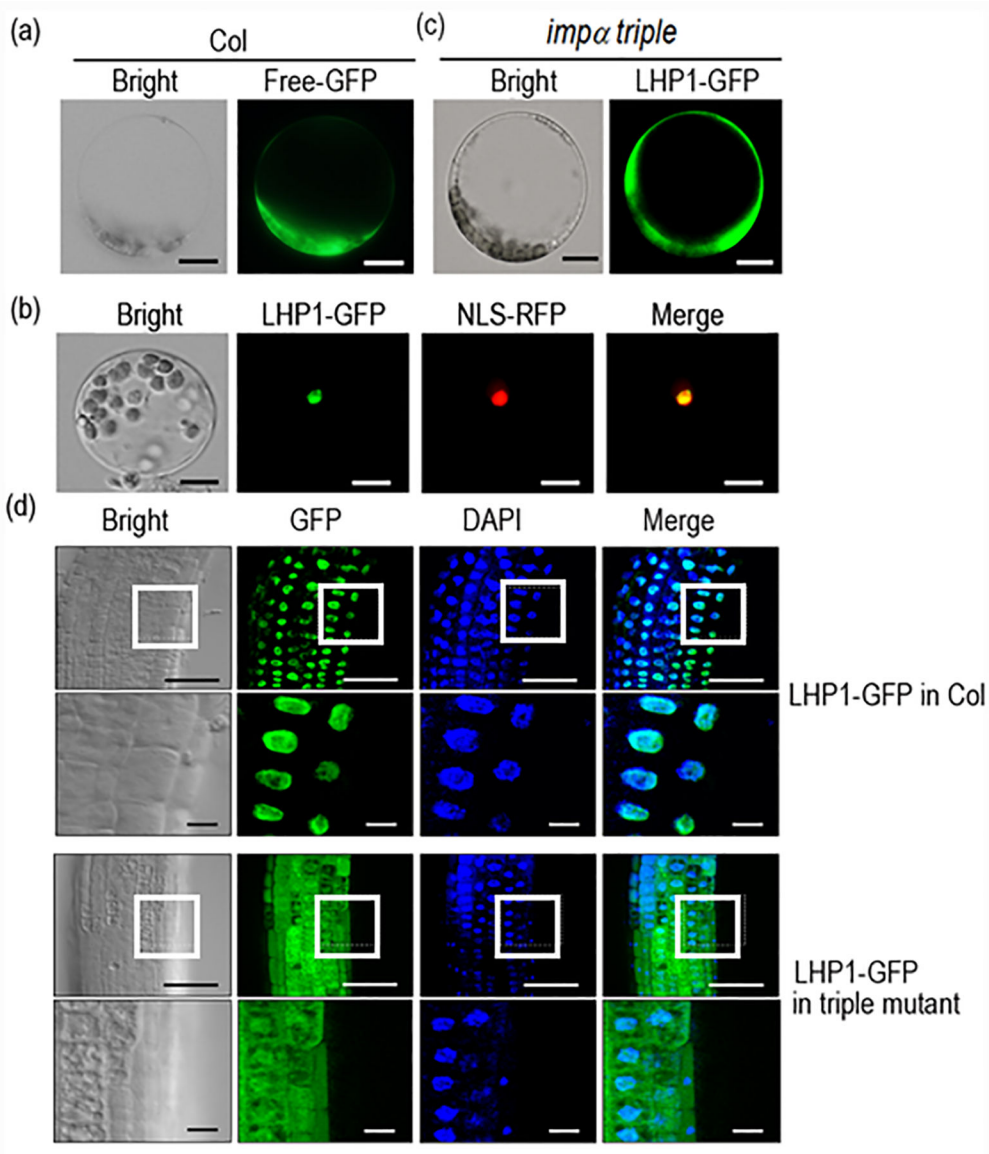

Figure 4.

