Probing formation of cargo/importin-α transport complexes in plant cells using a pathogen effector

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SUMMARY

Importin-αs are essential adapter proteins that recruit cytoplasmic proteins destined for active nuclear import to the nuclear transport machinery. Cargo proteins interact with the importin-α armadillo repeat domain via nuclear localization sequences (NLSs), short amino acids motifs enriched in Lys and Arg residues. Plant genomes typically encode several importin-α paralogs that can have both specific and partially redundant functions. Although some cargos are preferentially imported by a distinct importin-α it remains unknown how this specificity is generated and to what extent cargos compete for binding to nuclear transport receptors. Here we report that the effector protein HaRxL106 from the oomycete pathogen *Hyaloperonospora arabidopsidis* co-opt s the host cell’s nuclear import machinery. We use HaRxL106 as a probe to determine redundant and specific functions of importin-α paralogs from *Arabidopsis thaliana*. A crystal structure of the importin-α3/MOS6 armadillo repeat domain suggests that five of the six Arabidopsis importin-αs expressed in rosette leaves have an almost identical NLS-binding site. Comparison of the importin-α binding affinities of HaRxL106 and other cargos *in vitro* and in plant cells suggests that relatively small affinity differences *in vivo* affect the rate of transport complex formation. Our results suggest that cargo affinity for importin-α, sequence variation at the importin-α NLS-binding sites and tissue-specific expression levels of importin-αs determine formation of cargo/importin-α transport complexes in plant cells.

Keywords: importin-α, nucleo-cytoplasmic transport, nuclear localization sequence, oomycete effector, plant innate immunity, *Hyaloperonospora arabidopsidis, Arabidopsis thaliana*.

INTRODUCTION

In eukaryotic cells the nuclear envelope acts as a selective barrier separating nuclear from cytoplasmic processes. Coordination of nuclear and cytoplasmic events is mediated by nuclear pore complexes (NPCs) that span the nuclear envelope. Low-molecular-weight compounds such as solutes and proteins with a molecular weight of <40–60 kDa can traverse NPCs by passive diffusion (Stewart, 2007; Wang and Brattain, 2007). Proteins of higher molecular weight rely on nuclear transport receptors (NTRs) for passage through NPCs. Notably, many nuclear proteins of molecular weight below 40–60 kDa, such as several transcription factors, are also imported by NTRs, presumably ensuring more efficient nuclear import compared with passive diffusion (Ballesteros et al., 2001; Krebs et al., 2010). NTRs of the importin-α/β class are conserved from yeast to plant cells and transport many distinct cargo proteins into the nucleus. Importin-αs act as adapter proteins. The importin-α armadillo repeat domain binds to nuclear localization sequences (NLSs) of cargo proteins whilst an N-terminal α-helix makes direct contact to importin-β and is therefore called the importin-β-binding (IBB) domain (Cook et al., 2007). The IBB domain contains a sequence related to bipartite NLSs and, in the absence of importin-β, the IBB domain competes with NLS-cargos for binding to the armadillo repeat domain. On the cytoplasmic side of the NPC, binding of the IBB domain to importin-β negates...
this auto-inhibitory effect of the IBB domain and therefore facilitates cargo binding to importin-α (Kobe, 1999; Harrer-
eman et al., 2003). Active transport of the ternary importin-
α/cargo complex through the NPC is mediated by direct
interactions between importin-β and Phe/Gly-repeat nucle-
oporin proteins that line the inner side of the NPC (Terry
and Wente, 2009). On the nucleoplasmic side of the NPC the
ternary complex is destabilized by binding of the GTP-
bound form of the small GTPase Ran to importin-β, result-
ing in dissociation of the IBB domain from importin-β. This
re-establishes the auto-inhibitory effect of the IBB domain
on cargo binding and leads to release of cargo proteins on
the nucleoplasmic side of the NPC (Görlich et al., 1996;
Morianau et al., 1996; Harreman et al., 2003).

Nuclear import rates in yeast correlate with formation of
the importin-α/cargo ternary complex in the cytoplasm
(Hodel et al., 2006; Timney et al., 2006). Thus, nuclear
import kinetics are influenced by the cytoplasmic concen-
trations of both cargo proteins and NTRs, as well as the
affinity of a particular cargo NLS for the NTR. The best
characterized NLSs are Lys/Arg-rich sequence motifs that
fall into two subgroups, monopartite NLSs with the con-
sensus sequence ([K/R][K/R]X10–12[K/R]3–8a) (Chang et al., 2012;
Marfori et al., 2012). The importin-α armadillo repeats form
two NLS-binding sites on the concave side of the protein,
referred to as ‘major’ and ‘minor’ binding site. Whereas
monopartite NLSs make contact to both binding sites, mono-
partite NLSs bind to either the major or the minor site
(Marfori et al., 2011; Chang et al., 2013).

