CSIRO PUBLISHING
Functional Plant Biology
http://dx.doi.org/10.1071/FP16292

Studies of cytokinin receptor–phosphotransmitter interaction provide evidences for the initiation of cytokinin signalling in the endoplasmic reticulum

Sergey N. Lomin[A], *Yulia A. Myakushina*[A], *Dmitry V. Arkhipov*[A], *Olga G. Leonova*[B], *Vladimir I. Popenko*[B], *Thomas Schmülling*[C,E] *and Georgy A. Romanov*[A,D,E]

[A]Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya str. 35, 127 276 Moscow, Russia.
[B]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, 119 991 Moscow, Russia.
[C]Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences, Freie Universität Berlin, Albrecht-Thaer-Weg 6, D-14 195 Berlin, Germany.
[D]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Leninskie Gory 1, 119 992 Moscow, Russia.
[E]Corresponding authors. Emails: gromanov@yahoo.com; gar@ippras.ru; tschmue@zedat.fu-berlin.de

This paper originates from a presentation at the Fourth International Symposium on Plant Signaling and Behavior, Komarov Botanic Institute RAS/Russian Science Foundation, Saint Petersburg, Russia, 19–23 June 2016.

Abstract. Cytokinin receptors were shown recently to be localised mainly to the endoplasmic reticulum (ER); however, the activity of ER-located receptors was not proven. We have therefore tested the functionality of ER-located *Arabidopsis* receptors. The first step of cytokinin signal transduction is the transfer of a phosphoryl group from the activated receptor to a phosphotransfer protein. To determine the subcellular localisation of receptor–phosphotransmitter interaction *in planta*, BiFC experiments were performed. Receptors *ARABIDOPSIS* HISTIDINE KINASE 2 (AHK2), AHK3 and AHK4 (CRE1) and phosphotransmitters *ARABIDOPSIS* HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 1 (AHP1), AHP2 and AHP3 fused to split-eYFP were transiently expressed in *Nicotiana benthamiana* leaves. Receptor–phosphotransmitter pairs were shown to interact in every possible combination in a pattern reflecting the ER. Receptor dimers, an active form of the receptors, were also detected in the ER. According to BiFC and protease protection data, the catalytic part of AHK3 was located in the cytoplasm whereas the hormone binding module faced the ER lumen. This topology is consistent with receptor signalling from the ER membrane. Finally, the functionality of receptors in different membrane fractions was tested using an *in vitro* kinase assay visualising the phosphorylation of phosphotransfer proteins. The detected cytokinin-dependent phosphotransfer activity was confined mainly to the ER-enriched fraction. Collectively, our data demonstrate that ER-located cytokinin receptors are active in cytokinin signal transduction. Hence, intracellular cytokinins appear to play an essential role in cytokinin signalling. An updated model for the spatial organisation of cytokinin transport form activation, intracellular trafficking and signalling from the ER is proposed.

Additional keywords: *Arabidopsis thaliana*, cytokinin signaling, phosphotransfer, phosphotransmitter, protein interaction, receptor dimers.

Received 14 August 2016, accepted 11 January 2017, published online 14 March 2017

Introduction

Cytokinins are ubiquitous plant hormones that regulate many aspects of plant growth, development and physiology (Sakakibara 2006; Romanov 2009; Werner and Schmülling 2009; Hwang *et al*. 2012; Kieber and Schaller 2014). The cytokinin signal is perceived by membrane-spanning sensor histidine kinases feeding into a multistep phosphorelay (MSP) signal transduction pathway (reviewed by Müller 2011; Heyl *et al*. 2012; Lomin *et al*. 2012; Spíchal 2012; El-Showk *et al*. 2013). Cytokinin receptors from various species share a similar domain composition and 3D-structure (Lomin *et al*. 2012; Steklov *et al*. 2013), whereas the functional properties of the receptors, especially their profiles of ligand specificity, can be variable (Romanov 2009; Heyl *et al*. 2012; Lomin *et al*. 2012,

2015; Kuderová *et al*. 2015). One of the common features of cytokinin receptors is a drastic decrease in hormone binding ability at low (5–5.5) pH (Romanov *et al*. 2006; Lomin *et al*. 2015). This type of pH dependence served at the time as a first hint to suppose an intracellular receptor localisation. This initial hypothesis was further corroborated by detailed experiments showing that the bulk of cytokinin receptors is indeed located in the endoplasmic reticulum (ER) membranes (Caesar *et al*. 2011; Lomin *et al*. 2011; Wulfetange *et al*. 2011). However, the question whether or not endomembrane-located receptors are active and participate in cytokinin signalling has not been addressed, so still remains a matter of debate (Osugi and Sakakibara 2015; Zürcher and Müller 2016; Zürcher *et al*. 2016). Theoretically, it cannot be excluded that a minor part of cytokinin receptors, presumably located in the plasma membrane (PM), performs all the signalling, while the intracellular pool of receptors stays inert. For example, in mammalian cells, most (95%) of secreted lipoprotein lipase remains inactive within the ER (Doolittle *et al*. 2009). Determining whether or not ER-located cytokinin receptors are active would thus contribute to our understanding of the relative roles for extracellular and intracellular cytokinin as a hormonal signal.

Among the earliest events of intracellular cytokinin signalling is the cytokinin-induced activation of histidine kinase activity of receptors resulting in receptor dimer autophosphorylation and the subsequent transfer of the phosporyl group to mobile phosphotransfer proteins. Therefore, the subcellular localisation of the complex of functionally active receptors with phosphotransfer proteins would indicate the site of initiation of cytokinin signalling. At present, data on cytokinin receptor–phosphotransmitter interactions are rather limited. All available data were obtained in heterologous assay systems: *Escherichia coli* (Suzuki *et al*. 2001), yeast (Dortay *et al*. 2006) or insect (Mähönen *et al*. 2006) cells. Thus, the subcellular localisation of receptor–phosphotransmitter interaction *in planta* was not yet determined. Moreover, the phosphoryl-transmitting activity of cytokinin receptors located in plant membranes (total or fractionated) has not been demonstrated so far.

The aim of this study was to fill the above-mentioned gaps in our knowledge on cytokinin signalling. The receptor homo- and heterodimerisation and the binding of phosphotransmitters to cytokinin receptors were visualised in living plant cells. The phosphate feeding activity of cytokinin receptors within plant membranes has been demonstrated. These results support the functionality of ER-located cytokinin receptors. Thus, the ER seems to be an important site for cytokinin signalling and intracellular cytokinins may be considered as an active hormonal pool, possibly predominant at least in some tissues. On the basis of these results, and taking into account other recent data, we present an updated model for the spatial organisation of cytokinin trafficking, metabolic transformation to the active form and signalling within the cell.

Materials and methods

Plasmids and recombinant DNA techniques

For *Nicotiana benthamiana* transient transformation, the expression vectors pB7FWG2-AHK3, pB7m34GW–P_{AHK3}-AHK3-Myc, pSPYCE-35S-gAHK2, and pSPYNE-35S-gAHK2 (Wulfetange *et al*. 2011) containing the cDNA sequence of the *AHK3* and the genomic sequence of the *AHK2* gene, respectively, were used. The genomic *AHK3* and *AHK4* sequences were amplified by PCR (see primer sequences in Table S1, available as Supplementary Material to this paper) from the bacterial artificial chromosome (BAC) clones F17L21 (GenBank accession no. AC00455) and T23K3 (GenBank accession no. AC00706), respectively, and ligated into pSPYNE-35S and pSPYCE-35S (Walter *et al*. 2004). AHP1, AHP2, AHP3 and the mutated versions of AHP2 were amplified by PCR (see primer sequences in Table S1) from *Arabidopsis thaliana* (L.) Heynh. cDNA and pDEST^{TM}15-AHP2 (Dortay *et al*. 2006), and ligated into pSPYNE-35S and pSPYCE-35S. Genes in pSPYNE-35S, pSPYCE-35S and pB7FWG2 were positioned under control of the 35S cauliflower mosaic virus (CaMV) promoter. All constructs described above were used for *Agrobacterium tumefaciens* transformation (strain GV3101). For AHP expression in *Escherichia coli*, *AHP1* and *AHP3* genes were amplified by PCR (see primer sequences in Table S1) from *Arabidopsis* cDNA, and ligated into pDEST^{TM}15 (Invitrogen), replacing *AHP2* in pDEST^{TM}15-AHP2. pDEST^{TM}15-AHP1, pDEST^{TM}15-AHP2 and pDEST^{TM}15-AHP3 were used for *E. coli* transformation (strain *BL21*(*DE3*)*pLysE*).

Transient expression of receptor genes in tobacco plants

N. benthamiana tobacco plants were cultivated in a greenhouse on soil at 25°C under long day conditions (16 h of light/8 h of dark). The transient transformation of leaves of 4-week-old plants was carried out according to Sparkes *et al*. (2006). *A. tumefaciens* clones carrying target genes fused to fluorescent protein genes or a Myc-tag sequence and the helper clone carrying the *p19* gene of tomato bushy stunt virus (Voinnet *et al*. 2003) were grown for 16 h at 28°C until OD600 ~1 was reached. Agrobacteria were pelleted for 5 min at 10 000*g* at room temperature and resuspended in the infiltration solution (10 mM MES-KOH, pH 5.7, 10 mM $MgCl_2$, 0.15 mM acetosyringone), then pelleted again for 3 min at 10 000*g* and resuspended in 2–5 mL of the infiltration solution. The infiltration of tobacco leaves was performed with the following set of bacterial suspensions: pB7FWG2-AHK3-GFP (OD600 ~0.7) and p19 (OD600 ~1.0) for the AHK-AHP phosphotransfer assay; pSPYNE-35S-AHK/AHP (OD600 ~0.7), pSPYCE-35S-AHK/AHP (OD600 ~0.7), and p19 strain (OD600~1.0) for bimolecular fluorescence complementation (BiFC); and P_{AHK3}:*AHK3-Myc* (OD600 ~0.3) and p19 (OD600~0.1) for protease protection assay. As a control for ER localisation, some BiFC experiments were performed in conjunction with the ER marker CD3-959 (WAK2-mCherry-HDEL) (Nelson *et al.* 2007). Gene expression and interaction of the respective proteins in BiFC was detected 3–5 days after infiltration using an inverted fluorescence confocal microscope Leica DMI 6000 CS equipped with a Leica TCS SP5 laser scan unit (Leica Microsystems) and operated with the Leica application software. All confocal laser scanning microscopy images were obtained using a HC PL APO 20X/0.70 IMM CORR water-immersion objective at constant imaging conditions (i.e. gain, offset, and exposure time). The GFP signal was acquired by excitation at 488 nm

and detection in the 500–530 nm range. YFP fluorescence was acquired by excitation at 514 nm and detection in the 520–550 nm range. The mCherry signal was acquired by excitation at 561 nm and detection in the 580–640 nm range. For PM and nuclei staining, tobacco leaves were infiltrated with the dyes FM4-64 (Invitrogen) or 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) at concentrations of 50 µM and 50 µg mL^{-1} respectively. The following excitation lines and detection ranges were used: FM4-64, 488 nm and 625–665 nm; DAPI, 790 nm (two-photon excitation) and 400–470 nm.