LHP1-GFP is mis-localized in the *impα* triple mutant plants. (a-c) Subcellular localization of a) GFP in protoplasts prepared from Col-0 plants, b) LHP1-GFP in Col-0 plants, and c) LHP1-GFP in *impα* triple mutant plants. Transformed protoplasts were incubated for 36 h, and then observed by fluorescence microscopy. Yellow color indicates the overlap between GFP and red fluorescent signal. Scale bars = 10 μm. (d) Transgenic plants expressing LHP1-GFP were generated in Col-0 and the *impα* triple mutant background. Subcellular localization of LHP1-GFP was observed in primary roots by fluorescence microscopy. Each lower picture is enlarged by rectangles in each upper picture. Scale bars = 20 μm

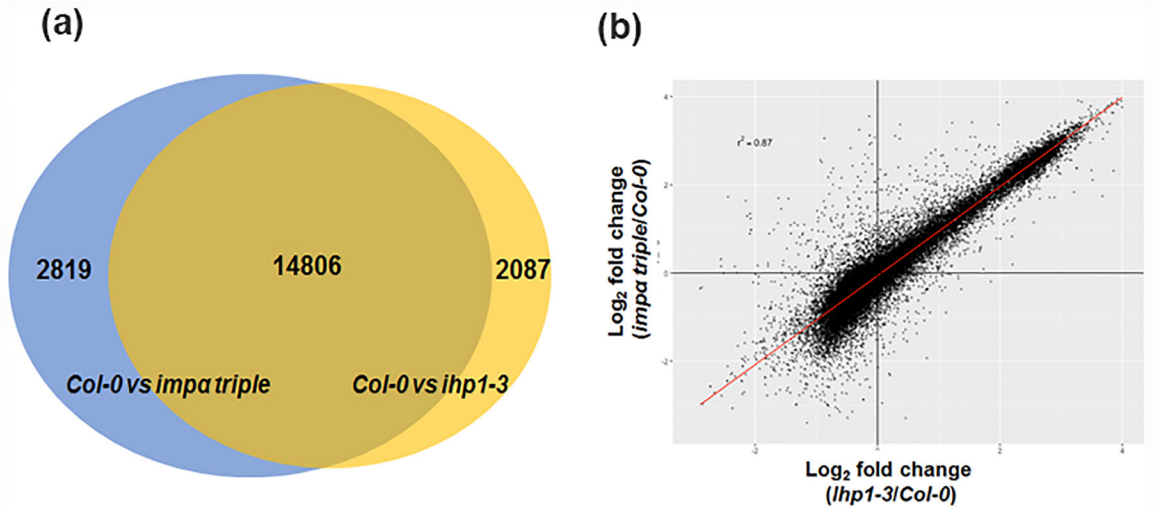

Figure 5.

Transcriptomic analysis between *lhp1–3* and *impα triple* mutant plants. (a) Differentially expressed genes obtained from RNA-seq (FDR <0.05) in the mutants (*lhp1–3* and *impα triple*) compared to wild type (Col-0) were used for the overlap analysis. Venn diagram shows that 87.64% genes differentially expressed in *lhp1–3* are also differentially expressed in *impα triple*. Statistical significance of the venn diagram overlap was determined hypergeometric test (p <0.001). (b) Scatter plot showing positive correlation between transcriptome of *lhp1–3* and *impα triple*. Log_2 fold change values of transcriptionally active 24,974 genes in *lhp1–3* and in the *impα triple* mutants (compared to the wild type) were plotted on x and y-axis respectively. Red line in the scatter plot represents the linear regression line with r^2 value of 0.87. Two biological replicates were used for the transcriptome analysis.