Adapted plant pathogens suppress host defences by translocating effector proteins into plant cells (Dou and
Zhou, 2012; Petre and Kamoun, 2014). Several effector
that manipulate nuclear processes have evolved NLSs and
coop-t the host’s importin-α/β system. In plant cells
infected with Agrobacterium tumefaciens the effector
VirD2 forms a covalently linked complex with the T-DNA in
the cytoplasm (Dürrenberger et al., 1989). A bipartite NLS
at the C-terminus of VirD2 interacts with several Arabidop-
sis importin-α and mediates transfer of the T-DNA complex
to the nucleus (Ballas and Citovsky, 1997; Bhattacharjee et al., 2008). Silencing of importin-α1 or -α2 in Nicotiana benthamiana attenuates nuclear import of sev-
eral effectors from the oomycete pathogen Phytophthora infestans and the Candidatus Phytoplasma asteris effector
SAP11 (Kanneganti et al., 2007; Bai et al., 2009). Importin-
α-mediated nuclear import is also essential for recognition of the Xanthomonas campestris transcription activator-like
(TAL) effector AvrBs3 by the pepper Bs3 gene (Van den Ack-
erveken et al., 1996; Szurek et al., 2001). AvrBs3 interacts
with plant importin-α via a C-terminal domain that is
conserved in other TAL effectors (Szurek et al., 2001;
Schornack et al., 2013).

A subcellular localization screen of effector candidates
from the Arabidopsis downy mildew pathogen Hyalopero-
nospora arabidopsisid (Hpa) revealed that 33% show
entirely nuclear localization (Caillaud et al., 2012). Despite
the prevalence of putative NLSs in effector sequences, a
directed Y2H screen of 83 effectors from Hpa and Pseudo-
monas syringae detected only two interactions between
plant importin-αs and effectors (Mukhtar et al., 2011). Hpa
effector HarXL1445 interacts with importin-α/MODIFIER
OF SNC1 6 (MOS6) whereas effector HarXL106 interacts
with MOS6, importin-α1, -α2 and -α4. However, results
from directed protein–protein interaction assays might not predict with certainty the formation of specific cargo/import-
lin-α complexes in plant cells.

Here we report that Hpa effector HarXL106 binds to the
MOS6 armadillo repeat domain via a bipartite NLS with
low micro-molar affinity, which is in the range of binding
affinities that has been determined for other cargo/importin-
α interactions (Marfori et al., 2012). We find that small
differences in NLS/importin-α binding affinities in vitro
result in significant changes in cargo/importin-α complex
formation in plant cells suggesting that there is significant
competition between cargo proteins for binding to importin-
αs. A crystal structure of the MOS6 armadillo repeat
domain suggests strong conservation of the NLS-binding
sites between MOS6 and four other Arabidopsis importin-
αs. HarXL106 binds equally well to these importin-α pro-
teins when they are expressed to comparable levels in
N. benthamiana. In Arabidopsis leaves, HarXL106 prefer-
entially forms protein complexes with the most highly
expressed importin-α1, -α2 and -α4. This suggests that
besides sequence variation in NLS-binding sites, importin-
α protein levels can determine which cargo/importin-α com-
plexes form in plant cells.

RESULTS

HarXL106 co-opts the host cell’s nuclear import system

An RFP-tagged version of HarXL106, lacking its predicted
secretion leader peptide (HarXL106 amino acids 25–285,
referred to as RFP-HarXL106 from here on), showed
entirely nuclear localization when transiently expressed in
N. benthamiana and when constitutively expressed in Ara-
bidopsis (Figures 1a and S1). NLS prediction algorithms
identified a putative bipartite NLS at amino acids 239–246
(RGKKQGTEAPDLPEGLTPKKRLKR) of HarXL106 (Kosugi
et al., 2009; Nguyen Ba et al., 2009). By testing a series of
N-terminal deletion constructs of HarXL106 for interaction
with MOS6 in a co-immunoprecipitation (co-IP) assay, we
confirmed that HarXL106 amino acids 228–285 (the C-
terminal 58 amino acids that encompass the predicted NLS)
were sufficient for binding to MOS6 (Figure S2). A con-
struct with a further N-terminal deletion, HarXL106 amino
acids 244–285, did not accumulate to detectable levels.
preventing us from testing its interaction with MOS6 by co-IP. This construct therefore served as a negative control to exclude non-specific binding of MOS6–GFP to the α-HA affinity resin (Figure S2). Fusion of the 58 C-terminal amino acids of HaRxL106 to RFP (‘RFP–Cterm58’ in Figure 1a) shifted the subcellular localization of RFP from nucleo-cytoplasmic to entirely nuclear, demonstrating that this region of HaRxL106 carries a functional NLS. In contrast, deletion of these 58 amino acids (RFP–HaRxL106ΔC) resulted in a nucleo-cytoplasmic distribution that was indistinguishable from RFP alone (Figure 1a). Fusion of a heterologous NLS (PKKKRKV) from the SV40 T-antigen to either the N- or C-terminus of the HaRxL106ΔC sequence restored entirely nuclear localization (Figure 1a). Despite deletion of the NLS-containing C-terminus, the RFP–HaRxL106ΔC construct still showed residual nuclear localization. This could either be due to a second NLS in the HaRxL106ΔC sequence, or due to elevated passive diffusion of the RFP–HaRxL106ΔC construct (predicted molecular weight 51.3 versus 57.8 kDa for RFP–HaRxL106). To test for presence of an additional NLS we replaced the two clusters of basic amino acids in the predicted bipartite NLS of HaRxL106 by the amino acid sequence NAAIRS, which is unlikely to interfere with protein secondary structure (Wilson et al., 1985; Marsilio et al., 1991). This RFP–HaRxL106 NAAIRS1+2 fusion protein was more efficiently excluded from nuclei than the RFP–HaRxL106ΔC fusion (Figure 1a), suggesting that the residual nuclear localization of the latter construct is due to passive diffusion into nuclei. We confirmed by an α-RFP western blot (Figure 1b) that all