Protease protection assay

Experiments were performed according to Sparkes *et al*. (2010) with modifications. One gram of agrobacteria-infiltrated tobacco leaf sectors expressing P_{AHK3}:*AHK3-Myc* construct was ground with a chilled mortar and pestle in 4 mL extraction buffer containing 40 mM HEPES-KOH pH 7.5, 0.4 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 0.4% PVP for 2 min. The homogenate was centrifuged at 1000*g* for 5 min at 4°C. Microsomal fraction was obtained by centrifugation at 100 000*g* for 1 h at 4°C in a step sucrose gradient. Approximately 200 µL of microsome fraction was recovered from the interphase between the 20 and 60% sucrose layers and gently resuspended in a fresh tube. For one probe, 15 µL of microsome fraction was mixed with 30 µL of buffer containing 75 mM Tris–HCl pH 8, 30 mM $CaCl_2$ and 0.15% Triton X100. The reaction was initiated by addition of 1 µL 2 mg mL^{-1} proteinase K solution, allowed to proceed for 30 min at 30°C, then stopped by addition of 1 mM PMSF, 50 mM EDTA and 16 µL loading buffer. Samples were treated at 100°C for 5 min and electrophoresed in 10% SDS–PAGE. Proteins were transferred afterwards to Immobilon-P transfer membranes (Millipore). The blots were treated with anti-Myc-tag monoclonal (mouse) (Millipore) and anti-BiP2 polyclonal (rabbit) antibodies (Agrisera) as described by Wulfetange *et al*. (2011).

AHK-AHP phosphotransfer assay

Experiments were performed according to Mähönen *et al*. (2006) with modifications. *GST-AHP1*, *GST-AHP2*, *GST-AHP3* genes were expressed in *E. coli* strain *BL21*(*DE3*) *pLysE*. When the bacterial culture density had reached OD600 ~1, transgene expression was induced by addition of 1 mM IPTG for 1 h. Then bacteria were pelleted by centrifuging at 3000*g* for 20 min at 4°C and resuspended in lysis buffer (50 mM Tris–HCl pH 7.6, 250 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2% Triton X100 and 1 mM PMSF). Cell lysates were prepared by sonication as in Verma *et al*. (2013), then GST-AHPs were bound to glutathione-agarose beads (affinity chromatography) (Sigma-Aldrich) for 3 h with shaking at 4°C. Beads were washed three times using the same lysis buffer. Finally, GST-AHPs were eluted with 50 mM Tris–HCl pH 7.6, 50 mM KCl, 10% glycerol, 10 mM reduced L-glutathione and 0.5 mM PMSF. Cytokinin receptor AHK3 was transiently expressed in tobacco leaves transformed with the pB7FWG2-AHK3-GFP construct. Six days later plant leaves were checked for GFP fluorescence, and in positive case membranes were isolated and fractionated by sucrose density centrifugation to obtain ER and PM fractions. The purity of membrane fractions was checked by immunoblotting with rabbit polyclonal antibodies (Agrisera) against the marker BiP2 (ER) and H^+-ATPase (PM), respectively, as described by Wulfetange *et al*. (2011). Phosphotransfer was carried out at 25°C for 10 min in 15 µL reaction mixture containing 50 mM Tris–HCl pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10% glycerol, 50 µM ATP, 5 µCi [γ^{32}P]ATP, 2 µg GST-AHP, 0.8 µg tobacco plant membranes and *trans*-zeatin in the nanomolar concentration range. The reaction was stopped by the addition of 5 µL 4× loading buffer (60 mM Tris–HCl pH 6.8, 10% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue) supplemented with 80 mM EDTA. Then, 15 µL of each sample without boiling were applied to 10% SDS–PAGE, and electrophoresed proteins were transferred onto PVDF membranes (BioRad). Radioactivity was detected by exposition to a phosphor screen for 10 min and analysed by Storm 860 Molecular Imager (GMI). Band intensities were measured using the ImageJ program, conventional specific activities correspond to detected radioactivity related to equal amounts of microsomes.

Results

Receptor–phosphotransmitter interaction in planta *occurs at the ER membrane*

To explore the subcellular localisation and putative interactions between cytokinin receptors and phosphotransmitters *in planta*, we performed bimolecular fluorescence complementation (BiFC) studies upon transient expression of individual proteins in *N. benthamiana* leaves. For these experiments, *Arabidopsis AHK2*, *AHK3* and *AHK4* receptor genes and *AHP1*, *AHP2* and *AHP3* phosphotransmitter genes were inserted in pSPYNE-35S and pSPYCE-35S vectors to obtain *C*-terminally split eYFP-tagged fusion proteins. Receptor and phosphotransmitter genes were co-expressed in tobacco leaves, and the protein interaction was tested by confocal microscopy. Specific eYFP fluorescence was detected for all AHK–AHP combinations without visible quantitative differences in signal emission (Fig. 1*a–r*). In the control experiments testing interaction of vectors lacking cytokinin receptor and/or phosphotransmitter genes (pSPYCE-35S-empty × pSPYNE-35S-AHK4, pSPYCE-35S-empty × pSPYNE-35S-AHP2, and pSPYNE-35S-empty × pSPYCE-35S-empty) no eYFP fluorescence was registered (Fig. 1*s–x*). The subcellular pattern of interaction was similar to the pattern of ER-associated proteins and included a strong perinuclear signal (Fig. 1), which is a hallmark of ER localisation (Marinos 1960; Koizumi *et al*. 2001; Grefen *et al*. 2008; McLellan *et al*. 2013). Control staining of nuclei with DAPI confirmed the perinuclear origin of BiFC fluorescence (see Fig. S1, available as Supplementary Material to this paper). At higher magnification, the fluorescence can be clearly seen not only around nuclei but also directly in the ER network (Figs 2, S2). Stacking of several tissue sections showed that the labelled ER network fills the entire volume of the cell (Fig. 2*g–i*).

To find further support of cytokinin receptor-phosphotransmitter protein interaction at the ER membrane, the co-expression of interacting AHK–AHP BiFC pairs with the ER marker WAK2-mCherry-HDEL (Nelson *et al*. 2007) was tested. The analysis yielded overlapping signals consistent with their co-localisation (Fig. 3*a–p*, *u*, *v*). In contrast, the signal overlap was minimal (if any) with PM membranes that were