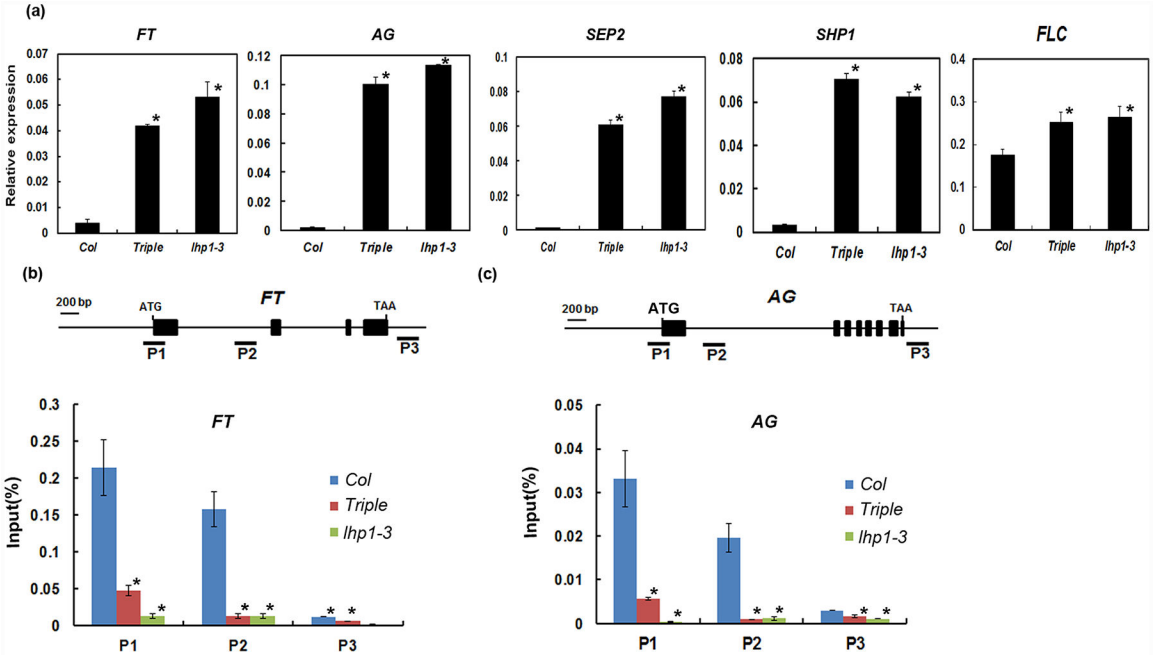

Figure 6.

Levels of LHP1 target genes are increased in the *impα* triple mutant plants. (a) Transcript levels of LHP1 target genes in Col-0, *impα* triple mutant, and *lhp1–3* mutant plants. Two-week-old seedling plants grown under LD (16 h light : 8 h dark) were harvested at ZT12, total RNAs were extracted, and indicated transcripts were quantified by qRT-PCR. Statistical significance was determined by Student's *t* test (*, $p<0.05$). Gene-specific primers are listed in Table S1. (b) (c) Chromatin immunoprecipitation (ChIP) analysis was carried out to investigate the enrichment of H3K27me3 over the *FT* and *AG* loci in Col-0, *impα* triple, and *lhp1–3* mutant plants. IP to INPUT ratios (log2) are plotted. Samples were harvested at ZT12. Gene-specific primers are listed in Table S1. Error bars indicate SD. Statistical significance was determined by Student's *t* test (*, $p<0.05$)