![Figure 1. The C-terminal 58 amino acids of HaRxL106 are sufficient and required for active nuclear import. (a) Confocal images of RFP and the indicated RFP–HaRxL106 fusion constructs in epidermal cells of N. benthamiana. The images were taken 48 h after infiltration with A. tumefaciens. Upper panels show RFP channel, lower panels show RFP channel overlaid on bright field images. Scale bars 50 μm. (b) Western blot of soluble proteins extracts for the RFP fusions used in (a). Samples were harvested 48 h post infiltration with A. tumefaciens and probed with α-RFP antibody. NS = non-specific signal of the α-RFP antibody. Coomassie stain shows RubisCO band as loading control.](image-url)
constructs were expressed and that RFP-HaRxL106 fusions were stable in N. benthamiana. Taken together, these data demonstrate that the C-terminal 58 amino acids of HaRxL106 mediate interaction with host importin-\(\alpha\)s and that the bipartite NLS is required and sufficient for active nuclear import of the effector.

**HaRxL106 binds to MOS6 directly and with low micro-molar affinity**

To test for direct interaction between HaRxL106 and importin-\(\alpha\)3/MOS6 in vitro, we generated E. coli expression constructs for the HaRxL106 effector domain (HaRxL106 amino acids 46–285, excluding the N-terminal signal peptide and the RxLR motif), an HaRxL106\(\Delta_C\) version of the same domain (amino acids 46–227) and a truncated version of MOS6 lacking its N-terminal IBB domain. We purified all proteins from the soluble fraction of E. coli crude extracts via an N-terminal His6 tag and tested for direct protein–protein interactions by separating protein mixtures on an analytical size exclusion chromatography column (Figure 2a,b). When His6-\(\Delta\)IBBMOS6 was mixed with His6-HaRxL106\(\Delta_C\), both proteins eluted in separate peaks (Figure 2a,b). Instead, when we separated mixtures of His6-\(\Delta\)IBBMOS6 and His6-HaRxL106, both proteins co-eluted from the column in a complex with a higher molecular weight than the importin-\(\alpha\) alone (Figure 2a,b). Therefore, the effector domain of HaRxL106 directly binds to the armadillo repeat domain of MOS6 and this interaction requires the HaRxL106 C-terminus encompassing the NLS.

Loss of importin-\(\alpha\)3/MOS6 attenuates constitutive immune signalling in the snc1 mutant background and mos6 mutants are more susceptible to compatible Hpa races and weakly virulent strains of P. syringae (Palma et al., 2005 and Figure S3). Formally, MOS6 and other importin-\(\alpha\)s could therefore also be virulence targets of HaRxL106. However,
our finding that HaRxL106 binds to the MOS6 armadillo repeat domain via a typical NLS supports the idea that HaRxL106 binds to importin-α to co-opt the host cell’s nuclear import system. Artificial NLSs with extremely high affinity for importin-α can interfere with cargo release in the nucleus and affect nuclear import (Kosugi et al., 2008; Marfori et al., 2012). We therefore determined the dissociation constant between ΔIBBMOS6 and the HaRxL106 effector domain by isothermal titration calorimetry (ITC). In vitro the two proteins interacted in a 1:1 molar ratio and we determined a $K_d$ for the ΔIBBMOS6/HaRxL106 complex in the low micro-molar range (0.54–0.85 μM, Figure 2c; for $D_H$ and $D_S$ values see Table S1). To relate this finding to other cargo importin-α interactions, we also determined the dissociation constants of ΔIBBMOS6 complexes with the HaRxL106ΔC–SV40NLS fusion as well as with the Phytoplasma effector SAP11 (Bai et al., 2009; Sugio et al., 2011). We found that both of these interactions had $K_d$ values that were only moderately higher than those for the ΔIBBMOS6/HaRxL106 complex (2.22–3.70 μM for HaRxL106ΔC–SV40NLS and 4.42–6.80 μM for SAP11, respectively; Figure 2c). Therefore, the HaRxL106 effector domain does not bind to MOS6 with unusually high affinity suggesting that the interaction is a canonical cargo/importin-α interaction.

A crystal structure of the MOS6 armadillo repeat domain suggests almost identical NLS-binding sites in five Arabidopsis importin-αs

We attempted to crystallize ΔIBBMOS6 in complex with either HaRxL106 or an HaRxL106 peptide containing the NLS, but we did not obtain protein crystals of sufficient quality for structure determination. The ΔIBBMOS6 protein on its own formed diffracting protein crystals and enabled us to determine the crystal structure of the ΔIBBMOS6 protein at 2.9 Å resolution (Figure 3a and Table S2; Data S4; PDB identifier 4TNM). Like other importin-α proteins from yeast, mammals and rice, ΔIBBMOS6 forms 10 armadillo repeats with strong conservation of residues that contribute to the major and minor NLS-binding sites (Marfori et al., 2011). We superposed the ΔIBBMOS6 structure onto the structure of rice importin-α1a in complex with a SV40NLS (Chang et al., 2012). This revealed that essentially all amino acids of rice importin-α1a, that make direct contact to the SV40NLS at the major and minor NLS-binding sites, are conserved in MOS6 (Figure 3b,c). The Arabidopsis genome encodes nine importin-αs (Merkle, 2011; Wirthmueller et al., 2013). Despite a high level of sequence conservation in the H3 helices that form the NLS-binding