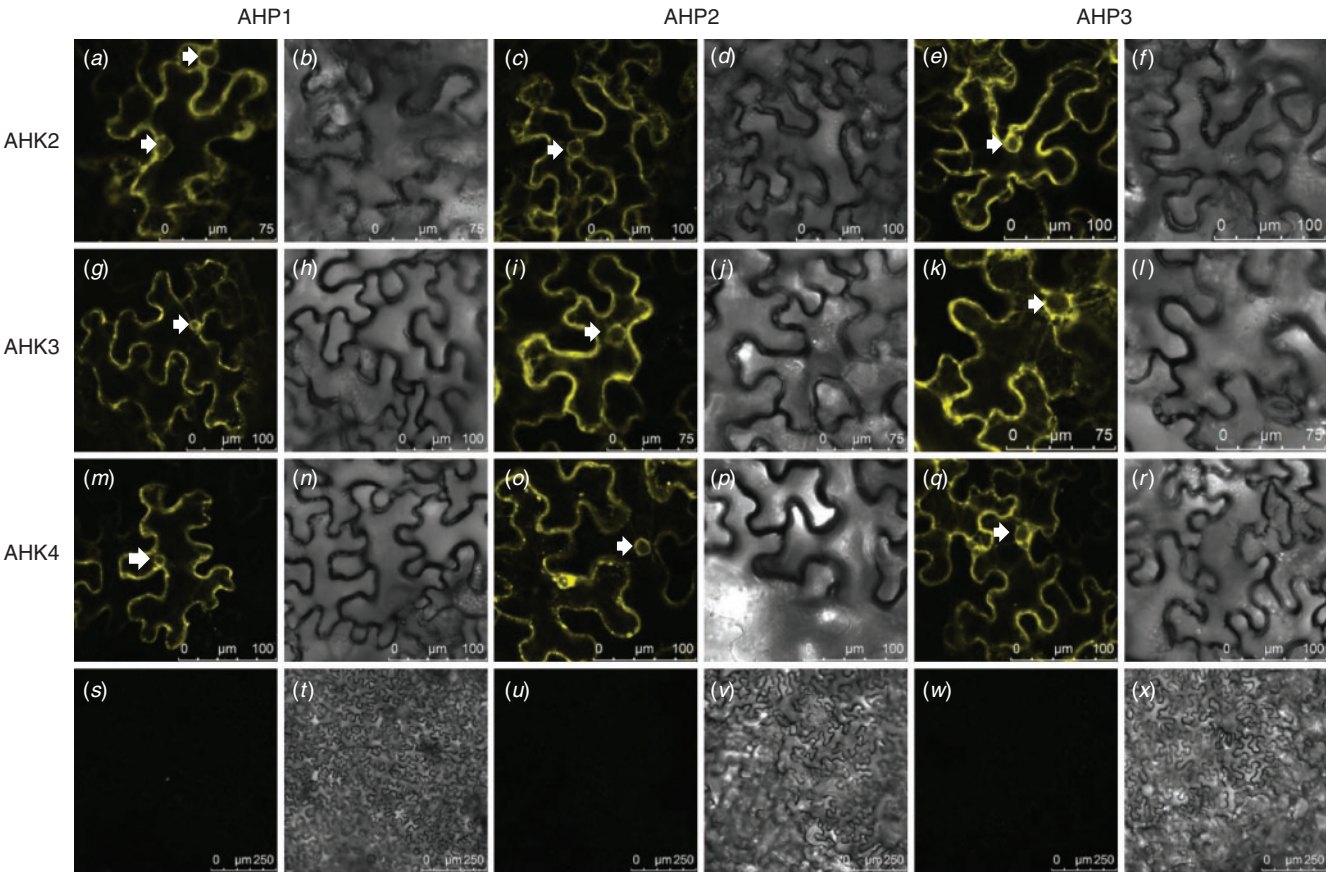

Fig. 1. Cytokinin receptor–phosphotransmitter interaction *in planta* determined by BiFC. Vectors pSPYNE-35S-AHK2–4 and pSPYCE-35S-AHP1–3 harbouring cytokinin receptor or phosphotransmitter genes were co-expressed in *N. benthamiana* leaves and cell fluorescence was visualised by confocal microscopy. Interaction pairs: (*a*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP1; (*c*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP2; (*e*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP3; (*g*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP1; (*i*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP2; (*k*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP3; (*m*) pSPYNE-35S-AHK4 × pSPYCE-35S-AHP1; (*o*) pSPYNE-35S-AHK4 × pSPYCE-35S-AHP2; (*q*) pSPYNE-35S-AHK4 × pSPYCE-35S-AHP3. Controls: (*s*) pSPYNE-35S × pSPYCE-35S; (*u*) pSPYNE-35S-AHK4 × pSPYCE-35S; and (*w*) pSPYNE-35S-AHP2 × pSPYCE-35S. (*b*, *d*, *f*, *h*, *j*, *l*, *n*, *p*, *r*, *t*, *v*, *x* – brightfield images corresponding to fluorescent ones on their respective left side. White arrows indicate nuclei.

stained with FM4-64 (Fig. 3*q–t*, *w*). Taken together, the subcellular pattern of AHK–AHP interaction *in planta* was consistent with the initiation of cytokinin signalling from the ER.

ER-located cytokinin receptors form dimers

Cytokinin receptors are long thought to function as dimers, similar to sensing histidine kinases in bacteria (West and Stock 2001). The dimeric form is considered to be the active form of histidine kinases. BiFC data have shown that AHK2 receptors form homodimers in the ER (Wulfetange *et al*. 2011). Also evidences were presented that AHK3 can form homo- or hetero- (with AHK4) dimers within the ER membrane (Caesar *et al*. 2011). Here we have tested by BiFC the interaction of different pairs of *Arabidopsis* cytokinin receptors. In each case a fluorescent signal was obtained, indicating the tight interaction of the expressed proteins (Fig. 4*a*, *e*, *i*). Some features, in particular a circular fluorescence around the nucleus (Fig. 4*m*, *n*) and overlap with the ER marker (Fig. 4*q–t*), indicated that this fluorescence originated in the ER. This pattern of ER-confined fluorescence contrasts with the pattern produced by interacting phosphotransfer proteins (Fig. 4*c*, *g*, *k*, *o*, *p*) which are known to reside in the cytoplasm and inside the nucleus (Punwani *et al*. 2010). We note that phosphotransmitter complexes colouring the nucleoplasm did not stain the nucleoli (Fig. S3).

Receptor topology within the ER is favourable for signalling

It is firmly established that phosphotransfer proteins, which circulate in the plant cell between cytoplasm and nucleus (Punwani *et al*. 2010), interact with the receiver domain located at the C-terminus of hybrid histidine kinases (Urao *et al*. 2000; Dortay *et al*. 2006; Pekárová *et al*. 2011; Bauer *et al*. 2013). Hence the *in vivo* interaction between cytokinin receptors and phosphotransmitters shown by BiFC (Figs 1–3) suggests that at least a significant portion of receptors adopt a topology favourable for signalling, meaning the *C*-terminus of the receptor facing the cytosol. Additional support was obtained from protease protection assays with microsomes isolated from tobacco leaves transiently expressing the AHK3 receptor harbouring a *C*-terminal Myc-tag. Freshly prepared ER

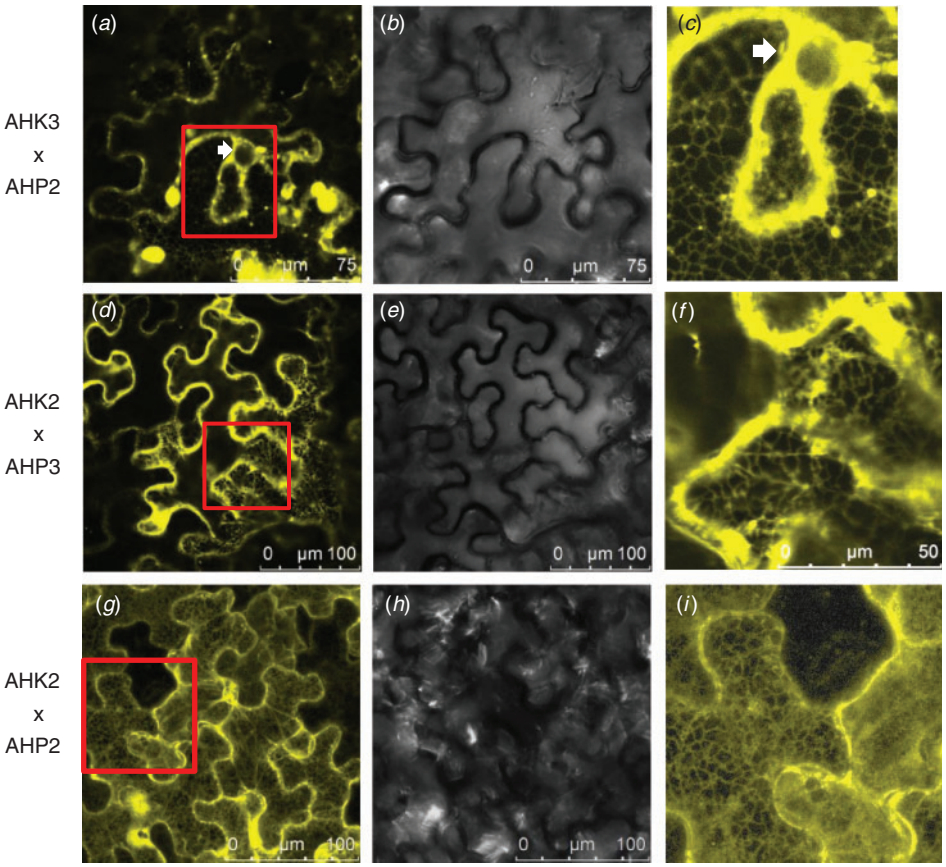

Fig. 2. High resolution subcellular localisation of cytokinin receptor–phosphotransmitter interaction as detected by BiFC. (*a*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP2; (*b*) brightfield image of (*a*); (*c*) enlarged view of the framed section in (*a*); (*d*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP3; (*e*) brightfield image of (*d*); (*f*) enlarged view of the framed section in (*d*). (*g*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP2; (*h*) brightfield image of (*g*); (*i*) enlarged view of the framed section in (*g*). (*g–i*) correspond to the image stack. Reticular structures typical for the ER network are clearly seen in the right column. White arrows indicate nuclei.

microsomes are topologically identical to the ER *in planta* (Ma *et al*. 2006), i.e. the outside of the microsomes corresponds to the ER membrane facing the cytoplasm. Incubation of such microsomes with proteinase K resulted in the loss of the Myc moiety from AHK3, but the ER lumen marker BiP was partly preserved, indicating the protection of lumen-localised peptides from protease digestion. When microsomes were permealised with the detergent Triton X-100, both bands disappeared after digestion (Fig. S4). These results are consistent with the conclusion derived from BiFC experiments and show that the C-terminus of most if not all cytokinin receptors is exposed to the cytoplasm. Accordingly, the hormone binding module at the *N*-terminus of the receptor should be oriented towards the ER lumen.

ER-located cytokinin receptors are active in AHK-to-AHP phosphotransfer in vitro

To study the receptor functionality, AHK–AHP phosphotransfer assays were performed *in vitro* with cytokinin receptors located within plant membranes. The *AHK3* receptor gene was overexpressed under control of the 35S CaMV promoter in tobacco leaves. Different membrane fractions enriched for ER or PM were obtained by centrifugation of total membranes from tobacco leaves in a discontinuous sucrose gradient. The degree of fraction purity was assessed by immunoblotting with specific antibodies against ER- and PM marker proteins (Fig. 5*a*). The estimated fraction enrichment was more than 2-fold for the ER marker and much higher for the PM marker. The phosphotransmitters AHP1, AHP2 and AHP3, all *N*-terminally fused to GST, were produced in *E. coli* and purified by affinity chromatography. Cytokinin-dependent phosphotransfer was carried out in a mixture of cell membranes, with one of the three purified AHPs, and a range of *trans*-zeatin concentrations from 0 to 500 nM. In the presence of labelled ATP, the phosphorylation of phosphotransfer proteins was clearly detected with membranes from leaves overexpressing the *AHK3* gene (Fig. 5*b*) but not detected when membranes from non-transformed leaves were used (Fig. 5*c*). AHP phosphorylation increased in a cytokinin-dependent manner with similar efficiency for all three AHP substrates. Both membrane fractions, ER- or PM-enriched, were principally able to carry