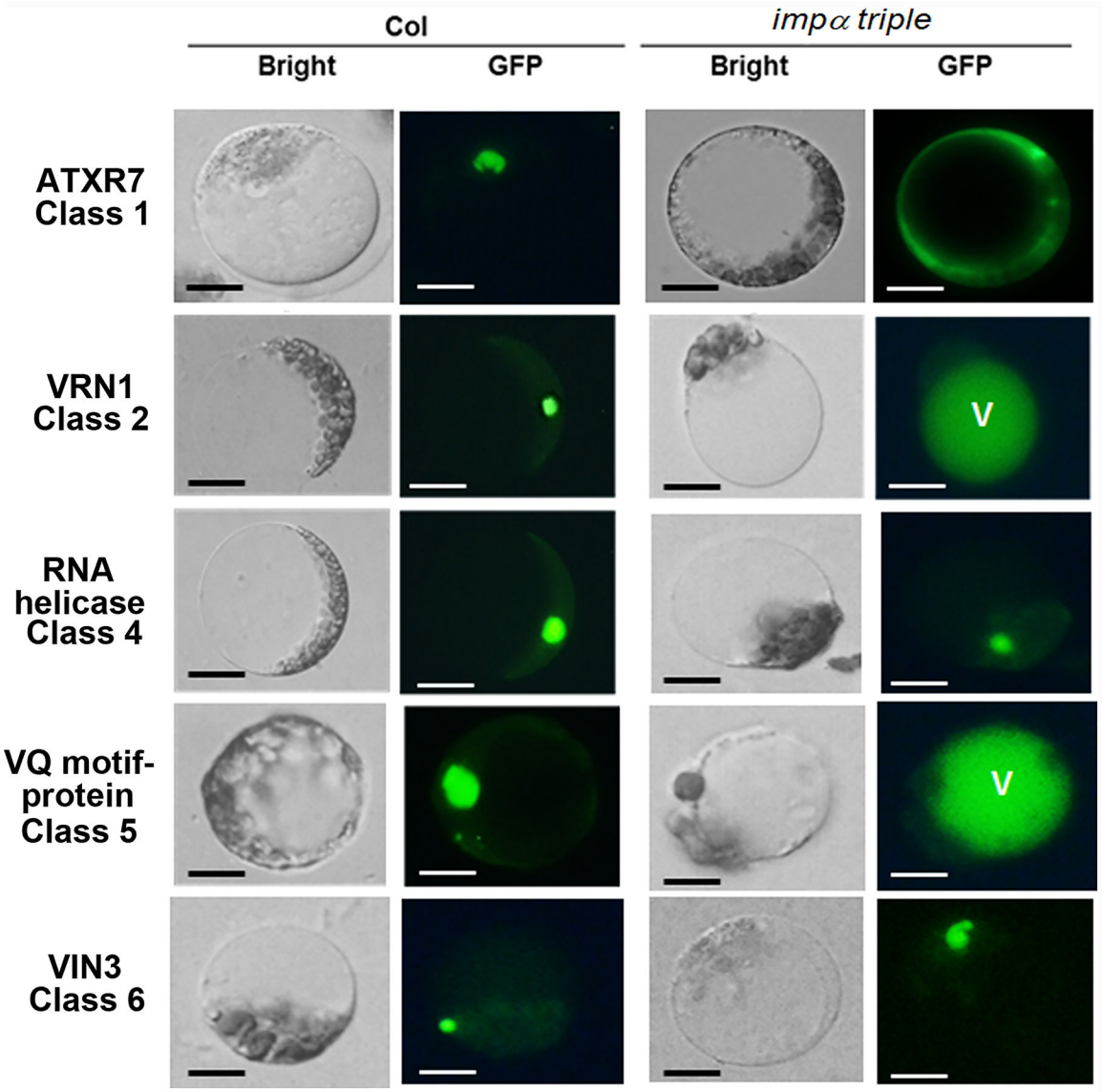

Figure 7.

IMPα−1, IMPα−2, and IMPα−3 recognize class 1, 2, and 5 NLSs. Subcellular localization of proteins of the indicated NLS-GFP class was observed by using transient expression assays. Proteins from each class linked to GFP were expressed in protoplasts prepared from Col-0 and *impα* triple mutant plants. Transformed protoplasts were incubated at 22 °C for 36 h and observed by fluorescence microscopy. V indicates vacuole. Scale bars = 10 μm.

Table 1.

Consensus sequence of six classes of IMPα-dependent NLSs. The sequences of IMPα-dependent NLSs were searched in the TAIR10 protein database and the candidates of IMPα-dependent NLSs were analysed using the PredictNLS program.

NLS class	Consensus sequence	Protein	Predicted NLS sequence
Class 1	KR(K/R)R **or** K(K/R)RK	LHP1(AT5G17690), ATXR7(AT5G42400)	R**KRKR**K, RKETLAL**KKRK**TVTRNKK
Class 2	(P/R)XXKR(^DE)(K/R)	VRN1 (AT3G18990)	PTPTPKI**PKKRGR**KKKNADPE
Class 3	KRX(W/F/Y)XXAF	Not found	
Class 4	(R/P)XXKR(K/R)(^DE)	RNA helicase, ATP-dependent, SK12/ DOB1 protein (AT2G06990)	PE**PRTKRRS**LKR
Class 5	LGKR(K/R)(W/F/Y)	VQ motif-containing protein (AT3G56880)	LG**LGKRK**RGPGVSGGKQTKRRSR
Class 6	KRX10–12K(KR)(KR) **or** KRX10–12K(KR)X(K/R)	VIN3 (AT5G57380)	**KRDIYKGKQGGNKRFK**SR