Figure 3. The armadillo repeat domain of MOS6 has the canonical importin-α fold.
(a) Crystal structure of the ΔIBBMOS6 protein in cartoon representation and superposition of the armadillo repeat domains of MOS6 (green) and rice importin-α1a (light blue, PDB 4B8O) (Chang et al., 2012).
(b) Superposition of ΔIBBMOS6 (green) and the ΔIBB variant of rice importin-α1a (light blue, PDB 4B8O) in complex with an SV40NLS (orange) bound at the major NLS-binding site. Residues of rice importin-α1a that contribute to the NLS-binding site and the corresponding MOS6 amino acids are shown in stick representation.
(c) Superposition of ΔIBBMOS6 (green) and the ΔIBB variant of rice importin-α1a (light blue, PDB 2YN5) in complex with the B54NLS (orange) bound at the minor NLS-binding site. Residues of rice importin-α1a that contribute to the NLS-binding site and the corresponding MOS6 amino acids are shown in stick representation. Residue labels in (b) and (c) correspond to the MOS6 sequence.
sites, knock-out of a single importin-α gene can lead to mutant phenotypes (Palma et al., 2005; Bhattacharjee et al., 2008). One possible determinant of specificity is variation in the importin-α NLS-binding sites that would lead to specific interaction with distinct NLSs. We determined the conservation of the NLS-binding sites of importin-α expressed in rosette leaves by homology modelling based on the 4iDBBMO6 structure. In RNA-sequencing experiments (Asai et al., 2014) we reliably detected sequencing reads of six importin-α genes in rosette leaf tissue (importin-α1, α2, α3/MOS6, α4, α6 and α9). Out of these, importin-α1, α2 and α4 had the highest expression levels, followed by importin-α9, α6 and α3/MOS6 (Figure 4a). We found that residues contributing to the MOS6 NLS-binding site are strongly conserved in importin-α1, α2, α4 and α6 (Figure 4b) whilst these residues are less conserved in importin-α9 (Figure 4c). Consistent with a conserved NLS-binding site, StrepII-3xHA (HS)-tagged HaRxL106 bound equally well to GFP-tagged importin-α1, α2, α4 and MOS6 in co-IPs (Figure 4d). In contrast, HaRxL106 did not co-IP with importin-α9 (Figure 4d). We further tested which importin-α co-purify with HaRxL106 in Arabidopsis. We IP-ed an YFP-HaRxL106 fusion protein from a stable transgenic line (see Data S4) and identified co-purifying importin-α proteins expressed in Arabidopsis rosette leaves. 

Figure 4. Conservation of the NLS-binding sites of importin-α proteins expressed in Arabidopsis rosette leaves. 
(a) Sequencing reads of the nine Arabidopsis importin-αs detected by RNA-Seq in Col-0 rosette leaves (Asai et al., 2014). Error bars show standard deviation (SD) of three biological replicates. 
(b) Conservation of residues contributing to the MOS6 NLS-binding sites in Arabidopsis importin-α1, α2, α4 and α6. The figure shows the MOS6 armadillo repeat domain and amino acids contributing to the inner concave site of the protein are shown in surface representation. Residues coloured in yellow are conserved in importin-α1, α2, α4 and α6. Orange colour indicates amino acids that diverge from MOS6 in at least one of the other importin-αs. For a sequence alignment of all Arabidopsis importin-α protein sequences, see Wirthmueller et al. (2013). 
(c) Conservation of residues contributing to the MOS6 NLS-binding sites in Arabidopsis importin-α9. Representation as in (b). 
(d) GFP fusion proteins of importin-α1, α2, α4, α6, MOS6 and free GFP were transiently co-expressed with StrepII-3xHA (HS)-tagged HaRxL106 in N. benthamiana. At 48 h post infiltration GFP-tagged importin-αs were IP-ed and co-purifying HS-HaRxL106 was detected by an α-HA western blot. Coomassie stains show Rubisco band in total protein extracts and IP-ed importin-αs in the IP blot. Similar results were obtained in two independent experiments. 

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proteins by liquid chromatography coupled with mass spectrometry (LC-MS/MS). In three independent replicates we consistently detected unique peptides from importin-α1, -α2 and -α4 in IPs of YFP-HaRxL106, whilst we found only a single importin-α peptide in one out of three control IPs from wild-type plants or a line expressing GFP (Table 1 and Data S1 and S2). Thus, in Arabidopsis rosette leaves, HaRxL106 appears to bind preferentially to the three importin-αs with the highest mRNA expression levels.