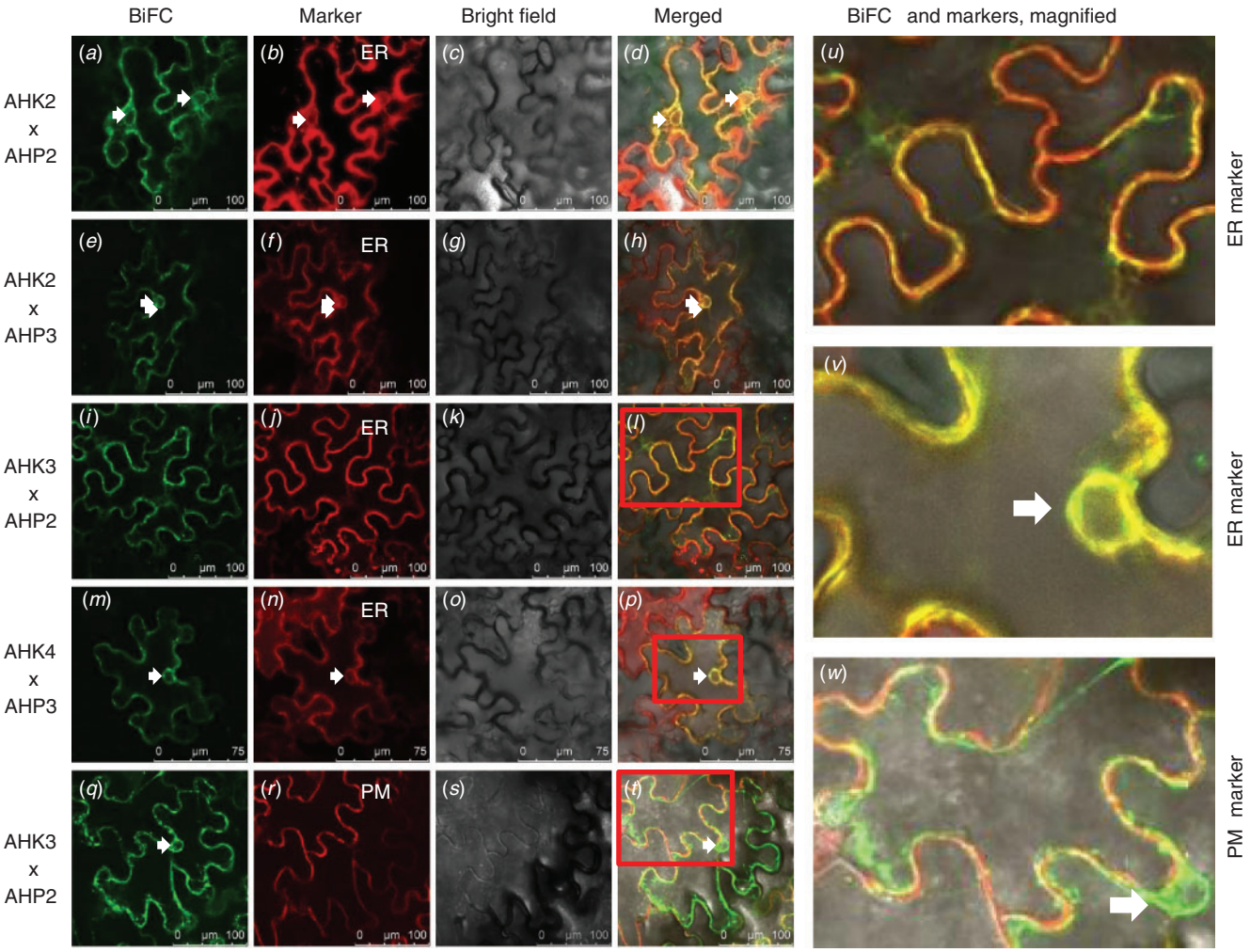

Fig. 3. Co-localisation *in planta* of cytokinin receptor–phosphotransmitter complexes (BiFC) with the ER marker protein WAK2-mCherry-HDEL. (*a–d*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP2 co-expressed with ER marker; (*e–h*) pSPYNE-35S-AHK2 × pSPYCE-35S-AHP3 co-expressed with ER marker; (*i–l*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP2 co-expressed with ER marker; (*m–p*) pSPYNE-35S-AHK4 × pSPYCE-35S-AHP3 co-expressed with ER marker; (*q–t*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHP2, PM was stained with FM4-64. (*a*, *e*, *i*, *m*, *q*) BiFC; (*b*, *f*, *j*, *n*) ER marker protein WAK2-mCherry-HDEL; (*r*) PM staining with FM4-64; (*c*, *g*, *k*, *o*, *s*) brightfield images for left figures in the respective row; (*d*, *h*, *l*, *p*, *t*) merged images for the respective row. White arrows indicate the nuclei. eYFP fluorescence in BiFC micrographs is shown in green to distinguish it from the co-localisation pattern (yellow colour in the merged images). Right column: enlarged views (*u*, *v*, *w*) of the framed sections in merged images (*l*, *p*, *t*, respectively). White arrows indicate the nuclei. Note that the yellow colour indicating genuine co-localisation occurs predominately with the ER- but not the PM marker.

out this cytokinin-dependent phosphorylation reaction, but the ER-enriched membranes demonstrated much higher activity (Fig. 5*b*). The quantitation of band intensity (Fig. 5*d–f*) showed that the specific activity (i.e. radioactivity transferred to phosphotransmitters by equal amounts of microsomes) of the ER-enriched fraction was more than 2-fold higher compared with the PM-enriched fraction. Hence, this approach demonstrates that the cytokinin receptors within the ER are able to phosphorylate phosphotransfer proteins upon hormone addition, meaning that they are functionally active.

Discussion

To date, the interaction between cytokinin receptors and phosphotransfer proteins has been analysed directly only in a single study in a heterologous test system (Dortay *et al.* 2006). These authors used the soluble *C*-terminal parts of *Arabidopsis* receptors AHK2, AHK3 and AHK4 together with a set of phosphotransfer proteins (AHP1 to AHP3, AHP5) and showed by means of a yeast two-hybrid system and *in vitro* pull-down assays a promiscuous interaction between receptors and phosphotransmitters: each receptor *C*-terminal part was able to bind any of the phosphotransfer proteins studied. This result was in accordance with functional tests made using bacterial cells (Suzuki *et al.* 2001) or insect membranes (Mähönen *et al.* 2006) as well as with genetic data on *Arabidopsis ahp* mutants (Hutchison *et al.* 2006) which revealed the functional redundancy of these phosphotransfer proteins. The receiver domains of other sensor histidine kinases (AHK5, CKI1, ETR1) of *Arabidopsis* were shown also to interact with different phosphotransmitters with a

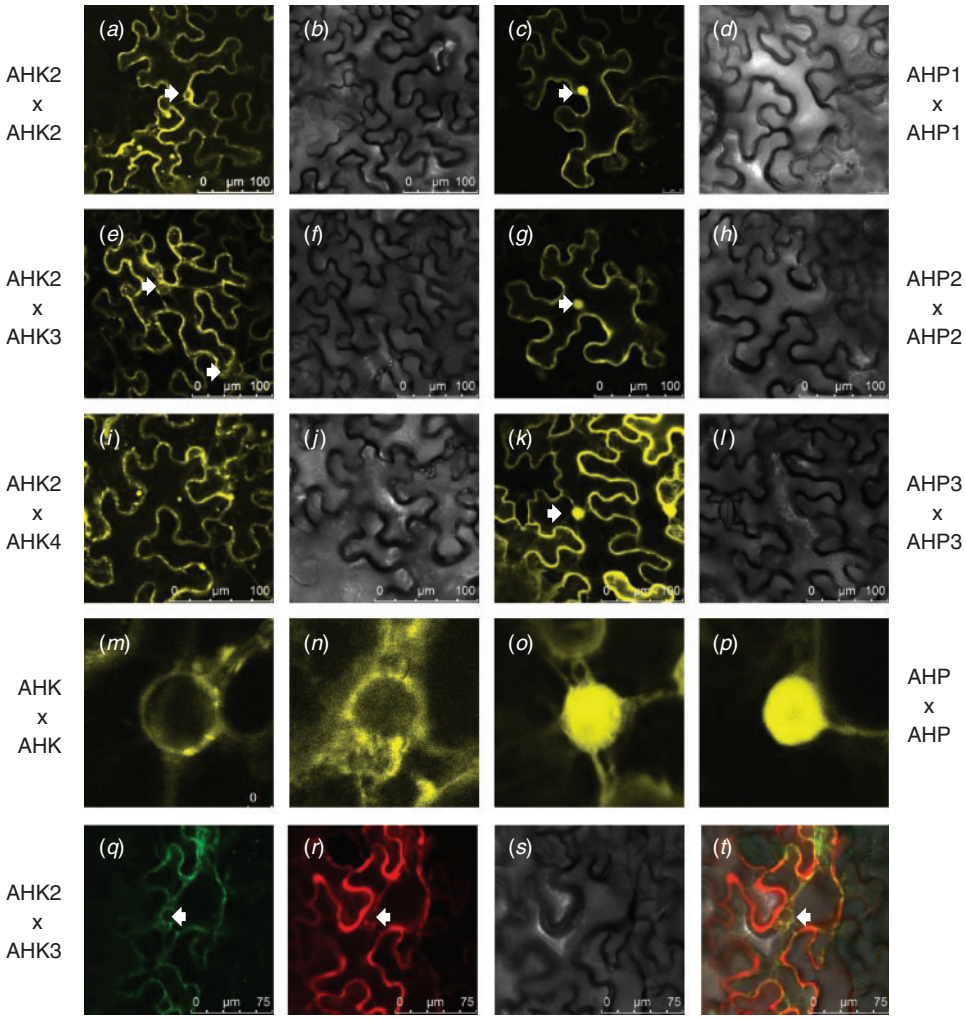

Fig. 4. Receptor dimers reside in the ER membrane. Vectors pSPYNE-35S-AHK2-4 and pSPYCE-35S-AHK2 harbouring cytokinin receptor genes were co-expressed in *N. benthamiana* leaves for BiFC analysis and cell fluorescence was detected by confocal microscopy (*a*, *e*, *i*). (*b*, *f*, *j*, *s*) brightfield images correspond to respective fluorescent ones on their left side. Similar vectors harbouring phosphotransfer protein genes were used as a control (*c*, *g*, *k*), with corresponding brightfield images (*d*, *h*, *l*). The nuclear fluorescence patterns of the expressed receptors (*m*, *n*) and phosphotransmitters (*o*, *p*) are compared. (*q*, *t*) pSPYNE-35S-AHK3 × pSPYCE-35S-AHK2 co-expressed with ER marker (*r*). White arrows indicate the nuclei.

varying degree of preference (Urao *et al*. 2000; Pekárová *et al*. 2011; Bauer *et al*. 2013).

In the work presented here, we investigated the interaction of the full-length *Arabidopsis* cytokinin receptors with different phosphotransfer proteins under close to natural conditions, i.e. in living plant cells, namely cells of the tobacco abaxial epidermis. Of course, it should be taken into account that all studied proteins were tagged and strongly overexpressed in tobacco leaves. However, precedent work has shown that the presence of a tag and the level of expression had no marked impact on the subcellular localisation of cytokinin receptors (Caesar *et al*. 2011; Lomin *et al*. 2011; Wulfetange *et al*. 2011). By using the BiFC method the interaction of receptors with phosphotransmitters was clearly visualised and the promiscuity of this interaction was demonstrated as well. Molecular modelling confirmed the promiscuous mode of interaction between receptor receiver domains and phosphotransfer proteins (DV Arkhipov, SL Lomin, GA Romanov, unpubl. data). The *in planta* BiFC study offered the advantage to test also for the subcellular localisation of the interaction of these macromolecules. According to our data, this interaction occurs principally at the ER membrane where the receptor dimers were detected as well. Contrary to prokaryotes where sensor histidine kinases usually form homodimers (West and Stock 2001), plant cytokinin receptors form both homo- and heterodimers. As phosphotransmitters are known to bind to the receptor at its *C*-terminus, the interaction between receptors and phosphotransmitters at the ER membrane might serve a proof for the orientation of the *C*-terminal part of the receptor towards the cytoplasm, meaning that the receptor orientation is appropriate for intracellular signalling. Taken together, our results of the receptor-AHP interaction study and the protease

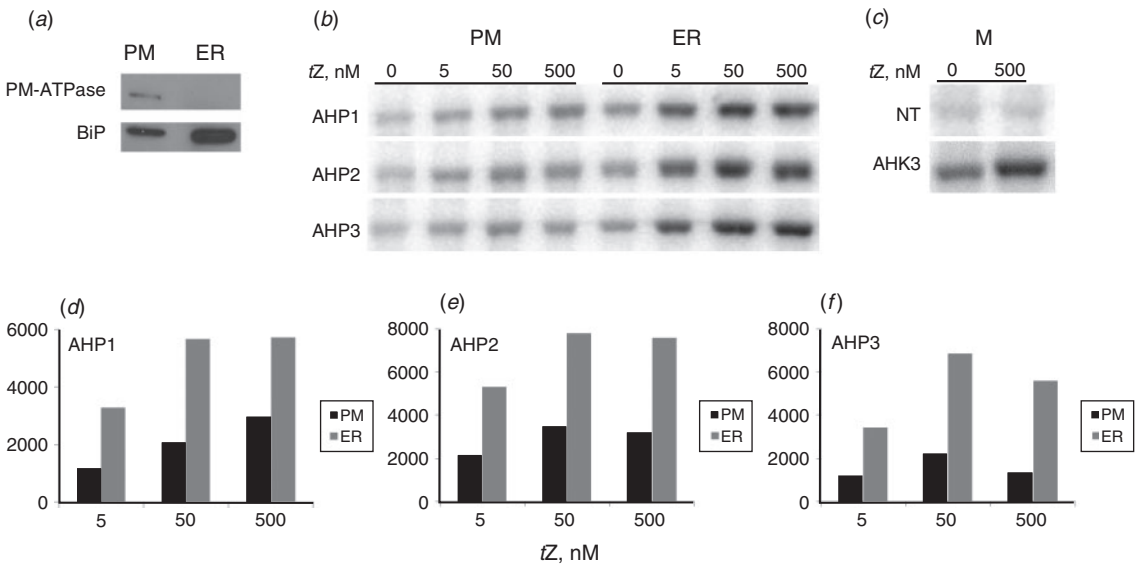

Fig. 5. AHK3–AHP phosphotransfer assay with ER- or PM-enriched plant membrane fraction (*a*) The purity of membrane fractions obtained from leaves overexpressing *AHK3* was tested by immunoblotting using antibodies against marker proteins. H^+-ATPase was used as PM marker, and BiP as ER marker. (*b*) AHK3-AHP phosphotransfer assay. The phosphotransfer assay was performed for 10 min at 25°C with 0.8 µg membrane proteins, 2 µg AHP, 50 µCi [γ^{32}P]ATP and *trans*-zeatin (*t*Z) between 0 and 500 nM. Then proteins were solubilised, resolved by SDS–PAGE, blotted on PVDF membranes and phosphorylated AHPs were detected by Storm 860 Molecular Imager (GMI). (*c*) Cytokinin receptor-AHP3 phosphotransfer assay with microsomal fractions (M) obtained from non-transformed (NT) or AHK3-overexpressing leaves in the absence or presence of 500 nM *t*Z. The reaction was performed for 20 min as described for (*b*). (*d*, *e*, *f*) Quantification of radioactivity (in arbitrary units) from AHP1, AHP2 and AHP3 bands shown in (*b*) after subtraction of background values obtained at 0 nM *t*Z. The ratios of maximal phosphotransmitter phosphorylation (at 50–500 nM *t*Z) to the corresponding basal level (at 0 nM *t*Z) were 2.66 ± 0.15 for the ER- and 2.32 ± 0.12 for the PM-enriched fraction. This basal phosphorylation can be caused by other histidine kinases (CKI1, ethylene receptor ETR1) as well as traces of endogenous cytokinins.

protection assay provided strong evidences for receptor localisation in and signalling from the ER membrane.

All phosphotransmitters used (AHP1, AHP2 and AHP3) were distributed between cytoplasm and nuclei, in accordance with previous data (Punwani *et al*. 2010). Phosphotransmitter complexes strongly accumulated in the nucleoplasm but left nucleoli unstained. Evidently, a part of phosphotransfer proteins interacting with the receptors should be transiently associated with the ER membrane too.

The prevalence of receptor dimers in the ER and even their association at this site with phosphotransmitters does not necessarily imply that the receptors are enzymatically active in this compartment. Therefore we have elaborated a special assay system to test the functional activity of receptors within different plant membranes. The results of the phosphotransfer assays *in vitro* further corroborated the localisation of cytokinin receptors in the ER and showed that these receptors transmit labelled phosphate to their natural substrate, the AHP. Previously, this phosphotransfer was studied using the AHK4 (CRE1) receptor fixed to membranes of SF9 insect cells (Mähönen *et al*. 2006). Here we demonstrate that a cytokinin receptor (exemplified by AHK3) inserted in a plant membrane has the ability to phosphorylate cognate MSP substrates (exemplified by AHP1, AHP2 and AHP3) in a cytokinin-dependent manner. Importantly, ER-enriched microsomes showed a higher receptor-to-AHP phosphotransfer than PM-enriched ones. It should be noted that the kinase reaction was carried out at pH 7.6, which is close to the pH in the cytoplasm and ER (Martinière *et al*. 2013). In contrast, *in planta* the outer side of the PM is normally much more acidic (Yu *et al*. 2000; Felle 2005) and might reduce ligand binding by cytokinin receptors (Romanov *et al*. 2006; Lomin *et al*. 2015). Therefore the contribution of the putative PM-located receptors to the total cytokinin signalling *in vivo* is presumably less than it may appear from *in vitro* assays. Further, it seems likely that at least a considerable portion of the activity of the PM-enriched fraction is due to its contamination with ER membranes (Fig. 5*a*). In addition, one should take into account that the ER comprises ~10–15 times more membranes than the PM (Wulfetange *et al*. 2011). Collectively, these data are consistent with the ER serving as the main cellular platform for active cytokinin receptors. In contrast, a role for the PM in cytokinin signalling cannot be concluded – but also not excluded – from the data; among *Arabidopsis* receptors, AHK4 seems to be the most plausible candidate to reside in the PM (Wulfetange *et al*. 2011).

In this and in precedent studies (Caesar *et al*. 2011; Wulfetange *et al*. 2011) the results supporting a predominant ER-location of cytokinin receptors were obtained using leaf tissues, mainly abaxial epidermis cells. This does not exclude a predominant PM-location of the receptors in some other organs/tissues or at different developmental stages (Kim *et al*. 2006; Lomin *et al*. 2011; Zürcher *et al*. 2016).

There are some advantages in the intracellular localisation of cytokinin receptors for hormone signalling. The probability of interaction with a phosphotransmitter would be higher when the receptors reside not only in the PM but are distributed

within the whole intracellular space where phosphotransmitters are synthesised and circulate. The ER-location of the receptor, compared with PM location, shortens the distance needed for phosphotransfer proteins to reach the nucleus thus increasing the velocity and the reliability of cytokinin signal transduction. This is particularly true for the perinuclear receptors which have been detected both as single fluorescent tags as well as part of a complex with phosphotransmitters and/or other receptors (forming homo- and heterodimers) in BiFC assays. As was stated above, also the intracellular pH is more favourable for ER-located receptors than the extracellular pH would be for PM-located ones. Finally, the receptor ER-localisation offers an opportunity for cytokinin signalling regulation and crosstalk also at the level of intracellular hormone trafficking and metabolic conversion as will be discussed in the following paragraphs.