Small differences in NLS-cargo/importin-α affinities in vitro significantly affect formation of transport complexes in plant cells

A previous study reported that although a double Lys to Ala mutation in the NLS of the yeast ribosomal protein Rpl25p resulted only in an approximately threefold reduced binding affinity to its cognate import receptor Kap123p/importin-64, this mutation significantly reduced nuclear import rates in yeast (Timney et al., 2006). The authors explained this discrepancy by non-specific competition for importin-β binding by other cytoplasmic proteins as it could be mimicked by an E. coli protein extract (Timney et al., 2006). As the HaRxL106/ΔIBBMOS6 complex has an approximately 4-8-fold lower $K_\text{d}$ when compared with ΔIBBMOS6 complexes with HaRxL106∆C-SV40NLS or SAP11, we tested if this difference in $K_\text{d}$ affects formation of MOS6/cargo complexes in N. benthamiana cells. To this end, we generated a MOS6-YFPC bimolecular fluorescence complementation (BiFC) expression construct and co-expressed this fusion protein with YFPΔ3-tagged cargo proteins in epidermal cells of N. benthamiana. Apart from an YFP signal in the nucleoplasm, which we observed for all BiFC pairs tested and therefore might result from spontaneous association of the YFP N- and C-terminal halves, we found that co-expression of YFPΔ3-HaRxL106 with MOS6-YFPC resulted in speckles at the nuclear rim (Figure 5a). Speckle formation was dependent on the HaRxL106 C-terminus as we did not observe them with the YFPΔ3-HaRxL106∆C construct. Although the SV40NLS is sufficient to restore entirely nuclear localization of HaRxL106∆C (Figure 1a), fusion of the SV40NLS to either the HaRxL106∆C N- or C-terminus did not result in speckles at the nuclear periphery in BiFC (Figure 5a). Similarly, we did not observe speckles in BiFC experiments between YFPΔ1-SAP11 and MOS6-YFPC. Although the molecular basis of speckle formation in this over-expression system remains unknown, we suggest that they may represent MOS6/HaRxL106 complexes that cannot be disassembled as efficiently as other importin-α/cargo complexes on the nucleoplasmic side of the NPC.

To exclude the possibility that the YFP speckles of the YFPΔ3-HaRxL106/MOS6-YFPC interaction are simply due to differences in protein levels compared with other YFPN- tagged cargos, we performed co-IPs between transiently expressed MOS6-GFP and HS-tagged cargo proteins in N. benthamiana cell extracts. IP of MOS6-GFP co-purified HS-HaRxL106, but not the corresponding HaRxL106∆C construct (Figure 5b). Although the SV40NLS was sufficient to restore nuclear import of the RFP-HaRxL106∆C protein (Figure 1a), we detected no or only very weak interactions between MOS6-GFP and HaRxL106∆C constructs that carry the SV40NLS either at the N- or C-terminus (Figure 5b). HS–SAP11 accumulated to lower levels than all other cargo proteins in the total extract and we did not detect SAP11 binding to MOS6 in co-IPs (Figure 5b). The BiFC and co-IP data demonstrate that in plant cells the NLS of HaRxL106 forms more stable complexes with MOS6 than those mediated by the SAP11 or SV40NLS. We next addressed if this property is unique to HaRxL106. A BLAST search with the NLS of HaRxL106 against the TAIR Arabidopsis protein database (v. 10) identified a Lys-rich sequence from the transcription factor bZIP5 (AT3G49760) as close match (Figure 5b). In BiFC experiments the YFPΔ3–bZIP5/MOS6–YFPC combination formed speckles at the nuclear periphery although they were less intense compared to those observed with YFPΔ3–HaRxL106 (Figure 5a). Taken together, the BiFC and co-IP results show that, despite the similar $K_\text{d}$ values we determined for select NLS-cargo/MOS6 complexes in vitro (Figure 2c), there are strong differences in transport complex formation in plant cells (Figure 5).

Table 1 Number of unique importin-α tryptic peptides identified by LC-MS/MS following immunoprecipitation of YFP–HaRxL106 from Arabidopsis

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<th>Experiment 1</th>
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<tr>
<td></td>
<td>GFP</td>
<td>YFP–HaRxL106</td>
<td>GFP</td>
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<tr>
<td>Importin-α1</td>
<td>4</td>
<td>–</td>
<td>–</td>
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<td>Importin-α2</td>
<td>24</td>
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<td>Importin-α3</td>
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NLS-cargos compete with other proteins for binding to importin-α in plant cells

Our finding that small differences in $K_d$ values determined in vitro translate into substantial differences in NLS/importin-α complex formation in plant cells could be due to competition by other cytoplasmic proteins for importin-α binding. As we used the non-auto-inhibited ΔIBB variant of MOS6 to determine $K_d$ values in vitro (Figure 2c), competition for MOS6 binding in plant cells could be either due to the auto-inhibiting function of the IBB domain or due to the presence of other competing proteins in the cytoplasm. As importin-αs are over-expressed in the transient expression system, negation of IBB auto-inhibition by endogenous importin-αs is likely to be negligible (Cardarelli et al., 2009). To distinguish between competition by the IBB domain and other cytoplasmic proteins we used the N. benthamiana transient expression system to test if HaRxL106, HaRxL106ΔC-SV40NLS and SAP11 differ in their abilities to form complexes with importin-α2 and ΔIBB importin-α2 (importin-α2 and MOS6 show comparable binding to HaRxL106, Figure 4d). The ΔIBB variant of importin-α2-YFP protein co-purified slightly more HS-HaRxL106 than the full-length importin-α2. This could either be due to the lack of auto-inhibition by the IBB domain or due to the higher protein levels of the ΔIBB importin-α2-YFP construct when compared with importin-α2-YFP (see CBB stain in Figure 6). However, ΔIBB importin-α2-YFP still co-purified HS-HaRxL106 more efficiently than HaRxL106ΔC-SV40NLS or SAP11 (Figure 6). Therefore, the differential complex formation in plant cells is not only a result of enhanced auto-inhibition by the IBB domain of over-expressed importin-αs but is due to additional competing factors in plant cell extracts. These findings suggest that endogenous NLS-cargos in plant cells compete with other proteins for binding to importin-α receptors and that NLS-cargo concentration and affinity for importin-αs determine formation of ternary transport complexes in the cytosol.