If one assumes that the bulk of active cytokinin receptors is located in the ER, one has to conclude that intracellular cytokinins substantially contribute to cytokinin signalling, the ER lumen being a main site for cytokinin recognition by receptors. The question is, however, how do cytokinins reach the receptor sensor module located inside the ER? Cytokinins are known to act in both paracrine and endocrine fashion (Faiss *et al*. 1997; Romanov 2009). In case of paracrine action, active cytokinins produced in a given cell or neighbouring cells connected through plasmodesmata easily find receptors located inside the same cell(s). But in case of endocrine (long-distance) action, cytokinins are transported principally as *N*9 ribosides *via* the xylem (mainly *t*ZR) and phloem (mainly iPR) (Hirose *et al*. 2008; Zürcher and Müller 2016). These transport forms have no own cytokinin activity (Hothorn *et al*. 2011; Lomin *et al*. 2015) and must be converted to free bases to become active. However, no specific plant enzyme that cleaves cytokinin ribosides and releases free bases have been found so far. The unique report on a related enzyme in potato, presumably apoplastic and displaying prevalently ribosyltransferase activity, suggested that cytokinin riboside phosphorylase activity is 'normally present at undetectably low levels' (Bromley *et al*. 2014). Indeed, isolated plant membranes, contrary to bacterial ones, showed no significant cytokinin riboside-cleaving activity (Lomin *et al*. 2015). Instead, LOG phosphoribohydrolases (Kurakawa *et al*. 2007; Kuroha *et al*. 2009) that function in the cytosol and cleave cytokinin nucleotides releasing free bases were described.

Here we propose a model for the conversion of the cytokinin transport form into an intracellular signal emitted from the ER (Fig. 6). According to this model, cytokinin ribosides derived from elsewhere in the plant enter the target cell, probably facilitated by ENT transporter (Hirose *et al*. 2008). Inside the cell, ribosides are phosphorylated by a presumed kinase activity, which has been detected in tracer experiments (Tokunaga *et al*. 2012), and thus converted to nucleotides – cytokinin riboside 5′-monophosphates. These nucleotides may serve as cytoplasmic inactive (storage) form for cytokinins. The next step depends on cytoplasmic LOG phosphoribohydrolases (Kurakawa *et al*. 2007; Kuroha *et al*. 2009), which play the main role in producing active cytokinin bases from nucleotides (Tokunaga *et al*. 2012). Active hormone released by LOG enzymes can penetrate into the ER lumen (Le Gall *et al*. 2004; Lizák *et al*. 2008) to trigger signalling from ER-located cytokinin receptors. Thus, in case of endocrine cytokinin action, the receptors are activated presumably by cytokinins released in the same or plasmodesmata-connected neighbouring cells, as in case of paracrine action. The two mentioned enzymatic activities (putative kinase and LOG) may participate in the regulation of cytokinin homeostasis and activity *in planta*. They may constitute additional targets of crosstalk between cytokinin and other regulatory systems as hormonal and non-hormonal factors may affect their activities. In particular, the expression of *LOG* genes is regulated by several environmental cues, including the availability of nutrients and abiotic stress (Ramireddy *et al*. 2014). Their regulation may contribute to translating information from the environment into a hormonal signal coordinating plant growth. These aspects of ER-location of active cytokinin receptors open new perspectives for further studies. Even so, at present we consider our model yet as one of several possible pathways for cytokinin trafficking and signalling within the cell, without pretension to be universal for this group of multifunctional hormones. For example, in the bryophyte *Physcomitrella*, extracellular iP and iPR were reported to be the main cytokinins responsible for bud induction (von Schwartzenberg *et al*. 2007).

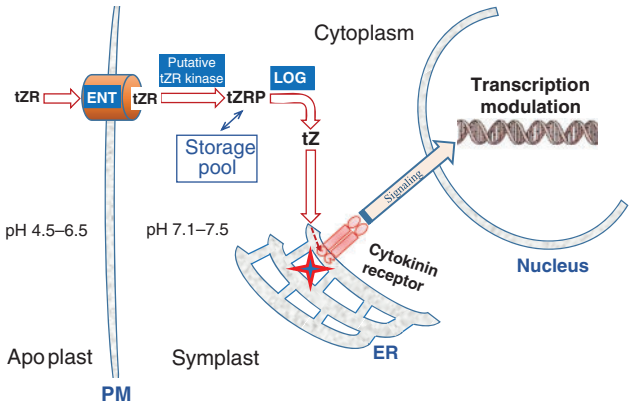

Fig. 6. A model for intracellular cytokinin trafficking and signalling, or how a former inactive transport form achieves receptor activation. The proposed pathway is exemplified by *trans*-zeatin-type cytokinins and leads to the activation of the initial transport form (*t*ZR, *trans*-zeatin riboside) *via* phosphorylation and successive conversion to the free base (*t*Z, *trans*-zeatin) inside the cell. The free base diffuses across the ER membrane activating cytokinin signal transduction and thus altering the transcription of nuclear genes. PM, plasma membrane; ER, endoplasmic reticulum; ENT, equilibrative nucleoside transporter, facilitates cytokinin ribosides to cross the plasma membrane (Hirose *et al*. 2008); LOG, LONELY GUY, a cytokinin nucleoside 5′-monophosphate phosphoribohydrolase (Kurakawa *et al*. 2007). The site of cytokinin signalling initiation (ER) is marked by a star.

Conclusion

The functionality of cytokinin receptors within ER membranes was tested in different ways. The receptors associated with the ER were shown to (i) specifically bind cytokinin (Lomin *et al*. 2011; Wulfetange *et al*. 2011); (ii) form homo- and heterodimers

(Wulfetange *et al*. 2011; this study); (iii) bind phosphotransfer proteins (this study); (iv) transfer the phosphoryl group from ATP to prosphotransfer proteins in a cytokinin-dependent manner (this study); and (v) possess a membrane topology appropriate for signalling from the ER (present study). Also the pH values at their respective site would be much more favourable for signalling in the case of ER-located receptors than for putative PM-located receptors (Romanov *et al*. 2006; Lomin *et al*. 2015). Taken together, these findings support the viewpoint that ER-located cytokinin receptors are active and represent an important source of cytokinin signalling in the plant cell. Accordingly, intracellular cytokinins could play an essential role in cytokinin signalling, at least in some tissue types. To reconcile these findings with data on cytokinin transport and metabolism required to supply cells with active cytokinins, we have presented an updated model explaining, how the inactive cytokinin transport form (riboside) coming from the apoplast becomes active cytokinin and reaches the ligand binding site of the receptor located in the ER lumen. This model suggests additional targets for crosstalk between cytokinin and other signalling pathways in higher plants.

Acknowledgements

This work was supported by the program 'Molecular and Cell Biology' of the Presidium of the Russian Academy of Sciences, and by grant no. 14-04-01714 and 16-34-01065 of the Russian Foundation for Basic Research. The research stays of SNL and GAR in Germany were supported by Freie Universität Berlin.

References

Bauer J, Reiss K, Veerabagu M, Heunemann M, Harter K, Stehle T (2013) Structure–function analysis of *Arabidopsis thaliana* histidine kinase AHK5 bound to its cognate phosphotransfer protein AHP1. *Molecular Plant* **6**, 959–970. doi:10.1093/mp/sss126

Bromley JR, Warnes BJ, Newell CA, Thomson JCP, James CM, Turnbull CGN, Hanke DE (2014) A purine nucleoside phosphorylase in *Solanum tuberosum* L. (potato) with specificity for cytokinins contributes to the duration of tuber endodormancy. *The Biochemical Journal* **458**, 225–237. doi:10.1042/BJ20130792

Caesar K, Thamm AM, Witthöft J, Elgass K, Huppenberger P, Grefen C, Horak J, Harter K (2011) Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *Journal of Experimental Botany* **62**, 5571–5580. doi:10.1093/jxb/err238

Doolittle MH, Neber SB, Ben-Zeev O, Ling-liao J, Gallagher CM, Hosseini M, Yin F, Wong H, Walter P, Péterfy M (2009) Lipase maturation factor LMF1, membrane topology and interaction with lipase proteins in the endoplasmic reticulum. *The Journal of Biological Chemistry* **284**, 33623–33633. doi:10.1074/jbc.M109.049395

Dortay H, Mehnert N, Bürkle L, Schmülling T, Heyl A (2006) Analysis of protein interactions within the cytokinin-signaling pathway of *Arabidopsis thaliana. FEBS Journal* **273**, 4631–4644. doi:10.1111/j.1742-4658.2006.05467.x

El-Showk S, Ruonala R, Helariutta Y (2013) Crossing paths: cytokinin signalling and crosstalk. *Development* **140**, 1373–1383. doi:10.1242/dev.086371

Faiss M, Zalubìlová J, Strnad M, Schmülling T (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *The Plant Journal* **12**, 401–415. doi:10.1046/j.1365-313X.1997.12020401.x

Felle HH (2005) pH Regulation in anoxic plants. *Annals of Botany* **96**, 519–532. doi:10.1093/aob/mci207

Grefen C, Städele K, Růžička K, Obrdlik P, Harter K, Horák J (2008) Subcellular localization and *in vivo* interactions of the *Arabidopsis thaliana* ethylene receptor family members. *Molecular Plant* **1**, 308–320. doi:10.1093/mp/ssm015

Heyl A, Riefler M, Romanov GA, Schmülling T (2012) Properties, functions and evolution of cytokinin receptors. *European Journal of Cell Biology* **91**, 246–256. doi:10.1016/j.ejcb.2011.02.009

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany* **59**, 75–83. doi:10.1093/jxb/erm157

Hothorn M, Dabi T, Chory J (2011) Structural basis for cytokinin recognition by *Arabidopsis thaliana* histidine kinase 4. *Nature Chemical Biology* **7**, 766–768. doi:10.1038/nchembio.667

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2006) The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *The Plant Cell* **18**, 3073–3087. doi:10.1105/tpc.106.045674

Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. *Annual Review of Plant Biology* **63**, 353–380. doi:10.1146/annurev-arplant-042811-105503

Kieber JJ, Schaller GE (2014) Cytokinins. *The Arabidopsis Book* **12**, e0168. doi:10.1199/tab.0168

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America* **103**, 814–819. doi:10.1073/pnas.0505150103

Koizumi N, Martinez IM, Kimata Y, Kohno K, Sano H, Chrispeels MJ (2001) Molecular characterization of two *Arabidopsis* Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiology* **127**, 949–962. doi:10.1104/pp.010636

Kuderová A, Gallová L, Kuricová K, Nejedlá E, Čurdová A, Micenková L, Plíhal O, Šmajs D, Spíchal L, Hejátko J (2015) Identification of AHK2- and AHK3-like cytokinin receptors in *Brassica napus* reveals two subfamilies of AHK2 orthologues. *Journal of Experimental Botany* **66**, 339–353. doi:10.1093/jxb/eru422

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**, 652–655. doi:10.1038/nature05504

Kuroha T, Tokunaga H, Kojima M, Ueda H, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H (2009) Functional analyses of *LONELY GUY* cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis. The Plant Cell* **21**, 3152–3169. doi:10.1105/tpc.109.068676

Le Gall S, Neuhof A, Rapoport T (2004) The endoplasmic reticulum membrane is permeable to small molecules. *Molecular Biology of the Cell* **15**, 447–455. doi:10.1091/mbc.E03-05-0325

Lizák B, Csala M, Benedetti A, Bánhegyi G (2008) The translocon and the non-specific transport of small molecules in the endoplasmic reticulum. *Molecular Membrane Biology* **25**, 95–101. doi:10.1080/09687680701670481

Lomin SN, Yonekura-Sakakibara K, Romanov GA, Sakakibara H (2011) Ligand-binding properties and subcellular localization of maize cytokinin receptors. *Journal of Experimental Botany* **62**, 5149–5159. doi:10.1093/jxb/err220

Lomin SN, Krivosheev DM, Steklov MY, Osolodkin DI, Romanov GA (2012) Receptor properties and features of cytokinin signaling. *Acta Naturae* **4**, 31–45.

Lomin SN, Krivosheev DM, Steklov MYu, Arkhipov DV, Schmülling T, Romanov GA (2015) Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. *Journal of Experimental Botany* **66**, 1851–1863. doi:10.1093/jxb/eru522

Ma B, Cui ML, Sun HJ, Takada K, Mori H, Kamada H, Ezura H (2006) Subcellular localization and membrane topology of the melon ethylene receptor CmERS1. *Plant Physiology* **141**, 587–597. doi:10.1104/pp.106.080523

Mähönen AP, Higuchi M, Törmäkangas K, Miyawaki K, Pischke MS, Sussman MR, Helariutta Y, Kakimoto T (2006) Cytokinins regulate a bidirectional phosphorelay network in *Arabidopsis. Current Biology* **16**, 1116–1122. doi:10.1016/j.cub.2006.04.030

Marinos NG (1960) The nuclear envelope of plant cells. *Journal of Ultrastructure Research* **3**, 328–333. doi:10.1016/S0022-5320(60)80019-6

Martinière A, Bassil E, Jublanc E, Alcon C, Reguera M, Sentenac H, Blumwald E, Paris N (2013) *In vivo* intracellular pH measurements in tobacco and *Arabidopsis* reveal an unexpected pH gradient in the endomembrane system. *The Plant Cell* **25**, 4028–4043. doi:10.1105/tpc.113.116897

McLellan H, Boevink PC, Armstrong MR, Pritchard L, Gomez S, Morales J, Whisson SC, Beynon JL, Birch PRJ (2013) An R × LR effector from *Phytophthora infestans* prevents re-localisation of two plant NAC transcription factors from the endoplasmic reticulum to the nucleus. *PLoS Pathogens* **9**, e1003670. doi:10.1371/journal.ppat.1003670

Müller B (2011) Generic signal-specific responses: cytokinin and context-dependent cellular responses. *Journal of Experimental Botany* **62**, 3273–3288. doi:10.1093/jxb/erq420

Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of *in vivo* organelle markers for co-localization studies in *Arabidopsis* and other plants. *The Plant Journal* **51**, 1126–1136. doi:10.1111/j.1365-313X.2007.03212.x

Osugi A, Sakakibara H (2015) Q&A: how do plants respond to cytokinins and what is their importance? *BMC Biology* **13**, 102. doi:10.1186/s12915-015-0214-5

Pekárová B, Klumpler T, Třísková O, Horák J, Jansen S, Dopitová R, Borkovcová P, Papoušková V, Nejedlá E, Sklenář V, Marek J, Zídek L, Hejátko J, Janda L (2011) Structure and binding specificity of the receiver domain of sensor histidine kinase CKI1 from *Arabidopsis thaliana. The Plant Journal* **67**, 827–839. doi:10.1111/j.1365-313X.2011.04637.x

Punwani JA, Hutchison CE, Schaller GE, Kieber JJ (2010) The subcellular distribution of the *Arabidopsis* histidine phosphotransfer proteins is independent of cytokinin signaling. *The Plant Journal* **62**, 473–482. doi:10.1111/j.1365-313X.2010.04165.x

Ramireddy E, Chang L, Schmülling T (2014) Cytokinin as a mediator for regulating root system architecture in response to environmental cues. *Plant Signaling & Behavior* **9**, e27771. doi:10.4161/psb.27771

Romanov GA (2009) How do cytokinins affect the cell? *Russian Journal of Plant Physiology* **56**, 268–290. doi:10.1134/S1021443709020174

Romanov GA, Lomin SN, Schmülling T (2006) Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *Journal of Experimental Botany* **57**, 4051–4058. doi:10.1093/jxb/erl179

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. *Annual Review of Plant Biology* **57**, 431–449. doi:10.1146/annurev.arplant.57.032905.105231

Sparkes IA, Runions J, Kearns A, Hawes C (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protocols* **1**, 2019–2025. doi:10.1038/nprot.2006.286

Sparkes I, Tolley N, Aller I, Svozil J, Osterrieder A, Botchway S, Mueller C, Frigerio L, Hawes C (2010) Five *Arabidopsis* reticulon isoforms share endoplasmic reticulum location, topology, and membrane-shaping properties. *The Plant Cell* **22**, 1333–1343. doi:10.1105/tpc.110.074385

Spíchal L (2012) Cytokinins – recent news and views of evolutionally old molecules. *Functional Plant Biology* **39**, 267–284. doi:10.1071/FP11276

Steklov MY, Lomin SN, Osolodkin DI, Romanov GA (2013) Structural basis for cytokinin receptor signaling: an evolutionary approach. *Plant Cell Reports* **32**, 781–793. doi:10.1007/s00299-013-1408-3

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001) The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinin. *Plant & Cell Physiology* **42**, 107–113. doi:10.1093/pcp/pce037

Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, Sakakibara H (2012) *Arabidopsis* lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. *The Plant Journal* **69**, 355–365. doi:10.1111/j.1365-313X.2011.04795.x

Urao T, Miyata S, Yamaguchi-Shinozaki K, Shinozaki K (2000) Possible His to Asp phosphorelay signaling in an Arabidopsis two-component system. *FEBS Letters* **478**, 227–232. doi:10.1016/S0014-5793(00)01860-3

Verma V, Sivaraman J, Kumar PP (2013) Expression, purification, and characterization of cytokinin signaling intermediates: Arabidopsis histidine phosphotransfer protein 1 (AHP1) and AHP2. *Plant Cell Reports* **32**, 795–805. doi:10.1007/s00299-013-1424-3

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949–956. doi:10.1046/j.1365-313X.2003.01676.x

von Schwartzenberg K, Núñez MF, Blaschke H, Dobrev PI, Novák O, Motyka V, Strnad M (2007) Cytokinins in the bryophyte *Physcomitrella patens*: analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. *Plant Physiology* **145**, 786–800. doi:10.1104/pp.107.103176

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, Kudla J (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *The Plant Journal* **40**, 428–438. doi:10.1111/j.1365-313X.2004.02219.x

Werner T, Schmülling T (2009) Cytokinin action in plant development. *Current Opinion in Plant Biology* **12**, 527–538. doi:10.1016/j.pbi.2009.07.002

West AH, Stock AM (2001) Histidine kinases and response regulator proteins in two-component signalling systems. *Trends in Biochemical Sciences* **26**, 369–376. doi:10.1016/S0968-0004(01)01852-7

Wulfetange K, Lomin SN, Romanov GA, Stolz A, Heyl A, Schmülling T (2011) The cytokinin receptors of *Arabidopsis* are located mainly to the endoplasmic reticulum. *Plant Physiology* **156**, 1808–1818. doi:10.1104/pp.111.180539

Yu Q, Tang C, Kuo J (2000) A critical review on methods to measure apoplastic pH in plants. *Plant and Soil* **219**, 29–40. doi:10.1023/A:1004724610550

Zürcher E, Müller B (2016) Cytokinin synthesis, signaling, and function – advances and new insights. *International Review of Cell and Molecular Biology* **324**, 1–38. doi:10.1016/bs.ircmb.2016.01.001

Zürcher E, Liu J, di Donato M, Geisler M, Müller B (2016) Plant development regulated by cytokinin sinks. *Science* **353**, 1027–1030. doi:10.1126/science.aaf7254

Supplementary data

Table S1. Sequences of primers used for PCR

Primer name	Primer sequence
F AHK3/XbaI	*5*`-ACGTCTAGAATGAGTCTGTTCCATG-3’
R AHK3/Cfr9I	*5*`- ATCCCGGGTGATTCTGTATCTGAAG-3’
F AHK4/XbaI	*5*`- ACGTCTAGAATGAGAAGAGATTTTGTG-3’
R AHK4/Cfr9I	*5*`- ATCCCGGGCGACGAAGGTGAGAT-3’
F AHP1/BcuI	*5*`-GCCACTAGTATGGATTTGGTTCAG-3`
R AHP1/XhoI	*5*`-CTCGAGAGATCCACCACCTCCACCAAATCCGAGTTCGAC-3`
F *AHP2/BcuI*	5`-GCCACTAGTATGGACGCTCTCAT-3`
R *AHP2/XhoI*	5`-GCCCTCGAGGTTAATATCCACTTGA-3`
F *AHP3/BcuI*	5`-GCCACTAGTATGGACACACTCATT-3`
R *AHP3/XhoI*	5`-ATCTCGAGAGATCCACCACCTCCACCTATATCCACTTGAGG-3`
F AHP1/NcoI	*5*`-TAACCATGGGGTGGAGGTGGTGGATCTATGGATTTGGTTCAG-3`
R *AHP1/BamHI*	5`-AAGGATCCTCAAAATCCGAGTTCGACG-3`
F *AHP3/NcoI*	5`-TAACCATGGGGTGGAGGTGGTGGATCTATGGACACACTCATT-3`
R *AHP3/BamHI*	5`-AGGGATCCTTATATATCCACTTGAGGG-3`