Figure 5. The HaRxL106 NLS mediates stronger complex formation with importin-αs than the SV40NLS in plant cells. (a) BiFC between MOS6-YFPC and the indicated YFPN-tagged NLS-cargo proteins in nuclei of N. benthamiana 48 h post infiltration. Images are representative of at least 10 nuclei analysed. Scale bars 5 μm. (b) MOS6-GFP was transiently co-expressed with the indicated StrepII-3xHA (HS)-tagged NLS-cargo proteins in N. benthamiana. At 48 h post infiltration MOS6-GFP was IP-ed and co-purifying HS-tagged proteins were detected by an α-HA western blot. Coomassie stains show Rubisco band in total protein extracts and IP-ed importin-αs in the IP blot. Similar results were obtained in two independent experiments.
DISCUSSION

Co-option of the importin-α/β nuclear transport pathway by HaRxL106 and other pathogen effectors

The contribution of importin-α3/MOS6 to plant immunity makes it a putative virulence target of pathogen effectors. Here we provide several lines of evidence suggesting that HaRxL106 binds to MOS6 and other importin-αs as a cargo protein but does not interfere with their function as NTRs: (i) HaRxL106 binds MOS6 exclusively via a peptide that fits to the consensus sequence of bipartite NLSs (Figure 1; Marfori et al., 2012) (ii) In vitro, the $K_d$ of the HaRxL106/MOS6 complex is only slightly lower than that mediated by the canonical SV40NLS (Figure 2c). In contrast synthetic NLSs, that interfere with nuclear transport, bind importin-αs with an affinity that is approximately one order of magnitude higher than that of the SV40NLS (Kosugi et al., 2008). (iii) In vivo the Arabidopsis transcription factor bZIP5 and HaRxL106 bind to MOS6 with similar efficiency (Figure 5). (iv) We have not observed that over-expression of HaRxL106 in N. benthamiana or A. thaliana leads to cell death, as one might expect if HaRxL106 were a strong inhibitor of nucleo-cytoplasmic transport.

Based on the NLS peptide-mediated mode of binding to importin-αs and the $K_d$ of the HaRxL106/MOS6 interaction we conclude that HaRxL106 is a cargo protein of Arabidopsis importin-αs. Notably, the molecular weight of several effectors that exploit the plant’s nuclear transport system is below the molecular weight exclusion limit of NPCs (Wang and Brattain, 2007). Like HaRxL106 (27 kDa), P. infestans NUK7 (47 kDa) and SAP11 (11 kDa) co-opt the importin-α/β pathway for efficient nuclear import (Howard et al., 1992; Shurvinton et al., 1992; Kanneganti et al., 2007; Bai et al., 2009). Therefore, even without an NLS, these effector proteins would be expected to enter the host cell nucleus by passive diffusion. Considering that effector protein levels might be relatively low in an infected cell, evolution of NLS sequences in these proteins may represent a mechanism for enhanced transport to ensure efficient delivery to the nucleus when compared with passive diffusion.

Functional affinity limits of NLS/importin-α interactions

Dissociation constants for several NLS/importin-α complexes from yeast, mammals and plants have been determined (Hübner et al., 1999; Hodel et al., 2001, 2006; Timney et al., 2006; Kosugi et al., 2008; Chang et al., 2012). Based on these results it has been suggested that $K_d$ values for canonical NLS-binding to importin-α are in the range of approximately 10 nM to 1 μM (Marfori et al., 2012). The $K_d$ values we determined for HaRxL106, HaRxL106ΔC–SV40NLS and SAP11 binding to the non-auto-inhibited MOS6 protein are at or beyond the upper limit of this interval and we would expect even higher $K_d$ values for complexes formed between full-length MOS6 and these cargo proteins. One explanation for this discrepancy may be the experimental method used to determine $K_d$ values. The
10 nm to 1 μm interval is mainly based on assays that require binding of one protein to a surface, such as plate binding assays (Hübner et al., 1999; Timney et al., 2006; Chang et al., 2012) or surface plasmon resonance (Kosugi et al., 2008). In contrast, we determined the $K_d$ values reported here by ITC. Two other reports have used ITC to determine dissociation constants for NLS/importin-α complexes. Ge et al. (2011) measured a $K_d$ of 3.03 ± 0.95 μM for binding of the NLS peptide from the rat transcription factor ChREBP to importin-α. Lott et al. (2011) obtained a $K_d$ of 48.7 ± 6.5 μM for binding of the NLS peptide from human phospholipid scramblase 4 to the non-auto-inhibited form of mouse importin-α. Thus, it appears that $K_d$ values in the low micro-molar range are probably due to different methods applied.

**Cargo proteins compete for binding to importin-α receptors in plant cells**

Although the NLSs from HaRxL106, SAP11, and the SV40NLS bind to the non-auto-inhibited form of MOS6 with comparable affinities *in vitro*, we observed substantial differences in cargo/importin-α complex formation in plant cells (BiFC, Figure 5a) and plant cell extracts (co-IP, Figure 5b). A 4-8-fold difference in $K_d$ values is unlikely to cause significant differences in complex formation unless there is competition for binding to the receptor. As the ΔIBB variant of importin-α2 co-purifies HaRxL106 much more efficiently than HaRxL106ΔC–SV40NLS and SAP11, this competitive effect is not mediated by the IBB domain (Figure 6). Our results are consistent with nuclear import experiments in yeast and in mammalian cells demonstrating that a 2-7-fold difference in $K_d$ values alters nuclear import kinetics (Efthymiadis et al., 1997; Xiao et al., 1998; Hodel et al., 2006; Timney et al., 2006). Timney et al. (2006) proposed that other cytoplasmic proteins non-specifically compete with binding of ribosomal cargo proteins to importin-β1 NTRs, thus explaining the discrepancy between *in vitro* and *in vivo* experiments. The same macromolecular crowding effect could also explain the difference between cargo/importin-α complex formation *in vitro* and in plant cells. However, we would expect that over-expression of cargos and importin-αs combined with several-fold dilution of other potentially competing proteins in a plant cell extract (typical protein concentration 6.5 mg ml$^{-1}$ versus estimated protein concentration in the cytosol 100–200 mg ml$^{-1}$ (Ellis, 2001; Zeskind et al., 2007)] diminishes macromolecular crowding. It is therefore surprising that we still observed differences in cargo/importin-α complex formation in co-IPs. It is conceivable that in addition to non-specific competition by bulk cellular proteins other NLS-cargos compete with binding to importin-αs and that competition is stronger in the approximately 4-8-fold higher $K_d$ range of the SV40 and SAP11 NLSs when compared to the NLS of HaRxL106.

**Conservation of the NLS-binding site in plant importin-αs**

The nine Arabidopsis importin-α proteins show approximately 26% overall sequence identity. However, when only the H3 helices of ARM repeats 1–8 that contribute the NLS-binding sites are considered, the sequence identity is approximately 45% (Wirthmueller et al., 2013). This conservation of the H3 helices allowed us to build homology models for the armadillo repeat domains of other Arabidopsis importin-αs based on the ΔIBBMOS6 structure. Superposition of individual models with the ΔIBBMOS6 structure revealed an almost complete conservation of the major and minor NLS-binding sites in five out of six importin-αs expressed in rosette leaves (importin-α1, -α2, -α3, -α4 and -α6) (Figure 4b,c). Our observation that HaRxL106 binds equally well to importin-α1, -α2, -α4 and MOS6 in plant cell extracts (Figure 4d) is in agreement with a conserved NLS-binding site on these importin-αs. Given this redundancy, it is interesting that genetic knock-out of a single importin-α gene can lead to mutant phenotypes (Palma et al., 2005; Bhattacharjee et al., 2008). We found that YFP–HaRxL106, IP-ed from transgenic Arabidopsis lines, predominantly interacts with importin-α1, -α2 and -α4, which have the highest expression levels in rosette leaves (Table 1 and Figure 4a). Tissue-specific differences in importin-α expression levels might therefore determine each importin-αs contribution to nuclear transport in the particular cell type. Bhattacharjee et al. (2008) reported that knock-out of importin-α4, but not -α1, -α2 or MOS6, leads to lower *A. tumefaciens* transformation rates in Arabidopsis root tissue. Based on available mRNA expression data (Hruz et al., 2008; Wirthmueller et al., 2013), importin-α4 has the highest expression level in root cells. Bhattacharjee et al. (2008) also found that several importin-α paralogs can complement the reduced transformation rates of the importin-α4 mutant when expressed under control of the tissue non-specific 35S promoter, supporting the hypothesis that tissue-specific expression levels of single importin-α genes might determine their contribution to nuclear transport. The NLS of yeast ribosomal protein Rpl25 has comparable affinities for the importin-βs Kap123p and Kap121p. However, due to higher cellular levels of Kap123p, this importin-β acts as the primary transport receptor in yeast (Timney et al., 2006). Our results suggest that: (i) protein levels of plant importin-αs; and (ii) the affinity of an NLS for a particular importin-α are two major factors that determine which NLS-cargo/importin-α complexes form in the plant cell cytoplasm. However, other possible sources of specificity such as different preferences for association of importin-αs with importin-βs or post-translational modification of importin-α/β and NLS flanking sequences have not thoroughly been addressed in
plants and might add a further layer of regulation to nuclear import.

**EXPERIMENTAL PROCEDURES**

**Plants and growth conditions**

Growth conditions for *N. benthamiana* and Arabidopsis have been described (Fabro et al., 2011; Segonzac et al., 2011). The *mos6*-1 and *mos6*-2 mutants have been described (Palma et al., 2005). The *mos6*-4 T-DNA insertion line (SALK 025919) was obtained from NASC. Transgenic Arabidopsis plants expressing YFP- and RFP-HaRxL106 were generated by transforming eco-type Col-0 with *A. tumefaciens* strain GV3101 pMP90<sup>HC</sup> carrying pENS-YFP-HaRxL106 and *A. tumefaciens* strain GV3101 pMP90 carrying pH7WGR2-HaRxL106, respectively (Logemann et al., 2006).