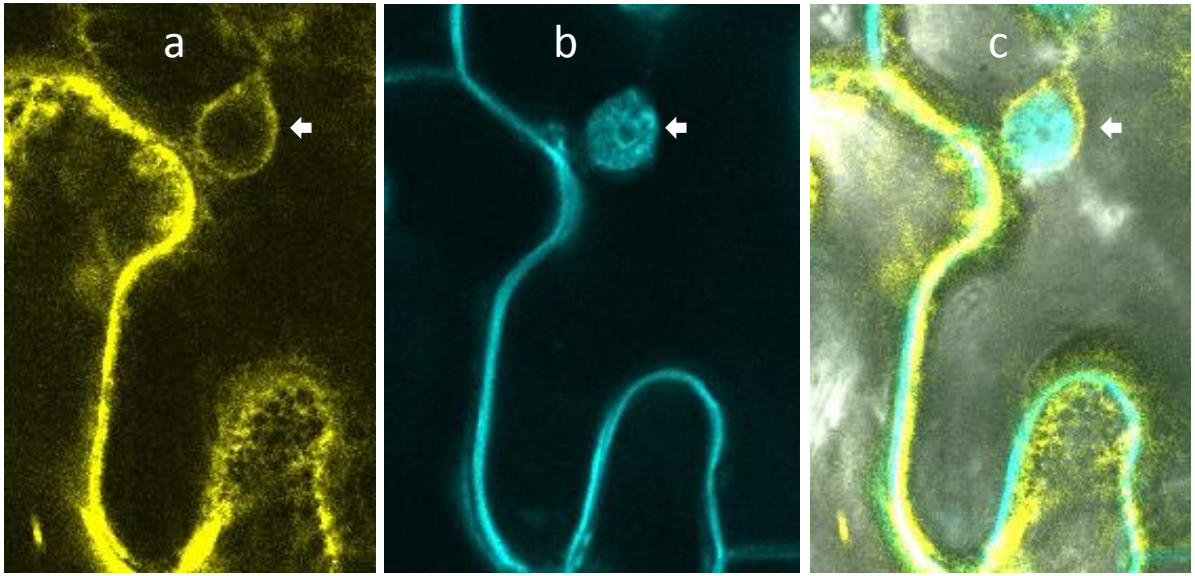

Fig. S1. Interaction pattern of AHK3 and AHP2 shown by BiFC in *N. benthamiana* leaves. (a) Fluorescence signal indicating AHK3 x AHP2 interaction. (b) DAPI staining. (c) Merged pictures of the sections shown in (a) and (b). White arrows indicate the nucleus. The nucleolus is clearly seen in (b) and (c). Apart nuclei, DAPI is known to stain also cell walls (b) (Jordan C.V. et al., Plant Mol. Biol., 2007, 65:163–175; Juntawong P. et al., The Plant J., 2013, 74:1016–1028). Note that BiFC staining and DAPI staining do not overlap.

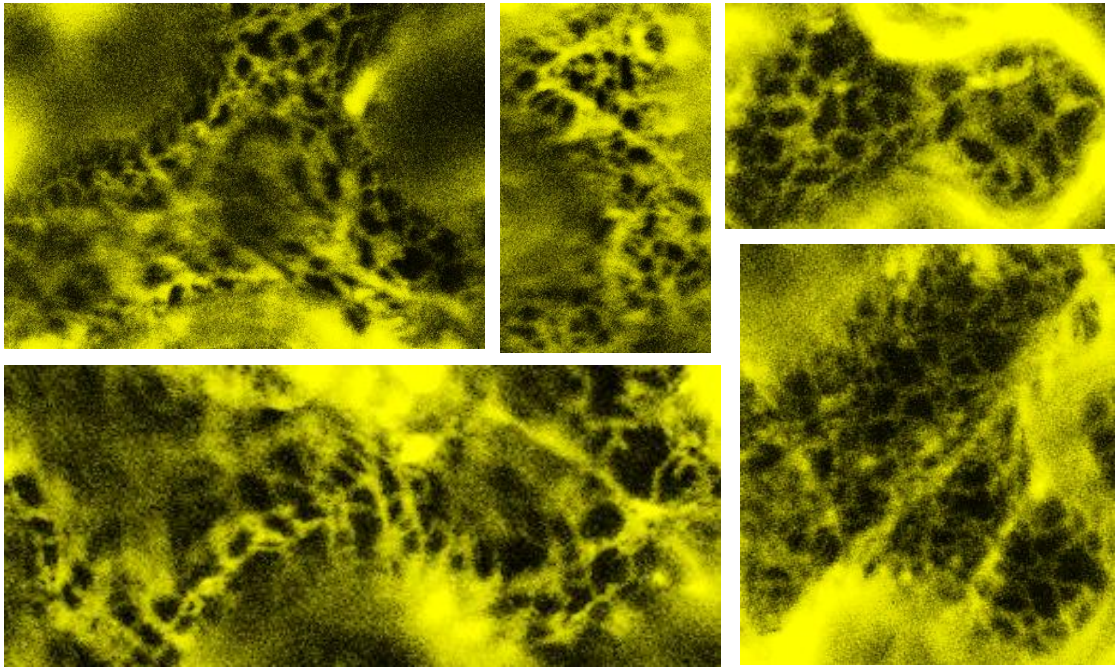

Fig. S2. Interaction AHK4 x AHP3 shown by BiFC in *N. benthamiana* leaves. Fluorescence signals indicating interaction mark the ER network.

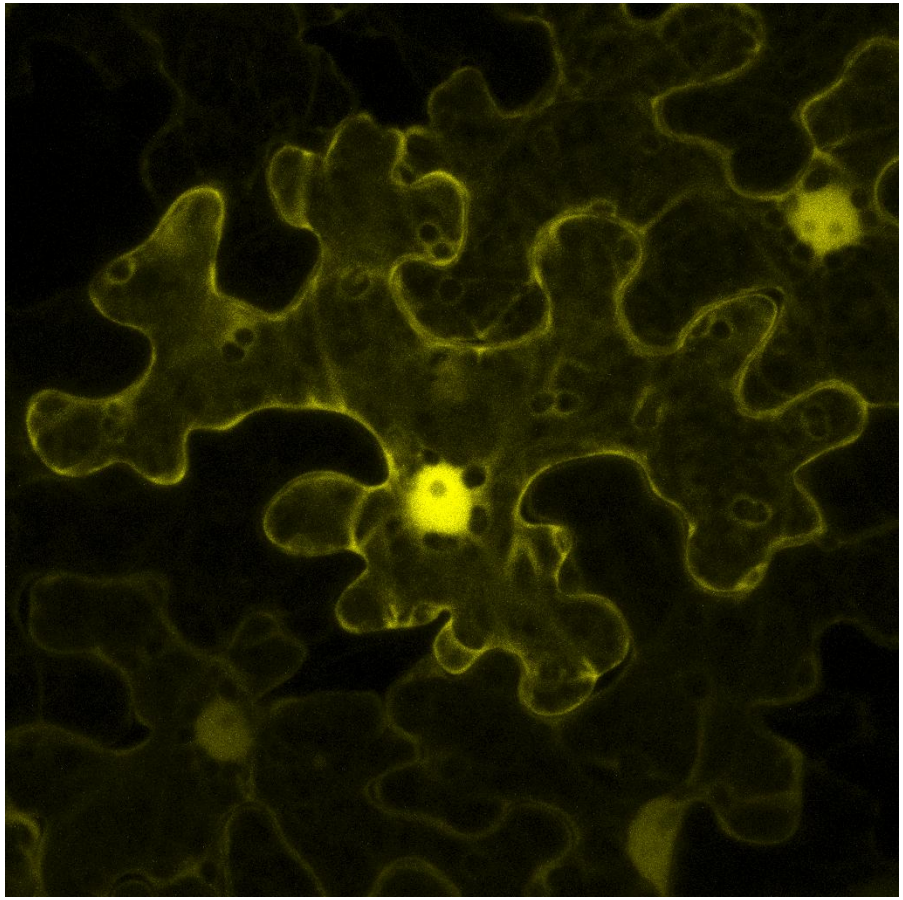

Fig. S3. An example of a fluorescent nucleus (AHP2 x AHP2, BiFC) where the nucleolus is clearly seen.

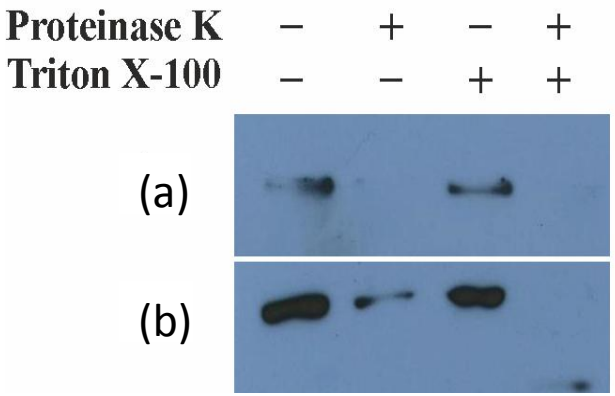

Fig. S4. Determination of AHK3 orientation by a protease protection assay. Microsomes from tobacco leaves expressing the *AHK3-Myc* gene were treated with proteinase K in the presence or absence of Triton X-100. The Myc-tag was fused to the C-terminus of AHK3. Microsomal proteins were solubilized, resolved by SDS-PAGE, and subjected to immunoblotting using (a) anti-Myc-tag monoclonal antibodies, or (b) rabbit polyclonal antibodies against the BiP marker of the ER lumen. The disappearance of the signal derived from the detection of the Myc-tag and the preservation of the BiP signal (second column) indicates the localization of these two markers at opposite sides of the membrane. The lowering of BiP content in (b) in the absence of Triton X-100 may be due to different reasons, the same trend was observed in the original protocol (Ma *et al*. 2006).