**Pathogen assays**

For bacterial growth assays 4-week-old plants were vacuum-infiltrated with bacterial suspensions of $1 \times 10^{6}$ cfu ml<sup>-1</sup> in 5 mM MgCl<sub>2</sub> and 0.0015% Silwett L-77 of *P. syringae* DC3000. ΔAvrPto/AvrPtoB (Lin and Martin, 2005) or ΔCEI (Alfano et al., 2000) and bacterial titers were determined at the day of infiltration and 3 days post inoculation by plating dilution series of extracts from infected leaves on selective media.

**Transient expression**

*A. tumefaciens* GV3101 and GV3103 bacteria were grown on selective media. Transgenic Arabidopsis plants expressing YFP- and RFP-HaRxL106 were generated by transforming eco-type Col-0 with *A. tumefaciens* strain GV3101 expressing the silencing suppressor 19 K at a ratio of 1:3<sup>[19K]</sup>. For co-expression the strains were mixed in a 1:1:3 [19K] ratio. Leaves of 3-week-old *N. benthamiana* plants were infiltrated with a syringe and leaves were harvested or imaged 48–72 h later.

**Protein extraction from *N. benthamiana*, co-IP and western blot**

Protein extracts were prepared by grinding *N. benthamiana* or Arabidopsis leaf material in liquid nitrogen to a fine powder followed by resuspension in extraction buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 5 mM DTT, 1 μM protease inhibitor cocktail (Sigma, http://www.sigmaaldrich.com), pH 7.5) at a ratio of 2 ml buffer per 1 g leaf material. The extracts were centrifuged at 17,000 g °C 20 min and the supernatant was either boiled in sodium dodecyl sulphate (SDS) sample buffer for western blots or used for co-IPs. For western blots protein samples were separated by SDS-PAGE and electro-blotted onto polyvinylidene difluoride membrane. Antibodies used were α-HA 3F10 (Roche, http://www.roche.com), α-GFP 210-PS-1GP (Amsbio, http://www.amsbio.com), α-RFP-biotin ab34771 (Abcam, http://www.abcam.com). For co-IPs a fraction of the supernatant was saved as ‘input’ sample and 20 μl GFP-beads (GFP-Trap-A; Chromotek, http://www.chromotek.com) or HA-beads (Sigma) were added to 1.4 ml of the remaining supernatant. The samples were incubated on a rotating wheel at 4°C for 2 h followed by collecting the beads by centrifugation at 1200 g and 4°C for 1.5 min. The beads were washed 3–4 times with 1 ml extraction buffer and then boiled in SDS sample buffer to elute protein from the beads.

**Isothermal titration calorimetry**

ITC experiments were performed using a MicroCal 205 calorimeter (Malvern, http://www.malvern.com) in high gain mode at 25°C with all proteins diluted in buffer 20 mM HEPES, 150 mM NaCl, pH 7.5. His6-ΔBB-MOS6 protein was pipetted into the sample chamber at 43–54 μM concentration and was titrated with His6-tagged HaRxL106, HaRxL106ΔC or SAP11 at concentrations between 320 and 940 μM. Two microtitre injections with 120 sec pause intervals were performed up to a cumulative volume of 38 μl. Binding isotherms were fitted to the integrated calorimetric data using Origin software (OriginLab, http://www.originlab.com). Control reactions titrating buffer into ΔIBBMOS6 showed that the heat of dilution was <0.1 Kcal mol<sup>-1</sup> of injectant and therefore comparable with the values obtained at the end point of each titration. At least one technical replicate for each ITC experiment was performed and gave similar results.

**Confocal microscopy**

*N. benthamiana* or Arabidopsis leaf discs were mounted onto microscopy slides in 60% glycerol and water and analysed on a Leica DM6000B/TC SPS confocal microscope (Leica Microsystems, http://www.leica-microsystems.com) with the following excitation wavelengths: YFP, 516 nm; RFP, 561 nm.

**Analytical size exclusion chromatography**

Analytical size exclusion chromatography was performed using a Superdex 200 HR 10/30 column (GE Healthcare, http://www.gelifesciences.com) in 50 mM HEPES, 150 mM NaCl, pH 7.0. His6-ΔBB-MOS6 protein was diluted to a concentration of 2 mg ml<sup>-1</sup> and incubated with a 1 μM excess of either His6-HaRxL106 or His6-HaRxL106ΔC for 1 h at 4°C. The samples were centrifuged at 17,000 g °C 20 min and 0.5 ml of the cleared supernatant was loaded on the column. The column was eluted at a flow rate of 0.5 ml min<sup>-1</sup> with two column volumes of buffer and 0.5 ml fractions were analysed by SDS-PAGE.

**Plasmids and oligo-nucleotides**

For a list of oligo-nucleotides and plasmids used in this study see Data S3 and S4.

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Figure S3. mos6 mutants are more susceptible to P. syringae strains with reduced effector repertoires.

Figure S4. Sequence alignment between HaRxL106 amino acids 232–279 and Arabidopsis H2IP5 amino acids 61–120.

Table S1. Stoichiometry, ΔH and ΔS values for ITC experiments shown in Figure 2(c).

Table S2. X-ray data collection, refinement, and validation statistics.

Data S1. Proteins identified by LC-MS/MS in immuno-precipitates of YFP-HaRxL106 transgenics and controls.

Data S2. Maxima of best Mascot ion scores and total spectrum counts for peptides identified by LC-MS/MS.

Data S3. List of oligo-nucleotides and pENTR plasmids used in this study.

Data S4. Experimental procedures.

REFERENCES


